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國立交通大學博士學位論文

Prevalence Analysis and Expressional Control of *Klebsiella pneumoniae* Fimbriae

克雷白氏肺炎桿菌線毛的分佈與表現

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1999 年，我離開從小居住的台北來到新竹，轉眼間已經過了 11 年了。現在回想起這一段日子，許多經過的事、遇見的人，心裡有感慨萬千，要感謝的人也實在太多。在這本論文完成同時，我想起大二升大三的暑假，我進入了實驗室進行專題研究，那是我大學生活的轉捩點，而後就這麼一路沒有間斷地直到現在。在這段過程中，首先我要感謝我的指導教授彭慧玲老師，給予我機會在實驗室進行研究。彭老師總是充滿耐性、細心地教導並且關心我的感受。我還記得當我是專題生時，老師每週都會撥空與我討論，從很基本的實驗細節與原理、學術論文的撰寫，以至於研究方向的邏輯，可以說是一點一滴、一字一句地指導我。彭老師總是給予學生很大的空間自由發展與嘗試。而開始專題研究後，過去認為枯燥無聊的教科書內容，變成了實際應用於研究的原理基礎，讓我重拾學習的興趣，可以說改變了我的人生。而在學術以外，我也要謝謝彭老師一直以來包容我個性上的缺點與不成熟，讓我有機會反省、學習與成長。

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論文摘要

克雷白氏肺炎桿菌是一株引起伺機性感染的革蘭式陰性細菌，大多於免疫力不全的病人身上，引起化膿性的潰瘍、菌血症、尿道以及呼吸道的感染。為了探討克雷白氏肺炎桿菌的致病機制，在本論文中，我們針對在感染初期扮演著重要角色的線毛的分佈情況及表現調控進行研究。

我們藉由分析克雷白氏肺炎桿菌 NTUH-K2044 的全基因體序列，共發現了九組獨立的線毛基因群。除了過去已知的第一型與第三型線毛基因組外，其餘的七組尚未有文獻報導，我們將其分別命名為 *kpa*、*kpb*、*kpc*、*kpd*、*kpe*、*kpf*、*kpg* 基因組。我們進一步分析了克雷白氏肺炎桿菌的臨床分離菌株，發現 *kpb* 與 *kpc* 基因組在 K1 莢膜血清型的菌株中有顯著較高的存在率。隨後，我們針對 Kpc 線毛進行了特性分析。在大腸桿菌中表現 Kpc 線毛的生合成基因組 *kpcABCD* 後，可以致使細菌製造出 Kpc 線毛並且增強其生物膜的形成能力。我們也發現 Kpc 線毛的表現受到了相變機制（phase variation）的調控，而此相變機制則是由 KpcI 專一性 DNA 重組酶所負責。最後，我們著重於第三型線毛的表現調控進行研究。我們發現位於第三型線毛基因組下游的 *mrkHIJ* 操作組可生合成出三個調控蛋白質來影響第三型線毛的表現。*mrkI* 基因被預測可生合成出 LuxR 類型的轉錄因子，而我們將 *mrkI* 在克雷白氏肺炎桿菌中進行剔除後，發現會破壞第三型線毛的基因轉錄。同時我們也證明，具有 PilZ 模組的 MrkH 與具有 EAL 模組的 MrkJ，可分別正向與負向調控第三型線毛的表現。此外，我們發現 Fur 攝鐵調控子會經

由調控 *mrkHIJ* 操作組來影響第三型線毛的表現。而二次代謝傳導物 c-di-GMP 也被證明可經由 Fur 與 MrkI 來活化第三型線毛的表現。我們還發現缺氧環境可能是影響第三型線毛表現的因子。

在本論文中，我們首度報導了克雷白氏肺炎桿菌線毛的基因體學分析，這個報導將有助於未來對於此細菌線毛黏附作用的研究（第二章）。我們也分析了 Kpc 線毛的功能性與相變調控表現（第三章），以及探討影響第三型線毛表現的多重調控因子（第四章）。

Thesis Abstract

Klebsiella pneumoniae is a Gram-negative pathogen which causes suppurative lesions, bacteremia and urinary as well as respiratory tract infections mostly in patients with underlying diseases. To investigate the pathogenicity of *K. pneumoniae*, we focused on the study of prevalence and expression of fimbriae, which are important virulence determinants during the initial infection.

By analyzing the whole genome sequence of *K. pneumoniae* NTUH-K2044, nine distinct fimbrial gene clusters were identified. Besides type 1 and type 3 fimbrial genes, the other seven are novel and designated *kpa*, *kpb*, *kpc*, *kpd*, *kpe*, *kpf*, and *kpg*. The following prevalence analysis among *K. pneumoniae* clinical isolates indicated that the *kpb* and *kpc* genes were more prevalent in the strains of capsular serotype K1. Subsequently, the Kpc fimbria, encoded by *kpcABCD* genes, was characterized. Induced expression of the recombinant *kpcABCD* genes in *Escherichia coli* resulted in Kpc fimbriation and increased bacterial biofilm formation. The Kpc fimbriae expression was also found to be regulated under phase variation mediated by the site-specific recombinase KpcI. Finally, the expressional control of type 3 fimbriae in *K. pneumoniae* CG43 was investigated. We described that the type 3 fimbriae expression was mediated by three regulatory proteins encoded by the *mrkHIJ* operon which located downstream to the type 3 fimbrial genes. Deletion of *mrkI*, which

encoded a LuxR-type response regulator, from *K. pneumoniae* was found to abolish the expression of type 3 fimbriae at transcriptional level. Moreover, MrkH, a PilZ domain protein, and MrkJ, an EAL domain protein, appeared to act as an activator and a repressor, respectively, for the type 3 fimbriae expression. Besides, we showed that the ferric uptake regulator Fur could activate the expression of type 3 fimbriae through regulation on the *mrkHIJ* operon. The second messenger c-di-GMP was also found to activate the expression of type 3 fimbriae through Fur and MrkI. Furthermore, we identified that oxygen-limitation was possibly an environmental stimulus for activating the type 3 fimbriae expression.

In this dissertation, we reported the first genomic analysis of fimbrial gene sequences in *K. pneumoniae*, which pave the way for future study of the bacterial adherence (Chapter 2). Subsequently, the functional role and phase-variable expression of Kpc fimbriae were characterized (Chapter 3). Moreover, a multi-factorial regulation of type 3 fimbriae expression was elucidated (Chapter 4).

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Abbreviations

bp	base pair(s)
CPS	capsular polysaccharide
CFU	colony forming unit
c-di-GMP	Bis-(3'-5')-cyclic dimeric guanosine monophosphate
DGC	di-guanylate cyclase
EDTA	ethylenediaminetetraacetic acid
ESBL	extended-spectrum β-lactamase
EMSA	electrophoretic mobility shift assay
h	hour(s)
IPTG	isopropyl 1-thio-β-D-galactopyranoside
kb	kilobase(s)
kDa	kilodalton(s)
LB	Luria-Bertani
μg	microgram
ml	mililiter
μl	microliter
mM	milimolar
μM	micromolar
OD	optical density
ONPG	o-nitrophenyl-β-D-galactopyranoside
PAGE	polyacrylamide gel electrophoresis
PDE	phosphodiesterase
qRT-PCR	quantitative real-time polymerase chain reaction
RFLP	restriction fragment length polymorphism
rpm	revolutions per minutes
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER 1

General Introduction

1.1. *Klebsiella pneumoniae*

Klebsiella pneumoniae is a Gram-negative bacterium that belongs to the gamma subdivision of the class *Proteobacteria* and exhibits relatively close genetic relatedness to the other genera of *Enterobacteriaceae*, including *Escherichia*, *Salmonella*, *Shigella*, and *Yersinia* (20). *Klebsiella* spp. are ubiquitous in nature and characterized as rod-shaped, non-motile, usually encapsulated bacteria that can live in water, soil, and plants, and infect humans and animals (243, 244). In humans, *K. pneumoniae* behaves like a commensal mainly in the nasopharyngeal and intestinal mucosae (244). In this respect, the genus *Klebsiella* is like *Enterobacter* and *Citrobacter* but unlike *Shigella* spp. or *E. coli*, which are common in humans but not in the environment (244).

1.1.1. *K. pneumoniae* infections

K. pneumoniae is an opportunistic pathogen frequently involved in severe nosocomial infections in immunocompromised individuals who are hospitalized and suffer from severe underlying diseases, such as diabetes mellitus or chronic pulmonary obstruction (244). *K. pneumoniae* is responsible for a variety of diseases including suppurative lesions, bacteriemia, urinary tract infections, pneumonia, and sometimes life-threatening septic shock (23, 149, 165, 236, 244). The clinical pattern

of *K. pneumoniae* infection in humans has changed since this organism was discovered in the 1880s (97, 98). Until the 1960s, *K. pneumoniae* was an important cause of community-acquired pneumonia (42), however, the incidence of this type of infection has dropped, and hospital-acquired *K. pneumoniae* infection now predominates (105, 244, 303). Since 1980s, *K. pneumoniae* is emerging as an important pathogen both in the community and the hospital setting (161). In the hospital environment with the extensive use of antibiotics, multiple drug resistance has been increasingly observed in *K. pneumoniae*, especially the extended-spectrum β-lactamase (ESBL)-producing strains (93, 96, 161, 208, 212). Carbapenems are considered to be the preferred agents for the treatment of serious infections caused by ESBL-producing *K. pneumoniae* because of their high stability to β-lactamase hydrolysis and observed retained susceptibility of ESBL producers (61). However, *K. pneumoniae* isolates resistant to carbapenems have been reported worldwide since 2000s (128, 169, 224, 329). The emergence of carbapenem-resistant enterobacteria is worrisome because of the option for antimicrobial treatment is further restricted.

The emergence of an invasive form of the community-acquired *K. pneumoniae* infection, which presents as primary bacteremic liver abscesses, endophthalmitis, and meningitis (50, 89, 90, 189, 246, 315), has been reported almost exclusively in Asia (100), especially in Taiwan (100, 302, 315). Although these invasive and highly

encapsulated *K. pneumoniae* strains are universally resistant to ampicillin, they are unable to produce ESBL and susceptible to most antibiotics (178, 179). In addition, approximately 50 to 75% of the patients with *K. pneumoniae* liver abscess also presented with diabetes mellitus (177, 217, 300). Although the preponderance of this severe invasive *K. pneumoniae* infection remains unknown, the involvement of both host and microbial factors during pathogenesis could be anticipated.

1.1.2. *K. pneumoniae* virulence factors

A number of bacterial factors that contribute to *K. pneumoniae* pathogenicity have been identified, which include capsular polysaccharide (CPS), lipopolysaccharide (LPS), iron acquisition systems, and adherence factors (244). Clinically isolated *K. pneumoniae* usually produced large amount of CPS and therefore forms large glistering colonies with viscid consistency. The abundant CPS that typically surrounds *K. pneumoniae* protects against the bactericidal action of serum and impairs phagocytosis (11, 63), and may be regarded as the most important virulence determinant of *K. pneumoniae*. Among the 77 described capsular (K) types of the serotyping scheme, serotypes K1, K2, K4 and K5 are highly virulent in experimental infection in mice and are often associated with severe infections in humans and animals (216, 220, 229, 281). Furthermore, the K1 and K2 serotypes

were found to be the most prevalent capsular serotypes in liver abscess-causing *K. pneumoniae* (100, 178).

Genetic determinants for K1 and K2 CPS biosynthesis and regulation have been reported (15, 58). In *K. pneumoniae* NTUH-K2044 of serotype K1, deletion of the mucoviscosity associated gene A (*magA*) abolishes the CPS biosynthesis and thus reduces the bacterial virulence (88). The gene *magA* is only contained in the K1 *cps* gene cluster and hence could be applied to rapidly detect *K. pneumoniae* strains of serotype K1 (328). A PCR analysis for the K2 capsule-associated gene A (*k2A*) has also been used to identify *K. pneumoniae* strains of serotype K2 (74, 330). The presence of *rmpA* (regulator of the mucoid phenotype A) gene correlated with abscess formation in patients with community-acquired *K. pneumoniae* bacteremia and attributed to be a risk factor for metastatic infection in patients with *K. pneumoniae* liver abscess (180, 331). The *rmpA* together with *rmpA2* gene both located on the large virulence plasmid pLVPK (48, 295) are able to enhance the CPS biosynthesis thereby confer *K. pneumoniae* a hypermucoviscosity phenotype (53, 171).

LPS comprising three parts, lipid A, core, and O antigen, is responsible for the resistance to complement-mediated killing as well as antimicrobial peptides attack, and the establishment of septic shock (7, 52, 87, 211). Antimicrobial peptides, such as

polymyxin B, are bactericidal agents that exert their effects by interacting with the LPS of Gram-negative bacteria. The polycationic peptide ring on polymyxin competes for and substitutes the calcium and magnesium bridges that stabilized LPS, thus disrupting the integrity of the outermembrane leading to cell death (118, 332). In our previous study, the genetic determinants for LPS modification and CPS level have been shown to involve in polymyxin B resistance of *K. pneumoniae* CG43 (52).

Iron starvation is one of the major barriers that virulent bacteria must overcome in order to proliferate in the host. Multiple iron-acquisition systems have been described in *K. pneumoniae* (136). Analysis of the genomic sequence of *K. pneumoniae* NTUH-K2044 revealed 10 putative iron-acquisition systems, whereas *K. pneumoniae* strain MGH78578 and CG43 possess only 6 and 8 of these systems, respectively (136, 185). Prevalence study and animal experiment have been performed to assess the role of iron-acquisition systems in *K. pneumoniae* pathogenicity (136, 220, 295). Adherence factors possessed by *K. pnuemoniae* including type 1 and type 3 fimbriae, which play crucial roles in adhesion to host cells, persistence, and biofilm formation, are focused in this thesis and introduced in detail below. Other *K. pneumoniae* virulence determinants involved in acid resistance (135), oxidative stress response (123), and allantoin metabolism (195) have also been reported.

Complete genome sequence of *K. pneumoniae* strains MGH78578 (230) and NTUH-K2044 (325), which are human pathogens respectively isolated from sputum and liver abscess, and a nitrogen-fixing endophyte strain 342 (95) have been determined. Genomic and phenotypic analyses have also been performed among 235 *K. pneumoniae* strains in order to identify the evolutionary emergence of virulent clones (37).

1.2. Fimbriae (Pili)

Successful establishment of infection by bacterial pathogens requires adhesion to host cells, colonization of tissues, and in certain cases cellular invasions, followed by intracellular multiplication, dissemination to other tissues, or persistence (242). Fimbriae, also called pili, are hair-like appendages that extend out of the bacterial cell surface and exert on bacterial attachment and invasion, biofilm formation, cell motility and transport of proteins and DNA across membranes. Based on their biosynthetic pathway, these non-flagellar appendages of Gram-negative bacteria can be categorized into five major classes: chaperone-usher (CU) fimbriae, curli, type 4 pili, type 3 secretion needle, and type 4 secretion pili (99). Of these five classes, the CU fimbriae are the most extensively studied and often constitute important virulence factors, responsible for specific host attachment and/or the evasion of host responses

(323, 333). CU fimbriae are assembled into linear, unbranched polymers consisting of several hundreds to thousands of pilus subunits (also known as pilins) that range in size from ~12 kDa to ~20 kDa. Generally, the major pilin constitutes the fimbrial rod and an adhesin located at the tip to mediate its specific binding activity. Besides, there are minor pilins which incorporate into the fimbrial rod and affect assembly and adherence activity for several types of fimbriae (18, 139, 155, 186, 310). The machinery of fimbrial assembly is highly conserved, which comprises a periplasmic chaperone and an outer-membrane usher proteins (99, 240, 263, 265, 299, 311, 314). The fimbrial subunits are secreted by general secretion pathway into the periplasm and protected by the fimbrial chaperone from degradation by protease, and then transported to the usher for the assembly.

An early hierarchical classification of CU systems based on conserved structural elements in the chaperones identified two distinct subgroups, FGL- and FGS-chaperone assembled pili, which correspond with the assembly of thin fibrillar and rod-like pili, respectively (147). However, recent phylogenetic analysis in 189 CU systems revealed 6 main clades: α-, β-, γ- (which is subdivided into γ_1, γ_2, γ_3, and γ_4), κ-, π- and σ-fimbriae, based on common usher ancestry, and supported by similarities in operon structure and morphology of organelles within the separate clades (226). Among these, the rod-like or typical fimbrial organelles are found in the α-, γ- and π-

fimbrial clades. These CU fimbrial genes, respectively encode pilins, chaperone, usher, and adhesin, generally transcribe in an operon manner, and multiple CU fimbrial gene clusters are often presented in a Gram-negative bacterial genome (192, 301, 305).

Most of *K. pneumoniae* strains possess two types of CU fimbriae, type 1 and type 3 fimbriae (belonged to the γ_1 and γ_4- fimbriae, respectively), which are introduced in detail below. The afimbrial adhesin CF29K (67, 70), KPF-28 fimbriae (72), and a capsule-like extracellular afimbrial adhesin (91) have also been reported for some *K. pneumoniae* strains.

1.2.1. *K. pneumoniae* type 1 fimbriae

Type 1 fimbriae are approximately 7 nm wide and 1-2 μm long surface organelles found in virtually all members of the family *Enterobacteriaceae* (119, 163). They are well known for the ability to bind to mannose-containing structures on host cells and extracellular matrix. Bacteria expressing type 1 fimbriae are able to cause mannose-sensitive agglutination of yeast cells or erythrocytes (mannose-sensitive haemagglutination, MSHA) from guinea pig. Furthermore, type 1 fimbriae has been shown to play a crucial role during urinary tract infections by mediating adhesion to mannose-containing receptors on the uroepithelium and promoting the formation of intracellular bacterial communities (62, 145, 162, 174, 219, 252, 285, 290, 323).

Type 1 fimbriae have been most extensively studied in *E. coli*, and the corresponding structures of *K. pneumoniae* are highly similar with regard to genetic composition and regulation (60, 107, 267, 290). However, there are significant genetic, serological, and functional differences between type 1 fimbria variants in the different species (60, 80, 108, 197). *E. coli* type 1 fimbriae are encoded by the *fimAICDEFGH* operon. The fimbrial rod consists of the major subunits FimA and the minor subunits FimI, FimF, and FimG. The adhesive properties of type 1 fimbriae are exerted by the FimH adhesin which locates at the tips of the fimbriae. FimC and FimD are respectively chaperone and usher that are required for the fimbrial assembly. Unique to the *K. pneumoniae fim* gene cluster is the *fimK* gene, which locates downstream to the *fimH* gene and encodes an EAL domain protein (252). Deletion of *fimK* has been shown to activate the type 1 fimbriae expression in *K. pneumoniae* (252). In addition, *fimB* and *fimE* genes located upstream to the *fim* operon encode DNA recombinases that mediate the expression of type 1 fimbriae (4, 29, 267, 290).

In *E. coli*, the regulatory network of type 1 fimbriae expression is extensively studied. Environmental conditions such as osmolarity and pH are involved in the modulation of the type 1 fimbriae expression (101, 276). Second messengers cAMP and (p)ppGpp as well as regulatory proteins, including Lrp, IHF, RpoS, NanR, NagC, CRP, and H-NS, have also been described to affect the expression of type 1 fimbriae

(1, 30, 75, 82, 83, 102, 157, 160, 218, 228, 283). However, whether these regulators exert similar effects in *K. pneumoniae* awaits further investigation. Several reports indicated that *K. pneumoniae* poorly expresses type 1 fimbriae *in vitro* (252, 267, 290), and the expression is phase-variable (267, 290). In addition, the thick capsule of *K. pneumoniae* has been shown to impede the activity of type 1 fimbriae and also to retard the assembly of type 1 fimbrial subunits from periplasm to cell surface (204, 260, 267), suggesting a cross-regulation of the expression of fimbriae and capsule for an efficient infection.

1.2.2. *K. pneumoniae* type 3 fimbriae

Type 3 fimbriae are 2-4 nm wide and 0.5-2 μm long surface organelles that are originally characterized in *Klebsiella* strains by their ability to mediate mannose-resistant agglutination of tannic acid-treated human erythrocytes (MR/K haemagglutination) (78, 106). Several studies have also demonstrated an important role for type 3 fimbriae in biofilm formation on biotic and abiotic surfaces (33, 39, 71, 148, 175, 232, 234, 273, 291). Biofilms are recognized as surface-attached bacteria embedded in a self-produced matrix, composed mainly of polysaccharide, but also containing proteins and nucleic acids (292). Biofilm formation promotes encrustation and protects the bacteria from the hydrodynamic forces of urine flow, host defenses

and antibiotics (316). The ability of bacteria to form biofilm on medical devices is believed to play a major role in development of nosocomial infections, including the catheter-associated urinary tract infections, which is frequently caused by *K. pneumoniae* (200, 244, 251, 316). In addition, type 3 fimbriae mediate adhesion to epithelial cells, from the respiratory and urinary tracts, and extracellular matrix proteins, such as collagen V, *in vitro* (133, 140, 148, 277, 296, 297).

Type 3 fimbriae are encoded by the *mrkABCDF* operon (8, 78, 139). MrkA and MrkF are the major and minor subunits, respectively, which constitute the fimbrial rod and facilitate biofilm formation (139, 175). MrkD is the adhesin that mediates binding specificity and biofilm formation on extracellular matrix-coated surfaces (134, 139, 148, 275), however its cognate receptor is still unknown. MrkB and MrkC are predicted to be chaperone and usher, respectively, which are responsible for the fimbrial assembly. A putative regulatory gene, *mrkE*, located upstream to *mrkA* has also been reported in *K. pneumoniae* IA565, which harbors a plasmid carrying the type 3 fimbrial genes (8). The *mrk* genes have been shown to reside at multiple genomic locations, including the chromosome (291), on conjugative plasmids (39, 232) and within a composite transposon (225). The spread of the type 3 fimbrial gene between *Enterobacteriaceae* strains by lateral gene transfer has also been described (39, 233).

1.3. Cyclic-di-GMP signaling

Bis-(3'-5')-cyclic dimeric guanosine monophosphate (Cyclic-di-GMP or c-di-GMP), has emerged as a second messenger specific to the domain of *Bacteria* (151, 239, 250, 256, 293). c-di-GMP controls a variety of cellular processes, mainly biogenesis and function of extracellular components, flagella and fimbriae, and exopolysaccharide synthesis. The intracellular level of c-di-GMP in bacteria is modulated through the activity of di-guanylate cyclases (DGCs) that convert two molecules of GTP to c-di-GMP, and phosphodiesterases (PDEs) that linearize c-di-GMP to pGpG, which is subsequently hydrolyzed to GMP. DGCs are characterized by the active site GG[D/E]EF amino acid motif in the enzyme catalytic site (127, 201, 237, 259), whereas PDEs contain either the EAL domain or HD-GYP domain (45, 57, 255, 271, 294). Formation of c-di-GMP requires dimerization of two GGDEF domains, and c-di-GMP degradation can be mediated either by the EAL domain or by the less common HD-GYP domain. DGCs and PDEs usually harbor an N-terminal signal input domain that regulates the activity of its C-terminal catalytic GGDEF or EAL/HD-GYP domain (122, 151, 269). Besides, GGDEF domains are often found together with EAL or HD-GYP domains in a single polypeptide. The coexistence of opposing enzymatic activities in these 'hybrid' proteins has long been controversial and only a few reports suggest that bifunctional proteins may exist (92,

168, 298). It has also been demonstrated that degenerated GGDEF domain may retain substrate-binding capacities and provide GTP-dependent control of the activity of an EAL domain in a single peptide (57, 137, 158). The mechanism of the intramolecular coordination of GGDEF and EAL/HD-GYP domains in composite protein remains to be shown.

GGDEF and EAL domain proteins are ubiquitous in bacteria but absent from archaea (103, 256). A single bacterial genome generally encodes many different members of these protein families (e.g. *E. coli* harbors 19 GGDEF and 17 EAL genes; *Vibrio cholerae* harbors 41 GGDEF, 22 EAL, and 9 HD-GYP genes) (103). Moreover, genomes were found to encode several GGDEF and EAL domain proteins with a particularly striking expansion in γ-*Proteobacteria*. This highly redundant and complex system are suggested to achieve signaling specificity through different modes of sequestration, including microcompartmentalization, temporal regulatory sequestration, and co-localization of the DGCs, PDEs, effector, and target molecules that constitute functional c-di-GMP signaling modules (151, 156, 238, 239, 256, 286, 317). A computational analysis of 11248 GGDEF and EAL domain proteins in 867 prokaryotic genomes also suggests that post-translational regulation and catalytic activity of these proteins play important roles in c-di-GMP signaling (278). As shown in Fig 1.1, in the genome of *K. pneumoniae* NTUH-K2044, ORFs encoding 11

GGDEF, 10 EAL, and 5 GGDEF-EAL domain proteins were found using a HMMER search (24, 81).

The response to fluctuating cellular levels of c-di-GMP is mediated by a variety of specific effector proteins or RNAs that control specific cellular processes (269). Four types of c-di-GMP effector proteins are currently known (122), and the most prevalent example of such effectors are c-di-GMP-binding proteins harboring a PilZ domain (12). The PilZ domain proteins studied so far seem to be activated by c-di-GMP and to function by protein-protein interactions (34, 122). In some cases, the PilZ domain is directly attached to the C-terminus of the GGDEF, EAL and/or HD-GYP domains (12), or is linked to a domain that generates a molecular output (12, 210, 231). BcsA, the catalytic subunit of cellulose synthase from *Gluconacetobacter xylinus*, is the first identified PilZ domain protein and activated via binding to c-di-GMP (205, 253, 319). The interaction of c-di-GMP with PilZ domains is further supported by binding and mutagenesis studies of several PilZ domain proteins (34, 56, 210, 245, 258). These studies demonstrate c-di-GMP binding with sub-micromolar affinity dependent on residues in the RxxxR and D/NxSxxG sequence motifs conserved in PilZ domains (27). The high-affinity binding of c-di-GMP of PilZ domain protein is also supported by NMR studies of PA4608 from *Pseudomonas aeruginosa* (247). Besides cellulose synthesis, PilZ proteins have been demonstrated

to regulate flagellar activity (34, 56), twitching motility (9), alginate synthesis (210), biofilm formation, and virulence in different bacteria (245). In *K. pneumoniae* NTUH-K2044, three PilZ domain proteins were found (Fig. 1.1); two of them are contained in the C-terminus of putative cellulose synthases, and the other one, named MrkH (153), is a putative protein of unknown function.

1.4. Thesis objectives

Fimbriae are the most well-known adherence factors in Gram-negative bacteria and play a crucial role in the initial step of infection, thus we focused on the study of *K. pneumoniae* fimbrial adherence in this thesis. The objectives are to investigate the prevalence and expression of fimbriae in *K. pneumoniae* for a better understanding of its pathogenic mechanism. Two highly virulent *K. penumoniae* strains isolated from liver abscess, NTUH-K2044 and CG43 (belonged to K1 and K2 serotypes, respectively), are studied in parallel. The study flow is listed as following:

Chapter 2 reports the identification of putative fimbrial gene clusters in the genome of *K. pneumoniae* NTUH-K2044. Besides type 1 and type 3 fimbrial genes, the others are novel and were designated Kpa, Kpb, Kpc, Kpd, Kpd, Kpf, and Kpg fimbriae. Prevalence analysis of the nine fimbrial gene clusters among *K. pneumoniae* clinical isolates was performed by PCR detection.

Chapter 3 characterizes the Kpc fimbriae which is prevalent in *K. pneumoniae* strains of serotype K1. Expression of Kpc fimbriae was found to increase bacterial biofilm-forming activity. A recombinase-mediated phase variation of the Kpc fimbriae expression is also elucidated.

Chapter 4 presents analyses of the regulation of type 3 fimbriae expression. Roles of three genes encoding putative regulators, MrkH, MrkI, and MrkJ, on the expression of type 3 fimbriae were studied. A global iron uptake regulator, Fur, also appeared to affect the type 3 fimbriae expression, and this regulation was found to be mediated by the c-di-GMP signaling. Furthermore, environmental stimuli including Fe^{2+} and oxygenation that influence the expression of type 3 fimbriae were analyzed.

Chapter 5 concludes with a comprehensive view and provides perspectives regarding further investigations on the fimbrial adherence of *K. pneumoniae*.

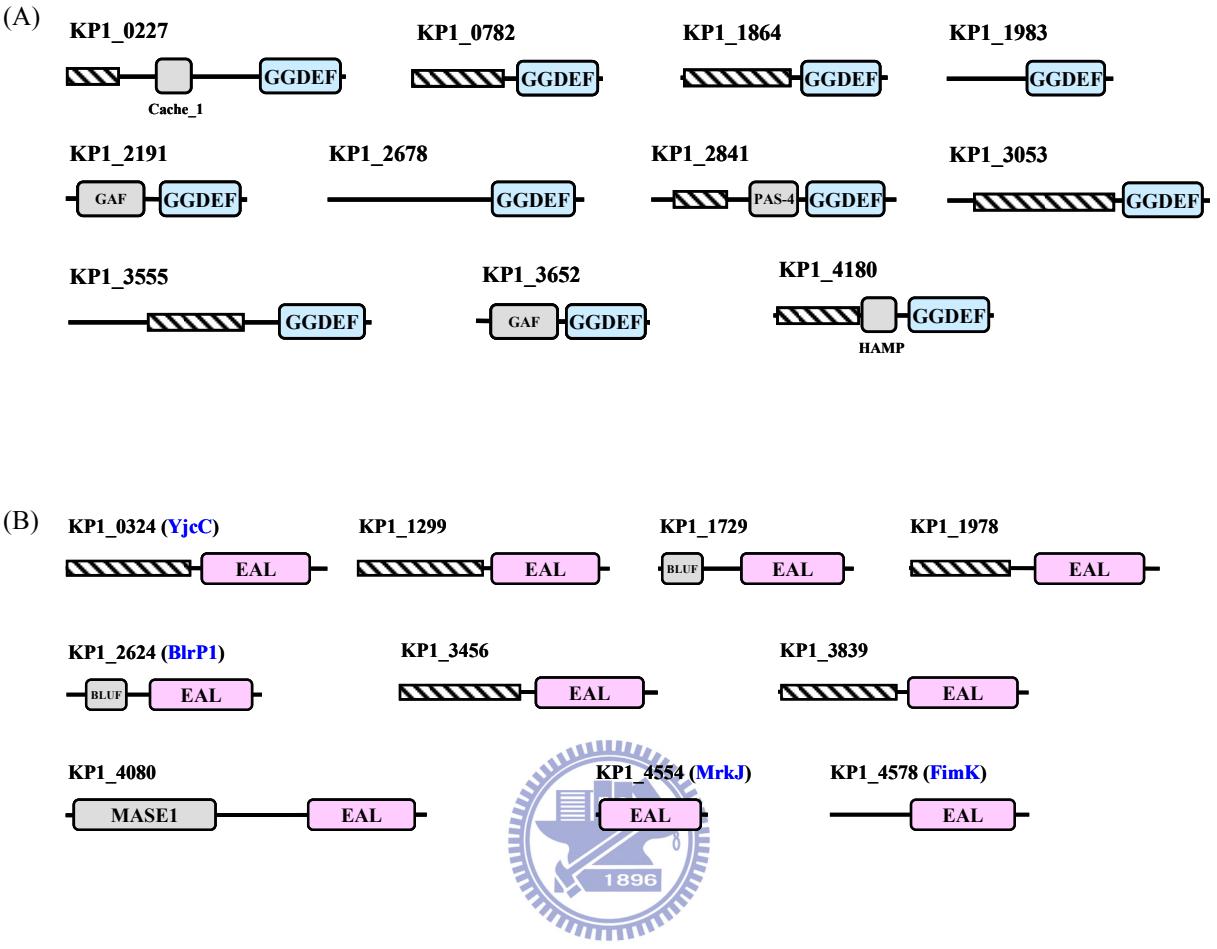

(A)
KP1_0227
Cache_1
GGDEF
KP1_0782
GGDEF
KP1_1864
GGDEF
KP1_1983
GGDEF
KP1_2191
GAF
GGDEF
KP1_2678
GGDEF
KP1_2841
PAS-4
GGDEF
KP1_3053
GGDEF
KP1_3555
GGDEF
KP1_3652
GAF
GGDEF
KP1_4180
HAMP
GGDEF
(B)
KP1_0324 (YjcC)
EAL
KP1_1299
EAL
KP1_1729
BLUF
EAL
KP1_1978
EAL
KP1_2624 (BlrP1)
BLUF
EAL
KP1_3456
EAL
KP1_3839
EAL
KP1_4080
MASE1
EAL
KP1_4554 (MrkJ)
EAL
KP1_4578 (FimK)
EAL
(C)
KP1_5220
HAMP
GGDEF
EAL
KP1_3659
GGDEF
EAL
KP1_2724
PAS
GGDEF
EAL
KP1_3998
MASE1
GGDEF
EAL
KP1_4976
GGDEF
EAL
(D)
KP1_4551
PilZ
KP1_5237 (AcsA)
Glycosyl transferase family 2
PilZ
KP1_5225 (BcsA)
Glycosyl transferase family 2
PilZ

Fig. 1.1. Domain architecture of putative c-di-GMP signaling proteins encoded by the *K. pneumoniae* NTUH-K2044 genome. The locus tag (KP1_number) of the genes encoding (A) GGDEF, (B) EAL, (C) GGDEF/EAL, or (D) PilZ domain proteins was indicated. Analysis of protein functional domain, as indicated, was performed using the Pfam database provided online (http://www.sanger.ac.uk /Software/Pfam/). The identities of the proteins are also shown in blue: four EAL domain proteins (YjcC, FimK, MrkJ, and BlrP1) that have been described in *K. pneumoniae* (22, 153, 170, 252) and two PilZ domain proteins (putative cellulose synthases BcsA and AcsA) (249). Predicted domain with unknown function is shown by a twilled box.

CHAPTER 2

Prevalence Analysis of the Fimbrial Gene Clusters in *Klebsiella pneumoniae*

2.1. Abstract

Using HMMER search for genes encoding the Pfam fimbrial components in the genome of *K. pneumoniae* NTUH-K2044, nine distinct fimbrial gene clusters were identified. In addition to *fim* and *mrk* gene clusters, encoding type 1 and type 3 fimbriae respectively, the other seven are novel and named *kpa*, *kpb*, *kpc*, *kpd*, *kpe*, *kpf*, and *kpg*. The presence of the fimbrial genes among 105 *K. pneumoniae* strains isolated from various infection sites was analyzed by PCR detection, and the *kpb* and *kpc* genes were found to be more prevalent ($P < 0.0001$) in the isolates of serotype K1. Besides, an RFLP analysis was performed among the 105 *K. pneumoniae* isolates which revealed most of the isolates possess a v1-like *mrkD* RFLP type.[a]

[a] A part of this chapter has been published:

Wu, C. C., Y. J. Huang, C. P. Fung, and H. L. Peng. 2010. Regulation of the *Klebsiella pneumoniae* Kpc fimbriae by the site-specific recombinase KpcI. Microbiology **156:**1983-92.

2.2. Introduction

A large number of fimbrial gene clusters are commonly present in a bacterial genome (192, 301, 305), which are believed to be expressed differentially in order to adhere to various host receptors during infection (144). In *Salmonella enterica* serovar Typhi (*S.* Typhi) CT18, 14 fimbrial gene clusters including a type 4 fimbrial operon, an orthologue of the *agf* (*csg*) operon, and 12 putative fimbrial operons of the chaperone-usher assembly class were identified (301). Prevalence study among different *Salmonella* isolates has revealed that an unique repertoire of fimbrial gene clusters is possessed by the strains of serotype Typhi, which is probably resulted in specific host adaptation (301).

Among these *Salmonella* fimbriae, only two of them, *fim* and *agf* (*csg*), have been demonstrated to express on the surfaces of serotype Typhimurium cells by electron microscopy (79, 112, 190, 289). The remaining 11 fimbrial operons are poorly expressed when bacteria are grown under standard laboratory conditions (143). However, expression of some of the fimbriae could be detected by flow cytometry while the Typhimurium cells recovered from bovine ligated ileal loops at 8 h after infection (143). Seroconversion to 11 types of fimbriae was found indicating that a transient expression of each of the fimbriae induced the host immune responses (142).

Moreover, the individual deletion of six of the fimbrial operons affected the bacterial persistence in intestines of mice (318), and a strain carrying mutations of four of the fimbrial operons resulted in a 26-fold increase of LD_{50} to mice (306). Cross-regulation between these fimbrial operons have also been described (130, 131, 326). Mutation of a fimbrial operon could activate the expression of another type of fimbriae (54, 274), further supporting a regulatory network involved in the expression of the multiple fimbriae.

Sequence variation of the fimbrial adhesin affects not only binding specificity but also fimbrial activity. We have previously found that the *mrkD* gene, encoding the type 3 fimbrial adhesin, from *K. pneumoniae* clinical isolates could be classified into four restriction fragment length polymorphism (RFLP) types: $mrkD_{v1}$, $mrkD_{v2}$, $mrkD_{v3}$, and $mrkD_{v4}$ (140). The MrkD sequence variation determines the binding specificity and the assembly efficiency of type 3 fimbriae (46, 140).

We initiated the study using bioinformatic tools to identify the fimbrial genes in the genome of *K. pneumoniae* NTUH-K2044, a liver abscess isolate of serotype K1 (325). Prevalence study of the fimbrial genes was then employed among 105 *K. pneumoniae* clinical isolates from different infection sites. Besides, the *mrkD*-RFLP types of these clinical isolates were also determined.

2.3. Results

2.3.1. Identification of the fimbrial gene clusters in *K. pneumoniae* NTUH-K2044

Nine fimbrial gene clusters were identified using the HMMER search of the genome of *K. pneumoniae* NTUH-K2044. Each contained at least four genes, encoding a putative major pilin, a chaperone, an usher and an adhesin for the biosynthesis of fimbriae belonging to the chaperone-usher assembly class. As shown in Fig. 2.1, these include the type 1 and type 3 fimbrial gene clusters *fim* and *mrk*, and seven novel ones, namely *kpa*, *kpb*, *kpc*, *kpd*, *kpe*, *kpf*, and *kpg*. Multiple sequence alignment by CLUSTAL W showed that the amino acid sequences of these pilins and adhesins shared 26.5-36.4% similarity; chaperones and ushers shared a higher similarity ranging from 49.3 to 55.4%. The *mrk-fim* fimbrial genes are clustered and transcribed divergently (Fig. 2.1). This gene organization including the *pecM*, *pecS* and *nicO* homologues has been found to be conserved in the genomes of *K. pneumoniae* CG43, C3091, MGH78578 and 342 (95, 230, 291). The gene clusters *kpf* and *kpg* are also linked physically but transcribed convergently.

BLAST analysis, using the sequences of the nine fimbrial gene clusters identified in *K. pneumoniae* NTUH-K2044 as templates, of the genome of *K. pneumoniae* strains MGH78578 and 342 (95, 230) showed that, except for *kpc* and *kpf*, the genes

were conserved in the three genomes. No homologue of the *kpc* genes was found in the strains MGH78578 and 342, while homologues of the *kpf* genes were found in the genome of MGH78578 but not of 342. Besides, except for the *kpc* genes, the other eight fimbrial gene clusters were also identified in the contig sequences of CG43 (unpublished results from Dr. S.-F. Tsai, National Health Research Institutes, Taiwan).

2.3.2. PCR screening for the presence of the fimbrial genes

To investigate the prevalence of the nine fimbrial gene clusters among *K. pneumoniae* strains, a total of 105 *K. pneumoniae* clinical isolates, namely the TVH strains, from different infection sites were collected. Two specific primer pairs corresponding to the pilin- and adhesin-encoding genes were designed for PCR detection. Prevalence was determined on the basis of the presence of the PCR amplicons (Fig. 2.2). The analysis revealed the presence of *kpa*, *kpd, kpe*, *kpg*, *fim,* and *mrk* genes in most of the isolates, and the prevalence percentages were 99, 82, 93, 97, 84 and 100%, respectively. The prevalence percentages for the *kpb*, *kpc* and *kpf* genes were lower at 52, 33 and 70% of the isolates, respectively. No obvious correlation between fimbrial type and disease could be identified. However, the *kpb* and *kpc* genes were shown to be more prevalent in K1 isolates (P<0.0001). As shown in Table 2.1, most of the clinical isolates of serotype K1 harbored *kpb* and *kpc* genes,

while non-K1 isolates carrying the *kpb* and *kpc* genes were much less frequent (32 and 1%, respectively). The close association of Kpc fimbriae with serotype K1 prompted the selection of the Kpc fimbriae for further study of the *K. pneumoniae* liver abscess pathogenic mechanism (Chapter 3).

2.3.3. PCR-RFLP analysis of the *mrkD* genes

A PCR-RFLP analysis previously described (140) was also employed to determine the *mrkD* type of the *K. pneumoniae* clinical isolates. The result showed that 87 of the 105 isolates possess *mrkD* gene of v1-like RFLP. The number of isolates harbored the v2-, v3-, and v4-like *mrkD* were respectively four, two, and four, while eight carried a novel *mrkD* RFLP type. The *mrkD*-RFLP types could not be associated with certain diseases. Interestingly, sequence variation was found in *mrkD* genes of same RFLP type. For example, *K. pneumoniae* NTUH-K2044, CG43, and TVH2 harbor *mrkD* gene of v1-RFLP type while frameshift mutations were found in *mrkD* gene of CG43 and TVH2. These sequence variations which resulted in a premature termination of the *mrkD* translation may affect the type 3 fimbrial assembly and adherence properties as reported (46, 140).

2.4. Discussion

Unlike some of the fimbrial gene clusters in *S.* Typhi CT18 and in *Escherichia coli* O157:H7 that contain either premature termination codons or frameshift mutations, the nine fimbrial gene clusters identified in *K. pneumoniae* NTUH-K2044 appeared to be intact. Although these fimbrial gene clusters identified using bioinformatic tools are putative ones, we anticipate that, besides the type 1 and type 3 fimbriae, the other seven are required for *K. pneumoniae* NTUH-K2044 infection at not yet identified environments.

A specific repertoire of fimbrial operons has been proposed as a complex virulence factor involved in *S.* Typhi infections (144). Although no obvious correlation between the disease and fimbrial type was noted, 28 out of 29 K1 isolates possessed an identical repertoire of fimbrial gene clusters suggesting a role of the fimbrial repertoire in pathogenicity of K1 isolates (Table 2.2). Consistent with the other reports (100, 178), most of the liver abscess isolates (14 out of 18) were capsular serotype K1 (Table 2.3). Aside from that, the capsular serotypes of the *K. pneumoniae* isolates appeared no correlation with the infection sites (Table 2.3).

As shown in Fig. 2.1, three ORFs encoding putative transcriptional factors, located upstream to the *kpb*, *kpd*, and *kpf* fimbrial genes, were designated *kpbR*, *kpdR*,

and *kpfR*, respectively. Regulator encoding genes are commonly found to be located adjacent to fimbrial operons (17, 94, 125, 141), such as *papB* for the *E. coli* P fimbrial gene cluster. PapB not only regulates the expression of P fimbriae but also affects the type 1 fimbriae expression (130, 131, 326). Besides, a mutant with a Tn*5* insertion in the putative promoter region of *kpgA* decreased the expression of type 3 fimbriae in *K. pneumoniae* 43816 (33). These findings suggested a cross-talk regulation is generally present for the control of the expression of different fimbriae. Nevertheless, functional roles of these putative fimbriae and their cognate regulators remain to be studied.

In many cases, mutation of a fimbrial operon did not affect or only moderately altered the bacterial virulence (25, 26, 191, 318). It may be due to a suboptimal experimental model or the gene-loss was functionally compensated by other adherence factors. It is also believed that multiple fimbriae may act synergistically or differentially to adhere to host cells during infection. Besides *S.* Typhi and Typhimurium, to our knowledge, genomic analysis of fimbrial gene clusters has also been described in *Pseudomonas aeruginosa* and *E. coli* O157:H7 (192, 222, 305). Herein, the genome-wide analysis of fimbrial gene clusters paved the way for further studies of *K. pneumoniae* fimbrial adherence.

Table 2.1. Prevalence of *kpb* and *kpc* genes in *K. pneumoniae* isolates

K. pneumoniae isolate	No. of ***kpb*** strain/total no. of strain isolated (%)		No. of ***kpc*** strain/total no. of strain isolated (%)	
	K1 serotype	other serotype	K1 serotype	other serotype
LA[a]	14/14	3/4	14/14	1/4
Bile		2/7		1/7
Urine	1/1	3/13	1/1	0/13
Sputum	3/3	3/11	2/3	1/11
Wound	6/6	5/15	6/6	1/15
Blood	5/5	4/17	5/5	2/17
Ascites		4/9		1/9
Total	29/29 (100)*	24/76 (32)*	28/29 (97)*	7/76 (1)*

*, $P < 0.0001$; [a], Liver abscess

Table 2.2. Repertoire of fimbrial genes among *K. pneumoniae* isolates with different K serotypes

Presence of the fimbrial genes									No. with the repertoire		
kpa	***kpb***	***kpc***	***kpd***	***kpe***	***kpf***	***kpg***	***fim***	***mrk***	**K1 serotype**	**K2 serotype**	**Other serotype**
+	+	+	+	+	+	+	+	+	28		5
+	–	–	+	+	+	+	+	+	0	1	21
+	–	–	–	+	+	+	+	+	0		8
+	+	–	+	+	+	+	+	+	1		6
+	–	–	+	+	–	+	+	+	0		6
+	–	–	–	–	–	+	–	+	0		6
+	+	–	+	+	–	+	+	+	0	5	4
+	–	–	+	+	–	+	–	+	0		1
+	–	–	+	+	–	+	–	+	0		2
+	–	–	–	+	–	+	+	+	0	1	1
+	+	+	+	+	–	+	+	+	0		1
+	+	–	–	+	+	+	+	+	0		1
+	+	–	+	+	–	+	–	+	0		1
+	+	–	–	+	+	+	–	+	0		1
+	+	–	+	+	–	–	–	+	0		1
+	–	+	+	+	–	+	–	+	0		1
–	+	–	–	–	–	+	–	+	0		1
+	–	–	+	+	+	–	–	+	0		1
+	–	–	+	+	–	–	–	+	0		1

Table 2.3. K serotypes of the *K. pneumoniae* clinical isolates

K serotypes (No. of isolates)	*K. pneumoniae* isolates (No.)						
	LA[a] (18)	Bile (7)	Urine (14)	Sputum (14)	Wound (21)	Blood (22)	Ascites (9)
K1 (29)	14		1	3	6	5	
K2 (7)	2	1	1		3		
K3 (1)						1	
K4 (3)		1			1	1	
K5 (3)			1		1	1	
K6 (4)	1					2	1
K7 (1)							1
K8 (2)				2			
K9 (4)		1	2	1			
K12 (1)						1	
K13 (4)			1		1		2
K14 (2)	1					1	
K15 (2)				1		1	
K16 (5)			1		3	1	
K17 (1)			1				
K20 (7)		2		1	3	1	
K22 (2)			1				1
K25 (2)			2				
K28 (3)				1	1	1	
K30 (1)							1
K31 (2)					1	1	
K38 (3)					1	2	
K39 (1)							1
K42 (1)			1				
K44 (2)				1		1	
K49 (2)		1	1				
K54 (2)						2	
K57 (1)				1			
K62 (1)				1			
K64 (5)		1	1	2			1
K70 (1)							1

[a] Liver abscess

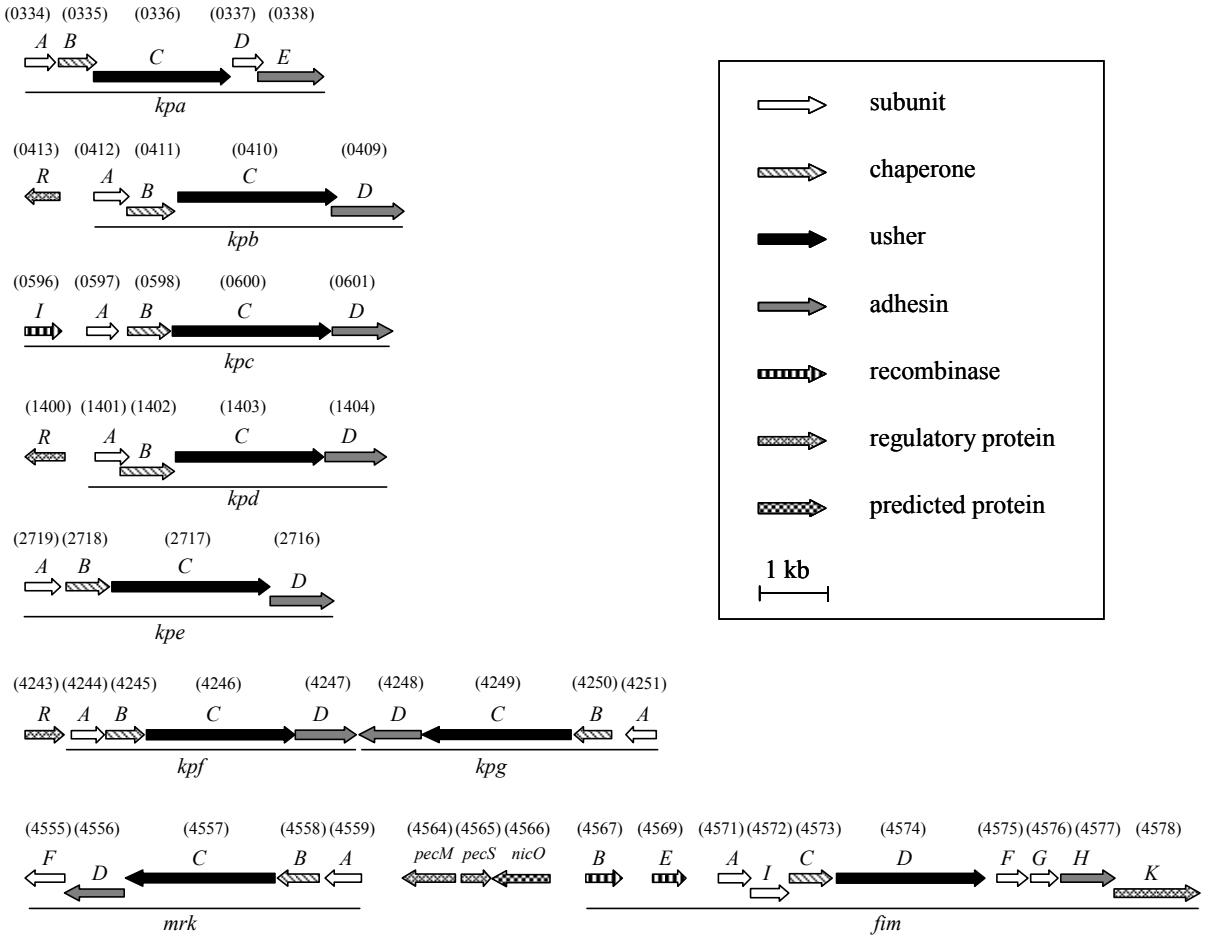


Fig. 2.1. Fimbrial gene clusters of the chaperone-usher-dependent assembly class in *K. pneumoniae* NTUH-K2044. The designation of putative fimbrial genes and the locus tag (KP1_number) of ORFs annotated in the *K. pneumoniae* NTUH-K2044 genome are indicated. A total number of nine fimbrial gene clusters and genes encoding putative regulators are as shown. Each of the putative fimbrial operons is underlined. The putative functions of the ORFs are also shown.

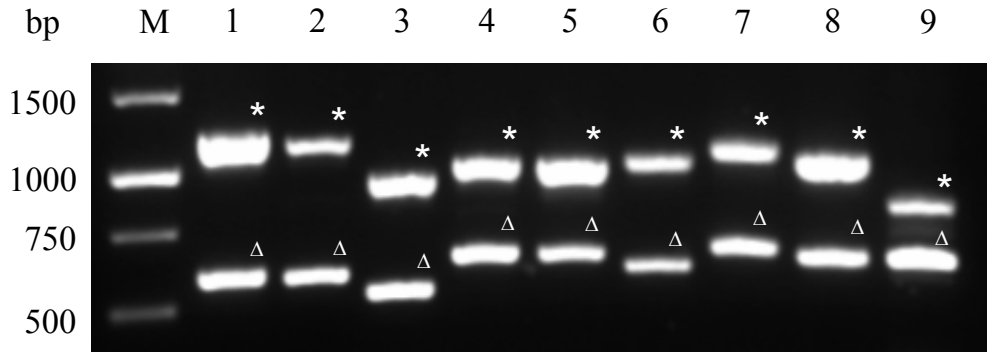


Fig. 2.2. PCR amplicons of the pilin and adhesin encoding genes in *K. pneumoniae* NTUH-K2044. Lane M: Gene Ruler 1-kb DNA molecular size markers (Fermentas, Vilnius, Lithuania). The DNA fragments represent pilin (Δ) and adhesin (*) encoding genes, lanes 1 to 9, respectively of Kpa, Kpb, Kpc, Kpd, Kpe, Kpf, Kpg, type 3, or type 1 fimbriae.

CHAPTER 3

Regulation of Kpc Fimbriae by the Site-specific Recombinase KpcI

3.1. Abstract

In this study, the Kpc fimbria of *Klebsiella pneumoniae* NTUH-K2044 was characterized. Induced expression of the recombinant *kpcABCD* genes in *Escherichia coli* resulted in Kpc fimbriation and increased biofilm formation, suggesting that the *kpc* genes are sufficient to encode a functional fimbrial apparatus. A putative site-specific recombinase encoding gene *kpcI* and a 302-bp intergenic DNA flanked by 11-bp inverted repeats, namely *kpcS*, were identified in the upstream region of the *kpcABCD* genes. Using LacZ as the reporter, a dramatic difference in promoter activity of *kpcS* in two different orientations was observed and assigned as ON and OFF phase accordingly. Expression of *kpcI* appeared to be able to invert the *kpcS in trans* from phase OFF to ON and vice versa. Using the two plasmid system, expression of *kpcA*, encoding the major component of the Kpc fimbriae, could be observed upon the induced expression of *kpcI*. These results indicate that KpcI is involved in the regulation of Kpc fimbriation in a phase-variable manner.[a]

[a] A part of this chapter has been published:

Wu, C. C., Y. J. Huang, C. P. Fung, and H. L. Peng. 2010. Regulation of the *Klebsiella pneumoniae* Kpc fimbriae by the site-specific recombinase KpcI. Microbiology **156:**1983-92.

3.2. Introduction

Expression of *Escherichia coli* type 1 and P fimbriae are regulated by a mechanism called phase variation, which referred to a reversible switch between "all-or-none" (ON/OFF) expressing phase (116, 121, 307). The phase variation, resulted in variation of fimbriae expression between individual cells of a clonal population, is a genetic or epigenetic mechanism that allows the variability to be heritable. Phase-expression is reversible between generations, and the reversion frequency exceeds that of a random mutation (116, 121, 307).

The phase-variation control of different fimbriae include conservative site-specific recombination (CSSR) for type 1 and MR/P fimbriae (4, 29, 181, 334), DNA methylation for P and Pef fimbriae (36, 125, 126, 223), and slipped-strand mispairing (SSM) for fimbriae of *Bordetalla pertusis* and *Haemophilus influenzae* (308, 320). The *E. coli* type 1 fimbriae represent the best-studied module for CSSR-mediated fimbriae expression. The oscillating ON-and-OFF expression of type 1 fimbriae is correlated with the inversion of a 314-bp DNA sequence (*fimS*) immediately upstream of *fimA*, the major pilin encoding gene. The promoter located within *fimS* that drives the expression of the type 1 fimbriae is flanked by 9-bp inverted repeats (IRs). The *fim* operon is only expressed when the *fimS* is in the ON

orientation, which allows transcription of the *fim* operon. The two genes located upstream to *fimS* respectively encode FimB and FimE site-specific DNA recombinases of the λ integrase family (86, 227). The recombinase FimB inverts *fimS* in the ON-to-OFF and OFF-to-ON directions, whereas FimE determines predominantly the ON-to-OFF direction of *fimS* (31, 101, 206). As a result, alternations of the expression ratio of FimB and FimE modulate the phase variation of type 1 fimbriae (1, 75, 283, 321). Other types of fimbriae controlled by DNA recombinase-mediated phase variation include *Proteus mirabilus* MR/P fimbriae (181, 334), enterotoxigenic *E. coli* CS18 fimbriae (132), and *Photorhabdus* Mad fimbriae (287). Unlike type 1 fimbriae, the phase variation of MR/P and Mad fimbriae is controlled by only one site-specific recombinase, MrpI and MadR, respectively, which catalyzed the DNA inversion in both directions (181, 287, 334).

Prevalence study among *K. pneumoniae* isolates has demonstrated that the putative fimbrial genes *kpcABCD* are highly prevalent in isolates of capsular serotype K1 (Table 2.1). In addition, a gene encoding a putative DNA recombinase was identified in the upstream region of the *kpcABCD* genes (Fig. 2.1) and was named *kpcI*. Whether KpcI modulates a phase variable expression of *kpcABCD* genes is investigated.

3.3. Results

3.3.1. Display of the Kpc fimbriae on *E. coli* surface

In order to determine whether the *kpcABCD* genes encode a fimbrial apparatus, an *E. coli* fimbriae display system was used. The *kpcABCD* genes were PCR-amplified and cloned into pET30a, designated pKPC-7, to allow controlled expression by IPTG induction. Numerous thin and rigid fimbriae on the surface of *E. coli* Novablue (DE3) harboring pKPC-7 could be observed, while the *E. coli* harboring pET30a was afimbriate (Fig. 3.1). Expression of KpcA, the putative major pilin, could also be detected by Western blot analysis (Fig. 3.2). These results indicated that the *kpcABCD* genes are sufficient to produce a fimbrial apparatus. However, the growth rate of *E. coli* harboring pKPC-7, in comparison with that of *E. coli* Novablue (DE3) [pET30a] was obviously decreased even without IPTG induction. This could be a result of the overexpression of *kpc* genes, especially the *kpcA* (Fig. 3.2, lane 3). In case the biased growth rate impeded the characterization of the Kpc fimbriae, another expression plasmid pETQ that can be used for protein expression under T5*lac* promoter control in *E. coli* and *K. pneumoniae* was used. The pETQ plasmid carrying the *kpcABCD* genes, pKPC-36, was then transformed into *E. coli* HB101 which is an afimbriate bacterium. Expression of the *kpcABCD* genes was

tightly controlled under the T5*lac* promoter, and no obvious difference in growth rate between *E. coli* HB101 harboring pKPC-36 and *E. coli* HB101 harboring pETQ was noted. Using polyclonal KpcA antiserum, Western blot analysis revealed that KpcA was expressed (Fig. 3.3A), and fimbriation on the surface of the recombinant *E. coli* HB101 was also observed by fluorescence microscopy (Fig. 3.3B). *E. coli* HB101 [pKPC-36] was thus used for the characterization of the recombinant Kpc fimbriae.

3.3.2. Expression of Kpc fimbriae increased biofilm-forming activity

Activity assessment of the recombinant Kpc fimbriae, including hemagglutination, cell adherence, and biofilm formation, was subsequently carried out. However, no hemagglutination activity against red blood cells from guinea pig, rabbit, or human (type A and type B) could be observed for the recombinant bacteria. Besides human epithelial cell lines Int407 (intestine), HCT-8 (intestine), Hep-2 (larynx), and T24 (bladder), two human hepatocellular liver carcinoma cell lines, HepG2 and SK-HEP-1, were also used to assess the cell adherence activity of the Kpc fimbriae. The cell adherence assay was performed as described by Huang *et al*. (140). Although the recombinant *E. coli* HB101 exhibited differential adherence to these cell lines, Kpc fimbriation did not increase the adherence activity of the bacteria to any of the cells. On the other hand, as shown in Fig. 3.4, *E. coli* HB101 harboring pKPC-36

exhibited a higher level of biofilm-forming activity on the abiotic surface than that observed for the *E. coli* HB101 [pETQ], as assessed by direct observation after crystal violet staining or by quantitative measurement ($P < 0.001$). However, *E. coli* HB101 harboring the type 1 fimbriae expression plasmid pAW69 or pAW67 (pETQ carrying the *kpcABC* genes) exhibited levels of biofilm-forming activity similar to those of bacteria carrying pETQ. This suggested a specific binding of the Kpc fimbriae to polystyrene wells and a positive role of Kpc fimbriae in biofilm formation. The expression of type 1 fimbriae encoded on pAW69 was also confirmed by yeast agglutination analysis (Fig. 3.5).

3.3.3. The KpcI recombinase is probably the regulator for the Kpc fimbriae

Multiple sequence alignment of the family of site-specific recombinases, including FimB/E of the type 1 fimbriae, MrpI of the MR/P fimbriae, FotS/T of the CS18 fimbriae, and KpcI, revealed four conserved residues, R42, H136, R139, and Y171, of the tyrosine recombinase family (Fig. 3.6) (5, 117). Between *kpcI* and *kpcA*, a 302 bp region flanked with a pair of 11-bp inverted repeats (IRs) was identified (Fig. 3.7). These findings suggested a recombinase-mediated phase variation control of Kpc fimbriae. KpcI is thus predicted to be able to invert the DNA segment between the two invert repeats to regulate the expression of Kpc fimbriae. The putative invertible

DNA segment was designated *kpcS* (switch region).

3.3.4. Expression of *kpcI* leads to the inversion of *kpcS*

A PCR-based switch orientation assay (Fig. 3.8A) was subsequently employed to detect the *kpcS* inversions in *K. pneumoniae* NTUH-K2044 grown in various growth conditions, including LB broth, TSB, and M9 minimal medium with or without agitation respectively at 25, 30, and 37°C. Since enteropathogenic *E. coli* bundle-forming pilus are expressed when the bacteria are grown in Dulbecco's Modified Eagle's Medium (DMEM) (313), several tissue-culture media, including DMEM, Roswell Park Memorial Institute (RPMI), Minimum Essential Medium (MEM), Basal Medium Eagle (BME), and McCoy' 5a medium, were also used to grow *K. pneumoniae* NTUH-K2044 (statically incubated at 37°C, in 5% CO_2). However, the *kpcS* inversion was not observed under all the above growth conditions. The expression of KpcA was also not detected by Western blot analysis, suggesting an "OFF" phase of *kpcS* (*kpcS*$_{OFF}$) in *K. pneumoniae* NTUH-K2044. In the *kpcS*$_{OFF}$ phase, a polypeptide of 196 amino acid residues, KpcI$_{196}$, could be deduced (Fig. 3.6). To determine whether expression of the KpcI recombinase could invert the DNA, pKPCI$_{196,}$ an expression plasmid encoding KpcI$_{196}$ driven by an *araBAD* promoter, was introduced into *K. pneumoniae* NTUH-K2044. As shown in Fig. 3.8B, the

addition of L-arabinose to induce expression of $KpcI_{196}$ was able to invert the DNA. After the inversion, the DNA was isolated, sequenced and named $kpcS_{ON}$ (Fig. 3.7). To assess the promoter activity of $kpcS_{ON}$ or $kpcS_{OFF}$, each DNA was cloned in front of the promoterless *lacZ* gene in pLacZ15 by transcriptional fusion, and the resulting plasmids were named pSY003 and pSY004, respectively. After the introduction of pSY003 or pSY004 into *K. pneumoniae* NTUH-K2044 Δ*lacZ* strain CCW01, the LacZ activity of the transformants was measured. As shown in Fig. 3.9, the $kpcS_{ON}$ phase activity was much higher than that of $kpcS_{OFF}$ and pLacZ15, implying that the orientation of *kpcS* determines the transcription of the *kpc* genes.

3.3.5. Effect of the recombinant $KpcI_{196}$ on the switching of *kpcS*

Unlike FimB/E and MrpI in which the inverted repeat left (IRL) is located in the non-coding region between the recombinase-encoding gene and the fimbrial operon (16), the IRL of *kpcS* is located in the coding region of *kpcI* (Fig. 3.7). The inversion of $kpcS_{OFF}$ is predicted to produce a polypeptide of 210 residues, $KpcI_{210}$ (Fig. 3.6). The DNA fragment containing $kpcI_{210}$ was amplified by PCR from the genomic DNA of the L-arabinose-induced *K. pneumoniae* NTUH-K2044 [$pKPCI_{196}$] and then subcloned into pBAD33 to yield the plasmid $pKpcI_{210}$. As shown in Fig. 3.10A, the induced expression of $KpcI_{210}$ in *K. pneumoniae* NTUH-K2044 could invert *kpcS* in

the OFF-to-ON direction. Since the growth condition for *K. pneumoniae* to switch ON *kpcS* is yet to be identified, a two-plasmid system in *E. coli* was used to analyze the activity of $KpcI_{196}$ and $KpcI_{210}$ towards *kpcS* inversion in both directions. $pKpcI_{196}$ and $pKpcI_{210}$ were respectively introduced into *E. coli* JM109 harboring either the "substrate plasmid" pKPC-ON or pKPC-OFF. As shown in Fig. 3.10B, the induced expression of $KpcI_{196}$ or $KpcI_{210}$ in the recombinant *E. coli* was able to invert *kpcS* in both ON-to-OFF and OFF-to-ON directions. These results suggested a similar catalytic activity possessed by the recombinant $KpcI_{196}$ and $KpcI_{210}$.

3.3.6. KpcI-mediated expression of KpcA

Although the recombinant KpcI was able to switch ON *kpcS*, the induced expression of KpcI in *K. pneumoniae* did not lead to the expression of KpcA (data not shown). This result implies the involvement of further regulators in the regulation of expression of Kpc fimbriae. To avoid possible factors in *K. pneumoniae* that impede the expression of KpcA, another two-plasmid system was constructed to analyze the KpcI-mediated phase variation in *E. coli*. The DNA fragment containing the $kpcS_{OFF}$-*kpcABCD* region was PCR amplified and cloned into plasmid yT&A to yield pAW73. Subsequently, $pKPCI_{196}$ and $pKPCI_{210}$ were respectively introduced into *E. coli* harboring pAW73. Upon induction with L-arabinose, the induced expression of

KpcI$_{196}$ or pKPCI$_{210}$ was able to switch ON the expression of KpcA as detected by KpcA antiserum (Fig. 3.11). This indicated that the KpcI-mediated phase variation was able to control the expression of KpcA.

3.3.7. The transcription of Kpc fimbrial genes is impeded in the *kpcS*$_{ON}$ *K. pneumoniae* cells

To investigate whether the production of KpcA could only be detected in the *E. coli* system (Fig. 3.11) but not in the phase ON *K. pneumoniae* cells, which may due to no transcription of mRNA, or the instability of *kpcA* mRNA or KpcA protein, plasmid pKPC-36 was introduced into *K. pneumoniae* NTUH-K2044. KpcA production was readily observed upon IPTG induction (Fig. 3.12), which implied that transcription of *kpcA* was low in the ON phase of *K. pneumoniae* cells.

The *kpcS*$_{ON}$ carried on pSY003 exerting with a remarkably high promoter activity (Fig. 3.9) contains the region between the two inverted repeats. It is possible that other factors participate in the transcriptional control of *kpcA* through binding to the 83-bp DNA between the IRR and *kpcA* start codon. To investigate this possibility, the DNA fragment encompassing *kpcS*$_{ON}$, IRR, and the 83-bp region was PCR amplified and then cloned in front of the promoterless *lacZ* gene in pLacZ15 by transcriptional fusion, and the resulting plasmid named pAW126 (Fig. 3.13). After the

introduction of pSY003 or pAW126 into the *K. pneumoniae* NTUH-K2044 Δ*lacZ* strain, the β-galactosidase activity of the transformants was measured. As shown in Fig. 3.13, the Δ*lacZ* strain harboring pAW126 exhibited a significantly lower level of β-galactosidase activity than that observed for Δ*lacZ* [pSY003]. This result suggested that in the ON phase, the transcription of *kpcA* is impeded by the DNA region between *kpcS* and *kpcA*.

3.4. Discussion

The expression of Kpc fimbriae in *K. pneumoniae* NTUH-K2044 could not be observed with various environmental stimuli, including temperature, starvation, and aeration. Furthermore, no Kpc fimbriae expression was found in the 35 *K. pneumoniae* clinical isolates which possessed *kpc* genes (Table 2.1) grown statically overnight in LB broth or M9 medium at 25 or 37°C. The heterologous expression system was hence used for functional characterization of the Kpc fimbriae. The displayed Kpc fimbriae were shown to confer the recombinant *E. coli* a higher biofilm-forming activity. Bacterial biofilm formation on indwelling devices, such as catheters or endotracheal tubes, is a significant medical problem. The Kpc fimbriae may play a role in the development of infections in catheterized patients, and the possibility awaits further investigation. Besides *kpcABCD* genes, the putative fimbrial genes *kpaABCDE* and *kpbABCD* genes have also been heterogeneous expressed on the afimbriate *E. coli* surface and the fimbriation assessed by transmission electromicroscopy (138).

Since a close association between liver abscess and K1 serotype has been reported, whether Kpc fimbriae could mediate a tissue-tropism in *K. pneumoniae* liver abscess is worth to study. However, the recombinant Kpc fimbriae on *E. coli* surface

did not increase the bacterial adherence to the human hepatocellular liver carcinoma cell lines, HepG2 and SK-HEP-1 (data not shown). The *kpcC* mutation had no apparent effect on *K. pneumoniae* NTUH-K2044 virulence as assessed using intragastrical inoculation to BALB/c mice (193). No anti-KpcA response could be identified in the sera of the *K. pneumoniae* liver abscess infection patients (193). How to assess the role of Kpc fimbriae in *K. pneumoniae* liver abscess pathogenesis remains challenging.

In *Pseudomonas aeruginosa*, the four types of fimbriae belonged to the chaperone-usher assembly class (CupA, CupB, CupC, and CupD fimbriae) do not express under laboratory growth conditions (167, 213, 222, 254, 304, 305). Transposon mutagenesis was thus employed to select for the mutants expressing either of the Cup fimbriae for further studies. A transposon-insertion mutant library derived from *K. pneumoniae* NTUH-K2044 CCW01 strain carrying P_{kpcA}-*lacZ* was also generated, and the mutants with color changes on the X-gal plate isolated. However, no blue colony was found in approximate 20,000 individual colonies of the mutant pool grown on X-gal plates.

As shown in Fig. 3.10, the recombinant KpcI possessed activity to flip *kpcS* in both directions; therefore, the induced expression of KpcI in *K. pneumoniae* may lead

to both ON-to-OFF and OFF-to-ON inversions that occur simultaneously in different cells in the bacterial population. However, whether KpcI could invert the *kpcS* from ON-to-OFF direction in *K. pneumoniae* remains to be investigated. In *E. coli* type 1 fimbriae, the *fimS* inversion which resulted in changes of the 3-untranslated region of *fimE* altered the mRNA stability and hence the FimE-mediated phase variation (284). Although the recombinant $KpcI_{196}$ and $KpcI_{210}$ possessed similar activities on the *kpcS* switch (Fig. 3.10), the possibility that different stability of the two *kpcI* transcripts in *K. pneumoniae* cells differentially control the *kpcS* inversion remains investigated.

Several reports have shown that DNA recombinases other than FimB/E cause the *fimS* switch (38, 327). The BLAST search revealed no fimbrial recombinase gene other than *fimB*, *fimE*, and *kpcI* in the *K. pneumoniae* NTUH-K2044 genome. Whether a cross-regulation occurs between type 1 and Kpc fimbriae by the recombinases was also analyzed. The expression plasmid carrying *fimB*, *fimE* or *kpcI* was individually introduced into *K. pneumoniae* NTUH-K2044, the orientation of *fimS* and *kpcS* were then determined under the induced-condition. However, inversion of the *fimS* or *kpcS* could only be observed by the expression of their cognate recombinase (data not shown).

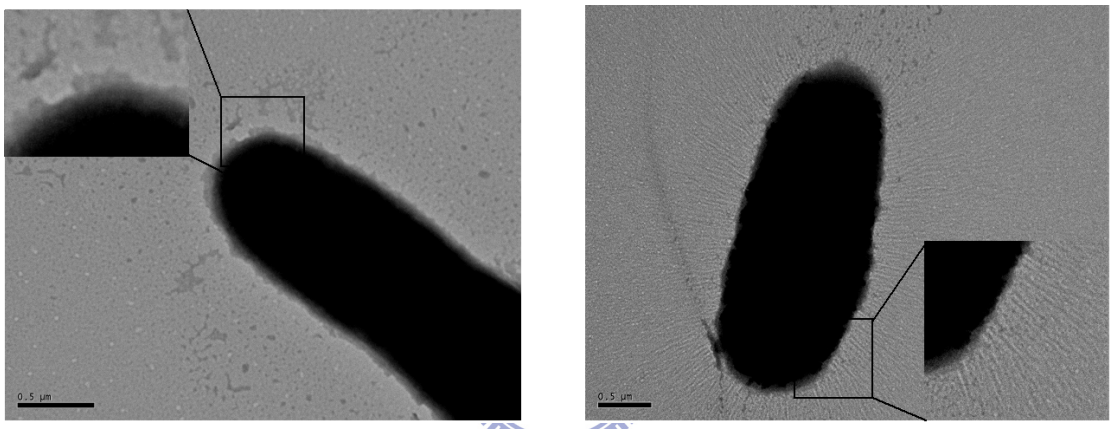


Fig. 3.1. Transmission electron micrographs of recombinant Kpc fimbriae. Left panel, *E. coli* Novablue (DE3) [pET30a]; right panel, *E. coli* Novablue (DE3) [pKPC-7]. Bars, 0.5 μm.

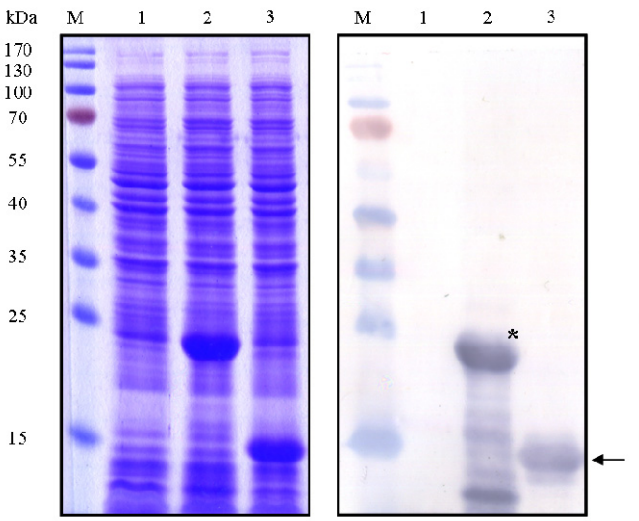


Fig. 3.2. Specificity of the KpcA antiserum. Proteins from total cell lysates of the recombinant bacteria were resolved in 15% (w/v) SDS-polyacrylamide gel and stained with Coomassie brilliant blue (left panel). The gel was subjected to Western blot analysis using KpcA antiserum (right panel). The recombinant protein His_6::KpcA (asterisk) and KpcA (arrow) are marked. M, protein marker; Lanes 1, *E. coli* NovaBlue(DE3) [pET30a]; 2, *E. coli* NovaBlue(DE3) [pKPCA]; 3: *E. coli* NovaBlue(DE3) [pKPC-7].

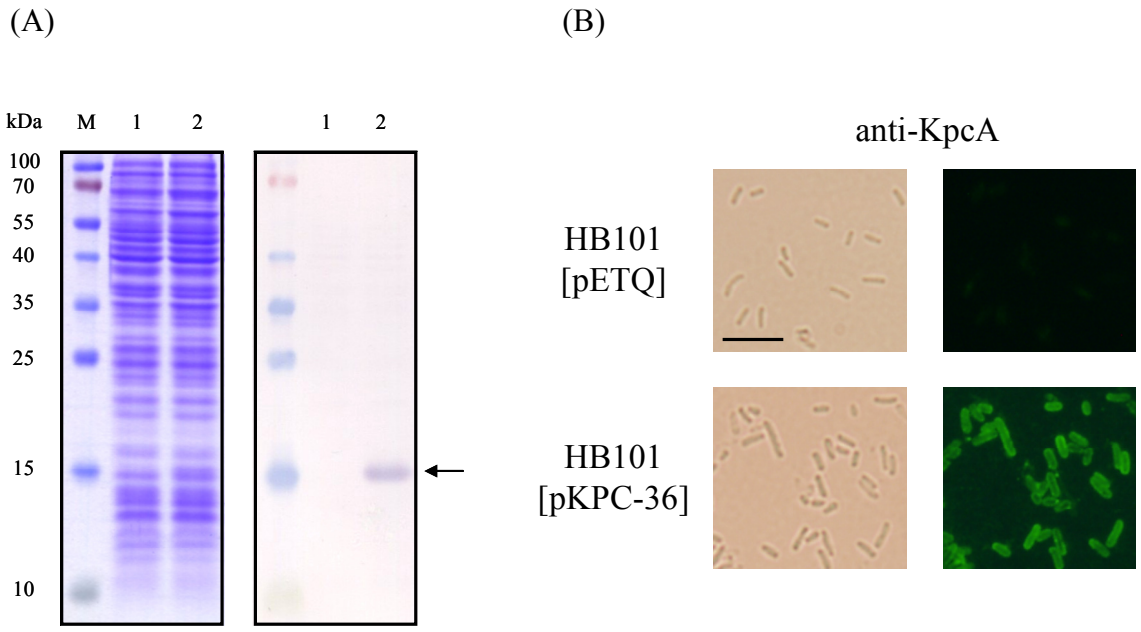


Fig. 3.3. Expression of Kpc fimbriae on recombinant *E. coli*. (A) Anti-KpcA Western blot analysis of *E. coli* HB101 harboring pETQ (lane 1) or pKPC-36 (lane 2). The expressed KpcA is indicated by an arrow. (B) Bright-field (left panel) and anti-KpcA immunofluorescence (right panel) microscopic analysis of *E. coli* HB101 harboring pETQ or pKPC-36 (magnification x630). Bar, 10 μm.

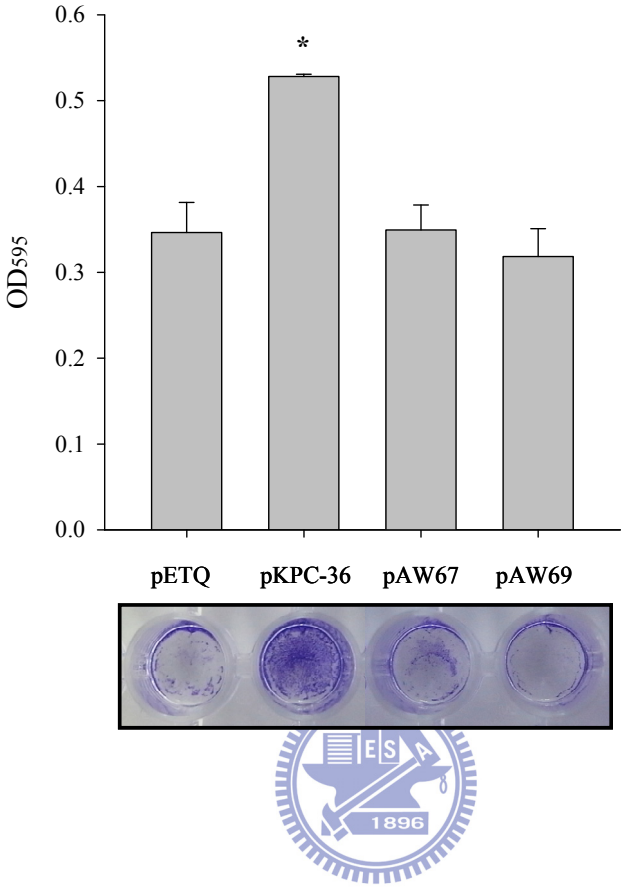


Fig. 3.4. Biofilm forming activity of *E. coli* expressing the Kpc fimbriae. The development of biofilms of *E. coli* HB101 harboring pETQ, pKPC-36, pAW67, and pAW69 was observed (the lower panel) and quantified (the upper panel) as described in Methods. A higher biofilm-forming activity could be observed for *E. coli* HB101 [pKPC-36]. The results are shown as the average of the triplicate samples. Error bars indicate standard deviations. *, $P < 0.001$ compared with HB101 [pETQ].

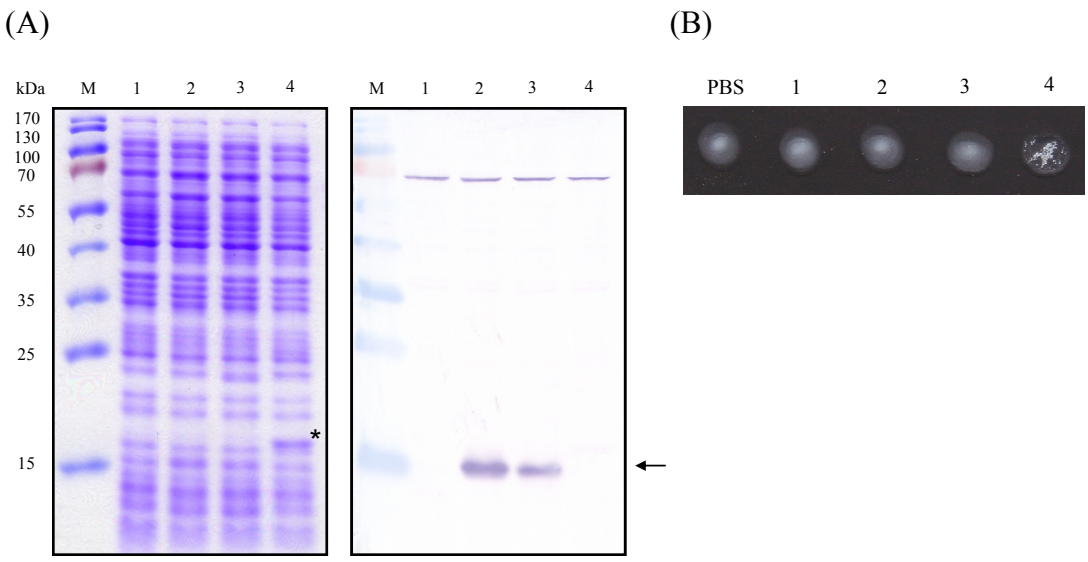


Fig. 3.5. IPTG-induced expression of the fimbrial genes in *E. coli* HB101. Plasmid pETQ (lane 1), pKPC-36 (lane 2), pAW67 (lane 3), or pAW69 (lane 4) was introduced into *E. coli* HB101, respectively. Log-phase grown bacteria were induced with 0.5 mM IPTG for 3 h. (A) The expression of KpcA, indicated by an arrow, was analyzed by SDS-PAGE and anti-KpcA Western blot analysis. The expression of the major pilin FimA (approximately 18.3 kDa) of type 1 fimbriae is marked by an asterisk. M, protein marker. (B) Yeast agglutination. Ten microliter bacterial suspension (10^7 CFU/ml) was mixed with ten microliter yeast suspension (10 mg/ml) on a glass slide. After gentle shaking on orbital shaker for 5 min, a strong yeast agglutinating activity of *E. coli* HB101 harboring pAW69 could be observed. PBS, negative control.

Fig. 3.6. Alignment of the amino acid sequences of the fimbrial recombinases. Identical residues are shaded, the predicted critical residues involved in the DNA recombination are marked by asterisks, and the tyrosine residue which is predicted to be directly involved in the phosphoryl transfer reaction is indicated by an arrow. The difference in the C-terminal fifteen residues between $KpcI_{196}$ and $KpcI_{210}$ is boxed.

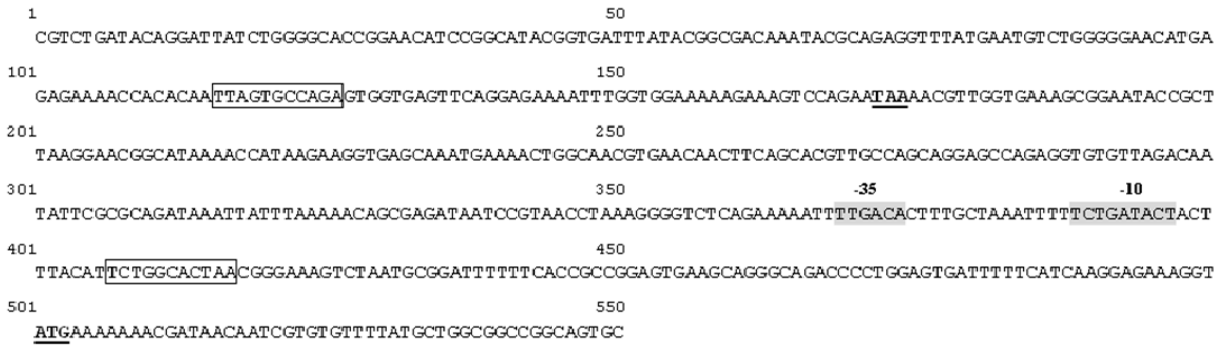
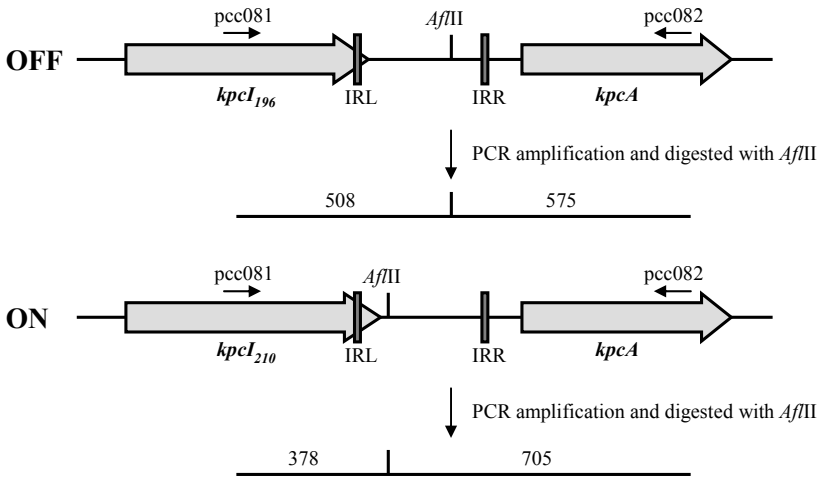


Fig. 3.7. Sequence analysis of the putative promoter region of the *kpc* gene cluster. The 500 bp upstream and 50 bp downstream regions of the *kpcA* translation start codon ATG (underlined) in the $kpcS_{ON}$ phase are shown. The 11 bp inverted repeats are outlined by square boxes. The predicted -10 and -35 promoter regions are shaded. The $kpcI_{210}$ translation stop codon TAA is also underlined.

(A)



(B)

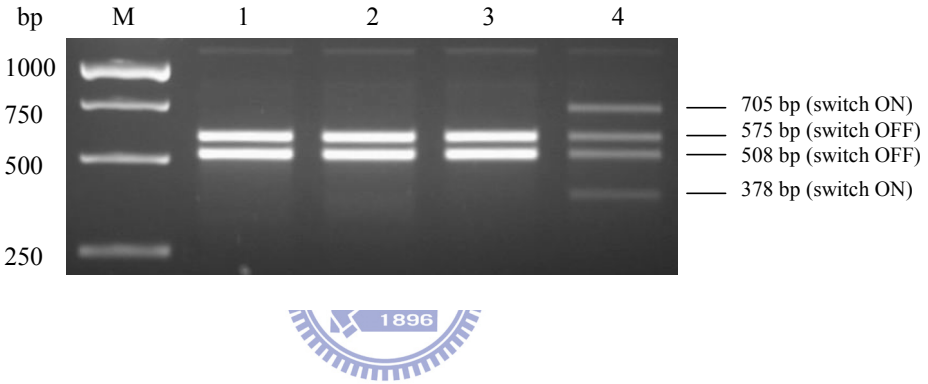


Fig. 3.8. KpcI$_{196}$-mediated inversion of *kpcS*. (A) Map of the invertible region, *kpcS*, in both OFF and ON orientations. The positions of the primers used in the PCR amplification, pcc081 and pcc082, and the sizes of the DNA fragments resulting from *Afl*II digestion are as indicated. IRL, inverted repeat left; IRR, inverted repeat right. (B) Expression of recombinant KpcI$_{196}$ resulted in a switch from the OFF to the ON phase. *K. pneumoniae* NTUH-K2044 transformed with pBAD33 or pKPCI$_{196}$ was grown in M9 broth supplemented with 0.4% glucose (lanes 1 and 3) or $_{L}$-arabinose (lanes 2 and 4) for 16 h with agitation at 37^{o}C. The grown bacterial cultures were collected and then subjected to the switch orientation assay of *kpcS*. Lanes: M, DNA molecular size markers; 1 and 2, pBAD33 vector as a control; 3 and 4, pKPCI$_{196}$.

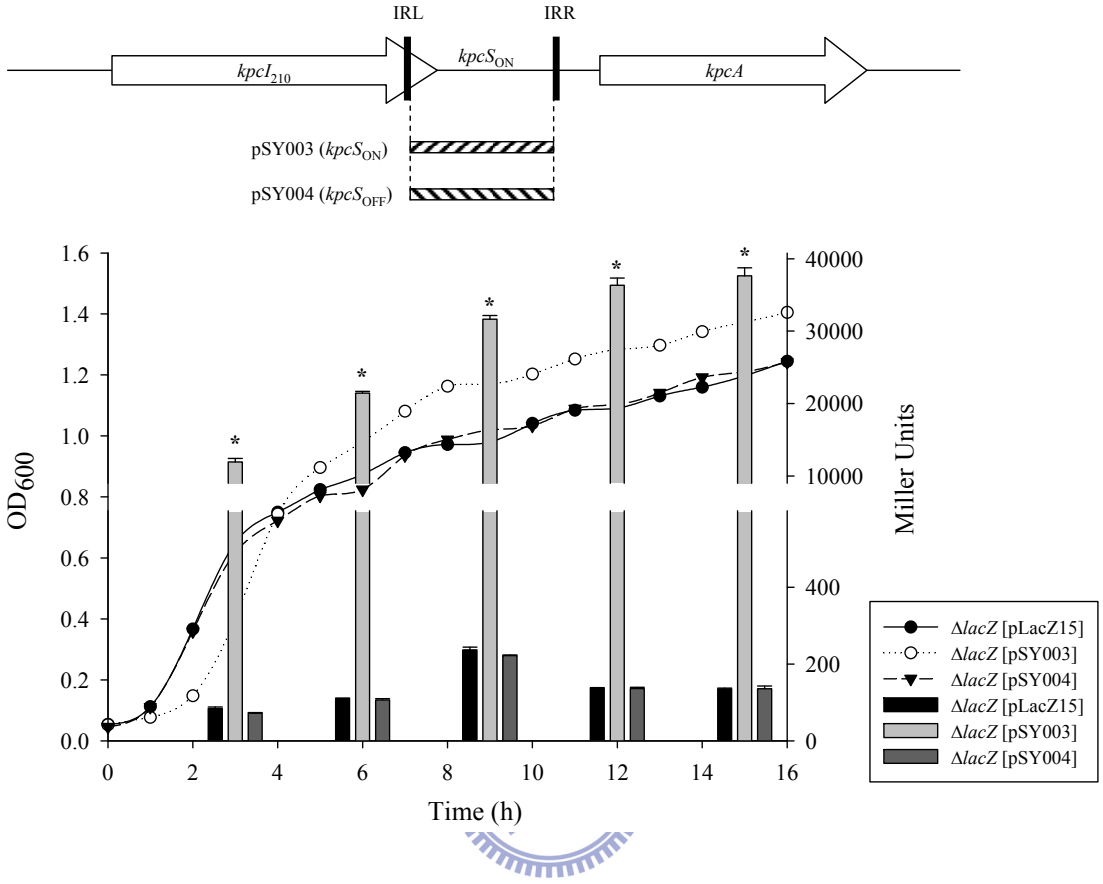
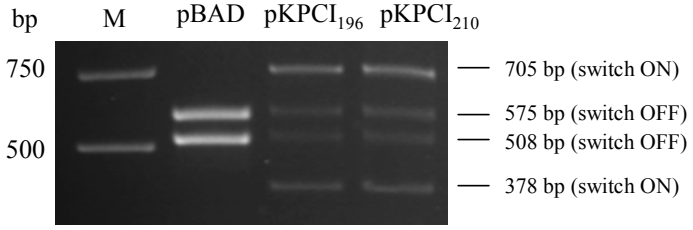


Fig. 3.9. Determination of the promoter activities of $kpcS_{ON}$ and $kpcS_{OFF}$. The β-galactosidase activities (Miller units) of $kpcS_{ON}$::*lacZ* and $kpcS_{OFF}$::*lacZ* in the *K. pneumoniae* NTUH-K2044 Δ*lacZ* strain CCW01 (Δ*lacZ*) carrying each of the reporter plasmids pSY003 ($kpcS_{ON}$), pSY004 ($kpcS_{OFF}$), or pLacZ15 (vector only as a negative control) were determined from log-phased cultures grown in LB broth. The results are shown as an average of triplicate samples. Error bars indicate standard deviations. *, $P < 0.0001$ compared with Δ*lacZ* [placZ15] in the same growth phase. The growth curve (OD_{600}) of the bacteria is also shown.

(A)



(B)

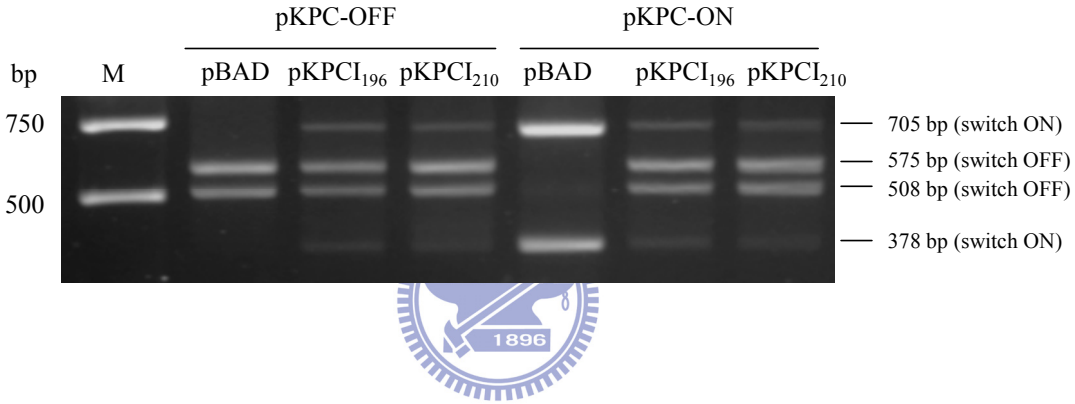


Fig. 3.10. The recombinant KpcI mediated the *kpcS* inversions in both directions. (A) Expression of recombinant $KpcI_{196}$ or $KpcI_{210}$ resulted in a switch from the OFF to the ON phase. *K. pneumoniae* NTUH-K2044 carrying pBAD33, $pKPCI_{196}$, or $pKPCI_{210}$ was grown in LB broth, supplemented with 0.4% $_L$-arabinose, for 16 h with agitation at 37°C. Lanes: M, DNA molecular size markers. (B) *E. coli* JM109 transformed with two plasmids was grown in LB broth, supplemented with 0.4% $_L$-arabinose, for 16 h with agitation at 37°C. The grown bacterial cultures were collected and then subjected to the switch orientation assay of *kpcS*. The fragment sizes corresponding to the position of the switches are shown to the right of the panel.

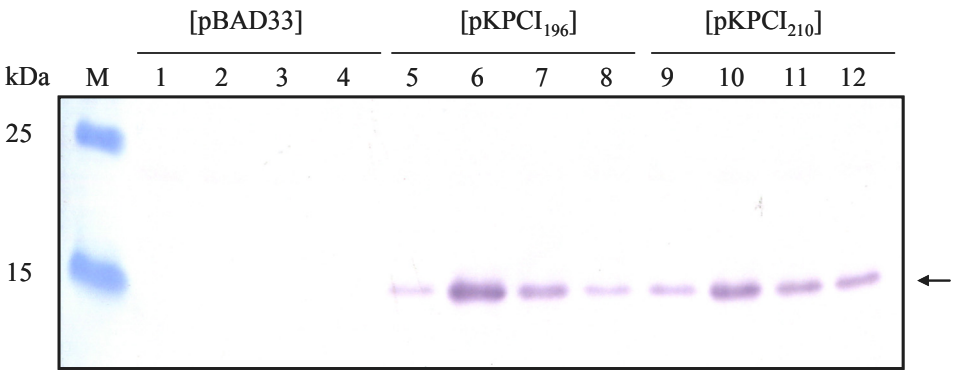


Fig. 3.11. KpcI-mediated expression of KpcA in *E. coli*. Plasmid pBAD33, $pKPCI_{196}$, or $pKPCI_{210}$, as marked above the panels, was introduced into *E. coli* JM109 [pAW73]. *E. coli* carrying the two plasmids was grown in LB broth, and when growth reached mid-exponential phase, the expression of KpcI was induced by varying concentrations of $_L$-arabinose: lanes 1, 5, and 9, no induction; lanes 2, 6, and 10, 0.002% ; lanes 3, 7, and 11, 0.02%; lanes 4, 8, and 12, 0.2% $_L$-arabinose induction. After 3 h induction, the bacteria were analyzed by anti-KpcA Western blot hybridization. The expression of KpcA is marked by an arrow.

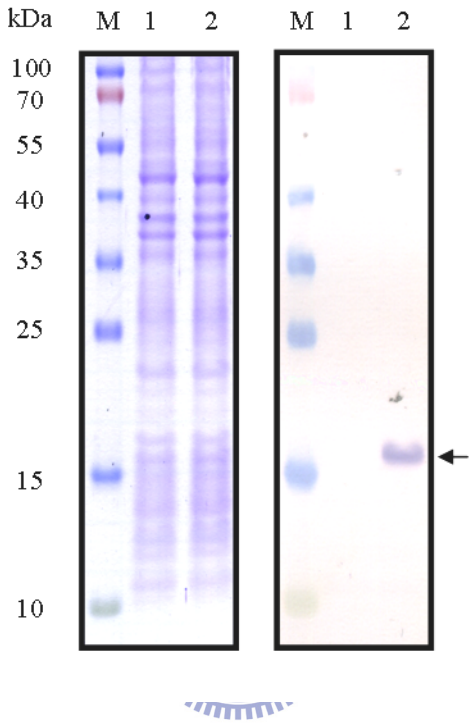


Fig. 3.12. The T5*lac* promoter driven expression of *kpcABCD* genes in *K. pneumoniae*. Plasmid pETQ (lane 1) and pKPC-36 (lane 2) were introduced into *K. pneumoniae* NTUH-K2044, respectively. The IPTG-induced expression of *kpcA* was analyzed by SDS-PAGE (left panel) and anti-KpcA Western blot hybridization (right panel). Expression of KpcA is indicated by an arrow. M, protein marker.

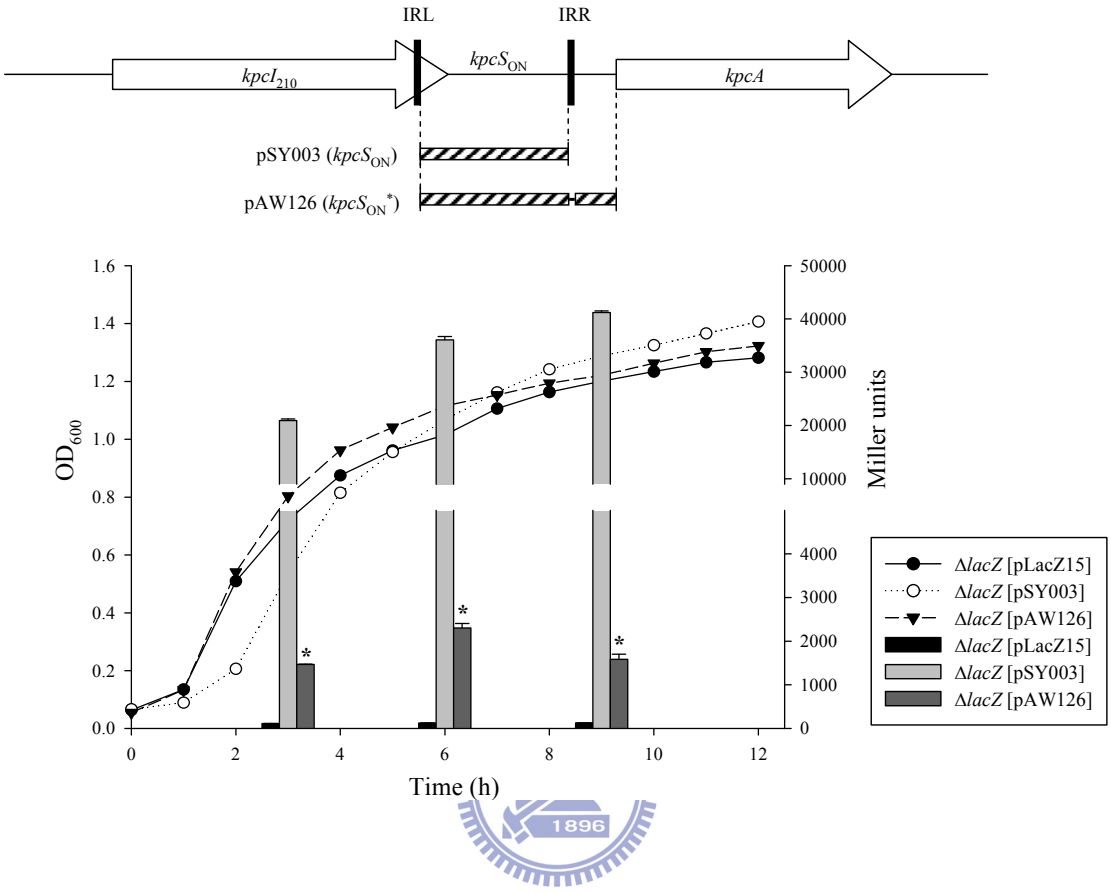


Fig. 3.13. Determination of the promoter activities of *kpcS*$_{ON}$ and *kpcS*$_{ON}^{*}$. The β-galactosidase activities (Miller units) of *kpcS*$_{ON}$::*lacZ* and *kpcS*$_{ON}^{*}$::*lacZ* in the *K. pneumoniae* NTUH-K2044 Δ*lacZ* strain CCW01 (Δ*lacZ*) carrying each of the reporter plasmids pSY003 (*kpcS*$_{ON}$), pAW126 (*kpcS*$_{ON}^{*}$), or pLacZ15 (vector only as a negative control) were determined from log-phased cultures grown in LB broth. The results are shown as an average of triplicate samples. Error bars indicate standard deviations. The growth curve (OD_{600}) of the bacteria is also shown. The results are shown as the average of the triplicate samples. Error bars indicate standard deviations. *, $P < 0.0001$ compared with Δ*lacZ* [pSY003] in the same growth phase.

CHAPTER 4

Regulation of the Expression of Type 3 Fimbriae in *Klebsiella pneumoniae* CG43

4.1. Abstract

Type 3 fimbriae play an important role in *Klebsiella pneumoniae* biofilm formation. Nevertheless, how the type 3 fimbrial operon, *mrkABCDF*, is regulated is largely unknown. Downstream to the *mrkF* are three putative regulatory genes named *mrkH*, *mrkI*, and *mrkJ*. MrkH is a PilZ domain protein of putative binding activity to the second messenger c-di-GMP. MrkI is predicted as a LuxR-type transcriptional regulator. MrkJ has been reported as a c-di-GMP phosphodiesterase. Reverse-transcription PCR analysis showed that *mrkH*, *mrkI*, and *mrkJ* could be transcribed in a polycistronic mRNA. Furthermore, deletion of *mrkI* from *K. pneumoniae* CG43S3 appeared to abolish the production of MrkA, the major pilin of type 3 fimbriae, as assessed by Western blot analysis. The following promoter-reporter assay of *mrkA* verified that MrkI regulated the type 3 fimbriae expression at transcriptional level. Moreover, mutation of a conserved aspartate residue (D56), which is predicted as a putative target site for phosphorylation, of MrkI affected the type 3 fimbriae expression. MrkA production was slightly increased by the *mrkJ*-deletion, whereas no obvious effect was found by the *mrkH*-deletion. Nevertheless, an increased expression of type 3 fimbriae could be observed upon the induced expression of MrkH.

Analysis of the putative promoter sequences of *mrkA* and *mrkHIJ* operon revealed the ferric uptake regulator Fur binding elements. Western blot analysis showed that the deletion of *fur* from *K. pneumonia*e CG43S3 abolished the expression of MrkA. Moreover, the promoter activity of *mrkA* and *mrkH* were reduced in the Δ*fur* strain. These suggested that Fur acted as an activator for the type 3 fimbriae expression. Interestingly, the overproduction of YdeH, an *Escherichia coli* c-di-GMP cyclase, appeared to activate the MrkA expression, whereas this activation was suppressed by deletion of *mrkI* or *fur* from *K. pneumoniae* CG43S3 [pYdeH]. Finally, we also found that the availability of oxygen could affect the expression of type 3 fimbriae. The findings concluded a multi-factorial regulation of the expression of type 3 fimbriae in *K. pneumoniae* CG43.

4.2. Introduction

Klebsiella pneumoniae type 3 fimbriae, which are encoded by the *mrkABCDF* operon, play an important role in biofilm formation on biotic and abiotic surfaces (71, 139, 148). By analyzing the available genome sequences of *K. pneumoniae*, three ORFs (namely *mrkH*, *mrkI*, and *mrkJ*) were found to locate downstream to the *mrkF* gene (Fig. 4.1). MrkH is predicted as a PilZ domain protein which is able to bind to c-di-GMP (12, 34, 56, 210, 245, 258), and MrkI is predicted as a LuxR-type transcriptional factor (153). MrkJ, an EAL domain protein, has been reported as a functional c-di-GMP phosphodiesterase (153). Deletion of *mrkJ* was found to increase the type 3 fimbriae expression and biofilm-forming activity which is speculated to be resulted from the accumulation of intracellular c-di-GMP (153). However, how MrkH and MrkI exert regulation on the expression of type 3 fimbriae awaits investigation.

Iron is essential to most bacteria for growth and reproduction by playing as a cofactor for electron transport chain and various enzymes (221). Under anaerobic conditions, iron is in the ferrous form (Fe^{2+}), which can be taken up by bacteria directly using transporter such as EfeUOB, FeoAB, MntH or SitABCD (14, 40, 111). Under aerobic conditions, Fe^{2+} is oxidized to the ferric (Fe^{3+}) state, which forms insoluble ferric-hydroxides at neutral pH resulting in poor iron availability (221).

Thus, bacteria utilized intricate iron transporting systems, which are able to dissolve and transport ferric iron under aerobic conditions. Such systems involve the secretion of high-affinity, low-molecular-weight, Fe^{3+}-chelating compounds called siderophores to form ferrisiderophore complexes with Fe^{3+} (166, 221). These complexes are subsequently taken up by bacteria through specific transport systems (166). Iron-uptake systems are also considered as virulence factors of pathogenic bacteria since iron availability is generally restricted *in vivo* (266).

Under aerobic conditions, however, excess iron tends to catalyze the generation of damaging free radicals which causes toxicity to bacteria (16). A tight regulation of iron-uptake systems is thus required. Genes responsible for the uptake and metabolism of iron are generally regulated by the ferric uptake regulator (Fur) in many bacteria (6, 41, 84, 120). Under iron-repletion conditions, Fur binds iron and dimerized, and the Fe^{2+}-Fur dimers bind to a 19-bp consensus DNA sequence, the Fur box (GATAATGATwATCATTATC; w=A or T), in target promoters (19, 85, 110). Binding of Fur at the promoters impedes the binding of RNA polymerase, thereby preventing transcription from these genes. Not only is Fur involved in regulating iron homeostasis, it is also participated in bacterial colonization, oxidative stress response, toxin secretion and virulence (41). In some cases, Fur functions as an activator and even to regulate certain genes in the absence of the iron (41). Recently, we have

shown that Fur repressed the expression of the mucoid factor RmpA and then decreased the biosynthesis of capsular polysaccharide (CPS) in *K. pneumoniae* CG43 (53).

Bis-(3'-5')-cyclic dimeric guanosine monophosphate (Cyclic-di-GMP or c-di-GMP) is an ubiquitous second messenger which regulates a variety of cellular processes, including biogenesis of fimbriae, flagella, and capsule, in bacteria (151, 239, 250, 256, 293). The elevated level of intracellular c-di-GMP has been reported to activate the expression of type 3 fimbriae and *fur* in *K. pneumoniae* and in *Escherichia coli*, respectively (153, 209). In this study, deletion effects of *mrkH*, *mrkI*, *mrkJ* and *fur* on the type 3 fimbriae expression were analyzed. The presence of Fur-mediated regulation on the transcription of *mrk* genes was also investigated.

4.3. Results

4.3.1. *mrkHIJ* is transcribed in a polycistronic mRNA

As shown in Fig. 4.1, three ORFs designated as *mrkH*, *mrkI*, and *mrkJ* were found to locate downstream to the type 3 fimbrial genes. *mrkH*, *mrkI*, and *mrkJ* appear to be transcribed in the same direction, and the intergenic regions of *mrkH-mrkI* and *mrkI-mrkJ* are only 5- and 143-bp, respectively, suggesting that the three genes could be transcribed in a transcriptional unit. To demonstrate the operon structure, reverse transcription PCR (RT-PCR) reactions were performed with total RNA from *K. pneumoniae* CG43S3 and specific primer pairs. As shown in Fig. 4.2, the PCR products b and c, respectively for the junction of *mrkH-mrkI*, and *mrkI-mrkJ*, were obtained, suggesting the three products derived from a single transcription unit. Expression of *mrkH*, *mrkI*, and *mrkJ* were also confirmed (Fig. 4.2- d, e, and f). In addition, a putative internal promoter located in the 143-bp intergenic region between *mrkI* and *mrkJ* could be predicted using the BPROM software (http://www.softberry.com/berry.phtml).

4.3.2. Deletion of *mrkI* represses the expression of type 3 fimbriae

To investigate whether MrkH, MrkI, and MrkJ play regulatory roles on the expression of type 3 fimbriae, specific gene-deletion mutants in *K. pneumoniae*

CG43S3 was generated individually. As shown in Fig. 4.3, the Western blot hybridization using MrkA antiserum revealed that the expression of MrkA was abolished in the Δ*mrkI* strain. A slightly increase of MrkA amount, approximately 1.67 fold, was found in the Δ*mrkJ* strain, which is consistent with the previous report (153), while the deletion of *mrkH* had no apparent effect.

To complement the *mrkI*-deletion effect, the *mrkI* gene was cloned into various plasmids including pACYC184 (44), pRK415 (159), pETQ and pBAD33 (114, 324), and then introduced into the Δ*mrkI* strain, respectively. However, the expression of MrkA could not be complemented by any of the *mrkI*-expression plasmids (data not shown). Thus, the *mrkI* gene and its approximately 1-kb adjacent regions were cloned into the suicide vector pKAS46 (282), and the resulting plasmid pWY45 was introduced into the Δ*mrkI* strain to generate a chromosomal integrated *mrkI*-complemented strain CCW41. The expression of MrkA could be restored in the CCW41 strain but not in the control strain CCW40 whose chromosome was integrated by pWY28 carrying only the adjacent regions of *mrkI* (Fig. 4.3). These results suggest that MrkI plays a positive role on the type 3 fimbriae expression.

4.3.3. Identification of the transcription start site of *mrkA*

Prior to examining the effect of MrkI on type 3 fimbriae expression, a rapid

amplification of 5' complementary DNA ends (5'-RACE) analysis of *mrkA* was performed to identify its promoter. As shown in Fig. 4.4A, PCR product of single DNA band was obtained using either primer pair. Sequence analysis of the PCR clones revealed that the transcription starts at the G nucleotide positioned -204 relative to the translational start site of MrkA (Fig. 4.4B). A conserved -10 and -35 promoter sequence of σ70 and a conserved ribosomal binding sequence (RBS) could be readily identified.

4.3.4. Deletion of *mrkI* decreases the transcription of *mrkA*

Mapping of *mrkA* transcription start allowed localization of the promoter region. As shown in Fig. 4.5, three promoter-reporter plasmids pmrkA-P1, pmrkA-P2, and pmrkA-P3, each carrying a *lacZ* transcriptional fusion to different promoter regions of *mrkA*, were respectively introduced into *K. pneumoniae* CG43S3 Δ*lacZ* and Δ*mrkI*Δ*lacZ* strains. The promoter activity measurements revealed that the deletion of *mrkI* reduced the activity of the P1 and P2. While the P3, of which the above-identified -10 and -35 sequences had been removed, had no promoter activity (Fig. 4.5). The result indicates that MrkI affects the expression of type 3 fimbriae at transcriptional level.

4.3.5. Activity of MrkI is probably affected by phosphorylation

The MrkI protein contains a LuxR-like helix-turn-helix (HTH) DNA binding domain. Several members of the LuxR family are response regulators (RR) which act as transcriptional activators or repressors. A common post-translational modification of RR is phosphorylation at a conserved aspartate residue (D) in the N-terminal receiver domain performed by a sensor histidine kinase (HK) or by autophosphorylation. A RR and its cognate HK form a "two-component system", which is a widespread signal transduction system in bacteria (104, 129, 203, 288). Phosphorylation of the conserved aspartate in the N-terminal receiver domain of the RR activates the protein by inducing conformational changes which facilitate interaction of the RR with the target DNA (202, 288). Furthermore, some RRs have been shown to autophosphorylate *in vitro* in the presence of acetyl-phosphate (51, 194, 272), which has been proposed to be a global signal in bacteria (207). As shown in Fig. 4.6A, a conserved aspartate residue (D56) which is the putative site for phosphorylation could be found in the N-terminal region of MrkI, suggesting MrkI is a RR activated by phosphorylation.

To determine the role of the conserved D56 on MrkI-mediated type 3 fimbriae expression, the site-directed mutants with single amino acid substitution of the

aspartate D56 to alanine (D→A) to prevent from phosphorylation (66, 196), or to glutamate (D→E) to mimic the phosphorylated state (113, 164, 172, 176) were created. As shown in Fig. 4.6B, introduction of the *mrkI* allele encoding $MrkI_{D56E}$ did not affect the expression of MrkA. By contrast, the MrkA production was dramatically reduced upon introduction into the chromosome with the mutant allele encoding $MrkI_{D56A}$. This result suggests that MrkI is a response regulator that is activated via phosphorylation of the D56 residue by an un-identified kinase.

4.3.6. Overproduction of MrkH increases the expression of type 3 fimbriae

The PilZ family of proteins have been reported as c-di-GMP effectors (122). In *K. pneumoniae* NTUH-K2044, three PilZ domain proteins could be found (Fig. 1.1). In addition to *mrkH*, the other two ORFs (locus tag: KP1_5225 and KP1_5237) were predicted to encode cellulose synthase subunits. In many bacteria, deletion or overexpression of *pilZ* genes have been reported to affect bacterial motility, biosynthesis of fimbriae and exopolysaccharide, biofilm formation, and virulence (9, 34, 56, 210, 245). As shown in Fig. 4.7, the conserved motifs RxxxR and D/NxSxGG, which play critical roles in c-di-GMP binding in many PilZ domain proteins (27), could also be found in MrkH.

Although deletion of *mrkH* did not affect the MrkA production (Fig. 4.3), the

introduction of a plasmid pMrkH carrying *mrkH* into *K. pneumoniae* CG43S3 was found to activate the expression of type 3 fimbriae (Fig. 4.8). Furthermore, the induced-expression of *mrkH* in *K. pneumoniae* NTUH-K2044, which generally carrying no detectable MrkA expression, also activated the MrkA production (data not shown), suggesting an involvement of MrkH in the regulation of type 3 fimbriae expression.

In addition to the C-terminal PilZ domain, MrkH contains an N-terminal sequence of unknown function. To further analyze its function, DNA fragments encoding the N-terminus ($MrkH_N$, residues 1 to 105) and the PilZ domain (PilZ, residues 82 to 234) of MrkH were isolated and cloned into pETQ to generate $pMrkH_N$ and pPilZ, respectively. A R111D mutation, predicted to abolish the c-di-GMP binding ability (27, 258), was also introduced into MrkH and $PilZ_{MrkH}$ ($MrkH^*$ and $PilZ^*$) and the DNAs were cloned into pETQ to generate $pMrkH^*$ and $pPilZ^*$, respectively. As shown in Fig. 4.8, the induced-expression of $MrkH^*$, PilZ, $PilZ^*$, or $MrkH_N$ in *K. pneumoniae* CG43S3 had no apparent effect on the expression of type 3 fimbriae, These results suggest that the N-terminus, the PilZ domain, and the R111 residue of MrkH are required for the MrkH-mediated activation of type 3 fimbriae expression. Whether MrkH could interact with c-di-GMP and how MrkH regulates gene expression await further investigation.

4.3.7. Deletion of *fur* represses the expression of type 3 fimbriae

Sequence analysis of the promoter regions of *mrkA* and *mrkHIJ* operon revealed putative 19-bp Fur-binding sequences (19, 85, 110), respectively located at -206 to -188 relative to the MrkA start codon and -119 to -101 relative to the MrkH start codon. As shown in Fig. 4.9A, both the Fur box-like sequences harbor 12/19 identity. To investigate if Fur plays a regulatory role on the expression of type 3 fimbriae, *K. pneumoniae* CG43S3 and its isogenic Δ*fur* strain were analyzed by Western blot analysis using MrkA antiserum. As shown in Fig. 4.9B, the deletion of *fur* abolished the expression of MrkA, and introduction of a *fur*-carrying plasmid, pfur, into Δ*fur* strain could resotre MrkA expression. Moreover, the promoter-reporter assay of P_{mrkA} showed that the deletion of *fur* reduced the activity of the *mrkA* promoter, and the reduction level was similar to the deletion effect of *mrkI* (Fig. 4.9C). These results suggest that Fur positively regulates the expression of type 3 fimbriae at transcriptional level.

4.3.8. Deletion of *fur* and *mrkI* represses the activity of P_{mrkH}

To investigate if Fur could also regulate the expression of *mrkHIJ* operon, the promoter reporter assay of P_{mrkH} was performed. The DNA fragment encompassing the putative promoter region of *mrkH* was cloned in front of the promoter-less *lacZ* of

placZ15 to generate pAW175. Since a putative RcsAB-binding sequences could also be identified in the upstream region of *mrkH* (Fig 4.2), the reporter plasmid pAW175 were introduced individually into *K. pneumoniae* CG43S3 Δ*lacZ* and its isogenic Δ*fur*, Δ*rcsA*, and Δ*rcsB* strains for promoter activity measurements. As shown in Fig. 4.10, the activity of the putative promoter of *mrkH* was decreased by the *fur*-deletion, while deletion of *rcsA* or *rcsB* did not cause apparent effect. The promoter activity of *mrkH* was also decreased in the Δ*mrkI* strain, suggesting an auto-regulation of MrkI on the *mrkHIJ* operon; while no obvious effect was found by the *mrkH*-deletion. Our results suggest that Fur and MrkI activate the promoter activity of *mrkH*. Since MrkI acted as an activator of the type 3 fimbriae (Fig. 4.3), it is likely that Fur indirectly regulates the type 3 fimbriae expression via MrkI.

4.3.9. Extracellular iron availability affects the expression of type 3 fimbriae

Since Fur has been reported to regulate gene expressions both in Fe^{2+}-dependent and -independent manners (41), we analyzed effects of iron-depletion and iron-repletion on the expression of type 3 fimbriae in *K. pneumoniae* CG43S3. As shown in Fig. 4.11, addition of 200 μM of the iron chelator 2, 2-dipyridyl (Dip) in LB medium (containing approximately 18 μM of iron) decreased the MrkA production. Moreover, the addition of 60 μM $FeSO_4$ in M9 medium (containing approximately 2

μM of iron) caused an apparent increase in the MrkA production. Besides, no obvious MrkA production was found in the Δ*fur* strains grown in all the above assay conditions. These results support the conclusion that Fur activates the expression of type 3 fimbriae in a Fe^{2+}-dependent manner.

4.3.10. Fur and MrkI are required for the c-di-GMP-activated expression of type 3 fimbriae

The second messenger c-di-GMP has been demonstrated to activate the expression of *fur* and type 3 fimbriae (153, 209). As shown in Fig. 4.12, the induced expression of the *E. coli* GGDEF domain protein YdeH (154), a c-di-GMP cyclase, in *K. pneumoniae* CG43S3 could increase the expression of type 3 fimbriae assessed by anti-MrkA Western blot hybridization. In the Δ*fur* or Δ*mrkI* strains, the increase of MrkA production by YdeH expression was no longer observed (Fig. 4.12). Expression of the AADEF mutant of YdeH ($YdeH_{AADEF}$), which has been reported to cause an impaired cyclase activity (280), had also eliminated the effect on the elevation of MrkA production (Fig. 4.12). These results indicated that the elevated cellular level of c-di-GMP by inducing expression of YdeH activates the expression of type 3 fimbriae; moreover, Fur and MrkI are likely involved in this c-di-GMP-mediated activation.

As shown in Fig. 4.13, the induced-expression of YdeH in Δ*mrkH* strain still

increased MrkA amount, implying that MrkH is not required for this activation. In addition to PilZ domain proteins, other protein effectors or RNA effectors for c-di-GMP signaling have been described in many bacteria (122). It is possible that effectors other than MrkH could bind to c-di-GMP and regulate the type 3 fimbriae expression in *K. pneumoniae*. The MrkH-activated expression of the type 3 fimbriae could still be observed in the Δ*fur*, Δ*mrkI*, or Δ*fur*Δ*mrkI* strain (Fig. 4.13), suggesting Fur or MrkI is not required for the MrkH-mediated activation.

4.3.11. Knockout of *fur* and *mrkI* decreases *K. pneumoniae* biofilm formation

Expression of type 3 fimbirae has been associated with *K. pneumoniae* biofilm formation (71, 139, 148). As shown in Fig. 4.14, the deletion of *mrkI* decreased the bacterial biofilm-forming activity to a similar level as that of the Δ*mrkA* strain, and the deleting effect of *mrkI* could be restored in the CCW41 strain. On the other hand, the deletion of *fur* only caused a slight reduction of *K. pnuemoniae* biofilm-forming activity. We have previously described that the deletion of *fur* causes an overproduction of CPS (53), another important determinant in biofilm formation, which may impede the assessment of the type 3 fimbriae-mediated biofilm-forming activity. Intriguingly, deletion of *mrkJ* resulted in a decrease of biofilm amount, which is opposite to the previous report (153). This discrepancy may be due to different *K.*

pneumoniae strains (CG43 and IApc35) and/or culturing conditions used in the experiments. Besides, the *mrkH*-deletion did not affect the bacterial biofilm-forming activity in the assay condition. These results suggested that both Fur and MrkI could regulate the type 3 fimbriae-mediated biofilm formation.

4.3.12. The expression of type 3 fimbriae under the oxygen-limiting conditions

Environmental stimulus that regulates the expression of type 3 fimbriae is yet to be identified. A previous study has demonstrated that *K. pneumoniae* grown in glycerol minimal medium possess stronger type 3 fimbrial activity than that grown in glucose minimal medium (275). It suggests that carbon sources may affect the expression of type 3 fimbriae. Thus, analysis of the type 3 fimbriae expression was performed while *K. pneumoniae* CG43S3 grown in M9 minimal medium supplemented with 0.4% glucose, glycerol, mannose, galactose, arabinose, or lactose. The bacteria were grown at 37^{o}C with agitation for 16 h and then subjected to Western blot analysis using MrkA antiserum. However, no apparent difference in MrkA production was noted (Fig. 4.15A).

Both the expression of type 1 and MR/P fimbriae have been reported to be increased during reduced oxygenation (173). The MR/P fimbriae do not express when *P. mirabilis* is grown in highly aerated broth culture, while increases in broth volume

results activation of the fimbriation (173). It is known that culturing condition with poor aeration results in a reduction of oxygen (173), and good aeration results from constant agitation and a large surface-to-volume ratio of the culture medium to the air; while poor aeration results from culturing a large volume in a small tube. To determine if oxygen level could affect the expression of type 3 fimbriae, we cultured bacteria in upright (rather than tilted) culture tubes or added a mineral oil overlay, which prevents direct oxygen exchange between broth and air. Overnight *K. pneumoniae* CG43S3 culture was 1:100 in volume subcultured into 1-, 3-, or 5-ml fresh LB broth and then subjected to incubation for another 20 h. As shown in Fig. 4.15B, the type 3 fimbriae expression was relatively low in the 1- and 3-ml cultures. In upright tube with 5-ml culture, the MrkA amount was obviously increased, and overlay with a mineral oil further increase the MrkA production. Although the oxygen level of the cultures remains to be determined, this result implies that the availability of oxygen affects the expression of type 3 fimbriae.

The reduced oxygenation effect on type 3 fimbriae expression was also analyzed in *K. pneumoniae* NTUH-K2044, and the CG43S3 mutant lacking *fur* or *mrkI* (Fig 4.15C). Compared to CG43S3, the expression level of MrkA in NTUH-K2044 was relatively low, suggesting the two clinical isolates regulate the type 3 fimbriae expression in different manners. The reduced oxygenation also caused a slight

increase of the MrkA production in NTUH-K2044. In the highly aerated culturing condition, either *mrkI*- or *fur*- deletion abolished the type 3 fimbriae expression; however the *fur*-deletion effect was partially restored upon reducing the aeration (Fig. 4.15C). These results suggest that the availability of oxygen affects type 3 fimbriae expression and also plays a role in the Fur-MrkI regulatory pathway.

4.4. Discussion

The expression of fimbrial genes are usually controlled by their adjacent genes encoding transcriptional regulators, such as PapB/I for P fimbriae (17, 94, 125, 141), FimW/Y/Z for *Salmonella* type 1 fimbriae (261), and MrpJ for MR/P fimbriae (173, 182). In *K. pneumoniae* CG43, deletion of *mrkI*, located downstream to the type 3 fimbrial genes *mrkABCDF*, resulted in a significant decrease of MrkA production (Fig. 4.3). The promoter-reporter assay also showed that the *mrkA* promoter activity was decreased by the *mrkI*-deletion (Fig. 4.5), suggesting that MrkI positively regulates the expression of type 3 fimbriae at transcriptional level.

Interestingly, a slight increase of type 1 fimbriae expression was also found in the Δ*mrkI* strain, which is assessed by mannose-sensitive yeast agglutination, Western blot analysis against FimA antiserum, and orientation analysis of the *fim* switch (Wei-Yun, Cheng, unpublished data). In addition, the deletion of *mrkA* caused an increase of type 1 fimbriae expression by an unknown mechanism (273). Whether MrkI plays a regulatory role in this counter-expression remains to be investigated. Besides, a Western blot analysis using FimA antiserum revealed that the deletion of *fur* from *K. pneumoniae* CG43S3 has no apparent effect on type 1 fimbriae expression.

Sequence analysis of MrkI revealed a LuxR-type transcriptional factor with an N-terminal regulatory domain and a C-terminal DNA-binding domain. Activation of the LuxR type regulator could be achieved by one of four mechanisms: (i) two-component system regulators that activated by phosphorylation on an aspartate residue (28, 202); (ii) regulators which are activated, or in very rare cases repressed, when bound to quorum-sensing molecules such as N-acyl homoserine lactones (235); (iii) autonomous effecter domain regulators, without a regulatory domain (77); (iiii) Multiple ligand-binding regulators (270). The sequence alignment and point mutation study showed that MrkI may be activated via phosphorylation at its D56 residue for the expression of the type 3 fimbriae (Fig. 4.6). In order to carry out an electrophoresis mobility shift assay (EMSA), the recombinant MrkI fused with MBP-tag has been constructed to resolve the problem of the aggregates resulted from the recombinant MrkI proteins fused with 6xHis-tag or GST-tag. The current data provided by Dr. Ching-Ting Lin (School of Chinese Medicine, China Medical University) indicated that this recombinant protein was able to bind P_{mrkA} only after the addition of the phosphodonor acetyl-phosphate in the reaction mixture. These results suggest that the phosphorylated MrkI directly regulates the expression of type 3 fimbriae. MrkI appeared to be an orphan regulator since no sensor kinase encoding gene could be found in the adjacent region. The MrkI cognate sensor remains to be

identified.

As shown in Fig. 4.2, the RT-PCR analysis revealed that *mrkHIJ* could be transcribed in a polycistronic mRNA. However, the deletion of *mrkI* or *mrkJ* resulted in an opposite effect on MrkA production (Fig. 4.3). This may result from additionally different regulation of *mrkJ* transcription by another promoter located in the 143-bp intergenic region between *mrkI* and *mrkJ*. The deletion of *mrkH* did not affect the type 3 fimbriae expression (Fig. 4.3); however, the overproduction of MrkH increased the amount of MrkA (Fig. 4.8). This suggested that, in particular growth conditions, MrkH also plays a role in the regulation of expression of type 3 fimbriae. An optimal growth condition for assessing the *mrkH*-deletion effect remains to be shown. As shown in Fig. 4.7 and Fig. 4.8, the R111 residue, the N-terminus of MrkH, and the C-terminal PilZ domain are all required for the MrkH-activated type 3 fimbriae expression.

The quantitative real-time-PCR (qRT-PCR) analysis indicated that the induced expression of MrkH significantly activated the mRNA level of *mrkA* (Dr. Ching-Ting Lin, unpublished data), suggesting that MrkH activates the expression at transcriptional level. The PilZ domain proteins have been reported to bind to c-di-GMP and then exert their function by protein-protein interaction (34, 122). In

Xanthomonas, the binding between PilZ domain proteins to an ATPase (PilB) and an EAL domain protein (FimX) regulate the type IV pilus biosynthesis (115). If protein-protein interactions occur between MrkH, MrkI, and MrkJ for the regulation of type 3 fimbriae expression awaits to be studied.

As shown in Fig. 4.9C and Fig. 4.10, the promoter activity of *mrkA* and *mrkH* were decreased by the *fur*-deletion. To ascertain if Fur could directly interact with the promoter regions of *mrkA* and *mrkH*, an EMSA has been performed by Dr. Ching-Ting Lin. The purified recombinant Fur protein was found to be able to bind to P_{mrkH} but not to P_{mrkA}, suggesting a direct Fur regulation on the *mrkHIJ* operon. Besides, the qRT-PCR analysis showed the expression of *mrkA*, *mrkH*, and *mrkI*, but not *mrkJ*, were reduced by the *fur*-deletion, and the deletion effects could be complemented by introducing a *fur*-expressing plasmid into the Δ*fur* strain (Dr. Ching-Ting Lin, unpublished data). These findings suggest that Fur indirectly regulates the type 3 fimbriae expression via MrkI.

We have previously demonstrated that the CPS biosynthesis of *K. pneumoniae* CG43S3 is regulated in coordination by multiple regulators RcsB, RcsA, RmpA, RmpA2, and Fur (53, 171, 183-185). Since Fur is also an activator for the expression of type 3 fimbriae, a cross-regulation by Fur on the expression of CPS and type 3

fimbriae could be predicted. To further indentify regulators involved in this cross-regulation, Western blot analysis using MrkA antiserum was performed among the individual strains lacking CPS regulatory genes including *rcsB*, *rcsA*, *kvhA*, *kvgA*, *rmpA*, *rmpA2*, or *rpoS*. However, only a slight decrease (approximate 43%) of MrkA production was found in the Δ*rcsB* strain (Fig. 4.16). Whether RcsB is involved in the regulation of type 3 fimbriae expression remains to be studied. Besides, the CPS biosynthesis was not affected by the *mrkI*-deletion (data not shown).

Fur has been implicated in iron uptake and metabolism, oxidative stress response, colonization, and virulence in many bacteria (41). Although Fur is predicted to exert similar function in *K. pneumoniae*, we have shown that, besides iron-uptake systems (185), Fur also participates in the regulation of CPS biosynthesis (53, 185) and type 3 fimbriae expression. Since iron-uptake systems, CPS, and type 3 fimbriae are well-known bacterial virulence factors, our findings suggest that Fur plays an important role in *K. pneumoniae* pathogenicity.

Iron availability influences the activity of the Fur protein as well as the transcription of *fur*. Fe^{2+}-Fur is an autorepressor, reducing *fur* expression in response to iron (68, 69, 124, 262). In *E. coli*, the expression of *fur* has been demonstrated to be modulated by many regulators including OxyR (309), SoxS (335), CRP (68), RstAB

(152), and ArcA (35). Transcription of *fur* is also activated in response to an elevated cellular c-di-GMP level (209). Since Fur acts as an activator for type 3 fimbriae in *K. pneumoniae*, regulators that modulate the expression of *fur* may subsequently affect the type 3 fimbriae expression. However, no obvious effect on the type 3 fimbriae expression was found for the *K. pneumoniae* strains lacking *rstA*, *rstB*, or *soxRS*.

Artificial manipulation of the cellular c-di-GMP content by the overproduction of GGDEF domain proteins has been reported to strongly stimulated the synthesis of adhesins and biofilm matrix components, whereas overproduction of EAL domain proteins produced the opposite phenotypes (64, 76, 122, 151, 250, 256, 257, 293, 322). We have also found that the induced-expression of YdeH c-di-GMP cyclase in *K. pneumoniae* CG43S3 activated the expression of type 3 fimbriae (Fig. 4.12). The qRT-PCR analyses also showed that the mRNA level of *fur*, *mrkA*, *mrkH*, *mrkI*, and *mrkJ* were significantly increased upon the induced expression of YdeH (Dr. Ching-Ting Lin, unpublished data). In addition, overexpression of MrkH, the PilZ domain protein, affects the type 3 fimbriae expression. These suggest c-di-GMP is involved in the regulation of type 3 fimbriae expression. Nevertheless, the exact mechanism awaits investigation.

By analyzing the genome sequence of *K. pneumoniae* NTUH-K2044, ORFs

encoding 11 GGDEF, 10 EAL, and 5 GGDEF-EAL domain proteins were found (Fig. 1.1). To our knowledge, most of these proteins have not been studied in *K. pneumoniae* yet, except MrkJ (153), FimK (290), YjcC (170), and BlrP1 (22), which are EAL domain proteins. A slight increase of MrkA production was found in the Δ*mrkJ* strain (Fig. 4.3), and the deletion of *fimK* has been shown to activate the expression of type 1 fimbriae but not type 3 fimbriae (290). The MrkA amount was obviously increased in the *K. pneumoniae* CG43S3 Δ*yjcC* strain which is highly susceptible to oxidative stress (Jing-Rou Hwang, unpublished data). BlrP1 is a light-regulated PDE, and its crystal structure complexed with c-di-GMP has been determined (22); however its biological role is unknown. Although the PDE activities of FimK and YjcC have not been determined yet, these EAL domain proteins seem to play differential roles in fimbriae expressions in *K. pneumoniae*.

Since oxidative stress has been shown to affect the expression of *yjcC* and *fur* (41, 170, 309), the type 3 fimbriae expression may also be regulated. This is supported by the study that *K. pneumoniae* OxyR, a central regulator for oxidative stress response, affected the bacterial colonization (123). As shown in Fig 4.11 and 4.15B, availability of iron and oxygen were found to activate the expression of type 3 fimbriae. To our knowledge, this is the first study to show environmental stimuli for type 3 fimbriae expression. Deletion of *fur* abolished the MrkA production regardless

of the iron availability (Fig 4.11). However, the *fur*-deletion effect on MrkA production was restored in an oxygen-limiting condition (Fig. 4.15C). Expression of *fur* has been shown to be controlled by ArcA (35), the primary regulator for the transition to anaerobiosis (109). Whether ArcA involves in the regulation of type 3 fimbriae expression remains to be studied. qRT-PCR analysis showed that, under highly aerated culturing condition, the mRNA level of *mrkI* was dramatically reduced in *fur*-deletion mutant; however this deletion effect was no longer observed in oxygen-limiting conditions (Dr. Ching-Ting Lin, unpublished data). This result suggested that anaerobic regulator such as ArcA and FNR may activate the expression of *mrkI* in oxygen-limiting conditions, and the possibility remains to be studied.

In summary, a model depicted as shown in Fig 4.17 is concluded. In *K. pneumoniae* CG43, the response regulator MrkI directly activates the expression of type 3 fimbriae upon phosphorylation of its D56 residue and auto-activates the expression of the *mrkHIJ* opeorn. Expression of MrkH increases the type 3 fimbriae expression through unknown mechanism, while MrkJ decreases the cellular level of c-di-GMP to repress the expression of type 3 fimbriae. The ferric uptake regulator Fur acts as an activator for the type 3 fimbriae expression through indirect activation of the expression of *mrkI*. The second messenger c-di-GMP activates the expression of *fur*, the *mrkHIJ* operon, and the type 3 fimbrial genes. The expression of type 3

fimbriae is activated in oxygen-limiting conditions, and an unknown regulator may activate the expression of *mrkI* to modulate the type 3 fimbriae expression during reduced oxygenation.

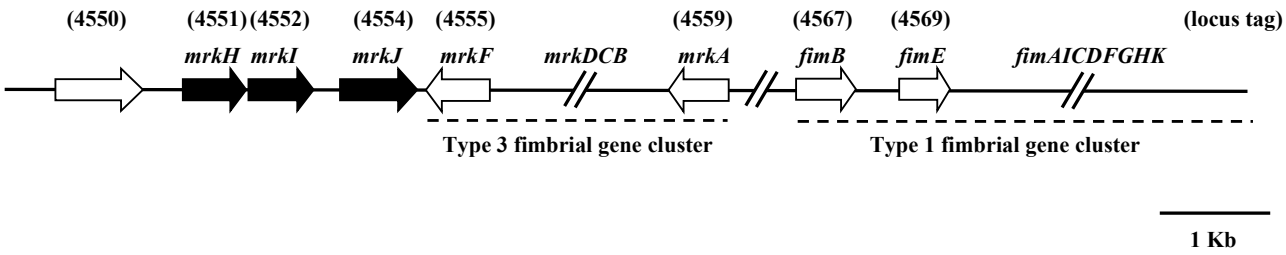


Fig. 4.1. Schematic gene organization of a chromosomal region encoding *K. pneumoniae* type 3 and type 1 fimbriae. The designation and the locus tag (KP1_number) of the ORFs are indicated. The three ORFs encoding putative regulatory proteins are shown in black.

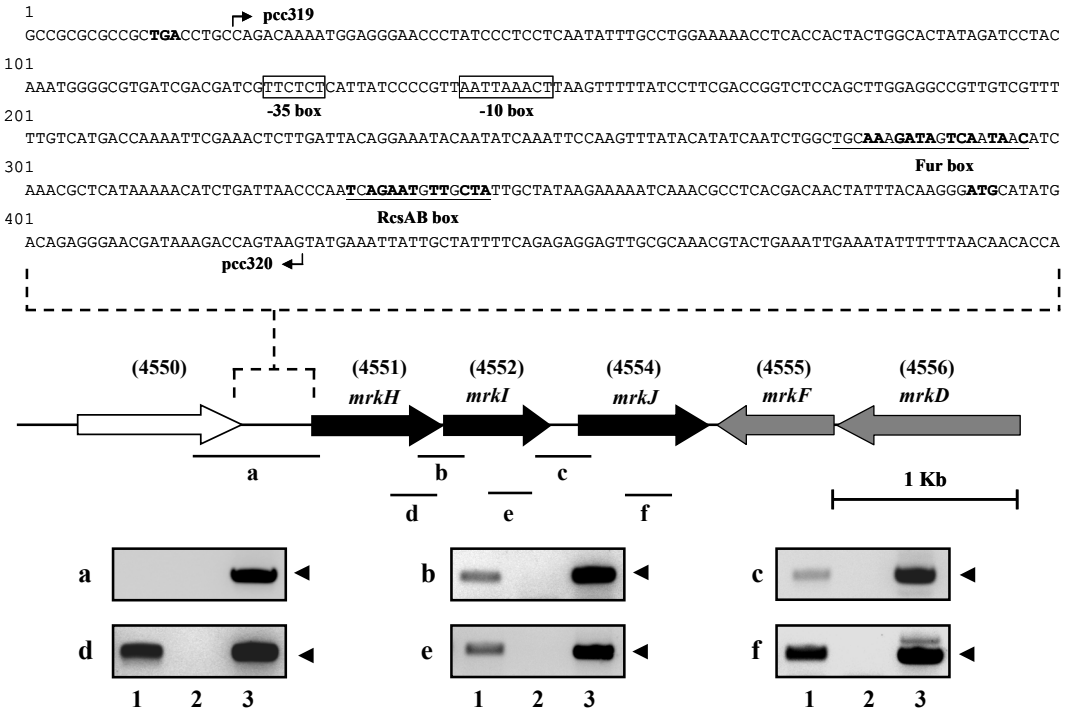


Fig. 4.2. The transcription units of *mrkH*, *mrkI*, and *mrkJ* defined by RT-PCR. The genetic organization of the downstream genes of the type 3 fimbrial gene cluster (*mrkABCDF*, and only *mrkD* and *mrkF* are shown as gray arrows) in *K. pneumoniae* is shown. The designation and the locus tag (KP1_number) of the ORFs are indicated. The three ORFs encoding putative regulatory proteins are shown in black. The upper panel of the figure shows the DNA sequence upstream of the *mrkH* gene. The start codon of *mrkH* and the stop codon of *KP4550* are shown in bold. The predicted -10, -35, and transcriptional factor binding boxes are indicated. Primers pcc319 and pcc320 used for the promoter-reporter construct are indicated by vertical arrows. The lower part of the figure shows the RT-PCR results by ethidium bromide–stained agarose gel. Panels a–f show the corresponding PCR products for primers located at ORF or the junction between ORFs. Lanes 1, RT-PCR products; 2, RT-PCR without reverse transcriptase, as a negative control; 3, PCR with genomic DNA as a template, as a positive control. Arrowheads indicate the expected sizes of RT-PCR products.

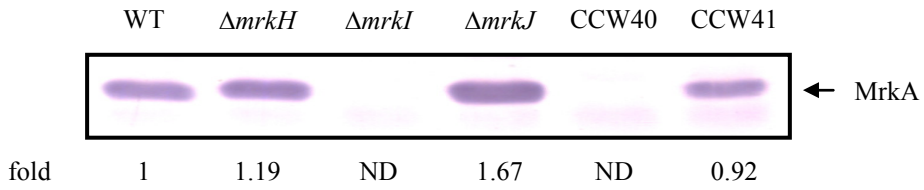


Fig. 4.3. Deletion of *mrkI* decreases the expressions of type 3 fimbriae. *K. pnuemoniae* CG43S3 (WT, wild-type), its isogenic gene-deletion strains (Δ*mrkH*, Δ*mrkI*, and Δ*mrkJ*), and the *mrkI*-complement strain CCW41 as well as the control strain CCW40 were grown overnight at 37°C with agitation in LB broth. Bacterial total protein, approximately five micrograms per lane, was separated by SDS-PAGE and then subjected to Western blot analysis using MrkA antiserum. The MrkA protein is indicated by an arrow. The fold change of MrkA amount calculated by ImageJ software is also shown. ND, not determined.

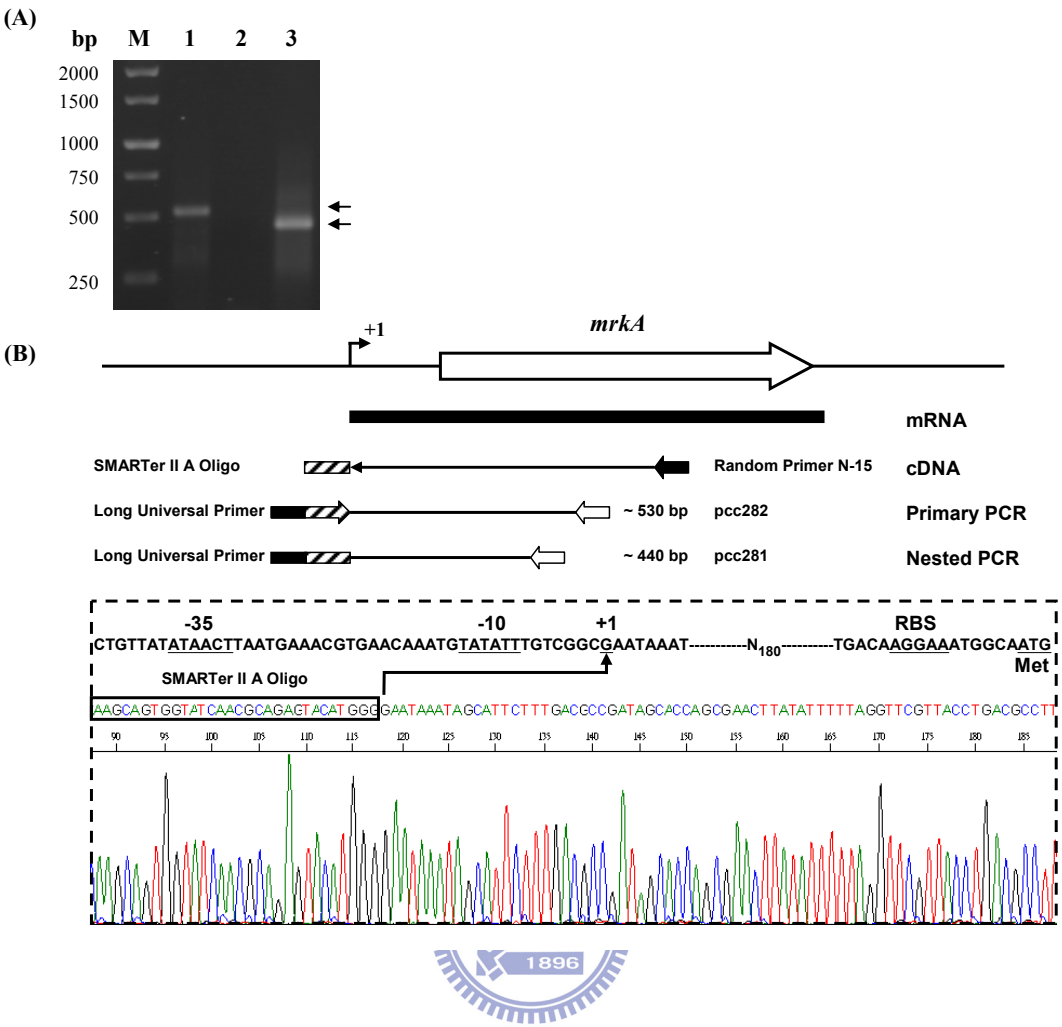


Fig. 4.4. Identification of *mrkA* transcription start site by 5'-RACE. (A) Electrophoresis of the 5'-RACE PCR products. M, DNA molecular size markers. The templates used in each PCR reaction include the cDNA from *K. pneumoniae* CG43S3 (Primary PCR) (lane 1), reverse transcription reaction mixture without transcriptase as a negative control (lane 2), or one hundred-fold diluted primary PCR mixture (Nested PCR) (lane 3). The arrows indicate the expected sizes of the PCR products. (B) Schematic representation of the *mrkA* loci and the 5'-RACE experimental design. The large arrow represents MrkA open reading frame. Relative position of the primers and expected sizes of the products in Primary and Nested PCR are indicated. The *mrkA* transcriptional start site is marked as +1. The potential -10, -35, ribosomal binding site, and the translational start site are underlined.

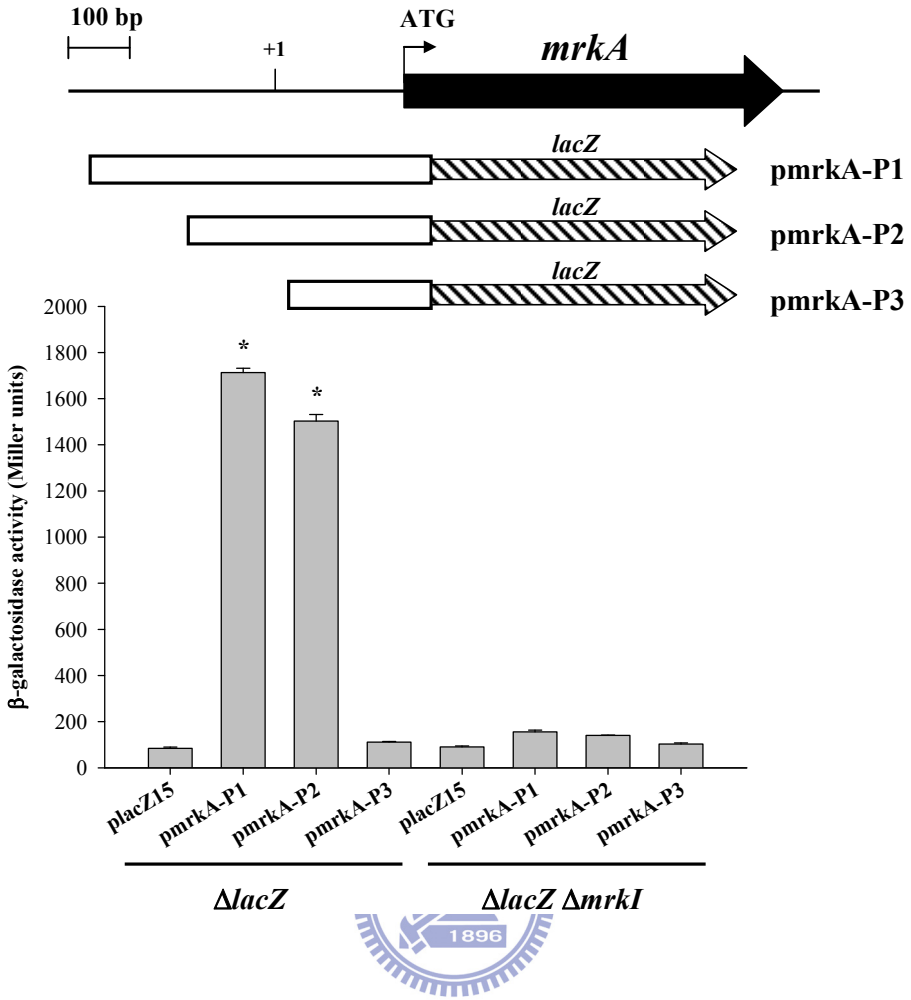
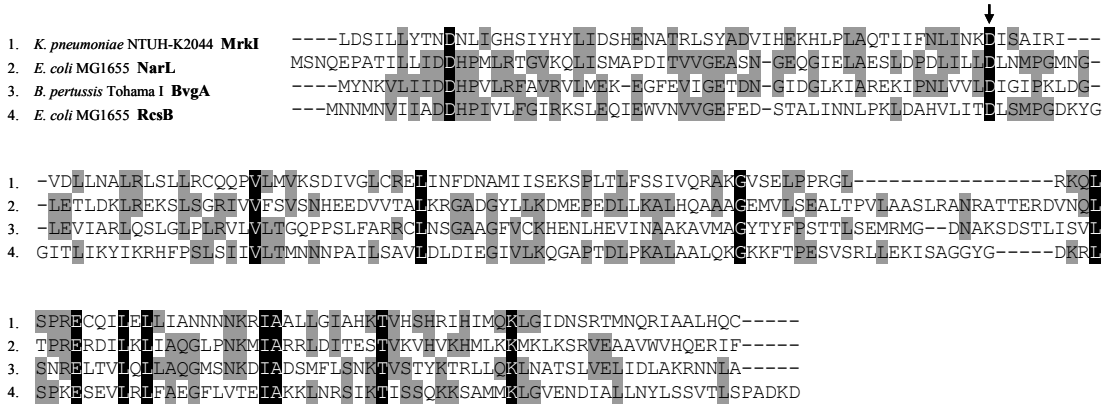


Fig. 4.5. Deletion of *mrkI* decreased the transcription of *mrkA*. The β-galactosidase activities of *K. pneumoniae* CG43S3Δ*lacZ* (Δ*lacZ*) and its isogenic *mrkI* deletion mutant (Δ*lacZ* Δ*mrkI*) carrying each of the reporter plasmids pmrkA-P1, pmrkA-P2, or pmrkA-P3 were determined from log-phased cultures grown in LB broth. The results are shown as average of the triplicate samples. Error bars indicate standard deviations. *, $P < 0.0001$ compared with Δ*lacZ* [placZ15].

(A)



(B)

Fig. 4.6. MrkI is probably a response regulator activated by phosphorylation. (A) Sequences of MrkI and LuxR-type transcriptional regulators NarL, BvgA, and RcsB were aligned by Vector NTI software. The conserved aspartate (D56) residue of MrkI as a putative target site for phosphorylation is indicated by an arrow. (B) D56 is important for MrkI functionality. *K. pneumoniae* CG43S3 (WT, wild-type), the Δ*mrkI* strain, and the mutant strains expressing $MrkI_{D56E}$ (D56E) or $MrkI_{D56A}$ (D56A) were grown overnight at 37°C with agitation in LB broth. Bacterial total protein, approximately five micrograms per lane, was separated by SDS-PAGE and then subjected to Western blot analysis using MrkA antiserum. The MrkA protein is indicated by an arrow.

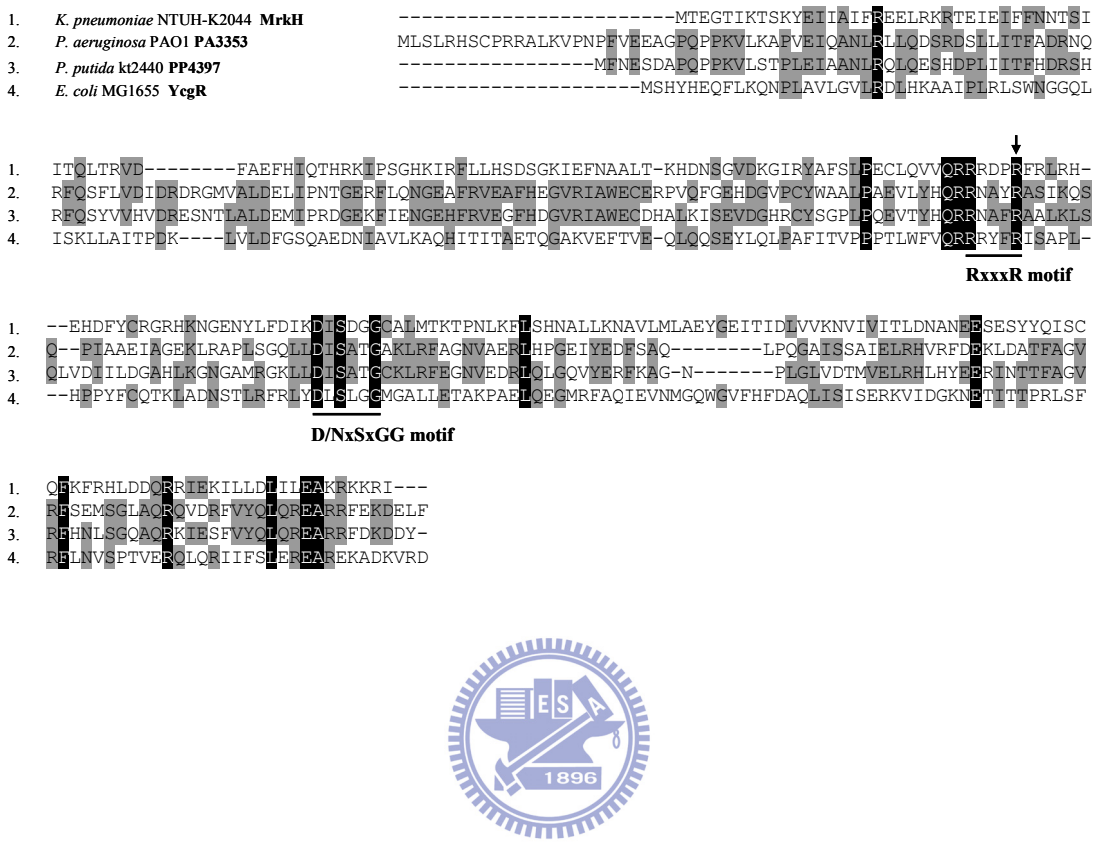


Fig. 4.7. Amino acid sequence alignment of PilZ domain proteins. Sequences of the PilZ domain proteins, including MrkH, PA3353, PP4397, and YcgR (27, 258), were aligned by the Vector NTI software. The conserved RxxxR motif and the D/NxSxGG motif are underlined (x, any residue). The critical lysine residue involving in c-di-GMP binding activity of YcgR (258) is indicated by an arrow.

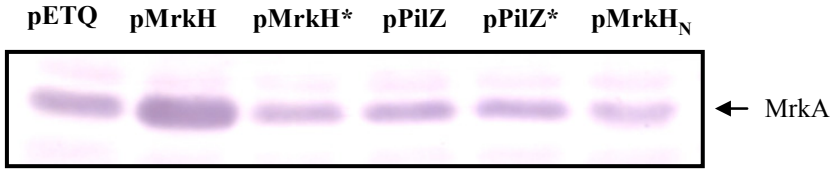


Fig. 4.8. MrkH-mediated activation of type 3 fimbriae expression. *K. pneumoniae* CG43S3 carrying expression plasmids, as shown in the upper panel, were grown in LB broth at 37^{o}C with agitation. When the bacterial growth reached mid-log phase, expression of the recombinant protein was induced by addition of 0.5 mM IPTG, and then subject to additional 3 h incubation. Bacterial total protein, approximately five micrograms per lane, was separated by SDS-PAGE and then subjected to Western blot analysis using MrkA antiserum. The MrkA protein is indicated by an arrow. pETQ, the vector only control.

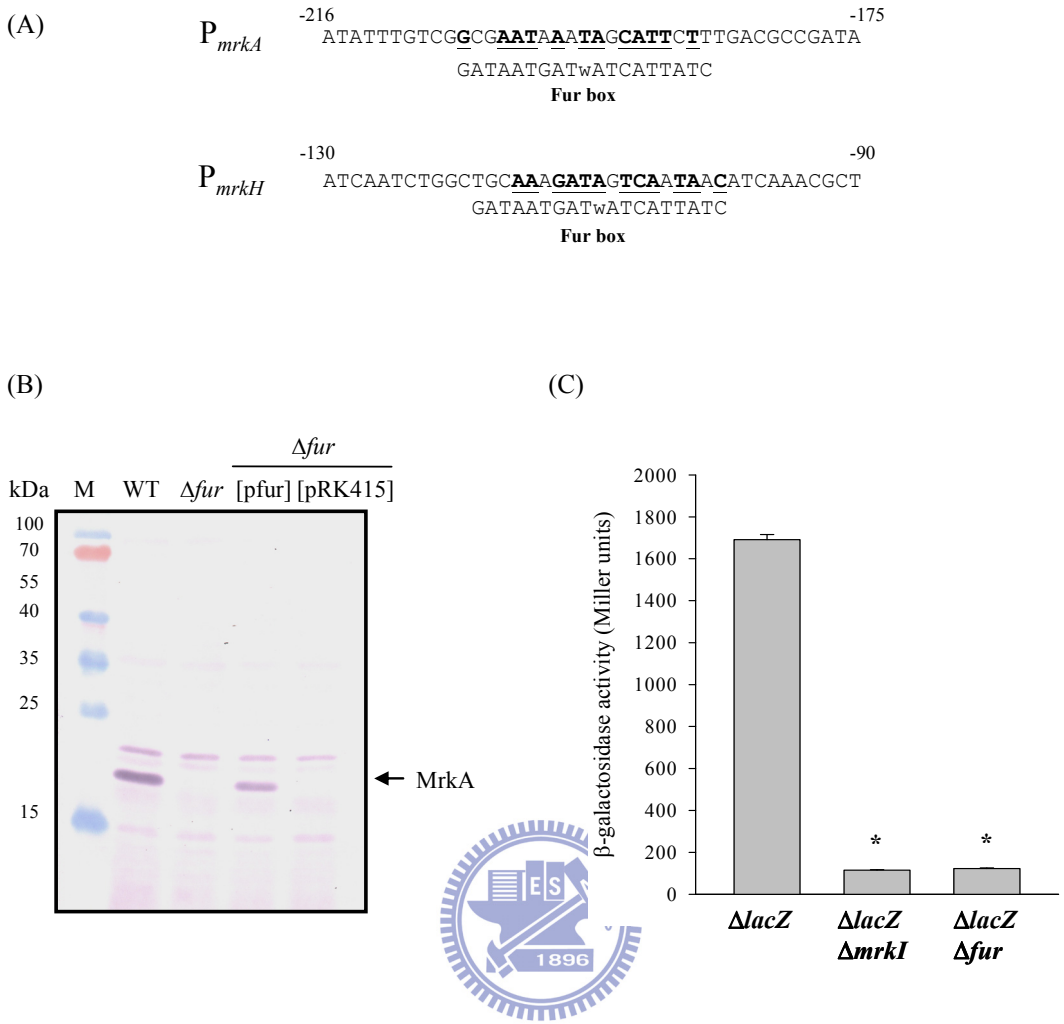


Fig. 4.9. Deletion of *fur* repressed the expression of type 3 fimbriae. (A) The predicted Fur-binding sequences on the promoter regions of *mrkA* and *mrkH*. The alignment with the 19-bp Fur box (w = A or T) is shown. (B) Anti-MrkA Western blot analysis of the total protein, approximately five micrograms per lane, isolated from *K. pneumoniae* CG43S3 strains. WT, wild-type. M, protein molecular size markers. The MrkA protein is indicated by an arrow. (C) Assessment of *mrkA* transcription using a promoter-reporter system. The β-galactosidase activities of *K. pneumoniae* CG43S3Δ*lacZ* and its isogenic deletion mutants (Δ*mrkI* and Δ*fur*) respectively carrying the reporter plasmid pmrkA-P2 were determined from log-phased cultures grown in LB broth. The results are shown as average of the triplicate samples. Error bars indicate standard deviations. *, $P < 0.0001$ compared with Δ*lacZ* [pmrkA-P2].

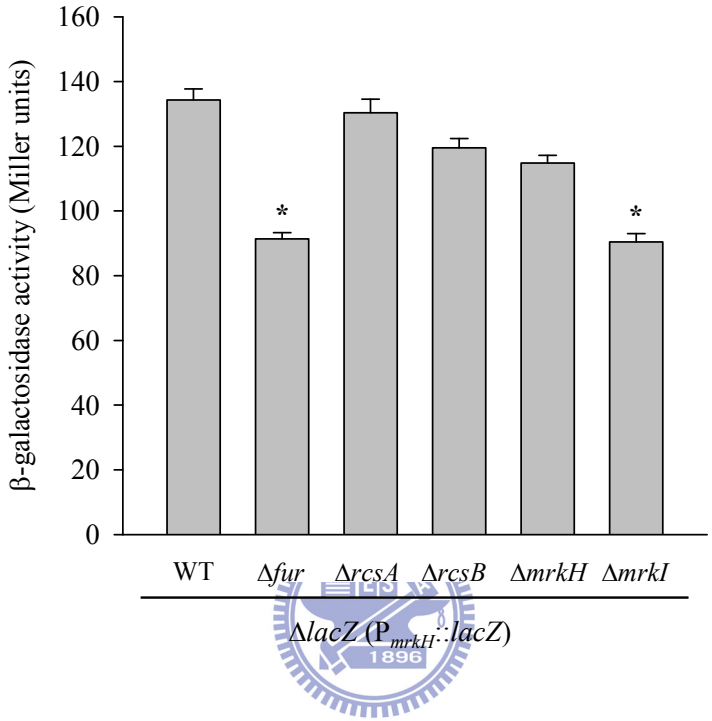


Fig. 4.10. The promoter activity of the upstream region of *mrkH* was regulated by Fur and MrkI. The β-galactosidase activities of *K. pneumoniae* CG43S3 Δ*lacZ* or its isogenic deletion strains respectively lacking *fur*, *rcsA*, *rcsB*, *mrkH*, and *mrkI* carrying pAW175 (P_{mrkH}::*lacZ*), were determined from log-phased cultures grown in LB broth. The results are shown as average of the triplicate samples. Error bars indicate standard deviations. *, $P < 0.0001$ compared with Δ*lacZ* [P_{mrkH}::*lacZ*].

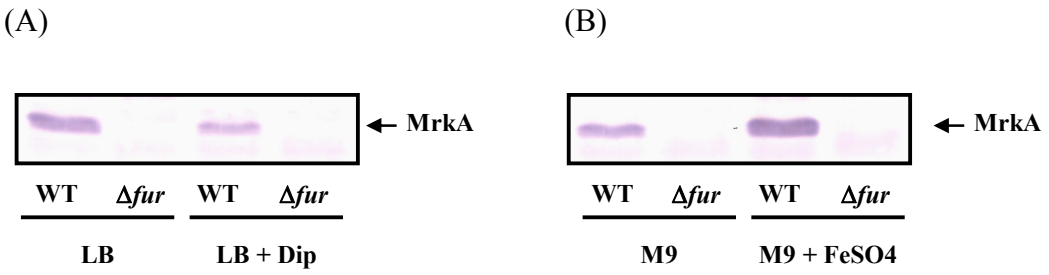


Fig. 4.11. Extracellular iron availability affected the expression of type 3 fimbriae. Fifty-microliter of overnight-grown *K. pneumoniae* CG43S3 wild-type and Δ*fur* cultures were added into 4-ml fresh LB (A) or M9 medium (B) supplemented with 200 μM 2, 2'- dipyridyl (Dip) or 60 mM $FeSO_4$ as indicated in the l panel. Then, the sub-cultured bacteria were grown overnight at 37°C with agitation, and the bacterial total protein, approximately five micrograms per lane, was separated by SDS-PAGE and then subjected to anti-MrkA Western blot analysis. The MrkA protein is indicated by an arrow.

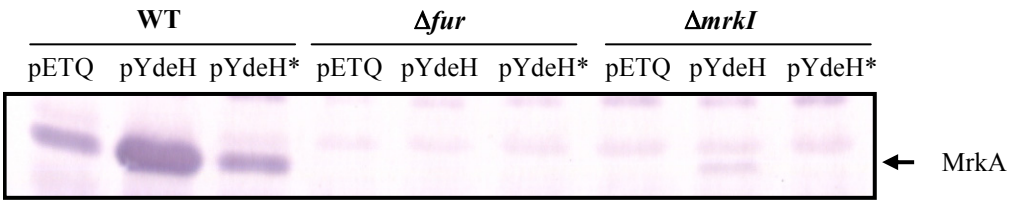


Fig. 4.12. Fur and MrkI were required for the c-di-GMP-activated type 3 fimbriae expression upon YdeH overproduction. Expression plasmids pETQ, pYdeH, or pYdeH* (the YdeH-AADEF mutant) were respectively introduced into *K. pneumoniae* CG43S3 (WT) and its isogenic mutant strains lacking *fur* or *mrkI*, as shown in the upper panel. The bacteria were grown in LB broth at 37^{o}C with agitation. When the bacterial growth reached mid-log phase, expression of the recombinant protein was induced by addition of 0.5 mM IPTG, and then subject to additional 3 h incubation. Bacterail total protein, approximately five micrograms per lane, was separated by SDS-PAGE and then subjected to Western blot analysis using MrkA antiserum. The MrkA protein is indicated by an arrow.

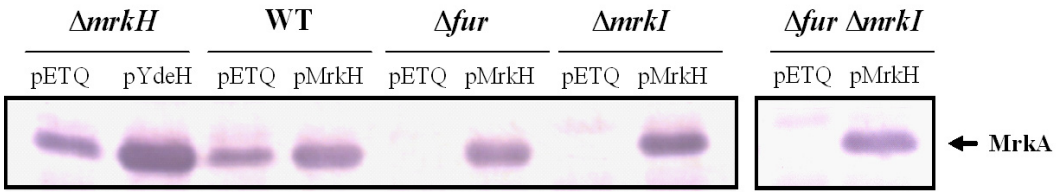


Fig. 4.13. Effects of *mrkH* deletion and overexpression on type 3 fimbriae expression. *K. pneumoniae* CG43S3 (WT) and its isogenic mutant strains lacking *mrkH*, *fur*, or *mrkI* were respectively transformed by expression plasmids pETQ, pYdeH, or pMrkH, as shown in the upper panel. The bacteria were grown in LB broth at 37°C with agitation. When the bacterial growth reached mid-log phase, expression of the recombinant protein was induced by addition of 0.5 mM IPTG, and then subject to additional 3 h incubation. Bacterial total protein, approximately five micrograms per lane, were separated by SDS-PAGE and then subjected to Western blot analysis using MrkA antiserum. The MrkA protein is indicated by an arrow.

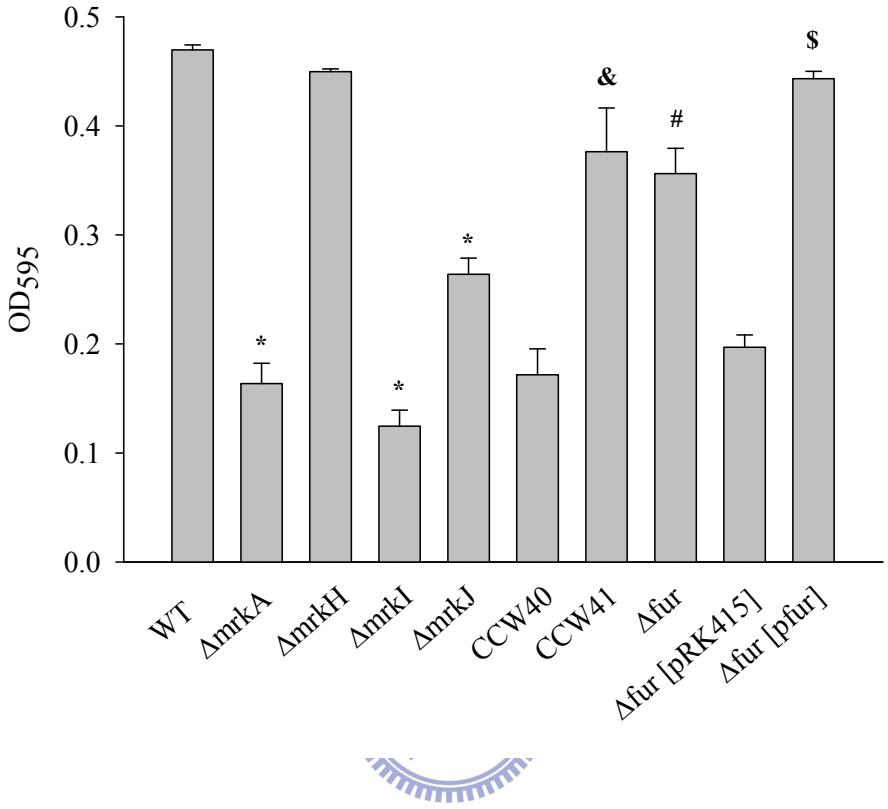


Fig. 4.14. Knockout of *fur*, *mrkI*, and *mrkJ* decreases *K. pneumoniae* biofilm formation. Overnight cultured *K. pneumoniae* strains, as shown in the lower panel (WT, the wild-type strain), were 100-fold diluted and inoculated into a 96-well microtiter dish. After 48 h static incubation at 37°C, the bacterial biofilm formation was quantified by crystal violet staining. The results are shown as average of the triplicate samples. Error bars indicate standard deviations. *, $P < 0.0001$ compared with the WT strain. #, $P < 0.005$ compared with the WT strain. &, $P < 0.005$ compared with the CCW40 strain. $, $P < 0.0001$ compared with the Δ*fur* [pRK415] strain.

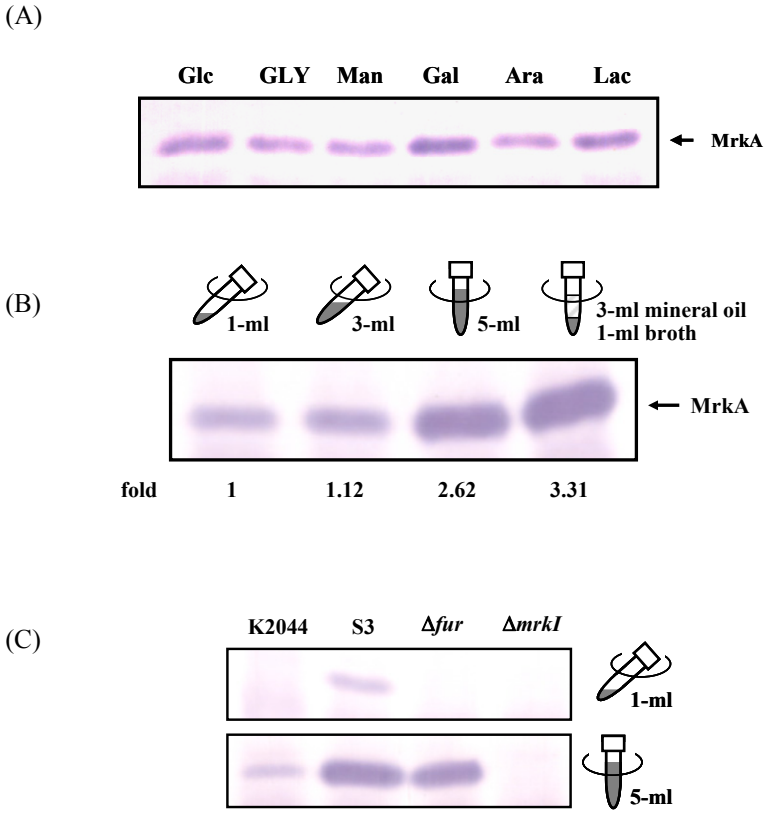


Fig. 4.15. Correlation between type 3 fimbriae expression and oxygenation. Bacteria were incubated for 20 h at 37°C with constant agitation (200 rpm), and the bacterial total protein, approximately five micrograms per lane, was then subject to Western blot analysis using MrkA antiserum. (A) Carbon sources did not cause apparent effect on MrkA production. *K. pneumonia*e CG43S3 was grown in M9 minimal medium supplemented with 0.4% glucose (Glc), glycerol (GLY), mannose (Man), galactose (Gal), arabinose (Ara), or lactose (Lac). (B) *K. pneumonia*e CG43S3 was incubated as 1-ml, 3-ml, or 5-ml cultures in 10-ml tubes as depicted in the diagram. Tubes were placed either tilted (45°) or upright in the incubator. As indicated, 1-ml cultures may overlay with 3-ml mineral oil to further reduce oxygen in the broth. The fold change of MrkA amount calculated by ImageJ software is shown in the lower panel. (C) Four *K. pneumoniae* strains including NTUH-K2044 (K2044), CG43S3 (S3), and CG43S3 isogenic mutants (Δ*fur* and Δ*mrkI*) were grown, as described above, and the two growth conditions used are depicted in the diagram.

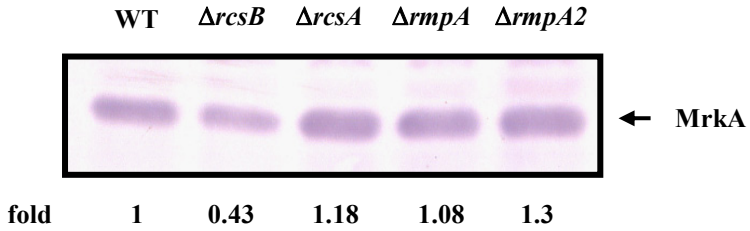


Fig. 4.16. Deletion of *rcsB* slightly decreased the expression of type 3 fimbriae. *K. pnuemoniae* CG43S3 (WT, wild-type) and its isogenic gene-deletion strains (Δ*rcsB*, Δ*rcsA*, and Δ*rmpA* and Δ*rmpA2*) were grown overnight at 37°C with agitation in LB broth. Bacterial total protein, approximately five micrograms per lane, were separated by SDS-PAGE and then subjected to Western blot analysis using MrkA antiserum. The MrkA protein is indicated by an arrow. The fold change of MrkA amount calculated by ImageJ software is also shown.

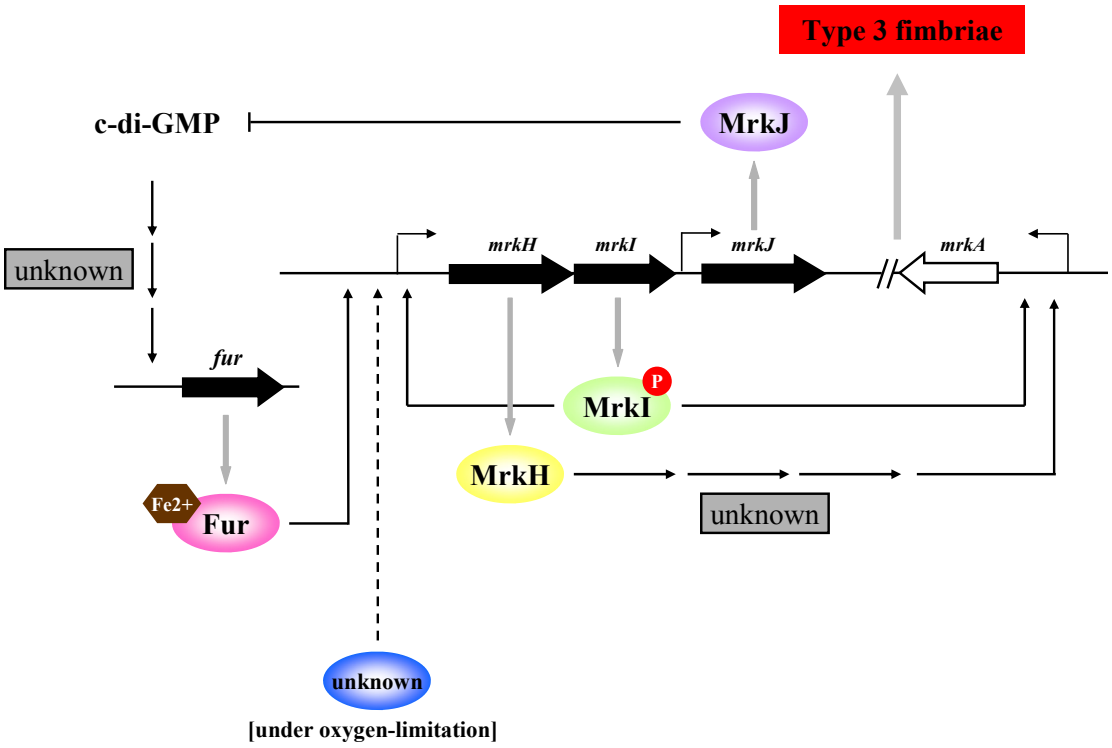


Fig. 4.17. A model illustrating the regulation of the expression of type 3 fimbriae mediated by Fur, MrkH, MrkI, and MrkJ in *K. pneumoniae* CG43. Increased cellular level of the second messenger c-di-GMP activates the expression of *fur* through an unknown mechanism. Fur binds Fe^{2+} and then interacts with P_{mrkH} to activate the expression of *mrkH* and *mrkI*, but not *mrkJ*. The production of MrkH activates the type 3 fimbriae expression at the transcriptional level, however the mechanism remains unclear. Expression of the response regulator MrkI, which is subsequently phosphorylated, directly activates the expression of the type 3 fimbrial genes, and MrkI probably auto-activates the expression of the *mrkHIJ* operon. Expression of MrkJ has been reported to decrease the cellular level of c-di-GMP to repress the type 3 fimbriae expression. In addition, an unknown regulator may increase the *mrkI* expression to activate the type 3 fimbriae expression during reduced oxygenation.

CHAPTER 5

Conclusion and Perspectives

Fimbriae are well-known adherence factors that play a crucial role in the attachment of pathogenic bacteria to host cells during infection. In this thesis, we have identified nine fimbrial gene clusters present in a *K. pneumoniae* genome through bioinformatic analysis (Chapter 2). Besides type 1 and type 3 fimbriae that have been respectively correlated with urinary tract infection and biofilm-related infection (252, 273, 290), the other seven types of fimbriae remain uncharacterized. It is speculated that these fimbriae play synergistic or differential roles in the bacterial pathogenicity and their expression are cross-regulated by a complex network. The three ORFs encoding putative transcriptional factors (KpbR, KpdR, and KpfR) are very likely involved in the regulation of Kpb, Kpd, and Kpf fimbriae expression, respectively.

The PCR analysis showed that the *kpb* and *kpc* fimbrial genes were more prevalent in the isolates of serotype K1, which regarded as a risk factor of *K. pneumoniae* liver abscess. We also demonstrated that the expression of *kpc* fimbrial genes, which resulted in fimbriation on the recombinant *E. coli*, could increase the biofilm formation. If Kpc fimbriae play a role in the pathogenic mechanism of *K. pneumoniae* liver abscess awaits investigation. Moreover, if the KpcI-mediated phase variation control of the expression of Kpc fimbriae (Chapter 3) acts as a switch under a specific liver environment would be much interest to be studied. The strain of *K. pneumoniae* which could readily express Kpc fimbriae and specific cell culture

system should allow solving the questions.

The nine fimbrial gene clusters possessed by *K. pneumoniae* NTUH-K2044 were classified into the chaperone-usher class. The ubiquitous chaperone-usher pathway fimbriae (CU fimbriae) often constitute important virulence factors in Gram-negative bacteria (323, 333), and the molecular mechanism of the assembly pathway has been extensively studied (21, 73, 146, 248, 264, 312). Based on these studies, compounds (namely pilicides), which target to CU fimbriae by interfering the interaction of chaperone/subunit complexes with usher, have been synthesized to block virulence of the fimbriate bacteria (2, 55, 241). The drugs that specifically target bacterial virulence factors, such as fimbriae, may exert less selective pressure on bacteria and hence minimize the risk for horizontal spread of drug-resistant genes (43, 59, 65).

Besides being useful as potential antibacterial agents, pilicides have been used as chemical tools to study details of pilus assembly and their role in disease processes (3, 241). Advanced techniques including atomic force microbiology (AFM) and optical tweezers have also been applied in studies including the interaction between fimbrial adhesin and its receptor, the measurement of adhesive force of adhesin, and the physical properties of fimbrial rod (10, 13, 150, 198, 199, 214, 268). We have also used AFM, optical tweezers, and *in situ* TEM techniques in the analyses of fimbrial

adherence (46), fimbriae-mediated DNA transfer (279), and bacterial surface changes (49, 188).

In Chapter 4, multi-factorial control of the expression of *K. pneumoniae* type 3 fimbriae was studied. We showed that MrkI acted as a transcriptional activator for the expression of type 3 fimbriae. The induced expression of the PilZ domain protein MrkH also increased the expression of type 3 fimbriae. By contrast, expression of the EAL domain protein MrkJ repressed the fimbriae expression possibly due to the accumulation of cellular c-di-GMP since the induced expression of c-di-GMP cyclase YdeH led to activation of the expression of type 3 fimbriae. Moreover, we showed that the c-di-GMP-mediated activation probably went through the expression of the ferric uptake regulator Fur to increase the expression of *mrkI*. Although c-di-GMP has been reported to exert regulation through RNA or protein effecters such as PilZ domain proteins, the exact mechanism of c-di-GMP-mediated expression of *fur* or type 3 fimbriae is yet to be characterized. Besides cellulose synthases, MrkH is the only PilZ domain protein found in *K. pneumoniae* NTUH-K2044 (Fig. 1.1). This suggests MrkH plays an important role in c-di-GMP signaling in *K. pneumoniae*. Intriguingly, MrkH was not required for the YdeH-activated type 3 fimbriae expression. If MrkH acts as a c-di-GMP effecter awaits further investigation. As shown in Fig. 1.1, numerous GGDEF and EAL domain proteins encoded by *K.*

pneumoniae were found. Nevertheless, most of them remain uncharacterized. If they also play a role in affecting the expression of type 3 fimbriae, type 1 fimbriae or CPS biosynthesis in *K. pneumoniae* is worth studying.

Finally, we have found that reduced oxygenation and Fe^{2+} level are probably the environmental stimuli that could activate the expression of type 3 fimbriae. To investigate the molecular mechanism for the type 3 fimbriae expression under reduced oxygenation is important since specific niches within the host can be oxygen limited. Apparently, the increasing knowledge of bacterial pathogenesis holds promise for future identification of the intervening targets.

CHAPTER 6

Experimental Section

6.1. Materials

6.1.1. Plasmids, primers, bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 6.1 and Table 6.2, and the primers used are listed in Table 6.3. *K. pneumoniae* NTUH-K2044, a highly invasive and hypermucous strain of serotype K1 (88), was provided by Dr. Jin-Town Wang, National Taiwan University Hospital. The 105 *K. pneumoniae* clinical isolates, provided by Dr. Chang-Phone Fung, were recovered from different tissue specimens of patients with a variety of infections at the Veterans General Hospital, Taipei, Taiwan, from 1991 to 1998. These strains have been identified and their serotypes determined, as previously described (100). *K. pneumoniae* CG43, a clinical isolate of serotype K2, is high virulent to mice (47). *E. coli* and *K. pneumoniae* strains were generally propagated at 37°C in Luria-Bertani (LB) broth. M9 minimal medium and tryptic soy broth (TSB) were also used. Bacterial growth was assessed by measuring the absorbance of 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). Mineral oil (M5310) was purchased from Sigma.

6.2 General Experimental Procedures

6.2.1. Bioinformatics

The 5.5 Mb *K. pneumoniae* NTUH-K2044 genome sequence (GenBank accession no. AP006725.1) has been determined and annotated (325). The fimbriae proteins were identified using the Pfam database (accession no. PF00419) and HMMER on the basis of a hidden Markov model (24, 81). The fimbrial gene clusters and the neighboring genes were analyzed by homology search using the BLAST program provided online by the National Center for Biotechnology Information.

6.2.2. PCR detection of fimbrial genes

The PCR mixture contained 20 mM Tris/HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 200 mM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP and dTTP) and 1 U recombinant Taq DNA polymerase (Violet Bioscience Inc.), along with *K. pneumoniae* genomic DNA and specific primers. The amplification cycle consisted of an initial 5 min hold at 95^{o}C, followed by 35 cycles of denaturation at 95^{o}C for 1 min, annealing at 50^{o}C for 1 min, and extension at 72^{o}C for 1.5 min, and finally an elongation step for 10 min at 72^{o}C. The amplified PCR product was then analysed by electrophoresis on a 1% agarose gel.

6.2.3. KpcA antiserum preparation

The coding region of *kpcA* was PCR amplified using primers pcc023 and pcc024 and cloned into pET30 to yield pKPCA. Plasmid pKPCA was then introduced into *E. coli* Novablue (DE3) for overexpression of the His_6::KpcA recombinant protein. The recombinant protein was expressed and purified according to the protocol in the pET manual (Novagen). KpcA antiserum was prepared by immunizing a New Zealand white rabbit with 0.5 mg of the purified His_6::KpcA recombinant protein and the immunized rabbit was exsanguinated on day 45.

6.2.4. Construction of the expression plasmid pETQ

The DNA fragment containing the T5*lac* promoter, multiple cloning sites and the *rrnB* T1 transcription terminator from pQE30 (Qiagen) was inserted into pET30a (Novagen) using restriction enzymes *Xba*I and *Xho*I. The 6 His-tag-coding sequence was subsequently removed by inverse PCR to yield plasmid pETQ.

6.2.5. Construction of fimbriae expression plasmids

The *kpcABCD* genes were PCR-amplified using primers pcc053 and pcc056, and the PCR product was cloned into the expression plasmids pET30a and pETQ to yield pKPC-7 and pKPC-36, respectively. The *kpcABC* genes were PCR-amplified using primers pcc202 and pcc223 and cloned into pETQ to yield pAW67. Genes encoding

type 1 fimbriae (*fimAICDEFGH*) were PCR-amplified using primers pcc167 and pcc169 and cloned into pETQ to yield pAW69. The DNA containing *kpcS*$_{OFF}$-*kpcABCD* was PCR-amplified by primers pcc081 and pcc056 and then cloned into yT&A (Yeastern Biotech Co.) to yield pAW73.

6.2.6. Transmission electron microscopy (TEM)

After IPTG (0.5 mM) induction of exponential-phase *E. coli* Novablue (DE3) harboring the expression plasmids pKPC-7 or pET30a for 3 h, the bacteria were collected by centrifugation and washed once with PBS. Twenty microliters of bacterial suspension (~10^8 CFU/ml) were added to formvar-coated copper grids (300 meshes) and negatively stained by 2% (w/v) phosphotungstic acid, pH 7.2. The grids were examined under a JEOL JEM 2000EXII transmission electron microscope at an operating voltage of 100 kV.

6.2.7. Immunofluorescence microscopy analysis

Induction of the *kpc* genes was carried out by adding 0.5 mM IPTG to an exponential-phase *E. coli* HB101 [pKPC-36] culture and incubating for an additional 3 h. Bacteria were collected and suspended in PBS (10^8 CFU/ml) and 10 μl of the suspension was applied to glass slide. After air-drying, 40 μl of the 1:100-diluted KpcA antiserum was added and the slide incubated at 25^{o}C for 1 h. After washing

with PBS, the slide was incubated with 40 μl 1:100 dilution of a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (Molecular Probes) in PBS for 1 h at 25°C. Finally, the slide was washed and examined by fluorescence microscopy.

6.2.8. Biofilm formation assay

Overnight grown bacteria were diluted 1:100 in LB broth supplemented with appropriate antibiotic and then inoculated into each well of a 96-well microtiter dish (Orange Scientific) for statically incubation at 37°C for 48 h. After removal of the bacteria, the plate was washed by deionized water once, and 150 μl of 1% (w/v) crystal violet was added to each well. The plate was incubated at room temperature on an orbital shaker for 30 min, and then washed three times. The dye was solubilized in 1% (w/v) SDS, and absorbance at 595 nm was determined.

6.2.9. Yeast-cell agglutination (YA)

Agglutination of yeast *Saccharomyces cerevisiae* AH109 was carried out as described (32). 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.

6.2.10. Switch orientation assay

The *kpcS* (switch region) was PCR-amplified from the tested strain using the primers pcc081 and pcc082. The amplified product was then purified and digested with *Afl*II. The restricted fragments were separated on 2% agarose gels and the pattern was visualized for the determination of orientation by staining with ethidium bromide. The DNA fragments containing $kpcS_{ON}$ and $kpcS_{OFF}$ were PCR-amplified from L-arabinose-induced *K. pneumoniae* NTUH-K2044 [$pKPCI_{196}$] using primers pcc081 and pcc082, and subsequently cloned into yT&A to yield the pKPC-ON and pKPC-OFF plasmids, respectively.

6.2.11. Construction of specific gene-deletion in *K. pneumoniae* NTUH-K2044

Specific gene deletion was individually introduced into the chromosome of *K. pneumoniae* NTUH-K2044 by allelic exchange strategy, as described above, using the suicide vector pKOV (187), which is a temperature-sensitive vector. The pKOV vector carrying two approximately 1000-bp DNA fragments flanking both sides of the deleted region was introduced into *K. pneumoniae* NTUH-K2044 by electroporation. The electroporated cells were plated on a LB plate containing 20 μg/ml chloramphenicol and then incubated at 30°C. The grown bacterial colony was inoculated into LB broth containing 20 μg/ml chloramphenicol and subjected to overnight incubation at 30°C with agitation. Overnight-grown bacteria was diluted

and plated onto a LB plate containing 20 μg/ml chloramphenicol and then incubated at 42°C. From the 42°C plate, bacterial colonies were picked for the confirmation of integration of the suicide vector using PCR. Overnight-grown bacteria with the integrated suicide vector were 1:100 diluted in LB broth supplemented with 5% sucrose (filter sterilized) for additional 12 h incubation at 30°C. The bacterial suspension was then diluted (~ 10^{-6}), and 100 μl of the suspension was spread onto an LB agar plate containing 5% sucrose. The chloramphenicol-sensitive colonies were selected, and the deletion was verified by PCR.

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

Specific gene deletion was individually introduced into the chromosome of *K. pneumoniae* CG43S3 by allelic exchange strategy (171). In brief, two approximately 1000-bp DNA fragments flanking both sides of the deleted region were cloned into the suicide vector pKAS46 (282), a suicide vector containing *rpsL*, which allows positive selection with streptomycin for vector loss. The resulting plasmid was then mobilized from *E. coli* S17-1 λ*pir* to *K. pneumoniae* CG43S3 or its derived strains by conjugation. The transconjugants, with the plasmid integrated into the chromosome via homologous recombination, were selected with kanamycin on M9 agar plates or LB agar plates containing 15 μg/ml of chlorhexidine. Overnight-grown transconjugant was collected, washed by saline once, and then 1:100 diluted in LB

broth supplemented with 500 μg/ml of streptomycin for additional 8-12 h incubation at 37°C. The bacterial suspension was then diluted (~ 10^{-6}), and 100 μl of the suspension was spread onto an LB agar plate containing 500 μg/ml of streptomycin. The streptomycin-resistant and kanamycin-sensitive colonies were selected, and the deletion was verified by PCR. The resulting *K. pneumoniae* mutants are listed Table 6.1.

To obtain the complementation plasmids, the DNA fragment containing *mrkI* and its flanking region was PCR amplified from the *K. pneumoniae* CG43 genomic DNA by primer wc07 and wc08, and the amplicon was cloned into yT&A (Yeastern Biotech). The *mrkI* coding region carried on the resulting plasmid was then removed by inverse PCR using primers wc09 and wc10. Subsequently, the DNA fragment containing the *mrkI* region or only the *mrkI*-flanking regions were subcloned into pKAS46 to yield pWY28 and pWY45, respectively. The plasmids were then individually introduced into *K. pneumoniae* CG43S3 Δ*mrkI* by conjugation. The resulting transconjugants, with pWY28 and pWY45 integrated into the chromosome via homologous recombination, were designated CCW40 and CCW41, respectively.

6.2.13. Construction of the reporter fusion plasmids and measurement of promoter activity

kpcS DNA was PCR-amplified from *K. pneumoniae* NTUH-K2044 by primers YCY001 and YCY002, and cloned in front of a promoter-less *lacZ* gene of the reporter plasmid placZ15 (156) to yield plasmids pSY003 and pSY004, containing *kpcS* without the two inverted repeats in opposite orientations. The approximately 375-bp DNA fragment containing the upstream region of *kpcA* was PCR-amplified with primers YCY001 and pcc264, from $kpcS_{ON}$ *K. pneumoniae* cells, and cloned into placZ15 to yield pAW126. The approximately 230 or 400-bp DNA fragments containing the upstream region of the *K. pneumoniae mrkABCDF* or *mrkHIJ* gene clusters were PCR-amplified with primers pcc273/pcc324 or pcc320/pcc321, respectively and cloned into placZ15 to yield pmrkA-P3 (pAW146) and pAW175. The resulting plasmids were mobilized from *E. coli* S17-1 λ*pir* to *K. pneumoniae* strains lacking *lacZ* by conjugation.

β-galactosidase activity was determined as previously described (156). In brief, overnight culture was 1:100 sub-cultured in LB broth to mid-log phase (OD600 of 0.7). Then 100 μl of the culture was mixed with 900 μl of 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 of chloroform and the mixture was shaken vigorously. After

incubation at 30°C for 10 min, the reaction was initiated by adding 200 μl of 4 mg/mL ONPG (o-nitrophenyl-β-D-galactopyranoside) (Sigma-Aldrich). Upon the appearance of yellow color, the reaction was stopped by adding 500 μl 1 M Na_2CO_3. OD_{420} was recorded and the β-galactosidase activity was expressed as Miller units (215). Each sample was assayed in triplicate, and at least three independent experiments were carried out, and the data shown were calculated from one representative experiment.

6.2.14. Identification of the operon structure by reverse-transcription PCR (RT-PCR)

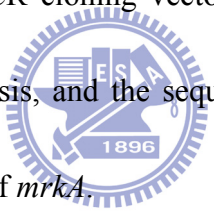
Total RNAs were isolated from early-exponential-phase grown *K. pneumoniae* CG43 cells by use of the RNeasy midi-column (QIAGEN) according to the manufacturer's instructions. RNA was DNase-treated with RNase-free DNase I (MoBioPlus) to eliminate DNA contamination. RNA of 1-μg was reverse-transcribed with the Transcriptor First Strand cDNA Synthesis Kit (Roche) using random primers in a reaction mixture of 20 μl. Reaction mixtures without reverse transcriptase were included as negative controls. The reaction mixtures were 1/50 diluted, and one-μl of each mixture was used as template for the PCR detections. PCR was carried out with initial denaturation at 95°C for 5 min, 25 cycles of denaturation at 95°C for 30 sec,

annealing at 52°C for 30 sec and elongation at 72°C for 45 sec, and a final 5 min of elongation at 72°C. The amplified products were resolved on 1% (w/v) agarose gels.

6.2.15. Identification of *mrkA* transcriptional start site

For the determination of *mrkA* 5'-mRNA ends, a 5'-RACE PCR was performed using 5'-RACE kit (Clontech) according to the manufacturer's instruction. In brief, total RNA was isolated from log-phased *K. pneumoniae* CG43S3 grown in LB medium using High Pure RNA Isolation Kit (Roche). One microgram of total RNA was treated with RNase-free DNase I (Roche) and recovered by phenol-chloroform extraction. For the first-strand cDNA synthesis, a 3.75-μl reaction mixture containing 1 μg of DNase-treated RNA and Random Primer Mix (N-15) was incubated at 72°C for 3 min and then transfered to room temperature for 5 min before the addition of 5X First-Strand buffer, deionized water, DTT, dNTP mix, RNase inhibitor, SMARTer II A oligonucleotide and SMARTScribeTM reverse transcriptase to a final volume of 10 μl. The reaction mixture was incubated at room temperature for 10 min, 42°C for 90 min and 72°C for 10 min to terminate the reaction, diluted with 20 μl Tricine-EDTA buffer and then stored at -20°C. For the primary PCR, a 50-μl reaction mixture containing diluted cDNA templates, gene-specific primer, Universal Primer Mix, dNTP mix, PCR buffer, deionized water and DNA polymerase was prepared. The

reaction mixture without reverse transcriptase served as a negative control template. The PCR program was 5 cycles of 30 s at 94°C, 3 min at 72°C, 5 cycles of 30 s at 94°C, 30 s at 70°C, 3 min at 72°C, and 25 cycles of 30 s at 94°C, 30 s at 68°C, 3 min at 72°C. For the nested PCR, the reaction mixture was essentially the same as the primary PCR mixture except 100-fold diluted primary PCR product as the template, gene-specific primer, and Nested Universal Primer were used. The PCR program was 25 cycles of 30 s at 94°C, 30 s at 60°C, 3 min at 72°C. The PCR products were resolved on an agarose gel by electrophoresis, and the DNA fragments were recovered and cloned into the PCR cloning vector yT&A. A total of seven clones were subjected to sequence analysis, and the sequencing results indicated the same residue as the transcriptional site of *mrkA*.



6.2.16. Construction and expression of the recombinant proteins

The DNA fragments containing the coding sequences of MrkH, $PilZ_{MrkH}$, $MrkH_N$ or YdeH were individually PCR-amplified with primer pair pcc212/pcc213, pcc335/pcc213, pcc212/pcc336 or pcc216/pcc217, and cloned into the expression vector pETQ to generate pMrkH, pPilZ, $pMrkH_N$ and pYdeH, respectively. The plasmids with site-directed mutations were constructed by either quick change or by inverse-PCR method. In quick change strategy, pYdeH was used as the template for

PCR-amplification with the complementary primer sets pcc224/pcc225 encompassing the mutation site by using Phusion DNA polymerase (Finnzymes). The PCR product was resolved on agarose gel, recovered, treated with *Dpn*I for 2 h to remove the template plasmid and transformed into *E. coli* JM109. The plasmid, pYdeH*, carrying the mutation allele encoding $YdeH_{AADEF}$ (YdeH*, G206A and G207A mutations) was then prepared from the transformant colony and confirmed by sequence analysis. For the inverse-PCR method, pMrkH was used as the PCR template to generate the mutant allele encoding $MrkH_{R111D}$ (MrkH*) recombinant proteins with primer pairs pcc335/pcc213. The PCR product was resolved on agarose gel, recovered, treated with *Dpn*I for 2 h, and subjected to T4 PNK treatment and self-ligation. The ligation product was transformed into *E. coli* JM109. The plasmid, pMrkH*, carrying the mutant allele encoding MrkH with the R111D mutation (MrkH*) was prepared from the transformant colony and confirmed by sequence analysis. The DNA fragment encoding $PilZ_{MrkH}$ with the R111D mutation (PilZ*) was PCR-amplified with primer pairs pcc335/pcc213, using pMrkH* as template, and then cloned into pETQ to yield pPilZ*.

6.2.17. Construction of the site-directed mutants derived from *K. pneumoniae* CG43S3

The DNA fragment carrying *mrkI* and its approximately 1-kb adjacent regions was PCR-amplified by primer pairs wc07/wc08 and cloned into yT&A. The resulting plasmid was used as template for the inverse-PCR with primer pair pcc337/pcc338 or pcc337/pcc339 to generate mutant alleles of *mrkI* with D56E or E56A mutations. The inverse-PCR strategy was performed as described above. Subsequently, the DNA fragments containing the D56E and D56A mutant alleles of *mrkI* were subcloned into pKAS46 to yield pAW197 and pAW198, respectively. pAW197 and pAW198 were then individually mobilized from *E. coli* S17-1 λ*pir* to the *K. pneumoniae* CG43S3 DmrkI strain by conjugation, and the subsequent selection was performed as described above. Each site-directed mutation in *K. pneumoniae* was confirmed by DNA sequencing.

6.2.18. Statistical methods

The results of the biofilm-forming activity and β-galactosidase activity assays were derived from a single experiment that was representative of three independent experiments. Each sample was assayed in triplicate and the data were presented as the mean ± standard deviation (SD). Differences between groups were evaluated by a

two-tailed Student's *t*-test. *P*-values less than 0.01 were considered statistically significant difference.

Table 6.1. Bacterial strains used in this study

Strain	Description	Reference or Source
K. pneumoniae		
NTUH-K2044	Clinical isolate of K1 serotype	(88, 325)
CCW01	NTUH-K2044 Δ*lacZ*	This study
CG43	Clinical isolate of K2 serotype	(47)
CG43S3	CG43, Sm^r	(171)
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
CCW40	CG43S3 Δ*mrkI* [pWY28], Km^r	This study
CCW41	CG43S3 Δ*mrkI* [pWY45], Km^r	This study
Δ*lacZ*	CG43S3 Δ*lacZ*, Sm^r	
Δ*lacZ* Δ*mrkI*	CG43S3 Δ*lacZ* Δ*mrkI*, Sm^r	This study
CCW51 ($MrkI_{D56E}$)	CG43S3 $mrkI_{D56E}$, Sm^r	This study
CCW54 ($MrkI_{D56A}$)	CG43S3 $mrkI_{D56A}$, Sm^r	This study
Δ*fur*	CG43S3 Δ*fur*, Sm^r	(53)
Δ*lacZ* Δ*fur*	CG43S3 Δ*lacZ* Δ*fur*, Sm^r	(53)

Table 6.1. (continued)

Strain	Description	Reference or Source
Δ*lacZ* Δ*rcsA*	CG43S3 Δ*lacZ* Δ*rcsA*, Sm^r	(185)
Δ*lacZ* Δ*rcsB*	CG43S3 Δ*lacZ* Δ*rcsB* , Sm^r	(185)
Δ*lacZ* Δ*mrkH*	CG43S3 Δ*lacZ* Δ*mrkH*, Sm^r	This study
Δ*rcsA*	CG43S3 Δ*rcsA*, Sm^r	(185)
Δ*rcsB*	CG43S3 Δ*rcsB*, Sm^r	(171)
Δ*rmpA*	CG43S3 Δ*rmpA*, Sm^r	(53)
Δ*rmpA2*	CG43S3 Δ*rmpA2*, Sm^r	(171)
E. coli		
JM109	*endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB*$^+$ Δ*(lac-proAB) e14-* [F' *traD36 proAB*$^+$ *lacI*^{q} *lacZ* Δ*M15*] *hsdR17* ($r_K^- m_K^+$)	New England Biolabs
S17-1 λ*pir*	*hsdR recA pro* RP4-2 (Tc::Mu; Km::Tn*7*)(λ*pir*)	(282)
Novablue (DE3)	F^- *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm* (DE3), Tc^r	Novagen
BL21 (DE3)	F^- *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm trxB*15::*kan* (DE3)	Novagen
HB101	F^- *thi*-1 *hsd*S20 ($r_B^- m_B^-$) *sup*E44 *rec*A13 *ara*-14 *leu*B6 *pro*A2 *lac*Y1 *gal*K2 *rps*L20 (str^r) *xyl*-5 *mtl*-1	Promega

Table 6.2. Plasmids used in this study

Plasmid	Description	Reference or Source
yT&A	Ap^r, T/A-type PCR cloning vector	Yeastern
pET30a-c	Km^r, His-tagged protein expression vector	Novagen
pKAS46	Ap^r, Km^r, suicide vector, *rpsL*	(282)
pBAD33	Cm^r, expression vector	(114)
placZ15	Cm^r, promoter selection vector, $lacZ^+$	(183)
pRK415	Tc^r, shuttle vector, mob^+	This study
pETQ (pETQ33)	Km^r, expression vector	This study
pKPCA (pETP3)	Km^r, 513-bp fragment encoding full length KpcA cloned into pET30a	This study
pKPC-7	Km^r, ~ 4.9-kb fragment containing the *kpcABCD* genes cloned into pET30a	This study
pKPC-36	Km^r, ~ 4.9-kb fragment containing the *kpcABCD* genes cloned into pETQ	This study
pAW67	Km^r, ~ 3.8-kb fragment containing the *kpcABC* genes cloned into pETQ	This study
pAW69	Km^r, ~ 8.1-kb fragment containing the *fimAICDEFGHK* genes cloned into pETQ	This study
$pKPCI_{196}$ (pSY008)	Cm^r, 591-bp fragment encoding full length $KpcI_{196}$ cloned into pBAD33	This study
$pKPCI_{210}$ (pAW142)	Cm^r, 633-bp fragment encoding full length $KpcI_{210}$ cloned into pBAD33	This study
pKPC-ON (pKPC-22)	Ap^r, 1087-bp fragment containing the $kpcS_{ON}$ region cloned into yT&A	This study
pKPC-OFF (pKPC-20)	Ap^r, 1087-bp fragment containing the $kpcS_{OFF}$ region cloned into yT&A	This study
pSY003	Cm^r, 280-bp fragment of the $kpcS_{ON}$ region cloned into placZ15	This study
pSY004	Cm^r, 280-bp fragment of the $kpcS_{OFF}$ region cloned into placZ15	This study

Table 6.2. (continued)

Plasmid	Description	Reference or Source
pAW126	Cm^r, 374-bp fragment of the upstream region of *kpcA* in the $kpcS_{ON}$ phase cloned into placZ15	This study
pAW73	Ap^r , ~ 5.5-kb fragment containing the $kpcS_{OFF}$-*kpcABCD* region cloned into yT&A	This study
pWY28	Km^r Ap^r, 1953-bp fragment containing the adjacent regions beside *mrkI* cloned into pKAS46	This study
pWY45	Km^r Ap^r, 2597-bp fragment containing the *mrkI* and its adjacent regions cloned into pKAS46	This study
pmrkA-P1 ($P_{L\text{-}mrkA}$)	Cm^r, 551-bp fragment of the upstream region of *mrkA* cloned into placZ15	Ying-Jung Huang
pmrkA-P2 ($P_{S\text{-}mrkA}$)	Cm^r, 402-bp fragment of the upstream region of *mrkA* cloned into placZ15	Ying-Jung Huang
pmrkA-P3 (pAW146)	Cm^r, 232-bp fragment of the upstream region of *mrkA* cloned into placZ15	This study
pAW197	Km^r Ap^r, 2597-bp fragment containing the $mrkI_{D56E}$ mutant allele cloned into pKAS46	This study
pAW198	Km^r Ap^r, 2597-bp fragment containing the $mrkI_{D56A}$ mutant allele cloned into pKAS46	This study
pMrkH (pAW45)	Km^r, 711-bp fragment encoding full length MrkH cloned into pETQ	This study
pMrkH* (pAW182)	Km^r, 711-bp fragment encoding full length $MrkH_{R111D}$ cloned into pETQ	This study
pPilZ (pAW190)	Km^r, ~ 480-bp fragment encoding the PilZ domain of MrkH cloned into pETQ	This study
pPilZ* (pAW191)	Km^r, ~ 480-bp fragment encoding the PilZ domain of $MrkH_{R111D}$ cloned into pETQ	This study
$pMrkH_N$ (pAW194)	Km^r, ~ 330-bp fragment encoding the N-terminal domain of MrkH cloned into pETQ	This study
pfur	Tc^r, 0.8-kb fragment containing a *fur* allele cloned into pRK415	(53)
pAW175	Cm^r, 407-bp fragment of the upstream region of *mrkH* cloned into placZ15	This study
pYdeH (pAW47)	Km^r, 894-bp fragment encoding YdeH, from *E. coli* W3110, cloned into pETQ	This study
pYdeH* (pAW71)	Km^r, 894-bp fragment encoding $YdeH_{AADEF}$ cloned into pETQ	This study

Table 6.3. Oligonucleotide primers used in this study

Primer	Sequence (5'--- 3') [a]	Enzyme cleaved	Complementary position (5' end)
prevalence analysis			
kpa-1 (pcc047)	CACGTATGTATTCGCCCT	none	+31 relative to the *kpaE* start codon
kpa-2 (pcc004)	TCAGTTATAAGTAAAGGTGATCACCCC	none	-24 relative to the *kpaE* stop codon
kpa-3 (dk001)	TTGTCGTTTCATATGGATATGGGAA	*Nde*I	-12 relative to the *kpaA* start codon
kpa-4 (dk002)	GATTCCCCTTTCTCCATTCAACA	none	+33 relative to the *kpaA* stop codon
kpb-1 (pcc005)	CACCGTGGGGCAAAAGC	none	-4 relative to the *kpbD* start codon
kpb-2 (pcc006)	TTAATCTTCCTGAATAACGACTTCCA	none	on the *kpbD* stop codon
kpb-3 (pcc021)	ATGAAAAAGACAATCGTAGCTGTA	none	on the *kpbA* start codon
kpb-4 (pcc022)	CGGGACCCTCGAGGGAAA	none	+20 relative to the *kpbA* stop codon
kpc-1 (pcc007)	CACCATGAAGGTGTTATTAAAATCCG	none	-4 relative to the *kpcD* start codon
kpc-2 (pcc008)	CTATTTATATGTCAACGTAAACGTCGC	none	on the *kpcD* stop codon
kpc-3 (pcc023)	ATGAAAAAAACGATAACAATCGTG	none	on the *kpcA* start codon
kpc-4 (pcc024)	CCCCTCGAGGGCACAGTGT	*Xho*I	+18 relative to the *kpcA* stop codon
kpd-1 (pcc009)	CACCATGAAAAAAATCATCGCA	none	-4 relative to the *kpdD* start codon
kpd-2 (pcc010)	TCAGTTATAAGTCACCACGAAGGTC	none	on the *kpdD* stop codon
kpd-3 (pcc025)	TAAGCGTGGTGATGAGGAGTG	none	-24 relative to the *kpdA* start codon
kpd-4 (pcc050)	GCCAGAAGCTTACGCCGC	*Hin*dIII	+78 relative to the *kpdA* stop codon
kpe-1 (pcc011)	CACCATGTCCTTTTTAACTCTCCTG	none	-4 relative to the *kpeD* start codon
kpe-2 (pcc012)	CTAGTCATAATGCAAGGTATAGGTCGC	none	on the *kpeD* stop codon

Table 6.3. (continued)

Primer	Sequence (5'--- 3') [a]	Enzyme cleaved	Complementary position
Prevalence analysis			
kpe-3 (pcc027)	TTGAGTATGAAAGAAAAAGGCACC		-6 relative to the *kpeA* start codon
kpe-4 (pcc028)	GGATCCGGTCTCGAGGAAGAG	*Bam*HI, *Xho*I	+70 relative to the *kpeA* stop codon
kpf-1 (pcc013)	CACCATGCGCCGACTTAGC		-4 relative to the *kpfD* start codon
kpf-2 (pcc014)	TTATTCAAAGGTCACGGTGATTTTG		on the *kpfD* stop codon
kpf-3 (pcc029)	TTGGCTATGAAAATGAAATCACTT		-6 relative to the *kpfA* start codon
kpf-4 (pcc030)	TTATCCGCCTCGAGCCGTC		+31 relative to the *kpfA* stop codon
kpg-1 (pcc015)	CACCATGAAATCTGTTTTTCGTCTAC		-4 relative to the *kpgD* start codon
kpg-2 (pcc016)	CTAGTTATACTCCAGGGCGAAAGTCA		on the *kpgD* stop codon
kpg-3 (pcc031)	ATGAAAAAACAACCTCGCTTTA		on the *kpgA* start codon
kpg-4 (pcc032)	TGACAATTAACACATAAGCTTTCTG	*Hin*dIII	+73 relative to the *kpgA* stop codon
mrk-1 (mrkD-N)	GGGACAGCAAACAACAAA		-27 relative to the *mrkD* start codon
mrk-2 (SL0080)	CGCATTAATCGTACGTCA		+4 relative to the *mrkD* stop codon
mrk-3 (mrkA-RTF)	GGTAAGTAATTTCGTAAGTCGCGT		-26 relative to the *mrkA* stop codon
mrk-4 (mrkA-RTR)	CTCTGACAAGGAAATGGCAATG		-19 relative to the *mrkA* start codon
fim-1 (pcc074)	TCGCTTCCCGCTGCAGGCC		-111 relative to the *fimH* start codon
fim-2 (SL0078)	GAACGCCTATCCCCTGCGCC		-168 relative to the *fimH* stop codon
fim-3 (pcc051)	GAAGGCACAACGGATCCCAA	*Bam*HI	-59 relative to the *fimA* start codon
fim-4 (pcc052)	CTTCCTTGCCTGACTCGGGT		+21 relative to the *fimA* stop codon

Table 6.3. (continued)

Primer	Sequence (5'--- 3') [a]	Enzyme cleaved	Complementary position
pcc053	CAAGGAGAAACATATGAAAAAAACGA	*Nde*I	-13 relative to the *kpcA* start codon
pcc056	CGATCAAACAGATCTTTCCACCA	*Bgl*II	+93 relative to the *kpcD* stop codon
pcc081	GGCGGGAGGCAGACAGCGAC		+281 relative to the *kpcI* start codon
pcc082	TGCGGCGAGGGTGTAGTCAGGAG		-103 relative to the *kpcA* stop codon
pcc149	ATGCCAGTAAAACGAAAACACC		on the *kpcA* start codon
pcc150	CTTTACATTCTGGCACTAATTGTGTG		+2 relative to the $kpcI_{196}$ stop codon
YCY001	GGATCCGTGGTGAGTTCAGGAGAAAATTTGG	*Bam*HI	-83 relative to the *kpcA* start codon
YCY002	AGATCTATGTAAAGTAGTATCAGAAAAATTTAGCAAAG	*Bgl*II	-374 relative to the *kpcA* start codon
pcc167	CAATCCGGTTCGTTATTTCGACATCGTTCAAAGG		-42 relative to the *fimA* start codon
pcc169	GCCAAACATGAATTCGATAACACCCGCGAATAC		+93 relative to the *fimK* stop codon
pcc202	GAATTCAAGGAGAAAGGTATGAAAAAAACGA	*Eco*RI	-12 relative to the *kpcA* start codon
pcc223	AGATCTCCAGCCAGCCGGATTTTAATAAC	*Bgl*II	+31 relative to the *kpcC* stop codon
pcc183	TTAAGGAGCAAGGCTTATGCCAGTAAAACGAAAAC		-16 relative to the *kpcI* start codon
wc07	AGATCCTACAAATGGGGCGTGA		-306 relative to the *mrkH* start codon
wc08	GGCCTGTTCACCTATTACGTTG		+136 relative to the *mrkJ* stop codon
wc09	CTCTTTTTGCGCTTGGCTTCTA		-5 relative to the *mrkH* stop codon
wc10	TTCTCCCGGTAAATCAGTAGCG		+4 relative to the *mrkI* stop codon

Table 6.3. (continued)

Primer	Sequence (5'--- 3') [a]	Enzyme cleaved	Complementary position
pcc212	GGATCCAAGGGATGCATATGACAGAGGG	*Bam*HI	-5 relative to the *mrkH* start codon
pcc213	AAGCTTACTGTCCAAGGTTGTCAGATTCTC	*Hin*dIII	+14 relative to the *mrkH* stop codon
pcc335	GGATCCATGCATGACAATAGCGGTGTCGATAAAGG	*Bam*HI	+244 relative to the *mrkH* start codon
pcc336	AAGCTTGCTGCACTACCTGCAGGCATTC	*Hin*dIII	+316 relative to the *mrkH* start codon
pcc216	GGATCCATGATCAAGAAGACAACGGAAATTG	*Bam*HI	on the *ydeH* start codon
pcc217	AAGCTTAAACTCGGTTAATCACATTTTGTTC	*Hin*dIII	+4 relative to the *ydeH* stop codon
pcc224	GAAACGGTTTATCGCTACGCGGCCGAAGAATTTATCATTATTG		+598 relative to the *ydeH* start codon
pcc225	CAATAATGATAAATTCTTCGGCCGCGTAGCGATAAACCGTTTC		+640 relative to the *ydeH* start codon
pcc337	CTTATTAATTAAATTGAAAATAATCGTCTGGGCC		+165 relative to the *mrkI* start codon
pcc338	GAGATTTCTGCCATCAGAATCGTCGATCTG		+165 relative to the *mrkI* start codon
pcc339	GCGATTTCTGCCATCAGAATCGTCGATCTG		+165 relative to the *mrkI* start codon
pcc281	CCGGAGACAGGTAAACGTTCGCATCGCT		+181 relative to the *mrkA* strat codon
pcc282	AGCAGCCTGGCAGTTAGAGACGTCAATGGTG		+270 relative to the *mrkA* strat codon
pcc273	AGATCTTTGACGCCGATAGCACCAG	*Hin*dIII	-188 relative to the *mrkA* start codon
pcc324	GGATCCGCGGTTGCCATTGCTGCAGAG	*Bam*HI	+38 relative to the *mrkA* start codon
pcc319	GGATCCAGACAAAATGGAGGGAACCCTATC	*Bam*HI	-376 relative to the *mrkH* start codon
pcc320	GGATCCTTACTGGTCTTTATCGTTCCCTCTG	*Bam*HI	+29 relative to the *mrkH* start codon

Table 6.3. (continued)

Primer	Sequence (5'--- 3') [a]	Enzyme cleaved	Complementary position
RT-PCR			
a1 (wc05)	GGATCCGCCTGGGTGCCCTTTTTCC	*Bam*HI	-640 relative to the *mrkH* start codon
a2 (wc03)	CCCTCTGTCATATGCATCCCTTG		+11 relative to the *mrkH* start codon
b1 (pcc275)	CACCCTGGATAACGCTAATGAAGAGAG		-150 relative to the *mrkI* start codon
b2 (pcc276)	CATAACTCAGACGGGTGGCATTTTC		+100 relative to the *mrkI* start codon
c1 (pcc277)	GAATCAGCGTATTGCCGCTCTCC		-177 relative to the *mrkJ* start codon
c2 (pcc256)	CATTCCACCGCGACCAGAGTAC		+110 relative to the *mrkJ* start codo
d1 (wc13)	ATGACCAAAACGCCGAATCTTA		+439 relative to the *mrkH* start codon
d2 (wc09)	CTCTTTTTGCGCTTGGCTTCTA		-5 relative to the *mrkH* stop codon
e1 (wc17)	GGATCCGGGCTGTGCAGAGAGTTGATAAA	*Bam*HI	+265 relative to the *mrkI* start codon
e2 (pcc280)	AACCGTTTTATGAGCAATGCCGAG		+495 relative to the *mrkI* start codon
f1 (pcc278)	CCATATCCTGAACCTGTTGCGCC		+273 relative to the *mrkJ* start codon
f2 (pcc279)	CGGACTCTTTGCGCATCAGGTG		+532 relative to the *mrkJ* start codon

[a] The nucleotide sequence recognized by each restriction enzyme listed are underlined.

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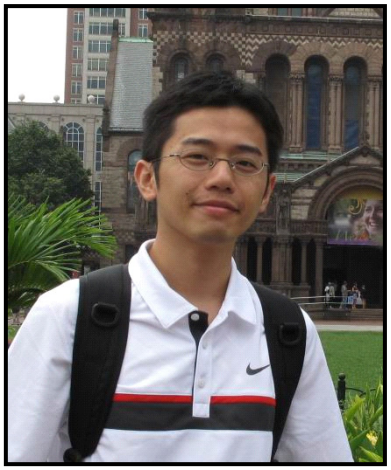
Vita



I am Chien-Chen Wu. I was born in Taipei, Taiwan in 1980. After graduated from Municipal Jianguo High School, Taipei, Taiwan in 1999, I was entered National Chiao-Tung University, Hsinchu, Taiwan and received my degree of Bachelor of Science from Department of Biological Science and Technology in 2003. I started my laboratory research focused on bacteriophage therapy in 2001, under the supervison of Professor Hwei-Ling Peng in the Laboratory of Molecular Regulation, and continued my graduate studies in the same laboratory. In 2004, I entered the Ph.D. program at Department of Biological Science and Technology and focused my studies on fimbriae adherence and regulatory mechanism of the c-di-GMP signaling pathway in *Klebsiella pneumoniae*. I am working towards the degree of Doctor of Philosophy and will receive it in 2010. My research interests include molecular mechanism of bacterial pathogenesis, bacterial genomics and signal transduction, and cellular microbiology.