國立交通大學

生物科技研究所

碩士論文

克雷白氏肺炎桿菌中纖毛表現之分析

Study on the fimbrial expression in *Klebsiella pneumoniae*

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Abbreviation

BLAST	basic local alignment search tool
BCIP	5-bromo-4-chloro-3-indolyl phosphate
ESBL	extended-spectrum beta-lactamase
2CS	two component system
kDa	kiloDalton
AFA	a-fimbrial adhesins
NFA	non-fimbrial adhesins
CPS	capsular polysaccharide
PG	peptidoglycan
IPTG	isopropyl-1-thio-β-D-galactopyranoside
LB	Luria-Bertani
mRNA	messenger RNA
NBT	nitro blue tetrazolium chloride
ONPG	*o*-nitrophenyl-β-D-galactopyranoside
PCR	polymerase chain reaction
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sufate

Lrp	leucine-responsive regulatory protein
IHF	integration host factor
CAP	catabolite gene activator protein
H-NS	histone-like protein
Dam	deoxyadenosine methylase
TFA	trifluoroacetic acid
PCD	programmed cell death

中文摘要

克雷白氏肺炎桿菌是伺機性感染的革蘭氏陰性菌。利用生物資訊分析方法 HMMER，我們在一株從台灣大學附設醫院的病患身上所分離出具有高致病性的 NTUH-K0244 菌株的基因體中找到九套線毛基因組。除了已被廣泛研究的第一型（*fim*）和第三型（*mrk*）線毛之外，其餘七套的功能以及特性都未曾被報導。我們分別將它們命名為 *kpa*、*kpb*、*kpc*、*kpd*、*kpe*、*kpf* 和 *kpg*。為了了解各套線毛在特定條件下的表現情形，我們建構啟動子的活性分析系統。在各種測試條件下，第一型以及第三型線毛的啟動子活性都比其他七套要高。此外，這兩套線毛的活性在添加葡萄糖或甘油的培養環境下皆有顯著上升的情形。相對地，在高滲透壓力、過氧化物的存在，或偏鹼環境的刺激下，都造成活性的降低。

另一方面，我們在 *kpd* 基因組上游找到一個未知功能的基因，經由胺基酸序列比對（BLAST），我們認為其轉譯蛋白可能以雙分子訊息傳遞系統中調控子的角色來調控 *kpd* 基因組表現，因而命名為 *kpdR*。有趣的是，在克雷白氏肺炎桿菌中大量表現 KpdR 蛋白時，我們發現 β–lactamase TEM-116 蛋白明顯增加因而大大提高細菌對 ampicillin 的抵抗力；相對的，其生物膜的形成受到抑制。雖然，我們分別在 CG43S3 和 NTUH-K2044 兩株菌中可利用核酸增殖反應（PCR）得到 $bla_{\text{TEM-116}}$，但經由比對現有的 NTUH-K2044 基因資料庫卻找不到該基因。今後，將確認 $bla_{\text{TEM-116}}$ 的位置，並建構 *kpdR* 及 $bla_{\text{SHV-1a}}$ 的突變菌株，進一步了解 KpdR 與細菌對抗 β－內酰胺類抗生素能力的關係。同時，我們在 *kpb* 及

kpf 基因組上游的位置分別找到類似的調控子基因，並分別命名為 *kpbR* 和 *kpfR*。而在克雷白氏肺炎桿菌中分別大量表現 KpbR 和 KpfR 蛋白時，我們發現細菌的生長都會受到抑制。

最後，我們發現移除座落於 *mrk* 及 *fim* 基因組之間的調控基因 *phgS* 與 *phgM*，不僅會顯著降低 *mrk* 啟動子的活性，*kpg* 線毛的啟動子活性也明顯下降；而進一步，分別在這兩個啟動子 P_{kpg} 和 P_{mrk} 片段中移除過去報導我們預測 PhgS 的辨認序列，原先受 PhgS 影響的現象已不復見，這些結果顯示 PhgS 和 PhgM 在調控 *kpg* 與 *mrk* 線毛的表現上扮演特定的角色。

Abstract

There are nine fimbiral operons identified in the genome of *Klebsiella pneumoniae* NTUH-K2044, a highly invasive strain isolated from Taiwan university hospital, by the HMMER search. The *fim* and *mrk* fimbriae had been described previously, but the others are novel fimbrial operons and are respectively named *kpa*, *kpb*, *kpc*, *kpd*, *kpe*, *kpf*, and *kpg*. The putative promoters of the nine operons were isolated, cloned in a LacZ reporter plasmid and the activities measured. In *K. pneumoniae* CG43S3Z01, the promoters of type 1 fimbriae (P_{fimA} and P_{fimB}) and type 3 fimbriae (P_{mrkA}) had higher level of activity than those of the other fimbrial promoters. The activity of P_{fimA}, P_{fimB}, and P_{mrkA} were enhanced under static cultures and the cultures addition with glucose or glycerol. On the other hand, the expression of *fimB* and *mrkA* were suppressed while the bacteria subject to higher osmotic pressure (> 200 mM NaCl), oxidative stress (60 μM H_2O_2), or the pH switch from 5.5 to 8.

Interestingly, the overexpression of KpdR, a putative response regulator located upstream of the *kpd* gene cluster, appeared to decrease the biofilm formation and increase expression of β-lactamase TEM-116 in both *K. pneumoniae* NTUH-K 2044 and CG43S3Z01. This implied a regulatory role of KpdR in the biofilm formation and also the resistance to the β-lactam drug. Searching for the gene of $bla_{\text{TEM-116}}$ and construction of the *kpdR* and $bla_{\text{SHV-1a}}$ mutants are in process. In the meantime,

over-expressions of two other putative regulators, KpbR and KpfR, in *K. pneumoniae* appeared to interfere growth of the bacteria.

Finally, the deletion of *phgS* or *phgM* was found to reduce not only the expression of type 3 fimbriae but also the activity of P_{kpgA}. Truncation of the predicted consensus sequences for PhgS binding from P_{kpgA} or P_{mrkA} abolished the PhgS dependent expression. It was suggested that a regulatory role of PhgS for the expression of the two fimbriae.

Introduction

The emergence of *Klebsiella pneumoniae* is a striking topic worldwide

Klebsiella pneumoniae, a gram-negative opportunistic pathogen, is a common cause of community-acquired and nosocomial infections (1). Although the incidence of community-acquired *K. pneumoniae* has decreased, the mortality rate due to *Klebsiella* pneumonia remains high (2). In western countries, most *K. pneumoniae* infections occur in lungs and urinary tract. While *K. pneumoniae* has been the leading cause of liver abscess in Taiwan (3). It has been shown that patients with diabetic mellitus in Taiwan are more susceptible to *K. pneumoniae* infection than those without diabetes (3). In addition to liver abscess that involves destructive clinical syndromes, the commonly emerging infections include metastatic meningitis and endophthalmitis, osteomyelitis, and brain abscess (3, 4). Although several virulence factors, such as polysaccharide capsule, different adhesins, lipopolysaccharide, and iron-scavenging proteins have been identified (1), the pathogenicity of *K. pneumoniae* has not been completely elucidated.

Individuals most at risk for *K. pneumoniae* infections are infants, the elderly, and those with compromised defense mechanisms. Patients that suffer from *Klebsiella* infections receiving antibiotics treatment commonly become colonized in the

gastrointestinal tract by antibiotic-resistant *K. pneumoniae* isolates (5, 6). These resistant strains produce extended-spectrum beta-lactamases and are of particular concern as the infections lead to relatively high mortality rates due to treatment failure and subsequent septicemia (7).

β-lactamases genes are prevalent in *Klebsiella pneumoniae*

Many Gram-negative bacteria produce β-lactamases for the resistance to β-lactam antibiotics. The enzymes evolved from bacterial penicillin-binding proteins which involved in peptidoglycan synthesis. The encoding genes are found on chromosome or on transmissible plasmids (8). Plasmid-encoded β-lactamases are often expressed in large amounts (9), while chromosomally encoded β-lactamases are typically expressed at low levels until induced by the presence of the substrates. In Gram-negative bacteria, β-lactamases are exported to the periplasm, whereas in Gram-positive bacteria they are secreted from the cell (10). β-lactamases enzymes exhibit diversity in both structure and function, and are divided into four classes (A-D) based on their primary sequence and catalytic mechanism. Class A is the most numerous and is a serine hydrolase as the Class C and D enzymes. The class B are zinc-dependent β-lactamases. Historically, these enzymes were described as penicillinases because they were able to catalyze penicillin hydrolysis. The ubiquitous TEM-1 enzyme, often encoded on cloning vectors to confer ampicillin resistance,

belongs to class A, and was the first β-lactamase for which the crystal structure was solved (11). Class A β-lactamases have a molecular mass of approximately 29 kDa and are comprised of 260-280 residues, of which nine appear to be highly conserved: four residues, Ser-70, Lys-73, Ser-130 and Glu-166 are critical for catalysis whereas the five remaining residues Gly-45, Pro-107, Asp-131, Ala-134 and Gly-236 likely play a role in structural integrity (12). Furthermore, three additional residues: Asn (in most sequences)-132, Lys/Arg-234 and Ser/Thr-235 also play important roles in enzyme activity. Sequence analysis revealed similarity between class A β-lactamases and the low molecular weight PBPs such as PBP4 of *Escherichia coli* (13). In addition, the crystal structure of class A β-lactamases revealed homology with the catalytic domain of PBP5 from *E. coli* (14). However, even though the PBPs and β-lactamases share strong structural homologies and have a common ancestry, β-lactamases are thought to have lost the ability to interact with PG while function efficiently as resistance enzymes (15).

The first plasmid-mediated β-lactamase in gram-negative bacteria, *tem-1*, was described in the early 1960s. Another common plasmid-mediated β-lactamase found in *K. pneumoniae* and *E. coli* is *shv-1*. Although SHV-1 was originally characterized as a plasmid-mediated β-lactamase (16), recent data showing that many clinical *K. pneumoniae* strains encode SHV-1 β-lactamase production from their chromosomes

suggest its production may be intrinsic to *K. pneumoniae*,. Many new β-lactam antibiotics have been designed to resist to the hydrolytic action. Resistance to these new beta-lactam antibiotics due to extended-spectrum beta-lactamases (ESBLs) also have emerged subsequently. ESBLs were commonly derived from *tem-1* and *shv-1* β-lactamases by mutations to alter the hydrolytic abilities and spectrums (17). Over 100 *tem* and *shv* types of β-lactamases have been characterized (http://www.lahey.org/studies/webt.asp).

Biofilm formation is linked to virulence and colonization

In addition to the prevalence of ESBLs, recent studies suggest that biofilm formation may also be an important virulence factor for *K. pneumoniae*. Biofilms are organized communities of bacteria living on surface environments. Growth as a biofilm is linked to virulence and colonization for a variety of bacterial pathogens, including *Pseudomonas aeruginosa*, which causes chronic, life threatening respiratory infections in cystic fibrosis patients (18, 19). It has been demonstrated in vitro that bacteria growing within biofilms are more resistant to antibiotic treatment than bacteria growing planktonically (20). While expression of β-lactamases have been shown to inhibit bacterial biofilm formation and this anti-biofilm effect was specific to class A and D β-lactamases (21). A model was proposed in which β-lactamases interfere with PG remodeling that is required for correct assembly of large

macromolecular complexes participating in surface attachment. The interference leads to affect the subsequent biofilm development (21).

Microbial adhesion to surfaces is the onset of the development of a biofilm. Adhesion to host tissue receptors is the first step in successful colonization by many Gram-negative pathogens. Adhesion fimbriae are specialized surface structures responsible for the successful recognition and binding of these bacteria to their host receptors. In addition, these fimbriae are responsible for maintaining this contact during the first stages of bacterial colonization. The resulting specific adherence may enhance bacterial persistence at the site of infections , but adherence also has been identified as a virulence factor that facilitates tissue attack, invasion, and mucosal inflammation.

Fimbriae are long filamentous polymeric protein structures located at the surface of bacterial cells. The word “fimbriae” comes from the Latin word for “thread” or “fibre” (22). These adhesive organelles are also referred to as “pili”, which comes from the Latin word for “hair” or “hairlike strucutre” (23). Practically all Gram-negative bacterial species that have been examined so far were found to produce one or more types of fimbriae. While a few Gram-positive bacteria were also shown to possess fimbriae (24). Fimbriae encoded by operon, located either on plasmid or chromosome, generally contains one or two genes encoding regulatory

proteins, and genes encoding a major subunit, minor subunit, a periplasmic chaperone, a relatively large outer membrane usher and an adhesin protein (25). Despite their functional differences, the conserved genetic organization implicates that these different fimbriae evolved from a common ancestor (25).

Besides fimbriae, amorphous adhesins also exist, called a-fimbrial adhesins (AFA) or non-fimbrial adhesins (NFA). These adhesins are present at the outer membrane of the bacterial cell as single proteins or large multi-unit aggregates (25). Another distinct group of adhesins is type 4 pili, which is present in a wide variety of Gram-negative bacterial pathogens on one pole of the bacterial cell. They are, besides adherence, associated with twitching motility (26, 27).

Polysaccharide capsule is a major pathogenicity factor

K. pneumoniae is enveloped by a prominent polysaccharide capsule that produce large, sticky colonies when plated on an agar plate with nutrient media. The strains with the hypermucoviscosity phenotype demonstrate extremely high viscosity determined by a string test (28). In Taiwan, the hypermucoviscosity-positive strains were more prevalent for cases of *K. pneumoniae* liver abscess than from other sites of *K. pneumoniae* infection (29).

There are 77 different capsular (K) antigens, and certain serotypes are associated

with certain infection sites (30). In animal models, K1 and K2 strains are the most virulent (1). The seroepidemiologic surveys indicated that *Klebsiella* K1 was rare among North American and European clinical isolates (30, 31, 32). However, the K1 serotype was found the most frequently in Taiwan (33).

Genome-wide study of the putative chaperone-usher assembly fimbriae in *K. pneumoniae* NTUH-K2044

In this study, two invasive strains of *K. pneumoniae* are studied and the properties compared. *K. pneumoniae* NTUH-K2044 of K1 antigen, isolated from the blood of a previously healthy 40 years-old man suffering from community-acquired primary liver abscess and metastatic meningitis, is a hypermucoviscous strain (28). *K. pneumoniae* CG43, a K2 serotype, is also a liver abscess isolate (34).

The sequence analysis revealed nine fimbrial operons identified by the HMMER search in the genome of *K. pneumoniae* NTUH-K2044. Except *mrk* (type 3 fimbriae) and *fim* (type 1 fimbriae), the other novel fimbrial operons were respectively named *kpa*, *kpb*, *kpc*, *kpd*, *kpe*, *kpf*, and *kpg*. Upstream of *kpb*, *kpd* and *kpf* gene clusters, respectively, a gene encoding putative response regulator that contains both receiver domain and DNA-binding domain was identified and named *kpbR*, *kpdR*, and *kpfR*. In-between the *mrk* and *fim* gene clusters, there are *pecS* and *pecM* homologous genes,

namely *phgS* and *phgM*.

PecS/M plays a critical role in virulence regulation in *Erwinia chrysanthemi*

PecS was originally discovered to negatively regulate the expression of cellulose and pectate lyase in *E. chrysanthemi* (35). The *pecS* mutant showed an increased rate of infection, which is likely resulted of the overproduction of extracellular macerating enzymes and resistance to oxidative stress (36). In addition to pectate lyase, PecS has also been shown to negatively affect the expression of cellulase, indigoidine, a type Ⅱ secretion pathway and flagella biosynthesis (35, 37, 38). Pectate lyases and cellulases are extracellular enzymes that attack components of the plant cell wall and are crucial in the pathogenesis of *E. chrysanthemi* (39). Indigoidine is a blue pigment upregulated in *pecS* mutants and is involved in the resistance to oxidative stress (35, 36). Moreover, PecS could positively regulate the expression of polygalacturonase (40, 41).

PecS was classified into the MarR/SlyA family of transcriptional regulators. MarR was the first member crystallized in the family (42). Although members of the MarR/SlyA family show little sequence homology, their structures share significant similarities. They include a common triangular shape with winged helix-turn-helix DNA-binding domain and multiple channels likely involved in the binding of small

regulatory molecules (39).

Cross-talk regulation between the expression of *kpg* and *mrk* genes

The factors mediating transcriptional regulation of fimbriae expression in response to environmental signals either belong to the family of "local" regulators or to the family of "global" regulators (43). The local regulators are encoded by the respective fimbrial operons and their function is restricted to the operon by which they are encoded. Global regulators control the expression of a variety of operons. The regulatory activity of global regulators is superimposed over the local control of each individual operon (44).

Global regulators which are known to be involved in fimbriae expression are, e.g., leucine-responsive regulatory protein (Lrp), integration host factor (IHF), catabolite gene activator protein (CAP), the histone-like protein (H-NS), and deoxyadenosine methylase (Dam) (43). Lrp, IHF and CAP are DNA bending proteins. The H-NS protein changes the degree of supercoiling of the DNA. Dam is an enzyme involved in the methylation of GATC sites (43). Many fimbiral operons were found to encode one or two local regulators. These genes probably act as transcriptional activators of the respective operons, but their precise mode of action is not well known.

Specific aims

This study could be classified into three parts. In part I, the optimal condition for the expression of the fimbriae is investigated using LacZ reporter system. Except for *fim* and *mrk* gene clusters that have been well characterized in *K. pneumoniae*, the conditions for the optimal expression of the fimbrial operons are mysterious.

As reported by Clegg, *et al.* in 2006 (45), the transposon insertion in the 5' non-coding sequence of *kpg* gene cluster lowered the expression level of type 3 fimbriae indicating a possible interacting regulation. It has been shown in our laboratory that the transcription of *mrk* is PhgS/M dependent (46). Thus, the part II is to investigate the role of PhgS in regulation of *kpg* and *mrk* expression.

In the vicinity to *kpb*, *kpd* and *kpf* fimbrial gene clusters, three two component system (2CS) response regulator encoding genes namely *kpbR*, *kpdR*, and *kpfR* were identified. Whether the response regulator(s) play a role on the expression of the fimbriae is investigated in part III.

Materials and Methods

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* JM109 was used for cloning and *Pseudomonas aeruginosa* PAO1 was a positive control strain in biofilm formation assay. Bacteria were grown at 37℃ in Luria-Bertani (LB) broth or the medium supplemented with appropriate antibiotics. Antibiotics were used where indicated at the following concentrations: ampicillin (100 μg/ml), kanamycin (25 μg/ml), and chloramphenicol (35 μg/ml). For β-galactosidase activity assay, the bacteria in different culture conditions were statically grown to early logarithmic phase (optical density at 600 nm 0.4 to 0.5).

DNA manipulation

Plasmids were purified by the High-Speed Plasmid Mini kit (Geneaid, Taipei, Taiwan). All restriction and DNA-modifying enzymes were used as recommended by the manufacturer (Fermentas, Hanover, MD, USA). PCR amplifications were performed with *Taq* DNA polymerase (MDBio, Inc, Taiwan) or Blend *Taq*^{TM}-Plus-DNA polymerase (Cosmo Bio Co., LTD.). PCR products and DNA fragments were purified using the Gel/PCR DNA Fragments Extraction kit (Geneaid). The primers used in this study were synthesized by MDBio, Inc, Taiwan. Transformation of *E. coli*

cells was performed by following the method of Dower (47).

Construction of plasmid-mediated LacZ reporter strains in *K. pneumoniae* CG43S3Z01

The putative promoter containing about 600 nucleotides upstream of the translation initiation codon of each of the fimbrial gene clusters (Fig. 1) were PCR amplified with respective primer pairs (Table 2) from the genomic DNA of *K. pneumoniae* NTUH-K0244, and the PCR product cloned into *Bam*HI site in front of the promolerless *lacZ* in the low-copied plasmid, pLacZ15 (48). To identifiy the Phg-binding site in P_{kpgA} and P_{mrkA}, the specific primers ZY105/pCC046, ZY106/pCC046 and ZY107/pMrkA5 were used to generate the truncated forms of the promoters.

Measurement of β-galactosidase activity

Miller assay was carried out as described (48). In summary, a culture was grown under the tested condition to early logarithmic phase (optical density at 600 nm 0.4 to 0.5). A 100 μl was removed and added to the reaction mixture which containing 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 0.1% SDS and 35 μl chloroform. The reaction mixture was then vortexed and incubated for 10 min at 30℃. Subsequently, 200 μl of 4 mg/ml

ONPG was added to the reaction and incubated at 30℃ until yellow color was apparent or for 1 h if there was no obvious color change. Finally, the reaction was stopped by adding 500 μl of stop solution (1 M Na_2CO_3) and the absorbance of the supernatant was measured at OD_{420} (ELx800, BIO-TEK). One unit of β-galactosidase is defined as the hydrolysis of 1 nmol ONPG per min per mg protein. Strains were tested under different conditions of temperature, pH, oxygen levels, carbon source, serums, osmolarity stress, and others.

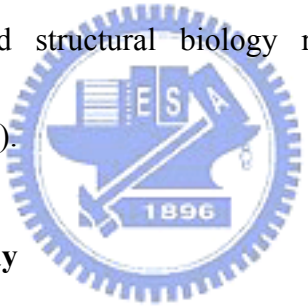
Over-expression of KpbR, KpdR and KpfR

The *kpbR*, *kpdR* and *kpfR* were respectively amplified with specific primers (Table 2) and cloned into a TA vector, pCR®2.1-TOPO® (Table 1), and then subcloned into pETQ33, which carrying an IPTG-inducible *tac*-promoter preceding the multiple cloning site, with *Eco*RI digestion. The resulted recombinant plasmids were then transformed into *E. coli* JM109, *K. pneumoniae* NTUH-K2044 and *K. pneumoniae* CG43S3 strains. Analysis of the overexpression was carried out while these transformants were induced with 0.5 mM IPTG and cultured at 37℃ for 4 h.

In-gel digestion

Stained protein bands were excised from gels and were rinsed with distilled water. The bands were destained with a solution containing 50 mM ammonium

bicarbonate and acetonitrile (1:1) for 15 min, and then the gels were transferred into another solution containing 50 mM ammonium bicarbonate and acetonitrile (3:2) for 15 min. After repeating the destain procedure, the protein was incubated in 500 μl acetonitrile to remove the water and dried using a speed vaccum concentrator. After immersed in 50 μl of 25 mM ammonium bicarbonate, the protein was digested with 3 to 5 μl trypsin (20 μg/ml) at 37℃ for 12 to 16 h. The digested peptides were then recovered using a solution containing 0.1% trifluoroacetic acid (TFA) in acetonitrile. The resulting peptide extracts were processed to MS or MS/MS analysis by core facilities for proteomics and structural biology research in Academia Sinica (http://proteome.sinica.edu.tw/).



Ampicillin susceptibility assay

Ampicillin susceptibility test was performed by the broth method (49). An overnight culture of bacteria was refreshed in LB broth with different concentrations of ampicillin. The transformants of *K. pneumoniae* NTUH-K2044 and *K. pneumoniae* CG43S3 carrying pETQ33 or *kpdR* overexpression plasmid were cultured with addition of 0.5 mM IPTG and 25μg/ml kanamycin. The optical density at 600 nm were measured after 16 h of incubation at 37℃.

Biofilm formation assay

Biofilm formation was assessed by the ability of the cells to adhere to the glass tubes. The indicator medium contained an aliquot of 1:20 diluted overnight bacteria culture in LB and was incubated at 37℃ for 48 h for biofilm formation. Quantification of biofilm formation was carried out as the reported protocol (50) with some modification. Essentially, the indicator medium (200 µl/well) in 96-well microtire dishes made of PVC (TPP 96 flat) contained an aliquot of 1:10 diluted overnight grown bacteria in LB and the plate was incubated at 37℃ for 12 h, 24 h or 48 h. The unadherent bacteria was washed with 200 µl H_20 and then 200 µl of 1% crystal violet was added to each well. After the plate was placed at room temperature for 30 min, ddH_2O was used to wash the plate three times. Finally, the crystal violet-stained biofilm was solubilized in 200 µl of 0.1% SDS and the absorbance determined at OD_{595} using spectrophotometer.

Yeast agglutination assay

The capacity of bacteria to express a *D*-mannose-binding phenotype was determined by their activity to agglutinate yeastcells (*Saccharomyces cerevisiae*) on glass slide. Bacterial cells were adjusted to about $1X10^9$ CFU and mixed with yeast cells (0.01%, in PBS) until the time agglutination occurred was observed in different concentration of bacteria (a series of two-fold dilution).

Qualitative analysis of the capsule polysaccharide (CPS)

Qualitative analysis of the CPS was performed by sedimentation test. An overnight culture of bacteria was refreshed in LB broth with 0.5 mM IPTG and 25μg/ml kanamycin. The sedimentation test was carried after 8 h of incubation at 37℃ and centrifuged at 3000 rpm for 10 min.

Preparation of anti-KpdA, anti-KpfA and anti-KpgA polyclonal antibodies

Five-week-old female BALB/c mice, purchased from Laboratory Animal Center in National Taiwan University, received injection in peritoneal cavity with about 50 μg/150 μl gel-extracted KpdA, KpfA or KpgA proteins respectively on day 1, 11 and 15. For the first injection, the same volume of Freund's complete adjuvant was used, and Freund's incomplete adjuvant was used for the following injections. The KpdA, KpfA and KpgA antisera were finally obtained by intracardic puncture. Indirect ELISA was performed to test the titer of serum from the tail on day 15 and the serum was harvested from the blood of the mice's heart.

Western blot analysis

As the method described (51), total cell lysates were resolved by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were electrophoretically transferred to polyvinylidene difluoride membranes

(Immobilon^{TM}-P, Millipore). Subsequently, the membranes were blocked with 5% skim milk in PBS, the membranes were incubated with a 10,000-fold diluted KpdR, KpfR, KpgR or MrkA anti-serum at room temperature for 2 h. Followed by incubation with a 10,000-fold diluted alkaline phosphatase-conjugated anti-mouse or anti-rabbit (to MrkA) immunoglobulin G at room temperature for 1 h, additional washes were applied triply and the bound antibodies were detected by using the chromogenic reagents BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (Nitro blue tetrazolium).

Localization of the *bla*$_{TEM-116}$ in *K. pneumoniae* NTUH-K2044

Extracted genomic DNA was treated with *Not*I, *Bam*HI, and *Eco*RV digestions and the fragmented DNA cloned to *Not*I, *Not*I/*Bam*HI, *Bam*HI/*Eco*RV, *Not*I/*Eco*RV, *Eco*RV, and *Bam*HI sites of pET30a individually. Then these clones in *E. coli* JM109 were selected with ampicillin and kanamycin.

Extraction of mRNAs from *K. pneumoniae*

Total RNA was isolated from mid-exponential phase of *K. pneumoniae* CG43S3 or NTUH-K2044 cells (OD_{600}=0.6~0.8) by extraction with the TRI reagent (Molecular Research Center, Cincinnati, OH). Contaminating DNA was eliminated from the RNA samples with RQ1 RNae-Free DNase (Promega, Madison, WI, USA).

Total RNA (1 μg) was reverse-transcribed using SMART PCR cDNA synthesis kit (CLONTECH Laboratories, Inc., Palo Alto, CA) to produce the cDNA pool.

Results

Part Ⅰ

Investigation of the fimbrial expression in *Klebsiella pneumoniae*

Comparative analysis of the fimbrial gene clusters present in *K. pneumoniae* CG43S3

In the sequenced genome of *K. pneumoniae* CG43S3 (http://genome.nhri.org.tw /kp/), we could find seven other fimbrial gene clusters, except *kpb* and *kpc*. An incomplete *kpb* gene cluster was found in *K. pneumoniae* CG43S3. The KpbD encoding gene, which encodes a putative adhsin of *kpb* fimbriae, could not be identified by PCR analysis in *K. pneumoniae* CG43S3. However, *kpbB* gene encoding a putative chaperone could be found.

In vitro expression of the fimbrial genes under different conditions

Bacteria only fully express virulence factors when the conditions are appropriate within a host. Therefore, bacteria must have a way of recognizing the environmental circumstances and be able to adapt their protein expression according to these conditions. Regulation of fimbiral gene expression triggered by environmental signals in enteric pathogens is well documented. The environmental conditions include temperature, carbon source, osmolarity, pH, and so on (43).

The promoter regions of each of the putative fimbrial gene clusters (except *kpc* and *kpe*) were cloned to the *lacZ* reporter plasmid pLacZ15 and the β-galactosidase activity was measured under different conditions (Table 3, 4, and 5). As shown, the

majority of the fusion promoters exerted extremely low level of activity. Only the putative promoter of *kpaA*, *fimA*, *fimB* and *mrkA* showed a certain level of detectable activity. A slightly higher expression was noted when the strains were grown in LB than in M9 medium (data not shown). As shown in Table 3, with exception of the P_{fimE} activity, all the others appeared to be higher in standing culture than in the aerated condition. As the temperature decreased from 37℃ to 30℃, the expression of *kpaA*, *kpfA*, *kpgA*, *fimA*, *fimB* and *mrkA* in standing culture increased. The promoter activity of *fimB* and *mrkA* at 30℃ were found to be about three times higher than those at 37 ℃. While the growth at mild acidic condition (pH 5.5), apparent increase of the expression of *fimB* and *mrkA* were also observed. Moreover, addition of 0.2% glycerol or 10% bovine albumin serums exerted a positive effect on the promoter activity of *fimB* and *mrkA*. Notably, the increased expression of *fimA* expression was significant with the addition of 10% bovine albumin serums (Table 4). However, as the growth at the condition adding with different concentrations of sodium chloride, H_2O_2 or bile salts treatments had negative effect on the expression of *fimB* and *mrkA* (Table 5).

The *phgS* deletion effect on the fimbrial expression

As has been previously shown the deletion of *phgS* or *phgM* led to a drastic drop of the P_{mrkA} activity in *K. pneumoniae* CG43S3Z01 (46). The effect of *phgS* deletion

on each of the promoter activity was hence investigated. As shown in Fig. 2, the activity of P_{mrkA} was drastically decreased. Interestingly, P_{kpgA} was also found to be reduced greatly in the *phgS* deletion mutant *K. pneumoniae* CG43S3Z01*phgS*⁻.

The possible interacting regulation for the expression of *kpg* and *mrk* in *K. pneumoniae* 43816 has been previously implicated by Clegg, *et al* (45). They reported an insertion in the upstream non-coding sequence of *kpg* fimbrial gene cluster decreased the expression of type 3 fimbriae (*mrk*) (45). We speculate that PhgS is likely the regulator for the interacting regulation between *kpg* and *mrk*.

Since *phgS/M* are homologous genes of *pecS/M* of *E. chrysanthemi*. PecS, belonging to the MarR family controls the synthesis of various virulence factors (39). The crystal structure of MarR had been recently elucidated (42). Sequence alignment with the representative members of the MarR family indicated that PhgS shares high similarity in the DNA-binding domain (Fig. 3). A consensus binding site for MarR family was therefore predicted. The analysis of 11 PecS target genes revealed a consensus binding site which further demonstrated by mutation analysis (38). The binding element consists of a 23-bp palindrome-like sequence $(C_{-11}G_{-10}A_{-9}N_{-8}W_{-7}T_{-6}C_{-5}G_{-4}T_{-3}A_{-2})T_{-1}A_0T_1(T_2A_3C_4G_5A_6N_7N_8N_9C_{10}G_{11})$. The very conserved part -6 to 6 allows a specific interaction with PecS and the relatively degenerated bases located apart significantly influence PecS affinity. In addition, the

four bases G_{-4}, A_{-2}, T_2 and C_4 are required for efficient binding of PecS and the presence of several binding sites on a promoter increases its affinity to PecS (38).

Comparative analysis with the conserved sequence, a putative palindrome with the conserved nucleotides were found to be present on P_{kpcI}, P_{kpgA}, and P_{mrkA}. Two putative PhgS-binding sites were found on P_{kpgA} (Fig. 4). As shown in Fig. 5, a putative PhgS binding site is located within P_{mrkA} which is about 380 bp upstream of the *mrkA* start codon. In order to verify if the consensus sequence is required for the PhgS dependent expression, truncation forms of P_{kpg} and P_{mrkA} including P_{kpgA1} (420 bp), P_{kpgA2} (488 bp), and P_{mrkAt} (386 bp) were generated. As shown in Fig. 6, the deletion of *phgS* appeared to be no effect on the promoter activity of either P_{kpgA1} P_{kpgA2}, or P_{mrkAt} implying that the truncated sequence is a PhgS binding element. Nevertheless, mutation analysis has to be carried out to support the possibility.

Part Ⅱ

Characterization of the putative regulators encoding by the genes respectively located in the vicinity to *kpb*, *kpd*, or *kpf* gene cluster

Characterization of the putative response regulator KpbR

The locus *kpbR* is located 451 bp upstream of the *kpb* gene cluster in a divergent transcription orientation. As shown in Fig. 7, next to *kpbR*, there is a putative entericidin locus in both KP strains. *E. coli* entericidin has been implicated to be required for the bacterial programmed cell death (52). Downstream of the entericidin loci *ecnA* and *ecnB*, a 500 bp DNA encoding elongation factor P was found (52). In *Citrobacter freundii*. *ecnR* encoding a member of the OmpR response regulator family was found upstream of *ecnB*. Next to *ecnB*, *sugE* gene which has been reported to encode a member of the SMR family of small multidrug resistance efflux pump (53) could be identified in the three bacteria (Fig. 7). Sequence analysis revealed that KpbR encodes a 2CS response regulator with an OmpR domain. The response regulator of approximately 25.1 kDa contains a carboxy-terminal DNA-binding helix-turn-helix motif and a conserved aspartate residue near the amino terminus (Fig. 8).

While overexpress KpbR in *E. coli* JM109, an apparent synthesis of a protein with the predicted molecular weight of KpbR could be observed (Fig. 9). However, transformation of pKpbR into NTUH-K2044 or CG43S3 appeared to inhibit growth of the bacteria. We speculate that KpbR may activate in some way the expression of the *ecnB* which in turn interfere with the bacterial growth.

Characterization of the putative response regulator KpdR

As shown in Fig. 10A, *kpdR* is located upstream of *kpd* gene cluster in a divergent transcription orientation with an intergenic sequence of 414 bp. The Blast analysis revealed that KpdR is a putative response regulator with a carboxyl DNA-binding motif and a conserved aspartate at the N-terminal CheY-like receiver domain. Notably, the over-expression of the KpdR in both *K. pneumoniae* strains enhanced the synthesis of a protein of about 28.5 kDa (Fig. 10B). The protein band was then isolated, subjected to in-gel digestion by trypsin and MS/MS analysis for protein identity. As shown in Fig. 10C, the specific peptide QQLIDWMEADKVAGPLLR matched to the sequence of β-lactamase TEM-116. Nevertheless, we found no sequence homolog of TEM-116 gene in either genome of *K. pneumoniae* NTUH-K2044 or CG43 (http://genome.nhri.org.tw/kp/). In order to confirm the protein identity, two pairs of TEM116 specific primers (56) were synthesized for amplification of L1 and L2 products as shown in Fig. 11A. As shown in Fig. 11B, the TEM-116 specific PCR products could be detected in *K. pneumoniae* NTUH-K2044 and CG43 strains indicating the TEM116 gene is present in both strains.

To investigate if the increased synthesis of TEM-116 affects the bacterial susceptibility to β-lactam antibiotics, ampicillin susceptibility test was carried out. As

shown in Fig. 12, the KpdR over-expression appeared to drastically increase the bacterial resistance to ampicillin. This further supported that over-expression of KpdR enhances the expression of TEM-116 β-lactamase.

While overexpression of FimB in *K. pneumoniae* CG43 (Fig. 13A), apparently diminished expression of MrkA, the major pilin of type 3 fimbriae was observed (Fig. 13B). However, the overexpression of KpdR had no apparent effect on the pilin synthesis as determined by western blot hybridization in Fig. 13. In *K. pneumoniae*, MrkA pilin of type 3 fimbriae has been shown as the major determinant for the bacterial activity to form biofilm (45). In case that the effect of the KpdR overexpression on type 3 fimbrial expression is too subtle to be detected, the effect on the biofilm formation was analyzed. The overexpression of KpdR reduced the biofilm formation when compared to that of wild-type bacteria carrying vector plasmid (Fig. 14). However, no apparent changes could be observed when the biofilm formation capability was quantitatively determined using 96-well PVC-microtire dish (data not shown). Moreover, the over-expression of KpdR slightly decreased the synthesis of the capsular polysaccharide (Fig. 15).

Characterization of the putative response regulator KpfR

KpfR is also a putative response regulator with a carboxyl DNA-binding motif

and a CheY-like receiver domain containing the conserved aspartate residue at the amino terminus (Fig. 16). The entire coding region of *kpfR* has been isolated and cloned into expression vector pETQ30a. However, the optimal condition for the expression of KpfR remains to be determined.

Discussion

Fimbriae are a focus for research as they often define the initial interaction with the host and are a key target for development of interventions such as vaccines. The investigation into the expression of these fimbriae is contributive to image of the disease dynamics during infection. We have found in this work that transcription of the fimbrial operons, except *kpd*, were enhanced by growing at 30℃. Temperature has been shown to be an important regulator of virulence gene expression in several genera of bacteria. Cytoplasmic membranes, nucleic acids, and ribosomes are suggested to be the cellular thermosensors (54). The major effects of lower temperature are a decrease in membrane fluidity and the stabilization of secondary structures of RNA and DNA, which may affect the efficiency of translation, transcription, and DNA replication. Specific transcription factors may involve in the expressions of these putative fimbriae at lower temperature.

As shown in Table 4, higher levels of promoter activity were observed for both P_{fimB} and P_{mrkA} as the bacteria were treated with glucose, glycerol, or serum. The mild acidic environment also appeared to enhance both P_{fimB} and P_{mrkA} activities. Some pH-responsive regulatory mechanisms involved alternative σ factors that sense an acidifying environment (54). However, whether the expression of *fim* or *mrk* involves alternative σ factors remains unknown. Bile salts are prevalent in the mammalian

intestine, a natural habitat of *K. pneumoniae*, where they act as detergents for the digestion of fats. In humans, 96% to 99% of the bile salts entering the small intestine are absorbed and returned to the liver, and only 1% to 4% pass the ileocecal valve and enter the colon (55). *K. pneumoniae* could have evolved adaptive strategy for dealing with bile salts. Nevertheless, no apparent effect of bile salts on the fimbrial expression was found.

Since there are multiple fimbriae in *K. pneumoniae*, investigation of the regulatory network in fimbrial expression is ponderable. Both *K. pneumoniae* NTUH-K2044 and CG43S3 appeared to express MrkA protein in LB static culture at 37℃ (Fig. 13). The activity of P_{mrkA} has been shown to be dropped greatly as *phgS* or *phgM* was deleted from *K. pneumoniae* CG43S3Z01 (46). As shown in Fig. 2, deletion of *phgS* or *phgM* was also found to lower the activity of P_{kpgA} (Fig. 2). A possibly interacting regulation between *kpg* and *mrk* has been discussed in *K. pneumoniae* 43816 (45). We demonstrated here that PhgS/M likely play, a role on the interacting regulation.

The entericidin locus has been implicated in determining programmed cell death (PCD) in bacteria (52). One of the best-studied PCD in bacteria is called toxin-antitoxin system, which consists of a pair of genes that specify two components: a stable toxin and an unstable antitoxin that interferes with the lethal action of the

toxin. In 1998, a novel chromosomal bacteriolytic module called entericidin locus was reported in *E. coli* and *C. freundii* (52). The entericidin locus contains two genes, *ecnA* and *ecnB*, which appear to encode two small, amphipathic α-helical lipoproteins. That putative antidote/toxin pair is positively controlled by RpoS and negatively controlled by the osmosensing EnvZ/OmpR. Under high osmolarity in stationary phase, the locus promotes bacteriolysis with the *ecnA* gene product acting as an antidote to EcnB (52). Since, we could not find *ecnA* next to *ecnB* gene in either *K. pneumoniae* NTUH-K2044 or CG43S3 genome, the involvement of the locus in regulation of KpbR is elusive.

In Korea, the $bla_{\text{TEM-116}}$ has been identified recently (56, 61). The prevalence study of TEM-type β-lactamase-producing *K. pneumoniae* indicated that $bla_{\text{TEM-116}}$ is the predominant type in Taiwan and Guangzhou (56, 57). Why the over-expression of KpdR enhances the β-lactamase TEM-116 expression is considerably urgent and important. The over-expression of KpdR also affected biofilm formation and capsular synthesis, which supporting the mechanism that was proposed the β-lactamase interferes with peptidoglycan remodeling complexes participating in surface attachment and subsequent biofilm development (44).

Reference

1. Podschun R, Ullmann U. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin. Microbiol. Rev. 1998; 11: 589-603.

2. Ko WC, Paterson DL, Saqnimeni AJ, Hansen DS, Von Gottberg A, Mohapatra S, Casellas KM, Goossens H, Mulazimoqlu L, Trenholme G, Kluqman KP, McCormack JG, Yu VL. Community-acquired *Klebsiella pneumoniae* bacteremia: global differences in clinical patterns. Emerg. Infect. Dis. 2002; 8: 160-6.

3. Wang JH, Liu YC, Lee SS, Yen MY, Chen YS, Wang JH, Wann SR, Lin HH. Primary liver abscess due to *Klebsiella pneumoniae* in Taiwan. Clin. Infect. Dis. 1998; 26: 1434-8.

4. Liu YC, Cheng DL, Lin CL. *Klebsiella pneumoniae* liver abscess associated with septic endophthalmitis. Arch. Intern. Med. 1986; 146: 1913-6.

5. Selden R, Lee S, Wang WL, Bennett JV, Eickhoff TC. Nosocomial *Klebsiella* infections: intestinal colonization as a reservoir. Ann. Intern. Med. 1971; 74: 657-64.

6. Tullus K, Berglund B, Fryklund B, Kühn I, Burman LG. Epidemiology of

fecal strains of the family *Enterobacteriaceae* in 22 neonatal wards and influence of antibiotic policy. J. Clin. Microbiol. 1988; 26: 1166-1170.

7. Jacoby GA. Genetics of extended-spectrum beta-lactamases. Eur. J. Clin. Microbiol. Infect. Dis. 1994; 13: S2-11.

8. Williams JD. Beta-lactamases and beta-lactamase inhibitors. Int. J. Antimicrob. Agents 1999; 12: S26-7.

9. Jacoby GA. Genetics of extended-spectrum beta-lactamases. Eur. J. Clin. Microbiol. Infect. Dis. 1994; 13: S2-11.

10. Rice LB, Carias LL, Hutton-Thomas R, Sifaoui F, Gutmann L, Rudin SD. Penicillin-binding protein 5 and expression of ampicillin resistance in *Enterococcus faecium*. Antimicrob. Agents Chemother. 2001; 45: 1480-6.

11. Jelsch C, Lenfant F, Masson JM, Samama JP. Beta-lactamase TEM-1 of *Escherichia coli*. Crystal structure determination at 2.5 Å resolution. FEBS Lett. 1992; 299: 135-42.

12. Matagne A, Brasseur J, Frere JM. Catalytic properties of class A beta-lactamases: efficiency and diversity. Biochem. J. 1998; 330: 581-98.

13. Massova I, Mobashery S. Kinship and diversification of bacterial

penicillin-binding proteins and beta-lactamases. Antimicrob. Agents Chemother. 1998; 42: 1-17.

14. Nicholas RA, Krings S, Tomberg J, Nicola G, Davies C. Crystal structure of wild-type penicillin-binding protein 5 from *Escherichia coli*: implications for deacylation of the acyl-enzyme complex. J. Biol. Chem. 2003; 278: 52826-33.

15. Frere JM. Beta-lactamases and bacterial resistance to antibiotics. Mol. Microbiol. 1995; 16: 385-95.

16. Matthew M. Plasmid mediated beta-lactamases of gram-negative bacteria: distribution and properties. J. Antimicrob. Chemother. 1979; 5: 349-58.

17. Bradford PA. Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev. 2001; 14: 933-51.

18. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science 1999; 284: 1318-22.

19. Fux CA, Costerton JW, Stewart PS, Stoodley P. Survival strategies of infectious biofilms. Trends Microbiol. 2005; 13: 34-40.

20. Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial

agents. Trends Microbiol. 2001; 9: 34-9.

21. Gallant CV, Daniels C, Leung JM, Ghosh AS, Young KD, Kotra LP, Burrows LL. Common beta-lactamases inhibit bacterial biofilm formation. Mol. Microbiol. 2005; 58: 1012-24.

22. Duguid, JP, Smith IW, Dempster G, Edmunds PN. Non-flagellar filamentous appendages ("fimbriae") and haemagglutinating activity in *Bacterium coli.* J. Pathol. Bacteriol. 1955; 70: 335-48.

23. Brinton CC. Non-flagellar appendages of bacteria. Nature. 1959; 183: 782-6.

24. Telford JL, Barocchi MA, Margarit I, Rappuoli R, Grandi G. Pili in Gram-positive pathogens. Nat. Rev. Microbiol. 2006; 4 (7): 509-19.

25. Kusters JG, Gaastra W. Fimbrial operons and evolution. In: Fimbriae: Adhesion, Genetics, Biogenesis, and Vaccines (Klemm, P., Ed.). pp. 179-96. CRC Press, Boca Raton.

26. Rudel T, Scheuerpflug I, Meyer TF. *Neisseria* PilC protein identified as type-4 pilus tip-located adhesion. Nature. 1995; 373: 357-9.

27. Woods DE, Straus DC, Johanson WG, Berry VK, Bass JA. Role of pili I adherence of *Pseudomonas aeruginosa* to mammalian buccal epithelial cells.

Infect. Immun. 1980; 29: 1146-51.

28. Fang CT, Chuang YP, Shun CT, Chang SC, Wang JT. A novel virulence gene in *Klebsiella pneumoniae* strains causing primary liver abscess and septic metastatic complications. J. Exp. Med. 2004; 199: 697-705.

29. Yu WL, Ko WC, Cheng KC, Lee HC, Ke DS, Lee CC, Fing CP, Chuang YC. Association between *rmpA* and *magA* genes and clinical syndromes caused by *Klebsiella pneumoniae* in Taiwan. Clin. Infect. Dis. 2006; 42: 1351-8.

30. Blanchette EA, Rubin SJ. Seroepidemiology of clinical isolates of *Klebsiella* in Connecticut. J Clin. Microbiol. 1980; 11: 474-8.

31. Cryz SJ, Mortimer PM, Mansfield V, Germanier R. Seroepidemiology of *Klebsiella* bacteremic isolates and implications for vaccine development. J. Clin. Microbiol. 1986; 23: 687-90.

32. Smith SM, Digori JT, Eng RH. Epidemiology of *Klebsiella* antibiotic resistance and serotypes. J. Clin. Microbiol. 1982; 16: 868-73.

33. Fung CP, Hu BS, Chang FY, Lee SC, Kuo BI, Ho M, Siu LK, Liu CY. A 5-year study of the seroepidemiology of *Klebsiella pneumoniae*: high prevalence of capsular serotype K1 in Taiwan and implication for vaccine efficacy. J. Infect. Dis. 2000; 181: 2075-9.

34. Yeh KM, Kurup A, Siu LK, Koh YL, Fung CP, Lin JC, Chen TL, Chang FY, Koh TH. Capsular serotype K1 or K2, rather than *magA* and *rmpA*, is a major virulence determinant for *Klebsiella pneumoniae* liver abscess in Singapore and Taiwan. J. Clin. Microbiol. 2007; 45 (2): 466-71.

35. Reverchon S, Nasser W, Baudouy JR. *PecS*: a locus controlling pectinase, cellulose and blue pigment production in *Erwinia chrysanthemi*. Mol. Microbiol. 1994; 11: 1127-39.

36. Reverchon S, Rouanet C, Expert D, Nasser W. Characterization of indigoidine biosynthetic genes in *Erwinia chrysanthemi* and role of this blue pigment in pathogenicity. J. Bacteriol. 2002; 184: 654-65.

37. Praillet T, Nasser W, Baudouy JR, Reverchon S. Purification and functional characterization of PecS, a regulator of virulence-factor synthesis in *Erwinia chrysanthemi*. Mol. Microbiol. 1996; 20: 391-402.

38. Rouanet C, Reverchon S, Rodionov DA, Nasser W. Definition of a consensus DNA-binding site for PecS, a global regulator of virulence gene expression in *Erwinia chrysanthemi* and identification of new members of the PecS regulon. J. Biol. Chem. 2004; 279: 30158-67.

39. Ellison DW, Miller VL. Regulation of virulence by members of the

MarR/SlyA family. Curr. Opin. Microbiol. 2006; 9: 153-9.

40. Nasser W, Shevchik VE, Hugouvieux-Cotte-Pattat N. Analysis of three clustered polygalacturonase genes in *Erwinia chrysanthemi* 3937 revealed an anti-repressor function for the PecS regulator. Mol. Microbiol. 1999; 34: 641-50.

41. Hugouvieux-Cotte-Pattat N, Shevchik VE, Nasser W. PehN, a polygalacturonase homologue with a low hydrolase activity, is coregated with the other *Erwinia chrysanthemi* polygalacturonases. J. Bacteriol. 2002; 184: 2664-73.

42. Alekshun MN, Lecy SB, Mealy TR, Seaton BA, Head JF. The crystal structure of MarR, a regulator of multiple antibiotic resistance, at 2.3 Å resolution. Nat. Struct. Biol. 2001; 8: 710-4.

43. Mol O, Oudega B. Molecular and structural aspects of fimbriae biosynthesis and assembly in *Escherichia coli*. FEMS Microbiol. Rev. 1996; 19: 25-52.

44. Newman EB, D'ari R, Lin RT. The leucine-Lrp regulon in *E. coli*: A global response in search of a raison d'Être. Cell 1992; 68: 617-9.

45. Boddicker JD, Anderson RA, Jagnow J, Clegg S. Signature-tagged mutagenesis of *Klebsiella pneumoniae* to identify genes that influence biofilm

formation on extracellular matrix material. Infect. Immun. 2006; 74: 4590-7.

46. Huang YJ. Characterization of type 3 fimbriae in *Klebsiella pneumoniae*. National Chiao Tung University. Department of Biological Science and Technology, 2007.

47. Dower WJ, Miller JF, Ragsdale CW. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. 1988; 16: 6127-45.

48. Miller JH. Experiments in Molecular Genetics. Cold Spring Harbor Laboratroy Press. Cold Spring Harbor, NY. 1972.

49. Baker CN, Stocker SA, Culver DH, Thornsberry C. Comparison of the E Test to agar dilution, broth microdilution, and agar diffusion susceptibility testing techniques by using a special challenge set of bacteria. J. Clin. Microbiol. 1991; 29 (3): 533-8.

50. Schembri MA, Klemm P. Biofilm formation in a hydrodynamic environment by novel fimh variants and ramifications for virulence. Infect Immun. 2001; 69: 1322-8.

51. Saulino ET, Thanassi DG, Pinkner JS, Hultgren SJ. Ramification of kinetic partitioning on usher-mediated pilus biogenesis. EMBO J. 1998; 17: 2177-85.

52. Bishop RE, Leskiw BK, Hodges RS, Kay CM, Weiner JH. The entericidin locus of *Escherichia coli* and its implications for programmed bacterial cell death. J. Mol. Biol. 1998; 280: 583-96.

53. Paulsen TT, Skurray RA, Tam R, Saier MH, Turner RJ, Weiner JH, Goldberg EB, Grinius LL. The SMR family: a novel family of multidrug efflux proteins involved with the efflux of lipophilic drugs. Mol. Microbiol. 1996; 19: 1167-75.

54. Phadtare S, Yamanaka K, Inouye M. Bacterial Stress Responses. ASM Press. Washington, DC 20036-2804. 2000; 33-45.

55. Bernstein C, Bernstein H, Payne CM, Beard SE, Schneider J. Bile salt activation of stress response Promoters in *Escherichia coli*. Curr Microbiol. 1999; 39: 68-72.

56. Lin TL, Tang SI, Fang CT, Hsueh PR, Chang SC, Wang JT. Extended-spectrum β-lactamase genes of *Klebsiella pneumoniae* strains in Taiwan: recharacterization of *shv-27*, *shv-41*, and *tem-116*. Microb Drug Resist. 2006; 12: 12-5.

57. Zuo B, Liu ZH, Wang HP, Yang YM, Chen JL, Ye HF. Genotype of TEM- and SHB-type beta-lactamase producing *Klebsiella pneumoniae* in Guangzhou

area. Zhonghua Yi Xue Za Zhi. 2006 Nov 7; 86(41):2928-32.

58. Lai YC, Peng HL, Chang HY. Identification of genes induced in vivo during *Klebsiella pneumoniae* CG43 infection. Infect Immun. 2001; 69: 7140-5.

59. Skorupski K, Taylor RK. Positive selection vectors for allelic exchange. Gene 1996; 169: 47-52.

60. Lin CT, Huang TY, Liang WC, Peng HL. Homologous response regulators KvgA, KvhA and KvhR regulate the synthesis of capsular polysaccharide in *Klebsiella pneumoniae* CG43 in a coordinated manner. J. Biochem. (Tokyo). 2006; 140: 429-38.

61. Jeong SH, Bae IK, Lee JH, Sohn SG, Kang GH, Jeon GJ, Kim YH, Jeong BC, Lee SH. Molecular characterization of extended-spectrum beta-lactamase produced by clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* from a Korean nationwide survey. J. Clin. Microbiol. 2004; 42 (7): 2902-6.

62. Tzouvelekis LS, Bonomo RA. SHV-type beta-lactamase. Curr. Pharm. Des. 1999; 5 (11): 847-64.

Table 1. Bacterial strains and plasmids used in this study

(A)

Strain or plasmid	Description	Reference
Strain		
K. pneumoniae		
CG43S3	CG43S3 Sm^r	(58)
CG43S3-Z01	CG43S3 $\Delta lacZ$	(60)
CG43S3*mrkA*$^-$	CG43S3-Z01 $\Delta mrkA$	(46)
CG43S3*phgS*$^-$	CG43S3-Z01 $\Delta phgS$	(46)
CG43S3*phgM*$^-$	CG43S3-Z01 $\Delta phgM$	(46)
E. coli		
JM109	*RecA supE44 endA1 hsdR gyrA96 relA1 thi* (*lac proAB*)*F* [*lacI*^{q} *lacZ M15 proAB traD36*]	Our Lab.
S17-1 λ *pir*	*HsdR recA pro* RP4-2 [Tc::Mu; Km::Tn7] (λ*pir*)	(59)
P. aeruginosa		
PAO1	Wild-type strain, laboratory strain serogroup 05	Our Lab.
Plasmid		
Yt&A vector	PCR cloning vector, Ap^r	Sigma
Pcr®2.1-TOPO®	PCR cloning vector, Km^r	Invitrogen
pLacZ15	A derivative of Pyc016, containing a promoterless *lacZ* from *K. pneumoniae* CG43S3 as the reporter, Cm^r	(60)
Petq33	Protein expression vector, Km^r	Our Lab.
PP*fimA*	~500-bp *Bam*HI fragment containing the putative *fimA* promoter, cloned into *Bam*HI site of placZ15	(46)
pP*fimB*	~500-bp *Bam*HI fragment containing the putative *fimB* promoter, cloned into *Bam*HI site of placZ15	(46)
pP*fimE*	~500-bp *Bam*HI fragment containing the putative *fimE* promoter, cloned into *Bam*HI site of placZ15	(46)
pP*mrkA*	~500-bp *Bam*HI fragment containing the putative *mrkA* promoter, cloned into *Bam*HI site of placZ15	(46)
pFimB	DNA fragment containing *fimB*, cloned into Petq33, Km^r	(46)

(B)

Plasmid	Description
pP*kpaA*	580-bp *Bam*HI fragment containing the putative *kpaA* promoter, cloned into *Bam*HI site of placZ15
pP*kpbA*	639-bp *Bam*HI fragment containing the putative *kpbA* promoter, cloned into *BamH*I site of placZ15
pP*kpbR*	639-bp *Bam*HI fragment containing the putative *kpbR* promoter, cloned into *Bam*HI site of placZ15
pP*kpdA*	602-bp *Bam*HI fragment containing the putative *kpdA* promoter, cloned into *Bam*HI site of placZ15
pP*kpdR*	602-bp *Bam*HI fragment containing the putative *kpdR* promoter, cloned into *Bam*HI site of placZ15
pP*kpfA*	746-bp *Bam*HI fragment containing the putative *kpfA* promoter, cloned into *Bam*HI site of placZ15
pP*kpgA*	578-bp *Bam*HI fragment containing the putative *kpgA* promoter, cloned into *Bam*HI site of placZ15
pP*kpgA*1	420-bp *Bam*HI fragment containing the putative *kpgA* promoter, cloned into *Bam*HI site of placZ15
pP*kpgA*2	388-bp *Bam*HI fragment containing the putative *kpgA* promoter, cloned into *Bam*HI site of placZ15
pP*mrkA*^{t}	386-bp *Bam*HI fragment containing the putative *mrkA* promoter, cloned into *Bam*HI site of placZ15
pKpdR	867-bp *Eco*RI fragment containing the *kpdR* of *K. pneumoniae* NTUH-K2044, cloned into pETQ33, Km^{r}
pKpbR	843-bp *Eco*RI fragment containing the *kpbR* of *K. pneumoniae* NTUH-K2044, cloned into pETQ33, Km^{r}
pKpfR	893-bp *Eco*RI fragment containing the *kpfR* of *K. pneumoniae* NTUH-K2044, cloned into pETQ33, Km^{r}

Table 2. Primers used in this study

Primer	Sequence	Complementary position
ZY001	5'-CAT ATGAAAAAATACAGCAGAGGAATGG-3'	-3 relative to the *kpdA* start codon
ZY002	5'-GCGGGTGGAGCATAACTTTG-3'	+371 relative to the *kpdD* stop codon
ZY003	5'-CATATGAAAATGAAATCACTTTGCCTGG-3'	-3 relative to the *kpfA* start codon
ZY004	5'-ATGGTGACTTTCGCCCTGGAG-3'	+45 relative to the *kpfD* stop codon
ZY005	5'-CATATGAAAAAACAACCTCGCTTTATAACC-3'	-3 relative to the *kpgA* start codon
ZY006	5'-CGAGAACGCCGAAGCTGG-3'	+77 relative to the *kpgD* stop codon
ZY007	5'- GATCCGAATTCGCCAAAACC -3'	+1289 relative to the *kpgC* start codon
ZY008	5'- GGTTTTGGCGAATTCGGATC -3'	-1284 relative to the *kpgC* stop codon
ZY009	5’-CTGCGGGATCCTGTGGGCTG-3’	-102 relative to the *kpbR* start codon
ZY010	5’-GACCAGCAAATGACTATCGCACC-3’	+52 relative to the *kpbR* stop codon
ZY011	5’-CCAGGGGATCCTTATGTTGATCTGC-3’	-13 relative to the *kpdR* start codon
ZY012	5’-CTGGGCGTGGCGAGTAATG-3’	+177 relative to the *kpdR* stop codon
ZY013	5’-CATGCAACATCTTTCATTAGGATCCTTC-3’	-30 relative to the *kpfR* start codon
ZY014	5’-CCGACGAGTGCCATTGCCAG-3’	+41 relative to the *kpfA* start codon
ZY101	5'-CGCTCATGAGACAATAACCC-3'	-56 relative to the $bla_{\text{TEM-116}}$ start codon (56)
ZY102	5'-CAGTGAGGCACCTATCTC-3'	-51 relative to the $bla_{\text{TEM-116}}$ stop codon (56)
ZY013	5’-CATTTTGCCTTCCTGTTTTTG-3’	+67 relative to the $bla_{\text{TEM-116}}$ start codon
ZY104	5’-CTACGATACGGGAGGGCTTACC-3’	-129 relative to the $bla_{\text{TEM-116}}$ stop codon
ZY105	5'-GTAGGATCCGACGAGCGCAC-3'	-442 relative to the *kpgA* start codon
ZY106	5'-GAATGGATCCGTTGTTGTTTAAAGG-3'	-373 relative to the *kpgA* start codon
ZY107	5'-CTGGATCCTGTTGCGGTC-3'	-358 relative to the *mrkA* start codon
pCC033	5’-CGTGCGCTGGATCCTGTT-3’	-532 relative to the *kpaA* start codon
pCC034	5’-TAAAAGATCTATGGCGGGTGC-3’	+50 relative to the *kpaA* start codon
pCC035	5’-GCATTGAGGCGGATCCACT-3’	-597 relative to the *kpbA* start codon
pCC036	5’-GGTCGCTAGATCTGCAGTGC-3’	+46 relative to the *kpbA* start codon
pCC039	5’-CGGATCCGCATATGCTGA-3’	-555 relative to the *kpdA* start codon
pCC040	5’-GCCGCTGACGGGAAGATCT-3’	+46 relative to the *kpdA* start codon
pCC043	5’-ACAGGCCGGATCCATGAC-3’	-681 relative to the *kpfA* start codon
pCC044	5’-GCTGGCCTTAGATCTGGCTAC-3’	+59 relative to the *kpfA* start codon
pCC045	5’-GGATCCGGTCTCTGGTTAACAT-3’	-526 relative to the *kpgA* start codon
pCC046	5’- CCATCCTCATAGGAGCGCTGCT-3’	+47 relative to the *kpgA* start codon

Table 3. Effect of culture condition on the fimbrial expression

Growth conditions \ Fimbriae		Promoter activity (Miller units)[a]								
		kpaA	*kpdA*	*kpdR*	*kpfA*	*kpgA*	*fimA*	*fimB*	*fimE*	*mrkA*
Aerated culture (37℃)		183.2 (±10.7)	21.7 (±1.9)	26.3 (±1.7)	23.0 (±2.8)	92.8 (±3.6)	520.4 (±27.3)	32.3 (±0.7)	96.1 (±5.5)	27.8 (±4.0)
Standing culture (37℃)		272.6 (±12.9)	24.8 (±1.1)	30.4 (±1.4)	46.3 (±2.1)	92.8 (±5.7)	510.8 (±25.6)	806.2 (±37.7)	24.3 (±1.1)	791.6 (±26.1)
Temperature	30℃	372.7 (±8.0)	16.8 (±0.6)	25.3 (±0.7)	90.8 (±2.8)	221.6 (±13.1)	594.4 (±32.9)	2555.9 (±53.0)	25.1 (±0.8)	2505.9 (±59.9)

a, the fimbrial expression was determined using LacZ as the promoter reporter. LacZ activity measurement was as described in Materials and Methods.

Table 4. Effects of glucose, glycerol, and bovine serum on the fimbrial expression

Growth conditions \ Fimbriae		Promoter activity (Miller units)[a]								
		kpaA	*kpdA*	*kpdR*	*kpfA*	*kpgA*	*fimA*	*fimB*	*fimE*	*mrkA*
Standing culture		272.6 (±12.9)	24.8 (±1.1)	30.4 (±1.4)	46.3 (±2.1)	92.8 (±5.7)	510.8 (±25.6)	806.2 (±37.7)	24.3 (±1.1)	791.6 (±26.1)
Glucose	15 mM	244.1 (±22.2)	22.0 (±1.9)	37.8 (±0.5)	43.0 (±1.3)	93.0 (±2.6)	504.2 (±27.1)	1217.4 (±32.7)	22.6 (±1.8)	1753.2 (±38.2)
	30 mM	215.1 (±11.9)	23.2 (±0.7)	33.2 (±1.1)	45.6 (±1.9)	88.3 (±2.1)	511.2 (±26.3)	1112.6 (±39.5)	20.7 (±1.1)	1563.8 (±33.3)
Glycerol	0.2%	345.1 (±9.7)	23.8 (±0.7)	34.4 (±1.1)	40.7 (±1.5)	114.1 (±9.1)	512.6 (±22.4)	3266.5 (±49.2)	25.6 (±1.8)	3431.0 (±58.7)
Bovine serum	10%	309.3 (±14.8)	23.0 (±1.1)	27.4 (±1.9)	51.9 (±6.0)	108.5 (±11.4)	1872.9 (±32.6)	1984.7 (±28.3)	19.9 (±1.4)	1617.2 (±27.7)

a, the fimbrial expression was determined using LacZ as the promoter reporter. LacZ activity measurement was as described in Materials and Methods.

Table 5. Effects of pH, osmotic pressure, H_2O_2, and bile salts on the fimbrial expression

Growth conditions \ Fimbriae		Promoter activity (Miller units)[a]								
		kpaA	*kpdA*	*kpdR*	*kpfA*	*kpgA*	*fimA*	*fimB*	*fimE*	*mrkA*
Standing culture		272.6 (±12.9)	24.8 (±1.1)	30.4 (±1.4)	46.3 (±2.1)	92.8 (±5.7)	510.8 (±25.6)	806.2 (±37.7)	24.3 (±1.1)	791.6 (±26.1)
pH	5.5	270.9 (±4.7)	-	-	26.9 (±3.2)	104.6 (±15.6)	174.0 (±17.3)	2170.2 (±88.6)	30.3 (±7.2)	2335.1 (±94.5)
	8.0	195.9 (±2.8)	27.9 (±1.8)	49.5 (±6.2)	32.2 (±1.4)	82.9 (±17.5)	368.8 (±28.4)	232.8 (±10.1)	25.9 (±2.8)	292.8 (±15.6)
	30 mM	215.1 (±11.9)	23.2 (±0.7)	33.2 (±1.1)	45.6 (±1.9)	88.3 (±2.1)	511.2 (±26.3)	1112.6 (±39.5)	20.7 (±1.1)	1563.8 (±33.3)
NaCl	0 mM	129.0 (±22.4)	23.6 (±1.4)	27.0 (±1.0)	23.0 (±2.3)	56.6 (±7.3)	202.5 (±16.1)	61.8 (±3.0)	13.8 (±1.0)	57.7 (±3.8)
	200 mM	120.1 (±15.6)	23.7 (±0.9)	26.8 (±1.8)	17.6 (±17.6)	59.2 (±6.0)	220.8 (±24.3)	432.5 (±22.7)	14.1 (±0.6)	381.2 (±24.3)
	800 mM	106.5 (±9.2)	18.9 (±1.4)	34.2 (±1.7)	14.8 (±1.1)	51.4 (±3.3)	165.4 (±12.9)	39.3 (±2.6)	12.5 (±0.7)	47.1 (±1.3)
	1.5 M	111.3 (±8.3)	-	-	15.2 (±2.4)	31.4 (±2.3)	154.9 (±10.1)	28.8 (±1.5)	14.3 (±2.6)	32.3 (±2.5)
H_2O_2	60 μM	191.5 (±19.8)	22.9 (±1.5)	32.1 (±1.5)	27.4 (±2.8)	100.2 (±2.3)	247.9 (±15.1)	606.0 (±18.4)	23.3 (±1.4)	575.2 (±19.1)
Bile salts	0.3%	103.0 (±10.0)	5.2 (±0.7)	8.1 (±0.2)	20.3 (±0.3)	43.1 (±3.8)	224.1 (±6.7)	553.7 (±15.0)	7.1 (±0.7)	469.7 (±17.6)

a, the fimbrial expression was determined using LacZ as the promoter reporter. LacZ activity measurement was as described in Materials and Methods.

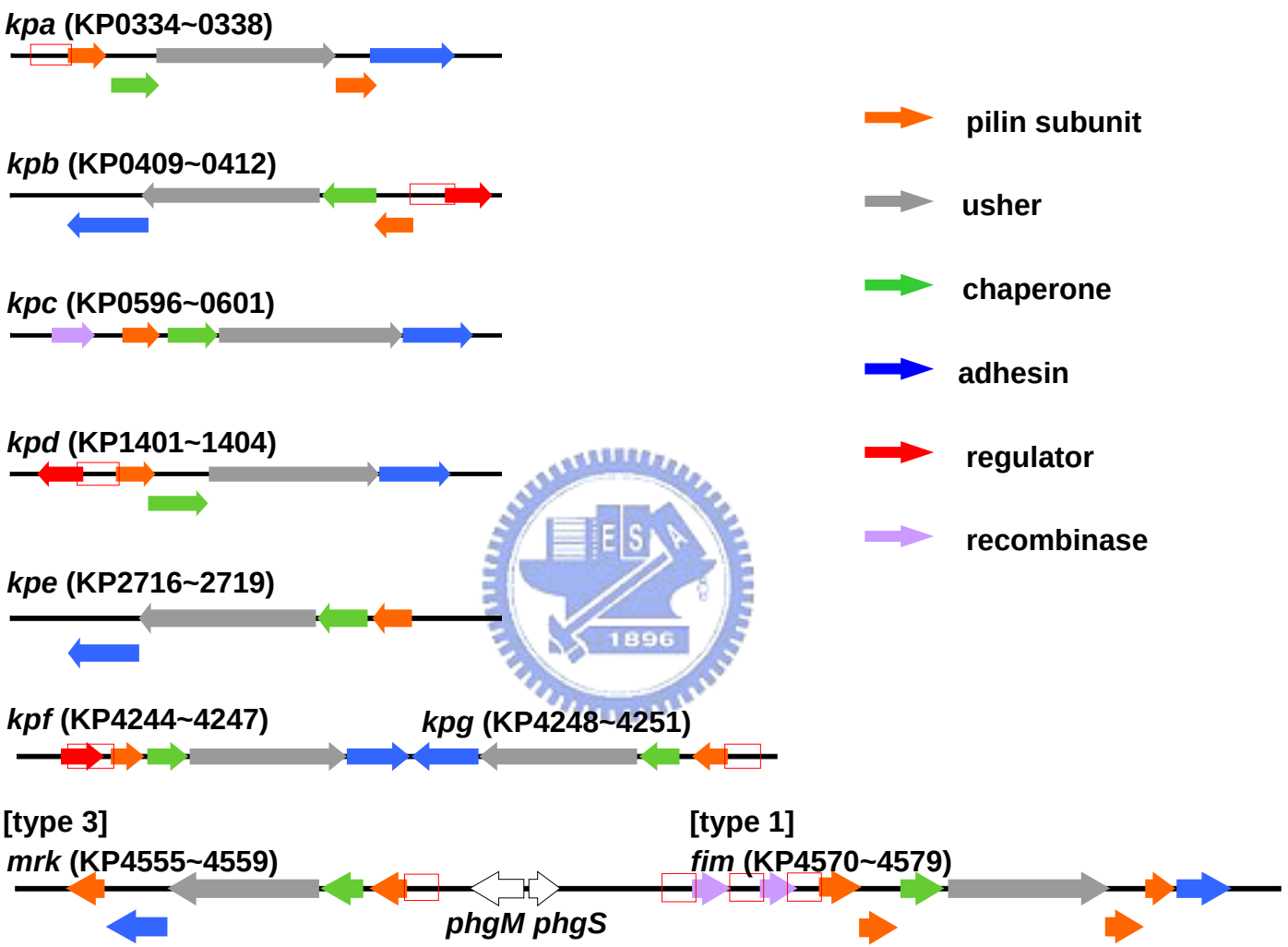


Figure 1. Gene organization of the putative fimbrial gene clusters in *K. pneumoniae* NTUH-K2044. The putative promoters are respectively boxed. The KP number is according to the annotation of the genome (http://genome.nhri.org.tw/kp/).

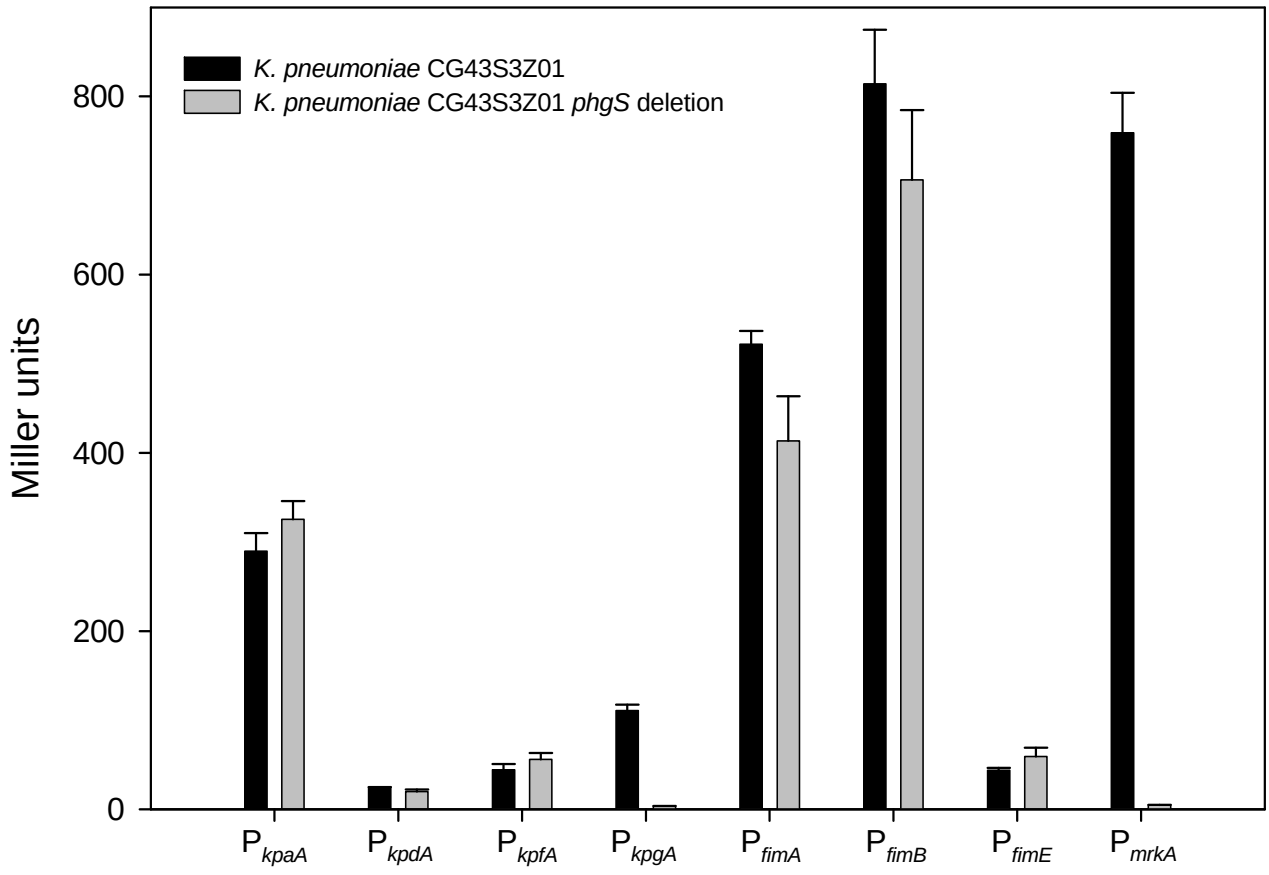


Figure 2. Measurements of the fimbriae in *K. pneumoniae* CG43S3Z01 and CG43S3Z01*phgS*¯. This assay was carried out as described in Materials and Methods while the bacteria was statically grown in LB at 37℃ to exponential growth phase.

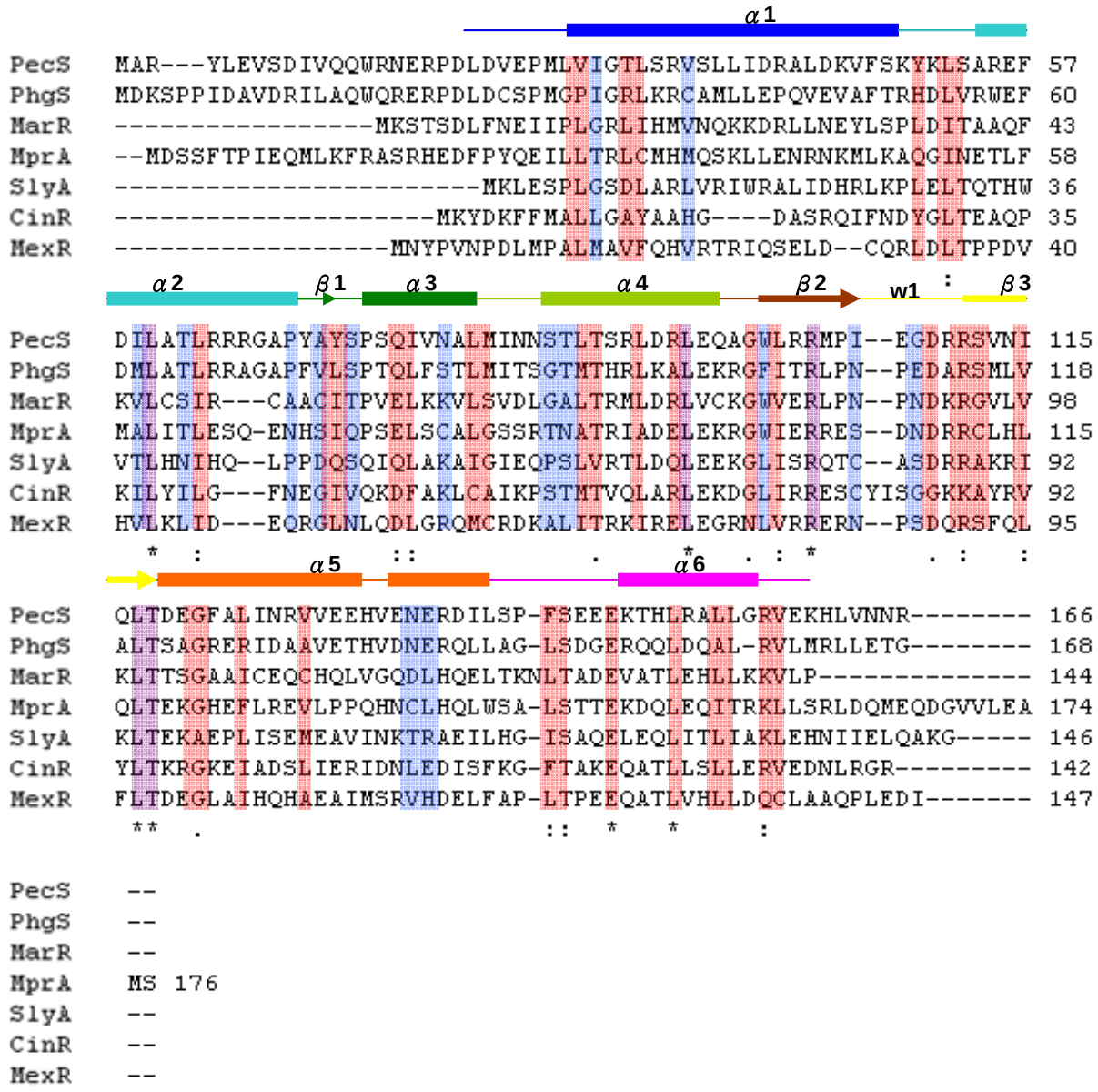


Figure 3. Sequence alignment of PhgS with other representative members of the MarR family. The secondary structure elements are illustrated on the basis of the reported MarR crystal structure (42). The tubes are for α-helices (α) and arrows for β-sheets (β); the single wing region (W1) is also indicated. The DNA-binding domain is boxed (α4). Residues that are identical in all homologs are colored in violet, highly conserved amino acids in pink and moderately conserved residues in blue. The proteins are from the following organisms: MarR, *E. coli*; MprA, *E. coli*; MexR, *P. aeruginosa*; SlyA, *Salmonella typhimurium*; PecS, *E. chrysanthemi*; CinR, *Butyrivibrio fibrisolvens*; PhgS, *K. pneumoniae*.

Figure 4. Nucleotide sequence of P_{kpgA}. The PecS consensus nucleotides are in bold letters and the predicted PhgS-binding sites are boxed. There are two putative -35 and -10 sites as predicted using the BPROM (prediction of bacterial promoters) on Softberry. The primers used to generate truncated forms of P_{kpgA} (pCC045 and pCC046) are underlined.

Figure 5. Nucleotide sequence of P_{mrkA}. The PecS nucleotides sequences are in bold letters and the predicted PhgS-binding sites are boxed. There are two putative -35 and -10 sites as predicted using the BPROM (prediction of bacterial promoters) on Softberry. The primer ZY107 is used to generate truncated form of P_{mrkA}.

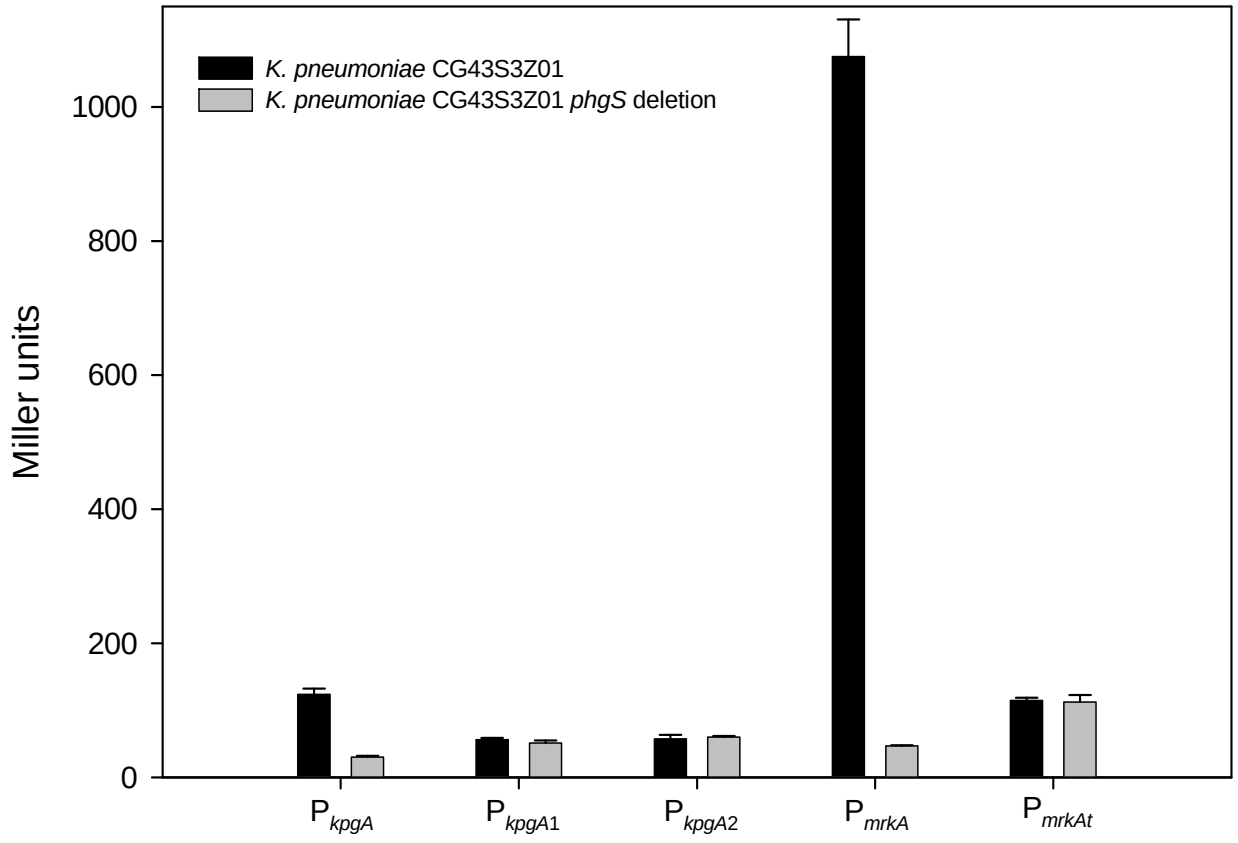
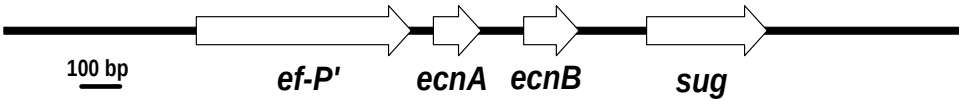
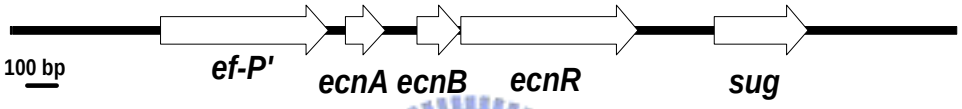


Figure 6. Activity of P_{kpgA}, P_{kpgA1}, P_{kpgA2}, P_{mrkA} and P_{mrkAt}. This assay was carried out as described in Materials and Methods while the bacteria was statically grown in LB at 37℃ to exponential growth phase.

E. coli



C. freundii



***K. pneumoniae* NTUH-K2044 and CG43S3**

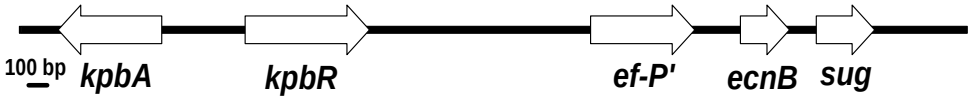


Figure 7. Schematic representation of the entericidin loci of *E. coli*, *C. freundii*, and *K. pneumoniae*. The entericidin locus downstream of the gene that encodes elongation factor P (52). The antitoxin encoding locus *ecnA* was not found in both *K. pneumoniae* NTUH-K2044 and CG43S3 (http://genome.nhri.org.tw/kp/).

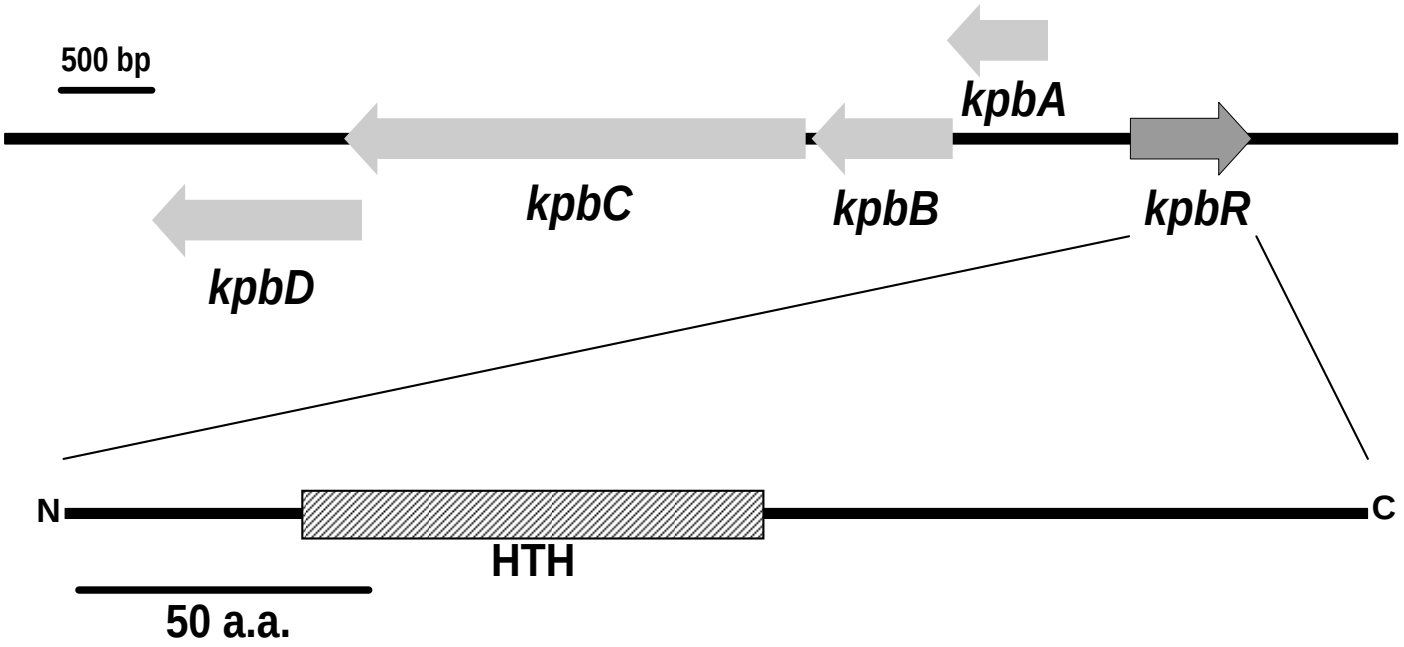


Figure 8. Organization of *kpbABCD* and *kpbR* genes, and the BLAST result of KpbR. There is a putative HTH-DNA binding domain at the N-terminal of KpbR.

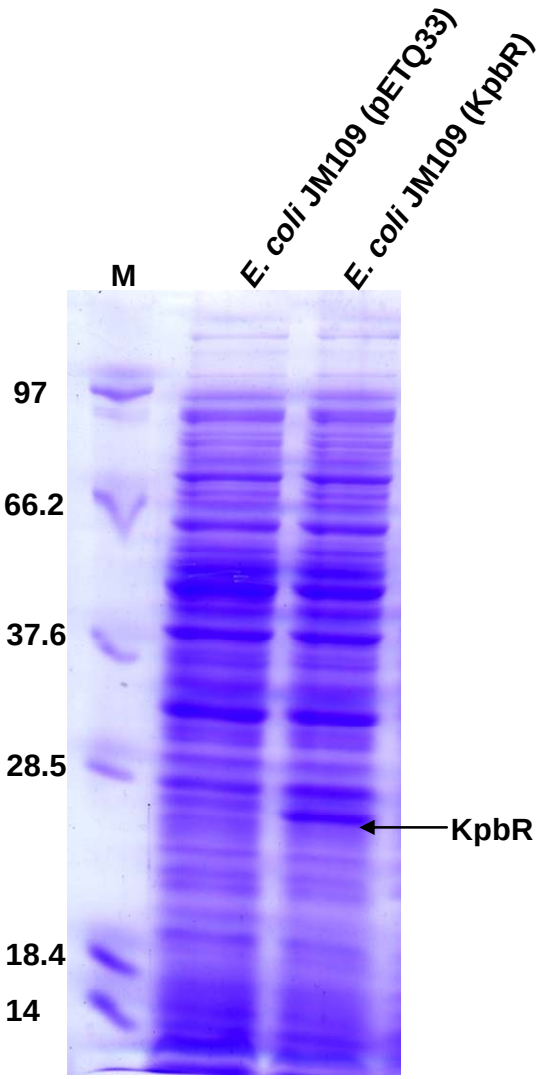
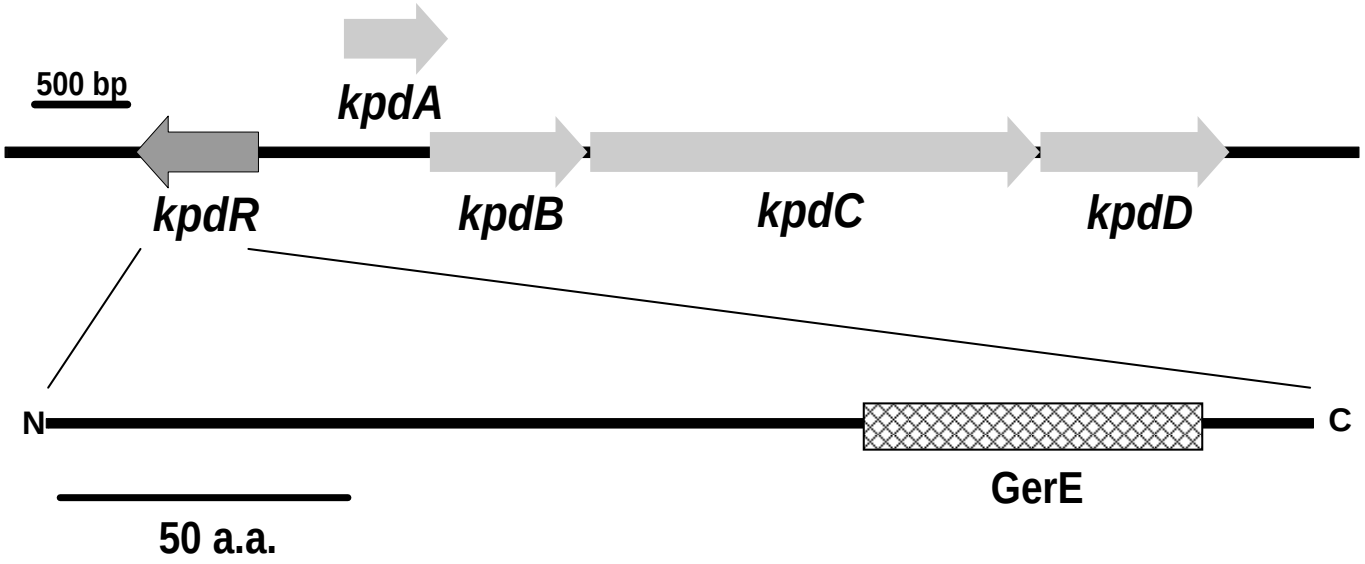
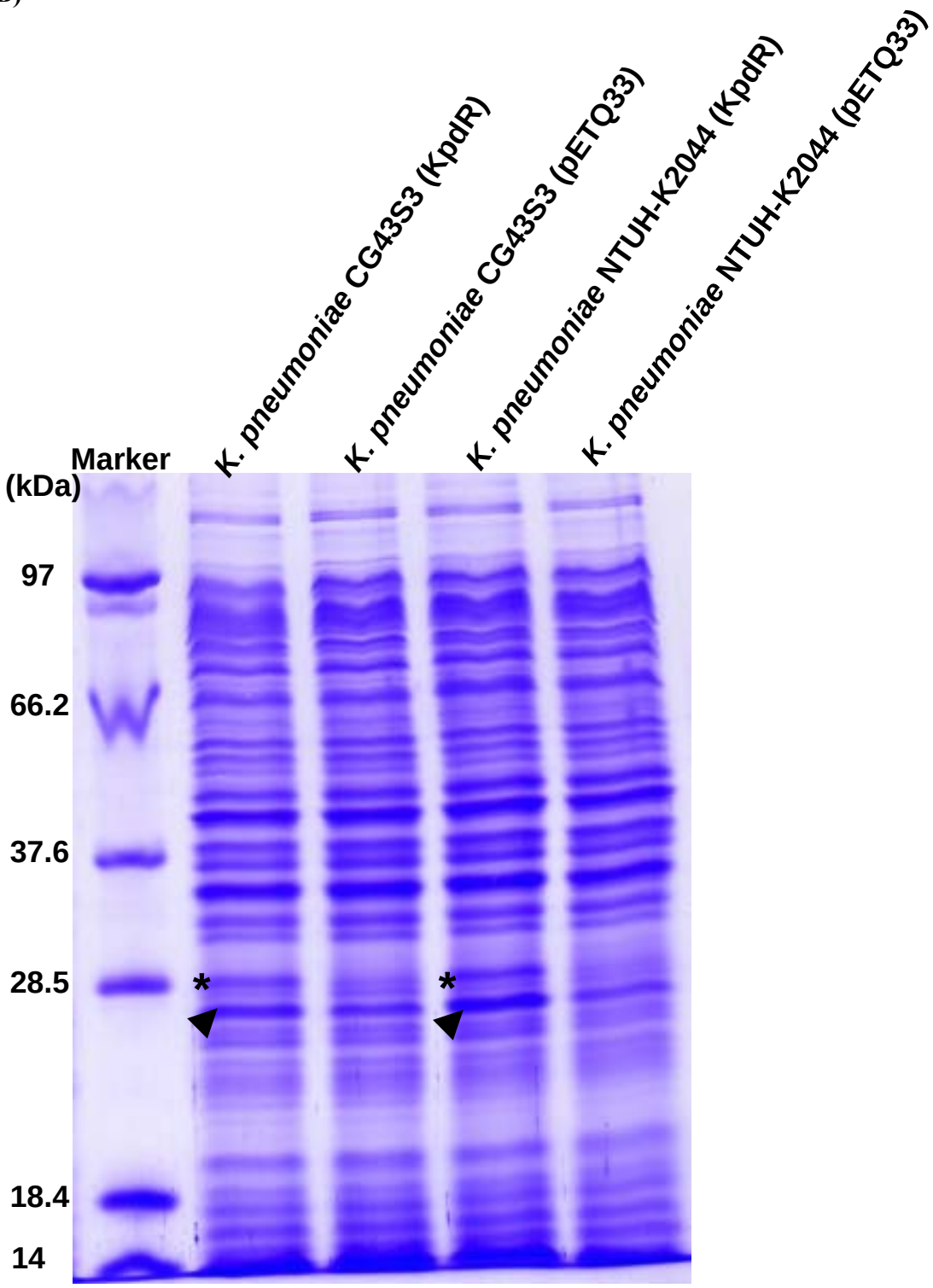


Figure 9. Over-expression of KpbR in *E. coli* JM109.

(A)



(B)


K. pneumoniae CG43S3 (KpdR)
K. pneumoniae CG43S3 (pETQ33)
K. pneumoniae NTUH-K2044 (KpdR)
K. pneumoniae NTUH-K2044 (pETQ33)
Marker
(kDa)
97
66.2
37.6
28.5
18.4
14
*
*

(C)

```
blaTEM-116     3 IQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRPEE
blaSHV-1a,     3 MRYIRLCIISLLATLPLAVHASPQPLEQIKQSESQLSGRVGMIEMDLASGRTLTAWRADE
                     *   *   *   * * * *  *   *  * **  *** ** ** **  *   *  *

blaTEM-116    63 RFPMMSTFKVLLCGAVLSRIDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVRELCS
blaSHV-1a,    63 RFPMMSTFKVVLCGAVLARVDAGDEQLERKIHYRQQDLVDYSPVSEKHLADGMTVGELCA
                 ********** ****** * *** *** * *** * *** **** **** ***** ***

blaTEM-116   123 AAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTMPV
blaSHV-1a,   123 AAITMSDNSAANLLLATVGGPAGLTAFLRQIGDNVTRLDRWETELNEALPGDARDTTTPA
                 ******** ****** * ***  *****   ** ******** ***** * * **** *

blaTEM-116   183 AMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGSRG
blaSHV-1a,   183 SMAATLRKLLTSQRLSARSQRQLLQWMVDDRVAGPLIRSVLPAGWFIADKTGAGERGARG
                  ** *******   *   *  **  **  * ***** ** ********** ****** **

blaTEM-116   243 IIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASLIKHW
blaSHV-1a,   243 IVALLGPNNKAERIVVIYLRDTPASMAERNQQIAGIGAALIEHW
                 * * ***  *  ******     * * *** *** *** ** **
```

Figure 10. (A) Organization of *kpdABCD* and *kpdR* genes, and the BLAST result of KpdR. There is a GerE domain at the C-terminal of the KpdR. This domain is a DNA-binding domain and present in transcription regulators of LuxR/FixJ family. **(B) Over-expression of KpdR in *K. pneumoniae* NTUH-K2044 and CG43S3.** KpdR is marked by arrow head and β-lactamase TEM-116 is labeled by star. **(C) Amino acid sequence alignment of β-lactamase TEM-116 (61) and β-lactamase SHV-1a (62).** Residues that are identical in these two β-lactamases are stared. The specific peptide sequence identified by MS/MS is highlighted.

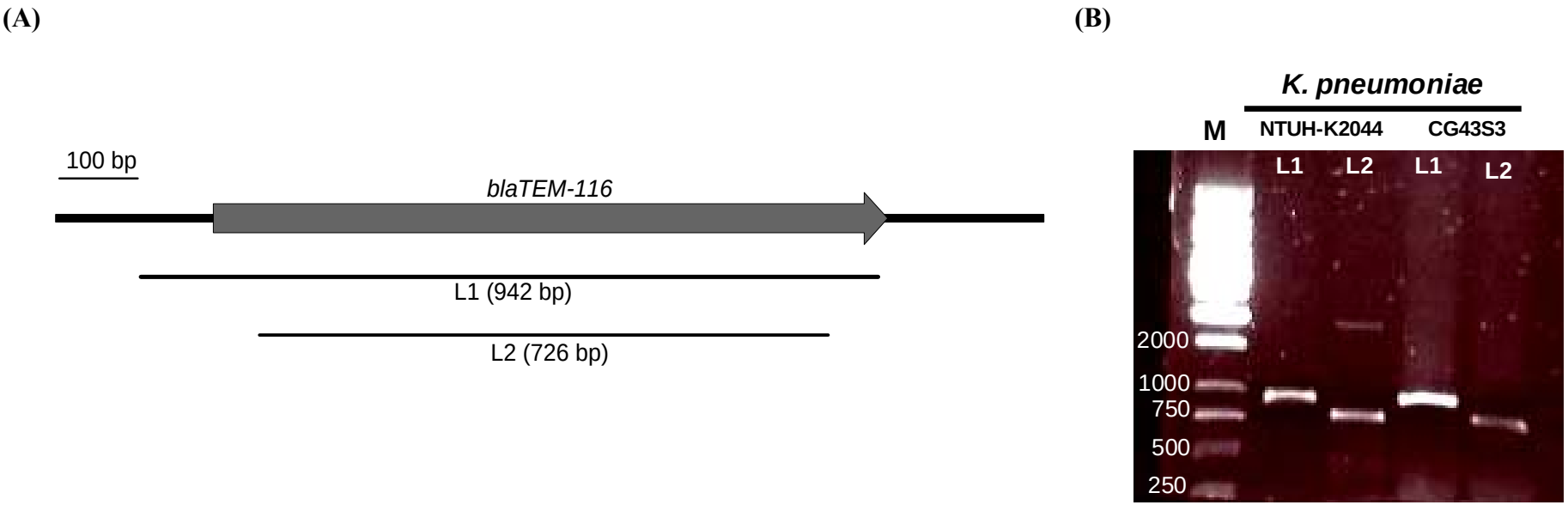


Figure 11. (A) L1 and L2 PCR products of *bla*$_{\text{TEM-116}}$. The specific primers used are ZY101/ZY102 for L1 (56) and ZY103/ZY104 for L2. **(B) PCR detection of *bla*$_{\text{TEM-116}}$ in *K. pneumoniae* NTUH-K2044 and CG43S3.** M: molecular weight marker.

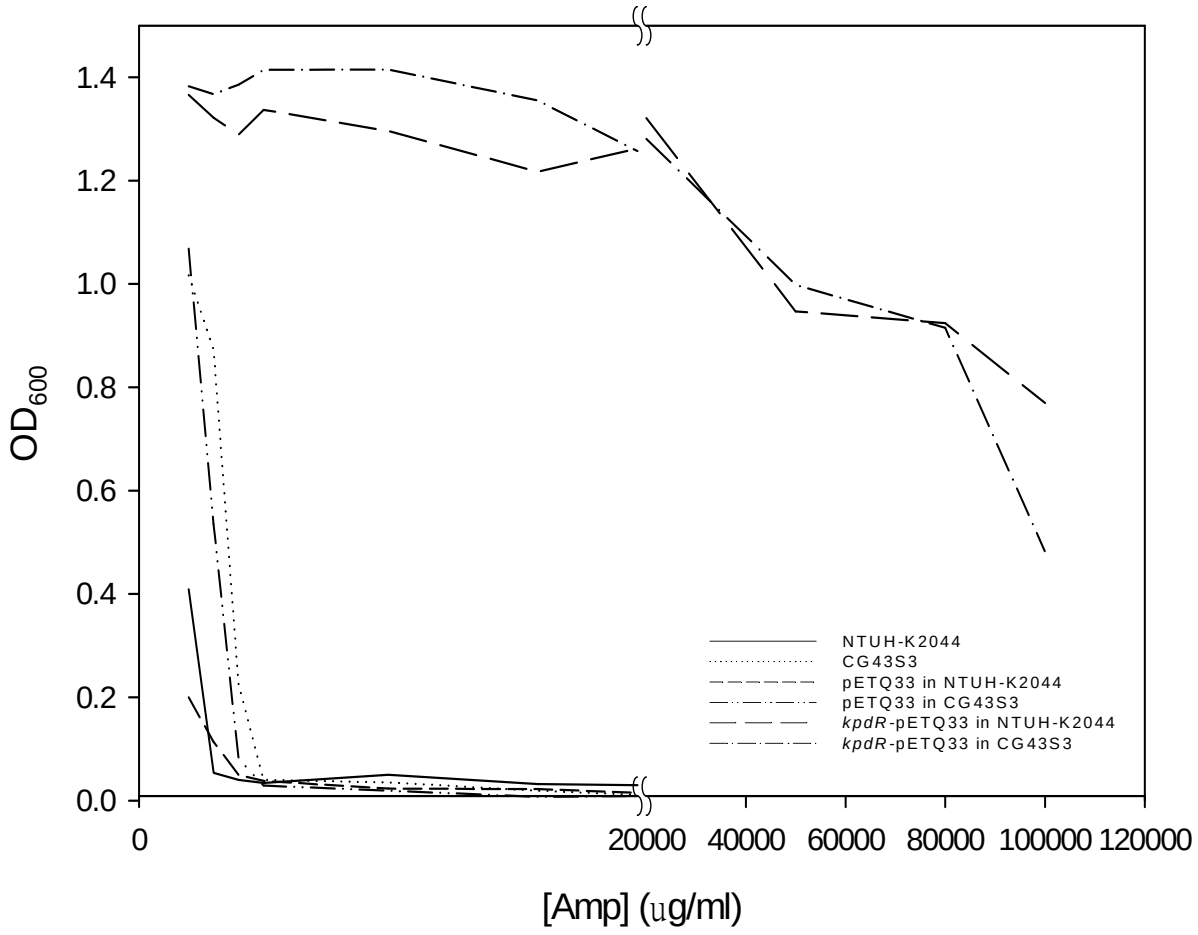
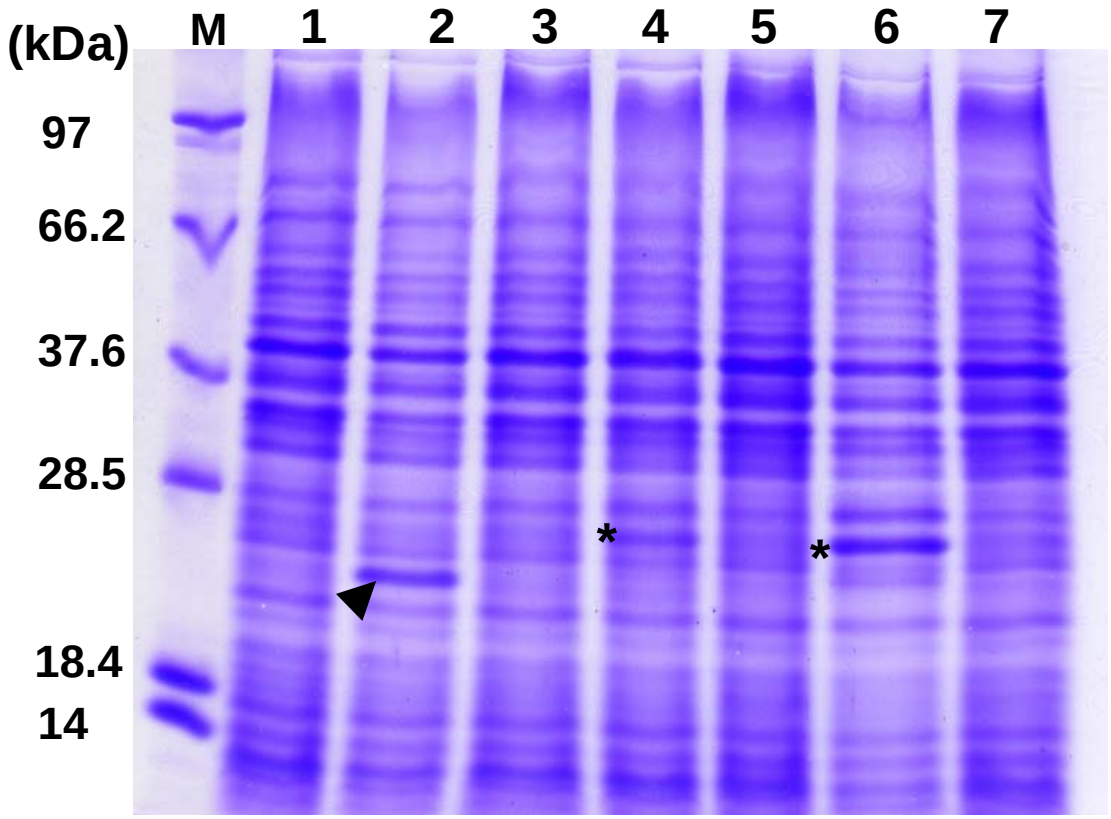


Figure 12. Ampicillin susceptibility assay. Overnight cultured bacteria was refreshed grown in LB with different concentrations of ampicillin and the optical density at 600 nm measured after 16 h of incubation at 37℃.

(A)



(B)

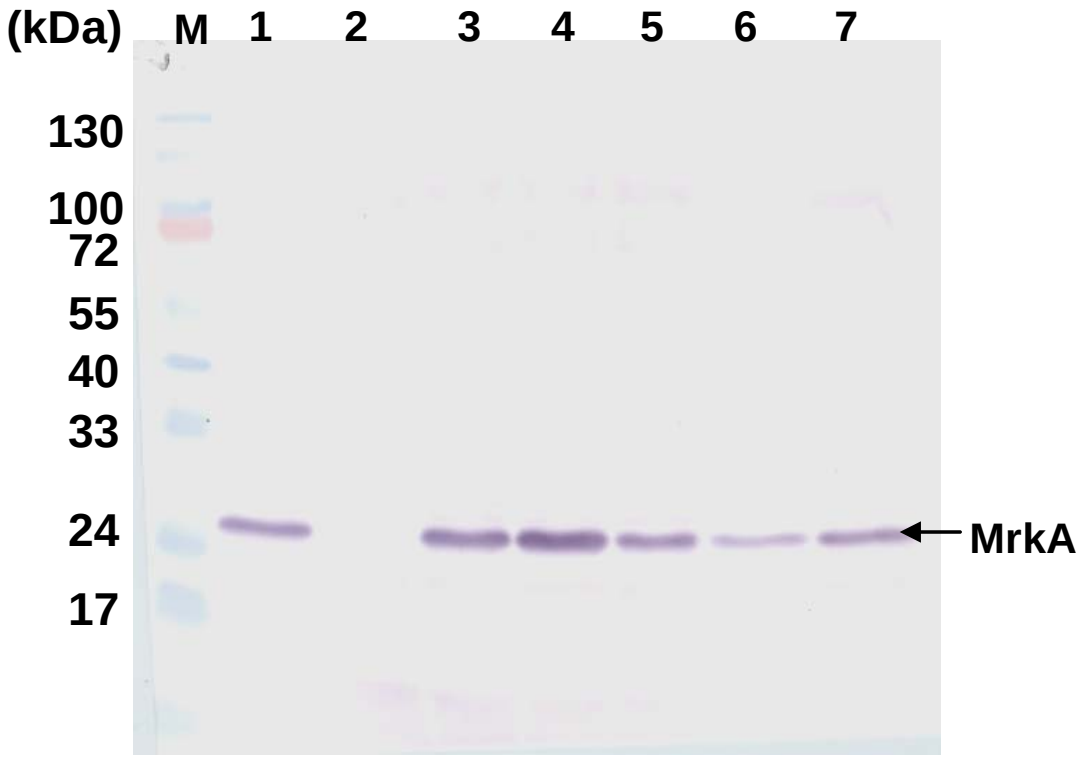


Figure 13. Effects of KpdR over-expression on the expression of type 3 fimbriae. Total cellular proteins were isolated from the overnight standing cultured bacteria in LB broth at 37℃ and the protein resolved on 12.5% SDS-polyacrymide gel by electriphoresis. Bacterial strains are numbered as following: 1, *K. pneumoniae* CG43S3; 2, *K. pneumoniae* CG43S3[pFimB]; 3, *K. pneumoniae* CG43S3[pETQ33]; 4, *K. pneumoniae* CG43S3[pKpdR]; 5, *K. pneumoniae* NTUH-K2044[pETQ33]; 6, *K. pneumoniae* NTUH-K2044[pKpdR]; 7, *K. pneumoniae* NTUH K-2044. (A) The protein gel was stained by Coomassie Blue. Arrow head indicates the over-expressed FimB and the over-expressed KpdR is marked with star. (B) The protein gel transferred onto PVDF and the expression of type 3 fimbriae recognized with anti-MrkA antibody. M: protein molecular weight marker.

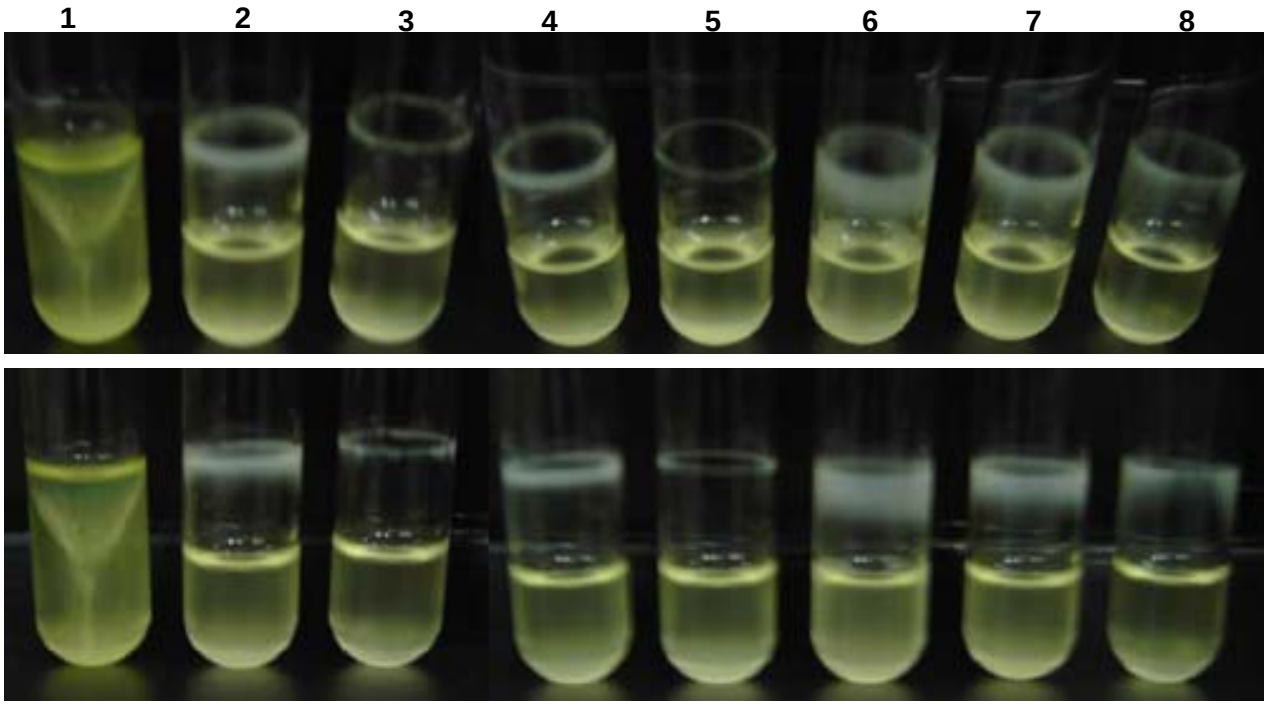


Figure 14. Biofilm formation capability. An overnight culture of bacteria was refreshed grown in 2ml LB broth statically at 37℃ for 48 h. Except the control sample (Table 1) in tube 1, 1 ml of each of the bacterial suspension was removed and the biofilm observed on these glass tubes. The upper panel shows integrity of the biofilm formation and thickness of the biofilm could be observed in lower panel. The bacteria are numbered as following: 1, *P. aeruginosa* PAO1; 2, *K. pneumoniae* CG43S3; 3, *K. pneumoniae* CG43S3[pFimB]; 4, *K. pneumoniae* CG43S3[pETQ33]; 5, *K. pneumoniae* CG43S3 [pKpdR]; 6, *K. pneumoniae* NTUH-K2044; 7, *K. pneumoniae* NTUH-K2044[pETQ33]; 8, *K. pneumoniae* NTUH-K2044 [pKpdR].

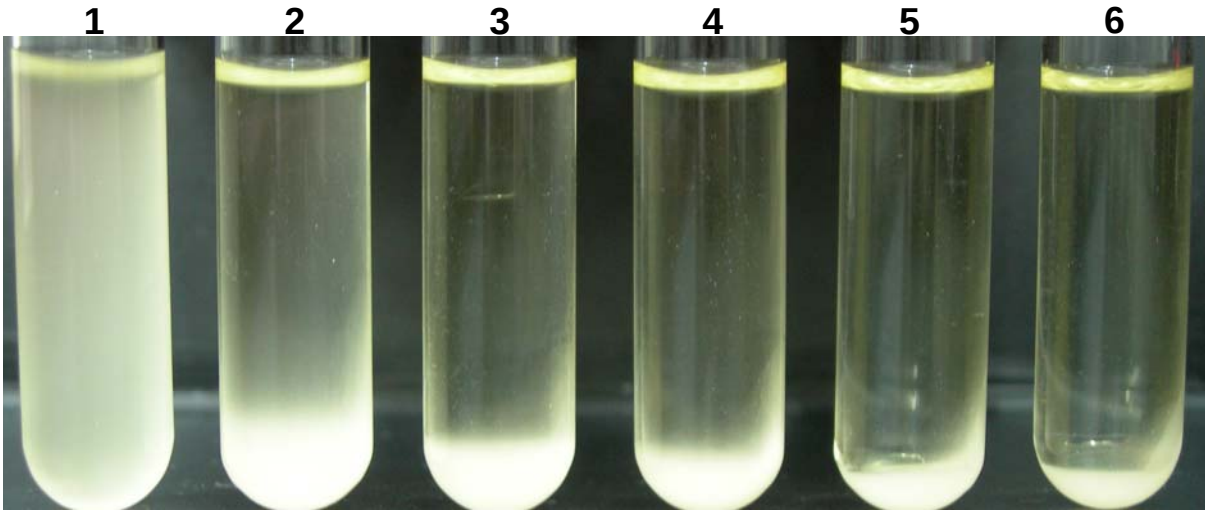


Figure 15. Effect of KpdR over-expression on the capsular synthesis. An overnight cultured bacteria was refreshed grown in LB with IPTG induction at 37℃ for 8 h. Then the bacteria was subjected to centrifugation at 3,000 rpm for 5 m. The bacteria are numbered as following: 1, *K. pneumoniae* NTUH-K2044; 2, *K. pneumoniae* NTUH-K2044[pETQ33]; 3, *K. pneumoniae* NTUH-K2044 [pKpdR]; 4, *K. pneumoniae* CG43S3; 5, *K. pneumoniae* CG43S3 [pETQ33]; 6, *K. pneumoniae* CG43S3 [pKpdR].

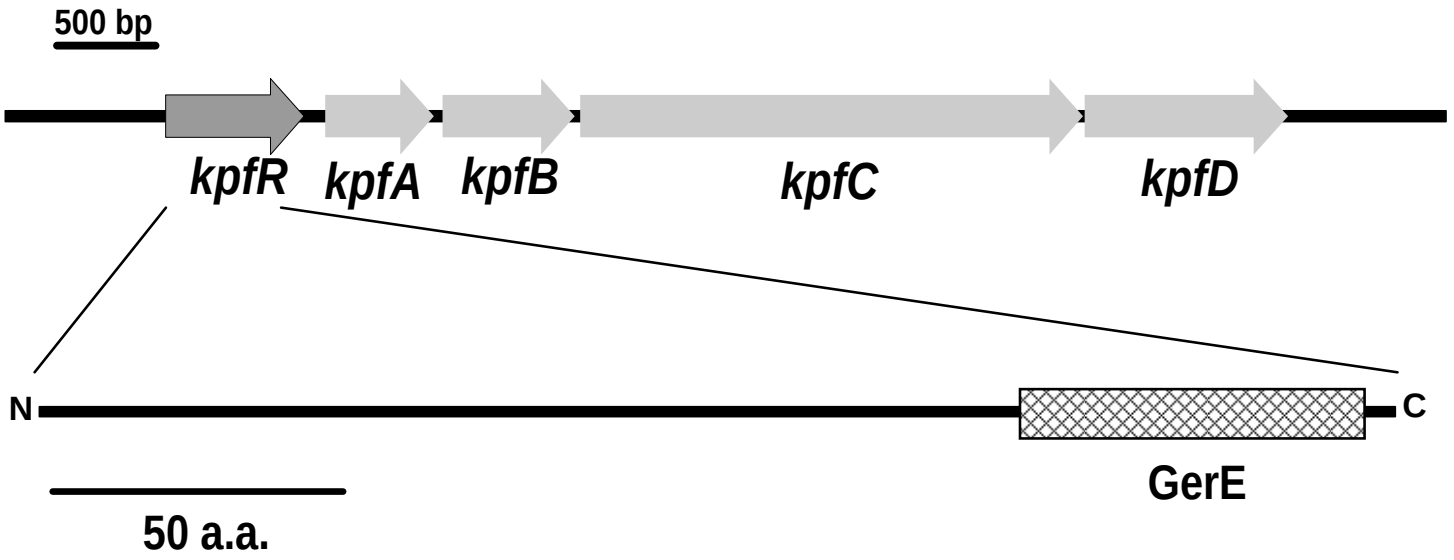


Figure 16. Organization of *kpfABCD* and *kpfR* genes, and the BLAST result of KpfR. There is a GerE domain close to the C-terminal of the KpfR. This domain is a DNA-binding domain and present in transcription regulators of the LuxR/FixJ family.