國立交通大學

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碩 士 論 文

探討大毒性質體 pLVPK 在克雷白氏肺炎桿菌致病過程中扮演的角色

Role of pLVPK in *Klebsiella pneumoniae* pathogenesis

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中文摘要

已知克雷白氏肺炎桿菌臨床分離株 CG43 中帶有一個 219 kb 與毒性相關的大型質體。為了了解此大型質體在克雷氏肺炎桿菌中致病過程中所扮演的角色，我們分析了 127 株克雷白氏肺炎桿菌臨床分離株的普及率。研究發現許多克雷白氏肺炎桿菌帶有大型質體，普及率為 56.69% (72/127)。利用 *rmpA*, *iutA*, *iroB*, *silS*, *terA* 此五個基因作為探針，以及螯鐵分子 siderphore 分泌，我們發現在所有的肝膿瘍臨床分離株之中均帶有一個與 pLVPK 相關的大型質體。然而兩者之間是否具有關係仍需要進一步研究。

在 pLVPK 之中，發現兩個相似的轉錄因子 *rmpA* 與 *rmpA2*。過去研究已知在克雷白氏肺炎桿菌 CG43 中，轉錄因子 *rmpA2* 的缺失會造成莢膜多醣體生合成減少，此現象與另一個轉錄因子 *rcsB* 無關。本實驗首先利用 RT-PCR 以及南方墨點法證明與 *rmpA2* 核酸相似度高達 78% 的 *rmpA* 基因是個活化基因。為了進一步研究 *rmpA* 基因的功能，我們建構 *rmpA* 缺失突變株。比較 *rmpA* 以及 *rmpA2* 缺失突變株，發現此兩種突變株具有類似的表現型， 例如在黏性、莢膜多醣體合成能力均會下降，而生物膜形成的能力會上升。 此外， 利用 LacZ 報導蛋白，分析莢膜多醣體基因的啟動子在這些突變株的表現差異。結果顯示：莢膜多醣體基因組開放骨架 1-2 或 16-17 的啟動子在 *rmpA* 突變株下，活性較野生株下降約 30%。然而，也發現 *rmpA* 的啟動子在 *rcsB* 突變株下，活性只有野生株的一半，因此我們推論 RcsB 可以活化 *rmpA* 的啟動子，此點與 *rmpA2* 啟動子不同。

Abstract

The large plasmid pLVPK, of 219-kb in size, has been shown to be required for the virulence of *Klebsiella pneumoniae* CG43, a highly virulent clinical isolate. To assess the role of pLVPK in *K. pneumoniae* pathgogenesis, the prevalence of large plasmids in 127 *K. pneumoniae* isolates of various origins was analyzed. Consistent with the previous findings indicating that many clinical isolates harbor large plasmids of 200 kb in size, the analysis revealed 56.69% (72/127) prevalence of large plasmids. Interestingly, by probing with the pLVPK specific genes *rmpA*, *iutA*, *iroB*, *silS*, and *terA*, together with the assays of siderphore synthesis, we have found all the liver abscess isolates harbored a pLVPK related plasmid. Nevertheless, whether the presence of the pLVPK like plasmid could be correlated with *K. pneumoniae* liver abscess requires more studies.

On pLVPK, two transcription factor encoding genes, *rmpA* and *rmpA2* were identified. In *K. pneumoniae* CG43, deletion of *rmpA2* has been reported to reduce the production of capsular polysaccharides (CPS) at transcriptional level in RcsB-independent manner. In the mutant CG43S3 *rmpA2*$^-$, expression of *rmpA*, an *rmpA2* homolog sharing 78% nucleotide sequence identity, could be demonstrated by RT-PCR and Southern blotting analysis. An *rmpA* deletion mutant was subsequently generated and the mutant was found to exert similar phenotypes to that of CG43S3 *rmpA2*$^-$, which including decrease of colony mucoidy, and reduction of glucouronic acid content, but increase of biofilm formation capability. In addition, promoter activity measurements using the *lacZ* as the reporter indicated that *rmpA* deletion, as well as the *rmpA2* deletion, reduce the activity of $P_{orf1\text{-}2}$::*lacZ* and $P_{orf16\text{-}17}$::*lacZ* approximately 30% in comparing with that of wild type. Nevertheless, activity of P_{rmpA} in *rcsB*$^-$ strain reduced to a half of that in wild type suggesting a positive regulatory role of RcsB on expression of P_{rmpA} and a regulation different from *rmpA2*.

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Abbreviation

BLAST	basic local alignment search tool
bp	base pair
CAS	chrome azurol S
CFU	colony forming unit
CPS	capsular polysaccharide
CV	crystal violet
LB	Luria-Bertani
ONPG	*o*-nitrophenyl-*β*-D-galactopyranoside
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
rpm	revolutions per minute
SDS	sodium dodecyl sufate
Tris	Tris(hydroxymethyl)-aminomethane

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Introduction

Klebsiella pneumoniae is a common pathogen of community-acquired and nosocomial infections. It causes a wide spectrum of infections, including septicemia, pneumonia, urinary tract infection, meningitis, and purulent abscess at various sites, especially liver abscess. In particular, a distinctive clinical syndrome, which is characterized by community-acquired *K. pneumoniae* bacteremia with primary liver abscess, metastatic meningitis, and endophthalmitis, has been recognized in Taiwan (19, 20, 39, 47). There are several virulence factors identified to participate in *K. pneumoniae* infections, which include capsular polysaccharides (CPS), lipopolysaccharides (LPS), adhesins, and iron acquisition systems (12, 19, 27, 32). In addition to these virulence factors, very little is known about the roles of other genes that may be associated with its pathogenesis. Most recently, the prevalence of multiple drug-resistant strains has significantly restricted the availability of antibiotics for effective treatment of the bacterial infection (34). Therefore, investigation the molecular mechanism involved in *K. pneumoniae* pathogenesis to provide novel drug targets brooks no delay.

Large plasmids associated with virulence, heavy metal resistance, and multiple antibiotic resistance have been reported in *Bacillus anthracis, Salmonella*, *E. coli*, *K. pneumoniae*, *Shigella,* and *Yersinia pestis* (9, 17, 18, 23, 25, 30, 38). For example, *B. anthracis* harbors the 181.7-kb plasmid pXO1, which is essential for the manifestation of the disease anthrax (17). In *Shigella*, the virulence plasmids pWR100, pMYSH6000, and pSS120 are determinants for invasiveness and the ability to cause disease. These plasmids carry the genes for invasions, molecular chaperons, motility, regulation, and a type III secretion pathway with a common 31-kb region (23). In *K.*

pneumoniae, the correlation of large plasmid and virulence has also been reported (24, 25). The large plasmid encodes the production of aerobactin which has been reported a virulence factor (25). Recently, a large plasmid named pLVPK (Large Virulence Plasmid of *K. penumoniae*) was found in *K. pneumoniae* CG43, a clinical isolate K2 serotype with LD_{50} as low as 10 C.F.U (18). Removal the 200-kb plasmid from *K. pneumoniae* CG43 resulted in a loss of colony mucoidy, a loss ability of secreting aerobactin, and 1000 fold decrease in virulence (18).

Sequence analysis of pLVPK showed that this plasmid consists of 219-kb containing 251 ORFs, and approximately 37% of the ORFs are functionally identified (5). Several gene clusters that may attribute to the bacterial virulence were noted, which include *iut*, *iro*, *pbr*, *pco*, *sil*, and *ter* gene clusters. The gene cluster *iucABCDiutA*, which is carried on a mobile region similar to the SHI-2 PAI of *Shigella flexneri* (23), encodes the proteins for aerobactin synthesis upon iron deprivation. The *iroBCDEN* gene cluster, firstly described in *S. enterica*, is known to participate in the uptake of catecholate-type siderophores, enterobactin (13). Secretion of aerobactin and enterobactin are essential for bacteria to survive in host cells (42). The regulation of siderophore synthesis is via Fur protein. Fur is a transcriptional repressor, which displays iron-dependent binding to conserved DNA sequences (Fur boxes) located in the promoters of iron-regulated genes (42). In most bacteria, including *K. pneumoniae*, the iron-complexed form of Fur binds to promoters of iron-uptake genes, thus repressing iron uptake in iron-replete conditions. The other four gene clusters encode the proteins respectively for lead-resistance genes (3), copper-resistance (4), silver-resistance (10), and tellurite resistance (38). Both the lead and silver resistance gene clusters, *pbrABC* and *silCBAP*, encode the proteins to form the efflux system, and PbrRS and SilRSE regulate respectivly the expression of *pbrRSABC* and *silCBAPsilRS* (3, 37).

In *K. pneumoniae*, CPS is an important virulence factor as a barrier against the antibacterial reactions in host cell (20). *K. pneumoniae* CPS has been classified into at least 77 serotypes (18). On basis of the mouse lethality assay, strains belonging to serotypes K1 and K2 are the most virulent (18). *Klebsialla* K2 CPS has be determined as [→]4-Glc-(1→3)-α-Glc-(1→4)-β-Man-(3←1)-α-GlcA]-(1→)$_n$ (21), which is produced by a similar biosynthetic pathway of Group 1 CPS in *E. coli* and made from three transcription units (2), including the *orf1-2*, involved in the synthesis CPS precursor, the *orf3-15*, encoding the core elements for synthesis CPS repeat units, and the *orf16-17*, encoding the enzymes involved in the biosynthesis of CPS precursors (2, 46, 47).

The regulatory strategy of *E. coli* group 1 CPS biosynthesis may serve as a model (43, 44), in which the *cps* gene cluster is regulated by RcsC/RcsD/RcsB and by Lon protease for degradation of RcsA (21). RcsC is a sensior protein, RcsB is a response regulator, and RcsD is a histidine-containing phosphotransfer protein that transfers the phosphoryl group from RcsC to RcsB. The activated RcsB together with the auxiliary protein, RcsA, subsequently coordinate the activation of the *cps* operon transcription (8, 41). In aligning many of the genes under the control by RcsAB, an RcsAB binding box TaAGaatatTcctA was identified (17, 44). Many reports suggest that Rcs system could sense alterations of cell envelop (such as osmotic shock) and metal signals (Zn^{2+} concentration) to positively control the genes involved in CPS synthesis and negative control the genes involved in flagellar synthesis, motility, and chemotaxis suggesting the Rcs system regulate the biofilm formation (14, 21, 40). In *E. coli*, curli encoding by *csgBADEFG* were necessary for biofilm formation. Rcs system negatively controlled the expression of *csgBADEFG* to affect the biofilm formation indirectly (40).

Two homologous genes, *rmpA* and *rmpA2*, which have been reported to be

associated with the mucoidy phenotype of *K. pneumoniae* were also identified on pLVPK (5). The RmpA encoding gene firstly reported by Nassif et al. (24), is a positive regulator for the production of K2 CPS in *K. pneumoniae* and colonic acid, Group 1 CPS in *E. coli* (24). In *E. coli*, *rmpA* gene has been shown to be able to complement the deficiency of *rcsA*-*lon* mutation, but not an *rcsA* mutation, indicating that *rmpA* expressed an *rcsA*-like activity (24). The *rmpA2*, which was named by the sequence identity with *rmpA*, has lately been reported (1, 42). The major difference between these two gene products is that the RmpA2 has an extended N-terminal region (42). In the previous studies, RmpA2 as well as RcsB were able to confer *E. coli* JM109 or HB101 the ability to produce *Klebsiella* K2 capsular in the presence K2 *cps* gene cluster (1, 24). Furthermore, RmpA2 could directly bind to the *cps* gene promoter via its C-terminal DNA binding motif. We have also shown that the expression of *rmpA2* was negatively autoregulated (18).

In this study, the relationship of pLVPK and the types of diseases was investigated. In addition, we attempt to elucidate the functional role of RmpA by using comparative analysis of the properties of wild type, the *rmpA* deletion mutant, and *rmpA2* mutant. Finally, the putative promoters of *rmpA*, *rmpA2* and *cps* genes were cloned respectively upstream to the promoterless *lacZ* to demonstrate a differential expression of *rmpA* and *rmpA2* in *K. pneumoniae* CG43.

Materials and methods

1. Bacterial strains, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1 and Table 2. *K. pneumoniae* CG43 is a clinical isolate recovered from a patient in the Chang Gung Memorial Hospital, Linkou and *K. pneumoniae* NTUH-K2044 of K1 serotype, was isolated at National Taiwan University Hospital. Other clinical isolates of *K. pneumoniae* used were recovered from different tissue specimens of patients with a variety of infections at the Veteran General Hospital, Taipei, from 1991 to 1998. The bacteria were propagated at 37℃ in Luria-Bertani (LB) broth or the medium supplemented with appropriate antibiotics which include kanamycin (25 μg/ml), ampicillin (100 μg/ml) and tetracycline (20 μg/ml).

2. Genomic DNA extraction and plasmid typing

Genomic DNAs of 127 *Klebsiella pneumoniae* isolates were extracted by the method of Kado and Liu (15). Cells from a single colony scraped from an agar plate were suspended in 50 μl lysis buffer (in 3% SDS, 50 mM Tris-HCl, pH 12.6). The mixture was incubated at 65℃ for 30 min and extracted with unbuffered phenol-chloroform (1:1). The genomic DNAs were separated by pulsed field gel electrophoresis (PFGE) (Rotaphor® TypV, Biometra).

3. Dot blotting hybridization and PCR detection of the *rmpA*, *terA*, *iutA*, *iroB*, and *silS* genes

The genomic DNAs were transferred to Hybond-N^+ membrane (Amersham-Pharmacia, Piscataway, NJ) respectively by dot blotting. The membrane

was then subject to denaturation, and hybridization with the appropriate probe for 16 h at 65℃. Finally, the signals on the membrane detected with CDP-star reagent (Amersham-Pharmacia, Piscataway, NJ**).** The specific primers used for PCR detection of *iutA*, *iroB*, *silS*, and *terA*, are listed in table 3.

4. Assessment of the siderphore biosynthesis activity

The *K. pneumoniae* liver abscess isolates were cultured in M9 minimal medium overnight, and 100 μl of bacteria diluted 1/100 in iron deficient medium containing 0.2 mM 2,2-dipyridyl in M9, was incubated at 37℃ for 30 min. after centrifugation 6000 rpm for 5 min, the supernatant was then mixed with chrome azurol S (CAS) solution containing in 100 ml with 1.5 ml iron solution (1 mM $FeCl_3 \cdot 6H_2O$), 7.5 ml 2 mM aqueous CAS solution, and 4.307 g piperazine dissolved in 6.25 ml 12 M hydrochloric acid (35).

5. Definition of mucoviscosity and colony size of 19 liver abscess clinical isolates

Strains with the mucoviscosity phenotype were defined as being virulent. To determine mucoviscosity phenotype, a standard bacteriologic loop was used to stretch a mucoviscous string from the colony. Strains were defined as mucoid positive and large colony when viscous strings were > 0.5 cm (a positive string test result) and colony diameter were > 3 mm (a large colony result). We have previously noted that the degree or strength of viscosity seems to be slightly diminished for colonies at their first subculture from frozen isolates, but their phenotype is well recovered at the second subculture and is in accordance with that from primary culture plates. Therefore, all isolates required 2 subcultures to get well-grown colonies before rmucoviscosity testing.

6. RT-PCR analysis

Total RNA was isolated from the *K. pneumoniae* cells in mid-log phased (OD_{600} = 0.6～0.8) by extraction with the 1 ml TRI reagent (Molecular Research Center, Cincinnati, OH). Contaminating DNA was eliminated from the RNA samples with RQ1 RNase-Free DNase (Promega, Madison, WI). The cDNA products were amplified with the specific primers rmpA07/rmpA08.

7. Bioinformatics analysis

Homology search analysis and gene annotation were performed with BLAST analysis in NCBI (http://www.ncbi.nlm.nih.gov) and VectorNTI (Invitrogen Vector NTI™ Advance). Promoter prediction was carried out by website (http://www.softberry.com/all.htm).

8. Recombinant DNA technique

The recombinant DNA experiment was carried out by standard procedures as described (33). Plasmid DNA was prepared by VIOGENE Miniprep Kit (Gene-Spin ^{TM}-V^2). Restriction endonucleases and DNA modifying enzymes were purchased form MBI (Fermentas, Hanover, MD), and were used according the recommendation of the suppliers.

9. Construction of the gene-deletion mutant and complement strains

The individual deletions in *rmpA* were introduced into chromosome of *K. pneumoniae* CG43 by allelic exchange strategy. The primer sets used for PCR amplification are listed in Table 3. Approximately 1000 bp sequence flanking both sides of the deleted region were cloned into plasmid pKAS46 (18), a suicide vector containing *rpsL*, which allows positive selection with streptiomycin for the loss of the

vector, to generate an in frame deletion plasmid, pRmpA1-4. The resulting plasmids were then mobilized to *K. pneumoniae* CG43S3 through conjugation from *E. coli* S17-1 λ*pir*. The transconjugants, carrying with pRmpA1-4 integrated in the chromosome via homologous recombination, were selected by ampocollin and kanamycin on minimal medium. One of colonies was grown in LB at 37℃ for overnight and then spread onto a LB plate containing 500 μg/ml streptomycin. The streptomycin-resistant and kanamycin sensitive colonies were selected and deletion of *rmpA* was verified by PCR and Southern blotting with a gene specific probe. The resulting bacteria with mutation in *rmpA* were named *rmpA*$^-$.

10. Construction the complement strains

A 1.1 kb fragment including the promoter and coding sequence was amplified with the specific primers rmpA05/ rmpA06 and then cloned to yT&A (PCR cloning vector), named pRmpA02. A *Hin*dIII/*Eco*RI fragment of pRmpA02 was subcloned to pRK415 and resulted in pRmpA03.

11. Southern blotting analysis

Chromosome DNA was prepared from overnight cultures of *K. pneumoniae* CG43S3 or the derived bacteria grown in LB medium. After the DNA digested with restriction endonuclease *Eco*RV, the DNA fragments were resolved on 1% agarose gels by electrophoresis and transferred to Hybond-N$^+$ membrane (Amersham-Pharmacia, Piscataway, NJ). Finially, the membranes were hybridized with *rmpA* probes to confirm the mutation of *rmpA*$^-$.

12. Quantification of biofilm formation

Biofilm formation was assessed by the ability oh the cells to adhere to the walls

of 96-well microtire dishes made of PVC (TPP 96 flat) with some modification of the reported protocol. Essentially, the indicator medium (200 µl/well) contained an aliquot of 1:10 diluted overnight bacteria culture and the plat was incubated at 37℃ for 36 h for biofilm formation. The unadherent bacteria was washed triply with 200 µl ddH_2O and then 200 µl of 1% crystal violet (CV) was added to each well. After the plate was placed at room temperature for 30 min, ddH_2O was utilized to wash the plate for three times again. Finally, the CV-strained biofilm was solubilized in 200 µl of 0.1% SDS and absorbance determined at OD_{595nm} using spectrophotometer (ELx800, BIO-TEK).

13. Extraction and quantification of CPS

CPS was extracted by using the method described (7). Briefly, bacteria were collected from 500 µl of culture medi and mixed with 100 µl of 1% Zwittergent 3-14 detergent (Sigma-Aldrich, Milwaukee, WI) in 100 mM citric acid (pH2.0). The mixture was incubated at 50℃ for 20 min. After centrifugation, 250 µl of the supernatant was transferred to a new tube and CPS was precipitated with 1 ml of absolute ethanol. The pellet was then dissolved in 200 µl distilled water and a 1,200 µl of 12.5 mM borax (Sigma-Aldrich, Milwaukee, WI) in H_2SO_4 was added. The mixture was vigorously vortexed, boiled for 5 min, cooled, and then 20 µl 0.15% 3-hydroxydiphenol (Sigma-Aldrich, Milwaukee, WI) was added and the absorbance at 520 nm was measured. The uronic acid content was determined from a standard curve of glucuronic acid (Sigma-Aldrich, Milwaukee, WI) and expressed as µg per 10^9 CFU.

14. Construction of the reporter gene fusion

To construct P_{rmpA} fusion plasmids, the PCR product amplified with primer pair

rmpAp01/rmpAp02 (Table 3) was cloned into yT&A (PCR cloning vector), and resulted in the plasmids pRmpA04. The *BamH*I/*Bgl*II fragment of pRmpA04 was then subcloned in front of the plasmidless *lacZ* gene, and resulted in the plasmid pRmpAZ15.

15. β-galactosidase activity assay

β-galactosidase was assayed according to the method of Miller (22). The bacteria in the early or late logarithmic growth phase (optical density at 600 nm 0.4 or 0.7) were taken 100 μl, and mixed with 900 μl Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol), 17 μl of 0.1% SDS and 35 μl chloroform and incubated for 10 min at 30℃. Subsequently, 200 μl of 4 mg/ml *o*-nitrophenyl-*β*-D-galactopyranoside (ONPG) was added and the mixture vortexed for 10 s, then incubated at 30℃ until yellow color was apparent. 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 OD_{420}. One unit of β-galactosidase is defined as the hydrolysis of 1 nmol ONPG per min per mg protein.

Results

Part 1 : Prevalence of pLVPK in *Klebisella pneumoniae* clinical isolates

1-1. Plasmid typing of 127 *K. pneumoniae* clinical isolates.

To identify the association of large plasmid and diseases, a total of 127 *K. pneumoniae* isolates respectively from liver abscess (TVH1-19), bile (TVH20-28), urine (TVH29-44), ascites (TVH45-58), sputum (TVH-59-78), wound (TVH79-102), and blood (TVH103-128) were collected. By using PFGE analysis, 106 clinical isolates were found to harbor large plasmids of at least 200 kb in size (Fig. 1). Among the 106 clinical isolates, 19 (100%) liver abscess isolates, 8 (88.89%) bile isolates, 12 (75%) urine isolates, 11 (78.57%) ascites isolates, 16 (70.37%) sputum isolates, 19 (79.17%) wound isolates, and 12 (80.77%) blood isolates were identified. This indicated that many of the *K. pneumoniae* clinical isolates harbor large plasmid and all of liver abscess isolates carry large plasmid. In addition, some of the isolates carry more than one large plasmid, such as *K. pneumoniae* TVH6, TVH9, and TVH16.

1-2. Distribution of *rmpA*, *terA*, *iutA*, *iroB*, and *silS* genes in clinical isolates.

Genomic DNAs of 127 *Klebsiella pneumoniae* isolates were extracted by standard techniques, and the DNAs used for dot blotting detection are *rmpA*, *terA*, *iutA*, *iroB*, and *silS* genes. As shown in Fig. 2 and Table 4, many of the isolates harbored *rmpA*, *terA*, *iutA*, *iroB*, and *silS* genes. Interestingly, all liver abscess isolates appear to carry the genes, *rmpA*, *iutA*, and *iroB*. In order to further confirm the results of the dot blotting, PCR analysis of 19 *K. pneumoniae* liver abscess isolates were performed. As shown in Fig. 3, the PCR products of expected size were obtained in all

the liver abscess isolates which further confirmed the finding of the dot blotting analysis. But the other isolates carried the 5 genes on the genome were respectively 33.33 % (bile), 31.25 % (urine), 21.43 % (ascites), 42.11 % (sputum), 45.83 % (wound), and 42.31 % (blood). These results suggested a close association of pLVPK with liver abscess isolates.

1-3. The liver abscess isolates harbor pLVPK-like plasmid.

To determine whether pLVPK plasmid is indeed carried on all the liver abscess isolates, the plasmids of these liver abscess isolates were isolated and resolved by PFGE, and the gel was subjected to Southern blotting analysis with an *rmpA* probe. As shown in Fig. 4, *rmpA* gene could be detected on both chromosome and the large plasmid of TVH10 and TVH16. These plasmids appeared to contain *rmpA* except those of TVH 5, TVH 13, and TVH 19. However, the discrepancy is not consistent with the finding of the PCR detection (Fig. 2). But these results indicated the pLVPK-like plasmids identified in liver abscess isolates may play a role in the *Klebsiella* infection process.

1-4. The siderophore biosynthesis activity of the *K. pneumoniae* isolates.

Iron is generally an essential element for all the microorganisms. To survive under an iron-deficient condition, bacteria produce siderophores which are low molecular mass of iron chelating compounds capable of binding iron with high affinity (41). Since both enterobactin and aerobactin iron acquisition gene clusters are contained in pLVPK, siderophore synthesis activity of the isolates were determined using CAS assay solution and compared. As shown in Fig. 5, comparing to *K. pneumoniae* CG43-101 curing of pLVPK, most of the liver abscess isolates produced siderophore in the iron deficient broth. However, no siderophore synthesis activity

could be detected in TVH13 and TVH15, as well as the plasmidless isolates TVH33, and TVH34.

1-5. The capsular polysaccahride biosynthesis activity and biofilm formation of the *K. pneumoniae* isolates.

In previous study, mucoid phenotype of *K. pneumoniae* was a large plasmid-encoded virulence factor (24, 25). As a reporter for pLVPK activity, biofilm formation activity of these isolates were also determined and compared. As shown in Fig. 6 and Table 5, all the liver abscess isolates displayed different levels of mucoidy, implying that pLVPK or the pLVPK-like plasmids affected differently the level of capsular polysaccharide biosynthesis. As shown in Fig. 7, biofilm formation capability of isolates appeared to be variable, which revealed that, in addition to the virulent plasmid, other factors encoding by the genes reside in bacterial chromosome also play roles in regulation of the biofilm formation and biosynthesis of the capsular polysaccharide.

Part 2 : Identification of the regulatory role of RmpA

2-1. RT-PCR demonstrated the expression of *rmpA* in *K. pneumoniae* CG43.

In previous study, the *rmpA2* were transcribed in *K. pneumoniae* CG43, but we didn't identity the present of *rmpA* gene. After pLVPK sequencing, we confirmed the existence of *rmpA* and *rmpA2* genes. To demonstrate expression of the rmpA encoding gene in *K. pneumoniae* CG43 and the derived *rmpA2*$^-$, total RNA were isolated and subject to RT-PCR and Southern blotting hybridization. As shown in Fig. 8 the *rmpA* transcript could be detected in both the *K. pneumoniae* CG43 and *rmpA2*$^-$, including an active expression of the *rmpA* gene.

2-2. Biological role of RmpA.

To investigate the function of RmpA, the *rmpA* deletion mutant was constructed by the allelic exchange and the deletion confirmed by using Southern blotting hybridization. The deleted DNA fragments included about 527 bp in *rmpA*. As shown in Fig. 9, 800 bp and 1327 bp signals were obtained respectively in the *rmpA*$^-$ and *K. penumoniae* CG43S3. The 4083 bp signals in the *rmpA*$^-$ and *K. penumoniae* CG43S3 were the fragments including the *rmpA2* gene. Both *rmpA*$^-$ and *rmpA2*$^-$ strain displayed similar colony in size with the *K. pneumoniae* CG43S3. In addition, the growth rate of *rmpA*$^-$ and *rmpA*$^-$*rmpA2*$^-$ appear to grow faster than both wild type and *rmpA2*$^-$ in LB medium, but not in M9 medium (Fig. 10A). Mucoidy of *rmpA*$^-$ and *rmpA2*$^-$ were significantly reduced as assessed using low-speed centrifugation. Introducing the plasmid carrying either *rmpA* or *rmpA2* into the mutants appeared to compensate for the deficiency (Fig. 10B).

The amount of CPS produced in wild type and mutant strains were further quantified by measuring the uronic acid content, which serves as an indicator of

Klebsiella K2 CPS. As shown in Fig. 10C, the *rmpA*$^-$, as well as the *rmpA2*$^-$, produced much less CPS than wild type strain and the deficiency could be restored by the complements. In previous studies, expression of CPS has been correlated with the regulation of biofilm formation. As shown in Fig. 11, capability of biofilm formation in *rmpA*$^-$ and *rmpA2*$^-$ like that in *rcsB*$^-$ were increased. Transformation of the plasmid carrying *rmpA* or *rmpA2* into the mutants appeared to be able restore the capability of biofilm formation. These results suggested that RmpA as well as RmpA2 could positively regulate the CPS biosynthesis.

2-3. RmpA regulates positively the expression of $cps_{orf1\text{-}2}$ and $cps_{orf16\text{-}17}$.

The reducing production of CPS by deletion of *rmpA* or *rmpA2* suggested a regulatory role on *cps* genes expression, while the three *lacZ* reporter fusion constructs (Fig. 12A), $P_{orf1\text{-}2}$, $P_{orf3\text{-}15}$, and $P_{orf16\text{-}17}$, were analyzed in the *rmpA*$^-$. Activity of $P_{orf1\text{-}2}$ and $P_{orf16\text{-}17}$ appeared to decrease 30 % in comparing with wild type bacteria, but in *rmpA2*$^-$ was only reduced 20 %. Compared the activity of $P_{orf3\text{-}15}$ in wild type with either *rmpA*$^-$ or *rmpA2*$^-$ strain, no obvious change of $P_{orf3\text{-}15}$ activity in *rmpA*$^-$ or *rmpA2*$^-$ strains was noted (Fig. 12B).

2-4. Expression of *rmpA* and *rmpA2* in *K. pneumoniae* CG43.

To analysis the expression of *rmpA* and *rmpA2*, the putative promoters of *rmpA* and *rmpA2* were isolated and respectively fusion with the promoterless *lacZ* in pLacZ15, and be transformed into *K. pneumoniae* LacZ16. As shown in Fig. 13, activity of P_{rmpA2} appeared to be much higher than P_{rmpA}. As shown in Fig. 14, 49.2% identity in nucleotide sequence lower than that of coding region. Analysis the putative promoters of *rmpA* and *rmpA2* showed Fur binding box and RcsAB binding box on P_{rmpA}. Alignment of the putative RcsA-RcsB binding site with 40-bp region of the

Erwinia amylovora ams (amylovoran biosynthesis), *E. coli cps* (16), and the *rmpA* promoter using VectorNTI (Invitrogen Vector NTI™ Advance) identified a 28 bp region of RcsAB box. To examine whether RcsAB affect *rmpA* expression, P_{rmpA} activity was analyzed in either *rcsA*$^-$ or *rcsB*$^-$. As shown in Fig. 15, *rcsB* deletion appeared to reduced P_{rmpA} activity to half of the wild type strain. Interestingly, the deletion of *rcsB* conferred no effect on the activity of P_{rmpA2}. But no obvious change of P_{rmpA} and P_{rmpA2} in *rcsA*$^-$ was noted. This indicated a differential regulation of the expression at *rmpA* and *rmpA2*.

Discussion

Most of the work on pathogenesis of *K. pneumoniae* has been limited to studying the polysaccharidic capsule, which protect against serum bactericidal activity and phagocytosis. Recently, many reports indicated the large plasmid is associated with virulence. In our study, many *K. pneumoniae* clinical isolates were also shown to be carried large plasmids. Furthermore, all liver abscess isolates (19 strains) and some other isolates (39 strains) could carry the pLVPK-like plasmid.

In Southern blotting analysis, the large plasmid in many liver abscess isolates carried *rmpA* gene could be detected except those of *K. pneumoniae* TVH 5, TVH 13, and TVH 19. However, the discrepancy, which is not consistent with the finding of the PCR detection (Fig. 3), is likely due to the concentration of the plasmids was too low to be detected. But the *rmpA* gene could be detected on both chromosome and the large plasmid of *K. pneumoniae* TVH10 and TVH16. To analyze sequence around the *rmpA* gene on pLVPK showed many insertion sequences (IS) around the *rmpA* gene suggesting *rmpA* gene could be located on a pathgenocity island by horizontal transfer to chromosome, such as SHI-2 island (23). The possibility is being investigated.

All liver abscess isolates carried the pLVPK-like plasmid suggesting the plasmids on those isolates have similar ability with pLVPK. In CAS assay, many liver abscess isolates could synthesis siderophore, except *K. pneumoniae* TVH13 and TVH15, indicating that siderophore synthesis pathway in some of the pLVPK carrying isolates may be deficient. In sedimentation test, all the liver abscess isolates containing pLVPK-like plasmid displayed more mucoid than the plasmidless isolates *K. pneumoniae* TVH33, and TVH34, suggesting the large plasmid is associated with the mucoid phenotype. Biofilm formation capability of isolates appeared to be

variable, which revealed that, in addition to the virulent plasmid, other factors encoding by the genes reside in bacterial chromosome also play roles in regulation of the biofilm formation, such as *rcsA* and *rcsB* reported to repress the biofilm formation (40).

Fang et al. has reported that the strains carrying *rmpA* were significantly associated with the hypermucoviousity phenotype and purulent tissue infections, such as liver abscess (20, 48). In the study, we have shown that the *rmpA* gene located on pLVPK or pLVPK-like plasmid could be correlated with liver abscess suggesting the RmpA located on pLVPK could regulate other virulence factors involved in the *K. pneumoniae* pathogenesis.

To investigate the functional role of *rmpA*, the *rmpA* deletion mutant was generated. The *rmpA* deletion mutant reduced the CPS production and increased the growth rate. Although *rmpA2*⁻ also affected negatively the CPS production, no effect on the bacterial growth indicating a differential role of the two transcription factors suggesting RmpA play more important role than RmpA2 on CPS synthesis. Nevertheless, analysis of expression of the *cps* gene revealed that RmpA as well as RmpA2 appeared to reduce the expression of *cps* gene *orf1-2* and *orf16-17*. Analysis of the ORF showed that *orf1* is a homolog of *S. typhimurium* LT2 *galF* and *E. coli galU*, which encodes the enzyme UDP-glucose pyrophosphorylase that regulates the supply of UDP-galactose and UDP-glucose, two major precursors for the biosynthesis of CPS (45, 46). While the *orf16* and *orf17* encoding respectively by the *manC* and *manB* genes, which encode mannoas-1-phosphate guanylytransferase (GDP-mannose pyrophosphorylase) and phosphomannomutase respectively that have been reported to be involved in the biosynthesis of mannose (2, 45, 46).

Notably, the *cps* expression was regulated by many factors, such RcsAB and RmpA2 (18). Previous study was determined that the *E. coli* co-transformed with

Klebsiella K2 *cps* gene cluster, *rmpA*, and *rcsB* could produce *Klebsiella* K2 CPS. But *E.coli* co-transformed with only K2 *cps* gene cluster and *rmpA* could not produce *Klebsiella* K2 CPS (24). Analysis of RmpA revealed a LuxR-like C-terminus as RcsA (21, 24), implying that RmpA in analogy with the role of RcsA interacts with RcsB to activate *cps* gene in *K. pneumoniae*. However, this hypothesis awaits to be investigated.

The activity of P_{rmpA} and P_{rmpA2} after time course showed that the expression of RmpA and RmpA2 were under different regulation. Analysis of the putative promoter of *rmpA* was found a putative RcsAB box and Fur box, but not *rmpA2*, suggesting the expression of *rmpA* could be regulated by RcsAB complex and Fur. This hypothesis was demonstrated by analysis the activity of P_{rmpA} in wild type and *rcsB*$^-$ strain. But comparison of the activity of P_{rmpA} or P_{rmpA2} under iron-limit and iron-rich medium were not obvious difference. We presumed that *rmpA* belonged to Rcs regulon is under the phosphoryl-RcsB control, but not *rmpA2*. To further identify the RcsAB binding region on P_{rmpA}, the deletion fragment of P_{rmpA} could be fused with LacZ reporter and analyzed.

In previous study, the RmpA2 could be autoregulated (18). Whether the RmpA could be autoregulated or cross-regulated with RmpA2, the activities of P_{rmpA} and P_{rmpA2} in *rmpA*$^-$ and *rmpA2*$^-$ strain were analyzed. The activities of P_{rmpA} in LacZ16, *rmpA*$^-$ and *rmpA2*$^-$ strain or P_{rmpA2} in LacZ16, *rmpA*$^-$ and *rmpA2*$^-$ strain were no obvious difference (Data not show) suggesting RmpA or RmpA2 could not directly cross-regulate the expression of RmpA or RmpA2 and RmpA could not be autoregulated. However, this hypothesis awaits to be investigated.

In conclusion, as shown in Fig. 16, we propose a regulatory circuit that RmpA belong to Rcs regulon, but not RmpA2. RcsB activates the expression of *rmpA*. The RmpA could interact with RcsB to activate CPS synthesis. But RmpA2 could directly

bind to *cps* genes without RcsB. In addition, expression of *rmpA* could be activated with osmotic shock or metal ion. The signal to activate the expression of *rmpA2* is unknown. However, this pathway is yet to be identified.

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Strain	Genotype or relevant property	Reference or source
E. coli:		
NovaBlue(DE3)	*endA1 hsdR17*($rk_{12}^{-}mk_{12}^{+}$) *supE44 thi-1 recA1 gyrA96 relA1 lac*[F′ *pro AB lac*^{q}Z△M15:: Tn*10*](DE3);Tet^{r}	Novagen
JM109	*RecA1 supE44 endA1 hsdR17 gyrA96 rolA1 thi* △ *(lac-proAB)*	Laboratory stock
S17-1λ*pir*	Tp^{r} Sm^{r} *recA, thi, pro, hsdR*$^{-}$*M*$^{+}$ [RP4-2-Tc::Mu:Km^{r}Tn*7*] (*pir*)	De Lorenzo *et al.*, 1994
ICC188λ*pir*	△*(ara-leu) araD* △*lac×74 galE galK phoA20*	Taylor *et al.*,1989
K. pneumoniae:		
CG43	Clinical isolate of K2 serotype	Laboratory stock
CG43-101	Curing the large plasmid pLVPK from CG43	Laboratory stock
CG43-S3	△*rspl*, Sm^{r}	Laboratory stock
rmpA^{-}	CG43-S3△*rmpA* Sm^{r}	This study
rmpA2^{-}	CG43-S3△*rmpA2* Sm^{r}	Laboratory stock
rcsA^{-}	CG43-S3△*rcsA* Sm^{r}	This study
rcsB^{-}	CG43-S3△*rcsB* Sm^{r}	Laboratory stock
LacZ16	CG43S3△*lacZ* Sm^{r}	Laboratory stock
rmpA^{-}*Z16*	LacZ16△*rmpA* Sm^{r}	This study
rmpA2^{-}*Z16*	LacZ16△*rmpA2* Sm^{r}	Laboratory stock
rcsA^{-}*Z16*	LacZ16△*rcsA* Sm^{r}	This study
rcsB^{-}*Z16*	LacZ16△*rcsB* Sm^{r}	Laboratory stock

Table 1. Bacterial strains used and constructed in this study

Plasmids	Relevant characteristic	Source or reference
yT&A vector	PCR cloning vector, Ap^r	Sigma
pKAS46	Suicide vector, Ap^r Km^r	Novagene
pRK415	Shuttle vector, *mob*$^+$, Tc^r	Laboratory stock
pLacZ15	A derivative of pYC016, containing lacZ as a reporter, Cm^r	Laboratory stock
pRmpA1-4	pKAS46 carrying a △*rmpA* fragment	This study
pRmpA02	A 1.1 kb PCR product of the *rmpA* locus with the putative promoter cloned into yT&A	This study
pRmpA03	A *Hin*dIII/*Eco*RI fragment of pRmpA02 cloned into the pRK415	This study
pRmpA04	A 0.5 kb PCR product of the *rmpA* putative promoter region cloned into yT&A	This study
pRmpAZ15	A *Bam*HI/*Bgl*II fragment of pRmpA04 cloned into the pLacZ15	This study
pRmpA2Z15	A 0.5 kb fragment of the rmpA2 putative promoter region cloned into the pLacZ15	Laboratory stock
porf1Z15	A 0.8 kb fragment of the *cps orf1-2* promoter region cloned into the pLacZ15	Laboratory stock
porf3Z15	A 0.9 kb fragment of the *cps orf3-15* promoter region cloned into the pLacZ15	Laboratory stock
porf16Z15	A 0.4 kb fragment of the *cps orf16-17* promoter region cloned into the pLacZ15	Laboratory stock
pYC084	A XbaI/HindIII fragment cloned into the pRK415	Laboratory stock
pAEE40	A 7 kb fragment of pLVPK cloned into pUC18	

Table 2. Plasmids used and constructed in this study

Primer	Sequence
rmpA1p-01	5'-GTCGGATCCATCGCCAAATA-3'
rmpA1p-02	5'-CAG TCA ACA CGG TGC TTT ACA T-3'
rmpA01	5'CTCTAGATAAGGCGGCCTTCG-3’
rmpA02	5’-ATAGTCGACGCTATGCTTTACA-3’
rmpA03	5’-TGGTCGACGAAAGATGGCTC-3’
rmpA04	5’-ATGAGCTCAATGTATGCCAAGG-3’
rmpA05	5’-GGCCGAAAGCAGTTAACTG -3’
rmpA06	5’-TTACCTAAATACTTGGCATGA GC -3’
rmpA07	5’- GGCCGAAAGCAGTTAACTG -3’
rmpA08	5’- TTACCTAAATACTTGGCATGAGC -3’
Yu-05	5’-CCTTCACATCCCCTCCCCTT -3’
Yu-06	5’-GTCGGATCCATCGCCAAATAA-3’
iutA01	5'- GAACAGCACAGAGTAGTTCA -3'
iutA02	5'- CAGTACACTGAAAACAAGATTG -3'
iroB01	5'- CTTTCTGTACCATCGCGATC -3'
iroB02	5'- TCATTCTTCAGCGAAGAGAT -3'
silS01	5'- ATCAGCCTGTCCACGATACT -3'
silS02	5'- GTCAAACATGACCCTGTCAG
terA01	5'- GGGGGCAATGCCCCTTTAATAGCT -3'
terA02	5'- AGACGGGCAATCGCACACAG -3'

Table 3. Primers used in this study

Origin of isolation (No. of isolates)	% of isolates carry large plasmid (No. of isolates)	% of isolates carry *rmpA* (No. of isolates)	% of isolates carry *iutA* (No. of isolates)	% of isolates carry *iroB* (No. of isolates)	% of isolates carry *silS* (No. of isolates)	% of isolates carry *terA* (No. of isolates)	% of isolates carry *mpA, iutA, iroB, sils,* and *terA* (No. of isolates)
liver abscess (19)	100 (19)	100 (19)	100 (19)	100 (19)	94.74 (18)	89.47 (17)	89.47 (17)
bile (9)	88.89 (8)	44.44 (4)	33.33 (3)	44.44 (4)	66.67 (6)	44.44 (4)	33.33 (3)
urine (16)	75 (12)	31.25 (5)	31.25 (5)	43.75 (7)	68.75 (11)	56.25 (9)	31.25 (5)
ascites (14)	78.57 (11)	28.58 (4)	21.43 (3)	21.43 (3)	71.43 (10)	21.43 (3)	21.43 (3)
sputum (19)	84.21 (16)	63.15 (12)	57.89 (11)	42.11 (8)	84.21 (16)	52.63 (10)	42.11 (8)
wound (24)	79.17 (19)	58.33 (14)	66.67 (16)	62.5 (5)	70.83 (17)	54.17 (13)	45.83 (11)
blood (26)	80.77 (21)	46.15 (12)	80.77 (21)	69.23 (18)	76.92 (20)	53.85 (14)	42.31 (11)
Total number	83.46 (106)	55.52 (70)	61.42 (78)	51.45 (73)	77.17 (98)	55.52 (70)	45.67 (58)

Table 4. Prevalence analysis of pLVPK in 127 *K. pneumoniae* clinical isolates

strains	mucoid	Colony size
TVH1	+	large
TVH2	+	large
TVH3	+	large
TVH4	+	large
TVH5	+	large
TVH6	+	large
TVH7	+	large
TVH8	+	large
TVH9	+	large
TVH10	+	large
TVH11	+	large
TVH12	+	large
TVH13	+	large
TVH14	+	large
TVH15	+	small
TVH16	+	large
TVH17	-	small
TVH18	+	large
TVH19	+	large

Table 5. String test and colony size of 19 liver abscess isolates.

Strains were defined as mucoid positive and large colony when viscous strings were > 0.5 cm (a positive string test result) and colony diameter were > 3 mm (a large colony result).

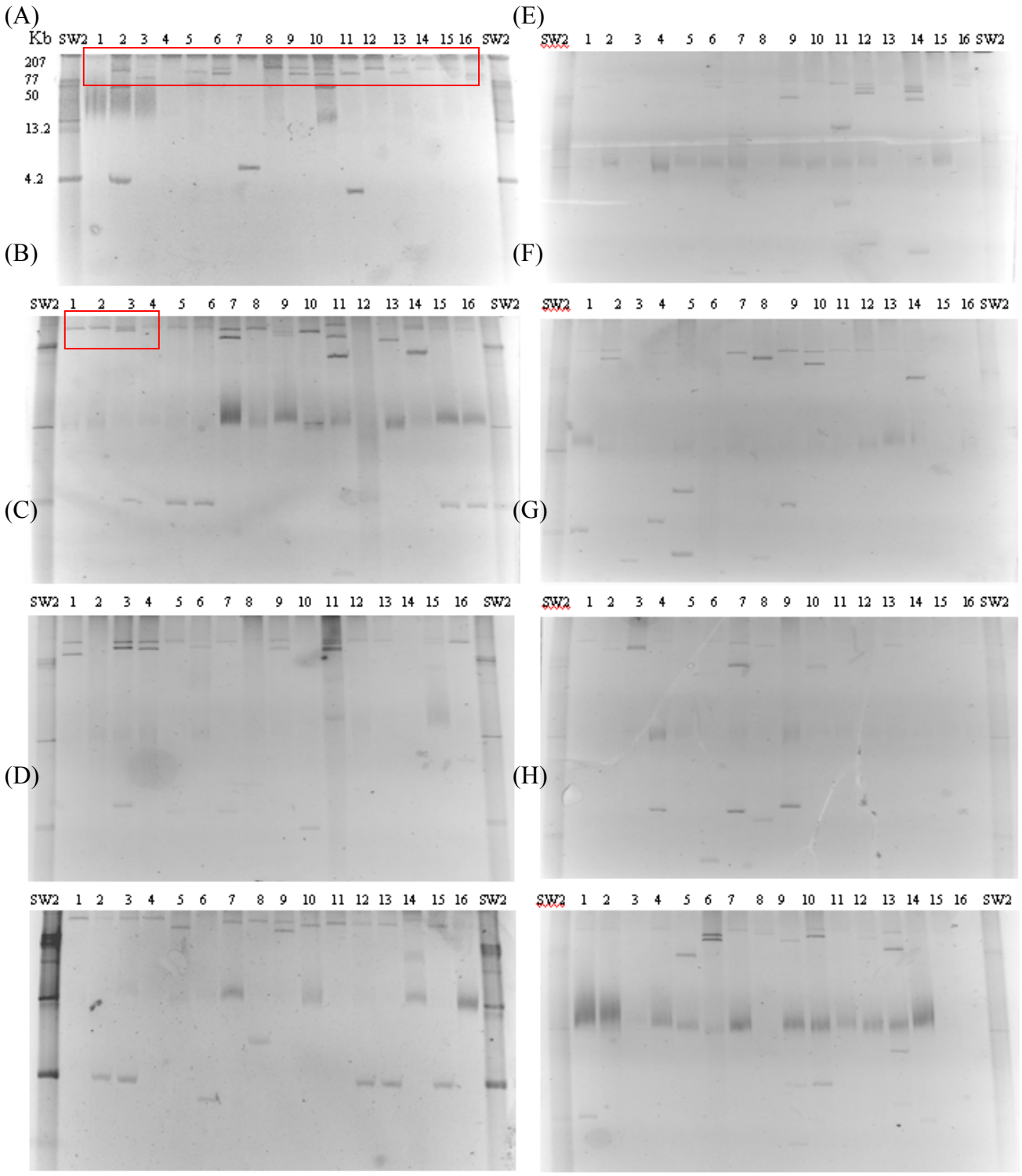


Figure. 1. Plasmid profile analysis of the 127 *K. pneumoniae* isolates by PFGE. Lanes 1 to 16 of (A)-(H), clinical isolates of *K. pneumoniae*; the size of *Erwinia* SW2 plasmids are marked. The boxed regions in (A) and (B) are liver abscess isolates.

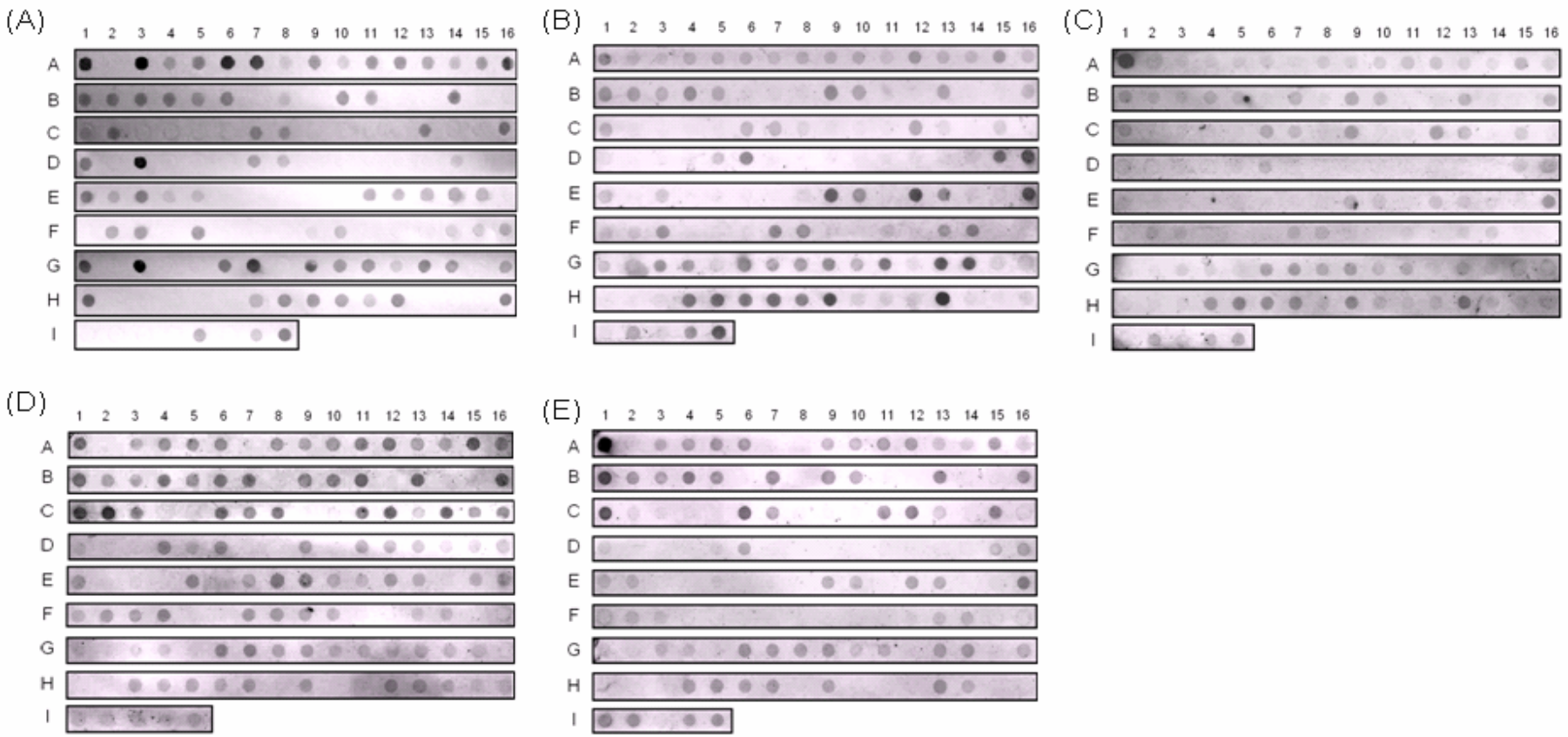


Figure 2. Dot-blotting analysis of 127 *K. pneumoniae* clinical isloates respectively probing with *rmpA*, *iutA*, *iroB*, *silS*, and *terA* gene.
The genomic DNAs were extracted and spotted on a Hybond-N^{+} membrane. The membrane was hybridized respectively with the probe specific to (A) *rmpA*, (B) *iutA*, (C) *iroB*, (D) *silS*, and (E) *terA*. The bacteria strains are: A-1, D-1,G-1 represent CG43S3; A-2, D-2, and G-2 represent CG43-101 (cured of pLVPK); A-3, D-3, and G-3 represent pAEE40 (the plasmid carry an *rmpA* gene); others are TVH 1 to TVH 128 in order. The boxed spots from A-4 to A-16 and B-1 to B-6 are the liver abscess isolates.

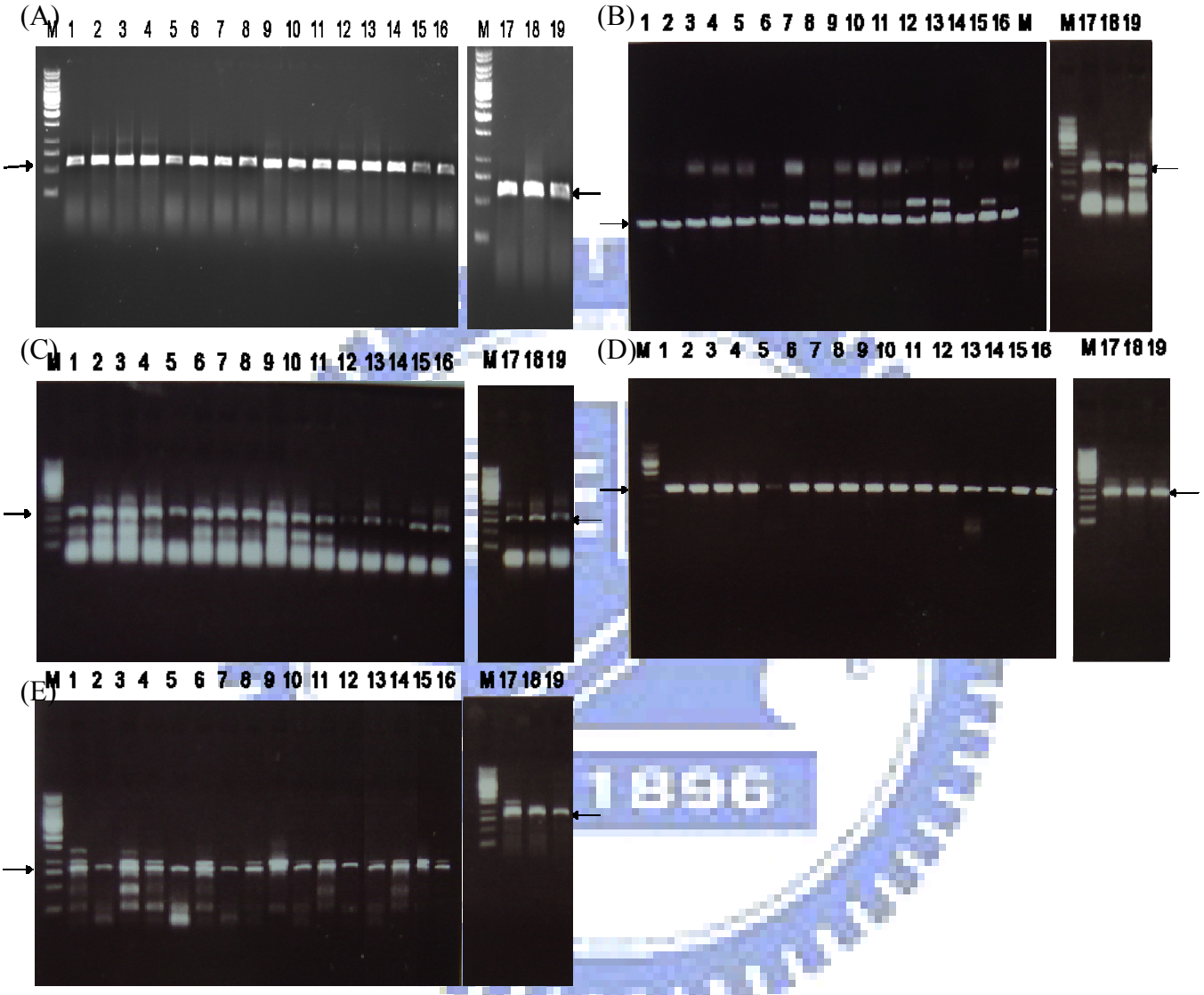


Figure 3. PCR detection of (A) *rmpA,* (B) *iutA* ,(C) *iroB* ,(D) *silS, and* (E) *terA* gene.

M: DNA molecular weight marker; PCR products in lanes 1 to 19 are *K. pneumoniae* TVH1 to *K. pneumoniae* TVH19; Arrows mark respectively the specific PCR products

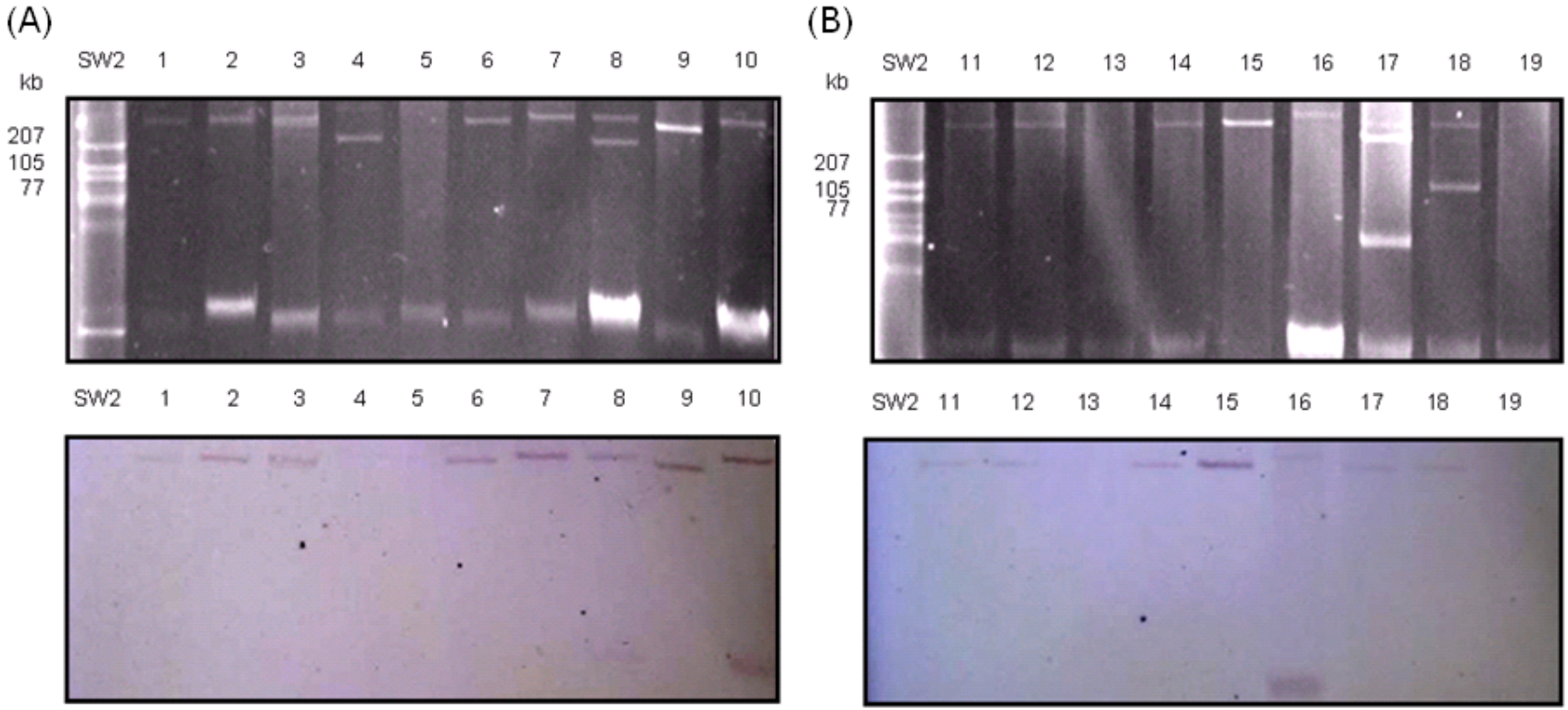


Figure 4. The presence of pLVPK in the liver abscess isolates.
(A) The plasmids isolated from *K. pneumoniae* liver abscess isolates were subjected to gel electrophoresis. Lane SW2, *Erwinia* SW2; lanes 1 to 19 are *K. pneumoniae* TVH1 to TVH19. (B) Southern blot hybridization with the probe specific to the *rmpA* gene.

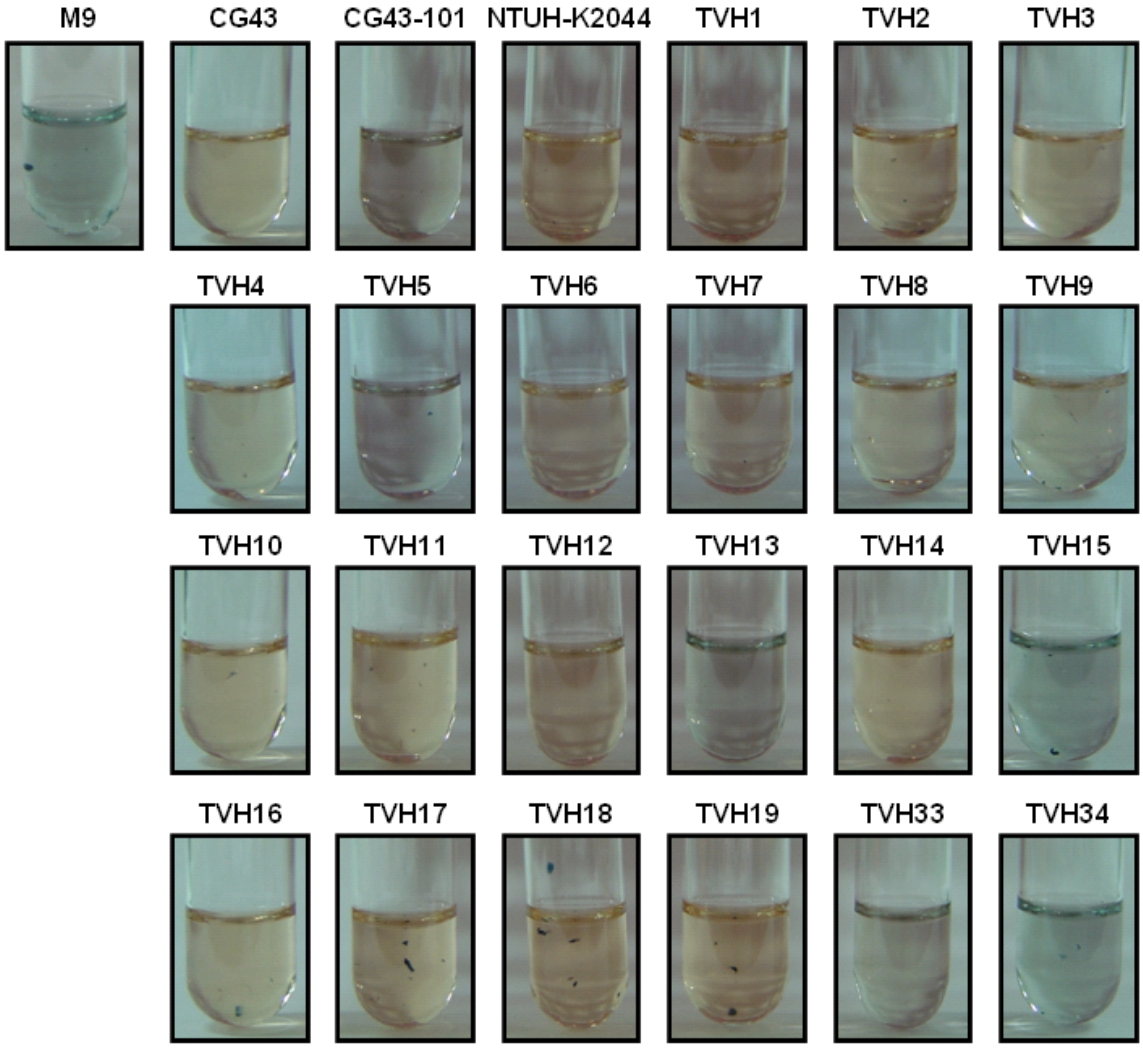


Figure 5. Siderophore synthesis activity of *K. pneumoniae* isolates in CAS solution.

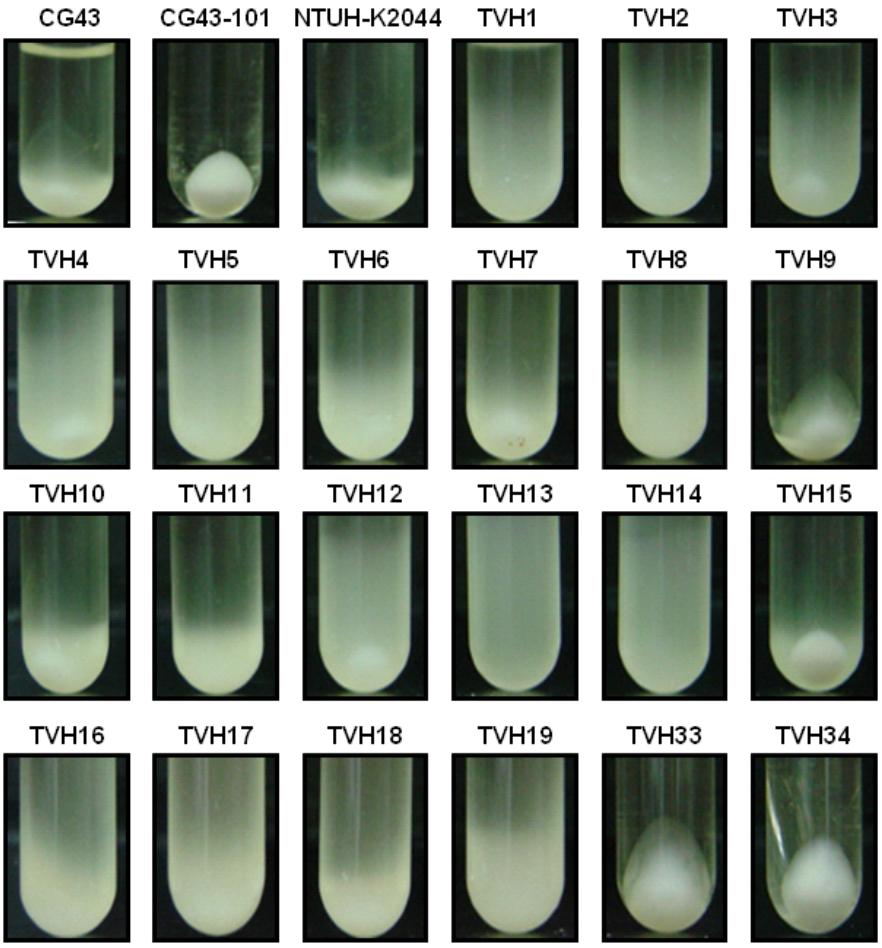


Figure 6. Sedimentation test of the *K. pneumonia* isolates.
K. pneumoniae TVH1 to 19 are liver abscess isolates. *K. pneumoniae* TVH33 and TVH34 are plasmidless strains.

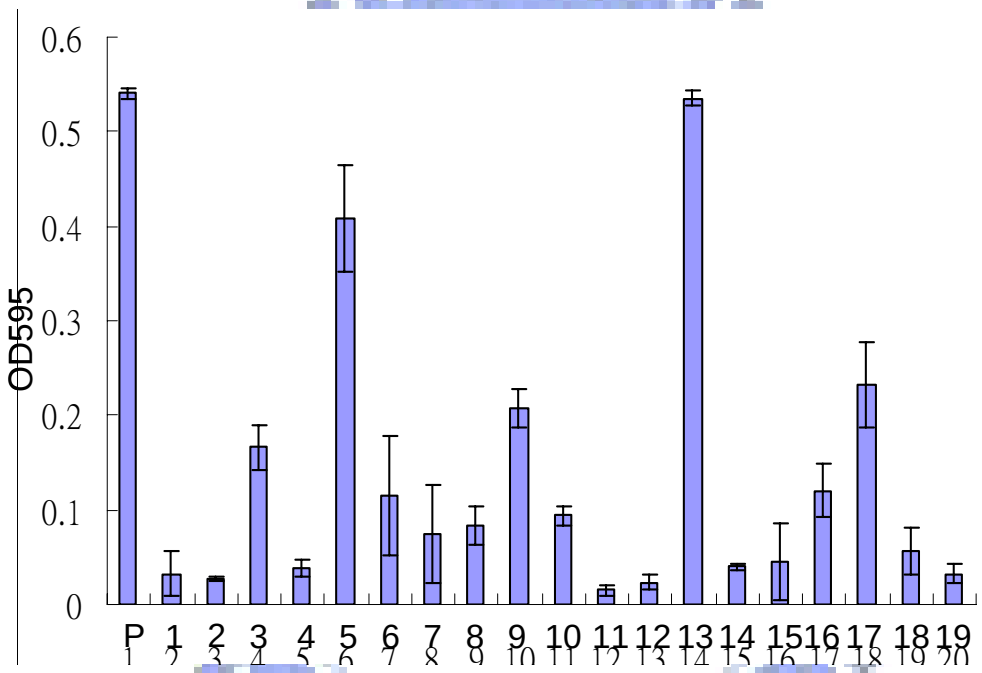


Figure 7. Biofilm formation of wild-type and *rmpA* mutant strain.
Lane P is *Pseudomonas aeruginosa* PAO1 as a positive control. Lanes 1 to 19 are *K. pneumoniae* TVH1 to TVH19.

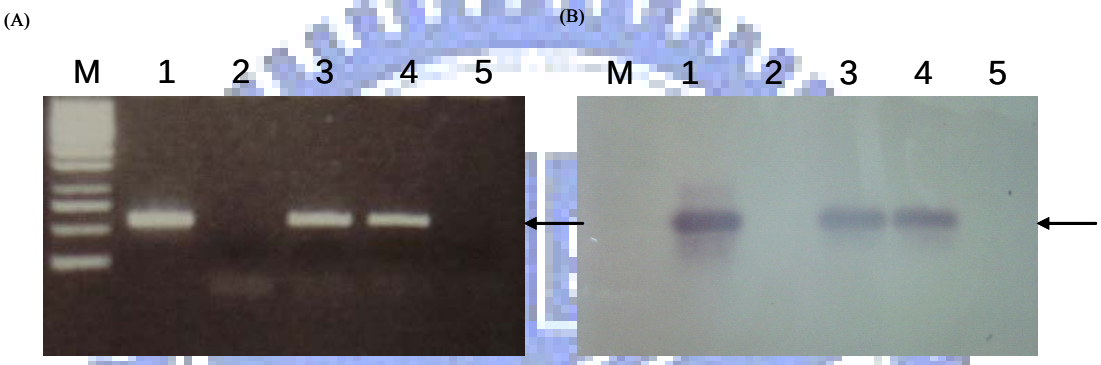


Figure 8. RT-PCR analysis of *rmpA* expression.
(A) Gel electrophoresis of RT-PCR of cDNA and total RNA from wild type and *rmpA2*- strain. Lane 1 is pAEE40 carrying the *rmpA* gene. Lanes 2 and 5 are total RNA isolated from *rmpA2*- and wild type respectively. Lanes 3 and 4 are cDNA from *rmpA2*- and wild type respectively. (B) Southern blotting hybridization with the probe specific to *rmpA*.

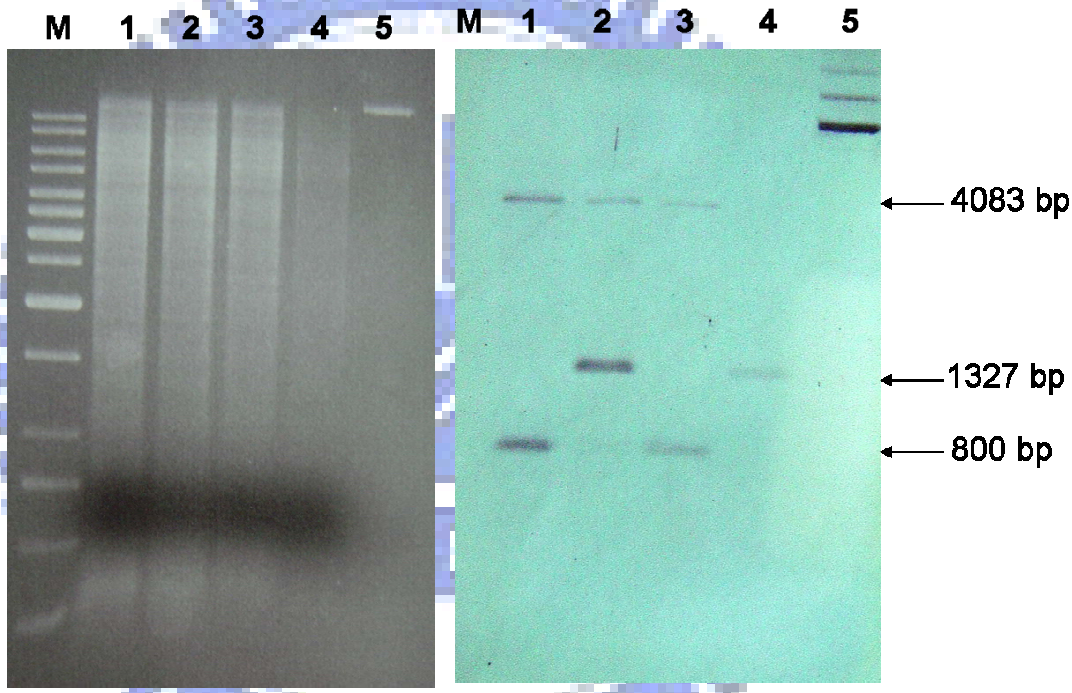


Figure 9. Construction of *rmpA* deletion mutant.
Lanes1 and 3 are the genomic DNA of *rmpA*⁻ strains digested by *EcoR*V. Lanes 2 and 4 are the genomic DNA digested by *EcoR*V. Lane 5 is the plasmid DNA of pAEE40.

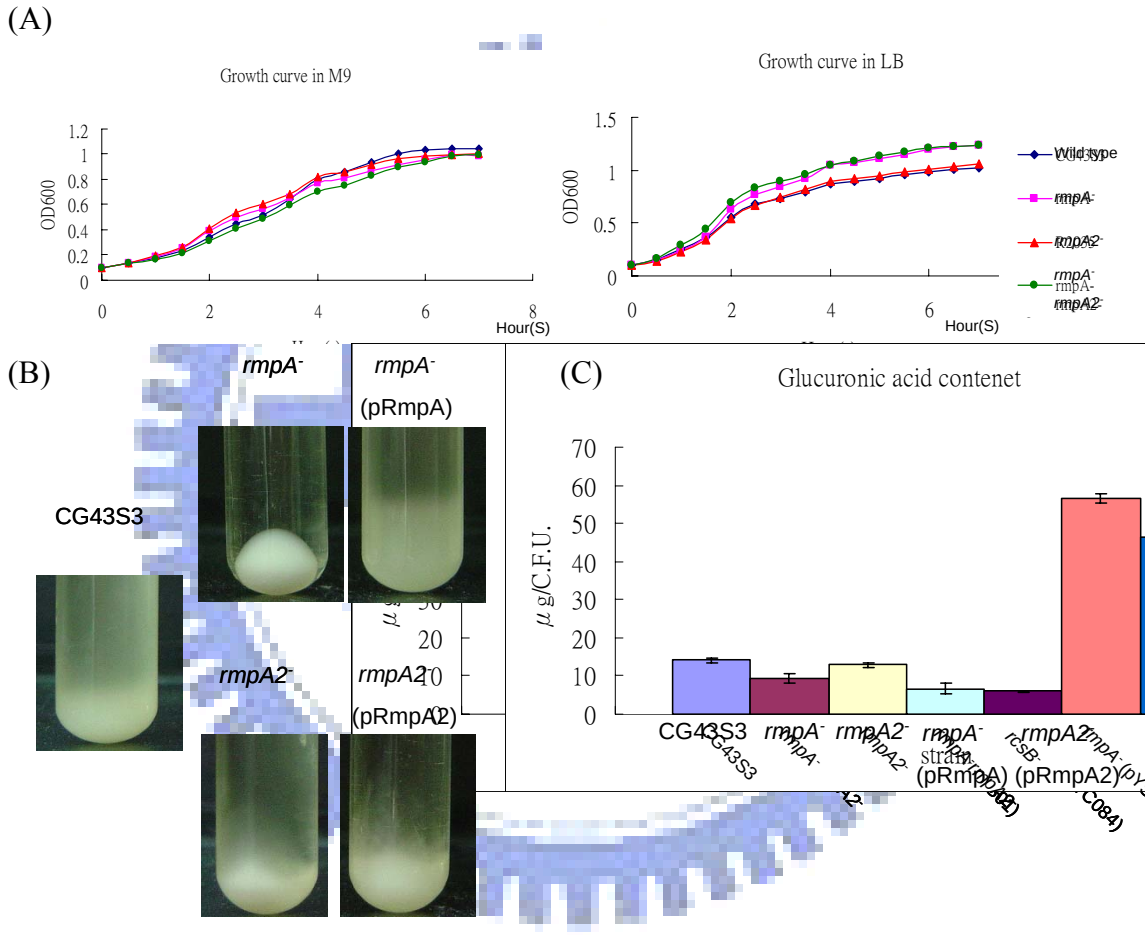


Figure 10. Phenotype analysis of the *rmpA*⁻ and *rmpA2*⁻ strains.
(A) Growth analysis in LB and M9 medium, (B) sedimentation test, and (C) quantification of CPS.

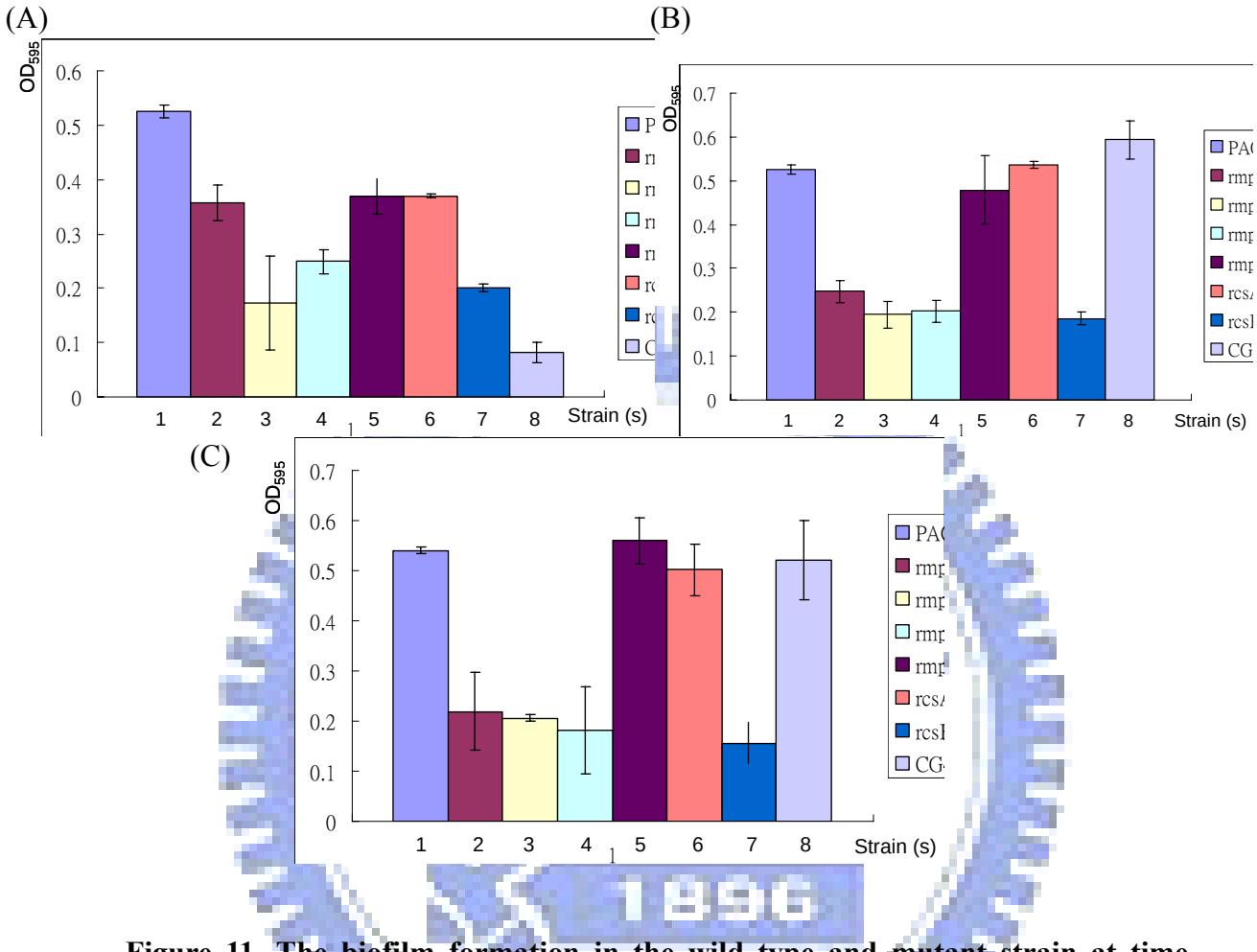


Figure 11. The biofilm formation in the wild type and mutant strain at time course (A) 12 H ,(B) 24 H, and (C) 36 H.
Lane 1: *P. aeruginosa* PAO1, lane 2: *rmpA*-, lane 3: *rmpA*$^-$ + pRmpA, lane 4: *rmpA2*$^-$, lane 5: *rmpA2*$^-$ + pRmpA2, lane 6: *rcsA*$^-$, lane 7: *rcsB*$^-$, and lane 8: *K. pneumoniae* CG43.

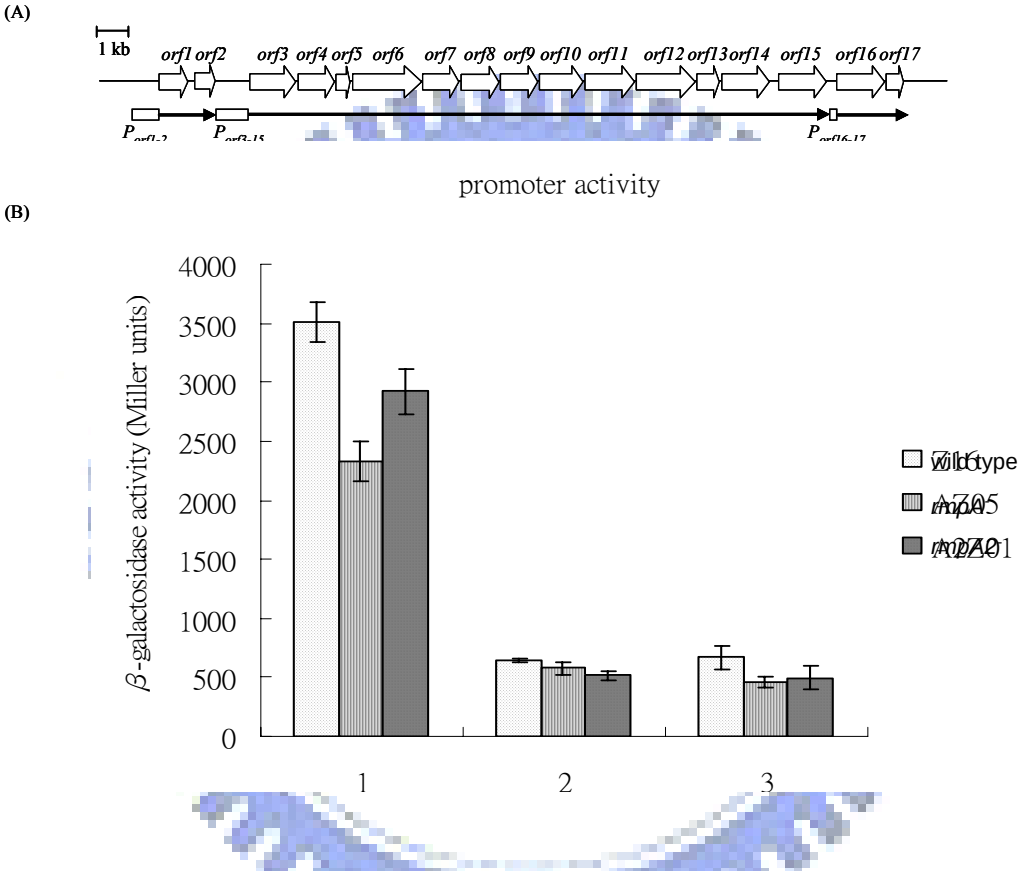


Figure 12. (A)The putative promoter regions of *K. pneumoniae* K2 cps gene cluster (B) Promoter activity analysis of *cps* genes in wild type, *rmpA*-, and *rmpA2*- stains were grown in LB at 37℃. The promoter activities were measured when these strain were cultured to late exponential phase.

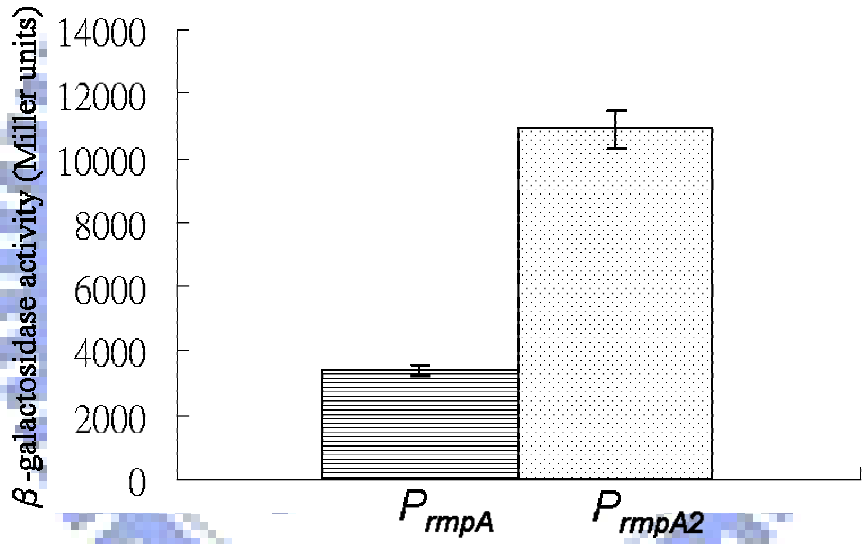


Figure 13. Activity of P_{rmpA} and P_{rmpA2} in*K. pneumoniae* LacZ16 in LB medium

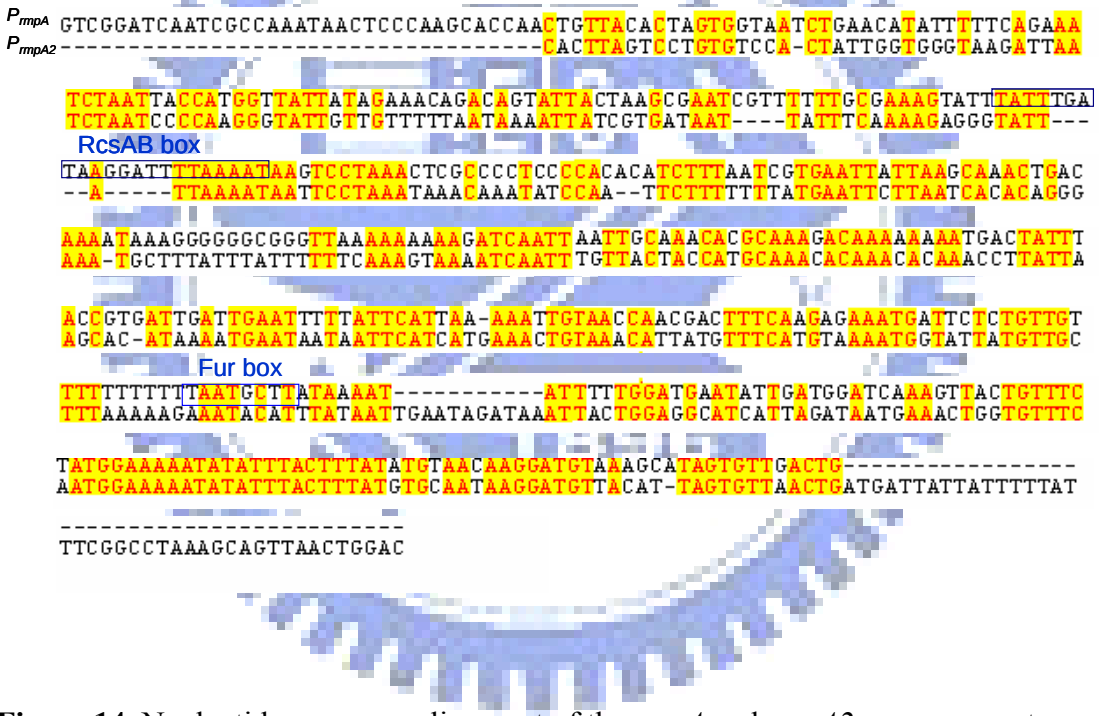


Figure 14. Nucleotide sequence alignment of the *rmpA* and *rmpA2* genes promoters.

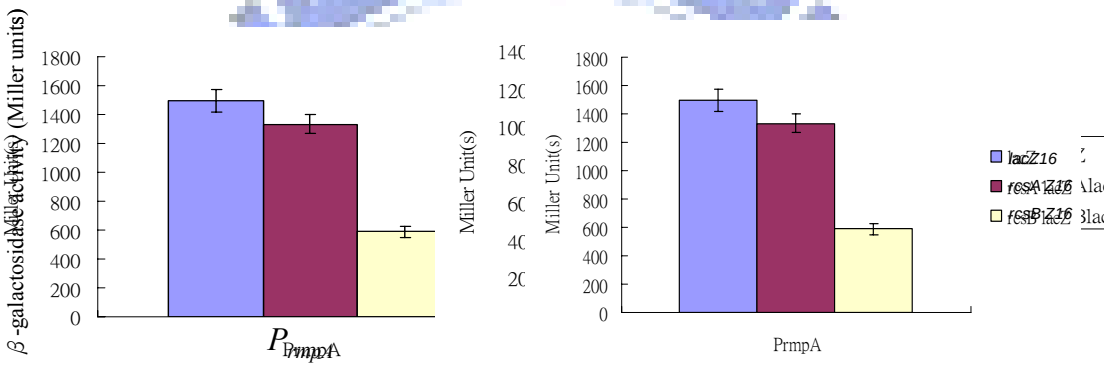


Figure 15. Activity of P_{rmpA} and P_{rmpA2} in *K. pneumoniae* LacZ16, *rcsA*⁻*Z16*, and *rcsB*⁻*Z16*.

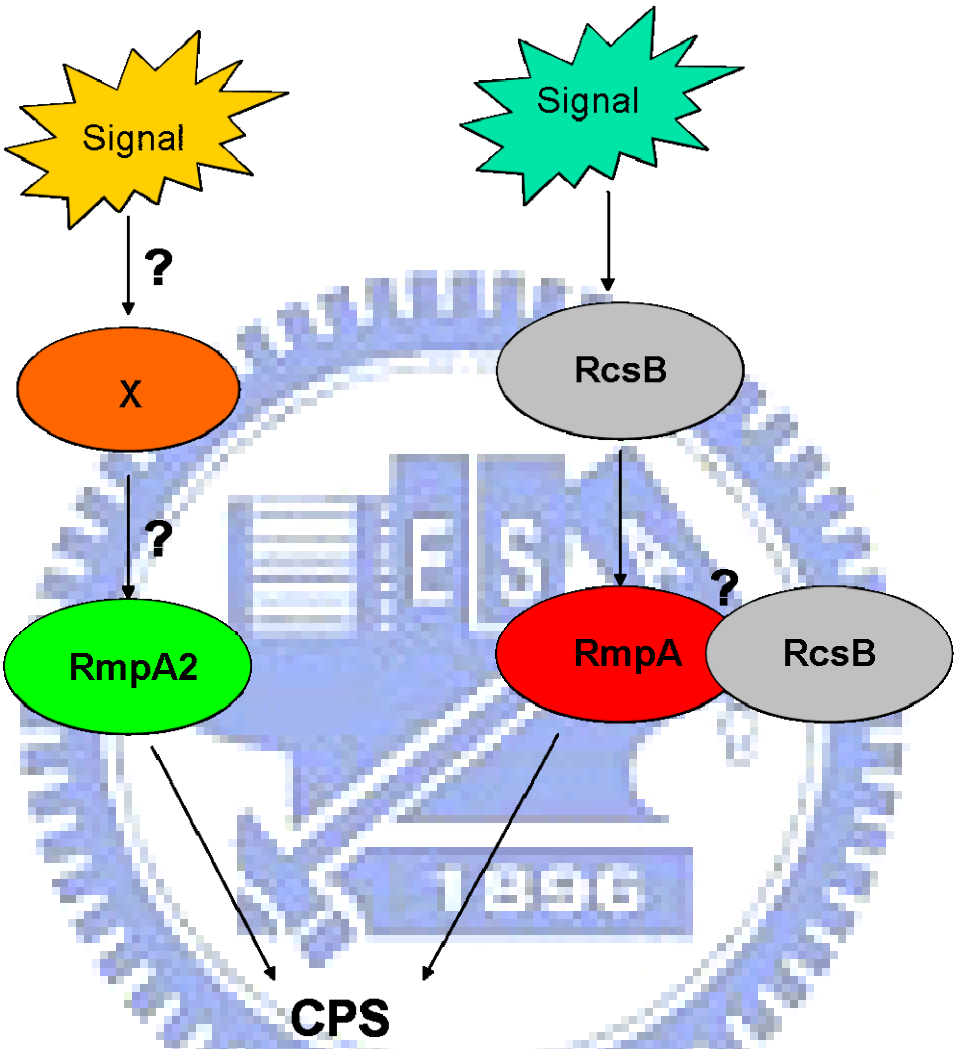
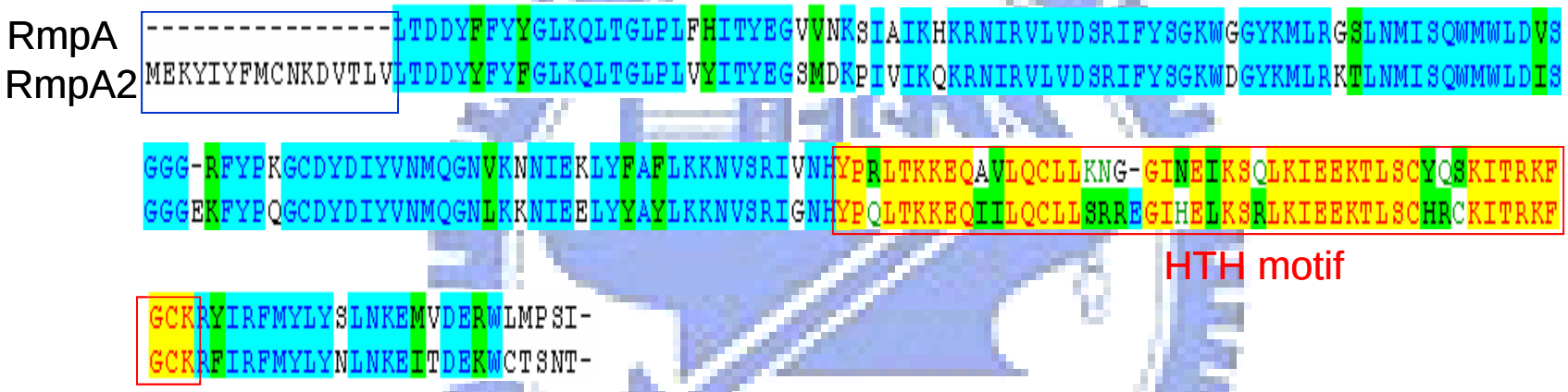


Figure 16. A proposed model of the regulation of RmpA and RmpA2. RcsB activates the expression of *rmpA*, but not of *rmpA2*. RmpA could interact with RcsB to activate CPS synthesis, and RmpA2 could directly bind the promoter of *cps* genes to activate the expression of *cps* genes.



Appendix 1. **Amino acid sequence aligment of RmpA and RmpA2.** RmpA and RmpA2 are homologs, both located on pLVPK, sharing 74% amino acid sequence identity. Their C-terminal domains exert considerable homology with the putative helix-turn-helix motifs of LuxR of *Vibrio fischeri*.