國 立 交 通 大 學

生 物 科 技 研 究 所

碩 士 論 文

Regulatory roles of MrkH, MrkI, MrkJ and CsgD on the expression of type 1 and type 3 fimbriae of *Klebsiella pneumoniae* CG43

克雷白氏肺炎桿菌 CG43 中 MrkH、 MrkI、MrkJ 和 CsgD 在調控第一型與第三型線毛表現所扮演的角色

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中華民國一百年六月

Abstract in Chinese

第一型和第三型線毛已被證實是克雷白氏肺炎桿菌的重要的致病因子。分析克雷白氏肺炎桿菌 CG43 基因體，我們發現這兩型線毛基因組相鄰座落，暗示二者的基因表現有協同調控的可能。本論文中，我們探討 MrkH、MrkI、MrkJ、CsgD 調控蛋白對此兩型線毛表現的影響。首先，分別構築 *mrkH*、 *mrkI* 、*mrkJ* 和 *csgD* 的基因剔除菌株，再藉由甘露醣競爭酵母菌凝集測試、西方墨點法、生物膜形成能力分析這些基因缺損的影響。結果顯示，*mrkI* 基因剔除菌中第一型線毛表現增加而第三型線毛的表現量減少；*mrkH* 基因的剔除也減少 MrkA 的表現量及降低生物膜活性，但不影響 FimA 的表現量；在 *mrkJ* 基因剔除株中，MrkA 的表現量有些許增加，但生物膜活性卻略微降低；另一方面，*csgD* 基因剔除後降低第三型線毛基因組啟動子活性。而在 *mrkI* 基因剔除株中，第三型線毛基因組啟動子的活性降低，但 *fimS* 翻轉變為正向的趨勢卻增加，此結果進一步證實 MrkI 調控蛋白在轉錄層次正向調控第三型線毛、負向調控第一型線毛的表現。此外，實驗顯示 MrkJ 重組蛋白具有磷酸二酯酶活性，這也暗示二級信使 c-di-GMP 在第三型線毛表現上扮演重要角色。最後，增加 c-di-GMP 結合蛋白 MrkH 表現能提高第三型線毛表現量，此結果更進一步支持了上述的假說。

Abstract

Klebsiella pneumoniae type 1 and type 3 fimbriae have been reported to be important virulence factors. Analysis of the *Klebsiella pneumoniae* CG43 genome revealed the two fimbriae encoding gene clusters are physically linked, which suggesting a coordinated -regulation for their expression. In the study, regulatory roles of the MrkH, MrkI, and MrkJ and CsgD on the expression of the two fimbriae were investigated. Firstly, the *K. pneumoniae* CG43S3-derived mutants respectively with a gene-deletion of *mrkH*, *mrkI*, *mrkJ*, and *csgD* were generated. The deleting effects on the fimbrial activities were then determined using analysis of mannose-sensitive yeast agglutination, western blot hybridization, and biofilm formation. An increased expression of type 1 fimbriae and a decreased expression of type 3 fimbriae were found for the Δ*mrkI* strain. The deletion of *mrkH* also decreased the expression of MrkA and biofilm formation activity but had no apparent effect on FimA expression. On the other hand, the *mrkJ* deletion appeared to slightly increase the MrkA expression but decrease the biofilm formation activity while the *csgD* deletion reduced the promoter activity of MrkA. The *mrkI* deletion reduced the *mrkA* promoter activity but increased OFF-to-ON inversion of the *fimS* suggesting MrkI is the transcription regulator for their reciprocal expression. Moreover, a phosphodiesterase activity of the recombinant MrkJ was demonstrated implying the second messenger c-di-GMP (bis-(3′-5′)-cyclic dimeric GMP) plays a regulatory role on the expression of type 3 fimbriae. The possibility was further supported by the notion that overexpression of the c-di-GMP binding protein MrkH apparently increased the type 3 fimbriae activity.

Acknowledgement

碩士班兩年是我人生很重要的轉捩點，開啟了研究生涯第一頁。最重要的是要感謝我的指導老師- 彭慧玲博士，每當我遇到困難裹足不前時，總是能推我一把給我勇氣，教導我如何思考，您就像媽媽一樣很溫暖，謝謝您這兩年來辛苦的教導。請您好好保重身體，要健康唷~

謝謝幽默的顗峰學長在我剛進實驗室時，耐心的帶領我設計實驗，讓我開始適應研究的生活；謝謝大胃王丸子太后和享受生活的雅雯學姊，總是讓我們吃到飽又哈哈大笑，很喜歡這快樂氣氛，真好！謝謝很有喜感的小新和健誠學長，對我平日的照顧以及給我實驗上寶貴的意見，也很懷念你們哥倆演內心戲的趣事；謝謝身材超棒的靜柔學姊，在我情緒低落的時候傾聽並給我鼓勵；謝謝志桓學長送我一些壓箱寶；謝謝可愛的哲充，你的某些想法真的很有趣，相信你內心應該還住著一個青春的小男孩，我不會忘記你愛的廣播電台；謝謝剛柔並濟的假花和親切的佩君學姊，常帶土產和水果填飽我們的五臟廟；謝謝跟我一起奮鬥兩年的品瑄、宜臻和欣怡，有你們讓我很幸福，永遠都要有 woman's talk 唷!非常聰明的豪君，我們一起與為了實驗奮鬥的心路歷程，永難忘懷。活潑大方的小波、很有安全感的郁勝、心思細密的舉豪和熱心的力成，實驗室的未來就交給你們了！誠心的感謝實驗室所有成員，有你們的幫忙才有現在的我，非常感謝大家!

最後，我想要謝謝我的家人、小豆和大牙牙，陪我走過人生很重要的階段，讓我一路走來不孤單，支撐我堅持到最後，我把碩士這份榮耀獻給愛我的人，謝謝你們。希望在未來的日子裡我們都可以過得幸福快樂，當我們再次相聚的時候還是有聊不完的趣事。

崴云 謹致於

交通大學生物科技研究所 中華民國 100 年 6 月

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Abbreviation

APS	ammonium persulfate
bp	Base pair (s)
c-di-GMP	Bis-(3'-5')-cyclic dimeric guanosine monophosphate
DGC	di-guanylate cyclase
EDTA	*N'N'N'N'*-ethylenediaminetetraacetate
ESBL	extended-spectrum β-lactamase
IPTG	isopropyl-β-D-thio-galactopyranoside
kDa	kiloDalton (s)
LB	Luria-Bertani broth
μg	microgram
μl	microliter
MR	Mannose resistance
NBT	nitro blue tetrazolium chloride
OD	optical density
ONPG	o-nitrophenyl-β-D-galactopyranoside
PAGE	polyacrylamide gel electrophoresis
PDE	phosphodiesterase
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PVDF	polyvinylidene fluoride
rpm	revolutions per minutes
SDS-PAGE	sodium dodecyl sulfate-polyacrylamid gel electrophoresis
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
TEMED	*N, N, N', N'*- tetramethylethyl –enediamide

Introduction

1. Clinical importance of *Klebsiella pneumoniae*

Klebsiella pneumonia, a member of the the *Enterobacteriaceae* family is a Gram-negative opportunistic pathogen that infects immunocompromised patients (16, 41). Since 1980s, *K. pneumoniae* is emerging as an important pathogen both in the community and the hospital setting. In the community the emergence of virulent strains with predominantly of K1/K2 capsular serotypes has been observed (41). *K. pneumoniae* causes a wide spectrum of infections, including septicemia, pneumonia, urinary tract infection, meningitis, and purulent abscess at various sites. Especially in Taiwan, *K. pneumoniae* has been attributed to be the major cause of liver abscess in diabetes mellitus patients (14, 92). 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 (47, 50, 89, 93).

In the hospital environment with the extensive use of antibiotics, multiple drug resistance has been increasingly observed in *K. pneumonia* isolated, especially the extended-spectrum β-lactamase (ESBL)-producing strains (24, 25, 41, 51, 53). The prevalence of ESBL is 25.0% for *Klebsiella* spp., 12.3% for *Escherichia coli* and less than 5% for other *Enterobacteriaceae*. Carbapenems has been considered to be the best option for the treatment of serious infections caused with ESBL-producing *K. pneumoniae* (18). The recent report of NDM-1 *K. pneumonia* which is a carbapenems-resistant strain and also produces a novel metallo-β-lactamase from a patient in New Delhi hospital has demanded a potent drug for effective clinical treatment (33, 44, 56, 94).

2. Adhesion properties

In addition to its antibiotic resistance feature (77), there are five major virulence factors identified to participate in *K. pneumonia* infections, which include capsular polysaccharides (32), lipopolysaccharides (88), iron-acquisition systems (60), and adhesion property (83). Adherence to host tissues is an essential early phase in many bacterial infections. Fimbriae, one of the adherence factors, are long, thread-like appendages on bacterial surface. They are found in as many as 500 copies per cell (43). Each fimbrial fiber is a polymer composed of hundreds of structural subunits called pilin. The adhesin which is located on the tip of fimbriae determines the specific binding to the host cell. The attachment to host surfaces is thought to increase the infection potentially by providing resistance to the mechanical clearance of the host defense system (84). Most clinical *K. pneumoniae* isolates are known to express two types of fimbrial adhesins, type 1 and type 3 fimbriae (27).

2.1 Type 1 fimbriae

Type 1 fimbriae, which facilitate colonization of uroepithelial cells (79), are heteropolymeric fibers produced by all members of the *Enterobacteriaceae* family and expressed by *fimACDFGHIK* gene cluster (42). They are right-handed, 6.9 nm wide pilus rod containing 500-3000 copies of the major structural subunit FimA, and of a linear tip fibrillum. The fibrillum is formed by the D-manose-specific adhesin FimH at the tip and by several copies of the subunits FimG and FimF (29, 40). In addition to mediating fimbriae attachment to the bladder epithelium, the adhesion FimH also enables bacteria to get internalized into bladder cells (13). The *fimC* and *fimD* genes respectively encode a fimbrial chaperone and usher protein. The *fimK* gene located directly downstream of *fimH* is only present in *K. pneumoniae* but not in *E. coli*. The *fimK* gene product has previously been shown to be involved in type 1

fimbria expression (70). The function of *fimI* gene product is unknown, but this product has been found to be essential for type 1 fimbriae biosynthesis in *E. coli* (91). Regulation of the type 1 fimbriae expression in *E. coli* is very complex, and several regulatory factors that act by altering the expression of *fimB* and *fimE* have been described. The *fimB* and *fimE* genes located upstream to the *fim* operon encode DNA recombinases that mediate the expression of type 1 fimbriae (1, 8, 72, 80). The recombinases FimB and FimE regulate the phase switch of type 1 fimbriae. FimB facilitates inversion from phase-OFF to phase-ON, as well as inversion from phase-ON to phase-OFF. FimE, on the other hand, causes only inversion from phase-ON to phase-OFF of type 1 fimbriae plays a significant role in the ability of *E. coli* infection of the urinary tract (19). Recently, type 1 fimbriae were also found to be essential for the ability of *K. pneumoniae* to cause UTI (urinary tract infection) (74).

2.2 Type 3 fimbriae

Type 3 fimbriae are 2 to 4 nm wide and 0.5 to 2 μm long organelles that are characterised by their ability to mediate mannose-resistant agglutination of tannic acid-treated human RBC (MR/K agglutination) (23). The fimbriae are similar to type 1 fimbriae, both are produced by the chaperone-usher assembly pathway (37). Type 3 fimbriae are encoded by *mrkABCDF* gene cluster (2, 23): MrkA and MrkF, the major fimbrial subunit protein and minor subunit, respectively (36), MrkD, the fimbrial adhesin of which the N terminal domain is responsible for receptor binding (86). Specifically, MrkD adhesin has also been shown to mediate adhesion to type IV and type V collagen (75); MrkB, a periplasmic chaperon; MrkC, an outer membrane usher protein which anchors the fimbriae to the bacterial cell surface. Most pathogenic *K. pneumoniae* strains produce type 3 fimbriae, which are essential for bacterial biofilm formation on biotic and abiotic surfaces (59, 81). Biofilms are organized communities

of bacteria attached to surfaces, composed of polysaccharides, nucleic acids and proteins known as extracellular polymeric substances (EPS) (11). In addition, type 3 fimbriae have been demonstrated to mediate bacterial attachments to several cell types including tracheal epithelial cells, renal tubular cells, extracellular matrix proteins, and components of basement membranes of human lung tissue (35, 86, 87) .

2.3 Curli fibers and CsgD

Curli fibers are a major adhesin factor to surfaces, and also affect cell aggregation and biofilm formation in many enterobacteria, such as *Salmonella* and pathogenic *E. coli* strains (20, 58, 62, 68). Expression of both curli fibers and cellulose depends on the CsgD protein, a response regulator of the LuxR family. The CsgD activates transcription of the *csgBAC* operon (5), which encodes curli structural subunits, and transcription of the *adrA* gene, a positive effector of cellulose biosynthesis (66). The AdrA is a member of the GGDEF protein family (26, 85) which can catalyze the synthesis of bis-(3′-5′)-cyclic dimeric GMP (c-di-GMP) and in turn stimulates the enzymes responsible for expression of cellulose and adhesive curli fibers (76). The expression of curli is also affected by environmental factors, such as a low growth temperature (<32℃), low osmolarity, and slow growth or starvation conditions (28, 30, 58, 69).

3. Cyclic-di-GMP signaling pathway

Almost 20 years after its discovery, bis-(3′-5′)-cyclic dimeric GMP (c-di-GMP) has come to be recognized as a ubiquitous second messenger in bacteria. The second messenger has been shown to control a variety of bacterial cellular processes, including motility, fimbriae expression, biofilm formation and cell cycle progression (31). The intracellular levels of cyclic-di-GMP is regulated by the GGDEF domain

proteins with diguanylate cyclase (DGC) activity and the EAL domain or HD-GYP domain proteins with cyclic di-GMP specific phosphodiesterase (PDE) activity (73). The synthesis of c-di-GMP from two GTP molecules is catalysed by the cooperative action of two GGDEF domains that each binds one GTP substrate. On the other hand, degradation of c-di GMP is catalysed by the highly specific HD-GYP or EAL-containing PDEs. Recent investigation has demonstrated that glutamate at position 352, conserved motif DDFG(T/A)GYSS (loop 6 domain) and Mg^{2+} or Mn^{2+} is required for PDE activity (67).

The recently study of BlrP1 (also known as KPN 01598) crystal structure from *K. pneumonia* shows that BlrP1 consist of, in addition to a phosphodiesterase EAL output domain, a BLUF photoreceptor domain which is able to senses blue light using a FAD chromophore (6). In 2006, PilZ- domain proteins were identified as a cellular c-di-GMP receptor using a bioinformatic approach (3). A nuclear magnetic resonance (NMR) structure of the PilZ-containing protein PA4608 from *P. aeruginosa* (63) and a crystal structure of the *Vibrio cholera* PlzD (7) have also been resolved and analyzed. Moreover, the residues in the RxxxR and D/NxSxxG which are conserved in the PilZ-domain proteins were shown to be able to bind c-di-GMP at the level of sub-micromolar affinity (17, 52, 71). However, it is still unclear how the binding of c-di-GMP to different PilZ-containing proteins affects the expression of their downstream target genes.

4. Specific aims : To investigate the functional role of MrkH, MrkI, MrkJ and CsgD on the expression of type 1 fimbriae and type 3 fimbriae in *K. pneumoniae* CG43

In *K. pneumoniae* CG43, a highly virulent liver abscess isolate of K2 serotype (15), the type 1 fimbriae encoding genes are physically linked with the type 3

fimbriae encoding genes *mrkABCDF*, which suggesting a coordinated regulation is involved in controlling their expression. Next to *mrkF*, three regulatory genes *mrkJ*, *mrkI*, and *mrkH* are found (Fig. 1A). The *mrkH* gene product is annotated to be a PilZ-domain protein, *mrkI* gene product is a transcriptional regulator containing an uncharacterized N-terminal region and a C-terminal LuxR-like DNA-binding domain, and MrkJ is an EAL-containing phosphodiesterase. Deletion of *mrkJ* was found to be able to increase the type 3 fimbriae production and biofilm formation activity, which resulted from the accumulation of intracellular c-di-GMP (38). Although no curli fimbriae genes *csgBAC* (12) could be identified in the published *K. pneumoniae* genomes, *csgD* was found to be clustered with *csgEFG* (65) in the genome of *K. pneumoniae* CG43 (Fig. 1B). In this study, functional roles of MrkH, MrkI, MrkJ and CsgD on the expression of type 3 fimbriae or and type 1 fimbriae in *K. pneumoniae* CG43 are investigated. We anticipate that a better understanding of the adhesion property allow identification of a potent target for the development of antibacterial agents.

Materials and methods

1. Plasmids, primers, bacterial strains and growth conditions

Bacterial strains, plasmids and primers used in this study are respectively listed in Table 1, Table 2 and Table 3. *K. pneumonia* CG43, a clinical isolate of serotype K2 recovered from Chang Gung Memorial Hospital, Linkou, Taiwan and is highly virulent to mice (15). *K. pneumonia* CG43S3, which is a derivative of CG43, is a streptomycin-resistant mutant (46). *K. pneumoniae* and *E. coli* strains were generally propagated at 37℃ in Luria-Bertani (LB) broth and M9 minimal medium. Bacterial growth was assessed by measuring the optical density at 600 nm (OD_{600}). The antibiotics used include ampicillin (100 μg/ml), chloramphenicol (20 μg/ml), kanamycin (25 μg/ml), tetracycline (12.5 μg/ml), chlorhexidine (15 μg/ml) and streptomycin (500 μg/ml).

2. DNA manipulation

Plasmids were purified by using the High-Speed Plasmid Mini kit (Geneaid, Taiwan). All DNA-modifying and -restriction enzymes were used as recommended by the manufacturer (Fermentas, USA). PCR amplifications were performed with Taq DNA polymerase (MDBio, Inc, Taiwan), Blend Taq DNA polymerase (TOYOBO, Japan) or High-Fidelity DNA Polymerase (Finnzymes, New England). PCR products and DNA fragments were purified using the Gel/PCR DNA Fragments Extraction kit (Geneaid, Taiwan). The primers used in this study were synthesized by MDBio, Inc, Taiwan. Transformation of *E. coli* cells was performed following the method of Dower (22).

3. Preparation of genomic DNA

Bacteria cultured at 37℃ in LB medium overnight were collected by centrifugation for 3 min (8,000 rpm). The pellet was resuspended in 800 μl lysis buffer (5 mM DTT, 100 μg/ml lysozyme, 200 mM NaCl, 20 mM EDTA, 40 mM Tris-HCl pH 8.0, 0.2% Triton X-100) and heated at 37℃ for 1 hr. Proteinase K solution 100 μg/ml was added and the mixture incubated at 50℃ overnight. After sitting on ice for 10 min, 250 μl of saturated NaCl was added, mixed by gently shaking for 10 min, and then precipitated by centrifugation for 10 min (13000 rpm). Finally, 1000 μl 99% alcohol was added to the collected supernatant (500 μl) and the mixture subjected to centrifugation for 10 min (13000 rpm). After gently rinsed with 75% alcohol, the pellet was dried and resuspended in sterile water.

4. Bioinformatics analysis

Homology search analysis and gene annotation were performed with the BLAST program provided by NCBI (http://www.ncbi.nlm.nih.gov) or VectorNTI (Invitrogen Vector NTI™ Advance). Functional domains of proteins were predicted using Pfam (http://pfam.sanger.ac.uk/) and promoter prediction was carried out by (http://www.softberry.com/all.htm).

5. Construction of specific gene-deletion in *K. pneumoniae* CG43

DNA fragments of 1 kb in length flanking both ends of the target genes *mrkH*, *mrkI*, *mrkJ*, or *csgD* were amplified by PCR with the respective primer sets (Table 3) and the amplified DNA fragments were cloned into the suicide vector pKAS46 (78), a suicide vector containing *rpsL*, which allows counter-selection with streptomycin for loss of the vector plasmid. The plasmids were transformed respectively into *E. coli* S17-1 λ*pir* (78) and then mobilized to the streptomycin-resistant strain *K. pneumoniae* CG43S3 (46) by conjugation. Several kanamycin resistant transconjugant were

selected and propagated in 4 ml LB overnight, and a small aliquot of the culture was plated on LB agar containing 500 μg/ml of streptomycin. The streptomycin resistant colonies were analyzed further for their susceptibility to ampicillin and kanamycin, a property reflecting the loss of the vector sequence. The streptomycin-resistant and kanamycin sensitive colonies were isolated and the deletion of *mrkH*, *mrkI*, *mrkJ*, or *csgD* was confirmed by PCR with the gene specific primers (Table 3). The resulting bacteria with mutation in *mrkH*, *mrkI*, *mrkJ*, or *csgD* were respectively named WYC09, WYC42, WYC12, and WYC45 (Table 1).

6. Yeast agglutination activity assay

The agglutination analysis of yeast *Saccharomyces cerevisiae* AH109 was carried out as described (9). Briefly, bacteria (~10^8 c.f.u./ml) were suspended in PBS with or without 2% mannose and then mixed with 10 mg/ml of yeast (Sigma, YSC2) on a glass slide. After 5 min incubation at room temperature on an orbital shaker, agglutination of yeast caused by bacteria could be assessed.

7. Construction of a complementation strain

The complementation plasmids which carrying respectively *mrkHJ* and *mrkHIJ* coding sequences were constructed by PCR cloning using the specific primers into yT&A (Yeastern Biotech, Taiwan), and named pWY05 and The *SacI*/ *XbaI* fragment from pWY05 and pWY24 was then subcloned to pRKAS46 and the resulting plasmids were individually transformed into *E. coli* S17-1 λ*pir*, and the transformants named Δ*mrkI*[pKAS46-*mrkHIJ*] and Δ*mrkI*[pKAS46-*mrkHJ*] (Table 1) and then mobilized to the streptomycin-resistant strain *K. pneumoniae* CG43S3 by conjugation.

8. Biofilm formation assays

The measurement of biofilm formation was performed according to the method described (57). Overnight grown bacteria were diluted (1:100) in LB medium, and 150 μl diluted bacteria were inoculated into each well of a 96-well microtiter dish (Orange Scientific, Belgium, cat #5530100 or TPP Scientific, America, cat #92096) and the plate incubated at 37℃ for 24 hr or 48 hr to allow the biofilm formation. Each well was then washed with water and 150 μl of 1% crystal violet was added and the incubation at room temperature continued for 30 min. After washing with water, 150 μl of 1% SDS was subsequently added to each well and the microtiter dish was shaken to dissolve the dye. The capability of biofilm formation was quantified by determining the absorbance at 595 nm (ELx800, BIO-TEK). The biofilm formation activity result represented the mean of three separate experiments.

9. Constructions of the recombinant His_6-tagged proteins

The DNA fragments which respectively contains the major pilin of type 1 fimbriae, MrkI-HTH domain and MrkJ-EAL domain were PCR amplified from the genomic DNA of *K. pneumoniae* CG43S3 with primers wc27 /wc12 and wc21/wc08 (Table 3). The amplified PCR products were cloned into the cloning vector yT&A (Yeastern Biotech, Taiwan), and then subcloned using proper restriction enzymes specific enzyme and then ligated into pET30 expression vector. The recombinant plasmid was then transformed into *E. coli* NovaBlue(DE3) or *E. coli* BL21(DE3).

10. Overexpression and purification of insoluble the His_6-tagged FimA

The bacterial cells were grown in 100 ml of LB medium at 37℃ with shaking until OD_{600} reached 0.6. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was then added to a final concentration of 0.5 mM and the growth was continued for 4 hrs at 37℃. Subsequently, the cells were harvested by centrifugation at 8000 rpm for 10

min, resuspended in lysis buffer (50mM Tris-HCl [pH8.0], 1mM EDTA and 100 mM NaCl), and the cell suspension disrupted by sonication and then the cell debris removed by centrifugation at 13000 rpm for 10 min. The recombinant FimA was insoluble. Finally, the His_6-tagged proteins were purified from the pellet via affinity chromatography using His-Bind resin (Novagen), and the elution was carried out with elution buffer (20 mM Tris-HCl, 0.5 M NaCl, 250 mM imidazole, 6N urea, [pH 7.9]). Aliquots of the collected fractions were analyzed by SDS-PAGE and the fractions containing most of the purified His_6-tagged protein were dialyzed against the 1 mL of 1X PBS buffer (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, [pH 7.4]) containing 6N urea. The 5.26mg/ ml anti-rabbit FimA was generated by Kelowna International Scientific Inc.

11. Western blot analysis of the expression of type 1 and type 3 fimbriae

Total cellular lysates from the bacteria grown overnight in LB medium were resolved by 12% SDS-PAGE to determine the expression of type 1 and type 3 fimbriae in *K. pneumoniae* CG43. The proteins were then electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After incubation with 5% skim milk at room temperature for 1 hr, the membrane was washed 3 times with 1X PBS. Subsequently, the membrane was incubated at room temperature for 2 hrs with diluted anti-FimA or anti-MrkA serum. After 3 washes with 1X PBS, a 5000-fold diluted alkaline phosphatase-conjugated anti-rabbit immunoglobulin G was added and the incubation continued for 1 hr. The blot was again washed and the bound antibodies were detected using the chromogenic reagents BCIP (5-bromo-4-chloro-3-indolyl phosphate), NBT (Nitro blue tetrazolium) and alkaline phosphatase buffer (10 mM, 5 mM and 100 mM Tris-HCl pH 9.5).

12. Construction of the LacZ reporter gene fusion

The putative promoters of *fimA*, *fimB*, *fimE* and *mrkA* were PCR amplified using the specific primers (Table 3) and the PCR products subcloned in front of the promoterless *lacZ* gene on placZ15 (48). The bacteria carrying each of the reporter plasmids were grown sharking overnight in LB medium, and the β-galactosidase activities were measured essentially as described (55). The data is representative of at last three independent experiments. Every sample was assayed in triplicate, and the average activity and standard deviation were presented.

13. β-galactosidase activity assay

β-galactosidase was assayed according to the method of Miller (54). The bacteria in the early or late logarithmic growth phase (optical density at 600 nm 0.5 or 0.8) 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 15 min at 28℃. Subsequently, 200 μl of 4 mg/ml o-nitrophenyl-β-D-galactopyranoside (ONPG) was added and the mixture vortexed for 10 sec, then incubated at 28℃ 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.

14. Motility assay

Essentially as described (49), 3 μl overnight-grown bacteria was inoculated onto trypton swimming plate (0.3% Bacto Agar, 0.5% NaCl, and 1% tryptone) and the plate incubated at 30℃ for 9 hrs. The diameter of the zone created by the swimming bacteria was measured.

15. Overexpression and purification of the His_6-tagged MrkJ

The bacterial cells were incubated in 100 ml of LB medium at 37℃ with shaking until OD_{600} reached 0.6. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was then added to a final concentration of 0.5 mM and the growth was continued for 4 hrs at 37℃. Subsequently, the cells were harvested by centrifugation at 8000 rpm for 10 min, resuspended in lysis buffer (50mM Tris-HCl [pH8.0], 1mM EDTA and 100mM NaCl), and the cell suspension disrupted by sonication and then the cell debris removed by centrifugation at 13000 rpm for 10 min. Finally, the His_6-tagged proteins were purified from the supernatant via affinity chromatography using His-Bind resin (Novagen), and the elution was carried out with buffer A (20 mM Tris-HCl, 500 mM NaCl, 250 mM imidazole, [pH 7.9]). Aliquots of the collected fractions were analyzed by SDS-PAGE and the fractions containing most of the purified His_6-tagged protein were dialyzed against the buffer containing 20 mM Tris-HCl [pH 8.5], 200 mM NaCl, and 10% glycerol.

16. Phosphodiesterase activity of MrkJ

Phosphodiesterase activity of the recombinant MrkJ was performed as previously described (39). In the assay buffer (50 mM Tris-HCl, 1 mM $MnCl_2$ [pH 8.5]) supplemented with 5 mM bis(p-nitrophenol) phosphate (bis-pNPP), 20μg of the purified MrkJ was added and the mixture incubated for 3 hrs at 37℃. Reactions were incubated for 3 hrs at 37℃, and the release of p-nitrophenol was measured at 410 nm.

Results

1. BLAST analysis and Pfam searches

As shown in Fig. 1A, the MrkH, MrkI, and MrkJ encoding genes are located downstream of the type 3 fimbriae operon *mrkABCDF* (Fig. 1A) and the intergenic region of *mrkH-mrkI* and *mrkI-mrkJ* are respectively 5 and 143 bp. The gene organization has been found to be conserved in the published *K. pneumoniae* genomes (97). As previously demonstrated by reverse transcription PCR (RT-PCR), the three ORFs *mrkH*, *mrkI*, and *mrkJ* could be transcribed in a transcriptional unit (96). These suggested a possibility of a coordinated expression of the physically linked genes *mrkABCDF* and *mrkHIJ*. The gene coding for *csgD*, which has been associated with bacterial virulence (31), is located within *yggR*, a putative ATPase, and *yqgF* which is an essential protein for Holliday junction resolvase (HJR) (4) (Fig. 1B).

Analysis using BLAST (Basic Local Alignment Search Tool) (38) and Pfam database (protein family database) revealed that *mrkH*, *mrkI*, *mrkJ* and *csgD* respectively encode c-di-GMP binding protein (PilZ domain protein), LuxR-type transcription regulator, c-di-GMP phosphodiesterase (EAL domain protein) and LuxR-type transcription regulator (Fig.1C). As shown in Fig. 2, the conserved RxxxR motif and D/NxSxGG motif, which play essential roles for c-di-GMP binding in many PilZ domain proteins (3, 10, 31, 52, 61, 71), is found in MrkH. The conserved DDGF(T/A)GYSS motif and glutamate residue critical for phosphodiesterase activity of many EAL domain protein, is also present in MrkJ (Fig. 3). In *K. pneumoniae*, several EAL domain proteins including BlrP1 (6), YjcC (45), FimK (80), MrkJ (38) have been reported. As shown in Fig.4, the sequence alignment revealed *K. pneumoniae* CsgD had 36% identity shared with *E. coli* CsgD and *Salmonella enterica*.

2. Generation of the *mrkH*, *mrkI*, *mrkJ* and *csgD* deletion mutants

The respective gene deletion was assessed using PCR analysis with the specific primer pairs, wc05/wc06 for Δ*mrkH*, wc07/wc08 for Δ*mrkI*, wc17/wc18 for Δ*mrkJ*, and pcc226/pcc227 for Δ*csgD*. As shown in Fig. 5, the amplicons of 1500-bp and 850-bp were obtained for wild type strain and Δ*mrkH* strain; 2500-bp and 1900-bp for wild type strain and Δ*mrkI* strain; 1300-bp and 620-bp for wild type strain and Δ*mrkJ* strain; 1300-bp and 680-bp for wild type strain and Δ*csgD* strain, respectivrly which confirmed the individual deletion for each of the mutants. The selection percentage for Δ*mrkH*, Δ*mrkI*, Δ*mrkJ* and Δ*csgD* were respectivrly 57%, 64%, 10% and 21%.

To determine if the gene deletion effects the bacterial growth, growth curve of the Δ*mrkH*, Δ*mrkI*, Δ*mrkJ* and Δ*csgD* in LB or M9 medium were determined. The four mutant strains appeared to show similar growth curve as the wild type strain in LB medium (Fig. 6A) or M9 medium (Fig. 6B).

3. Analysis of the deletion effects on the activity of type 1 and type 3 fimbriae

The activity of type 1 fimbriae was assessed using yeast agglutination analysis. As shown in Fig. 7A, agglutination could be observed for Δ*mrkA*, Δ*mrkH*, Δ*mrkI* and Δ*mrkI*[pKAS46-*mrkHJ*] strains. The *mrkI* deletion effect was able to be complemented by introducing pKAS46-*mrkHIJ* into Δ*mrkI* strain. Addition of 2% mannose could inhibit the agglutination activity of Δ*mrkA*, Δ*mrkI* or Δ*mrkI*[pKAS46-*mrkHJ*] indicating a mannose-sensitive agglutination activity (Fig. 7B).

Expression of *K. pneumonia* type 3 fimbriae has been reported to be able to promote the biofilm formation (21, 36). Compared to wild type, the biofilm formation activity of Δ*mrkA*, Δ*mrkH* or Δ*mrkI* was apparently reduced while the activity slightly decreased for Δ*mrkJ* in either type of microtiter dish (Fig. 8A and C or Fig. 8B and D)

and in 24 hrs or 48 hrs incubation (Fig. 8A and B or Fig. 8C and D). The deletion effect of *mrkI* was able to be complemented by introduction pKAS46-*mrkHIJ* into Δ*mrkI* strain. However, deletion of *csgD* did not affect biofilm formation activity except a reduced biofilm formation was observed in TPP-microtiter dish for 48 hrs.

4. Analysis of the deletion effects on the expression of FimA pilin of type 1 fimbriae and MrkA pilin of type 3 fimbriae

In order to obtain a good amount of the recombinant FimA protein, the recombinant plasmid pETfimA-23 (Table 2) was used to transform *E. coli* Novablue (DE3) and expression of the recombinant FimA was analyzed. As shown in Fig. 9A, an IPTG-induced overexpression of the His_6-FimA could be observed, however, most of the recombinant proteins were in the pellet-fraction. Urea (6N) was employed to unfold the aggregated protein. The purified His_6-FimA of approximately 28 kDa (Fig. 9A) was used to immunize rabbit to raise anti-FimA antibody. The specific of anti-FimA antibody was tested by a 10000-fold diluted anti-FimA at room temperature for 1 hr. As shown in Fig. 9B, the anti-FimA antibody could specifically bind to the recombinant FimA.

Western blot analysis using the prepared FimA antiserum and the MrkA antiserum obtained from Dr. HY Chang's lab (NTHU, College of Life Science (96)) was then performed to determine the expression of type 1 and type 3 fimbriae. As shown in Fig. 10, approximately same amount of the bacterial total proteins were applied to gel stained by coomassie brilliant blue. Compared to wild type, expression of MrkA was abolished in Δ*mrkH*, Δ*mrkI*, Δ*mrkA* while slightly decreased in Δ*csgD*. MrkA expression could be restored in Δ*mrkI*[pKAS46-*mrkHIJ*] strain. By contrast, a slight increase on the expression of MrkA was observed in Δ*mrkJ* strain. On the other hand, the expression of FimA was increased in Δ*mrkI* and Δ*mrkA* strains. Interestingly,

expression of FimA was observed in Δ*mrkI* as well as in Δ*mrkA* strains further suggesting a reciprocal expression of the two fimbriae.

5. MrkI affected the *fimS* inversion

As shown in Fig. 11A, two primer pairs pcc248/ pcc249 and pcc247/ pcc249 were designed to respectively assess ON-phase or OFF-phase of *fimS*. Compare to wild type, the level of ON-phase of *fimS* represented by a 478-bp amplicon in Δ*mrkI* and Δ*mrkI*[pKAS46-*mrkHJ*]strain increased (Fig. 11B). The *mrkI* deletion effect could be complemented by introduction of pKAS46-*mrkHIJ* into Δ*mrkI* strain, suggesting MrkI negatively regulates the *fimS* promoter activity. By contrast, a slight decrease of ON-phase of *fimS* level was found for Δ*mrkJ* and Δ*csgD* strains. However, no apparent change of ON-phase or OFF-phase of *fimS* (599-bp amplicon) level in Δ*mrkH* was observed (Fig. 11B).

6. MrkA, FimA, FimB and FimE promoter activity analysis

As shown in Fig. 12A, the putative promoters of 551-bp, 358-bp, 400-bp and 270-bp noncoding DNA respectively located upstream of *mrkA*, *fimA*, *fimB* and *fimE* were individually fused with the promoterless *lacZ* gene of the reporter plasmid placZ15 (Table 2). Thus, the activity of P_{mrkA}, P_{fimA}, P_{fimB} and P_{fimE} could be determined by the activity of LacZ. The promoter reporter plasmids were then individually transformed into the parental strain Δ*lacZ* (CG43S3Z01), or each of the specific gene deletion strains Δ*lacZ*Δ*mrkH* (CG43S3Z01Δ*mrkH*), Δ*lacZ*Δ*mrkI* (CG43S3Z01Δ*mrkI*) and Δ*lacZ*Δ*csgD* (CG43S3Z01Δ*csgD*). The deletion of *mrkH* had no apparent effect on the activity of P_{fimA}, P_{fimB} or P_{fimE} (Fig. 12A left panel) which is consistent with the result of *fimS* analysis (Fig. 11). On the contrary, the *mrkH* deletion dramatically decreased P_{mrkA} activity (Fig. 12A right panel). As shown in Fig.

12B, similar deletion effect on the promoter activity was observed for *mrkI* compared to *mrkH*. However, *mrkJ* deletion had no apparent effect on either of the promoter activity (Fig. 12C). Although no apparent change of the P_{fimA}, P_{fimB} or P_{fimE} activity by the *csgD* deletion (Fig. 12D left panel), reduced activity of P_{mrkA} in Δ*lacZ*Δ*csgD* was observed (Fig. 12D right panel).

7. The recombinant MrkJ exhibited a phosphodiesterase activity

To examine whether *mrkJ* encodes a functional phosphodiesterase, the MrkJ expression plasmid was transformed into *E.coli* MG1655 for motility analysis. As shown in Fig.13, *E. coli* MG1655[pRK415-MrkJ] exhibited the highest level of motile activity, while *E. coli* MG1655[pRK415-Ydeh] which expresses c-di-GMP cyclase activity (95), had the lowest level of motility activity. This implied that MrkJ encodes a functional phosphodiesterase activity to reduce the cellular c-di-GMP leading to increase the bacterial swimming activity.

The in vitro analysis was performed using the purified recombinant MrkJ and phosphodiesterase-specific substrate bis(*p*NPP). As shown in Fig. 14, an IPTG-induced overexpression of the His_6-MrkJ could be observed in *E. coli* BL21 (DE3), however, the recombinant proteins were found mostly in pellet-fraction but some in supernatant fractions. The recombinant MrkJ was then purified from the supernatant fraction for the assay of phosphodiesterase activity. The purified His_6-MrkJ of approximately 34 kDa was found to exhibit a 40-fold increase of the *p*-nitrophenol release compared to the reaction with BSA (Fig. 15). This further supported that MrkJ is a phosphodiesterase playing a role in modulation of the level of the secondary messenger c-di-GMP.

Discussion

Type 1 and type 3 fimbriae are important factors for bacterial invasion, biofilm formation, cell motility and persistence in specific cell surface. Regulation of type 1 fimbriae is well known which is mediated by the DNA recombinase FimB and FimE to control the inversion DNA sequence of *fimS* (34). By contrast, the regulation of type 3 fimbriae is poorly understood. Downstream to the type 3 fimbrial gene clusters, *mrkH*, *mrkI*, and *mrkJ* have recently been demonstrated to be transcribed in a transcription unit (96). This also implies that *mrkHIJ* acts as a regulatory operon for the expression of type 3 fimbriae. If *mrkHIJ* operon is also involved in the type 1 fimbriae expression is hence investigated.

1. MrkH is a positive regulator for type 3 fimbriae

The deletion of *mrkH* from *K. pneumoniae* CG43S3 caused an increased of mannose-resistant yeast agglutination implying that MrkH controls an unknown type of sugar-mediated binding adhesion activity. On the other hand, a reduced level of biofilm formation was found for the *mrkH* deletion strain which suggesting a positive regulatory role on the expression of type 3 fimbriae. A decreased expression of MrkA and P_{mrkA} activity in the Δ*mrkH* strain further supports that MrkH play a positive role on the expression of type 3 fimbriae through influencing P_{mrkA} activity. Science MrkH is a putative c-di-GMP binding protein, how the second messenger-mediated regulation carried out remains be investigated.

2. An inverse regulatory role of MrkI on the expression of type 1 and type 3 fimbriae

The deletion of MrkI from *K. pneumoniae* CG43S3 caused an increase of

mannose-sensitive yeast agglutination, ON-phase *fimS* inversion, and FimA expression. This implies MrkI plays an inhibitory role for the expression of type 1 fimbriae via altering of the *fimS* direction. However, promoter activity measurement (Fig. 12B) revealed that none of the promoters P_{fimA}, P_{fimB}, or P_{fimE} were affected by the deletion of *mrkI*. If MrkI indirectly affects the expression of type 1 fimbriae remains to be clarified. On the other hand, a reduced level of biofilm formation, P_{mrkA} activity, promoter activity and MrkA pilin expression were found for the *mrkI* deletion strain suggesting MrkI plays as an activator at the transcription level for the expression of type 3 fimbriae. RT-qPCR analysis of the *mrkI* deletion effect and an electrophoresis mobility shift assay (EMSA) of MrkI binding to P_{mrkA} performed by Dr. Ching-Ting Lin (School of Chinese Medicine, China Medical University) indicated that the recombinant MrkI was able to bind P_{mrkA} further supporting that MrkI reciprocally regulates the expression of the fimbriae at the transcription level.

3. MrkJ exerted a PDE activity

MrkJ has been reported in *K. pneumoniae* IApc35 as a functional c-di-GMP phosphodiesterase (38). Here, overexpression of MrkJ in *E. coli* appeared to increase the motility further supporting that MrkJ function as a PDE to decrease the cellular c-di GMP level. In the *mrkJ*-deletion mutant, a slightly increased of MrkA product was found whereas no obvious effect on the expression of FimA. This implied MrkJ plays a negative role in regulating the expression of type 3 fimbriae. Nevertheless, biofilm forming activity was decreased by the deletion of mrkJ indicated that MrkJ may play an indirect role to affect type 3 fimbriae activity.

4. CsgD is also a positive regulator for the expression of type 3 fimbriae

Deletion of *csgD* which encoding a LuxR-type transcription regulator slightly

reduced the MrkA expression and P_{mrkA} activity, suggesting CsgD is a positive regulator at the transcription level for the expression of type 3 fimbriae. However, no apparent effect of the *csgD* deletion on type 1 fimbriae was observed. Different regulatory role from the CsgD of *E. coli* or *Salmonella* is speculated because *K. pneumoniae* is non-flagellated bacteria.

In summary, this study indicated that MrkH, MrkI and CsgD play as activators whereas MrkJ plays as an inhibitor for the expression of type 3 fimbriae. In addition, MrkI played a negative role for the expression of type 1 fimbriae. The deletion of *mrkA* has been shown to cause an increase of type 1 fimbriae expression, however, by an unknown mechanism (82). It is concluded that MrkI is probably the regulator determining the reciprocal expression between the two types of fimbriae.

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Table 1. Bacteria strains used in this study

strains	Genotypes or relevant properties	Reference or source
Escherichia coli		
JM109	*endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB* $^{+}$ *Δ(lac-proAB) e14- [F' traD36 proAB*$^{+}$ *lacI*^{q} *lacZ ΔM15] hsdR17(*$r_K^- m_K^+$*)*	Laboratory stock
S17-1 λpir	*hsdR recA pro* RP4-2 (Tc::Mu; Km::Tn7)(λpir)	
BL21(DE3)	F$^-$ *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm trx*B15::*kan* (DE3)	Novagen
Novablue (DE3)	F$^-$ *ompT hsd*$_{SB}$ ($r_B^- m_B^-$) *gal dcm* (DE3), Tc^r	Novagen
Klebsiella pneumoniae		
CG43S3	Clinical isolate of K2 serotype, Sm^r	(46)
Δ *mrkH*	CG43S3 Δ*mrkH* , Sm^r	This study
Δ *mrkI*	CG43S3 Δ*mrkI*, Sm^r	This study
Δ *mrkJ*	CG43S3 Δ*mrkJ*, Sm^r	This study
Δ *mrkI* [pKAS46-*mrkHJ*]	CG43S3 Δ*mrkI* [pKAS46-*mrkHJ*], Sm^r	This study
Δ *mrkI* [pKAS46-*mrkHIJ*]	CG43S3 Δ*mrkI* [pKAS46-*mrkHIJ*], Sm^r	This study
Δ *lacZ*	CG43S3 Δ*lacZ*, Sm^r	This study
Δ *lacZ* Δ*mrkH*	CG43S3 Δ*lacZ* Δ*mrkH*, Sm^r	This study
Δ *lacZ* Δ*mrkI*	CG43S3 Δ*lacZ* Δ*mrkI*, Sm^r	This study
Δ *lacZ* Δ*mrkJ*	CG43S3 Δ*lacZ* Δ*mrkJ*, Sm^r	This study
Δ *lacZ* Δ*csgD*	CG43S3 Δ*lacZ* Δ*csgD*, Sm^r	This study

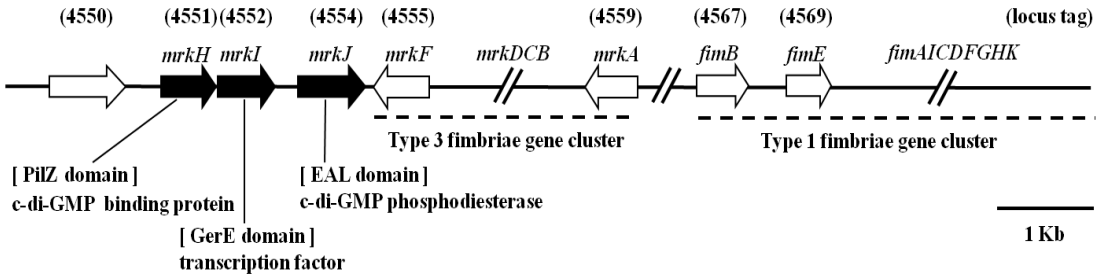
Table 2. Plasmids used in this study

Plasmid	Relevant characteristic	Reference or source
yT&A	Ap^r, T/A-type PCR cloning vector	Yeastern
pET30a	Km^r, His-tagged protein expression vector	Novagen
pKAS46	Ap^r, Km^r, suicide vector, *rpsL*	(78)
placZ15	Cm^r, promoter selection vector, $lacZ^+$	(48)
pRK415	Tc^r, shuttle vector, mob^+	This study
pETfimA-23	Km^r, ~629-bp fragment region of fimA cloned into pET30a	Chien-Chen Wu
pmrkA-P	Cm^r, 551-bp fragment of the upstream region of *mrkA* cloned into placZ15	Ying-Jung Huang
pfimA-P	Cm^r, 270-bp fragment of the upstream region of *fimA* cloned into placZ15	Ying-Jung Huang
pfimB-P	Cm^r, 358-bp fragment of the upstream region of *fimB* cloned into placZ15	Ying-Jung Huang
pfimE-P	Cm^r, 400-bp fragment of the upstream region of *fimE* cloned into placZ15	Ying-Jung Huang
pWY02	Ap^r, 1039-bp fragment of the upstream and 1111-bp fragment of downstream region of *mrkH* cloned into yT&A	This study
pWY24	Ap^r, 1005-bp fragment of the upstream and 993-bp fragment of downstream region of *mrkI* cloned into yT&A	This study
pWY04	Ap^r, 928-bp fragment of the upstream and 983-bp fragment of downstream region of *mrkJ* cloned into yT&A	This study
pWY22	Ap^r, 1133-bp fragment of the upstream and 1178-bp fragment of downstream region of *csgD* cloned into yT&A	This study
pKAS46-*mrkHIJ*	Km^r Ap^r, ~2500-bp fragment of the *mrkHIJ* and its adjacent regions cloned into pRKS46	This study
pKAS46-*mrkHJ*	Km^r Ap^r, ~1900-bp fragment of the *mrkHJ* and its adjacent regions cloned into pRKS46	This study

Table 3. Oligonucleotide primers used in this study

Primer	Sequence (5'--- 3')
wc05	GGATCCGCCTGGGTGCCCTTTTTCC
wc06	AAGCTTGGAGCGCATTCAGCAGATCG
wc07	AGATCCTACAAATGGGGCGTGA
wc08	GGCCTGTTCACCTATTACGTTG
wc17	GGATCCGGGCTGTGCAGAGAGTTGATAAA
wc18	AAGCTTGCAGGAAAGCGCGGTCAGC
pcc226	GGATCCGAGCTTCTGGTCAACACGCC
pcc227	AAGCTTCGATGAACGGCTGAGTACGGT
pfimA3	TTGGATCCATTTTGACTCGTTG
pfimA4	GGGGCCAAACTGTTTAGATCTT
pfimB4	TGTCGGCGGGATTCCTCATGG
pfimB5	CAAGATCTTGAGCATACCACAGC
pfimE4	TGACGAGCAGCCTGGATCCT
pfimE5	CAGATCTAATAGCCTTGACGCCAC
pmrkA4	GGAGATCTCATGGGCTGCCCT
pmrkA5	GCGGATCCCATTGCTGCAGA

(A)



(B)

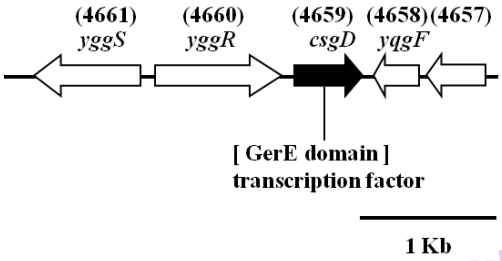


Fig. 1. Gene organization and domain analysis of *mrkH*, *mrkI*, *mrkJ* and *csgD*

The locus tag (KP1_number) is also indicated (A and B). The predicted function and the conserved protein domains, analyzed using bioinformatic tools provided online by NCBI (http://www.ncbi.nlm.nih.gov/) and Pfam (http://pfam.sanger.ac.uk/), are respectively marked in (blue) and green.

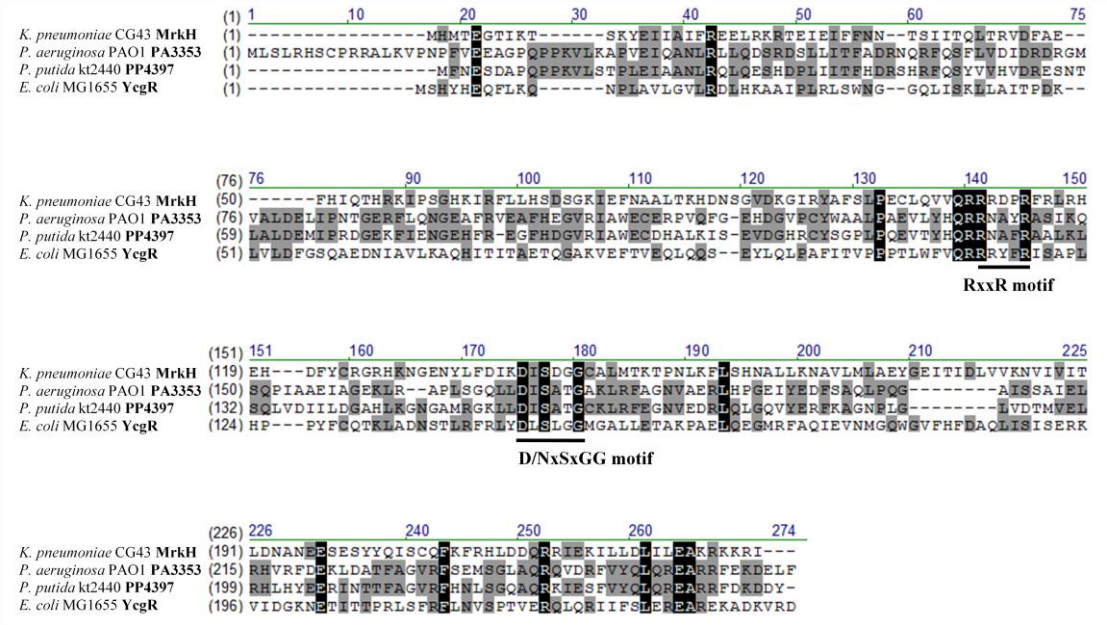


Fig. 2. Comparative sequence alignment of the PilZ domains

Sequences of the PilZ domain proteins, including MrkH, PA3353, PP4397, and YcgR (7, 71), were aligned by Vector NTI software. The conserved RxxxR motif and D/NxSxGG motif (x, any residue) are underlined. The critical lysine residue required for the c-di-GMP binding activity of YcgR (71) is indicated by an arrow (96).

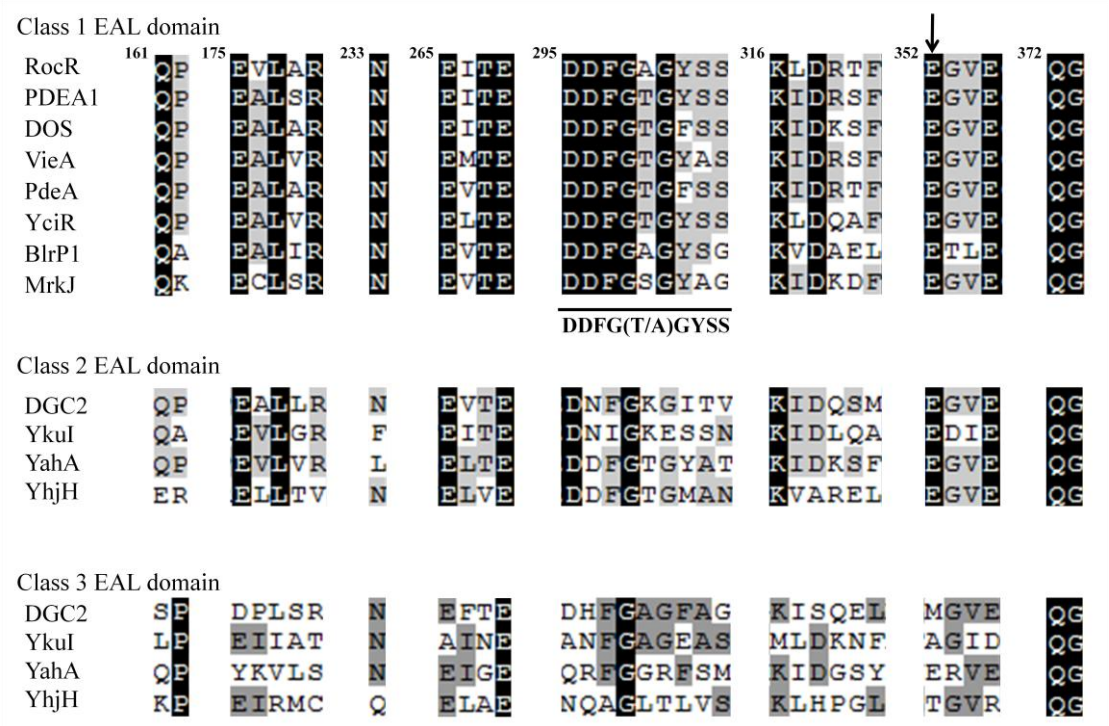


Fig. 3. Comparative sequence alignment of the EAL domains

Sequences of the EAL domain proteins, including MrkJ, RocR, PDEA1, DOS, VieA, PdeA, YciR, BlrP1, DGC2, YkuI, YahA, YhjH, YcgF, YdiV, LapD, and CsrD (67), were aligned. The conserved EAL motif, DDFG(T/A)GYSS and glutamate residue which were suggested to be essential for the enzymatic activity (64, 67, 90) are framed respectively. The numbering of the residues is based on the RocR sequence (64) and the glutamate residue is indicated by an arrow.

Fig. 4. Comparative sequence alignment of the CsgDs

The sequence aligment revealed *K. pneumoniae* CsgD had 36% identity shared with *E. coli* CsgD and *S.* Typhimurium.

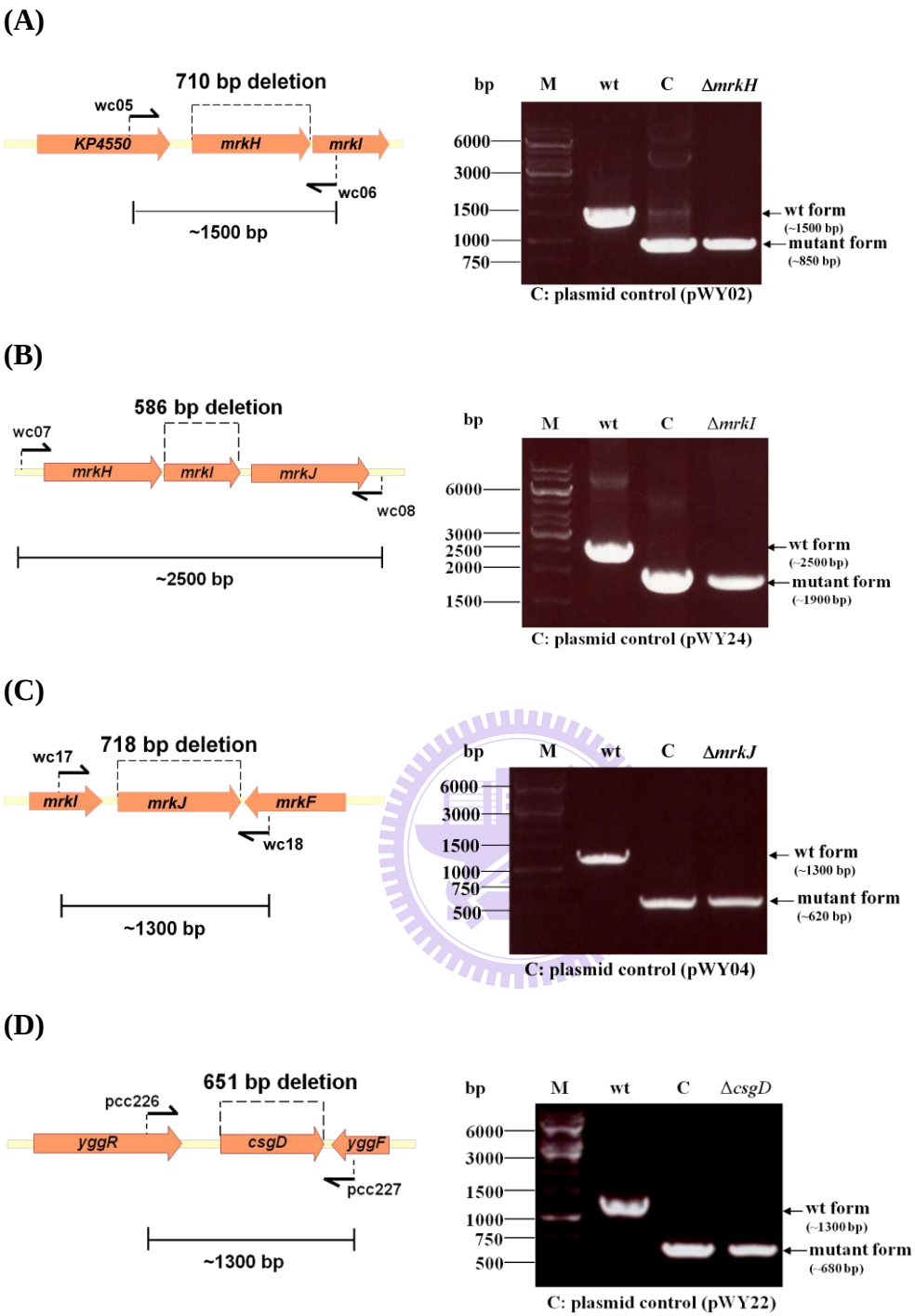
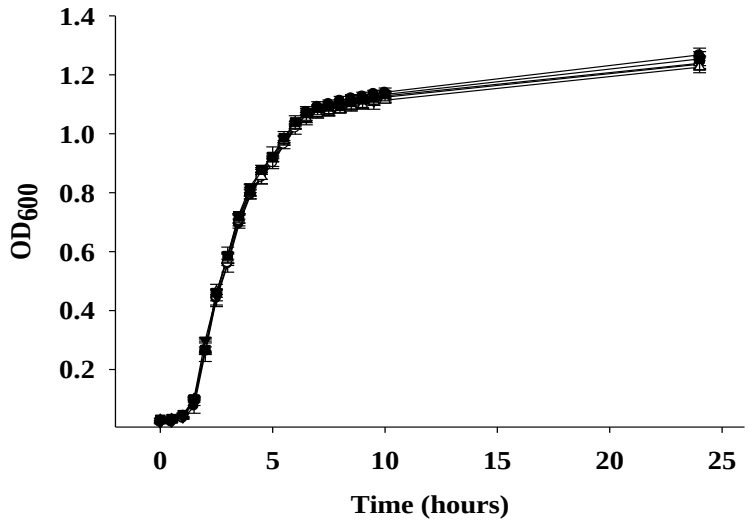


Fig. 5. Schematic depiction of the deletion-construct on the left panel and PCR analysis of the deletion mutants on the right panel

The mutant was confirmed using PCR with a specific primer pair located upstream and downstream of the target gene. (A) Δ*mrkH* , (B) Δ*mrkI* , (C) Δ*mrkJ* and (D) Δ*csgD*. M, DNA molecular size marker; wt, wild-type bacteria; C, plasmid control.

(A)



(B)

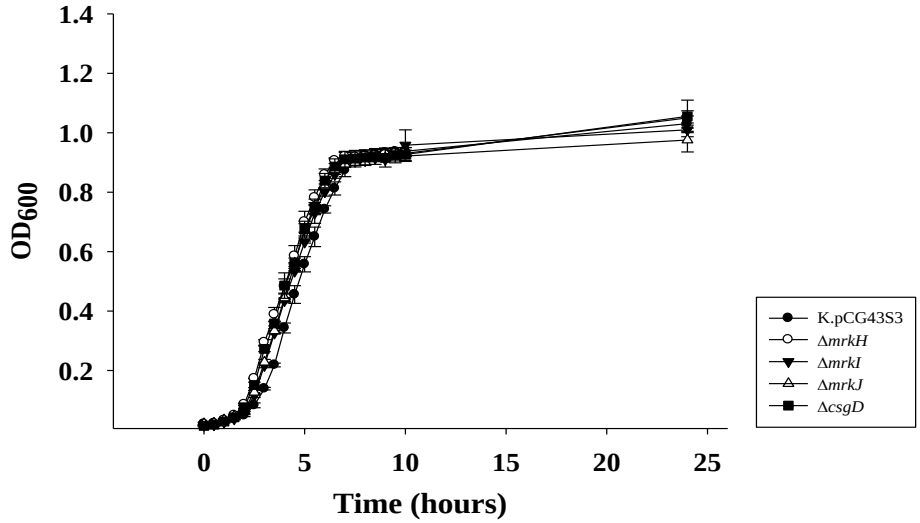
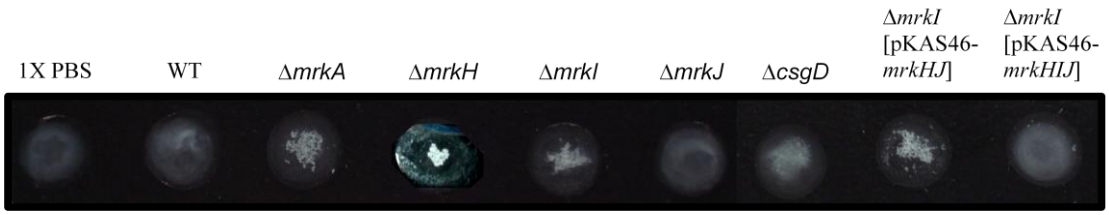


Fig. 6. Growth of CG43S3 and the derived mutants in LB and M9 media

Forty-microliter of overnight-grown bacterial suspension were subcultured into 4-ml fresh LB medium (A) or M9 medium (B). Growth of the wild type bacteria, Δ*mrkH*, Δ*mrkI*, Δ*mrkJ* and Δ*csgD* strains at 37℃ with agitation were determined by measuring the optical density at 600 nm.

(A)



(B)

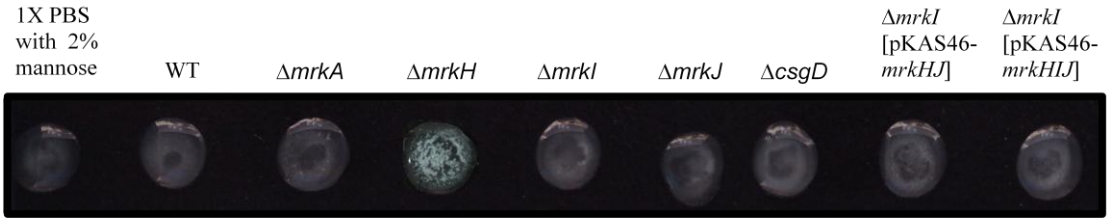


Fig. 7. Yeast agglutination activity assay

Overnight-grown bacterial suspension (~10^8 c.f.u./ml) and yeast solution (10 mg/ml) were mixed in 1:1 (v/v) ratio on a glass-slide and then incubated at room temperature on a orbital shaker for 30 min. Mannose-sensitive yeast agglutination, which represents type 1 fimbriae activity, was monitored visually by aggregation and precipitation of the cells after the addition of 2% mannose.

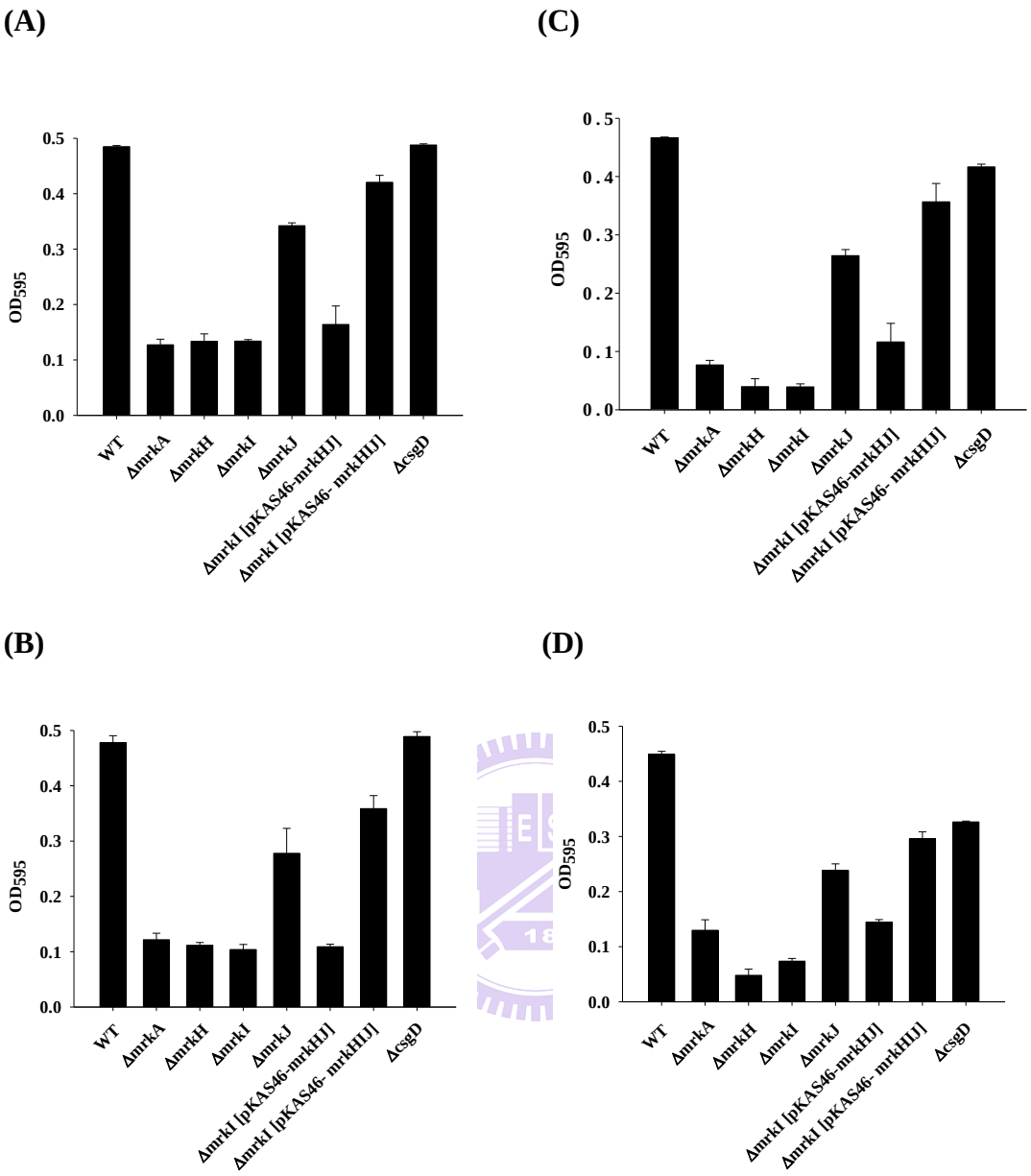


Fig. 8. Biofilm formation in polystyrene Material at 24hrs or 48hrs

Overnight cultured bacteria were 100-fold diluted and inoculated into the 96-well microtiter dish (A), (C) Orange (cat#5530100) (B), (D) TPP (cat#92096). After 24 hrs (A), (B) or 48hrs (C), (D) static incubation at 37℃, the bacterial biofilm formation was quantified by measuring the optical density of the crystal violet stained biofilm at 595 nm. The values correspond to the average of triplicate samples from a single trial.

(A) **(B)**

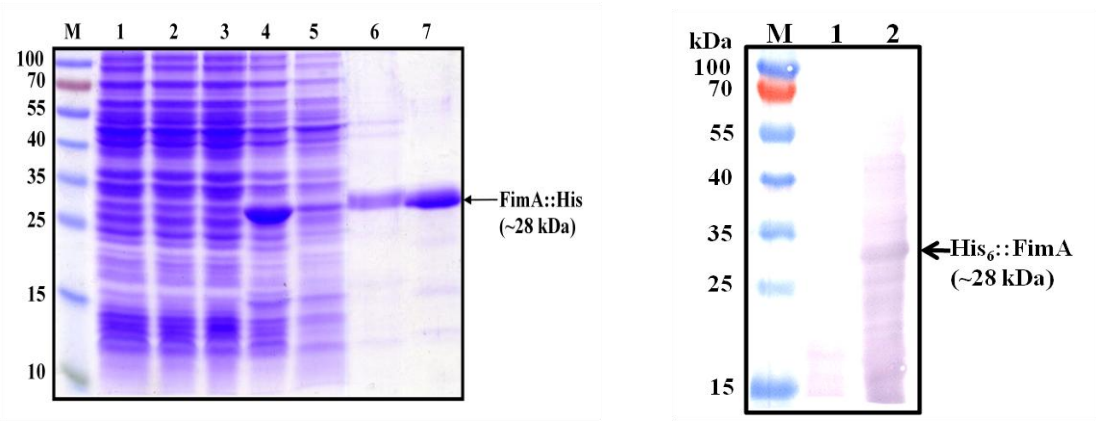


Fig. 9. SDS-PAGE analysis of the expression of the recombinant FimA in *E. coli* Novablue (DE3) and purity of the protein

(A) The sample in each lane was prepared from *E. coli* Novablue (DE3) cells carrying each of the following plasmids: Lanes 1, 2: pET30a; 3, 4, 5, 6, 7: pET30a-*fimA*. For the induced cells, 0.5 mM IPTG was added and the bacteria cultured for 5 hrs at 37℃. M: protein size markers. Lanes 1, 3: noniduced total cell lysate; 2, 3, 4, 5, 6, 7: the total cell lysates induced with 0.5 mM IPTG. Lane 5 is the supernatant fraction, lane 6 the pellet fraction and lane 7 the purified FimA::His_6 (3.0 ug). (B) Western blot analysis by rabbit anti-FimA antibody. Lanes 1: pET30a; 2: pET30a-*fimA* in Novablue (DE3). Lanes 1, 2, 3: the total cell lysates induced with 0.5 mM IPTG. M: protein size markers. The arrow indicated the recombinant FimA detected by antiserum.

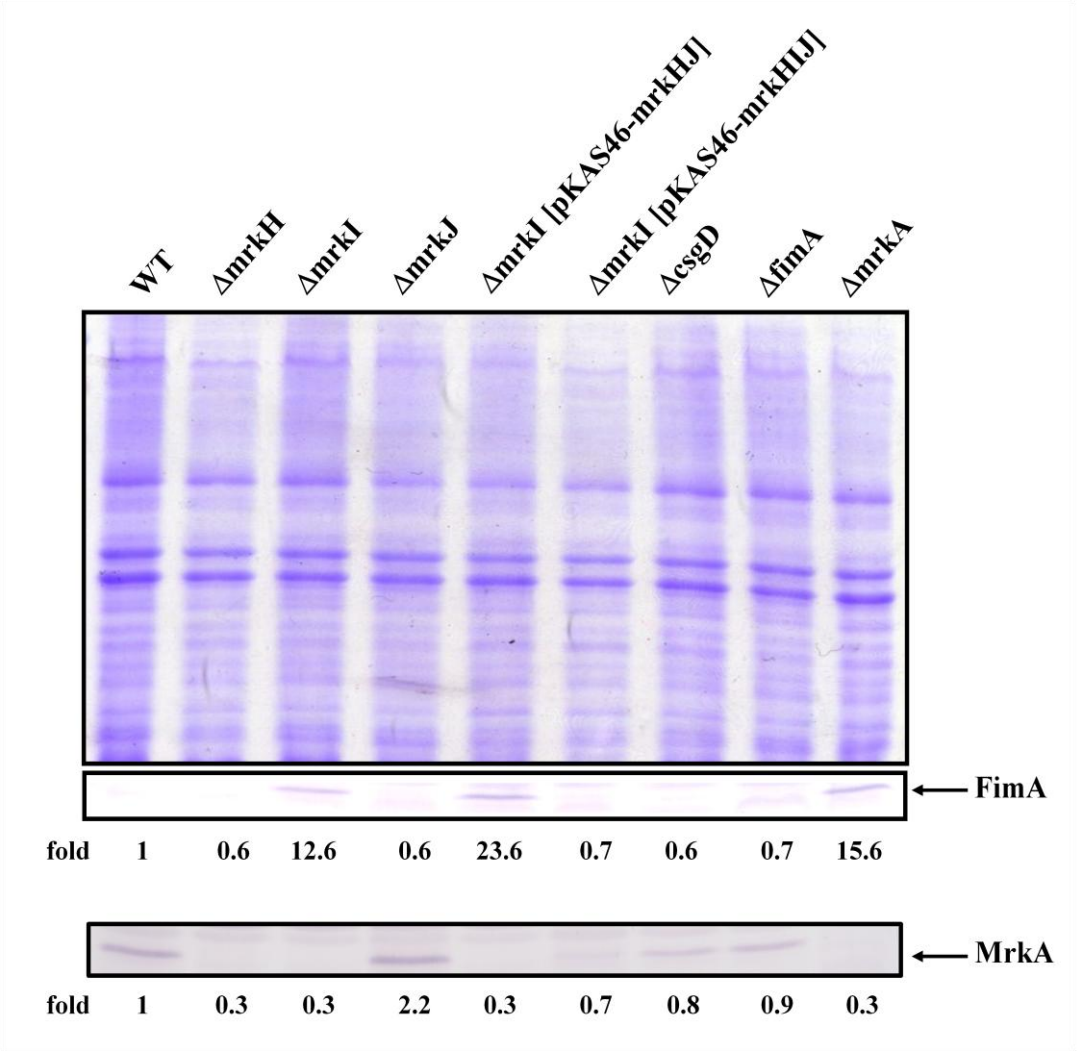


Fig. 10. Western blot hybridization analysis of the deletion mutants

K. pnuemoniae strains were grown overnight at 37℃ with agitation in LB broth. Total protein of bacterial cultures, 10μg per each lane, were separated by SDS-PAGE, transferred to PVDF membrane and finally subjected to hybridization against the antibodies of anti-FimA (the upper panel) or anti-MrkA (the lower panel). The expression of MrkA and FimA is indicated by arrows. WT stands for the wild-type strain. The fold change of FimA or MrkA amount calculated by ImageJ software is also shown.

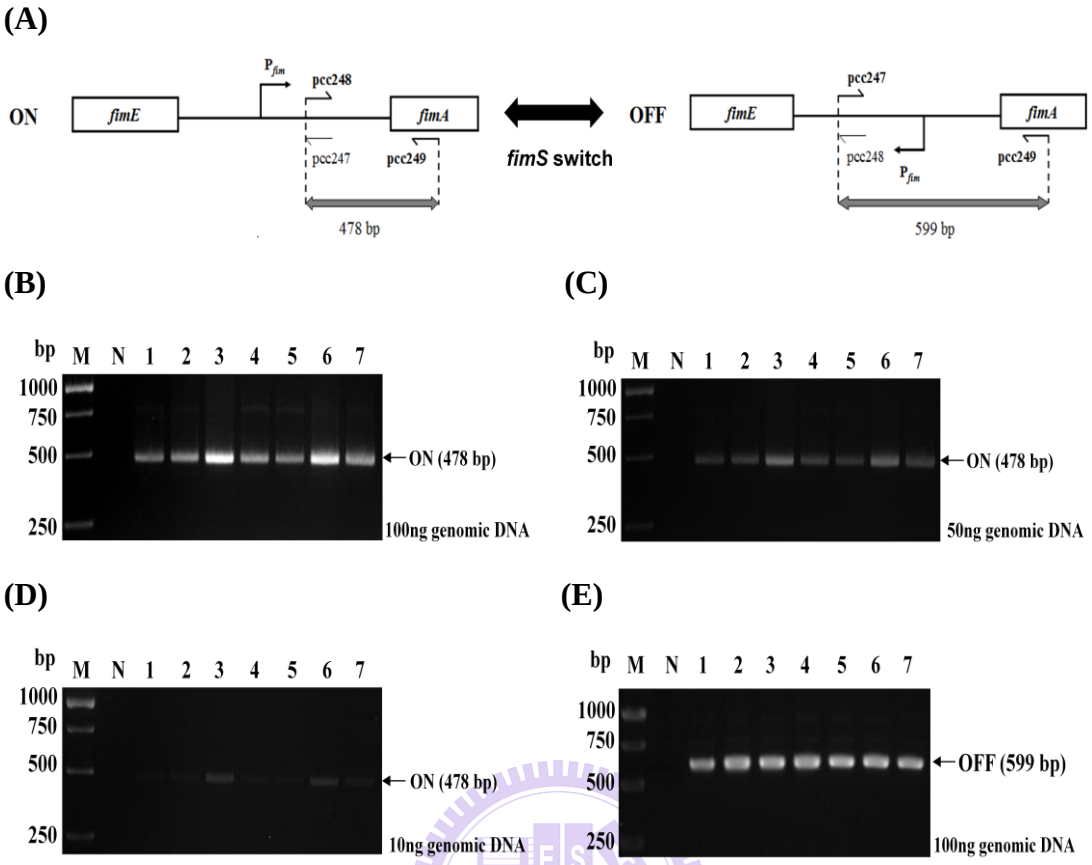
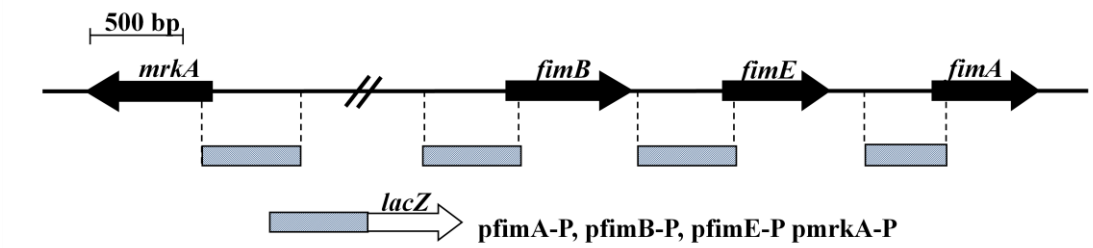
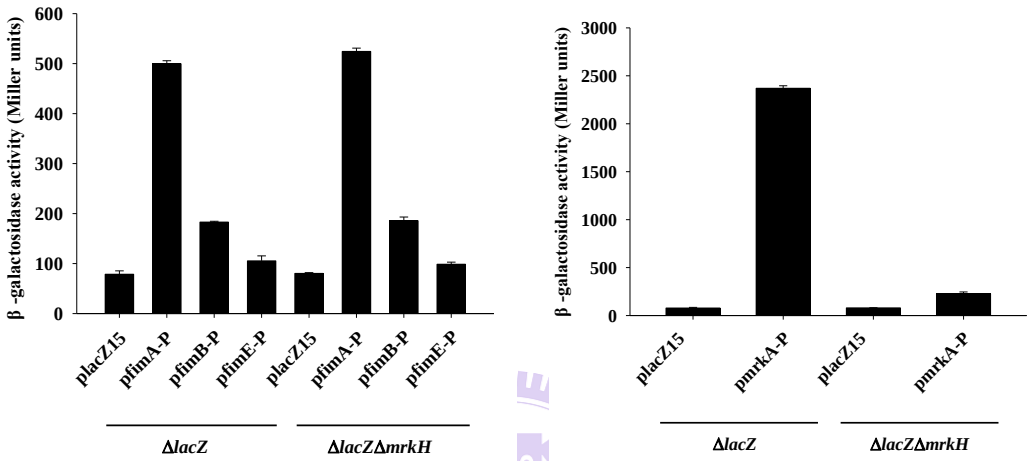


Fig. 11. The *fimS* promoter analysis

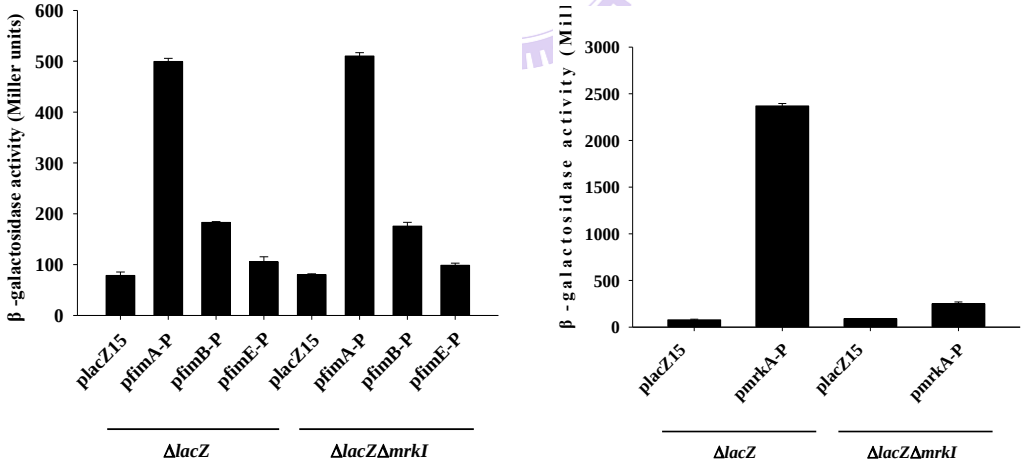
(A) Schematic depiction of the PCR-based assay for assessing the *fimS* inversion. Location of the primers (pcc247, pcc248, and pcc249) used and the size of the PCR amplicons in the ON- or OFF-phase are indicated. (B), (C), (D) and (E) PCR detection of the ON and OFF orientations of the *fimS*. The bacterial chromosomal DNA of 100ng (B) and (E), 50ng (C) and 10ng (D) was used as templates for the PCR analysis. M, DNA molecular size markers; N, a PCR result with no template DNA; Lanes 1, the wild-type strain; 2, Δ*mrkH*; 3, Δ*mrkI*; 4, Δ*mrkJ*; 5, Δ*csgD*; 6, Δ*mrkI* [pKAS46-*mrkHJ*]; 7, Δ*mrkI* [pKAS46- *mrkHIJ*].


500 bp
mrkA
fimB
fimE
fimA
lacZ
pfimA-P, pfimB-P, pfimE-P pmrkA-P

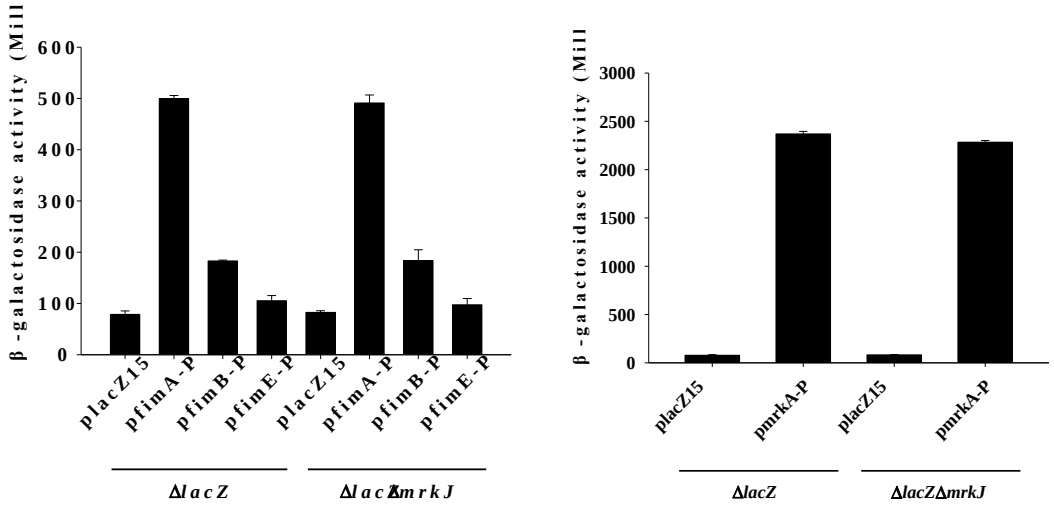
(A)


β -galactosidase activity (Miller units)
600
500
400
300
200
100
0
placZ15
pfimA-P
pfimB-P
pfimE-P
placZ15
pfimA-P
pfimB-P
pfimE-P
ΔlacZ
ΔlacZΔmrkH
β -galactosidase activity (Miller units)
3000
2500
2000
1500
1000
500
0
placZ15
pmrkA-P
placZ15
pmrkA-P
ΔlacZ
ΔlacZΔmrkH

(B)


β -galactosidase activity (Miller units)
600
500
400
300
200
100
0
placZ15
pfimA-P
pfimB-P
pfimE-P
placZ15
pfimA-P
pfimB-P
pfimE-P
ΔlacZ
ΔlacZΔmrkI
β -galactosidase activity (Mill
3000
2500
2000
1500
1000
500
0
placZ15
pmrkA-P
placZ15
pmrkA-P
ΔlacZ
ΔlacZΔmrkI

(C)



(D)

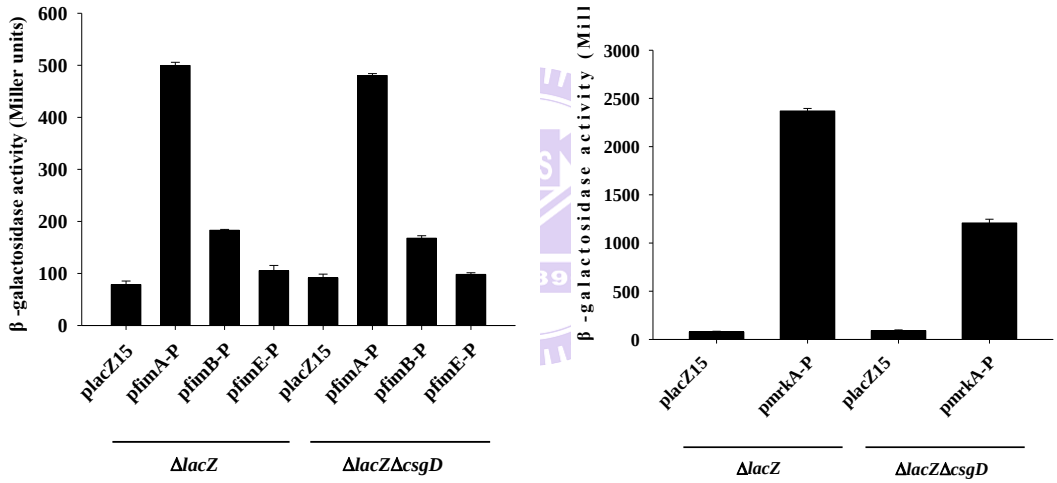


Fig. 12. The promoter activity measurement using LacZ as a reporter

The β-galactosidase activity of $P_{fimA::lacZ}$, $P_{fimB::lacZ}$, $P_{fimE::lacZ}$ and $P_{mrkA::lacZ}$ in log-phased cultures of (A) *K. pneumoniae*CG43S3Δ*lacZ* or CG43S3Δ*lacZΔmrkH*, (B) *K. pneumoniae*CG43S3Δ*lacZ* or CG43S3Δ*lacZΔmrkI*, (C) *K. pneumoniae*CG43S3Δ*lacZ* or CG43S3Δ*lacZΔmrkJ*, (D) *K. pneumoniae*CG43S3Δ*lacZ* or CG43S3Δ*lacZΔcsgD* which carrying respectively the reporter plasmids pmrkA-P, pfimA-P, pfimB-P, and pfimE-P were determined.

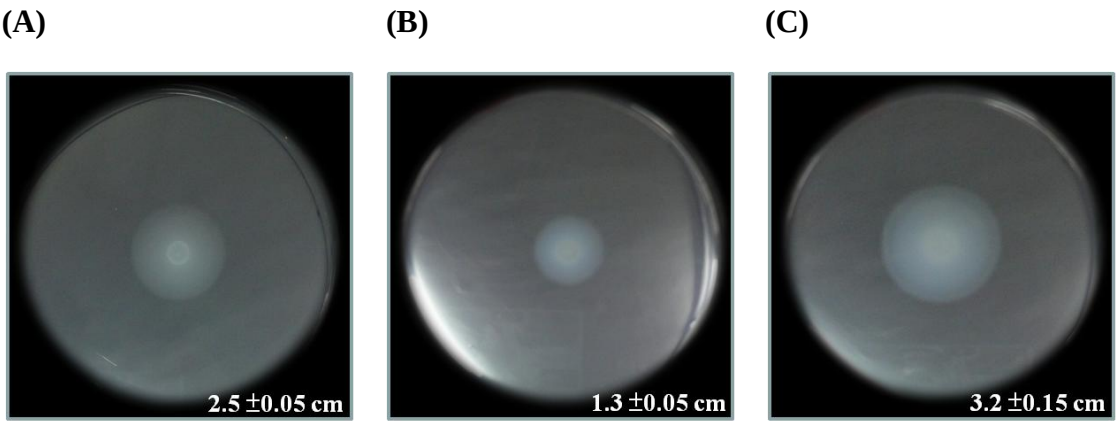


Fig. 13. Motility assay

The photograph was taken after the bacteria were incubated for 9 hrs on the swimming plate at 30℃. (A) *E. coli* MG1655[pRK415], (B) *E. coli* MG1655[pRK415-ydeh], (C) *E. coli* MG1655[pRK415-*mrkJ*]. The diameter is 2.5± 0.05 cm, 1.3 ±0.05 cm and 3.2 ±0.15 cm, respectively.

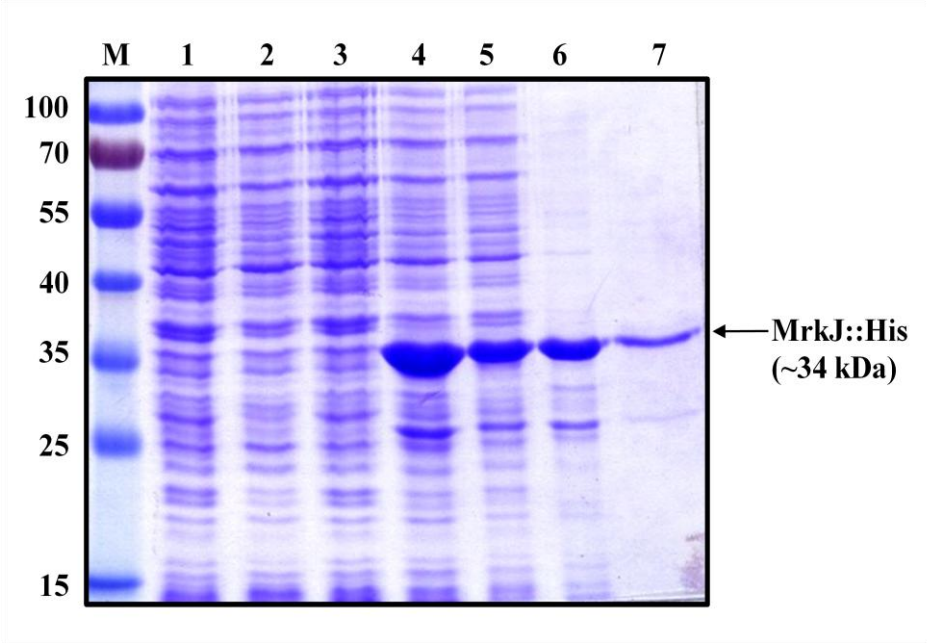


Fig. 14. SDS-PAGE analysis of the expression of the recombinant MrkJ in *E. coli* BL21(DE3) and purity of the protein

The sample was prepared from *E. coli* BL21(DE3) cells carrying each of the following plasmids: lanes 1, 2: pET30a; 3, 4, 5, 6, 7: pET30a-*mrkJ*. For the induced cells, 0.5 mM IPTG was added and the bacteria cultured for 5 hrs at 37℃. M: protein size markers; Lanes 1, 3: cell lysate of non-induced cells; Lanes 2, 3, 4, 5, 6, 7: cell lysates of the 0.5 mM IPTG induced bacteria; Lane 5: the supernatant fraction; Lane 6 the pellet fraction; Lane 7 the purified MrkJ::His_6 (1.25μg).

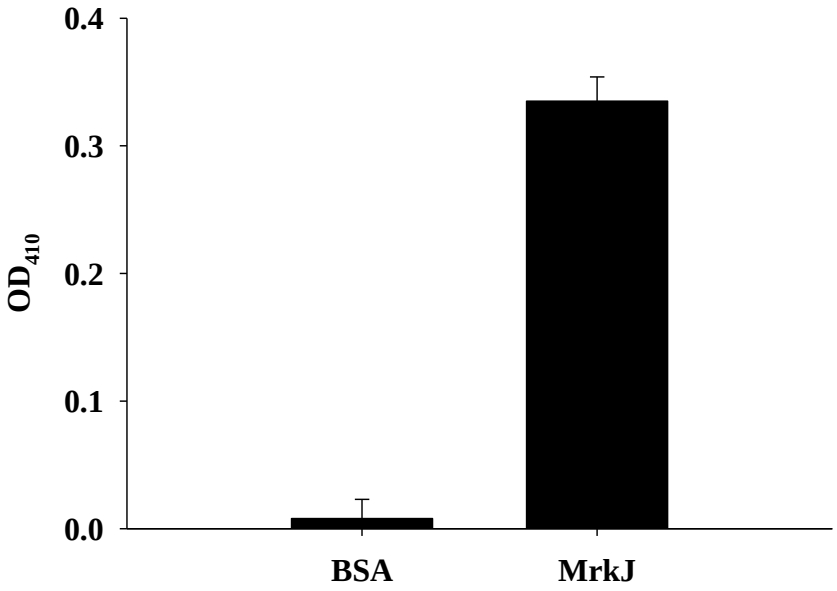


Fig. 15. Phosphodiesterase activity of the recombinant MrkJ

In vitro phosphodiesterase activity assays comparing the ability of partially purified BSA and MrkJ to cleave the phosphodiesterase-specific substrate bis(pNPP). Release of p-nitrophenol was determined at 410 nm.