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**Genome wide analysis of expression of the fimbrial operons in  
*Klebsiella pneumoniae***

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## I. Abstract

### Ia. Abstract in Chinese

關鍵詞：克雷白氏肺炎桿菌，*kpa*，*kpb*，*kpc*，*kpd*，*kpe*，*kpf* 和 *kpg* 纖毛，FimA、MrkA、MrkD、MrkF 多株抗體，第三型纖毛表現載體，MrkD、KpbD、KpfD 黏附蛋白，FimB、PhgS、PhgM、FimK、KpdR 調控蛋白。

在克雷白氏肺炎桿菌 NTUH K-2044 基因體中，除了已知的第一型及第三型纖毛外，我們另外發現了七套未被發表過的纖毛基因組，分別命名為 *kpa*，*kpb*，*kpc*，*kpd*，*kpe*，*kpf* 和 *kpg*。為了了解這九套纖毛基因組在黏附特異性及表現調控上的異同，這三年完成的工作如下：

1. 以 ClustalW 工具分別進行纖毛蛋白質的序列分析，可發現這九套纖毛之間並沒有明顯的親緣關係。纖毛基因存在分佈率的研究結果顯示，不同來源的105株克雷白氏肺炎桿菌分離株，大多都含有*kpa*、*kpe*、*kpg*、*fim*，以及*mrk*纖毛基因組 (87% to 100%)。此外，我們發現*kpb*和*kpc*纖毛基因組的分佈則與K1血清型有密切的相關性 ( $P < 0.0001$ )；在35株K1荚膜血清型分離株中，有32株具有相同的纖毛基因組合。然而，這九套纖毛基因組的存在率與疾病種類並沒有明顯的關聯性。
2. 我們分別將這九套纖毛基因組起使子轉殖到LacZ通報系統，然而，除了第一、三型纖毛外，其他七套基因組起使子因活性太低而無法進一步分析；我們也將七套纖毛基因組自克雷白氏肺炎桿菌 NTUH K-2044中選殖出來，並且以不會生成纖毛的大腸桿菌為宿主，分別表現於大腸桿菌表面，以SDS-PAGE 分析可以偵測到 *kpb* 和 *kpc*纖毛的表現，目前在準備製造KpbA和KpcA抗體，未來將以免疫螢光顯微鏡觀察來確認二者的表現；另外，我們還把八套纖毛的黏附蛋白基因選殖置換第三型纖毛表現載體上的*mrkD*黏附蛋白基因，以血球凝集測試發現KpbD和KpfD會凝集未經單寧酸處理的兔子和天竺鼠紅血球，以KpcD 或FimH取代MrkD黏附蛋白時會減少第三型纖毛的單元聚合；最後，我們還製備了MrkA、MrkD、MrkF 及 FimA 多株抗體，可分別第一、三型纖毛的表現。
3. 經由穿透式電子顯微鏡、西方墨點法以及線毛活性的測定，證實黏附蛋白 MrkD基因序列的變化影響了第三型線毛的組裝和活性；藉雷射鐳夾量，

我們發現MrkDv3對膠原蛋白的黏附力最大；最後，我們也證明MrkF為第三型纖毛的結構蛋白質，具有MrkF 組成會減少第三型纖毛的單元聚合而使第三型纖毛較短而糾結，可以提高細菌生物膜的形成。

4. 我們證明第一型及第三型纖毛的表現有互相拮抗的效應，在CG43或NTUHK-2044菌株中可以偵測到第三型纖毛的表現，而相對的只有在MrkA基因被剔除時，才能偵測到第一型纖毛的表現；而在大量表現FimB的菌株會活化第一型纖毛的表現，卻無法偵測到第三型纖毛的表現。
5. 啟動子活性測試顯示-*phgS* 和*phgM*的基因缺損會降低 $P_{kpg}$ 的活性；而剔除活化夾膜多醣合成基因表現的調控蛋白RcsB基因，可以提高 $P_{fimA}$  活性而不影響  $P_{mrkA}$ 的活性。另外，在大量表現FimB的菌株中發現 $P_{mrkA}$ 活性降低。
6. 大量表現KpdR可降低生物膜的生成，同時增加TEM-116  $\beta$ -lactamase 酵素的生成而提高細菌對 $\beta$ -lactam抗生素的抗性；而大量表現FimK能降低夾膜多醣的合成，其作用機制仍待證明；大量表現KpbR和KpfR卻抑制細菌的生長，我們將改以甘露醣誘導表現載體進一步分析這些轉錄調控蛋白的功能。

## I. Abstract

### Ib. Abstract in English

**Keywords :** *Klebsiella pneumoniae*, *kpa*, *kpb*, *kpc*, *kpd*, *kpe*, *kpf*, and *kpg* fimbriae, FimA, MrkA, MrkD, MrkF polyclonal antibodies, type 3 fimbriae expression plasmid, MrkD, KpbD and KpfD adhesins, FimB, PhgS, PhgM, FimK and KpdR regulatory proteins.

In the genome of *Klebsiella pneumoniae* NTUH K-2044, there are nine putative fimbrial operons encoding respectively type 1 and type 3 fimbriae, and seven novel fimbriae *kpa*, *kpb*, *kpc*, *kpd*, *kpe*, *kpf*, and *kpg*. The accomplished experiments in analysis of the expression of these fimbriae are as following:

(1) Multiple sequence alignment of the fimbrial genes by ClustalW revealed that the four major components of the nine fimbrial operons are distinct to each other. The prevalence analysis of the nine fimbrial gene clusters among 105 *K. pneumoniae* clinical isolates from various types of disease indicated that *kpa*, *kpe*, *kpg*, *fim*, and *mrk* genes were contained in most of the isolates (87% to 100%). In addition, *kpb* and *kpc* genes were present in a higher frequency ( $P < 0.0001$ ) in K1 isolates. In addition, 32 of 35 K1 isolates appeared to possess identical repertoire of fimbrial operons. However, no obvious correlation between the fimbrial types and diseases could be identified.

(2) Each of the putative promoters has been cloned respectively into a LacZ reporter vector. However, except the promoters of type 1 and 3 fimbrial genes, the promoter activities of the other seven were too low to be detected. We have also tried to isolate each of the fimbrial operons by PCR-based cloning from *K. pneumoniae* NTUH K-2044 and the fimbriae displayed on the surface of an afimbriated *E. coli*. Heterologous expression of the recombinant type 3 fimbriae, and *kpb* and *kpc* fimbriae could be demonstrated by SDS-PAGE analysis. Polyclonal antibodies against KpbA and KpbC are currently prepared for immunofluorescence microscopy analysis. Furthermore, the filament pattern analysis revealed that the replacement of *mrkD* on type 3 fimbriae displaying plasmid with either *kpcD* or *fimH* reduced polymerization of the MrkA filament. The displayed KpfD adhesin

could agglutinate both erythrocytes of guinea pig and rabbit. Finally, polyclonal antibodies against MrkA, MrkD, MrkF and FimA have been obtained and used for the analysis of the expression of type 1 and type 3 fimbriae.

(3) As demonstrated using TEM and western blotting analysis, and the assessments for fimbrial activity, allelic variations of *mrkD* adhesin were shown to affect fimbriation and activity of type 3 fimbriae. We have also employed optic tweezers to measure the adhesive force to collagen and the result revealed that the fimbriae expressing MrkDv3 had the highest binding force to collagen. Finally, MrkF was shown to be a component of type 3 fimbriae and the incorporation of MrkF conferred the recombinant type 3 fimbriae shorter and meshwork like morphology which led to a higher level of biofilm formation activity.

(4) We have demonstrated a counter-expression between type 1 and 3 fimbriae. The expression of type 3 fimbriae could be readily detected in either *K. pneumoniae* CG43 or NTUH-K2044. Expression of type 1 could only be detected in *mrkA* deletion mutant. Overexpression of FimB was shown to switch on the expression of type 1 fimbriae but block the type 3 fimbrial expression.

(5) Promoter activity measurement using LacZ as reporter indicated that the deletion of either *phgS* or *phgM* reduced the activity of  $P_{kpg}$ . The deletion of *rscB*, which encoding the positive regulator for CPS synthesis, increased the activity of  $P_{fimA}$  but had no apparent activity of  $P_{mrkA}$ . Whereas overexpression of FimB was shown to reduce  $P_{mrkA}$  activity.

(6) Over-expression of KpdR reduced the biofilm formation and increased the expression of  $\beta$ -lactamase TEM-116 in both *K. pneumoniae* NTUH-K 2044 and CG43S3. The FimK overexpression was shown to reduce the capsular polysaccharide biosynthesis. However, overexpression of either KpbR or KpfR was shown to inhibit the bacterial growth. A pBAD-based mannose inducible expression vector will be used to further investigate functional roles of the regulatory proteins.

## II. Background and Significance

The biosynthesis and binding properties of type 1 fimbriae have been well studied in *Escherichia coli* (15, 16, 17, 18). Although with less information, type 1 fimbriae have also been isolated and characterized in *K. pneumoniae* (4, 8, 9). Type 3 fimbrial adhesin, referred to as mannose-resistant *Klebsiella*-like (MR/K) hemagglutinin, has been shown to be produced by a few of *K. pneumoniae* strains (1, 9, 10). In addition to the two fimbrial adhesins, three more including the fimbrial antigen KPF-28, the afimbrial adhesin CF29K, and an afimbrial adhesin composed of capsule-like extracellular material have recently been reported in *Klebsiella* isolates (2, 3, 6).

Adhesin specificity plays a major role in determining the host range and tissue tropism in bacterial infection (21). Depending on the expression of type 1 or P fimbriae, UPEC (uropathogenic *E. coli*) vary significantly in their abilities to colonize and persist within the bladder or kidneys (27). PapB, a regulator for phase variation of P fimbriae, and its functional homologue, SfaB of S fimbriae, both were shown to be able to increase the frequency of ON-to-OFF phase for type 1 fimbriae (12, 26). This indicated the presence of a regulatory network in controlling differential expression of type 1-, P-, and S-fimbriae in UPEC. In *E. coli*, type 1 fimbriae has been demonstrated to be essential for the bacteria in establishment and persistence of urinary tract infections (15, 27). Most likely, the fimbriae are also required for *K. pneumoniae* to colonize urinary tract. In addition to uroepithelial cells, the adherence to respiratory epithelia, intestinal and endothelial cells of the *K. pneumoniae* strains expressing type 3 fimbriae have been demonstrated (20, 22). However, the specificity and regulation of fimbrial adherence in *K. pneumoniae* are largely unknown.

Prevalence analysis of *Salmonella enterica* sp. Typhi isolates revealed a unique repertoire of fimbrial gene clusters possibly due to specific selective pressures (23, 24). Expression analysis using flow cytometry of eleven *S. Typhimurium* fimbrial operons indicated further that *in vivo* growth conditions drastically alter the

expression of repertoire of fimbrial antigens (14). Thus, differential expression of the nine fimbriae for *K. pneumoniae* to adhere to different cells or abiotic surfaces, or for the bacterial self-aggregation or biofilm formation could be foreseen. Using bioinformatic analysis, nine sets of fimbriae encoding operons of the chaperone-usher assembly pathway which includes type 1-, type 3- and seven novel-type fimbriae, namely *kpa*, *kpb*, *kpc*, *kpd*, *kpe*, *kpf*, and *kpg* in the genome of *K. pneumoniae* NTUH K-2044 (<http://genome.nhri.org.tw/kp/index.php>), a K1 isolate of pyogenic liver abscess (5), were identified. The specific aims proposed to be accomplished included:

- (1) Analysis of the fimbrial gene clusters in *K. pneumoniae* using the tools of bioinformatics
- (2) Investigation of the prevalence of the fimbrial gene clusters in *K. pneumoniae* clinical isolates
- (3) Determination of the regulatory control of expression of the fimbrial operons
- (4) Characterization of the adherence specificities displayed by each of the fimbrial adhesins

### III. Material and Methods

**Bacterial strains and growth condition.** *K. pneumoniae* NTUH-K2044, of K1 serotype, was provided by Dr. Jin-Town Wang from National Taiwan University Hospital (5). Clinical isolates of *K. pneumoniae* used in this study were recovered from different tissue specimens of patients with a variety of infections at the Veteran General Hospital, Taipei, from 1991 to 1998. The strains were identified and serotype determined. The bacteria were grown in Luria-Bertani (LB) broth or agar at 37°C and stored at -80°C before use.

**PCR amplification for prevalence analysis.** The PCR mixture contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 μM (each) deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), and 1 U of recombinant *Taq* DNA polymerase (Violet Bioscience Inc.) along with each of the *K. pneumoniae* genomic DNA and the specific primers. The primer pairs designed for the respective adhesin and pilin encoding genes of each fimbrial operons are shown in Table 1. The amplification cycle consisted of an initial 1 min hold at 95°C followed by 35 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min, and finally an elongation step for 10 min at 72°C. The amplified PCR product was then analyzed by electrophoresis on a 1% agarose gel.

**Hemagglutination activity.** The agglutination assay was carried out as described<sup>1</sup> with some modifications. In brief, overnight grown bacteria were suspended in PBS to approximately 10<sup>9</sup> CFU/ml and the collected erythrocytes of guinea pig and rabbit washed twice with PBS. A series of two-fold dilution of the bacterial suspension were mixed with an equal volume of 3% (vol/vol) erythrocytes in PBS. The mixture was incubated at room temperature for 30 min to allow erythrocytes settle to the 96-well

V-bottomed microtiter plates.

**Antiserum preparation.** The coding regions corresponding to FimA and MrkA, the major pilin subunit of type 1 fimbriae and type 3 fimbriae, were amplified by PCR and cloned into expression vector pET30, respectively. The expression plasmid was then transformed into *E. coli* Nova-Blue (DE3) and overexpression of the recombinant pilin was induced by addition of 0.5 mM IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside). The MrkA and FimA proteins fusion with His-tag were then purified using a nickel charged resin (Novagen, Madison, WI). In order to raise antibody, five-week-old female BALB/c mice, purchased from the animal center of National Taiwan University, were immunized intraperitoneally with 5  $\mu$ g of the purified MrkA or FimA. Ten days later, the mice were immunized again with 5  $\mu$ g of the MrkA or FimA protein. Finally, the antisera were obtained by intracardiac puncture.

**Western blot analysis of the expression of type 1 and type 3 fimbriae.** The clinical isolates of *K. pneumoniae* were grown overnight in either LB, for optimal expression of type 1 fimbriae, or GCAA (minimal medium supplemented with 1% glycerol and 0.3% casamino acids) broth for optimal expression of type 3 fimbriae. Total cellular proteins of the bacteria were resolved by SDS-PAGE and electrophoretically transferred from the gels onto PVDF membranes (Immobilon<sup>TM</sup>-P, Millipore). The membranes were then blocked with 5% skim milk at room temperature for 1 h, and washed 3 times with 1 X phosphate-buffered saline (PBS). Subsequently, the membranes were incubated with diluted anti-FimA or anti-MrkA serum at room temperature for 1 h. After 3 washes with 1 X PBS, a 3000-fold diluted alkaline phosphatase-conjugated anti-mouse immunoglobulin G

was added and the incubation continued for one more hour. The blot was again washed and the bound antibodies were detected by using the chromogenic reagents BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (Nitro blue tetrazolium).

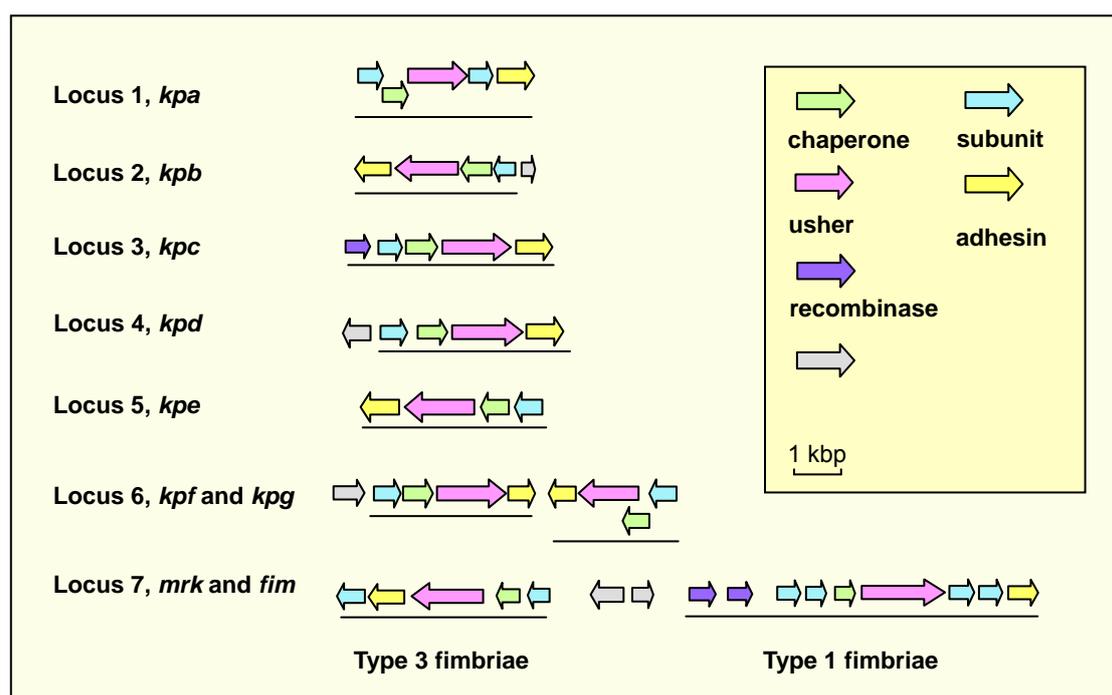
**Type 3 filament analysis** Total proteins were isolated from the recombinant bacteria *E. coli*, which has been cultured for 20 h in GCAA medium, and resolved by 7.5% SDS-PAGE. The filament polymer was then assessed using Coomassie blue staining or western blotting analysis against anti-MrkA polyclonal antibody.

**Transmission electron microscopy (TEM).** Twenty microliters of bacterial suspension ( $10^8$  cfu/ml) were added to collodion-coated copper grids (300 mesh) and negatively stained by 2% phosphotungstic acid, pH 7.2. The grids were then examined under a JEOL JEM 2000EXII transmission electron microscope at an operating voltage of 100 kV.

**Promoter activity measurement.**  $\beta$ -galactosidase activity assay was carried out essentially as described by Miller. The data presented were derived from a single experiment which was representative of at least three independent experiments. Every sample was assayed in triplicate, and the average activity and standard deviation were presented.

## IV. Progress Report and Discussion

**Specific Aim 1- Analysis of the fimbrial gene clusters in *K. pneumoniae* using the tools of bioinformatics.** Using HMMER to search for the Pfam fimbrial family and BLAST analysis of the *K. pneumoniae* NTUH K-2044 genome, nine distinct putative fimbrial operons including type 1 and type 3, and seven novel types of fimbriae, namely *kpa*, *kpb*, *kpc*, *kpd*, *kpe*, *kpf*, and *kpg* were identified (Fig. 1).



**FIG. 1. Fimbrial gene clusters in *K. pneumoniae* NTUH-K2044**

(<http://genome.nhri.org.tw/kp/index.php>). Each of the putative fimbrial operons is underlined.

The BLAST analysis also revealed that, except the *kpbABCD* and *kpeABCD* operons, homologs could be identified in other bacteria for *kpa*, *kpc*, *kpd*, *kpg*, and *kpf* gene clusters. The *kpaABCDE* operon, is likely an orthologue of *Salmonella* Typhimurium LT2 *sthABCDE* operon, which has been reported to be required for intestinal persistence in mice. In *kpa* operon, both *kpaA* and *kpaD* genes appeared to

be capable of encoding a fimbrial subunit suggesting that they are respectively the major and minor subunits of the fimbrial rod. The *kpcABCD* are homologous to the putative fimbrial genes, plu2159 to plu2156, of *Photorhabdus luminescens* subsp. laumondii TTO1. The *kpdABCD* encodes a homologous operon of Reut02000958 to Reut02000961 in *Ralstonia metallidurans* CH34 (unfinished sequence, GenBank: AAAI00000000). The physically linked *kpfABCD* operon and *kpgABCD* genes appeared to be respectively an orthologue of *Edwardsiella tarda* KG8401 *etfABCD* operon, and an orthologue of the fimbrial operon, plu2159 to plu2156, in *P. luminescens* subsp. laumondii TTO1.

Upstream of the *kpbABCD* operon, a divergently transcribed gene encoding a transcriptional regulator with a DNA-binding domain was identified and designated *kpbR* (Fig. 1). A DNA recombinase encoding gene, namely *kpcI*, located upstream of *kpcABCD*, was found to be an orthologue of several fimbrial invertases including MrpI, MrxI (AAM91928) and MrfI. As shown in Fig. 2, an 11-bp inverted repeat was identified in the putative promoter region of *kpc* operon, implying a phase-variation control by DNA inversion for the operon expression. Moreover, TCS (two component system) response regulator encoding genes, namely *kpdR* and *kpfR*, were found to be located upstream of *kpdABCD* and *kpfABCD* operons (Fig. 1).

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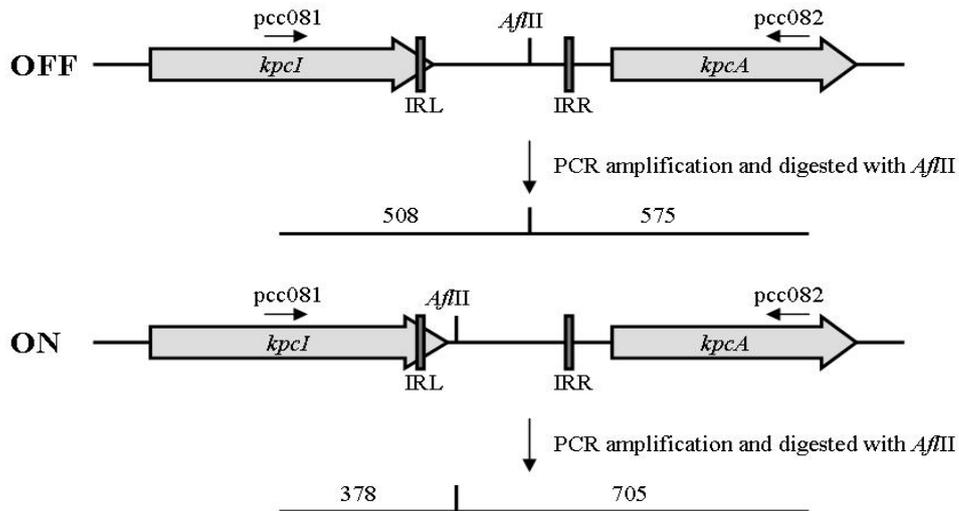
1                               50
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101                               150
GAGAAAACCACACAAATTAGTGCCAGAAATGTAAGTAGTATCAGAAAAATTTAGCAAAGTGTCAAATTTTTCTGAGACCCCTTTAGGTTACGGATTATCT
201                               250
CGCTGTTTTTAAATAATTTATCTGCGCGAATATTGTCTAACACACCTCTGGCTCCTGCTGGCAACGTGCTGAAGTTGTTCACGTTGCCAGTTTTTCATTTG
301                               350                               -10                               OmpR                               RpoD17
CTCACCTTCTTATGGTTTTATGCCGTTCTTAAGCGGTAATTCGGCTTTTACCAACGTTTATTCTTGGACTTTCTTTTTCACCAAAATTTTCTCCTGAACT
401                               450                               Lip                               MetR
CACCACCTCTGGCACTAACGGGAAAGTCTAATGCGGATTTTTCACCGCCGGAGTGAAGCAGGGCAGACCCCTGGAGTGATTTTTCATCAAGGAGAAAGGT
501                               550
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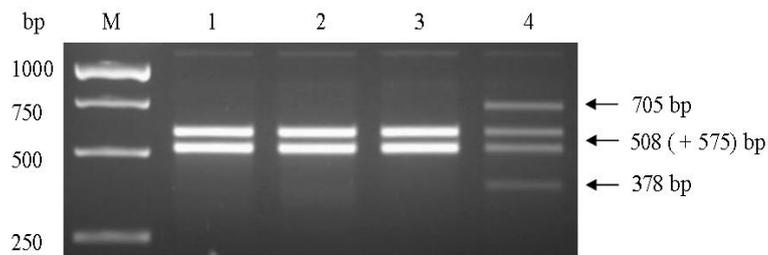
**FIG. 2.** DNA sequence of the putative promoter region of *kpc* operon.

While introducing into the bacteria NTUH-K2044 with a *kpcI* containing plasmid pKC-19, inversion of the noncoding DNA upstream of *KpcA* start codon was observed (Fig. 3) which implying the presence of a phase variation control for the expression of *kpc* fimbriae.

(A)



(B)



**FIG. 3.** (A) Schematic presentation of the predicted invertible DNA in-between *kpcI* and *kpcA*. (B) Effect of *KpcI* expression on the DNA inversion. The isolated PCR products were digested with *AflIII* and resolved on 1.2% agarose gel. Lanes 1, NTUH-K2044[pAW03] in 0.4% glucose; 2, NTUH-K2044[pAW03] in 0.4% mannose; 3, NTUH-K2044[pKC-19] in 0.4% glucose; NTUH-K2044[pKC-19] in 0.4% mannose.

**Specific aim 2- Investigation of the prevalence of the fimbrial gene clusters in *K.***

***pneumoniae* clinical isolates.** Prevalence analysis via PCR detection of the respective pilin and adhesin encoding genes in 105 *K. pneumoniae* clinical isolates from various types of diseases (collected by Dr. CP Fung from the Veteran General Hospital Taipei) revealed that *kpa*, *kpe*, *kpg*, *fim*, and *mrk* genes were contained in most of the isolates (87% to 100%). As shown in Table 1, the distribution of *kpb*, *kpc*, *kpd*, and *kpf* genes were 50%, 32%, 83%, and 70%, respectively. In addition, 32 of 35 K1 isolates appeared to possess identical repertoire of fimbrial operons. No obvious correlation between the fimbrial types and diseases could be identified.

**TABLE 1.** Frequency and repertoire of fimbrial genes among *K. pneumoniae* isolates with different K serotypes.

Presence of gene									No. with genes (%)		
<i>kpa</i>	<i>kpb</i>	<i>kpc</i>	<i>kpd</i>	<i>kpe</i>	<i>kpf</i>	<i>kpg</i>	<i>fim</i>	<i>mrk</i>	K1 serotype	K2 serotype	Other serotypes
(A) Distribution of fimbrial genes in combination with each other among different K serotype bacteria											
+	+	+	+	+	+	+	+	+	32 (91)		6 (7)
+	-	-	+	+	+	+	+	+	1 (3)	1	25 (29)
+	-	-	-	+	+	+	+	+	0		9 (11)
+	+	-	+	+	+	+	+	+	1 (3)		10 (12)
+	-	-	+	+	-	+	+	+	1 (3)		7 (8)
+	-	-	-	-	-	+	-	+	0		6 (7)
+	+	-	+	+	-	+	+	+	0	5	4 (5)
+	-	-	+	+	-	+	-	+	0		2 (2)
+	-	-	+	+	-	+	-	+	0		2 (2)
+	-	-	-	+	-	+	+	+	0	1	1 (1)
+	+	+	+	+	-	+	+	+	0		1 (1)
+	+	-	-	+	+	+	+	+	0		1 (1)
+	+	-	+	+	-	+	-	+	0		1 (1)
+	+	-	-	+	+	+	-	+	0		1 (1)
+	+	-	+	+	-	-	-	+	0		1 (1)
+	-	+	+	+	-	+	-	+	0		1 (1)
-	+	-	-	-	-	+	-	+	0		1 (1)
+	-	-	+	+	+	-	-	+	0		1 (1)
+	-	-	+	+	-	-	-	+	0		1 (1)
(B) No. of isolates (n = 123) presenting specific fimbriae-related DNA sequences (%)											
122 (99)	64 (52)	40 (33)	103 (84)	116 (94)	88 (72)	120 (98)	106 (86)	123 (100)			

Nevertheless, *kpb* and *kpc* gene clusters were found to be more prevalent ( $P < 0.0001$ ) in the isolates of K1 serotype (Table 2). The results indicated that the specific combination and regulation of these fimbrial operons may play important roles in *K. pneumoniae* K1 pathogenesis.

**TABLE 2.** Prevalence and distribution of *kpb* (A) and *kpc* (B) genes.

(A)			(B)		
<i>K. pneumoniae</i> isolates	No. of strains/total no. of strains isolated (%)		<i>K. pneumoniae</i> isolates	No. of strains/total no. of strains isolated (%)	
	K1 serotype	Other serotypes		K1 serotype	Other serotypes
Liver abscess	14/14	4/5	Liver abscess	14/14	2/5
Bile		4/9	Bile		1/9
Urine	1/2	4/14	Urine	1/2	0/14
sputum	5/5	5/15	sputum	4/5	1/15
Wound	6/6	6/18	Wound	6/6	1/18
Blood	7/8	4/18	Blood	7/8	2/18
Ascites		4/9	Ascites		1/9
<b>Total</b>	<b>33/35 (94)*</b>	<b>31/88 (35)*</b>	<b>Total</b>	<b>32/35 (91)*</b>	<b>8/88 (11)*</b>

\*,  $P < 0.0001$

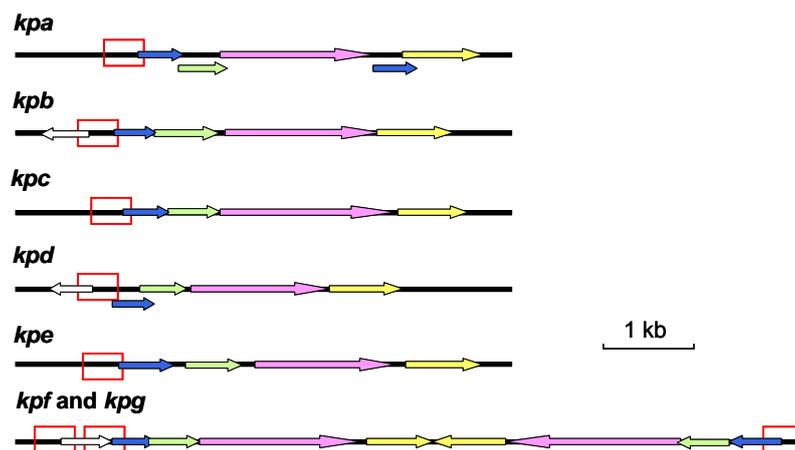
Interestingly, western blotting hybridization using anti-FimA and anti-MrkA antibodies, respectively, revealed that only 15.7% and 46.3% of the isolates expressed *in vitro* the major subunit of type 1 and type 3 fimbriae. Nevertheless, type 1 fimbriae were found to be prevalently expressed (71%) in liver abscess isolates of K1 serotype suggesting a not yet identified role of type 1 fimbrial expression for the infection of K1 isolates causing liver abscess (Table 3).

**TABLE 3.** *In vitro* expression of type 1 and type 3 fimbriae.

Expression of fimbriae	<i>K. pneumoniae</i> isolates (No.)			
	K1 isolates, KLA (14)	K1 isolates, nonKLA (21)	K2 isolates (7)	Other serotypes (81)
% of isolates expressing fimbriae under the specific conditions (No.)				
Type 1 fimbriae	71 (10)	0 (0)	14 (1)	1 (8)
Type 3 fimbriae	14 (2)	10 (2)	43 (3)	62 (50)

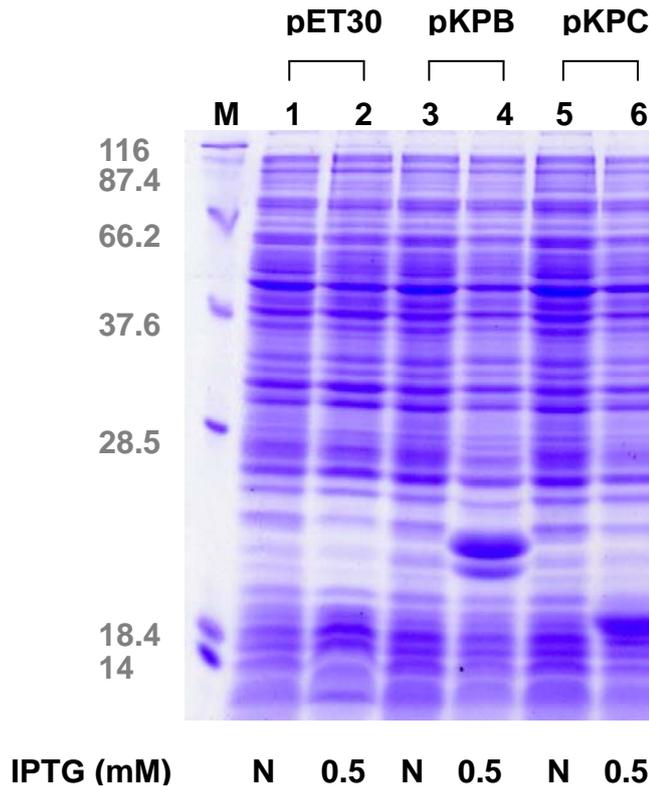
**Specific aim 3- Determination of the regulatory control of expression of the**

**fimbrial operons.** In order to measure the expression of each of the fimbriae, the putative promoter regions of each of the nine fimbrial gene clusters have been isolated from *K. pneumoniae* NTUH-K2044, and cloned into the reporter plasmid placZ15 containing a promoterless *lacZ* gene, which allow determine the signals that affect the fimbriae expression and the promoter activity of each of the fimbriae (Fig. 3). The promoter activities were measured in the presence of different cultural conditions such as changes of nutrient sources, growth temperature, osmolarity, and iron concentration.



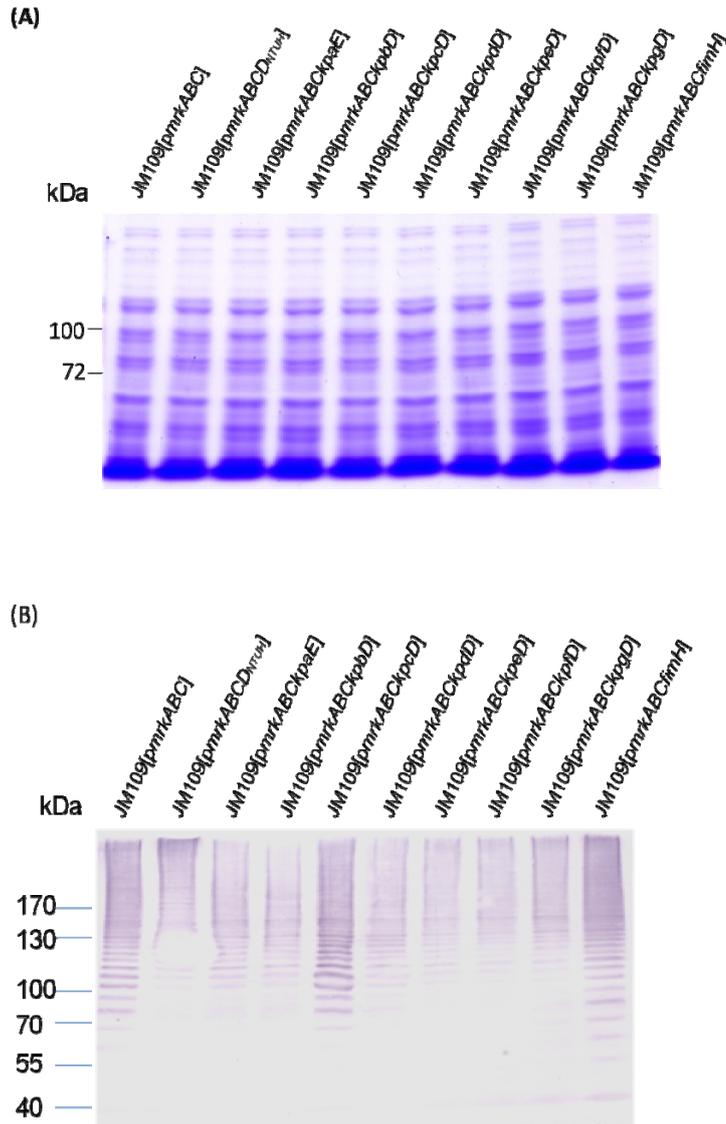
**FIG. 4. Schematic diagram of the putative promoters of *kpa*, *kpb*, *kpc*, *kpd*, *kpe*, *kpf*, and *kpg* operons**

The heterologous expression constructs in *Escherichia coli* for each of the pilins have been generated. As shown in Fig. 4, expression of the major pilin of *kpb* and *kpc* could be observed by SDS-PAGE analysis.



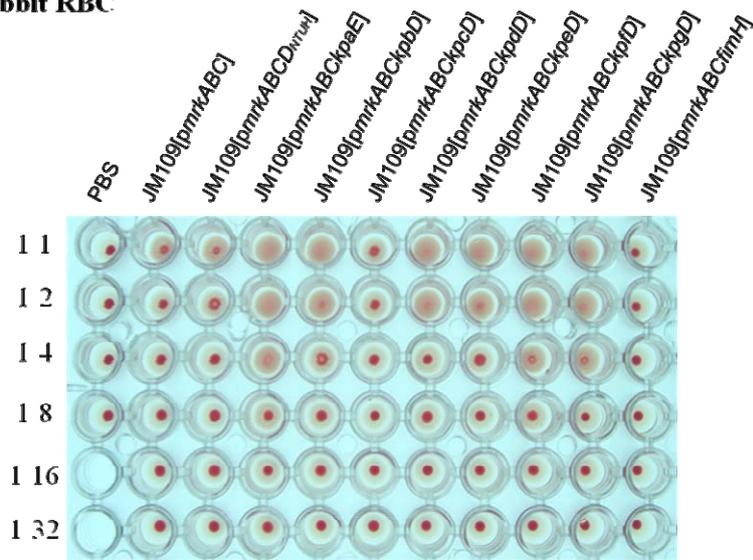
**FIG. 5. Heterologous expression of *kpb*- and *kpc*-encoded fimbriae.** The plasmids pKPB and pKPC respectively contain *kpb* and *kpc* genes and the expression was induced by 0.5 mM IPTG for 3 h. Protein marker (M); *E. coli* Novablue (DE3) containing pET30 (lanes 1 and 2), pKPB (lanes 3 and 4), or pKPC (lanes 5 and 6). The arrows respectively mark the expression of major subunit of *kpb* and *kpc* fimbriae.

The filament pattern analysis revealed that the replacement of *mrkD* on type 3 fimbriae displaying plasmid with either *kpcD* or *fimH* reduced the polymer formation of the MrkA filament (Fig. 6A and B). In addition, the hemagglutination assay indicated that the displayed KpfD adhesin could bind to erythrocytes of guinea pig and rabbit (Fig. 7A and B). In analogy with the recent structural analysis of type 1 fimbriae, further investigation of the interacting domain of each of the adhesins with MrkA pilin could be predicted.

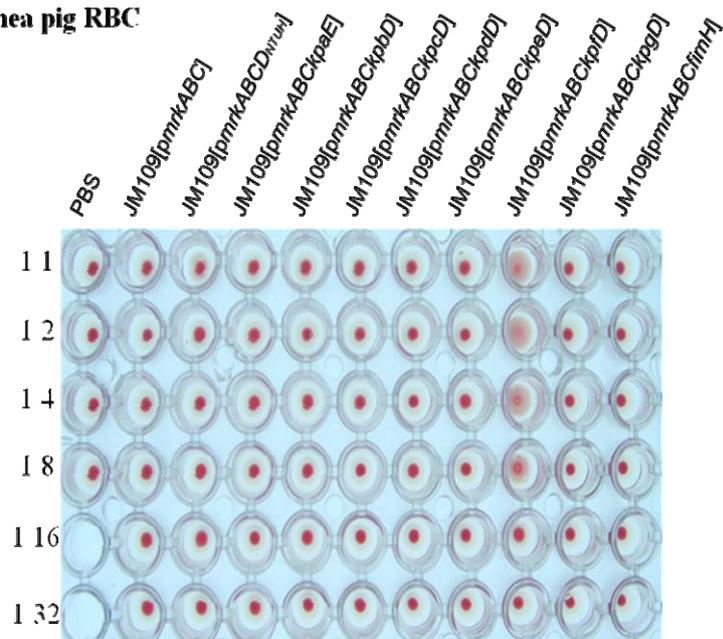


**FIG. 6. Analysis of polymerization pattern of the recombinant type 3 fimbriae.** The total proteins were isolated from the recombinant bacteria *E. coli*, which has been cultured for 20 h in GCAA medium, and resolved by 7.5% SDS-PAGE. The filament polymer was assessed using Coomassie blue stain (A) or western blotting analysis against anti-MrkA polyclonal antibody (B). Molecular mass markers are also indicated.

(A) Rabbit RBC



(B) Guinea pig RBC



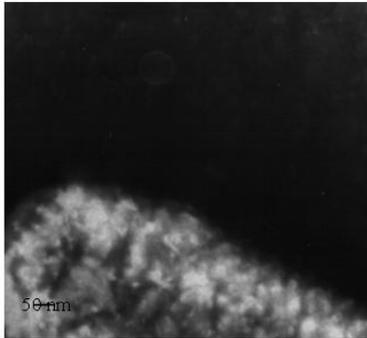
**FIG. 7. Hemagglutination assay.** Aliquots ( $10^9$  CFU/ml) of the recombinant *E. coli*, which has been cultured for 20 h in GCAA medium, were suspended in PBS and then diluted by 2-fold gradient in PBS (from 1:1 to a 1:32). Fifty microliters of the bacterial suspension was then added to each well with an equal volume of 3% rabbit RBC(A) or guinea pig RBC(B), and the plate stood at room temperature till the appearance of agglutination.

**Specific aim 4- Determination of the regulatory control of expression of the fimbrial operons.**

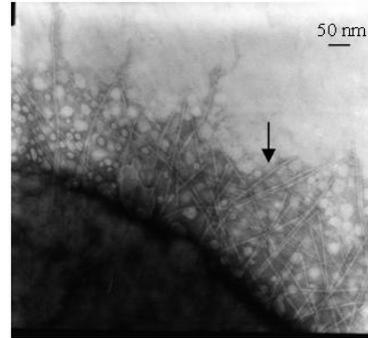
*Four novel mrkD alleles were identified and the growth of type 3 fimbriae is MrkD adhesin dependent* . Four *mrkD* variants were identified from 17 meningitis isolates using PCR-RFLP analysis, and each of the PCR products was cloned, sequences determined, and named *mrkD<sub>V1</sub>*, *mrkD<sub>V2</sub>*, *mrkD<sub>V3</sub>*, and *mrkD<sub>V4</sub>* (under the GenBank accession number *AY225462*, *AY225463*, *AY225464*, and *AY225465*). The TEM analysis (Fig. 8) revealed different forms of fimbriae indicating the filament growth is adhesin dependent and effects of the allelic variation on the fimbrial activity had been demonstrated (13).

*MrkF is a component of type 3 fimbriae*. Fimbriation analysis of the *E. coli* displaying with various recombinants of type 3 fimbriae including *mrkABC*, *mrkABCD*, *mrkABCF*, *mrkABCDF* using transmission electronic microscopy (Fig. 9), western blotting analysis, and co-immunoprecipitation, demonstrated that MrkF is a component of type 3 fimbriae.

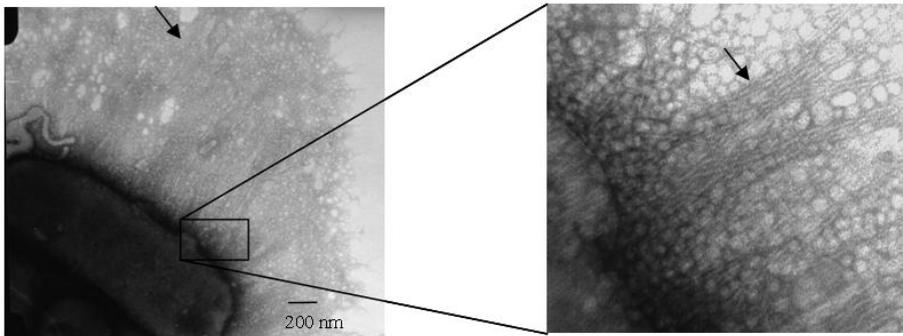
(A) *E. coli* JM109 [pGEM-T]



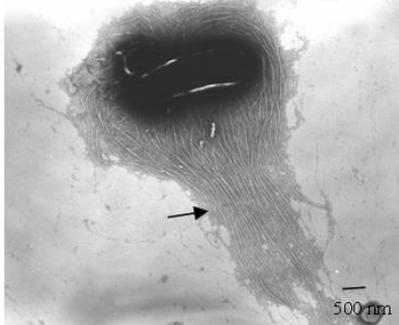
(B) *E. coli* JM109 [pmrkABC]



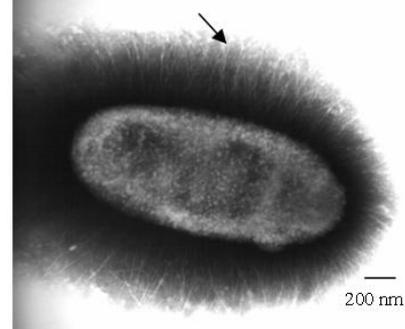
(C) *E. coli* JM109 [pmrkABCF]



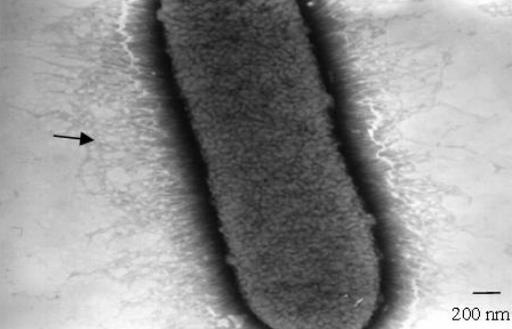
(D) *E. coli* JM109 [pmrkABCD<sub>v1</sub>F]



(E) *E. coli* JM109 [pmrkABCD<sub>v2</sub>F]



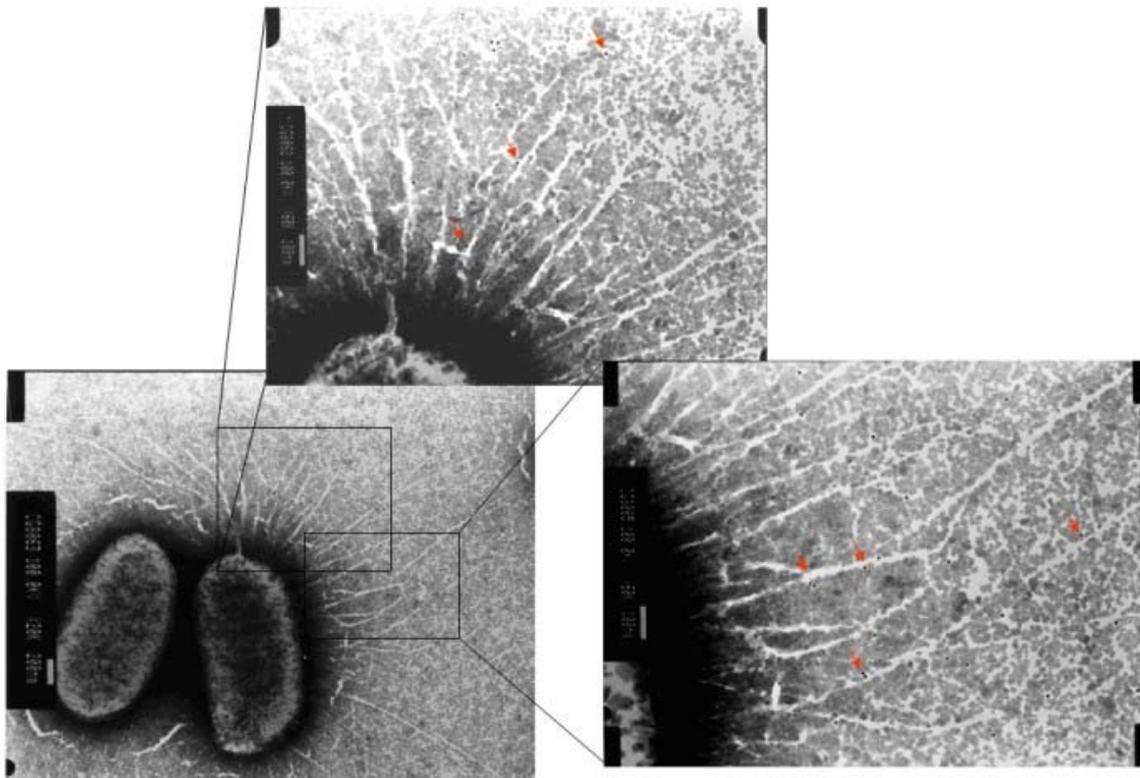
(F) *E. coli* JM109 [pmrkABCD<sub>v3</sub>F]



(G) *E. coli* JM109 [pmrkABCD<sub>v4</sub>F]



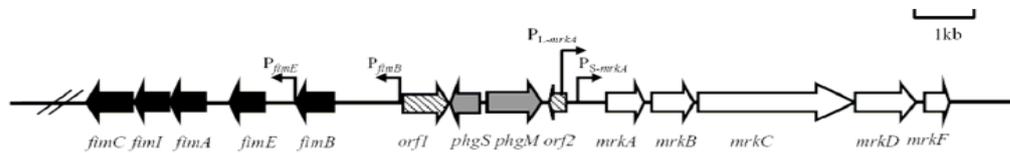
**FIG. 8.** Transmission electron micrographs of the type 3 fimbriae displaying on *E. coli*. One drop of bacterial suspension was placed on a carbon-coated copper grid, and negatively stained with 2 % phosphotungstic acid. (A) *E. coli* JM109 [pGEM-T], (B) *E. coli* JM109 [pmrkABC], (C) *E. coli* JM109 [pmrkABCF], (D) *E. coli* JM109 [pmrkABCD<sub>v1</sub>F], (E) *E. coli* JM109[pmrkABCD<sub>v2</sub>F], (F) *E. coli* JM109 [pmrkABCD<sub>v3</sub>F], (G) *E. coli* JM109 [pmrkABCD<sub>v4</sub>F].



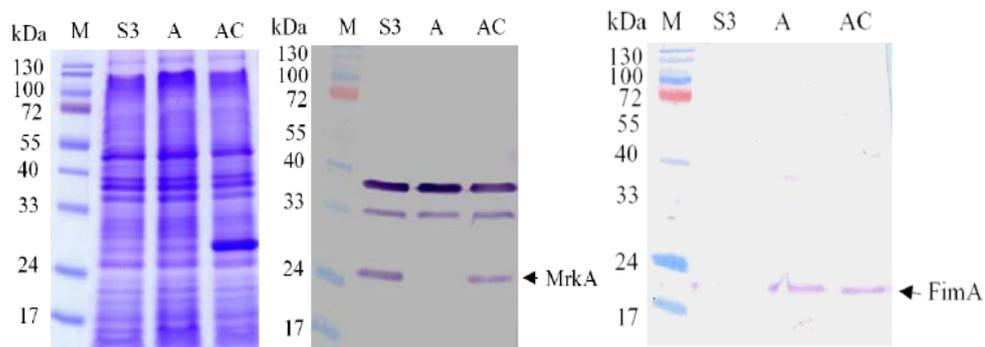
**FIG. 9.** Type 3 fimbriae labeled using anti-MrkF immuno-gold. The magnified views showed the position of MrkF labeled with 10 nm gold particle.

***A cross-talk regulation is present for the expression of type 1 and 3 fimbriae.*** As shown in Fig. 10A, the gene clusters respectively encoding type 1 and type 3 fimbriae are physically linked. Deletion of *mrkA*, which encoding the major pilin of type 3 fimbriae, increased the expression of type 1 fimbriae (Fig. 10B). As shown in Fig. 10C, The overexpression of *fimB* recombinase enhanced the expression of type 1 fimbriae but reduced the expression of type 3 fimbriae (Fig.10C).

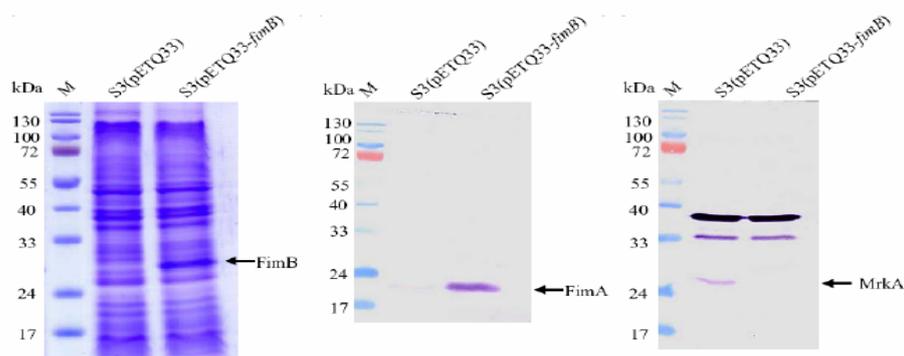
(A)



(B)

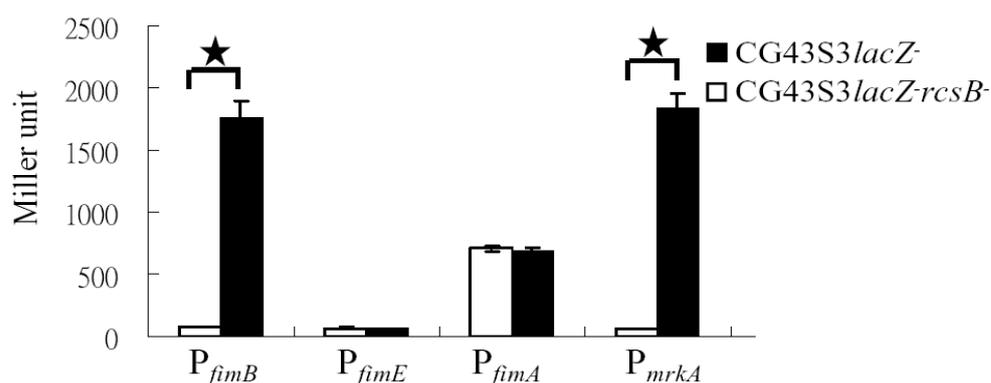


(C)



**FIG. 10.** (A) Organization of the *fim-mrk* gene clusters of type 1 fimbriae and type 3 fimbriae. (B) and (C) Western blot analysis of the expression of FimA and MrkA proteins.

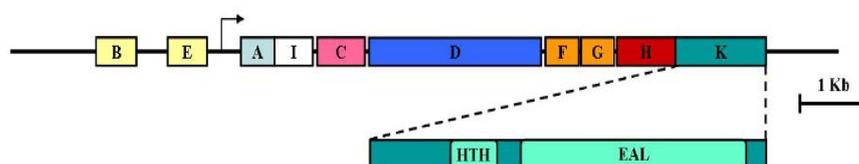
Promoter activity measurement as shown in Fig. 11, the deletion of *rscB* appeared to reduce the activity of  $P_{fimB}$ , which is consistent with the recent finding in *E. coli*<sup>5</sup> that RcsB acts as a positive regulator for the expression of type 1 fimbriae. Nevertheless, *rscB* deletion also reduced  $P_{mrkA}$  activity (Fig. 11) indicating that the regulator responsible for the counter-expression of type 1 and 3 fimbriae has yet to be identified.



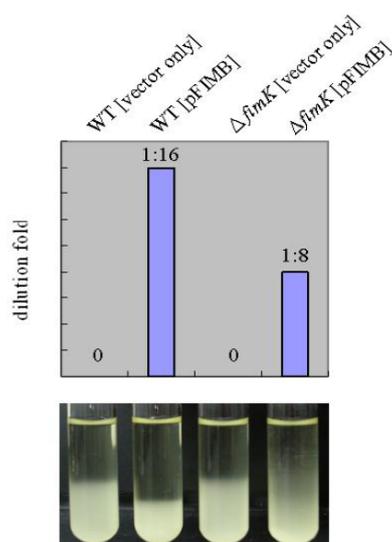
**FIG. 11.** Deletion effects of *rscB* on the activity of  $P_{fimA}$ ,  $P_{fimB}$ ,  $P_{fimE}$ , and  $P_{mrkA}$ . A significant difference ( $P < 0.001$ ) was marked by a star.

***FimK probably acts to regulate the expression of type 1 fimbriae and cps.*** The *fimK* gene, located at the end of the type 1 fimbriae gene cluster, encodes a protein with a DNA binding domain at the N-terminus and an EAL domain at the C-terminus (Fig. 12A), which suggesting a c-di-GMP phosphodiesterase activity of FimK protein. As shown in Fig. 12B, deletion of *fimK* increased the activity of type 1 fimbriae but decreased the CPS amount.

(A)



(B)

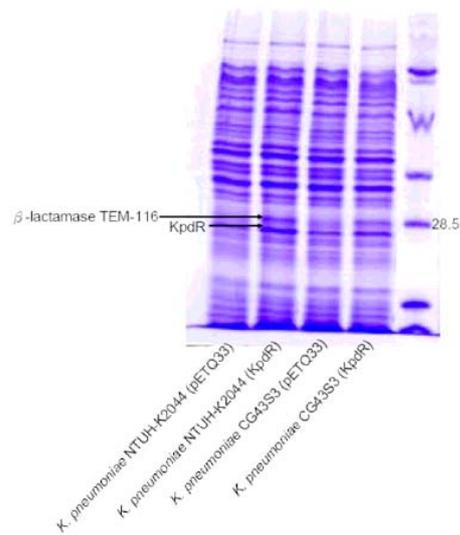


**FIG. 12.** (A) Gene organization of type 1 fimbriae in *K. pneumoniae* NTUH-K2044. FimK contains HTH domain (77.6% aligned) and EAL domain (100% aligned). (B) Assessment of type 1 fimbrial activity by yeast agglutination test and sedimentation test to determine the CPS synthesis (lower panel).

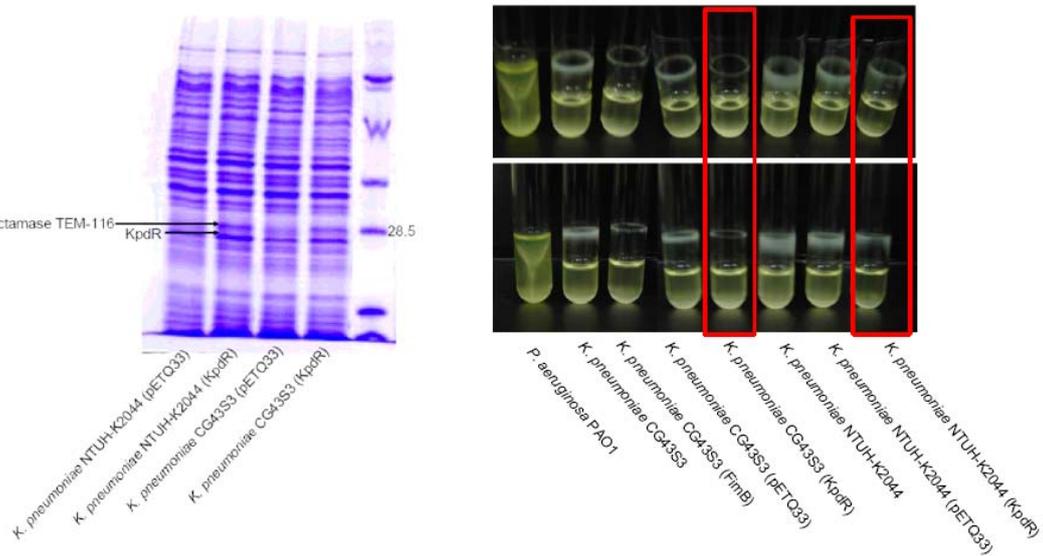
***Overexpression of KpdR enhanced the synthesis of  $\beta$ -lactamase TEM-116 but reduced the biofilm formation.*** The overexpression of the 2CS response regulator encoding gene *kpdR*, located upstream of the *kpd* fimbriae gene cluster, increased the synthesis of an unknown protein (Fig. 13A). Subsequently, the MS/MS analysis of the protein revealed a perfect match to  $\beta$ -lactamase TEM-116. Consistent with the report by Gallant CV *et al.*, 2005 (7), the increase synthesis of TEM-116 not only

increased the bacterial resistance to  $\beta$ -lactam antibiotic but also decreased the biofilm formation (Fig. 13B).

(A)



(B)



**FIG. 13.** (A) Overexpression of KpdR in both *K. pneumoniae* NTUH-K2044 and *K. pneumoniae* CG43S3. The bacteria were grown overnight in LB broth with addition of 25  $\mu$ g/ml Kanamycin and 1 mM IPTG at 37 $^{\circ}$ C. (B) Biofilm formation analysis. Bacteria were grown at 37 $^{\circ}$ C in LB broth with addition of 25  $\mu$ g/ml Kanamycin and 1 mM IPTG.

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## VI. Self-Assessment

We have carried out the experiments as planned, published two papers (1, 2), and several manuscripts (3~5) are in the final stage to be submitted for publication. In addition, many interesting findings allow the anticipation of important contributions to unravel the regulation of fimbrial expression at genomic level, the cross-talk regulatory circuit in-between the nine fimbrial gene clusters, and also their roles in infections, which hold promise to provide intervening targets for antimicrobial agents.

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