國立交通大學

生物科技研究所

碩 士 論 文

克雷白氏肺炎桿菌 CG43 中 KvhAS 雙分子調控系統的功能分析

Functional analysis of the Two-component System KvhAS in *Klebsiella pneumoniae* CG43

研究生：梁婉君

學號：9128511

指導教授： 彭 慧 玲 博士

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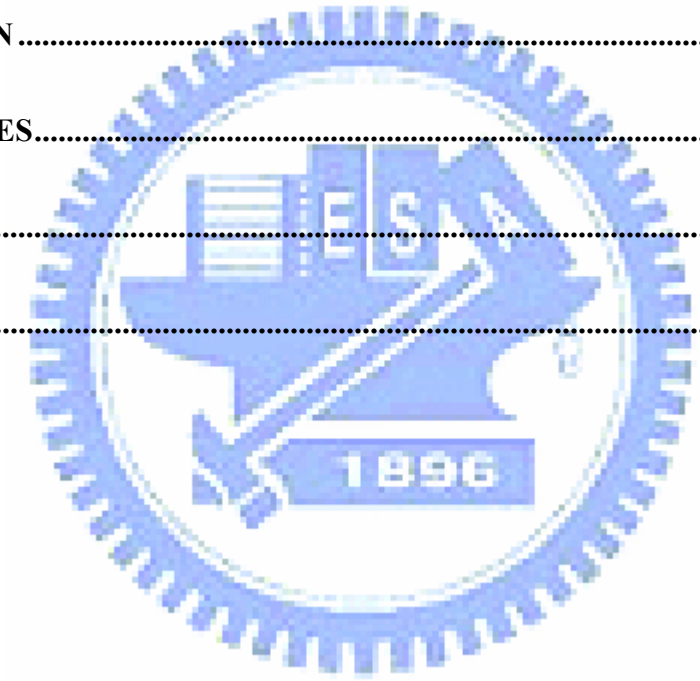

摘要

根據本實驗室過去的 EMSA(Electrophoretic mobility shift assay)研究，我們發現 KvhAS 及其同源雙分子調控系統 KvgAS 和 KvhR 可能具有互相調控關係。為了更進一步研究彼此的交互作用，因此構築了一系列的突變株，包括 *kvhA*⁻*kvgA*⁻、*kvhA*⁻*kvhR*⁻、*kvgA*⁻*kvhR*⁻及 *kvhA*⁻*kvgA*⁻*kvhR*⁻。經過比較這些突變株的生長及菌體低速離心狀態後，將這些突變株分成兩群：第一群菌帶有 *kvgA* 或 *kvhR* 突變，呈現快速生長及低黏度的表現型；而以老鼠腹膜炎模式分析，刪除 *kvgA* 或 *kvhR* 會導致老鼠半致死率增加。第二群菌包含 *kvhA*⁻ 和 *kvhA*⁻*kvhR*⁻ 突變株，這些突變株呈現和母株 LacZ16 相同的生長速率及菌體黏度。將含有 *kvhA* 的質體轉型入 *kvhA*⁻*kvhR*⁻雙基因突變株，我們發現 *kvhA*⁻*kvhR*⁻突變株會由第二群轉成第一群；且同時含有 *kvhA* 及 *kvhR* 基因突變時，細菌對人類血清的抵抗能力下降。這些結果顯示 KvhA 和 KvhR 之間可能有交互作用，而這些突變株在菌體黏度及致病力表現不同程度的差異，以莢膜多糖體(capsular polysaccharide)為最可能引起變化的因子。因此，我們利用 LacZ 為報導蛋白，分析莢膜多糖體基因的啟動子在這些突變株的表現差異。結果顯示：莢膜多糖體基因組開放骨架 1~2 或 16~17 的啟動子活性在第一群突變株中表現降低，而在 *kvhR*⁻降低的活性會因為 *kvhA* 基因同時突變而回復；然而，開放骨架 3~15 的啟動子活性在任一突變株中並沒有顯著差異。另外，當壓力調節的主控子 RpoS 基因突變時，*kvhAS* 的啟動子活性明顯上升，進一步分析發現在 *kvhAS* 基因的上游-379 至-476 片段可能是 RpoS 調控的區域，而此區也是 KvhA 的自我調控區域。我們還發現 *kvhAS* 的啟動子活性會隨鎂離子濃度升高而上升，這個結果暗示 KvhAS 和 PhoPQ 有交互作用。除此之外，大量表現 KvhA 蛋白質會改變菌體對不同抗生素的感受性，這結果顯示著 KvhA 可能調控細菌對藥物的感受性。

ABSTRACT

Previous EMSA (Electrophoretic mobility shift assay) study in *Klebsiella pneumoniae* CG43 genome has shown a possibility of the presence of a regulatory circuit in-between the homologous two components (2CS) KvhAS, KvgAS and KvhR. In order to investigate further the regulatory interaction, a series of mutants including *kvhA*$^-$*kvgA*$^-$, *kvhA*$^-$*kvhR*$^-$, *kvgA*$^-$*kvhR*$^-$ and *kvhA*$^-$*kvgA*$^-$*kvhR*$^-$ derived from *K. pneumoniae* LacZ16 were constructed. Comparative analysis of the growth and phenotype of the mutants allowed us to classify the mutants into two groups: the Group I carrying either *kvgA* or *kvhR* mutation displayed a less mucoid and a faster growth rate by comparing to the parental strain LacZ16. In a mouse peritonitis model, the deletion of either *kvgA* or *kvhR* resulted in an increase of LD_{50}. Group II includes *kvhA*$^-$ and *kvhA*$^-$*kvhR*$^-$ mutants, which exert a similar phenotype and growth rate with that of the parental strain. Complementation of the *kvhA*$^-$*kvhR*$^-$ with a *kvhA* containing plasmid converts the mutant into Group I. Only with both *kvhA*$^-$ and *kvhR*$^-$ mutations, the bacteria revealed a decreasing activity of resistance to human serum. The results suggested that a cross-talk is present in-between KvhA and KvhR. Mutations of these genes affect both bacterial mucoidity and virulence at different levels indicating that change of capsular polysaccharide (CPS) is most likely the causing factor. We therefore attempt further to analyze promoters of the CPS

encoding genes in the mutants using LacZ as a reporter. The promoter activity of either $P_{orf1\text{-}2}$ or $P_{orf16\text{-}17}$ in Group I mutants appeared to be reduced, which suggests that KvgA and KvhR are the regulators for *cps* gene expression. The decreasing activity in *kvhR*$^-$ was found to be reversed in *kvhA*$^-$*kvhR*$^-$, indicating a direct interaction between KvhA and KvhR. In addition, we have shown that mutation of the global stress regulator RpoS increased the promoter activity of *kvhAS*. The putative regulatory region of *kvhAS* by RpoS was localized between -379 and -476 bp upstream of *kvhAS*, which appeared to contain also the auto-regulating region of KvhA. The promoter activity of *kvhAS* was found to increase by the addition of $MgSO_4$, suggesting an interaction with PhoPQ regulon. Moreover, changes of resistance activity to antibiotics were noted by overexpression of KvhA in the bacterial, implying its involvement in drug resistance.

INTRODUCTION

Klebsiella pneumoniae is a commonly reported nosocomial Gram-negative bacteria. As an opportunistic pathogen, it often causes pneumonia, septicemia, bacteremia, suppurative lesion, wound infection, burn infection, and urinary or respiratory tract infections in chronic alcoholics or immunocompromised patients. There are five major virulence factors identified to participate in *K. pneumoniae* infections, which include capsular polysaccharide, lipopolysaccharide, adhesin, iron-acquisition system, and serum resistance factors (Podschun and Ullmann, 1998). Besides these virulence factors, very little is known about other genes that may participate in the pathogenesis of *K. pneumoniae*.

To persist in nature, bacteria must be able to compete and survive under various growth conditions. To accomplish this task, they possess regulatory systems that permit them to recognize and adapt to a changing environment. These adaptations are brought about largely through changes in gene expression that are coordinated by global regulatory networks. Pathogenic bacteria often use two-component systems (2CSs) to control expression of the genes encoding bacterial toxins, adhesins, and other virulence-associated molecules that promote their survival in the host (Locht, 1999). The 2CSs composed of a membrane-bound sensor protein and a cytoplasmic

response regulator are commonly used to regulate bacterial gene expression in response to environmental changes. On stimulation by an extracellular signal, a histidine kinase domain of the sensor protein is autophosphorylated and the signal transduction occurs with transfer of the phosphoryl group from the phosphorylated kinase to an aspartic acid residue in the receiver domain of the cognate response regulator. Phosphorylation of the regulator protein, which is often a transcription factor containing a C-terminal DNA-binding domain, increases its binding affinity to target promoters and thereby influences the expression of the target genes (Foussard *et al*., 2001; Chang *et al*., 1998; Mizuno, 1998) .

A novel two-component system *kvgASXR* (*K*lebsiella virulence gene) has been isolated by PCR-supported genomic subtractive hybridization from a highly virulent strain *K. pneumoniae* CG43 (楊淑理, 民國八十九年), which is not found in the genome of *K. pneumoniae* strain MGH78578 (http://genome.wustl.edu/gsc/). A 2CS exhibiting high sequence identity with that of the *kvgAS* was isolated later in our laboratory also from *K. pneumoniae* CG43 and designated *kvhAS* (*kvg* homolog), which is present in the genome of *K. pneumoniae* MGH78578 (賴旻初, 民國八十九年). In contrast to *kvgAS*, which is present in approximately 15% of the laboratory-collected clinical isolates (Lai *et al.*, 2000), *kvhAS* prevails in almost all the strains. As well as the KvgAS, KvhAS displayed a moderate similarity to BvgAS

(*Bordetella* virulence gene) of *B. pertussis* and EvgAS (*Escherichia* virulence gene), the *E. coli* ortholog of BvgAS (Konig *et al.*, 2002; Bock *et al.*, 2001; Utsumi *et al.*, 1992). These sensors are classified as unorthodox type sensor, each of which is composed of an N-terminal periplasmic region and a C-terminal cytoplasmic region containing four functional domains of a linker, transmitter, receiver and an output, the Hpt module. A multistep phosphorelay occurs among the alternating histidine and aspartate residues (His-Asp-His-Asp) before the response regulator is activated (Stock, 2000). Although EvgAS is highly homologous to BvgAS, domain swapping of the liberated signaling modules had shown that the C-terminal Hpt domain of the sensor protein endows a specificity for the corresponding regulator, and the receiver domain of the response regulator determines the specific interaction (Perraud *et al.*, 1998, 2000).

Lipopolysaccharide (LPS) and capsular polysaccharide (CPS) are two of the virulence factors of *K. pneumoniae*. The production of a hydrophilic CPS (K antigen) is characteristic of the genus *Klebsiella*, and is involved mainly in the resistance, by acting as a physical barrier, to phagocytosis by polymorphonuclear cells (Kabha *et al.*, 1995 ; Held *et al.*, 2000). *Klebsiella* K2 CPS biosynthetic pathway is similar to that of the group I CPS in *E. coli*, and the regulatory strategy of colanic acid biosynthesis employed by *E. coli* may therefore serve as a model (Whitfield *et al.*, 1999, 2003).

Colanic acid is produced by enzymes encoded by the *cps* genes, which are regulated by *rcsC/yojN/rcsB* and also by *lon* protease for degradation of RcsA. RcsC is a sensor protein, RcsB is its cognate response regulator, and YojN is a histidine-containing phosphotransfer protein that transfers the phosphoryl group from RcsC to RcsB. The activated RcsB together with the co-activator RcsA subsequently coordinate the activation of the *cps* operon transcription (Chen *et al*., 2001). In addition to Rcs system, the *rmpA2* gene was isolated from a 200-kb virulence plasmid of *K. pneumoniae* CG43 also encodes an activator for CPS synthesis. Deletion of *rmpA2* was shown to reduce the mucoidy and virulence of the bacteria (Lai *et al.*, 2003).

In bacteria, *rpoS* gene encodes the alternative sigma factor σ^s (RpoS) subunit of RNA polymerase to control the expression of genes involved in cell survival during the cessation of growth (stationary phase) and provide cross-protection to various stresses (Hengge-Aronis, 2002). Expression of σ^s increases dramatically at the onset of stationary phase, which is regulated at the transcriptional, post-transcriptional and post-translational levels (Loewen *et al.*, 1998). A most complex regulatory system is also essential for *Salmonella* virulence in mice, which includes the GacA/GacS two component system and quorum sensing control to affect *rpoS* expression (Ibanez-Ruiz *et al*., 2000). The recent study of DNA microarray analysis suggested that *rpoS* expression is affected by multiple two-component systems (Venturi, 2003; Sugiura *et*

al., 2003). In *E. coli*, CpxR-P in conjunction with RpoS activates expression of the *cpxRA* two-component system, suggesting an involvement of the Cpx system in stationary-phase survival (De Wulf, 1999).

While the physically linked sequences of *kvhAS* compared with that of the *evgAS*, several homologous genes were identified (黃騰逸, 民國九十一年). The putative acid-resistance genes *yfdXL* which show a high homology with the *evgAS*-regulated *yfdX* were identified downstream and upstream of *kvhAS* respectively. The *emrKY* and *yhiUV* which are located nearby the EvgAS genes were also identified, however, elsewhere in *K. pneumoniae* MGH78578 genome. Overexpression of the *evgA* response regulator up-regulates the drug transporter genes *emrKY* and *yhiUV* in *E.coli*, which led to resistance to a wide range of toxic compounds, such as erythromycin, doxorubicin, novobiocin, crystal violet, rhodamine 6G, TPP, benzalkonium, SDS, and deoxycholate (Nishino *et al.*, 2001, 2002; Eguchi *et al*., 2003). The 2CS BvgAS positively controlled transcription of several virulence genes, which include the genes for toxins and adhesins, in *B. pertussis* and *B. bronchiseptica*. On the other hand, the BvgAS system negatively controlled the expression of a set of genes, the majority of these genes encode putative metabolic functions involved in redox reactions and amino acid transport (Schneider *et al*., 2002). It has also been demonstrated that the expression of *bvgAS* genes is regulated by the addition of $MgSO_4$, nicotinic acid or

elevated growth temperatures (Scarlato *et al*., 1991). As an ortholog of EvgAS and BvgAS, KvhAS is likely playing a similar function.

The emergence and spread of multiple antibiotic-resistant *K. pneumoniae*, especially the extended-spectrum β-lactamase-producing strains, urge a need for new antibiotics with an innovative mode of action (Gupta *et al*., 2003). It has been proven that two-component systems play an important role in sensing cell disruption by drugs and thereby activating expression of some resistance factors (Ohki *et al*., 2003). Higher eukaryotes including mammals often use a distinct signal transduction system that incorporates serine, threonine, and tyrosine phosphorylation. As for the selectivity of antimicrobial agents, histidine kinases appear to be potent targets (Matsushita *et al*., 2002). Previous studies have shown the possibility of the presence of a regulatory circuit connected by *kvhAS*, *kvgAS* and *kvhR*, a response regulator located at the downstream of *kvgAS*. In this study, we attempt to elucidate the relationship between these 2CS paralogs (Fig. 1). A series of mutant strains including *kvhA*, *kvgA*, *kvhR* were constructed in order to assign functional role of the 2CSs in the bacteria. The environmental signal(s) sensed by KvhS and the target genes under control by *kvhAS* were also investigated.

MATERIALS AND METHODS

Plasmids, bacterial strains, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1 and 2. Tryptone, yeast extract, and agar were purchased from Difco Laboratories (Detroit, Mich). *E. coli*, *K. pneumoniae* CG43 and its derivatives were propagated at 37°C in Luria-Bertani (LB) broth supplemented with the appropriate antibiotics unless otherwise indicated. The density of the bacterial culture was determined by measuring the absorbance at 600 nm (OD_{600}).

Recombinant DNA technique. The recombinant DNA experiment was carried out by standard procedures as described (Joseph *et al.*, 2001). Plasmid DNA was prepared using VIOGENE Miniprep kit. Restriction endonucleases and DNA modifying enzymes were purchased from New England Biolab (Beverly, MA), MBI (Hanover, MD), or Roche Molecular Biochemicals (Mannheim, Germany), and were used according to the recommendation of the suppliers.

Construction of the gene-deletion mutants. *K. pneumoniae* CG43LacZ16 derived mutants with deletion specific at either of *kvhA*, *kvgA*, and *kvhR* genes were constructed by allelic exchange strategy (Skorupski *et al.*, 1996). The pKAS46 derivative containing a deletion in *kvgA* constructed previously was delivered from *E.*

coli S17-1 *λ-pir* into *kvhA*$^-$ and *kvhR*$^-$ respectively by conjugation. The transconjugants were selected by plating with a 10^5-fold dilution of the culture on a minimal medium containing 25 μg/ml kanamycin and 100 μg/ml ampicillin for the integration of the plasmid. Subsequently, the resulting strains were cultured without selection to late logarithmic phase followed by selection on the plates containing 500 μg/ml streptomycin for loss of the vector sequence. In addition, a plasmid containing a deletion in *kvhR* was transferred from *E. coli* S17-1 *λ-pir* into *kvhA*$^-$ and *kvhA*$^-$*kvgA*$^-$ respectively. The plasmid containing a tetracycline gene insertion in *rpos* was transferred also from *E. coli* S17-1*λ-pir* into *kvhA*$^-$. The transconjugants were selected on appropriate medium and the mutations were all confirmed by PCR and Southern analysis.

Expression and purification of the histidine-tagged KvhA. The recombinant His_6-tagged response regulator, His_6-KvhA, was constructed (pHP4005) and the plasmid was transformed into *E. coli* BL21-RIL (DE3). The transformants carrying pHP4005 were cultured in LB broth to log phase, and expression of the His_6-KvhA recombinant protein was induced with 0.5 mM IPTG for 4 h. The IPTG (isopropyl-β-D-thiogalactopyranoside)-induced bacterial cells were harvested by centrifugation at 4°C, 6000 rpm for 15 min, resuspended in 1 X binding buffer (5 mM imidazole, 0.5 M NaCl and 20 mM Tris-HCl pH 7.9), and then disrupted by

sonication. To purify His_6-KvhA, the cell lysate was centrifuged at 13,500 rpm for 20 min at 4°C, and the supernatant was then applied to a column containing His-Bind Resin (Novagen, Madison, WI). The column was washed with 1 X binding buffer, and then washed with 1 X wash buffer (60 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl pH7.9). Finally, the His_6-KvhA protein was eluted with 1 X elution buffer (1 M imidazole, 0.5 M NaCl, and 20 mM Tris-HCl pH 7.9), dialyzed with electrophoretic mobility shift assay buffer (20 mM Tris-HCl pH 8.0, 4 mM $MgCl_2$, 50 mM KCl, 1 mM $CaCl_2$, 1 mM dithiothreitol), and then concentrated by PEG-20,000 at 4°C.

Electrophoretic mobility shift assays. The probes used in the electrophoretic mobility shift assay were the restriction fragments of the upstream region of *kvhR*. The restricted fragments were separated on a 0.8% agarose gel and recovered, and 5' end-labelled with [γ-^{32}P]-ATP (25 μCi/ml) using T4 polynucleotide kinase. The reaction mixture was incubated for 60 min at 37°C. The His_6-KvhA was incubated in 1 X gel shift binding buffer (20 mM Tris-HCl, pH 8.0, 0.4 mM $MgCl_2$, 50 mM KCl, 1 mM $CaCl_2$, 1 mM DTT, and 10% glycerol) for 30 min at 25°C (Karimova *et al.* 1996). Bovine serum albumin (1 μg) and the radioactively-labelled DNA fragments were then added and incubated at 37°C for 20 min. The samples were applied to 5% non-denaturing polyacrylamide (acrylamide: bis-acrylamide, 19: 1) gels that were

pre-electrophoresed at a constant voltage of 100 V for 1 h at 4°C. Electrophoresis was performed at 100 V for 6 h at 4°C and the gels were read by InstantImager™ (Packard Instrument Company).

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 resuspended in 0.2 ml of saline in 10-fold graded doses. The LD_{50}, based on the number of survivors after 10 days, was calculated and expressed as CFU (colony forming units) as described (Reed *et al.*, 1938).

Resistance to serum killing. Fresh blood collected from healthy volunteers was centrifuged to obtain nonimmune human sera (NHS). NHS was incubated at 56°C for 30 min to obtain heat-inactivated human sera (HI-NHS). The serum-resistant activity of bacteria was determined essentially as described (Lai *et al.*, 2003).One hundred microliters of overnight grown bacteria re-suspended in saline was mixed with 100 µl of NHS or HI-NHS, and the mixture was incubated at 37°C for 30 min. The number of viable bacteria in the mixture was then determined by plating onto LB agar.

Extraction and quantification of CPS. CPS was extracted by the method as described previously (Domenico *et al.*, 1989).Five hundred microliters of bacteria

cultured in LB broth overnight were mixed with 100 µl of 1% Zwittergent 3-14 detergent in 100 mM citric acid (pH 2.0), and then the mixture was incubated at 50°C for 20 min. After centrifugation 13,500 rpm for 10 min, 250 µl of the supernatant was transferred to a new tube, and CPS was precipitated with 1 ml of absolute ethanol at 4°C for 20 min and then centrifugated 13,500 rpm for 25 min. The pellet was dried at 37°C and dissolved in 200 µl of distilled water, and a 1,200-µl volume of 12.5 mM borax in H_2SO_4 was added. The mixture was vigorously vortexed, boiled for 5 min, and cooled, and then 20 µl of 0.15% 3-hydroxydiphenol was added and the absorbance at 520 nm was measured. The uronic acid content was determined from a standard curve of glucuronic acid and expressed as micrograms per 10^9 CFU (Blumenkrantz *et al.*, 1973).

Capsular polysaccharides purification. 1.5 ml bacteria cultured in LB broth overnight at 37°C were harvested by centrifugation at 13,200 rpm for 10 min. The pellet was resuspended with 500 µl phosphate-buffered saline (PBS) and mixed with 500 µl phenol, subsequently extracted at 65°C for 5 min then put on ice for 5 min and repeated for three times. The sample was centrifuged at 10,000 *g* for 5 min and transferred aqueous phase to dialyze against water overnight. Subsequently, the sample was treated with DNase and RNase at 37°C for 2 h and proteinase K at 50°C overnight then dialyzed against water overnight again as described (Kalka-Moll *et al.*,

2001).

Capsular polysaccharides pattern analysis by Alcian Blue-silver stain. CPS was resolved on 10% (w/v) acrylamide gel and immediately immersed in alcian blue solution (0.005% alcian blue, 40% ethanol, and 5% acidic acid in dH_2O) for 30 min, then changed to fresh solution overnight. The gel was rinsed in dH_2O for 5 min and oxidized in 0.7% (w/v) sodium metaperiodate for 10 min, then washed five times with dH_2O for 5 min. The gel was stained by 10% silver solution (Bio-Rad silver concentrate) for 10 min, and then washed with dH_2O for 5 min. The color was developed with 1.5% (w/v) developer (Bio-Rad) and agitated until dark precipitation formed, then stopped with 5% acetic acid for 10 min and rinsed in dH_2O (Karlyshev and Wren, 2001).

Anti-phagocytosis assay. THP-1 (ATCC TIB202), originally isolated from a child with acute leukemia, are mature cells in the monocyte/macrophage lineage with a normal diploid karyotype. These nonadherent cells were maintained in continuous culture in RPMI 1640 cell culture media (GIBCO/BRL, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (GIBCO/BRL) in an atmosphere of 5% CO_2 at 37°C. THP-1 cells were centrifuged at 1000 rpm for 5 min and resuspended with fresh medium, then 0.5 ml of cells (10^6/ml) were plated onto per well of 24-well

tissue culture plates and treated with phorbol 12-myristate 13-acetate (PMA) (Calbiochem Co., La Jolla, Calif.) for 48 h to induce maturation of the monocytes and became macrophage-like; differentiated macrophages were identified by morphological features and their ability to adhere to plastic. Before experimentation, THP-1 cells were washed twice with PBS to remove nonadherent cells and resuspended in 0.5 ml RPMI without fetal bovine serum, then incubated with 50 μl of overnight cultured bacteria previously washed one time and diluted in PBS (6×10^8/ml) for 2 h. Subsequently, wells were washed twice with PBS and incubated for 2 h with fresh RPMI medium containing gentamycin (100 μg/ml) to kill extracellular bacteria. Then wells were washed three times with PBS and phagocytosed bacteria were quantified by plating them on LB agar plates after THP-1 lyses with 500 μl of 0.1% Triton X-100 as described (Perez-Perez *et al.*, 1995; Cortes *et al.*, 2002).

β-galactosidase activity assay. β-galactosidase was assayed according to the method of Miller (Miller. 1972). Fresh M9 medium was inoculated with one-tenth volumes of the overnight M9 cultured bacteria. Samples of bacteria were taken 100 μl in the early or late logarithmic growth phase (optical density at 600 nm 0.4 or 0.7), and mixed with 900 μl Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol), 17 μl of 0.1% SDS and 35 μl chloroform for 10 min at 30°C. Subsequently, 200 μl of 4 mg/ml *o*-nitrophenyl-β-D-galactopyranoside

(ONPG) was added and vortexed for 10 s, then incubated at 30°C until yellow color was apparent. Finally, reaction was stopped by adding 500 µl of stop solution (1M Na_2CO_3) and the OD_{420} of supernatant was measured. One unit of β-galactosidase is defined as the hydrolysis of 1nmol ONPG per min per mg protein. Signals were added in the early logarithmic growth phase, and after incubated 1 h, β-galactosidase activity was tested as described above.

Drug 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. Standard antibiotic disks were obtained from Difco (Detroit, Mich), Becton Dickinson (Sparks, Md) and Oxoid Ltd (Basingstoke, Hampshire), and the following disc concentrations were used: erythromycin, 15 µg; novobiocin 5 µg; ceftazidime 30 µg; cefamandole 30 µg; cefotaxime 30 µg; cephalothin 30 µg; fosfomycin 50 µg; nalidixic acid 30 µg; methicillin 5 µg; penicillin 10 µg; piperacillin 100 µg; ticarcillin 75 µg; carbenicillin 100 µg; sulfamethoxazole 23.75 µg + trimethoprim 1.25 µg; polymyxin B 300 IU.

RESULTS

Characterization of the mutants derived from LacZ16. Previous EMSA study has shown that KvgA bound to either the promoter of *kvhA* or *kvhR*, suggesting presence of a regulatory circuit among *kvhAS, kvgAS* and *kvhR* (林靖婷，民國九十年). The gene organizations are shown in Fig. 1. In order to investigate further the interaction, a series of mutants were constructed on *K. pneumoniae* LacZ16 deriving *kvhA*$^-$*kvgA*$^-$, *kvhA*$^-$*kvhR*$^-$, *kvgA*$^-$*kvhR*$^-$, and *kvhA*$^-$*kvgA*$^-$*kvhR*$^-$. Comparative analysis of the growth and sedimentation rates of the mutants allowed the classification of these mutants into two groups: the Group I carrying either *kvgA* or *kvhR* mutation that displayed a less mucoid phenotype and a faster growth rate than that of the parental strain LacZ16. Group II includes *kvhA*$^-$ and *kvhA*$^-$*kvhR*$^-$ mutants, which exert similar growth rate and phenotype with that of the parental strain LacZ16 (Fig. 2A and 2B).

The reduction of mucoidy in strains of Group I was also evident as determined by the inability of the colony to form a string using a toothpick. The amount of CPS produced in these mutants were further quantified by measuring the glucuronic acid contents. As shown in Table 4, Group I strains synthesized much less K2 CPS than the parental LacZ16 strain. A slight reduction of CPS and colony mucoidy also appeared in *kvhA*$^-$*kvhR*$^-$, which belongs to Group II. Complementation of the *kvhA*$^-$*kvhR*$^-$ with

pA415, a DNA fragment corresponding to the entire *kvhA* locus with its putative promoter amplified by A201/AS02 primer set and cloned into pRK415, converted both phenotype and growth of the mutant from Group II into Group I (Fig. 3A and 3B).

The His_6-KvhA binds specifically to *kvhR* promoter. The response regulator KvhA, like many other response regulators of the two-component system, possesses a helix-turn-helix motif at the C-terminus. In order to demonstrate its interaction with *kvhR* promoter, pHP4005 which is pET30C containing the entire *kvhA* was transformed into *E. coli* BL21-RIL (DE3), and the IPTG-induced His_6-KvhA was purified by affinity chromatography (Fig. 4). Binding capability of the His_6-KvhA to *kvhR* promoter was assessed by electrophoretic mobility shift assay. As presented in Fig. 5, specific DNA-binding activity of the His_6-KvhA was demonstrated. The mobility of His_6-KvhA appeared to be retarded with the addition of ^{32}P-labelled P_{kvhR}. The DNA binding appeared to be specific because the shifted band disappeared in the presence of an excess amount of unlabelled P_{kvhR}, but not in the presence of pUC19 DNA.

The CPS and LPS patterns of the mutants. The CPS and LPS patterns of each mutants were analyzed by electrophoresis on 10% (w/v) polyacrylamide with a

without SDS supplement and 15% (w/v) SDS-PAGE respectively. The resolved LPS was visualized by silver staining, and the CPS was visualized by staining with Alcian Blue followed by silver staining. We have found that *K. pneumoniae* CPS was barely detectable without the use of Alcian Blue. The CPS pattern of *kvhA*$^-$ appeared to be the same as that of wild type, however, *kvhA*$^-$*kvhR*$^-$ showed less high molecular weight CPS, which migrated as a broad smear at the top of the gel (Fig. 6). When the *kvhA*$^-$*kvhR*$^-$ mutant was complemented with a *kvhA* carrying plasmid, an obvious increase of the low molecular weight CPS components was noted. In addition, the Group I mutants included *kvgA*$^-$, *kvhR*$^-$, *kvhA*$^-$*kvgA*$^-$, *kvgA*$^-$*kvhR*$^-$ and *kvhA*$^-$*kvgA*$^-$*kvhR*$^-$ showed more high molecular weight CPS comparing with that of the parental strain LacZ16 (Fig. 6). As shown in Fig. 7, LPS was clearly visible in all the mutants, showing the characteristic O-antigen banding pattern.

Anti-phagocytosis activity of the mutants. To investigate the mutation effect on the bacterial susceptibility to macrophage, the cell line THP-1 was used. After 2 h incubation with each of the mutant bacteria, the THP-1 cells were washed and the ingested bacteria were quantified by plating onto LB plates. As shown in Fig. 8, the Group I mutants *kvgA*$^-$, *kvhR*$^-$, *kvhA*$^-$*kvgA*$^-$, *kvgA*$^-$*kvhR*$^-$ and *kvhA*$^-$*kvgA*$^-$*kvhR*$^-$ significantly lost their resistant activity to phagocytosis, whereas, the *kvhA*$^-$ and *kvhA*$^-$*kvhR*$^-$ possessed a higher anti-phagocytosis activity. Complement of the mutant

kvhA$^-$*kvhR*$^-$ with a *kvhA* carrying plasmid appeared to reduce its anti-phagocytosis activity.

Effects of *kvhA*, *kvgA* and *kvhR* mutation on the bacterial virulence. In a mouse peritonitis model, the virulence of the mutants were compared. As shown in Table 5, deletion of *kvgA* or *kvhR* resulted in an increase of the LD_{50}. The *kvhA* mutation, however, had no apparent effects. In the complement-mediated killing test, only *kvhA*$^-$*kvhR*$^-$ appeared to show a decreasing resistance to human serum. Complementation of the mutant with pA415, the *kvhA* carrying plasmid, slightly restored the resistance. While in the heat inactivated human sera, the sensitivity to complement-mediated killing was also reduced.

Effects of *kvhA*, *kvgA* and *kvhR* on CG43 *cps* gene expression. In *E. coli* K-12, the amount of colanic acid produced has been reported to be correlated with the transcriptional level of *cps* genes (Arakawa *et al.*, 1995). We reasoned that the production of CPS affected by *kvhA*, *kvgA* and *kvhR* might also be resulted from differential *cps* genes expression in *K. pneumoniae* CG43, which carries a K2 capsular type. Thus, five *lacZ* reporter fusion constructs, porf1Z15 and porf2Z15, which comprise 800 bp and 440 bp non-translated region upstream of *cps* gene *orf1-2* ($P_{orf1\text{-}2}$*::lacZ*) respectively, porf3Z15 and porf4Z15, which carry 950 bp and 500 bp

non-translated region upstream of the *cps* operon *orf3-15* ($P_{orf3\text{-}15}$::*lacZ*) respectively, and porf162Z15 ($P_{orf16\text{-}17}$::*lacZ*), which contains the putative promoter of *cps* gene *orf16* and *orf17* were generated (Fig. 9). The *lacZ* fusion plasmids were then transformed respectively into *K. pneumoniae* LacZ16 derived mutants. As shown in Fig. 10, the β-galactosidase activities of porf1Z15 (A), porf2Z15 (B), porf3Z15 (C), porf4Z15 (D) and porf162Z15 (E) were measured. The promoter activities of either $P_{orf1\text{-}2}$ or $P_{orf16\text{-}17}$ in Group I mutants appeared to be reduced, and the decreasing activity in *kvhR*$^-$ was found to be reversed in *kvhA*$^-$*kvhR*$^-$. However, no change of the promoter activity of $P_{orf3\text{-}15}$ in any of the mutants was noted.

Effects of the *rpoS* and *kvhA* mutations on the promoter activity of *kvhAS*. To investigate the regulatory roles of RpoS and KvhA on *kvhAS* expression, three putative promoters of *kvhAS*, pA15, which contains 467 bp non-translated region upstream of *kvhAS*, pF15, which contains 379 bp upstream of *kvhAS*, and pE15, which comprises 180 bp non-translated region upstream of *kvhAS*, were cloned into placZ15 using *lacZ* as a reporter (Fig. 11). The *lacZ* fusion plasmids were then respectively transformed into *K. pneumoniae* LacZ16 and its derived mutants *kvhA*$^-$, *rpoS*$^-$, and *rpoS*$^-$*kvhA*$^-$. As shown in Fig. 12, the promoter activity of pA15 increased about 2-fold in *rpoS*$^-$. However, the pE15 and pF15 promoter activities remained to be similar in any of these strains. The results suggested that the putative regulatory region of *kvhAS*

by RpoS is located -379 to -476 bp upstream of *kvhAS*, which contains also the auto-regulating region of KvhA.

Identification of the signaling molecules for *kvhAS* expression. The β-galactosidase activity of pA15 in wild-type LacZ16 grown in M9 was measured by supplying the culture with either of 25 mM $MgSO_4$, 50 mM $MgSO_4$, 10 mM nacin, 2 mM EDTA, 0.2 mM 2,2-dipyridyl, 0.2 mM paraquat or 0.4 mM paraquat for 1 h at 37°C. As shown in Fig. 13, the promoter activity of *kvhAS* was found to increase by the addition of $MgSO_4$. However, the rest of the reagents showed no apparent effects on *kvhAS* expression.

Effect of the *phoP* mutation on the promoter activity of *kvhAS*. PhoPQ is a two-component system that governs virulence, mediates the adaptation to Mg^{2+}-limiting environments, and regulates numerous cellular activities in several gram-negative species (Groisman, 2001). To investigate the relationship between *kvhAS* and *phoPQ*, pA15, pE15 and pF15 were respectively transformed into *K. pneumoniae* LacZ16 and its derived mutant *phoP*$^-$. As shown in Fig. 14, the promoter activities of pA15 and pE15 decreased in *phoP*$^-$, however, the pF15 promoter activity in *phoP*$^-$ remained the same as that in wild type. The results suggested that the putative regulatory element on *kvhAS* by an unknown activator is located -379 bp to

-476 bp and -1 bp to -180 bp upstream of *kvhAS*. Most likely, some unknown repressor binding region is within -180 bp to -379 bp upstream of *kvhAS*. These regulatory factors are likely under-controlled by PhoP or even cross-talking to each other.

Effect of KvhA overexpression on bacterial resistance activity to antibiotics. In order to investigate the effect of KvhA overexpression on the bacterial drug resistance, pHAm-c, a DNA fragment carrying the entire *kvhA* locus cloned into pETm-c, and pHANm-c, a DNA fragment corresponding to the *kvhA* locus without helix-turn-helix (HTH) DNA-binding motif, which was amplified by AS07/HA01 primer set and cloned into pETm-c, were constructed. The plasmids pETm-c, pHAm-c and pHANm-c were then transformed into *K. pneumoniae* CG43S3 respectively. The strains were exposed to various antimicrobial agents in a disk diffusion assay. The data were summarized in Table 6, and the photographs were shown in Fig. 15. Overexpression of KvhA conferred the bacteria resist more to several antibiotics including cephalothin, piperacillin, ticarcillin, and carbenicillin of β-Lactam group. However, the bacteria with an ovexpression of KvhA appeared to be more susceptible to fosfomycin and sulfonamides (trimethoprim + sulfamethoxazole), which are inhibitors of cell wall synthesis and metabolism respectively. While removing the HTH domain from KvhA, the overexpression effects on the bacterial resistance

activity to antibiotic were abolished.

DISCUSSION

Cross-talk among the 2CSs that a sensor transfers a phosphate group to a 'non-cognate' response regulator within a genome has recently been noted (Verhamme *et al*. 2002). We have previously isolated novel 2CSs *kvhAS*, *kvgAS* and *kvhR* from *K. pneumoniae* CG43. The deduced amino acid sequence of *kvhA* shares 58.5 % and 57.0 % similarity with that of *kvgA* and *kvhR* respectively, suggesting they are paralogous response regulators. Previous EMSA study has also shown a possibility of the presence of a regulatory circuit in-between these homologous two-components. We further demonstrate the relationship of these 2CS paralogs and also the functional role of KvhA in this study.

On the basis of the comparative analysis of the mutant phenotypes, the deletion of either *kvgA* or *kvhR* reduced the bacteria CPS production which leads to the phenotypes of less mucoid and a faster growth rate. We have also shown that both KvgA and KvhR are likely involved in regulating not only the amount but also the form of CPS. Although *kvhA* deletion showed no apparent effect on the production of CPS, the mutation however, rendered a null effect of the *kvhR* mutation. We believe that KvhA interacts with KvhR in some way to suppress the activity of KvhR.

The hydrophilic CPS (K antigen) is involved mainly in resistance to

phagocytosis by polymorphonuclear cells by acting as a physical barrier (Alberti *et al.*, 1996; Alvarez *et al.*, 2000). The assay of anti-phagocytosis activity and virulence determination in a mouse peritonitis model, deletion of either *kvgA* or *kvhR* resulted in an increase of LD_{50}. The results indicated that *kvgA* and *kvhR* may be involved partly in the bacterial pathogenicity. LPS consists of lipid A, core, and O-polysaccharide antigen that are essential for the microorganism to resist complement-mediated killing (Cortes *et al.*, 2002). Only with both *kvhA* and *kvhR* mutations revealed a decrease of resistant activity to human serum. Although the mutants display LPS core and O-antigen, *kvhA*$^-$*kvhR*$^-$ seems to product less or poor O-antigen so that susceptible to human serum.

To investigate further the *kvgA*$^-$, *kvhR*$^-$ mutation effect on the *cps* gene expression, the Group I mutants *kvgA*$^-$, *kvhR*$^-$, *kvhA*$^-$*kvgA*$^-$, *kvgA*$^-$*kvhR*$^-$ and *kvhA*$^-$*kvgA*$^-$*kvhR*$^-$ appeared to reduce the expression of *cps* gene *orf1-2* and *orf16-17*. The putative regulatory region of *orf1-2* in Group I mutants is located between -1 to -400 bp upstream of *orf1-2*, and the putative regulatory region of *orf16-17* by Group I mutants is located between -1 to -450 bp upstream of *orf16-17*. The decreasing *cps* gene expression in *kvhR*$^-$ was found to be reversed in *kvhA*$^-$*kvhR*$^-$. Analysis of the ORF showed that *orf1* is a homolog of *S. typhimurium* LT2 *galF* and *E. coli galU*. While *orf16* and *orf17* correspond respectively to the *manC* and *manB* genes, which encode

mannose-1-phosphate guanylyltransferase (GDP-mannose pyrophosphorylase) and phosphomannomutase respectively. Both enzymes have been reported to be involved in the biosynthesis of mannose (Arakawa *et al.*, 1995). KvgA and KvhR may hence play a role as regulators for mannose synthesis. Notably, the *cps* expression regulated by many factors, such as RcsC, YojN, RcsB, RcsA and RmpA2 has been demonstrated (Lai *et al.*, 2003). We believe that KvgA and KvhR may very likely interact with either Rcs system or RmpA2.

The mutation of the global stress regulator RpoS appeared to enhance the promoter activity of *kvhAS*, suggesting a negative regulation. Although a predicted RpoS binding site is present about -270 bp upstream of *kvhAS*, the RpoS regulatory region determined in Fig. 11 is located between -379 and -476 bp upstream of *kvhAS*, which will be further investigated *in vitro* by EMSA. The expression of *kvhAS* appeared to be positively auto-regulated by KvhA, which was shown to share a common regulatory region with RpoS. Previous study has shown that *kvgAS* was positively regulated by RpoS. It is hence expressions of *kvhAS* and *kvgAS* are working likely differentially to adapt a diverse environment.

Whole-genome transcriptional profiles of *E. coli* had shown that *evgA* is down-regulated by paraquat (Pomposiello *et al*., 2001). Previous data obtained in our

laboratory had also shown that addition of paraquat or 2', 2' dipyridyl to *K. pneumoniae* cells activates the expression of *kvgAS* (Lai *et al*., 2003). However, these reagents conferred no apparent effects on *kvhAS* expression. The expression of *kvhAS* was found in this study to increase by addition of $MgSO_4$. In contrast, *phoPQ* was activated at low Mg^{2+} concentration. In *E. coli*, a novel interaction was demonstrated between the EvgAS and PhoPQ systems (Eguchi, 2004). The genome-wide analyses suggested the presence of a signaling network connecting the Rcs system with the PhoPQ system for a coordinate regulating of the extracellular polysaccharide synthesis in response to the external concentration of divalent cations (Hagiwara, 2003). We therefore proposed that the expression of *kvhAS* was affected by PhoP indirectly through an interaction with some unknown factors.

Overexpression of KvhA conferred the bacteria a significant resistance to β-lactams, indicating that the response regulator-mediated drug resistance is of great potential as the major determinant for the bacterial drug resistance. In *E. coli*, overexpression of 2CSs BaeR, EvgA, RcsB and DcuR have been shown to confer a high-level β-lactam resistance (Hirakawa, 2003). The *cps* genes were induced by a subset of β-lactams but not by agents inhibiting proteins synthesis or DNA replication, indicating that *cps* expression is specific and not due to stresses accompanying cell death or by a general inhibition of peptidoglycan synthesis (Sailer *et al*., 2003). Thus,

the presence of a signaling network built by KvhAS, PhoPQ and Rcs systems is likely responsible to regulate the cell surface structures, by modifying lipopolysaccharide (LPS) and/ or capsular polysaccharide (CPS), in response to the external states. Finally, study on searching for the target genes regulated by KvhAS using cDNA subtraction method is currently underway.

In conclusion, as shown in Fig. 16, we propose a regulatory circuit that RpoS activates the expression of *kvgAS* but represses that of *kvhAS*. Both *kvhAS* and *kvhR* are under-controlled by *kvgAS* and there may be a cross-talk between each other to regulate CPS synthesis and LPS modification. However, the pathway is yet to be identified. In addition, *kvhAS* senses a high-level concentration of Mg^{2+} and in response to affect the drug susceptibility.

REFERENCES

楊淑理，國立清華大學碩士論文，肺炎克雷白氏株特異性基因的鑑定- *kvgASQR* 基因群的序列及表現分析，中華民國八十九年六月。

賴旻初，國立交通大學碩士論文，克雷白氏肺炎桿菌 *kvgAI* 基因之選殖與表現分析，中華民國八十九年八月。

林靖婷，國立交通大學碩士論文，克雷白氏肺炎桿菌 *kvgAS* 雙分子訊息傳遞系統的功能探討，中華民國九十年六月。

黃騰逸，國立交通大學碩士論文，克雷白氏肺炎桿菌 CG43 中 KvhAS 雙分子調控系統的特性分析，中華民國九十一年六月。

賴怡琪，國立清華大學博士論文，克雷白氏肺炎菌 CG43 致病基因相關的搜尋（RmpA2 調控莢膜生合成的機轉），民國九十一年七月。

Alberti S, Alvarez D, Merino S, Casado MT, Vivanco F, Tomas JM, Benedi VJ. Analysis of complement C3 deposition and degradation on *Klebsiella pneumoniae*. Infect Immun. 1996 Nov;64(11):4726-32.

Alvarez D, Merino S, Tomas JM, Benedi VJ, Alberti S. Capsular polysaccharide is a major complement resistance factor in lipopolysaccharide O side chain-deficient

Klebsiella pneumoniae clinical isolates. Infect Immun. 2000 Feb;68(2):953-5.

Arakawa Y, Wacharotayankun R, Nagatsuka T, Ito H, Kato N, Ohta M. Genomic organization of the *Klebsiella pneumoniae cps* region responsible for serotype K2 capsular polysaccharide synthesis in the virulent strain Chedid. J Bacteriol. 1995 Apr;177(7):1788-96.

Blumenkrantz N, Asboe-Hansen G. New method for quantitative determination of uronic acids. Anal Biochem. 1973 Aug;54(2):484-9.

Bock A, Gross R. The BvgAS two-component system of *Bordetella* spp.: a versatile modulator of virulence gene expression. Int J Med Microbiol. 2001 May;291(2):119-30.

Chang C, Stewart RC. The two-component system: Regulation of diverse signaling pathways in prokaryotes and eukaryotes. Plant Physiol. 1998 Jul;117(3):723–31.

Chen MH, Takeda S, Yamada H, Ishii Y, Yamashino T, Mizuno T. Characterization of the RcsC-->YojN-->RcsB phosphorelay signaling pathway involved in capsular synthesis in *Escherichia coli*. Biosci Biotechnol Biochem. 2001 Oct;65(10):2364-7.

Cortes G, Borrell N, de Astorza B, Gomez C, Sauleda J, Alberti S. Molecular analysis

of the contribution of the capsular polysaccharide and the lipopolysaccharide O side chain to the virulence of *Klebsiella pneumoniae* in a murine model of pneumonia. Infect Immun. 2002 May;70(5):2583-90.

De Lorenzo V, Timmis KN. Analysis and construction of stable phenotypes in Gram-negative bacteria with Tn5- and Tn10- derived minitransposons. Methods Enzymol. 1994;235:386-405.

De Wulf P, Kwon O, Lin EC. The CpxRA signal transduction system of *Escherichia coli*: growth-related autoactivation and control of unanticipated target operons. J Bacteriol. 1999 Nov;181(21):6772–8.

Domenico P, Schwartz S, Cunha BA. Reduction of capsular polysaccharide production in *Klebsiella pneumoniae* by sodium salicylate. Infect Immun. 1989 Dec; 57(12):3778-82.

Eguchi Y, Oshima T, Mori H, Aono R, Yamamoto K, Ishihama A, Utsumi R. Transcriptional regulation of drug efflux genes by EvgAS, a two-component system in *Escherichia coli*. Microbiology. 2003 Oct;149(Pt 10):2819–28.

Foussard M, Cabantous S, Pedelacq J, Guillet V, Tranier S, Mourey L, Birck C, Samama J. The molecular puzzle of two-component signaling cascades. Microbes

Infect. 2001 Apr;3(5):417-24.

Groisman EA. The pleiotropic two-component regulatory system PhoP-PhoQ. J Bacteriol. 2001 Mar;183(6):1835-42.

Gupta A, Ampofo K, Rubenstein D, Saiman L. Extended spectrum beta lactamase-producing *Klebsiella pneumoniae* infections: a review of the literature. J Perinatol. 2003 Sep;23(6):439-43.

Hagiwara D, Sugiura M, Oshima T, Mori H, Aiba H, Yamashino T, Mizuno T. Genome-wide analyses revealing a signaling network of the RcsC-YojN-RcsB phosphorelay system in *Escherichia coli*. J Bacteriol. 2003 Oct;185(19):5735-46.

Held TK, Jendrike NR, Rukavina T, Podschun R, Trautmann M. Binding to and opsonophagocytic activity of O-antigen-specific monoclonal antibodies against encapsulated and nonencapsulated *Klebsiella pneumoniae* serotype O1 strains. Infect Immun. 2000 May;68(5):2402-9.

Hengge-Aronis R. Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase. Microbiol Mol Biol Rev. 2002 Sep;66(3):373-95, table of contents.

Hirakawa H, Nishino K, Hirata T, Yamaguchi A. Comprehensive studies of drug resistance mediated by overexpression of response regulators of two-component signal transduction systems in *Escherichia coli*. J Bacteriol. 2003 Mar;185(6):1851-6.

Hirakawa H, Nishino K, Yamada J, Hirata T, Yamaguchi A. Beta-lactam resistance modulated by the overexpression of response regulators of two-component signal transduction systems in *Escherichia coli*. J Antimicrob Chemother. 2003 Oct;52(4):576-82. Epub 2003 Sep 01.

Ibanez-Ruiz M, Robbe-Saule V, Hermant D, Labrude S, Norel F. Identification of RpoS (σ^S)-regulated genes in *Salmonella enterica* Serovar typhimurium. J Bacteriol. 2000 Oct;182(20):5749-56.

Joseph S, Russell DW. Molecular Cloning: a laboratory manual—3rd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 2001.

Kabha K, Nissimov L, Athamna A, Keisari Y, Parolis H, Parolis LA, Grue RM, Schlepper-Schafer J, Ezekowitz AR, Ohman DE, et al. Relationships among capsular structure, phagocytosis, and mouse virulence in *Klebsiella pneumoniae*. Infect Immun. 1995 Mar;63(3):847-52.

Kalka-Moll WM, Wang Y, Comstock LE, Gonzalez SE, Tzianabos AO, Kasper DL.

Immunochemical and biological characterization of three capsular polysaccharides from a single *Bacteroides fragilis* strain. Infect Immun. 2001 Apr;69(4):2339-44.

Karimova G., Bellalous J, Ullmann A. Phosphorylation-dependent binding of BvgA to the upstream region of the *cyaA* gene of *Bordetella pertussis*. Mol Microbiol. 1996 May;20(3):489-96.

Karlyshev AV, Wren BW. Detection and initial characterization of novel capsular polysaccharide among diverse *Campylobacter jejuni* strains using alcian blue dye. J Clin Microbiol. 2001 Jan;39(1):279-84.

Konig J, Bock A, Perraud AL, Fuchs TM, Beier D, Gross R. Regulatory factors of *Bordetella pertussis* affecting virulence gene expression. J Mol Microbiol Biotechnol. 2002 May;4(3):197-203.

Lai YC, Lin GT, Yang SL, Chang HY, Peng HL. Identification and characterization of KvgAS, a two-component system in *Klebsiella pneumoniae* CG43. FEMS Microbiol Lett. 2003 Jan 21;218(1):121-6.

Lai YC, Peng HL, Chang HY. RmpA2, an activator of capsule biosynthesis in *Klebsiella pneumoniae* CG43, regulates K2 cps gene expression at the transcriptional level. J Bacteriol. 2003 Feb;185(3):788-800.

Lai YC, Yang SL, Peng HL, Chang HY. Identification of genes present specifically in a virulence strain of *Klebsiella pneumoniae*. Infect Immun. 2000 Dec;68(12): 7149-51.

Locht C. Molecular aspects of *Bordetella pertussis* pathogenesis. Int Microbiol. 1999 Sep;2(3):137-44.

Loewen PC, Hu B, Strutinsky J, Sparling R. Regulation in the rpoS regulon of *Escherichia coli*. Can J Microbiol. 1998 Aug;44(8):707-17.

Matsushita M, Janda KD. Histidine kinases as targets for new antimicrobial agents. Bioorg Med Chem. 2002 Apr;10(4):855–67.

Miller J H. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 1972.

Mizuno T. His-Asp phosphotransfer signal transduction. J Biochem (Tokyo). 1998 Apr;123(4)555-63.

Nishino K, Yamaguchi A. EvgA of the two-component signal transduction system modulates production of the YhiUV multidrug transporter in *Escherichia coli.* J Bacteriol. 2002 Apr184(8):2319–23.

Nishino K, Yamaguchi A. Overexpression of the response regulator *evgA* of the two-component signal transduction system modulates multidrug resistance conferred by multidrug resistance transporters. J Bacteriol. 2001 Feb;183(4):1455–8.

Ohki R, Giyanto, Tateno K, Masuyama W, Moriya S, Kobayashi K, Ogasawara N. The BceRS two-component regulatory system induces expression of the bacitracin transporter, BceAB, in *Bacillus subtilis*. Mol Microbiol. 2003 Aug;49(4):1135-44.

Perez-Perez GI, Shepherd VL, Morrow JD, Blaser MJ. Activation of human THP-1 cells and rat bone marrow-derived macrophages by *Helicobacter pylori* lipopolysaccharide. Infect Immun. 1995 Apr;63(4):1183-7.

Perraud AL, Kimmel B, Weiss V, Gross R. Specificity of the BvgAS and EvgAS phosphorelay is mediated by the C-terminal HPt domains of the sensor proteins. Mol Microbiol. 1998 Mar; 27(5):875-87.

Perraud AL, Rippe K, Bantscheff M, Glocker M, Lucassen M, Jung K, Sebald W, Weiss V, Gross R. Dimerization of signalling modules of the EvgAS and BvgAS phosphorelay systems. Biochim Biophys Acta. 2000 May 23;1478(2):341-54.

Podschun R, Ullmann U. *Klebsiella spp.* as nosocomial pathogens: epidemiology, taxonomy, typing methods and pathogenicity factors. Clin Microbiol Rev. 1998 Oct;

11(4):589-603.

Pomposiello PJ, Bennik MH, Demple B. Genome-wide transcriptional profiling of the *Escherichia coli* responses to superoxide stress and sodium salicylate. J Bacteriol. 2001 Jul;183(13):3890-902.

Reed LJ, Muench H. A sample method of estimating the fifty percent endpoints. Am. J. Hyg. 1938, 27:493-497.

Sailer FC, Meberg BM, Young KD. Beta-Lactam induction of colanic acid gene expression in *Escherichia coli*. FEMS Microbiol Lett. 2003 Sep 26;226(2):245-9.

Scarlato V, Arico B, Prugnola A, Rappuoli R. Sequential activation and environmental regulation of virulence genes in *Bordetella pertussis*. EMBO J. 1991 Dec;10(12):3971-5.

Schneider B, Stubs D, Gross R. Identification and genomic organization of gene loci negatively controlled by the virulence regulatory BvgAS two-component system in *Bordetella bronchiseptica*. Mol Genet Genomics. 2002 Jun;267(4):526–35.

Skorupski K, Taylor RK. Positive selection vectors for allelic exchange. Gene. 1996 Feb 22;169(1):47-52.

Stock AM, Robinson VL, Goudreau PN. Two-component signal transduction. Annu Rev Biochem. 2000;69:183-215.

Sugiura M, Aiba H, Mizuno T. Identification and classification of two-component systems that affect *rpos* expression in *Escherichia coli*. Biosci Biotechnol Biochem. 2003 Jul;67(7):1612-5.

Taylor RK, Manoil C, Mekalanos JJ. Broad-host-range vectors for delivery of TnphoA: use in genetic analysis of secreted virulence determinants of *Vibrio cholerae*. J. Bacteriol. 1989 Apr;171(4):1870-8.

Utsumi R, Katayama S, Ikeda M, Igaki S, Nakagawa H, Miwa A, Taniguchi M, Noda M. Cloning and sequence analysis of the evgAS genes involved in signal transduction of *Escherichia coli* K-12. Nucleic Acids Symp Ser. 1992;(27):149-50.

Venturi V. Control of *rpoS* transcription in *Escherichia coli* and *Pseudomonas* : why so different? Mol Microbiol. 2003 Jun;49(1):1–9.

Verhamme DT, Arents JC, Postma PW, Crielaard W, Hellingwerf KJ. Investigation of in vivo cross-talk between key two-component systems of *Escherichia coli*. Microbiology. 2002 Jan;148(Pt 1):69-78.

Whitfield C, Paiment A. Biosynthesis and assembly of Group 1 capsular polysaccharides in *Escherichia coli* and related extracellular polysaccharides in other bacteria. Carbohydr Res. 2003 Nov 14;338(23):2491-502.

Whitfield C, Roberts IS. Structure, assembly and regulation of expression of capsules in *Escherichia coli*. Mol Microbiol. 1999 Mar;31(5):1307-19.