中文摘要:

關鍵詞：克雷白氏肺炎桿菌；訊號傳遞基因組；*kvgAS* ; *kvhR*；*kvhAS*；電泳膠遲滯實驗；LacZ 報導系統; RpoS; 2D-PAGE

克雷白氏肺炎桿菌 (*Klebsiella pnenmoniae*) 是常見的伺機性病原菌，本實驗室在具高毒性的 CG43 菌株內得到兩套特有的訊號傳遞基因組- *kvgASR* 以及 *kvhAS*。由序列分析結果發現， KvgS 和 KvhS 可能是負責接受外來訊息的感應蛋白，KvgA、KvhA 和 KvgR 的相似度很高，可能是扮演訊息傳遞系統中調控蛋白的角色。三年來, 我們實驗室由反轉錄聚合酶連鎖反應 (RT-PCR) 和南方墨點法 (southern blot) 證實 *kvgAS* 及 *kvhAS* 與其他細菌中負責訊息傳遞系統相同，具有基因組 (operon) 的結構，為了探討這些訊號傳遞基因組之間的關係，我們建構了 LacZ 報導系統，並且也構築了一系列基因缺損株，這些包含 *lacZ*⁻ (Z01) 、*kvgA*⁻ (A18)、 *kvhA*⁻ (Ah01) 、 *kvhR*⁻ (R16) ，以及由 *lacZ* 基因缺損株所衍生出來的一系列基因突變株 *kvgA*⁻ (AZ18), *kvgS*⁻ (S01), *kvgR*⁻ (RZ16), *kvhA*⁻ (AhZ01) 和 *kvhS*⁻ (HS01) 和壓力蛋白 RpoS 突變株 *rpoS*⁻ (RpoS01)。經由電泳膠遲滯實驗以及 LacZ 報導系統證明 KvgA 轉錄因子可以正向自我調控，並且可以活化 KvhAS 和 KvhR 的表現。利用 LacZ 報導基因活性偵測發現 Paraquat 以及 EDTA 可以增強 KvgAS 以及 KvhR 啟動子的活性表現，因此推測 KvgAS 以及 KvhR 可能具有調控抗氧化及抗壓力的功能，而以 LacZ 報導系顯示 RpoS 為 KvgAS 啟動子的活化子，卻同時為 KvhAS 的抑制子。過去一年來的結果整理如下:

- 確認 RpoS 在 *kvgAS* 以及 *kvhAS* 啟動子上的調控區域。
- KvgAS 以及 KvhAS 可以調控在 RpoS 調控基因組中的與抗壓力有關的 *katG* 以及 *sodC* 基因表現。
- 在克雷白氏肺炎桿菌中大量表現 KvhA 會使細菌對 fosfomycin 以及 sulfonamides 抗生素的敏感度增加。
- 為了研究這些訊號傳遞基因組之間的調控關係，構築了一系列由 *lacZ* 基因突變株所衍生的雙重或三重突變株 *kvhA*⁻*kvgA*⁻, *kvhA*⁻*kvhR*⁻, *kvgA*⁻*kvhR*⁻ 以及 *kvhA*⁻*kvgA*⁻*kvhR*⁻。
- 比較這些突變株之間的生長速率以及型態上的差異可區分為兩個群組:
  - 群組 1 是帶有 *kvgA*⁻ 或者 *kvhR*⁻ 基因缺損的突變株，這些突變株與野生株的細菌比較起來具有較少的黏性以及較快的生長速度，並且在利用小鼠腹膜炎的毒性測試分析，這些突變株的 LD50 皆上升。
  - 在此群組中也可發現莢膜多醣體的含量會減少，而分析莢膜多糖體基因的啟動子 $P_{orf1\text{-}2}$ 以及 $P_{orf16\text{-}17}$，可以發現兩者在這些突變株的表現量都降低。
  - 群組 2 是帶有 *kvhA*⁻的單一突變株以及 *kvhA*⁻*kvhR*⁻的雙重突變株，這兩個突變株與野生株具有相似的表現型。

- 另外我們也利用 LacZ 為報導基因以及蛋白質二維電泳膠分離鑑定了可受 *kvgAS* 以及 *kvhAS* 調控的下游基因。

綜合三年的研究成果，我們發現 KvgA, KvhA 以及 KvhR 之間有相互調節關係，這些訊號傳遞基因組不僅負責調控莢膜多醣體生合成基因組的表現，其中 KvhA 在細菌細胞壁合成的調節上也扮演重要的角色。

ABSTRACT

Key words: *Klebsiella pneumoniae*; two-component system (2CS); *kvgAS*; *kvhR*; *kvhAS*; Electrophoretic mobility shift assays; LacZ reporter system; RpoS; 2D-PAGE

Klebsiella pneumoniae is a common opportunistic pathogen. The gene clusters, *kvgAS*, *kvhR* and *kvhAS,* have previously been isolated from a highly virulent strain *K. pneumoniae* CG43 and identified as the members of two-component systems (2CS). According to sequence analysis, KvgS and KvhS are sensory histidine kinases which allow bacteria to sense and respond to the changes in their environment. KvgA, KvhA and KvhR are responsible for regulating the expression of the signal induced genes. In the past three years, both *kvgAS* and *kvhAS* have been demonstrated respectively having an operon structure by RT-PCR and southern blot analysis. A LacZ reporter system was constructed and the *K. pneumoniae* CG43 *lacZ*$^-$ derived mutants, *kvgA*$^-$ (AZ18), *kvgS*$^-$ (S01), *kvgR*$^-$ (RZ16), *kvhA*$^-$ (AhZ01) and *kvhS*$^-$ (HS01) were also obtained. Both electrophoretic mobility shift assay (EMSA) and LacZ reporter system analysis have shown that the KvgA acts as a positively regulator for not only its own promoter expression, but also the *kvhR* and *kvhAS* expression. Paraquat and EDTA affected both *kvgAS* and *kvhR* promoters by increasing the LacZ activities which implied that the 2CS is responsible for an anti-oxidation and/or anti-stress regulation. The global stress regulator- RpoS mutant was finally constructed. We have found that RpoS acts as an activator for *kvgAS* expression, but as a repressor for the expression of *kvhAS*. We summarized the results accomplished in the past year as following:

- Identification of the RpoS regulatory elements contained within P_{kvgA} and P_{kvhA}.
- Expressions of the stress resistance genes *katG* and *sodC* in RpoS regulon were under control by KvgA and KvhA.
- Over-expression of KvhA appeared to affect the bacteria susceptibilities to fosfomycin and sulfonamides, which are inhibitors of cell wall synthesis and metabolism, respectively.
- A series of *K. pneumoniae* Z01 derived mutants including *kvhA*$^-$*kvgA*$^-$, *kvhA*$^-$*kvhR*$^-$, *kvgA*$^-$*kvhR*$^-$ and *kvhA*$^-$*kvgA*$^-$*kvhR*$^-$ were constructed.
- Comparative analysis of the growth and phenotype of the mutants allowed us to classify the mutants into two groups:
  - Group I carrying either *kvgA* or *kvhR* mutation displayed a less mucoid and a faster growth rate by comparing to the parental strain Z01. In a mouse peritonitis model, the deletion of either *kvgA* or *kvhR* resulted in an increase of LD_{50}.
  - Decreasing amount of uronic acid, the composition of capsule

polysaccharide (CPS), was found in the Group I mutants. The activity of the CPS promoter, either $P_{orf1\text{-}2}$ or $P_{orf16\text{-}17}$, also appeared to be reduced in the Group I mutants.

- Group II includes *kvhA*$^-$ and *kvhA*$^-$*kvhR*$^-$ mutants, which exert a similar phenotype and growth rate with that of the parental strain.

- We have isolated several likely target genes that are regulated by *kvgAS* and *kvhAS* using a promoter trap and 2D-PAGE technology.

It is concluded that the regulatory interaction is present in-between the three paralogs 2CS KvgAS, KvhAS and KvhR. The identity and involvement of these genes in the regulatory network are being investigated. These 2CS are not only involved in regulation of the CPS biosynthesis in *K. pneumoniae* CG43, but also responsible for regulating the bacterial susceptibility of the antibiotics, the inhibitors of cell wall synthesis and metabolism.

INTRODUCTION

Klebsiella pneumoniae is a common nosocomial pathogen, which causes suppurative lesions, septicemia, urinary and respiratory tract infection in immunocompromised patients (8, 11, 15). The wide spread of extended spectrum β–lactamase producing *K. pneumoniae* (ESBLKp) has prompted the search for new drugs to intervene the bacterial infections (40). Recently, development of an antimicrobial approach using two-component systems (2CS) as a drug target to block the signalling pathway has been evaluated (23).

During infection, bacterial 2CS, each consisting of a sensor histidine kinase and a response regulator, acts to recognize specific signals and convert this information into specific transcriptional or behavioral responses. 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 (24, 26, 33, 35, 39). The number of 2CS varies dramatically in bacteria genomes. For example, *Bacillus subtilis* genome appears to encode 70 2CS proteins (13, 29), whereas only 11 and 9 2CS proteins, respectively were found in *Helicobacter pylori* and *Hemophilus influenzae* (26). The large number of sensors and response regulators are believed to organize as a signal transduction network in order for the bacteria to respond to complex environmental stimuli.

The presence of a cross talk and regulatory circuit in between the bacterial 2CS has been increasingly identified. In *E. coli*, CheA histidine kinase phosphorylates either of the two response regulators, CheY and CheB in the reaction of chemotaxis (14). The sporulation control of *B. subtilis* is a multi-component phosphorelay system that Spo0F receives a phosphoryl group from either KinA or KinB and subsequently transfers it to the HPt domain of Spo0B, which then phosphorylates the terminal response regulator Spo0A (17). In *Salmonalla*, the transcription of regulator PmrA can be induced by another regulator PhoQ under a low magnesium condition for the polymyxin resistance (41).

We have previously isolated a novel 2CS operon *kvgAS*, which was not found in the genome of *K. pneumoniae* MGH78578 (http://genome.wustl.edu/gsc/), from *K. pneumoniae* CG43, a clinical isolate with a high virulence to laboratory mice, using a DNA subtractive hybridization technique (1, 21). An additional 2CS-encoding operon that exhibits a high sequence similarity with *kvgAS* was also identified in the bacteria. The operon was subsequently isolated and designated *kvhAS* as *kvgAS* homolog (2, 4,

5). On the basis of sequence analysis, KvgS and KvhS are sensory histidine kinases. KvgA and KvhA are responsible for regulating the expression of the signal induced genes. Functional motif analysis revealed that KvgAS and KvhAS are both unorthodox type 2CS, of which the sensor contains a transmitter, a receiver, and an Hpt domain to carry out a cascade transfer of the phosphate group (31). The two 2CS gene clusters, *kvgAS* and *kvhAS,* display a significant homology with *Bordetella pertussis bvgAS* and *Escherichia coli evgAS*. In contrast to *kvhAS*, which was found in 100 *K. pneumoniae* blood isolates collected in the laboratory, the *kvgAS* was only identified in 15% of the strains by dot blot analysis (1, 2, 21).

BvgAS locus governs the expression of several virulence factors including filmentous hemagglutinin (*fha*), pertussis toxin (*ptx*), and adenylate cyclase toxin/hemolysin (*cya*) of this organism (8, 16, 18, 34, 37). Recent studies showed that *evgAS* can regulate the transcription of a putative efflux pump, *emrKY*, and the over-expression of EvgA conferred the bacteria a multidrug resistance phenotype (19, 27, 36). Sequence comparison to those of *bvgAS* and *evgAS* suggested that the orthologous *kvgAS* and *kvhAS* might play a similar role and their expression subject to a similar modulation.

We have shown previously that both *kvgAS* and *kvhAS* are organized as an operon using RT-PCR and southern blot analysis (3, 21). We have shown also that addition of paraquat or 2',2' dipyridyl into *K. pneumoniae* cells activated the expression of *kvgAS* (3, 21). It is hence predicated that KvgAS is a stress-responsive 2CS, but the role of KvhAS has yet to be identified. Furthermore, the $KvgA_t$ could actually and specifically bind to the putative promoters of *kvhAS* and *kvhR*, a response regulator that located at downstream of *kvgAS,* by using electrophoretic mobility shift assay suggesting the presence of a cross-talk among *kvgAS, kvhR* and *kvhAS*.

To gain further a understanding of the functional roles of the 2CSs, 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 established to identify the signals that sensed by *kvgAS* and *kvhR* and the regulatory circuits of *kvgAS, kvhR* and *kvhAS.* Moreover, we have also employed the *lacZ*-based promoter trapping system and a proteomic approach to identify the target sequences under control by the 2CSs. It is hence include that we draw an interacting map of the 2CS paralogs KvgAS, KvhR and KvhAS in the bacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. The *E. coli* strains, *K. pneumoniae* CG43S3 and its derivatives were routinely cultured at 37°C in Luria-Bertani (LB) medium or M9 minimal medium supplemented with appropriate antibiotics.

Construction of the LacZ reporter system. In order to assess each of the promoter activity, placZ15, a promoter-trap vector with LacZ as the reporter and CG43S3-Z01, *K. pneumoniae* CG43S3 with a *lacZ* gene deletion, were constructed (Table 1). Briefly, 1 kb DNA fragments flanking the *lacZ* gene were PCR amplified using specific primer sets (lac05-lac06 and lac03-lac07) as listed in Table 2. The generated DNA fragments were ligated and subcloned into a suicide vector pKAS46 (a generous gift of Dr. Skorupski, University of New Hampshire). The resulting plasmid placZ16 was transformed into *E. coli* S17-1λ*pir* and then mobilized to the streptomycin-resistant strain *K. pneumoniae* CG43S3 by conjugation. A kanamycin resistant transconjugant was initially picked and propagated in 2 ml LB overnight, and a small aliquot of the culture was plated on LB agar containing 500 μg/ml streptomycin. A total of 50 streptomycin resistant colonies were further ascertained for their susceptibility to kanamycin. The *lacZ* mutation was confirmed by plating the bacteria onto a X-gal containing medium and by Southern hybridization (data not shown), and the mutant was designated as *K. pneumoniae* CG43S3-Z01. The promoterless *lacZ* gene was also amplified from *K. pneumoniae* CG43S3 with the primers lac01 and lac02 (Table 2), and then inserted into the promoter-trap vector pYC016 (19). The resulting reporter plasmid was designated as placZ15.

Construction of the gene-deletion mutants. *K. pneumoniae* CG43S3-Z01 derived mutants with deletion specific at either of *kvhA*, *kvgA*, and *kvhR* genes were constructed by allelic exchange strategy. The pKAS46 derived plasmids containing a deletion in the DNA fragments of *kvgA*, *kvhA* and *kvhR* were constructed as described above. The resulting plasmids were transformed into *E. coli* S17-1 *λpir* and then delivered from the bacteria into *K. pneumoniae* CG43S3-Z01 by conjugation. The resulting *kvgA*, *kvhA* and *kvhR* mutants in *K. pneumoniae* CG43S3-Z01 were designated as AZ18, AhZ01 and RZ01 respectively. To identify the interaction role among KvgA, KvhA and KvhR, the double mutants and triple mutants were also constructed. The pKAS46 derivative containing a deletion in *kvgA* constructed previously was delivered from *E. coli* S17-1 *λpir* into either *kvhA* or *kvhR* mutants respectively by conjugation. The plasmid carrying a deletion of *kvhA* was also

transformed into *E. coli* S17-1 *λpir* and then mobilized to *kvhR* mutant by conjugation. However, the triple mutant was also constructed as described above, the pKAS46 derivative containing a deletion in *kvgA* constructed previously was delivered from *E. coli* S17-1 *λpir* into *kvhA*$^-$*kvhR*$^-$ double mutant 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. The transconjugants were selected on appropriate medium and the mutations were all confirmed by PCR and Southern analysis. In addition, the resulting double mutants in were designated as A2h01 (*kvgA*$^-$ *kvhA*$^-$), A2R01 (*kvgA*$^-$ *kvhR*$^-$) and AhR01 (*kvhA*$^-$ *kvhR*$^-$) respectively. The triple deletion mutant was A2hR01 (*kvgA*$^-$ *kvhA*$^-$*kvhR*$^-$).

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: cephalothin 30 μg; fosfomycin 50 μg; piperacillin 100 μg; carbenicillin 100 μg; sulfamethoxazole 23.75 μg + trimethoprim 1.25 μg.

Extraction and quantification of CPS. CPS was extracted by the method as described previously (12). 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 centrifugation 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.

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 as described (30).

Promoter activity assay. The putative promoter regions of *kvgAS*, *kvhAS*, *kvhR*, *katG, soxR, sodC*, *oxyR*, *orf1-2, orf3-15* and *orf16-17* were PCR amplified from *K. pneumoniae* CG43S3 by the designed primer sets (Table 2) and subcloned into placZ15 to fuse with the *lacZ* reporter gene. One-tenth of the overnight culture of the bacteria carrying each of the plasmids with the respective promoter were refreshed and grown in M9 medium to an OD_{600} about 0.6 to 0.7. The β-galactosidase activity assay was carried out essentially as the method of Miller (25). The assays were performed in triplicate and repeated at least three times.

Preparation of the recombinant $KvgA_t$ and KvhA. The coding region of *kvgA* and *kvhA* 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 pkvgA1 and pHP4004, respectively. The plasmid pkvgA1 was digested with *Cla*I to remove the receiver domain of KvgA and then the DNA binding domain, $KvgA_t$, was subcloned into the *Sal*I-*Not*I sites of pET30c, which resulted in the expression plasmid pkvgA4. The plasmid pHP4004 was digested with *Bam*HI and the entire *kvhA* fragment subcloned into pET30c, and resulted in pHP4005. The expression plasmids pkvgA4 and pHP4005 were then transformed into *E. coli* BL21-RIL respectively. The transformant carrying either pkvgA4 or pHP4005 was cultured in LB medium to log phase, and expression of either the recombinant $KvgA_t$ or KvhA protein was induced with 1 mM IPTG for 3 h at 37°C. The overexpressed His-$KvgA_t$ protein formed an inclusion body and the His-KvhA appeared to be in soluble form. The bacteria carrying pkvgA4 were lysed by sonication and the pellet was resuspended and denatured with 6 N urea. After purification by affinity chromatography with His-Bind resin (Novagen), the denatured protein was finally refolded through dialysis against a gradient of decreasing concentrations of urea in the reaction buffer (20 mM Tris-HCl, pH 8.0, 4 mM $MgCl_4$, 50 mM KCl, 1 mM $CaCl_2$ and 1 mM dithiothreitol). The bacteria carrying pHP4005 were purified from the soluble fraction by affinity chromatography and dialyzed against the reaction buffer. Subsequently, the His-$KvgA_t$ and His-KvhA were concentrated with PEG20,000 and the concentration determined by Bradford assay (9). Finally, the molecular weight of the proteins and the purity of protein were analyzed by SDS-polyacrylamide gel electrophoresis.

Electrophoretic mobility shift assay (EMSA). DNA fragments comprising a serial of the promoter regions were obtained by PCR amplification with respective primer sets a09-a14 (pA16), a09-a15 (pA28), a09-a17 (pA26), a09-a18 (pA23), A201-A203 (pAh01), AP01-AP02 (pAh02), AP02-AP04 (pAh03), K01-K02 (pKatG02), D01-D02 (pSodC02), X01-X02 (pSox02), and O01-O02 (pOxyR02) and digested by either *Bam*HI or *Bgl*II, and then labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The purified His-$KvgA_t$ or His KvhA was incubated with the radioactively labeled DNA in a 20 μl solution containing 20 mM Tris-HCl (pH 8.0), 4 mM $MgCl_4$, 50 mM KCl, 1 mM $CaCl_2$ and 1 mM dithiothreitol at 37°C for 20 min. The samples were then loaded onto a running gel of 5% nondenaturing polyacrylamide in 0.5X TBE (45 mM Tris-HCl, pH 8.0, 45 mM boric acid, 1 mM EDTA). Gels were electrophoresed with a 20-mA current at 4°C and detected by either autoradiograph or by InstantImager™ (Packard Instrument Company).

KatG (HPI) activity measurement. One-tenth of the overnight culture of the bacteria were refreshed and grown in LB medium to an OD_{600} about 0.6 to 0.7. The crude extract (approximately 2 to 5 mg of total protein/ml) of the bacteria was washed and resuspended in 50 mM potassium phosphate buffer, pH 7.0. The diluted hydrogen peroxide (0.5 ml of 34 mM H_2O_2 freshly diluted in 50 mM potassium phosphate buffer, pH 7.0) was then added, and the absorbance of the samples at 240 nm was measured every 15 s for 1 min. The specific activity of catalase (micromoles of H_2O_2 decomposed per minute per milligram of total protein) was then calculated (38). After measuring the total catalase activity, the crude extracts were heated in a 55°C water bath for 15 min, and then the assay was repeated. This was done to quantify the activity of the heat stable HPII. The HPI activity was estimated by subtracting the HPII activity.

Superoxide dimutase (SOD) activity assay. Each of the extracted proteins (200 μg/lane) was separated by native PAGE and the gel soaked in 100 ml of solution A (25 mg NBT and 10 mg riboflavin in 100 ml distilled water) for 25 min. The solution A was then replaced by solution B (1% TEMED in distilled water), and the gel was placed in a shaker for 30 min. After incubation in solution B, the gel was illuminated for 20 min or until white bands appeared and then the gel was fixed in solution C (30% methanol and 2% glycerol) for 1 h. The three SOD types were distinguished by their different sensitivity to inhibitors (7). The inhibitors, 2 mM KCN or 5 mM H_2O_2, was applied to the gel for 30 min prior to incubation in solution A.

RESULTS AND DISCUSSIONS

Summary of the previously reports. In the previously stusy, we have demonstrated that the operon structures for both *kvgAS* and *kvhAS* were demonstrated by RT-PCR and southern blot analysis (3, 21). Both KvgA and KvhA are capable of specifically binding their own promoters (3, 4). The $KvgA_t$ could actually and specifically bind to the putative promoters of *kvhAS* and *kvgR* strongly suggesting the presence of a cross-talk between *kvgAS*, *kvhR* and *kvhAS* (3). The KvgAS system played a role in countering free radical stresses and sensing iron-limiting conditions.

A LacZ reporter system including placZ15, a *lacZ* reporter in pYC016, and Z01, a *K. pneumoniae* CG43 *lacZ* deletion mutant, was constructed to analysis the transcriptional regulation of the 2CSs. A series mutants of the *kvgA*, *kvhR* and *kvhA* also were constructed to assess each of the regulator mutation effect on the promoter activity of the 2CSs. Both electrophoretic mobility shift analysis and promoter activity assay using LacZ as the reporter revealed that the KvgA acts as a positive autoregulator and also an activator for the expression of *kvhAS.* Deletion of the global stress regulator RpoS was found to reduce *kvgAS* expression, which suggested that RpoS is an activator for *kvgAS* expression. In contrast, RpoS appeared to play a negatively regulatory role on expression of *kvhAS* in the bacteria.

Identification of the RpoS regulatory regions of P_{kvgA} and P_{kvhA}. To identify the RpoS regulatory region, the β-galactosidase activities of *K. pneumoniae* CG43S3-RpoS01 carrying each of the plasmids pA23, pA26 and pA28 were measured. The activity of *K. pneumoniae* CG43S3-RpoS01 [pA23] was similar to that of *K. pneumoniae* CG43S3-Z01 [pA23], while the activities of *K. pneumoniae* CG43S3-RpoS01 carrying either pA26 or pA28 increased slightly comparing with that of *K. pneumoniae* CG43S3-RpoS01 [pA16] (Fig. 1A). The RpoS regulatory region of P_{kvhAS} was also defined by measuring the activities of pAh02 and pAh03 in Z01 and RpoS01. Both pAh02 and pAh03 activities in *K. pneumoniae* CG43S3-Z01 showed a diminished level compared to that of pAh01. Although an σ^S binding sequence 5'-CGATAT-3' contained within the DNA fragment of pAh02 was noted, no apparent change of the pAh02 activity was observed in RpoS01 (Fig. 1B).

Regulatory roles of KvgA, KvhR and KvhA on expression of the stress related genes. The promoter activities of the four genes *katG*, *sodC*, *soxR* and *oxyR* that are responsible for the adaptive response while the bacteria are subject to attack by either hydrogen peroxide or superoxide anions, were assayed in wild type, *kvgA* and *kvhA* mutants. Both *kvgA* and *kvhA* deletions appeared to affect the promoter activities of

pKatG02 and pSodC02, which revealed lower activities in both *kvgA* and *kvhA* mutant. (Fig. 2A and B). No apparent change of the β-galactosidase activities of pSox02 and pOxyR02 was found in either of the bacteria (Fig. 2C and D). To identify if either *kvgA* or *kvhA* deletion affects expressions of the *katG* and *sodC* at the translational level, the activities of HPI and CuZnSOD were also measured. No apparent changes of the activities of HPI and CuZnSOD were found in either of the bacteria. The interaction maps of KvgAS, KvhAS and RpoS have demonstrated in this study and prepared to submit. The β-galactosidase activities of pYfiD02, pSodC02 and pSox02 in *K. pneumoniae* CG43S3-RZ16 appeared to be lower than that of *K. pneumoniae* CG43S3-Z01 (Fig. 3).

Effect of KvhA overexpression on bacterial resistance activity to antibiotics. In order to investigate the effect of *K. pneumoniae* CG43-S3 [pHAm-c] on the bacterial drug resistance, the strains including *K. pneumoniae* CG43-S3, *K. pneumoniae* CG43-S3 [pHAm-c], and Ah01 were exposed to various antimicrobial agents in a disk diffusion assay. The data were summarized in Table 3, and the photographs were shown in Fig. 4. Overexpression of KvhA conferred the bacteria resist more to several antibiotics including cephalothin, piperacillin, 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. Besides of these, the effects on bacterial resistance activity to antibiotics were abolished.

Characterization of the mutants derived from *K. pneumoniae* CG43S3-Z01. In order to investigate further the regulatory interactions of the 2CSs, a series of *K. pneumoniae* CG43S3-Z01 derived double and triple mutants of *kvhA, kvgA* and *kvhR* were further constructed as described above, which included A2h01 (*kvgA*$^-$ *kvhA*$^-$), A2R01 (*kvgA*$^-$ *kvhR*$^-$), AhR01 (*kvhA*$^-$ *kvhR*$^-$) and A2hR01 (*kvgA*$^-$ *kvhA*$^-$*kvhR*$^-$). Comparative analysis of the growth and sedimentation rates of the mutants, while the tested strains were cultured in LB broth at 37°C and subjected to centrifugation at 4,000 rpm for 3 min, 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 Z01. The group II includes *kvhA*$^-$ and *kvhA*$^-$*kvhR*$^-$ mutants, which exert similar growth rate and phenotype with that of the parental strain Z01 (Fig. 5A and B). 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. As shown in Table 4, Group I strains synthesized much less K2

CPS than the parental Z01 strain. A slight reduction of CPS and colony mucoidy also appeared in *kvhA*$^{-}$*kvhR*$^{-}$, which belongs to Group II. In a mouse peritonitis model, virulence of the mutants were also compared. Either *kvgA* or *kvhR* deletion in bacteria resulted in an increase of the LD_{50}. The *kvhA* mutation, however, had no apparent effect. The results indicated that *kvgA* and *kvhR* may be involved partly in determining the bacterial virulence to BALB/c mice, and both KvgA and KvhR were likely the regulators to control biosynthesis of the CPS. Although *kvhA* deletion showed no apparent effect on the amount of CPS, the mutation, however, rendered a null effect of the *kvhR* mutation. We believed that KvhA interacts with KvhR in some way to suppress the activity of KvhR.

Regulatory roles of KvgA, KvhA and KvhR on expression of the *cps* genes in *K. pneumoniae* CG43. In *E. coli* K-12, the amount of colanic acid produced has been reported to be correlated with the transcriptional level of *cps* genes (6). We reasoned that the production of CPS affected by *kvgA*, *kvhA* and *kvhR* might be resulted from differential *cps* genes expression in *K. pneumoniae* CG43. Thus, three *lacZ* reporter fusion constructs, pOrf12, which comprised 800-bp non-translated region upstream of *cps* operon contains the *orf1* and *orf2* ($P_{orf1\text{-}2}$*::lacZ*), pOrf315, carrying 950-bp non-translated region upstream of the *orf3* to *orf15* ($P_{orf3\text{-}15}$*::lacZ*), and pOrf1617 ($P_{orf16\text{-}17}$*::lacZ*), which contains the putative promoter of *cps* gene *orf16* and *orf17* were generated (Fig. 6A). The *lacZ* fusion plasmids were then transformed respectively into *K. pneumoniae* CG43S3-Z01 derived mutants and the β-galactosidase activities of pOrf12, pOrf315 and pOrf1617 were measured. As shown in Fig. 6B, the activity of either $P_{orf1\text{-}2}$ or $P_{orf16\text{-}17}$ in the group I mutants appeared to be reduced. The decreasing activity in *kvhR*$^{-}$ was not found in *kvhA*$^{-}$*kvhR*$^{-}$. No change of the promoter activity of $P_{orf3\text{-}15}$ in any of the mutants was noted. In contrast, the β-galactosidase activity of pOrf1617 measured in either *kvhA*$^{-}$ or *kvhA*$^{-}$ *kvhR*$^{-}$ mutant reavealed a slightly higher activity in comparison with that of *K. pneumoniae* CG43S3-Z01. Analysis of the ORFs showed that *orf1* encodes UDP-glucose-1-phosphate uridylyltransferase, which is a homolog of *E. coli galU*. The enzyme catalyzes the synthesis of UDP-glucose for LPS biosynthesis. While *orf16* and *orf17* correspond respectively to the *manC* and *manB* genes in *E. coli*, which encode mannose-1-phosphate guanylyltransferase (GDP-mannose pyrophosphorylase) and phosphomannomutase (6). Both enzymes have been reported to be involved in the biosynthesis of mannose. KvgA and KvhR may hence play a regulatory role for UDP-glucose and mannose synthesis.

Identification of the target genes of the KvgAS two-component system in

***Klebsiella pneumoniae* CG43 using a promoter trapping system.** 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 *kvgA*$^{-}$ mutant (AZ18), which can be determined by plating the bacteria on LB agar containing X-Gal for blue/white selection (Fig. 7). While the plasmid conferred Z01 a blue phenotype, but AZ18 a white phenotype implying that the promoter in the plasmid 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 sequence determined (Table. 5). Three of these clones carry respectively the promoter regions of *iucABCDiutA* operon, encoding an aerobatin iron-acquisition system, and *murB*, the gene encoding UDP-N-acetylenolpyruvylglucosamine reductase in peptidoglycan biosynthesis. Northern blot analysis and ampicillin susceptibility assay revealed that KvgA deletion increased slightly the promoter activity of *iucABCDiutA* and the ampicillin susceptibility, which support the selected results of the *lacZ*-based promoter trapping system (Fig. 8 and Fig. 9).

Proteomic analysis of KvgAS, KvhR and KvhAS. Using 2D-PAGE (pH 3 to 10) analysis, the protein profiles of the *kvgA, kvhR* and *kvhA* mutant strains were obtained (Fig. 10). The majority of the proteins were clustered between pH 4 to 7 and their molecular weights are within 30 to 100 kDa as shown. As marked in Fig. 10, the protein spots appeared to be induced or repressed were isolated from the gel. After trypsin digestion, the proteins were analyzed through the MALDI/TOF analysis. The peptide profiles through Mascot program of ExPASY (http://www.matrixscience.com/search_form_select.html) then subject to database searching. The results were shown in Table 6.

Notably, the *cps* expression appeared to be regulated by many factors, such as RcsC, YojN, RcsB, RcsA and RmpA2 (21). We believe that KvgA and KvhR may very likely interact with either Rcs system or RmpA2 to regulate the *cps* synthesis. In conclusion, we propose a regulatory circuit that *kvhAS* and the paralogs 2CS components appeared to coordinate in some way to regulate CPS synthesis in bacteria.

In conclusion, the response regulator KvgA exerted a positive regulatory role on both expressions of *kvgAS* and *kvhAS*. Interestingly, the global regulator RpoS in responding to stress, appeared to be an activator for expression of *kvgAS*, but a repressor for *kvhAS* expression. Finally, we have shown that KvgA and KvhA were able to regulate the expression of the stress related genes, *katG* and *sodC*, in the RpoS regulon. It is hence concluded that KvgAS and KvhAS are parts of the RpoS regulon

in *K. pneumoniae* CG43. We propose a regulatory circuit that *kvhAS* and the paralogs 2CS components appeared to coordinate in some way to regulate CPS synthesis in bacteria. KvhAS is also involvement with regulating the antibiotic suscepbility in bacteria. Comparative analysis of the *K. pneumoniae* wild type strain, the *kvgA* mutant and the *kvhA* mutant using a promoter trapping system and two-dimensional gel electrophoresis of the proteome have carried out towards understanding of the 2CS functions.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Descriptions	Reference or source
Strains		
K. pneumoniae		
CG43S3	CG43 Sm^r	(20)
CG43S3-Z01	CG43S3 Δ*lacZ*	This study
CG43S3-AZ18	CG43S3-Z01 Δ*kvgA*	This study
CG43S3-AhZ01	CG43S3-Z01 Δ*kvhA*	This study
CG43S3-RZ01	CG43S3-Z01 Δ*kvhR*	This study
CG43S3-A2h01	CG43S3-Z01 Δ*kvgA* Δ*kvhA*	This study
CG43S3-A2R01	CG43S3-Z01 Δ*kvgA* Δ*kvhR*	This study
CG43S3-AhR01	CG43S3-Z01 Δ*kvhA* Δ*kvhR*	This study
CG43S3-A2hR01	CG43S3-Z01 Δ*kvgA* Δ*kvhA* Δ*kvhR*	This study
E. coli		
JM109	*RecA1 supE44 endA1 hsdR17 gyrA96 rolA1 thi* Δ *(lac-proAB)*	Laboratory stock
BL21-RIL	F^- *ompT* $hsdS_B(r_B-m_B-)$*gal dcm* (DE3)	Laboratory stock
S17-1 λ *pir*	*hsdR recA pro* RP4-2 [Tc::Mu; Km::Tn*7*] (λ*pir*)	(32)
Plasmids		
pKAS46	Positive selection suicide vector, *rpsL* Ap^r Km^r	(40)
pET30c	His-tagging protein expression vector, Km^r	Novagen
placZ15	A derivative of pYC016, containing *K. pneumoniae* CG43 *lacZ* as a reporter, Cm^r	This study
pR14	pKAS46 carring a Δ*kvhR* fragment	This study
pkvgA4	Deletion of the receiver domain of *kvgA* gene digested by *Cla*I, and cloned into pET30c, Km^r	This study
pHP4004	A fragment of *K. pneumoniae* CG43 *kvhA* gene generated by PCR, and cloned into pUC-T, Ap^r	This study
pHP4005	A fragment of *K. pneumoniae* CG43 *kvhA* gene digested by *Bam*HI, and cloned into pET30c, Km^r	This study
pA16	420-bp *Bam*HI fragment containing the putative *kvgAS* promoter, cloned into placZ15	This study
pA23	195-bp *Bam*HI/*Bgl*II fragment containing the putative *kvgAS* promoter, cloned into *Bam*HI site of placZ15	This study
pA26	318-bp *Bam*HI/*Bgl*II fragment containing the putative *kvgAS* promoter, cloned into *Bam*HI site of placZ15	This study
pA28	360-bp *Bam*HI/*Bgl*II fragment containing the putative *kvgAS* promoter, cloned into *Bam*HI site of placZ15	This study
pAh01	500-bp *Bam*HI fragment containing the putative *kvhAS* promoter, cloned into placZ15	This study
pAh02	347-bp *Bam*HI/*Bgl*II fragment containing the putative *kvhAS* promoter, cloned into *Bam*HI site of placZ15	This study
pAh03	180-bp *Bam*HI/*Bgl*II fragment containing the putative *kvhAS* promoter, cloned into *Bam*HI site of placZ15	This study
pKatG02	430-bp *Bam*HI/*Bgl*II fragment containing the putative *katG* promoter, cloned into *Bam*HI site of placZ15	This study
pSox02	180-bp *Bam*HI/*Bgl*II fragment containing the putative *soxR* promoter, cloned into *Bam*HI site of placZ15	This study
pSodC02	160-bp *Bam*HI/*Bgl*II fragment containing the putative *sodC* promoter, cloned into *Bam*HI site of placZ15	This study
pOxyR02	400-bp *Bam*HI fragment containing the putative *oxyR* promoter, cloned into placZ15	This study

pRP05	500-bp *Bam*HI fragment containing the putative *kvhR* promoter, cloned into placZ15	This study
pA415	A 1.3 kb *Eco*RI fragment containing *kvhA* locus with the putative promoter cloned into pRK415	This study
pRC01	A 1.1 kb *Eco*RI fragment containing *kvhR* locus with the putative promoter cloned into pACYC184	This study
pOrf12	800-bp *Bam*HI fragment containing the putative *orf1-2* promoter, cloned into placZ15	This study
pOrf315	950-bp *Bam*HI fragment containing the putative *orf3-15* promoter, cloned into placZ15	This study
pOrf1617	400-bp *Bam*HI fragment containing the putative *orf16-17* promoter, cloned into placZ15	This study

TABLE 2. Primer used in this study

Primer no.	Sequence	Complementary position
lac01	5’-GCGAACGACAAGATCTGACTTA-3’	-24 relative to the *lacZ* start codon
lac02	5’-ATTATGCCGTTCTAGAGGCG-3’	+103 relative to the *lacZ* stop codon
lac03	5’-TGAAACGCAAGGATCCGAGC-3’	+1444 of the *lacZ* coding region
lac05	5’-CAGGTGGAGGAGCTCGAAAG-3’	-907 relative to the *lacZ* start codon
lac06	5’-AAACGGGATCCGCTGGCA-3’	+117 of the *lacZ* coding region
lac07	5’-GCAGTGCGCCTCTAGATCGT-3’	+2498 of the *lacZ* coding region
a02	5'-CAATATCATAGCCAGCA-3'	+45 relative to the *kvgA* stop codon
a03	5'-ATTGCTTCACTCACCCT-3'	-32 relative to the *kvgA* start codon
a08	5’-GAGAGCTCGATTATTTCATCGA-3’	-834 relative to the *kvgA* start codon
a09	5’-CATATTGTGGATCCTGCTGTTC-3’	+22 of the *kvgA* coding region
a10	5’-CGATGCGGGATCCAATGCCTTTA-3’	+296 of the *kvgA* coding region
a11	5’-AACAAGATCTAGCTTTTGAT-3’	+699 relative to the *kvgA* stop codon
a14	5’-ATTTTCAGGATCCACCACCTT-3’	-409 relative to the *kvgA* start codon
a15	5'-TGCGTTGGATCCGTGATTAG-3'	-204 relative to the *kvgA* start codon
a17	5'-GGTAACTAACGGATCCACTC-3'	-320 relative to the *kvgA* start codon
a18	5'-CAC,AAT,AAC,TGG,ATC,CGA,AT-3'	-371 relative to the *kvgA* start codon
AS02	5'-CAGCCATGCTTTCTCCTT-3'	+156 relative to the *kvhA* stop codon
AS07	5'-ATCAGGATCCACGCCCCC-3'	-18 relative to the *kvhA* start codon
AS04	5’-ATCTGCAGAATATCCCGT-3’	+1532 of the *kvhS* coding region
AS12	5’-TCCTGCAATGCTGGAATT-3’	-1245 relative to the *kvhA* start codon
AS16	5’-GCCCGGGTTATTTTTATC-3’	-52 relative to the *kvhA* start codon
AS23	5’-CATGGCGGTTCGTCTTAT-3’	-1 relative to the *kvhS* start codon
A201	5’-GTGAAAAAGCTTCGTTCA-3’	-516 relative to the *kvhA* start codon
A203	5’-CAACGACAGCTCTTCCAA-3’	+69 of the *kvhA* coding region
AP01	5’-GAACGCCGGATCCTACAGC-3’	-188 relative to the *kvhA* start codon
AP02	5’-GCTGTCGAGATCTGCCGC-3’	+98 of the *kvhA* coding region
AP04	5’-CATCAGATGGATCCAAACCC-3’	-355 relative to the *kvhA* start codon
K01	5’-CGGATCCATTGTTGGATG-3’	+36 of the *katG* coding region
K02	5’-CACGCTGATAGATCTGTATTC-3’	-422 relative to the *katG* start codon
D01	5’-GCGAGGGATAAGATCTCG-3’	+34 of the *sodC* coding region
D02	5’-CAGCAGTGGATCCGCATC-3’	-121 relative to the *sodC* start codon
X01	5’-GTCAACAGCATTTGGATCCG-3’	+40 of the *soxR* coding region
X02	5’-CGAGATCTTCATCAATCCATT-3’	-136 relative to the *soxR* start codon
O01	5’-CTACCAGGTATTCAAGATCTC-3’	+31 of the *oxyR* coding region
O02	5’-GTATCGGATCCTGTCGCTGC-3’	-371 relative to the *soxR* start codon

Table 3. Effect of *kvhA* overexpression and the deletion mutant in *K.pneumoniae* CG43S3 on the antibiotics resistance.

Antibiotics (μg/disk)	Zone (mm)[a] / *K.pneumoniae* CG43S3		
	Wild type	overexpression mutant of *kvhA*	deletion mutant of *kvhA*
fosfomycin 50	22	31	21
Trimethoprim 1.25 + sulfamethoxazloe 23.75	23	26	24
Cephalothin 30	21	13	20
Piperacillin 100	25	16	26
Carbenicillin 100	14	7	14

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

TABLE 4. Characterizations of *K. pneumoniae* CG43S3-Z01 derived mutants.

Strains	CPS amounts (Mean quantity ± SD[a])	Mucoid phenotype[b]	LD_{50}(CFU)
Z01 (*lacZ*$^-$)	22.8 ± 3.8	++	3×10^3
AZ18 (*kvgA*$^-$)	15.7 ± 0.3	-	5×10^4 ~5×10^5
AhZ01 (*kvhA*$^-$)	24.4 ± 2.4	++	3×10^3
RZ01 (*kvhR*$^-$)	13.6 ± 1.5	-	$>3\times10^5$
A2h01 (*kvgA*$^-$*kvhA*$^-$)	11.9 ± 2.2	-	$>4\times10^5$
A2R01 (*kvgA*$^-$*kvhR*$^-$)	12.9 ± 0.8	-	$>4\times10^5$
AhR01 (*kvhA*$^-$*kvhR*$^-$)	17.6 ± 0.9	+	$>3\times10^5$
A2hR01(*kvgA*$^-$*kvhA*$^-$ *kvhA*$^-$)	16.1 ± 0.3	-	$>4\times10^5$

[a] Values are the averages of triplicate samples and are given as micrograms of uronic acid per 10^9 CFU.

[b] Assessed by string formation test after 48 h grown on LB medium. Symbols: －, negative; ＋, positive; ＋＋, strong.

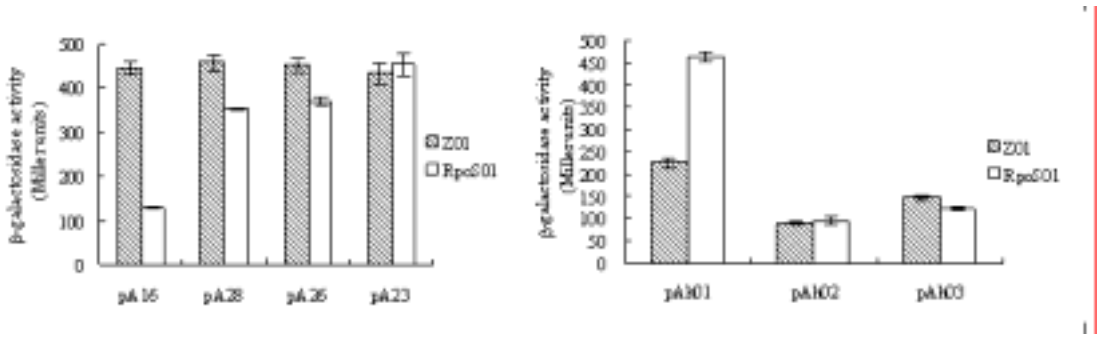


Fig. 1 **The *rpoS* deletion affected both expressions of *kvgAS* and *kvhAS*.** The promoter activities of *kvgAS* and *kvhAS* were determined by β-galactosidase activity assay. The plasmids carrying (A) P_{kvgAS} promoter regions, pA16, pA28, pA26 and pA23, and (B) P_{kvhAS} promoter regions, pAh01, pAh02 and pAh03, were transferred into Z01 and RpoS01 respectively by conjugation. The cells were grown in M9 medium to an OD_{600} of 0.7, and the β-galactosidase activity was determined as previously described. The values represent the average of three independent experiments.

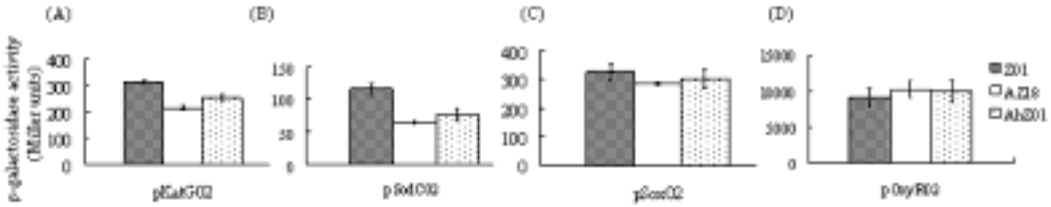
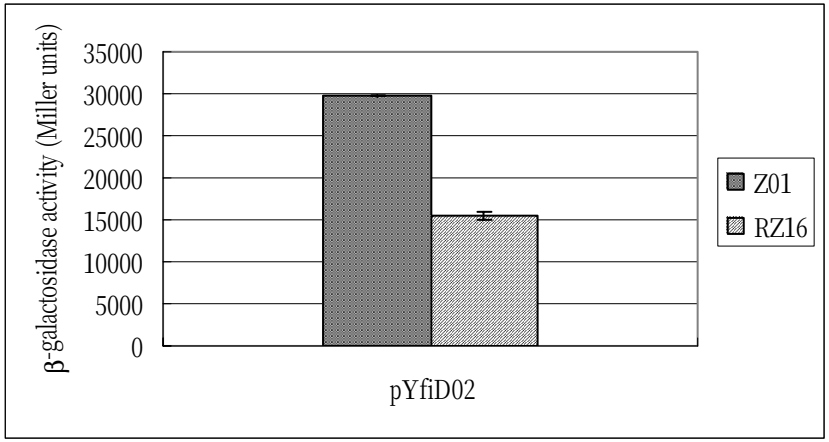
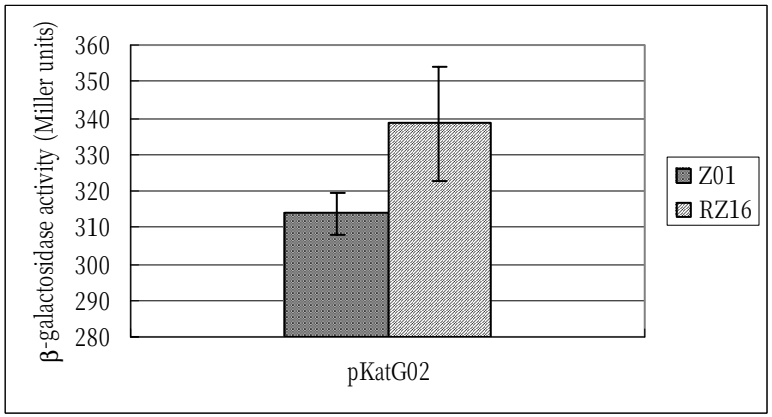


Fig. 2 Deletion effect of either *kvgA* or *kvhA* on expressions of the stress related genes. The promoter activities of the stress related genes, *katG, sodC, soxR* and *oxyR*, were determined by β-galactosidase activity assay. The plasmids carrying each of the promoters, pKatG02 (A), pSodC02 (B), pSox02 (C) and pOxyR02 (D), were transferred into Z01, AZ18 and AhZ01 respectively by conjugation. The cells were grown in M9 medium to an OD_{600} of 0.7, and the β-galactosidase activity was determined as previously described. The values represent the average of three independent experiments.

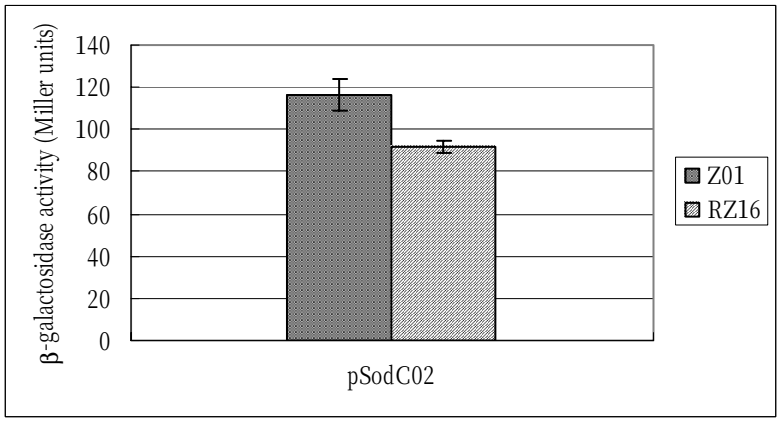
(A)


β-galactosidase activity (Miller units)
35000
30000
25000
20000
15000
10000
5000
0
pYfiD02
Z01
RZ16

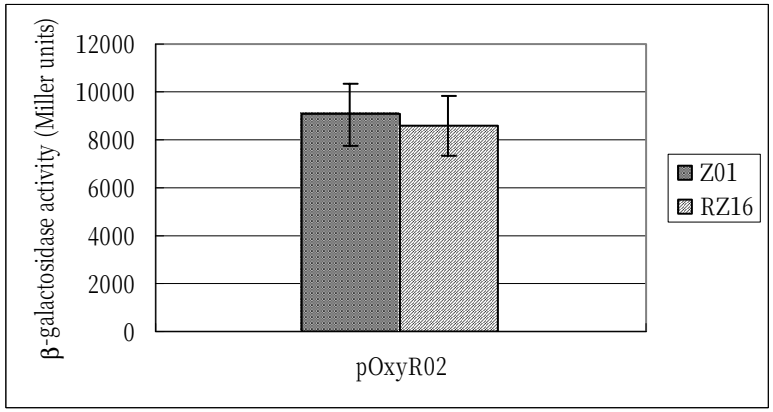
(B)


β-galactosidase activity (Miller units)
360
350
340
330
320
310
300
290
280
pKatG02
Z01
RZ16

(C)


β-galactosidase activity (Miller units)
140
120
100
80
60
40
20
0
pSodC02
Z01
RZ16

(D)


β-galactosidase activity (Miller units)
12000
10000
8000
6000
4000
2000
0
pOxyR02
Z01
RZ16

(E)

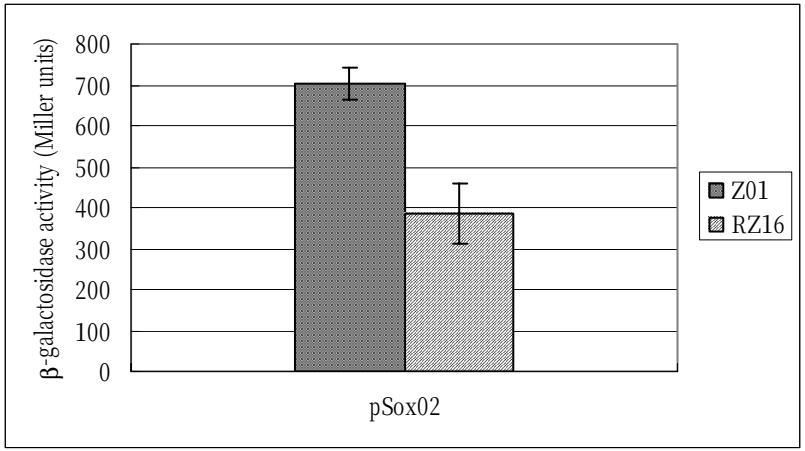


Fig. 3. The promoter activities of *yfiD, katG, sodC, oxyR* and *soxRS*. The promoter activities of the *yfiD*, *katG sodC, oxyR* and *soxRS* were detected by β-galactosidase activity assay. Plasmids pYfiD02 (A), pKatG02 (B), pSodC02 (C), pOxyR02 (D) and pSox02 have been transferred into Z01 and RZ16 respectively by conjugation. The cells were growth in M9 medium for OD600 about 0.7, and the level of β-galactosidase activity was determined as previously described. The values are the averages of three independent experiments.

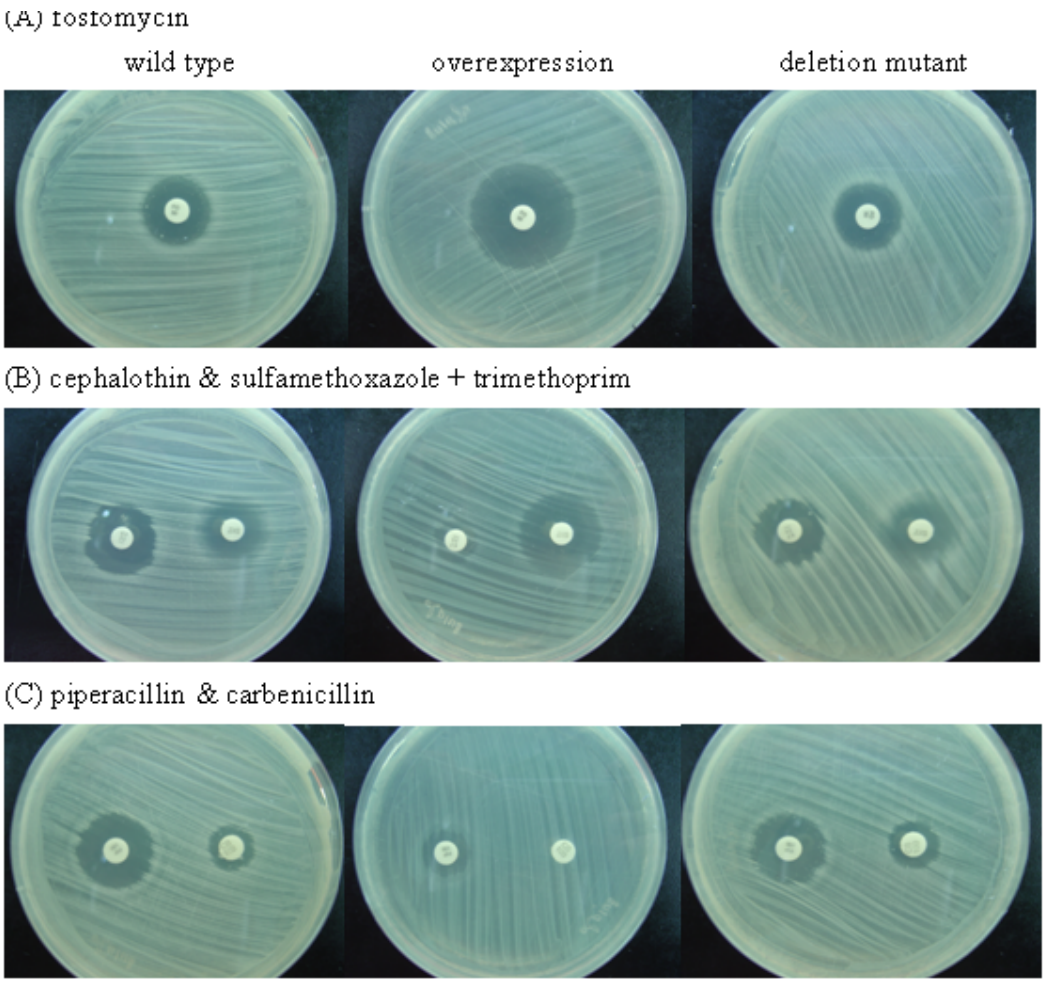
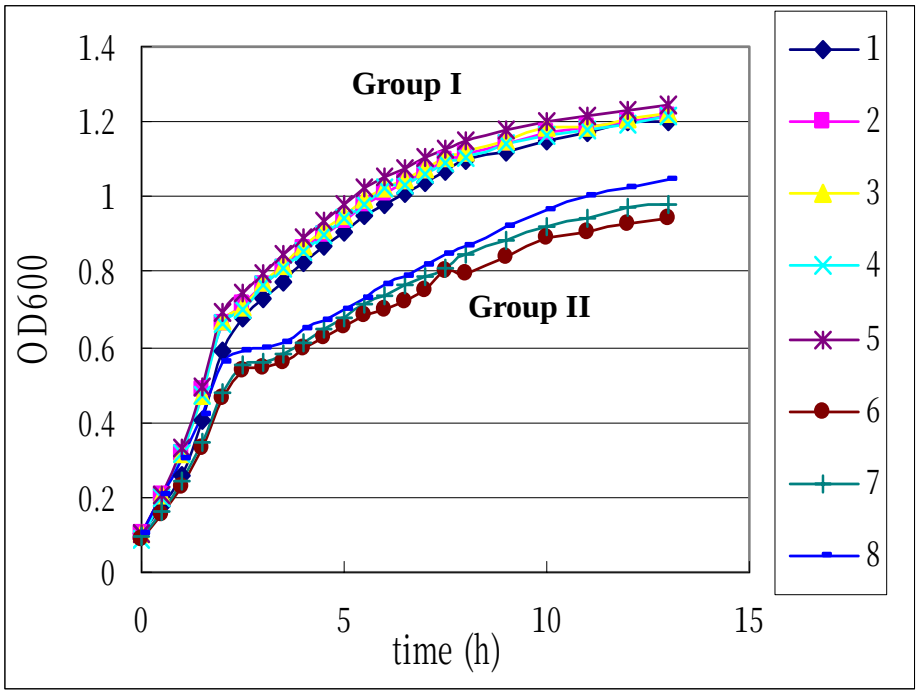


Fig. 4. Phenotypes of antibiotics-resistant as determined in *Klebsiella pneumoniae.*

(A) The fosfomycin disk (50 μg each) is at the center; (B) cephalothin (left) and sulfamethoxazole + trimethoprim (right); (C) piperacillin (left) and carbenicillin (right). The plates at left column were agarose culture exposed to wild type *K.pneumoniae* CG43. The plates in the middle column were exposed to *K.pneumoniae* CG43 [pHAm-c]. The plates at right column were exposed to Ah01.

A. Growth curve



B. Sedimentation test

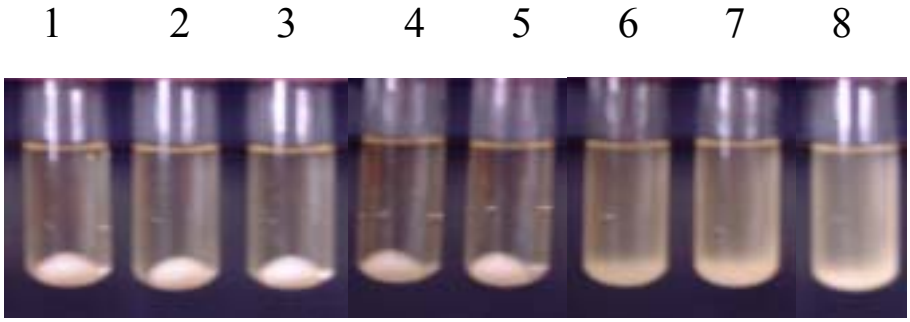


Fig. 5. The growth curve (A) and sedimentation test (B) of a series of mutants derived from Z01. The strains tested were cultured in LB broth at 37℃ and subjected to centrifugation at 4,000 rpm (1,500 *g*) for 3 min. According to collective analysis of both assays, two groups can be identified, in which the Group I includes 1: *kvgA*$^-$, 2: *kvhR*$^-$, 3: *kvhA*$^-$*kvgA*$^-$, 4: *kvgA*$^-$*kvhR*$^-$ and 5: *kvhA*$^-$*kvgA*$^-$*kvhR*$^-$, and Group II contains 6: LacZ16, 7: *kvhA*$^-$ and 8: *kvhA*$^-$*kvhR*$^-$.

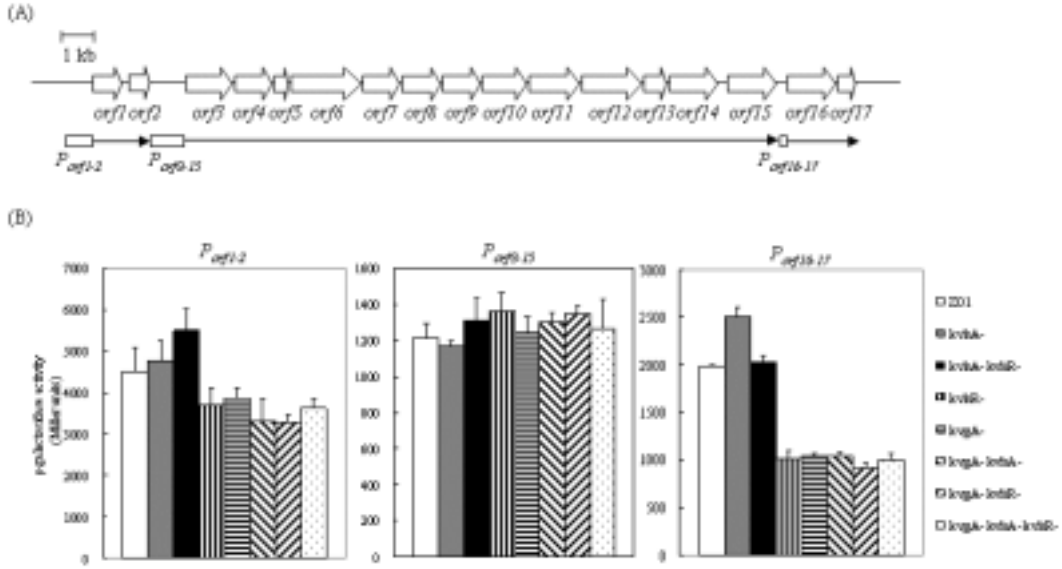
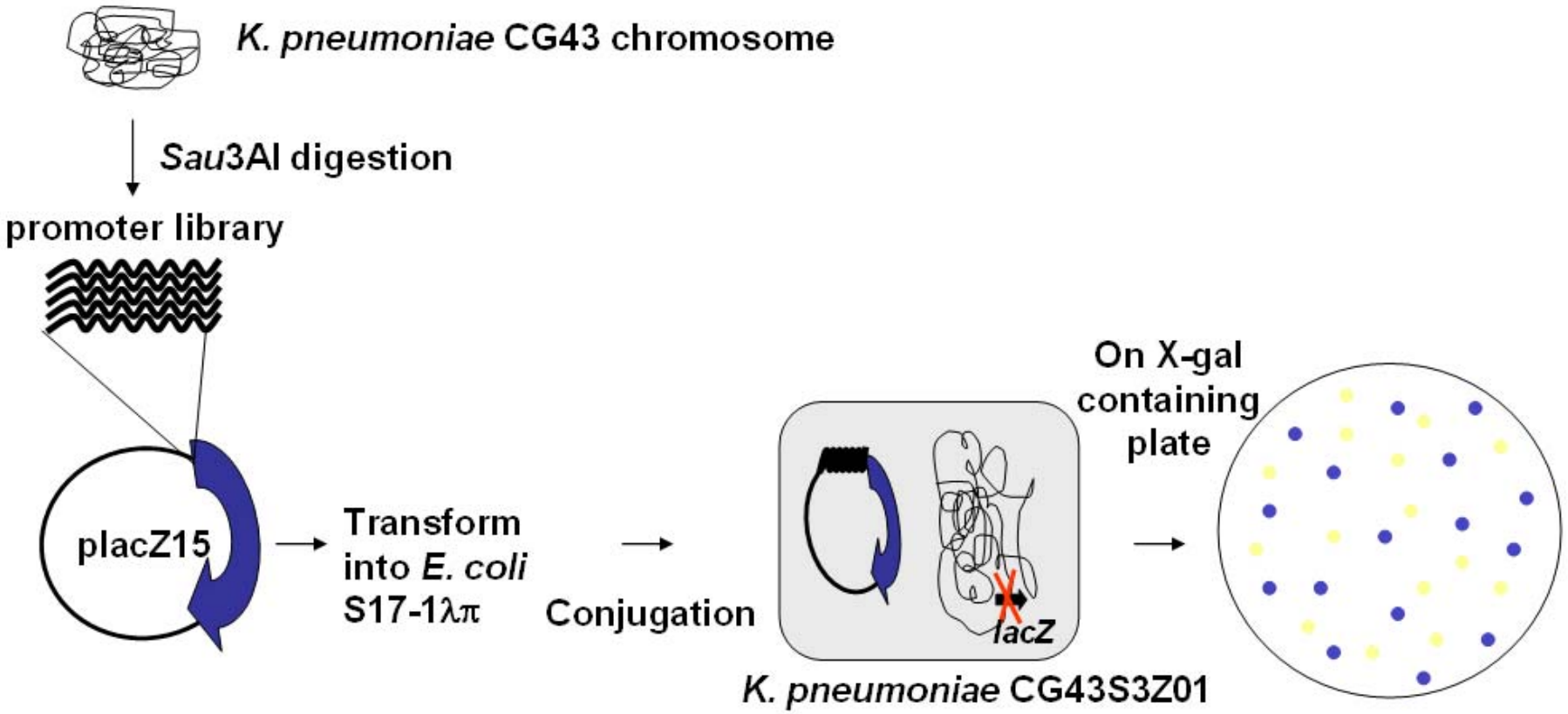


Fig. 6. The effects of *kvhA*, *kvgA* and *kvhR* mutations on the putative promoters of *K. pneumoniae* K2 *cps* genes. (A) The putative promoter regions of *K. pneumoniae* K2 *cps* gene cluster were cloned into placZ15 as *lacZ* transcriptional fusions. The primers used for PCR amplification and the extents of subclones used in this study are indicated. pOrf12 ($P_{orf1\text{-}2}$*::lacZ*) comprises the region controlling the expression of *orf1* and *orf2*, pOrf315 ($P_{orf3\text{-}15}$*::lacZ*) carries the putative promoter region responsibles for initiating transcription of the operon containing *orf3* to *orf15*, and pOrf1617 ($P_{orf16\text{-}17}$*::lacZ*) contains the putative promoter region of *orf16* and *orf17*. (B) The β-galactosidase activities of pOrf12, pOrf315, pOrf1617 were measured in the mutants. The cells were grown in M9 medium to an OD600 of 0.7, and the β-galactosidase activity was determined as previously described. The values represent the average of three independent experiments.


K. pneumoniae CG43 chromosome
Sau3AI digestion
promoter library
placZ15
Transform
into E. coli
S17-1λπ
Conjugation
lacZ
K. pneumoniae CG43S3Z01
On X-gal
containing
plate

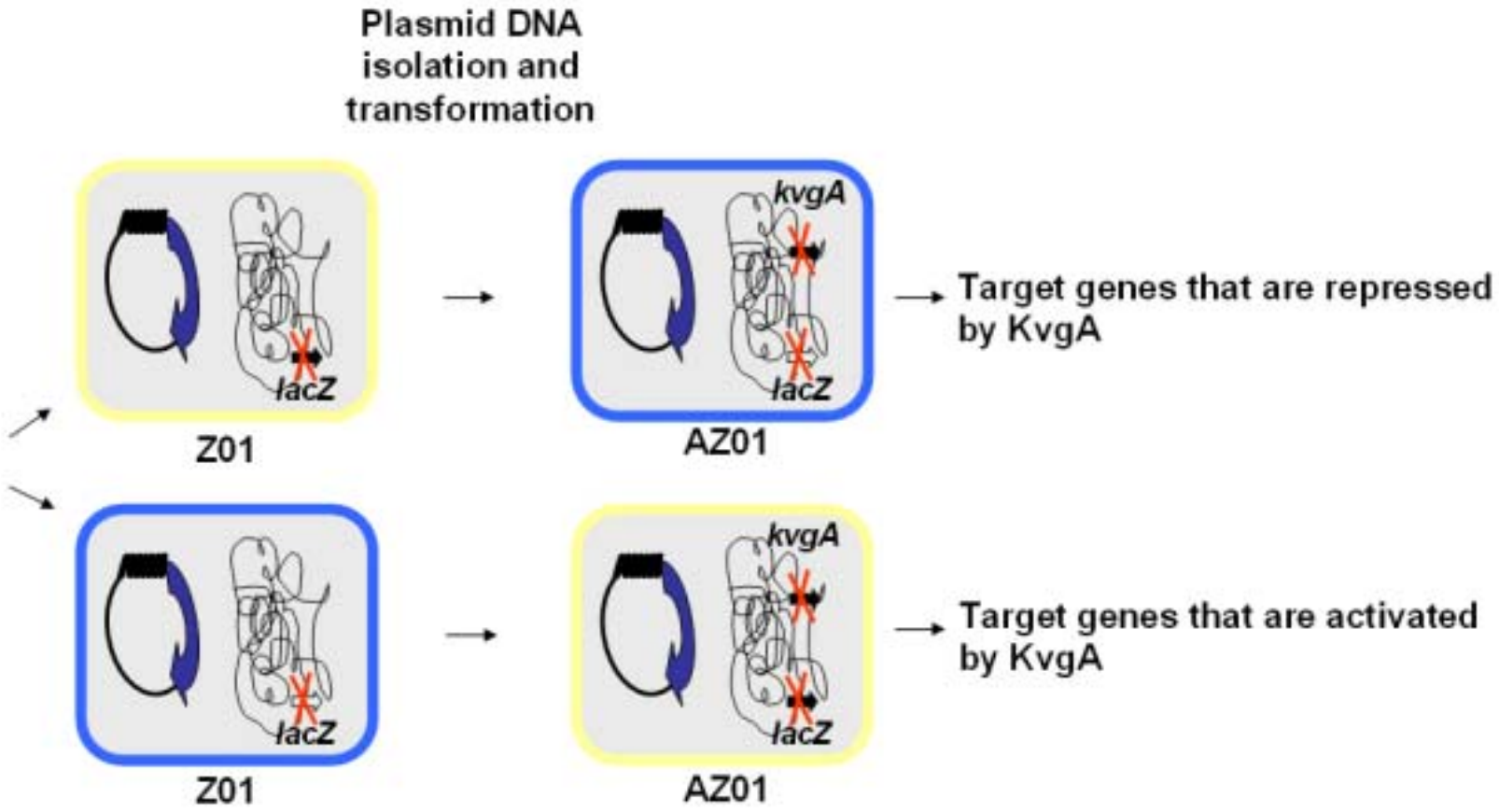


Fig. 7. Selection strategy of the *lacZ*-based promoter trapping system.

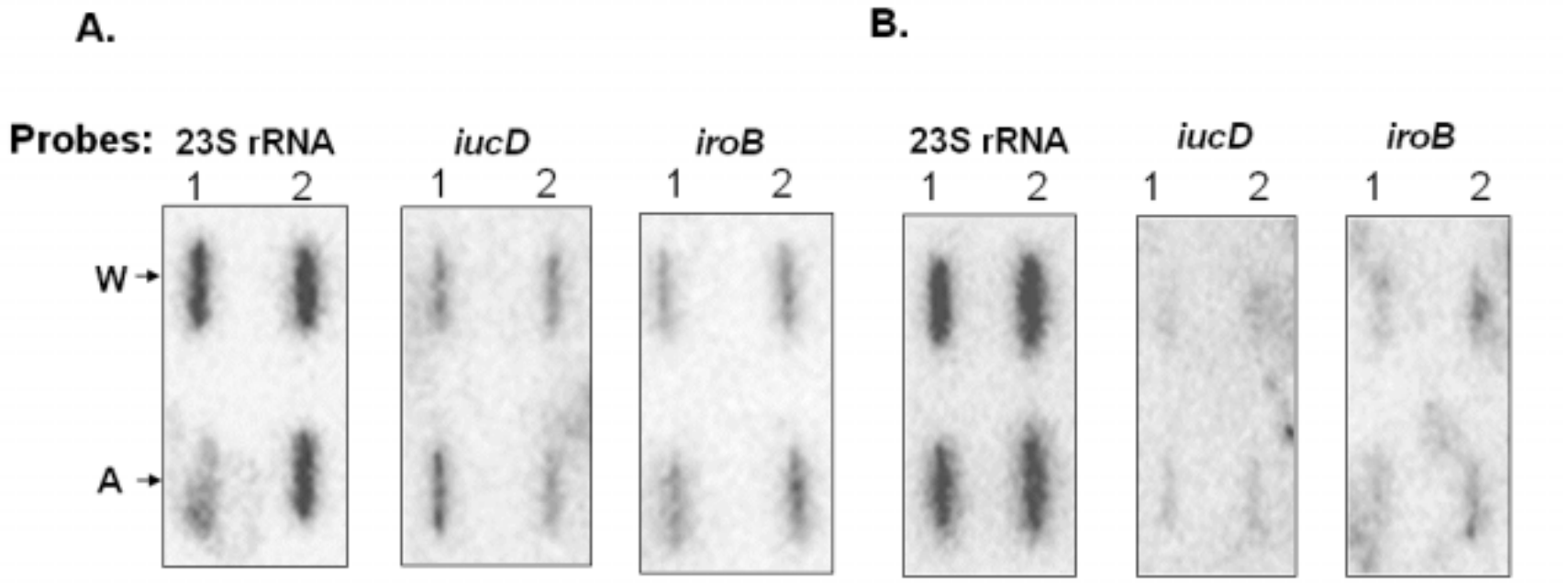


Fig. 8. Northern blotting analysis of *iro* and *iuc* operon expression in *K. pneumoniae* CG43S3-U9451 and *kvgA* mutant. Total RNA was extracted from the bacteria grown in LB (A) and M9 medium (B). Twenty micrograms of the total cellular RNA was loaded on a Hybond-N+ membrane and the membrane hybridized with the probes specific to either *iucD* or *iroB*. 1: No treatment 2: Treated with 200 μM 2',2'-dipyridyl. The abbreviations on the left are: W, CG43S3-U9451 (wild type); A, CG43S3-U9451A16 (*kvgA* mutant).

TABLE 5. Molecular characterization of the *kvgA* repressed genes

No.	β-galactosidase activity[a] (mean±SD)			homolog		
	Wild type	*kvgA* mutant	Fold reduction	Gene (%identity)	Bacteria	Position/length
krg01	58.8±1.3	203.7±13.1	3.4	***iucA*** promoter region (100%)	*K. pneumoniae*	22/100
				cps gene cluster for ORF12 (100%)	*K. pneumoniae*	831/1740
				oligogalacturonate lyase (64%)	*V. vulnificus* YJ016	255/522
				2,5-diketo-D-gluconate reductase (83%)	*E. coli* O157:H7	67/825
				abgR (83%)	*E. coli*	47/906
krg30	71.6±8.3	302.3±21.6	4.2	DNA primase (60%)	Bacteriophage P4	468/1086
				ATP-independent RNA helicase *dbpA* (81%)	*E. coli* CFT073	333/1371
krg32	43.6±7.4	288.5±10.8	6.6	***murB*** promoter region	*E. coli* CFT073	120
				RepA/RepC (100%)	*K. pneumoniae*	252/1008
				SocE (50%)	*Myxococcus xanthus*	346/1458
				hypothetical protein (100%)	*Erwinia amylovora*	125/405
krg89	36.4±3.2	200.7±20.1	5.5	*cysF* (96%)	*Klebsiella aerogenes*	22/1415
				lacZ (89%)	*K. pneumoniae*	153/3105
				iucA promoter region (100%)	*K. pneumoniae*	22/100
				uvrB homolog promoter region (100%)	*Frankia alni*	21/173

a The β-galactosidase activities (in Miller units) of the stationary-phase bacteriagrown in LB were determined

Wild type

kvgA mutant

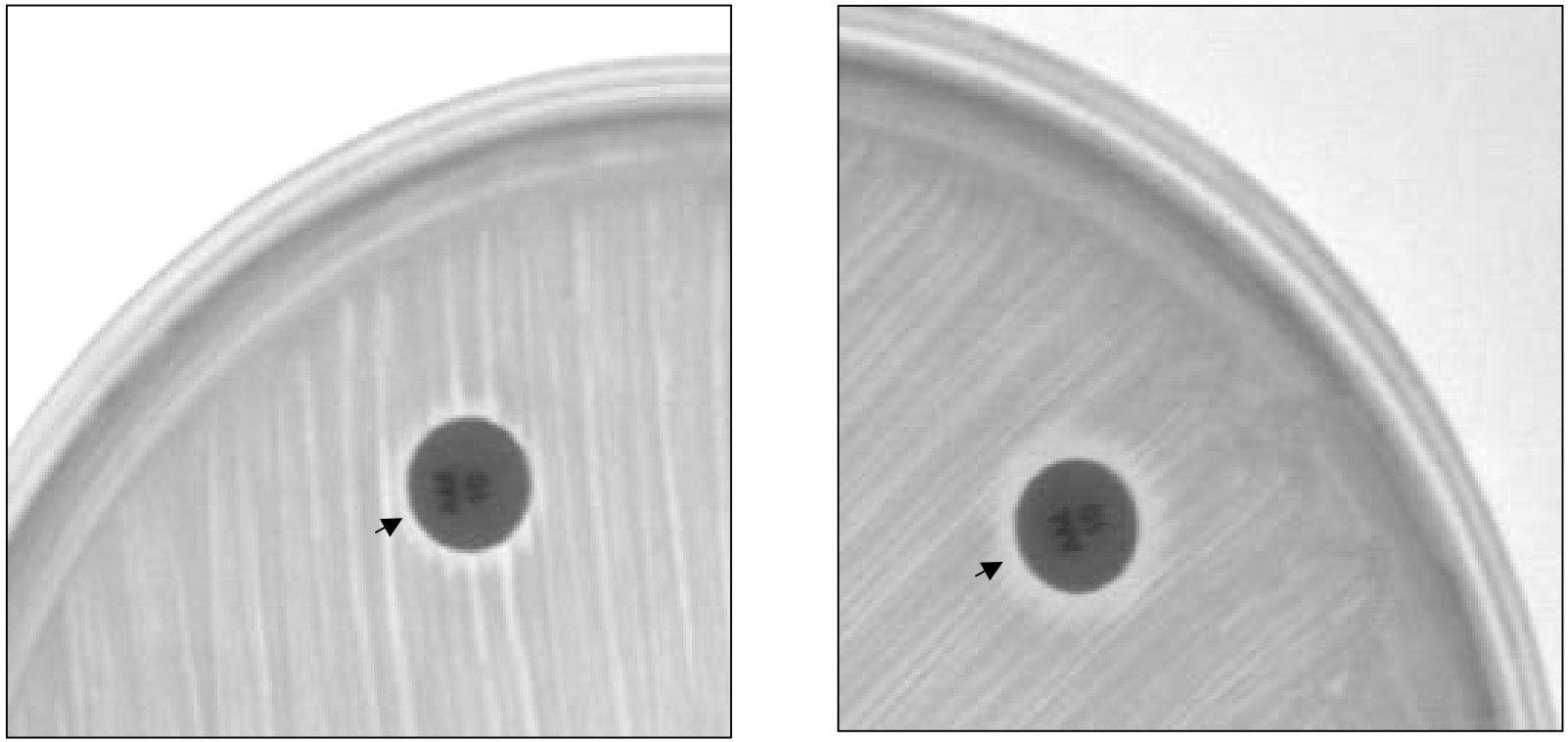
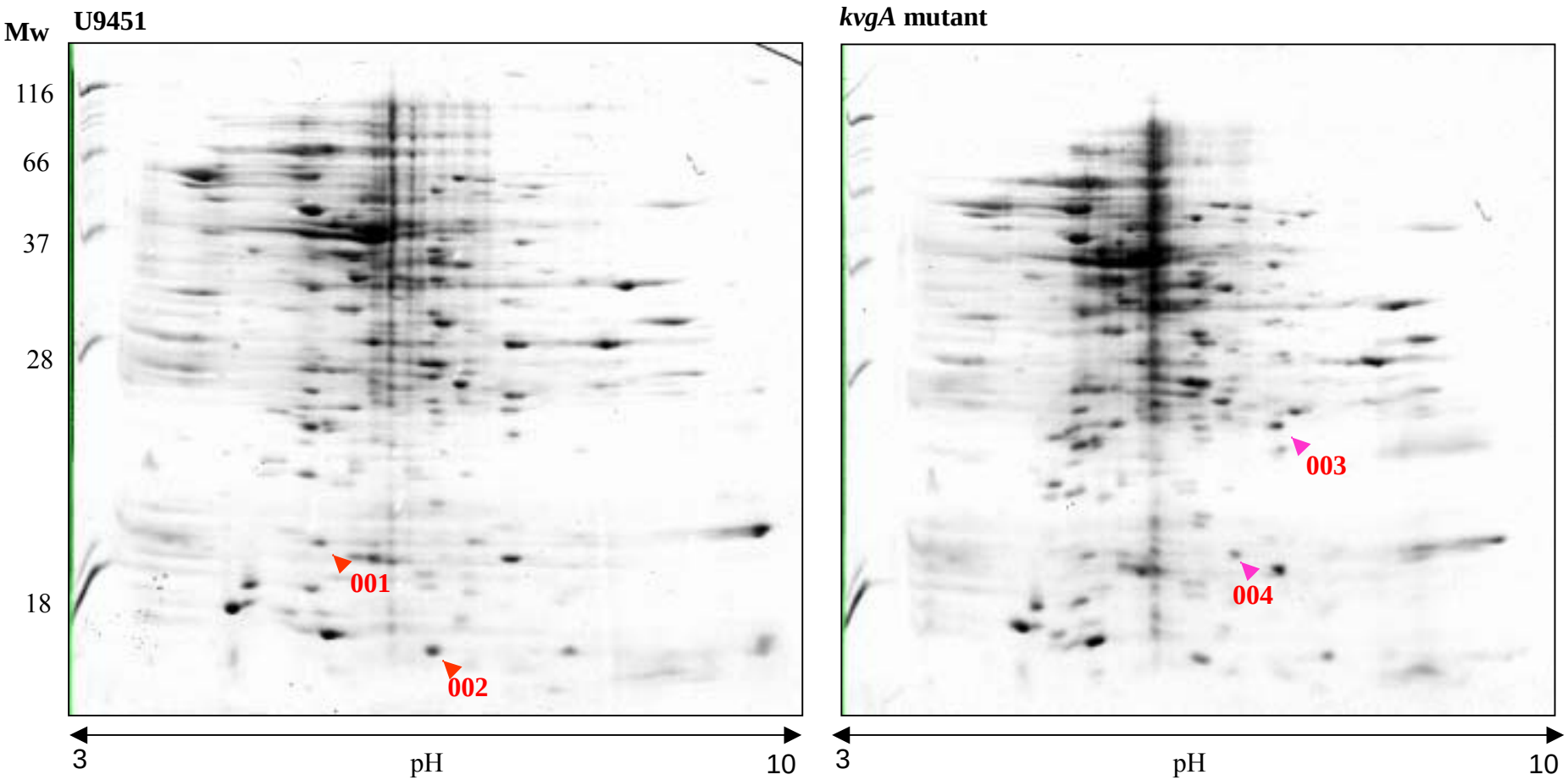
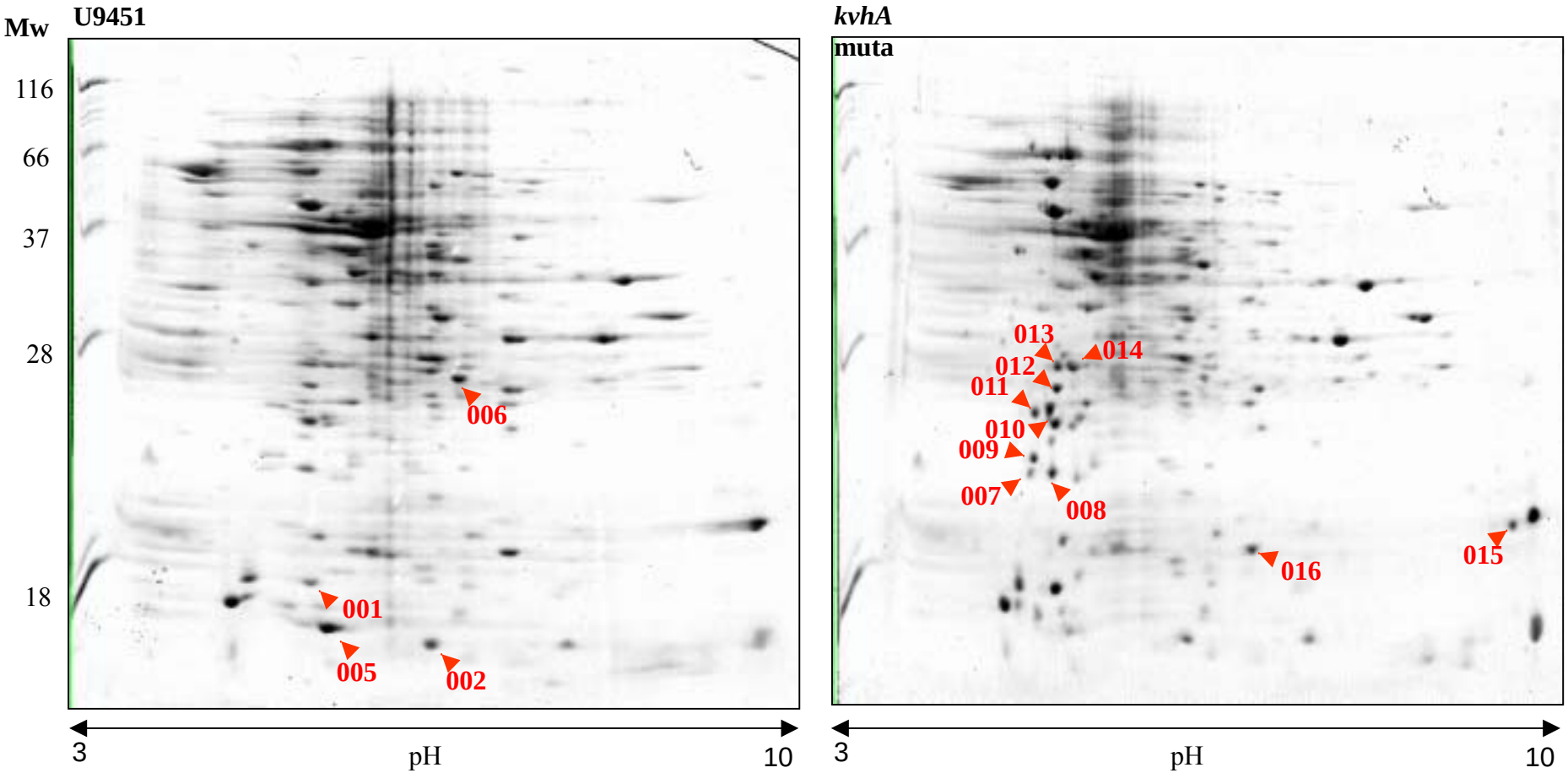


Fig. 9. Ampicillin susceptibility assay. An overnight-grown suspension of either *K. pneumoniae* CG43S3-Z01 (wild type) or AZ18 (*kvgA* mutant) was spread over the LB medium. An ampicillin disk (10 μg) was then pressed on the top of the agar and the agar plate incubated overnight for the formation of inhibition zone.


Mw
U9451
116
66
37
28
18
001
002
3
pH
10
kvgA mutant
003
004
3
pH
10


Mw
U9451
116
66
37
28
18
006
001
005
002
3
pH
10
kvhA
muta
013
014
012
011
010
009
007
008
016
015
3
pH
10

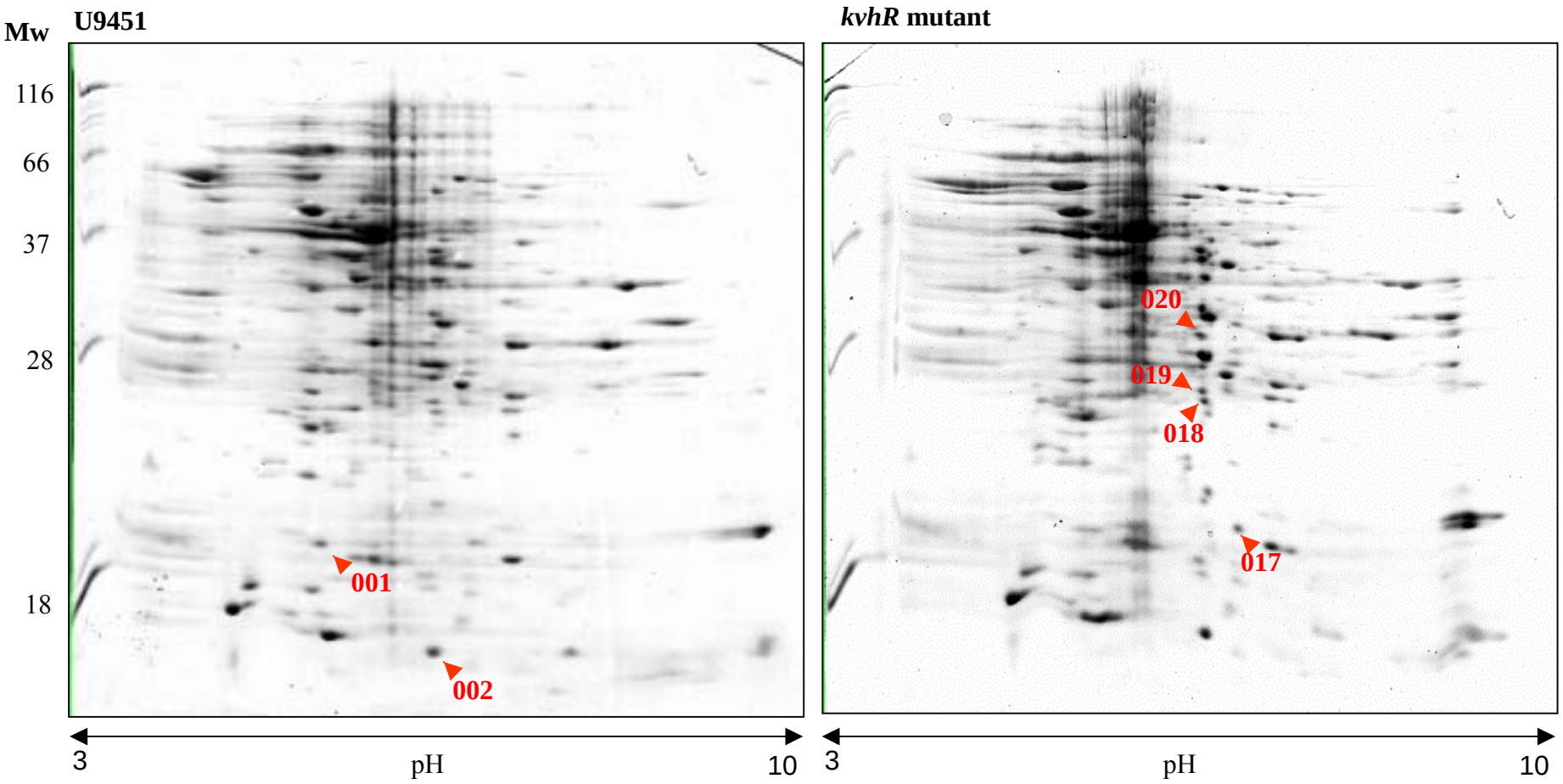

Mw
U9451
kvhR mutant
116
66
37
28
18
001
002
020
019
018
017
3
pH
10
3
pH
10

Fig. 10. 2D protein gel electrophoresis patterns (pH gradient 3 to 10) of *K. pneumoniae* CG43S3-U9451 and its isogenic mutants, *kvgA*, *kvhA* and *kvhR*. The protein extracts (500 μg of protein per sample) of late-log phase culture in M9 medium were analyzed and strained with Coomassie blue. The arrows indicate spots with differential expression in wild and mutants.

TABLE 6.

Sports number	Protein name and/or descriptions	Expression difference in bacteria			
		U9451	*kvgA* mutant	*kvhA* mutant	*kvhR* mutant
001	thioredoxin-dependent hydroperoxide peroxidase	+	-	-	-
002	AidB protein	+	-	-	-
005	Hypothetical protein YtmB	-		+	
007	V-type sodium ATP synthase subunit E (Na(+)-translocating ATPase subunit E)	-		+	
008	Orotidine 5'-phosphate decarboxylase (OMP decarboxylase)	-		+	
009	PTS system, glucose-specific IIA component (EIIA-GLC) (Glucose- permease IIA component) (Phosphotransferase enzyme II, A component)	-		+	
010	Tellurium resistance protein *terE*	-		+	
011	Tellurium resistance protein *terD*	-		+	

成果自評

在此計畫三年的執行期間，我們證明了 *kvgAS* 以及 *kvhAS* 與其他細菌中負責訊息傳遞系統相同具有基因組 (operon) 結構的特性，並且篩選出包含 *kvgASQR* 基因片段的噬菌體株，經由次選殖以及定序了解其上下游的 DNA 序列以及利用華盛頓大學已公佈的克雷白氏肺炎桿菌 MGH78578 的染色體序列比對 *kvhAS* 上下游的 DNA 序列，利用這些結果來推測 *kvgASQR* 以及 *kvhAS* 可能調控的基因。並且我們也成功的構築並且純化出調控蛋白 KvgA 以及 KvhA，利用電泳膠遲滯實驗以及 LacZ 報導系統不僅證明了 KvgA 和 KvhA 具有專一結合本身啟動子的特性，更進一步發現 KvgA 和 KvhA 皆可進行正向的自我調控，並且 KvgA 對於 KvhAS 以及 KvhR 的表現扮演一個活化子的角色。顯示 *kvgAS*、*kvhR* 與 *kvhAS* 這些訊息傳遞基因組可能具有相互作用。同時也構築了一系列基因缺損株，這些包含 *lacZ*$^-$ (Z01) 、*kvgA*$^-$ (A18)、 *kvhA*$^-$ (Ah01) 、 *kvhR*$^-$ (R16) ，以及由 *lacZ* 基因缺損株所衍生出來的一系列基因突變株 *kvgA*$^-$ (AZ18), *kvgS*$^-$ (S01), *kvgR*$^-$ (RZ16), *kvhA*$^-$ (AhZ01) 和 *kvhS*$^-$ (HS01) 和壓力蛋白 RpoS 突變株 *rpoS*$^-$ (RpoS01)。藉由偵測這些突變株的型態及基因表現上的差異，我們得以進一步了解我們發現 KvgA, KvhA 以及 KvhR 之間有相互調節關係，這些訊號傳遞基因組不僅負責調控莢膜多醣體生合成基因組的表現，其中 KvhA 在細菌細胞壁合成的調節上也扮演重要的角色。

因此目前本計劃的預期目標我們已積極完成，因此我們相信經由這三年所完成的成果，我們可以了解這些訊息傳遞系統 *kvgAS*, *kvhR* 以及 *kvhAS* 在克雷白氏肺炎桿菌 CG43 中所扮演的角色以及調控機制，並且利用這些結果提供可能干擾此細菌致病的標的。

而這三年內我們已發表的一篇著作於國外的期刊上，目前也有三篇著作準備投稿中，其成果如下所示:

1. Lai, Y. C., G. T. Lin, S. L. Yang, H. Y. Chang, and H. L. Peng. 2003. Identification and characterization of KvgAS, a two-component system in *Klebsiella pneumoniae* CG43. FEMS. Microbiol. Lett. **218:**121-126.
2. Regulation of the RpoS dependent two-component systems KvgAS and KvhAS in *Klebsiella pneumoniae* CG43. (Prepared to be submitted)
3. The paralogus two-component systems, KvgAS, KvhAS and KvhR, coordinately regulate the capsular polysaccharide synthesis in *Klebsiella pneumoniae* CG43. (Prepared to be submitted)
4. KvgA and KvhA of the two-component signal transduction systems modulate production of *Klebsiella pneumonia* MurA, a UDP-N-acetylglucosamine enolpyruvyl transferase involved in peptidoglycan synthesis and fosfomycin resistance. (Prepared to be submitted)

並且參加國內外相關的研討會，藉由壁報展示其研究成果與相關研究學者進

行討論，壁報題目及所參與的會議如下:

1. 2002 細菌學研討會 Transcriptional analysis of the two component system, *kvgASQR*, in *Klebsiella pneumoniae* CG43. Ching-Ting Lin, Hwei-Ling Peng
2. 2003 細菌學研討會 Functional analysis of the two-component System *kvhAS* in *Klebsiella pneumoniae* CG43. 梁婉君, 林靖婷, 黃騰逸, 彭慧玲
3. 2003 ASM. Functional Characterization of KvgAS and KvhAS Two-Component Systems in *Klebsiella pneumoniae* CG43 by Proteomic and Promoter Trapping Strategies. Ching-Ting Lin, Ten-Yi Huang and Hwei-Ling Peng
4. 2004 細菌致病機制研討會 Proteomic analysis of the two component systems, KvgAS and KvhAS, in *Klebsiella pneumoniae* CG43. Ching-Ting Lin, Hwei-Ling Peng
5. 2004 ASM. Identification of the target genes of the KvgAS two-component system in *Klebsiella pneumoniae* CG43 by a promoter trapping system. Ching-Ting Lin and Hwei-Ling Peng