國立交通大學生物科技學系

博士論文

克雷白氏肺炎桿菌中第三型纖毛的特性分析

Characterization of type 3 fimbriae in *Klebsiella pneumoniae*

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中文摘要

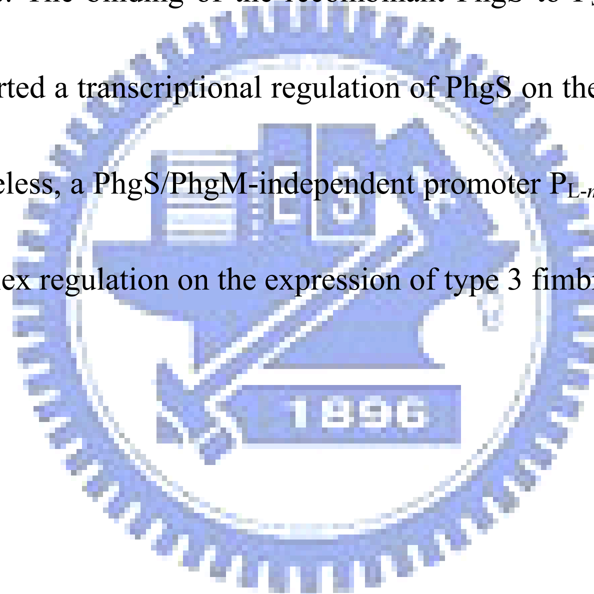
附著寄主細胞，是細菌達成感染的第一步驟。已知，第三型纖毛的表現與克雷白氏肺炎桿菌感染寄主細胞的部位密切相關。本論文主題在探討第三型纖毛的表現與克雷白氏肺炎菌致病過程的關聯：首先，我們以PCR-RFLP的技術分析第三型纖毛黏附蛋白基因型，結果在十七株腦膜炎菌株中發現四種新的黏附蛋白基因型，分別命名為$mrkD_{V1}$，$mrkD_{V2}$， $mrkD_{V3}$和$mrkD_{V4}$。接著，我們以大腸桿菌JM109 為表現載體構築了表現第三型纖毛的重組質體，分別帶有$mrkD_{V1}$，$mrkD_{V2}$，$mrkD_{V3}$和$mrkD_{V4}$基因型。而藉穿透式電子顯微鏡來觀察這些重組大腸桿菌表現的纖毛，我們發現*mrkD*基因型的變異會影響第三型纖毛的長度和型態。同時，測試這些重組細菌黏附第四型和第五型膠原蛋白的能力、生物膜的形成與黏附細胞的活性，結果顯示，*E. coli* JM109[pMrkABCD$_{V3}$F]在這些測試中，活性皆高於帶有其他三種黏附蛋白基因型的大腸桿菌。我們也發現MrkDv3 黏附蛋白上的RGD序列可以決定*E. coli* JM109[pMrkABCD$_{V3}$F]黏附HCT-8 細胞的專一性。其次，我們也探討第三型纖毛在克雷白氏肺炎桿菌CG43 中的表現調控：核酸序列分析顯示第一型纖毛和第三型纖毛的基因群相連。在LB或GCAA培養液中，都可以偵測到第三型纖毛的表現；相反的，只有在*mrkA*缺損或是*fimB*大量表現的情況下，才能偵測到第一型纖毛的表現。而在*fimB*大量表現時，第三型纖毛的表現會明顯下降，這樣的結果暗示著這兩種纖毛的表現有互相調控的關係。而在第一型纖毛和第三型纖毛基因群之間，我們發現有兩個基因和*Erwinia chrysathemi*調控毒性因子的*pecS*和*pecM*相似，分別命名為*phgS*和*phgM*。在測量啟動子活性時，我們發現$P_{S\text{-}mrkA}$的活性在*phgS*或*phgM*缺損株中，都明顯降低，這暗示著PhgS/PhgM可以調控第三型纖毛的表現。同時，以電泳膠遲滯實驗證明重

組蛋白PhgS可以結合$P_{S\text{-}mrkA}$也顯示PhgS具有轉錄調控的功能。另外，我們還找到一段不受PhgS/PhgM影響的啟動子$P_{L\text{-}mrkA}$，顯示第三型纖毛的表現調控可能並不單純。

Abstract

As generally known, attachment of pathogens to their host is a prerequisite step of infection. The study reports the involvement of type 3 fimbriae, which has been shown as the primary adhesion factor in *Klebsiella pneumoniae*, in the bacterial pathogenesis. Firstly, four novel *mrkD* alleles namely $mrkD_{V1}$, $mrkD_{V2}$, $mrkD_{V3}$ and $mrkD_{V4}$, were identified in seventeen *K. pneumoniae* meningitis strains. A type 3 fimbriae display system in *Escherichia coli* was subsequently constructed to determine the effect of MrkD allelic variation on the fimbrial activity. The TEM analysis indicated that the proper growth of the filament and fimbrial morphology were MrkD adhesin dependent. The assessments via measurements of collagen IV and V binding activity, biofilm formation, and cell adherence revealed that the *E. coli* JM109[pmrkABCD$_{V3}$F] had the highest level of fimbriae activity and the adhesion to HCT-8 cells is probably through the interaction of the RGD sequence on MrkD$_{V3}$ with integrin. Secondly, regulation of expression of type 3 fimbriae in *K. pneumoniae* CG43 is investigated. Sequence analysis revealed that type 1- and type 3-fimbriae gene clusters are physically linked in the genome of *K. pneumoniae* CG43. The expression of type 3 fimbriae in LB or GCAA medium could be readily demonstrated in the bacteria. In contrast, the expression of type 1 fimbriae was evident only in the *mrkA* deletion mutant or in the *fimB* overexpression mutant. Whereas, the

overexpression of *fimB* diminished the expression of type 3 fimbirae suggesting a cross regulation is present for the expression of the two fimbriae. In-between the two gene clusters, homologues of *Erwinia chrysanthemi* virulence regulatory genes *pecS* and *pecM* were identified and named *phgS* and *phgM* respectively. The promoter activity measurement revealed that the deletion of *phgS* or *phgM* reduced the activity of the putative promoter $P_{S\text{-}mrk}$, which suggesting a PhgS/PhgM-dependent expression of type 3 fimbriae. The binding of the recombinant PhgS to $P_{S\text{-}mrk}$ demonstrated by EMSA also supported a transcriptional regulation of PhgS on the expression of type 3 fimbriae. Nevertheless, a PhgS/PhgM-independent promoter $P_{L\text{-}mrk}$ was also identified indicating a complex regulation on the expression of type 3 fimbriae.



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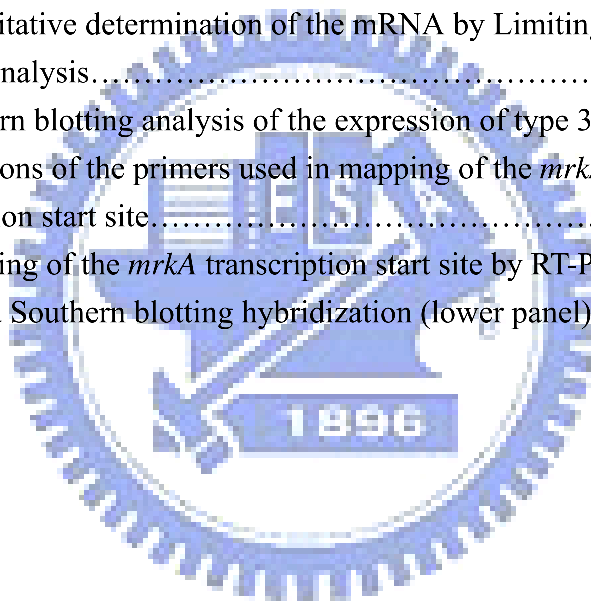


Abbreviations

Amp	Ampcilin
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
Cm	Chloramphenicol
CRP	cAMP Receptor Protein
EMSA	Electrophoretic mobility shift assay
GCAA	Minimal medium containing 1% glycerol and 0.3% casamino acid
HA	hemagglutination
IVET	In vivo expression technology
Km	Kanamycin
LD-RT-PCR	Limiting-dilution-reverse transcription-PCR
LRP	Leucine-responsive regulatory protein
NBT	Nitro blue tetrazolium
PBS	Phosphate-buffered saline
RGD	Arg-Gly-Asp
RFLP	Restriction fragment length polymorphism
Sm	Streptomycin
STM	Signature-tagged mutagenesis
TEM	Transmission Electron Microscopy
Tc	Tetracycline

Chapter I

Introduction

Klebsiella pneumoniae is an important cause of community-acquired pneumonia that commonly results in high fatality if untreated (Han, 1995; Podschun and Ulmann, 1998). The vast majority of *K. pneumoniae* infections are associated with hospitalization, which has been estimated to cause up to 8% of all nosocomial bacterial infections in developed countries (Bergogne-Berezin, 1995; Schaberg *et al*., 1991). Nasopharynx and intestinal tract have been thought as the primary reservoirs and colonization of the bacterium in perigenital area is an important phase for subsequent infection of the urinary tract (Mayhall *et al*., 1980; Seidler *et al*., 1975). In Taiwan, *K. pneumoniae* has been attributed to be the major cause of liver abscess especially in diabetes mellitus patients (Chang and Chou, 1995; Wang *et al*., 1998). The infections often cause serious complications such as septic endogenous endophthalmitis, metastatic infections of brain and lung, and necrotizing fasciitis (Cheng *et al*., 2003). Several factors are known to participate in the pathogenesis of *Klebsiella* spp., including capsular polysaccharides, lipopoly- saccharides, iron-acquisition systems, and adhesins (Podschun and Ulmann, 1998). Most recently, in vivo expression technology (IVET) and Signature-tagged mutagenesis (STM) assays have been applied to identify other factors involved in the bacterial pathogenesis (Boddicker *et al*., 2006; Lai *et al*., 2001; Struve *et al*., 2003). However, the pathogenic mechanisms remain largely unknown.

Adherence to host tissues is an essential early phase in many bacterial infections. The attachment to host surfaces is thought to increase the infection potential by providing resistance to the mechanical clearance by the host defense system (Switalski *et al*., 1989). In *K. pneumonia*e, the adhesion process is frequently mediated by fimbriae and the specific interaction is determined by adhesins, a component on the tip of fimbriae. Among the five adhesins identified in *K. pneumoniae*, type 1 fimbriae are present frequently in pyelonephritis isolates of *Escherichia coli* (Iwahi *et al*., 1983). Type 3 fimbriae, referred to as the mannose-resistant *Klebsiella* hemagglutin (Mr/KH), have been shown to be produced by some uropathogenic isolates of *K. pnenumoniae* (Old, 1972). The nonfimbrial adhesin CF29K, which is involved in the adherence to human intestinal cell line Caco-2 (Di-Martino *et al*., 1995), and a fimbrial antigen KPF-28 that has been shown to be a determinant for the colonization on human gut (Di-Martino *et al*., 1996), are both encoded by a β-lactamase-producing plasmid. The fifth has recently been described as a nonfimbrial adhesin to mediate an aggregative adhesion pattern to intestinal cell lines (Favre-Bonte *et al*., 1995).

Bacterial adherence determines the tissue tropism during infection and the process is generally mediated by adhesin (Clegg and Gerlach, 1987; Westerlund-Wikstrom and Korhonen, 2005). Specific deletion of the *fimH* gene did

dramatically reduce the number of fimbriae expressed on the cell surface (Schembri *et al*., 2002). The effect of allelic variation of the adhesive molecule on adhesive activity of type 1 and type P fimbriae also has been reported. For instance, a minor mutation in *fimH*, the adhesin encoding gene of type 1 fimbriae, rendered approximately 70% of the uropathogenic *Escherichia coli* an increasing ability to recognize monomannose (Man 1). While 80% of the feces isolates bind only to trimannose (Man 3) receptors (Firon *et al*., 1987). The variation of PapG, the adhesin of P fimbriae, also appeared to alter the fimbrial receptor specificity (Stromberg *et al*., 1991).

Type 3 fimbriae is often found on *Klebsiella* strains as well as in a variety of enteric bacteria (Old and Adegbola, 1983). Similar to the operon structure coding for type 1 fimbriae, type 3 fimbriae is encoded by *mrk* gene cluster (Allen *et al*., 1991). The *mrk* operon includes *mrkA*, which encodes the major fimbrial subunit; *mrkB* and *mrkC*, respectively coding for chaperone and usher proteins required for assembly and anchorage of the fimbriae; *mrkF*, encoding a protein which has been shown to affect the stability of the fimbriae; and *mrkD* which encodes the adhesin responsible for the activity of MR/K hemagglutination. Until recently, only three *mrkD* variants, a plasmid-encoded MrkD$_{1p}$ and chromosomally occurred MrkD$_{1C1}$ and MrkD$_{1C2}$, each with somewhat different binding properties to type IV and type V collagen, have been

reported (Sebghati *et al*., 1998). Although a protein receptor has been suggested (Hornick *et al*., 1992), the identity of MrkD receptor remains unknown.

In addition to the major subunit pilin protein and the adhesin protein on the tip of a fimbria, there are other proteins, minor subunits, found in the filamentous hair. For example, type 1 fimbriae contains minor subunits, FimF and FimG, which are required for integration of FimH adhesin into the fimbirae at the tip location (Krogfelt and Klemm, 1988). Their roles in determining morphology and assembly of the fimbrial structure have been demonstrated (Klemm and Christiansen, 1987). The minor subunits of P pilus, PapF, PapK and PapH have also been reported to function as adaptor, initiator, and terminator, respectively, for the growth of the filament (Jacob-Dubuisson *et al*., 1993; Verger *et al*., 2006). In addition, PapE was shown to be responsible for binding to fibronectin indicating another role of the minor subunit (Westerlund and Korhonen, 1991). MrkF has been reported to affect type 3 fimbrial activity by stabilizing the structure of the filament (Allen *et al*., 1991). We have shown previously by TEM analysis that MrkF was able to serve as an initiator for the growth of the filament and to control the length of the fimbriae (廖心瑋，民國九十五年). Furthermore, as determined by the measurements including collagen binding activity, biofilm formation, and autoaggregation phenotype, MrkF also played a regulatory role for the activity of type 3 fimbriae (廖心瑋，民國九十五年).

Bacteria can often express multiple adhesins during infection in order to establish the attachment to specific niches. In the genome of *Salmonella enterica* serovar Typhi CT18, twelve fimbrial gene clusters of the chaperone-usher-dependent assembly class have been identified. Through prevalence analysis of these fimbrial gene clusters among serotypes of *S. enterica* subspecies, *S.* Typhi was shown to possess a unique repertoire of fimbrial gene sequences possibly due to specific selective pressures (Townsend *et al*., 2001). Using of flow cytometry to detect expression of eleven *S.* Typhimurium fimbrial operons also showed that *in vivo* growth conditions drastically alter the repertoire of fimbrial antigens expressed in *S.* Typhimurium (Humphries *et al*., 2003). In uropathogenic *E. coli*, the mutant deficient in both type 1 and P fimbrial expression produced F1C fimbriae, which was not produced by wild-type strain during growth in aerated or static culture conditions (Snyder *et al*., 2005). This also suggested that the presence of a regulatory network in controlling differential expression of these fimbriae.

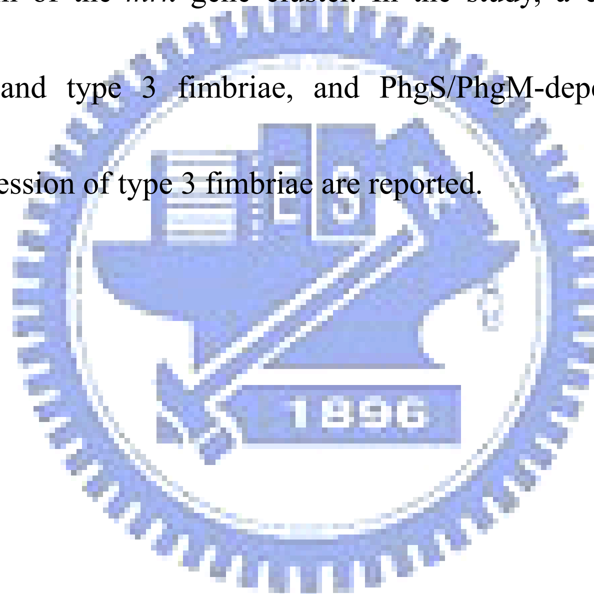
The two most commonly associated fimbriae with *E. coli* urinary tract infections are type 1 and type P fimbriae (Holden *et al*., 2006). The expression of type 1 fimbriae requires two recombinases, FimB, to promote the inversion of the promoter DNA in off-to-on phase, and FimE to mediate an on-to-off orientation (Gally *et al*., 1996). On the other hand, the phase-variable expression for P fimbirae is controlled

by alternative DNA methylation patterns (Blomfield, 2001). PapB, a positive regulator for type P fimbriae expression, has been shown to affect the expression of type 1 fimbriae via inhibition of the FimB-promoted recombination but enhancement of the expression of *fimE* (Xia *et al*., 2000). The DNA microarray analysis also demonstrated a coordinate expression between type 1 fimbriae and P fimbriae (Snyder *et al*., 2005). Moreover, a study further determined that the cross-regulation between P and type 1 fimbriae occurs in *E. coli* clinical isolates with the level of PapB that are produced from its natural promoter on the chromosome (Holden *et al*., 2006).

K. pneumoniae has become an increasingly common pathogen of adult community-acquired bacterial meningitis (Lee *et al*., 2003). In part I of the study, we intend to investigate the association between variation of MrkD adhesin of type 3 fimbriae and the meningitis-associated *K. pneumoniae* isolates. Since the expression of type 3 fimbriae is generally low in *K. pneumoniae* clinical isolates, a type 3 fimbriae display system carrying respectively each of the MrkD variants was constructed and the influences of the *mrkD* allelic variation on the fimbrial activity investigated.

Type 1 and type 3 fimbriae are commonly reported in *K. pneumoniae*. While the regulation of type 1 fimbriae has been clear, that of type 3 fimbriae remains to be investigated. In part II of the study, we aim to investigate the regulation of expression

of type 3 fimbriae in *K. pneumoniae* CG43, a highly virulent clinical isolate of K2 serotype (Chang *et al*., 1996). A lamda phage clone containing *mrkABCDF* gene cluster has previously been isolated from a genomic library (Peng *et al*., 1992) and the sequences determined. The *fim* gene cluster coding for type 1 fimbriae and divergently transcribed *phgS* and *phgM*, homologues of *Erwinia chrysanthemi* virulence regulator encoding genes *pecS* and *pecM* (Reverchon *et al*., 1994), were identified upstream of the *mrk* gene cluster. In the study, a coordinate expression between type 1 and type 3 fimbriae, and PhgS/PhgM-dependent PhgS/PhgM -independent expression of type 3 fimbriae are reported.



Chapter II

Materials and methods

2.1 Bacterial strains, plasmids and media

Bacterial strains and plasmids used in this study are listed in Table 1. The growth media were LB (Luria Broth) and GCAA (minimal medium containing 1% glycerol and 0.3% casamino acid) (Gerlach *et al*., 1989) supplemented with appropriate antibiotics. The bacteria were grown at 37°C unless otherwise indicated.

2.2 PCR-RFLP analysis of the mrkD genes

Genomic DNA of the *K. pneumoniae* isolates were prepared as the template and the primers used are corresponding respectively to the 5' and the 3'-end of $mrkD_{1p}$ coding region (Gerlach and Clegg, 1988). The PCR products were then digested with *Sau3*AI and the restriction fragments resolved on a 2% agarose by gel electrophoresis.

2.3 Cell adhesion assay

Three epithelial cell lines including human laryngeal carcinoma cell line Hep-2, ileocecal epithelial cell line HCT-8, and embryonic intestinal epithelial cell line Int-407 were used. According to the cellular adherence assay (Oelschlaeger and Tall, 1997), the cells were seeded into 24 well plate (TPP, Trasadingen, Switzerland) and incubated to confluent growth in 5% CO_2 for 48 h. Approximately 10^7 bacteria were then added to each well containing about 10^5 cells, and the incubation continued for 1 h. To determine if the RGD motif contained in $MrkD_{V3}$ plays a role in cell adhesion,

the hexapetides GRGDSP (Calbiochem 03340035) and GRADSP (Calbiochem 03340052), and anti-integrin monoclonal antibody $\alpha5\beta1$ (Chemicon JBS5) were added. Finally, the plates were washed three times with phosphate-buffered saline (PBS), and the cells were lysed with 0.1% Triton X-100. The cell-adhesive bacteria were measured by recovery of the bacteria from the lysates.

2.4 Preparation of the recombinant proteins FimA, MrkA and PhgS

The genes *fimA* and *mrkA*, respectively encoding the major pilin subunit of type 1 fimbriae and type 3 fimbriae, and *phgS* were isolated by PCR cloning from *K. pneumoniae* CG43S3 (Lai *et al*., 2003) and ligated into pET30 expression vector. The recombinant plasmid was then transformed into *E. coli* NovaBlue(DE3) and overexpression of the recombinant protein was induced by addition of 0.5 mM IPTG (isopropyl-β-*D*-thiogalactopyranoside). Finally, the recombinant proteins FimA, MrkA, and PhgS, N-terminal fusion with His-tag respectively, were purified using the affinity column charged with nickel resins (Novagen, Madison, WI, USA).

2.5 Antisera preparation

Five-week-old female BALB/c mice, purchased from the animal center of National Taiwan University, were immunized intraperitoneally with 5 μg of the purified FimA

or MrkA. Ten days later, the mice were immunized again with 5 μg of the protein and the antisera obtained by intracardic puncture.

2.6 Western blot analysis of the expression of type 1 and type 3 fimbriae

Total cellular lysates from the bacteria grown overnight in either LB or GCAA broth were resolved by SDS-PAGE to determine the expression of type 1 and type 3 fimbriae in *K. pneumoniae* CG43. The proteins were then electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After incubation with 5% skim milk at room temperature for 1 h, the membrane was washed 3 times with 1X PBS. Subsequently, the membrane was incubated at room temperature for 1 h with diluted anti-FimA or anti-MrkA serum. After 3 washes with 1X PBS, a 3000-fold diluted alkaline phosphatase-conjugated anti-mouse immunoglobulin G was added and the incubation continued for one more hour. The blot was again washed and the bound antibodies were detected by using the chromogenic reagents BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (Nitro blue tetrazolium).

2.6 Construction of the type 3 fimbriae expression plasmid

The recombinant plasmid pmrkABC carries the *mrkABC* genes PCR amplified from *K.*

pneumoniae CG43. Each of the *mrkD* variants was then subcloned respectively into pmrkABC, which resulted in the plasmids pmrkABCD$_{V1}$, pmrkABCD$_{V2}$, pmrkABCD$_{V3}$ and pmrkABCD$_{V4}$ (陳美甄，民國九十一年). The gene coding for MrkF, which helps to stabilize type 3 fimbriae (Allen *et al*., 1991), was then inserted downstream to each of the *mrkD* alleles. The plasmids were named pmrkABCD$_{V1}$F, pmrkABCD$_{V2}$F, pmrkABCD$_{V3}$F, and pmrkABCD$_{V4}$F, respectively.

2.7 Transmission electron microscopy (TEM)

Twenty microliters of bacterial suspension (10^8 cfu/ml) were added to collodion-coated copper grids (300 mesh) and negatively stained by 2% phosphotungstic acid, pH 7.2. For immunogold TEM analysis, the bacteria coated on the grids (10-nm diameter) were reacted with the raised anti-MrkA polyclonal antibody (1:50 dilution) and the anti-mouse IgG -gold conjugate of 1:65 dilution before staining with 2% phosphotungstic acid. The grids were examined under a JEOL JEM 2000EXII trans -mission electron microscope at an operating voltage of 100 kV (Hornick *et al*., 1995).

2.8 RT-PCR analysis to assess an operon structure of mrkABCDF.

K. pneumoniae NTUH K-2044 was grown statically in LB medium at 37°C for 20 h.

Total RNA was isolated from *K. pneumoniae* cells by extraction with the TRI reagent (Molecular Research Center, Cincinnati, Oh, USA) and the residual DNA was eliminated with RQ1 RNase-free DNase (Promega, Madison, WI, USA). The cDNAs used for PCR amplification were each synthesized from 1μg of total RNA using the random hexamer primer from a RT-PCR kit (Stratagene, La Jolla, CA, USA). The primers used to amplify the major pilin MrkA of type 3 fimbriae are MrkA-RT-1 (5'-CTC TGA CAA GGA AAT GGC AAT G-3') and MrkA-RT-2 (5'-GGT AAG TAA TTT CGT AAG TCG CGT-3'). The primers for MrkD adhesin are MrkD-RT-1 (5'-ATG TCG CTG AGG AAA TTA CTA ACG-3') and MrkD-RT-2 (5'-GCT GAA ACG CAT GCC GAT-3'). The primers for MrkF are MrkF-RT-1 (5'-ATG AAG GGA TTG CCG AAA AA-3') and MrkF-RT-2 (5'-GCT CCA TCC GGC AAG GTA-3'). The primers specific for intergenic region between mrkD and mrkF are MrkDF-RT-1 (5'-AGG AGA CCC GCT ACA TCA CC-3') and MrkF-RT-2. The PCR was carried out with initial denaturation at 95°C for 10 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 46°C for 1 min, and elongation at 72°C for 1 min, and finally 10 min of elongation at 72°C. Finally, the amplified products were resolved on 1% agarose gels.

2.9 Mr/K hemagglutination assay

The hemagglutination assay was performed as described (Gerlach and Clegg, 1988). Briefly, overnight grown bacteria were collected and suspended in PBS to approximately 10^9 CFU/ml. Human erythrocytes (group A) were treated with 0.01% tannic acid for 15 min at 37°C and subsequently washed twice with PBS. A series of four-fold dilution of the bacterial suspension with 2% D-mannose were mixed with an equal volume of 3% (vol/vol) tanned-erythrocytes in PBS. The mixture was incubated at room temperature for 30 min to allow erythrocytes settle to the bottom of the glass tube.

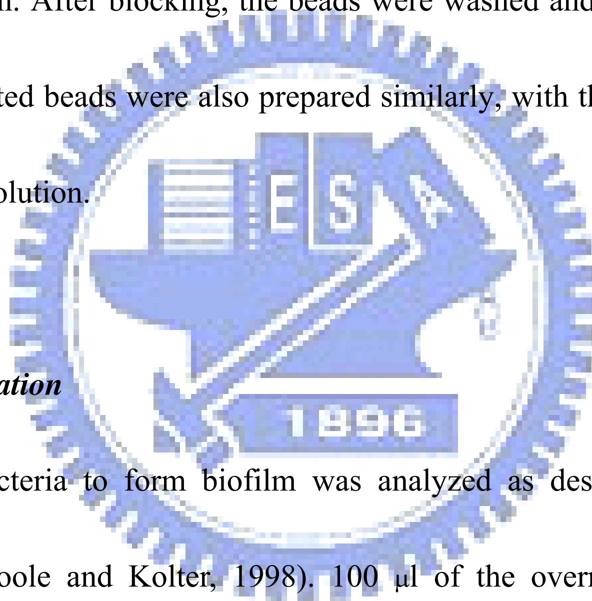
2.10 Binding to type IV- and type V-collagen

The binding assay was carried out as described (Sebghati *et al*., 1998). Essentially, the wells of flatbottom microtiter plate (Nunc, Rochester, NY, USA) were coated following incubation overnight at 4°C with optimal concentrations of type IV collagen (Sigma C7521) or type V collagen (Sigma C3657). The non-specific bindings were prevented by incubation for 2 h at 22°C with a 1% (wt/vol) solution of bovine serum albumin (BSA). Subsequently, each well was added with 100 μl bacteria (10^8 cfu/ml) and the incubation continued for 2 h at 22°C with gentle shaking. The unattached bacteria were removed by washing three times with 0.05% Tween-20 in of PBS. Finally, the attached bacteria were washed off by 0.1% Triton X-100 and the

adhesion was determined by the recovery of the bacteria.

2.11 Preparation of collagen-coated beads

Polystyrene beads (Polysciences, Warrington, PA, USA) of 1 lm in diameter were added into PBS containing 10 μg collagen and then incubated at 4°C for 16 h. The beads were then blocked with the blocking reagent (2% BSA in PBS) at room temperature for 1 h. After blocking, the beads were washed and re-suspended in 100 ml PBS. BSA-coated beads were also prepared similarly, with the replacement of the collagen to BSA solution.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2.12 Biofilm formation

The ability of bacteria to form biofilm was analyzed as described with a minor modification (O'Toole and Kolter, 1998). 100 μl of the overnight grown bacteria diluted 1/100 in GCAA medium were inoculated into each well of a 96-well microtiter dish and incubated at 37°C for 48 h. After washing, 150 μl of crystal violet (1%) was added to each well and incubated for 30 min at room temperature. The plate was then washed, the dye was solubilized in 1% SDS, and the absorbance at 595 nm was determined. The mean of three separate experiments represents the biofilm formation capability.

2.13 Construction of mrkA, mrkF, phgS, and phgM deletion mutants

DNA fragments of 1 kb in length flanking both ends of the target genes *mrkA*, *phgS*, and *phgM* were amplified by PCR with the primer sets (Table 2) and the amplified DNA fragments were cloned into the suicide vector pKAS46(Lai *et al*., 2003). The plasmids were transformed respectively into *E. coli* S17-1pir (de Lorenzo and Timmis, 1994) and then mobilized to the streptomycin-resistant strain *K. pneumoniae* CG43S3 (Lai *et al*., 2003) by conjugation. A kanamycin resistant transconjugant was selected and propagated in 2 ml LB overnight, and a small aliquot of the culture was plated on LB agar containing 500 μg/ml streptomycin. The streptomycin resistant colonies were screened further for their susceptibility to ampicillin and kanamycin, a property reflecting the loss of vector sequence. To construct *mrkF* deletion mutant, the amplified DNA fragments were cloned into the suicide vector pwA01 (Table 1). The resulting plasmid was transformed to the *K. pneumoniae* NTUH-K2044 by conjugation. A kanamycin resistant transconjugant was selected and propagated in 2 ml LB overnight, and a small aliquot of the culture was plated on LB agar containing 20% sucrose. The colonies resistant to sucrose were screened further for their susceptibility to kanamycin, a property reflecting the loss of vector sequence. Occurrence of a double recombination event was verified by PCR with primers

flanking *mrkA*, *mrkF*, *phgS*, or *phgM*, and confirmed with Southern hybridization.

2.14 Yeast-cell agglutination

Agglutination of yeast *Saccharomyces cerevisiae* AH109 was carried out as described (Blumer *et al*., 2005). Briefly, the bacteria were suspended in PBS or PBS with 2 % mannose (2 x 10^9/ml) and then mixed with 10 mg/ml of yeast on a glass slide. The degree of clumping was assessed by eyes and the agglutination count was expressed as the highest dilution of the bacteria causing visible agglutination of yeast.

2.15 Promoter activity measurements

The putative promoters of *fimB*, *fimE* and *mrkA* were PCR amplified using the specific primers (Table 2) and the PCR products subcloned in front of the promoterless *lacZ* gene on placZ15 (Lin *et al*., 2006). The bacteria carrying each of the reporter plasmids were grown statically overnight in LB medium, and the β-galactosidase activities were measured essentially as described (Miller, 1972). The data presented were derived from a single experiment, which is representative of at last three independent experiments. Every sample was assayed in triplicate, and the average activity and standard deviation were presented.

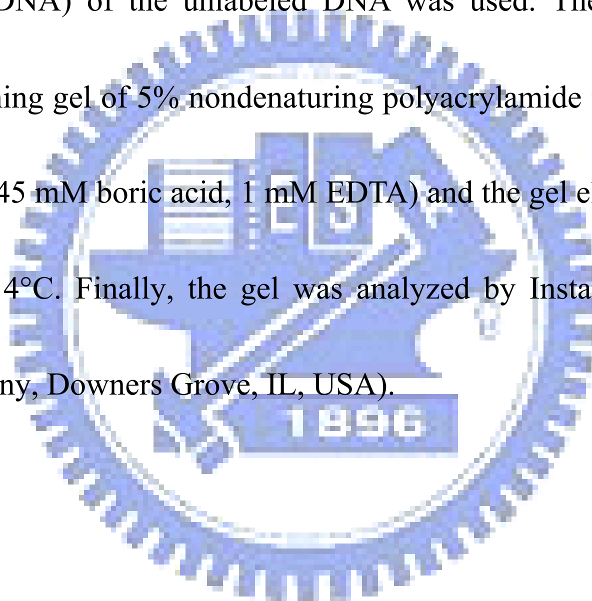
2.16 Limiting-dilution RT-PCR (LD-RT-PCR) analyses

LD-RT-PCR was performed as described (Schwan *et al*., 2005; Schwan *et al*., 2002). The cDNA was prepared as described in RT-PCR analysis to assess an operon structure of *mrkABCDF* and then subjected to PCR amplification. Each of the cDNA was diluted in four-fold graded dose and then subjected to PCR amplification. The primers used to amplify the major pilin MrkA of type 3 fimbriae are MrkA-RT-1 (5'-CTC TGA CAA GGA AAT GGC AAT G-3') and MrkA-RT-2 (5'-GGT AAG TAA TTT CGT AAG TCG CGT-3'). The primers specific for 23S-rRNA are Kp-23S-rRNA-1 (5'-CCC CCG AAG ATG AGT TCA CG-3') and Kp-23S-rRNA-2 (5'-GGC GAT GTC CGA ATG GGG AA-3'). The PCR was carried out with initial denaturation at 95°C for 10 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 46°C for 1 min, and elongation at 72°C for 1 min, and finally 10 min of elongation at 72°C. Finally, the amplification products were resolved on 1.5% agarose gels.

2.17 Electrophoretic mobility shift assay (EMSA)

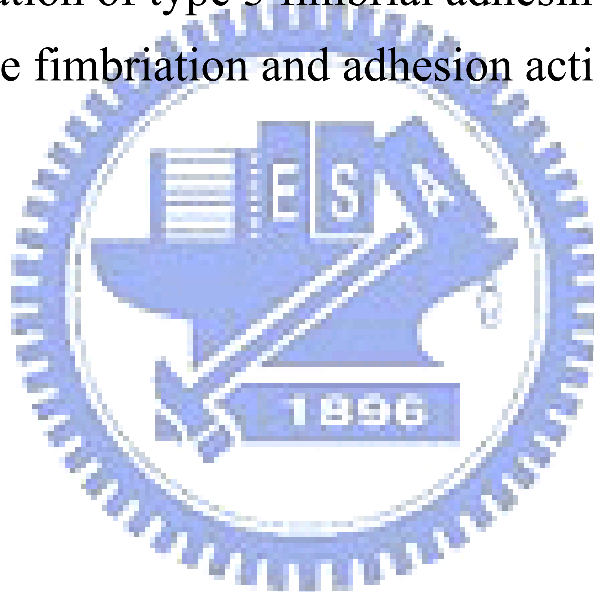
Since the overexpressed PhgS protein formed insoluble inclusion bodies, 6 M urea was used to dissolve the insoluble protein and then the denatured protein purified. The purified PhgS was refolded via dialysis against PBS buffer before applying to EMSA.

The DNA fragment comprising the putative promoter $P_{S\text{-}mrkA}$ was obtained by PCR amplification and then labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase as described (Lai *et al*., 2003). The purified PhgS was then incubated with the radioactively labeled DNA in 20 μl containing 20 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 50 mM KCl, 1 mM $CaCl_2$ and 1 mM dithiothreitol at 37℃ for 20 min. To demonstrate the binding specificity, excess amount (approximately 100 times more than the labeled DNA) of the unlabeled DNA was used. The samples were then loaded onto a running gel of 5% nondenaturing polyacrylamide in 0.5× TBE (45 mM Tris-HCl, pH 8.0, 45 mM boric acid, 1 mM EDTA) and the gel electrophoresed with a 20-mA current at 4°C. Finally, the gel was analyzed by InstantImager^{TM} (Packard Instrument Company, Downers Grove, IL, USA).



Chapter III

Allelic variation of type 3 fimbrial adhesin MrkD affects the fimbriation and adhesion activity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3.1 Abstract

Four novel *mrkD* alleles namely $mrkD_{V1}$, $mrkD_{V2}$, $mrkD_{V3}$ and $mrkD_{V4}$, were identified in seventeen *Klebsiella pneumoniae* meningitis strains using PCR-RFLP and sequence determination. Comparative analysis revealed a highly variable region containing an RGD motif in the receptor domain of $MrkD_{V3}$. In order to determine if the sequence confers the *K. pneumoniae* $mrkD_{V3}$ the highest level of the fimbrial activity, a type 3 fimbriae display system was constructed in *Escherichia coli*. The *E. coli* JM109[$pmrkABCD_{V3}F$] displaying meshwork-like fimbriae also had the most fimbrial activity, supporting a possible role of the varied sequences. In a dose-dependent manner, the GRGDSP hexapeptide appeared to inhibit the adhesion of the *E. coli* JM109[$pmrkABCD_{V3}F$] to HCT-8, an ileocecal epithelial cell line. In addition, the adhesion activity was reduced by the addition of anti-$\alpha5\beta1$ integrin monoclonal antibody, indicating that the RGD containing region in $MrkD_{V3}$ is responsible for the binding of type 3 fimbriae to integrin.

3.2 Results and discussion

3.2.1 Identification of four novel mrkD alleles

Recently, the incidence of *K. pneumoniae* meningitis in newborns and adult patients have been reported worldwide (Lee *et al*., 2003). Since the role of type 3 fimbriae in determining the tissue tropism has been suggested (Hornick *et al*., 1992; Schurtz *et al*., 1994), the presence of a specific type 3 fimbrial adhesin *mrkD* allele in meningitis isolates was investigated. Using the primers specific to $mrkD_{1p}$, PCR analysis showed that all the 17 meningitis isolates carry *mrkD* gene, and four different *mrkD* RFLP types were obtained. Each of the PCR products was then cloned and their sequences determined. The BLASTX (http://www.ncbi.nlm.nih.gov/BLAST/) analysis revealed 4 novel *mrkD* alleles, designated $mrkD_{V1}$, $mrkD_{V2}$, $mrkD_{V3}$, and $mrkD_{V4}$ (under the GenBank accession number AY225462, AY225463, AY225464, and AY225465). Notably, fourteen of the isolates carry $mrkD_{V1}$ RFLP and others include one each of the variants $mrkD_{V2}$ (VHm2), $mrkD_{V3}$ (VHm5) and $mrkD_{V4}$ (VHm10). This suggests that the *K. pneumoniae* carrying $mrkD_{V1}$ RFLP is a prevalent strain. All the isolates carry *mrkD* gene implying a possible correlation of type 3 fimbriae with the disease. Nevertheless, more isolates are needed to establish the association.

3.2.2 Amino acid sequence analysis

Comparative analysis with sequence of *K. pneumoniae* MGH78578 (http://genome.wustl.edu/) revealed an identical *mrkD* except that a G deletion was found in *mrkD*$_{V1}$ at the position 355. The nucleotide deletion caused a frame shift and resulted in a truncated protein, of which the pilin domain was replaced with a garbled sequence of 57 amino acid residues at the C-terminus. It is hence the name MrkD$_{V1T}$ for the truncated form of the adhesin. As shown in Fig. 1, the conserved receptor binding and pilin domains, and the cysteine residues could be identified in each of the MrkD variants. The comparison indicated that MrkD$_{V2}$ and MrkD$_{V4}$ share the most identity, which is 88.1%. Less were found between MrkD$_{V2}$ and MrkD$_{V3}$ with 79.3% identity, and MrkD$_{V3}$ and MrkD$_{V4}$ with 80.2%. In the receptor domain of MrkD$_{V3}$, a varied sequence from residues 120 to 140, and an RGD motif of integrin recognition site (Ruoslahti and Pierschbacher, 1987) were identified (Fig. 1). In addition, the residues which have been proposed to facilitate the interaction of MrkD with other fimbrial component (Sebghati and Clegg, 1999), were unique in MrkD$_{V3}$ (C102 and R200). The D-R-N (residues 68 to 70) of MrkD$_{1P}$ that has been shown to affect the fimbrial activity (Sebghati and Clegg, 1999) appeared to be replaced by different residues in MrkD$_{V2}$, MrkD$_{V3}$ and MrkD$_{V4}$. These implied regulatory roles of the

varied sequences for the fimbriae activity.

3.2.3 Type 3 fimbriae activity of the meningitis isolates

It has been reported that type 3 fimbriae of *K. pneumoniae* mediate a specific adherence to different kinds of human epithelial cells (Schurtz *et al*., 1994; Tarkkanen *et al*., 1997). To examine influences of the *mrkD* allelic variation on the fimbrial adhesive activity, three epithelial cell lines Hep-2, HCT-8, and Int-407 were used. As shown in Fig. 2A, the bacteria VHm5 of $mrkD_{V3}$ allele exerted a relatively higher level of the cell adhesion activity. While, 14 of the $mrkD_{V1}$ strains revealed different levels of activity. The subsequent analysis using western blotting hybridization with the anti-MrkA antiserum indicated that the expression of type 3 fimbriae could only be observed in VHm2 ($mrkD_{V2}$), VHm5 ($mrkD_{V3}$), and 6 of the $mrkD_{V1}$ strains (Fig. 2B). These implied that, besides type 3 fimbriae, other factor(s) such as capsular polysaccharide, which has been reported to impede the bacterial adherence to cells (Sahly *et al*., 2000), is/are involved in determining the cellular adherence activity.

3.2.4 Expression of the recombinant type 3 fimbriae

To rule out the possibility that other factors resided in *K. pneumoniae* interfere with the activity of type 3 fimbriae, an *E. coli* type 3 fimbriae display system was

established. The production of type 3 fimbriae on the surface of the recombinant bacteria was confirmed by western blot analysis (Fig. 3). The TEM analysis (Fig. 4) revealed that no fimbriae on the surface of JM109[pGEMT-easy] could be observed. Only in a small portion, approximately one tenth of the bacteria JM109[pmrkABC], some short and erect fimbriae were found. In addition, several long fimbriae were found on the surface of *E. coli* JM109[pmrkABCF], suggesting that MrkF, as a minor fimbrial subunit, is able to function as an initiator for the growth of the filament. In the absence of MrkD adhesin, however, the growth of filament could not be properly terminated and hence appeared lengthy. The speculation is supported by the appearance of extremely long and bundle fimbriae on the surface of *E. coli* JM109[pmrkABCD$_{V1T}$F], which could be caused by an interaction of the truncated MrkD$_{V1T}$ with the usher protein leading to uncontrollable length of the fimbriae.

Different from the uniform fimbrial pattern observed on JM109[pmrkABCD$_{V2}$F] and JM109[pmrkABCD$_{V4}$F], the fimbriae on the surface of JM109[pmrkABCD$_{V3}$F] are entangled and give rise to a meshwork-like morphology. The sequence comparison in Fig. 1 indicated that unique residues of MrkD $_{V3}$ are probably the determinants in facilitating MrkD interaction with other fimbrial protein for the distinct morphology.

3.2.5 The mrkF gene is a component of mrkABCDF operon

The common structural characteristics of major pilin subunit are (i) two cysteine residues; (ii) a conserved pattern of alternating hydrophobic residues at position 4, 6, and 7 from the COOH terminus; (iii) a penultimate tyrosine; and (iv) a Gly at position 14 from COOH terminus (Girardeau *et al*., 2000). As shown in Fig. 5, sequence analysis of MrkF revealed a signal peptide using LipoP in ExPASy proteomic tools and all the characteristics of major subunit. In addition, MrkF also possessed sequence motifs that are conserved among fimbrial subunits (Girardeau and Bertin, 1995). The intergenic sequence of 16 bp between *mrkD* and *mrkF* also implied *mrkF* is a component of the *mrk* operon structure of *mrkABCDF*. The RT-PCR analysis of *mrk* expression in *K. pnemoniae* NTUH-K2044 revealed a co-expression of *mrkA*, *mrkD*, *mrkF*, and the intergenic region between *mrkD* and *mrkF* (Fig. 6). This indicated *mrkF* belongs to *mrk* operon. The previous study using *E. coli* display system has shown MrkF played a role in regulating the length of the filament and the fimbrial activity (廖心瑋，民國九十五年). A *mrkF* deletion mutant is being constructed, and the fimbrial morphology and activity will be compared to that of wild-type bacteria toward understanding the role of MrkF.

3.2.6 Activity assessments of the recombinant fimbriae

As shown in Table 3, the bacteria JM109[pmrkABCD$_{V3}$F] and JM109[pmrkABCD$_{V4}$F] expressed approximately 16 HA units, and JM109[pmrkABCD$_{V2}$F] had less of the activity. Whereas, JM109[pmrkABCD$_{V1T}$F] as well as the bacteria carrying pGEMT-easy, pmrkABC, or pmrkABCF exhibited no hemagglutination. This suggested that the MrkD$_{V1T}$ truncation alters conformation of the MrkD receptor binding domain and hence no hemagglutination activity could be detected. As shown in Fig. 7A, JM109[pmrkABCD$_{V3}$F] expressed the highest level of adhesive activity to either of the three cell lines. Allelic variation of MrkD has been shown to affect the binding activity and specificity to collagen (Schurtz *et al*., 1994). The Fig. 7B showed that JM109[pmrkABCD$_{V3}$F] also revealed the strongest binding activity to collagen IV and V, and JM109[pmrkABCD$_{V4}$F] had a medium level activity. Moreover, the biofilm formation analysis revealed that JM109 [pmrkABCD$_{V3}$F] retained the highest activity (Fig. 7C). JM109[pmrkABCD$_{V2}$F] and JM109[pmrkABCD$_{V4}$F] also exhibited a comparable activity of biofilm formation. These support the finding that type 3 fimbriae is a major determinant for *K. pneumoniae* biofilm formation (Jagnow and Clegg, 2003). As shown in Fig. 1, the sequence comparison indicated that the D-R-N (residues 68 to 70) of MrkD$_{1P}$ and the variation sequence in the receptor domain of MrkD$_{V3}$ are probably the determinants

the fimbrial activity. Interestingly, an autoaggregation phenotype was observed only for JM109[pmrkABCD$_{V3}$F] (Fig. 7D) suggesting the meshwork like fimbriae increased the interaction of the bacteria. The alteration of receptor-binding domain of FimH has been shown to affect the autoaggregation (Schembri *et al.*, 2001). It is also likely that the varied sequence in the receptor domain of MrkD$_{V3}$ also confers the bacteria an autoaggregation property.

Since the number of fimbrial filaments varies among bacterial cells, the above-mentioned activity measurements could only determine the average binding activity between a given bacterial population and the target molecules. To determine precisely the direct interacting force between a fimbria and its target molecules, the optical tweezers was also used to investigate whether the MrkD really plays a role in presenting the highest activity of highest JM109[pmrkABCD$_{V3}$F]. Therefore, a typical record of the bead's displacement during the measurement is illustrated in Fig. 8. The adhesive force of a single fimbria, which expressed with each of *mrkD* alleles, to collagen IV measured using optical tweezers were 2.03 ± 0.03 pN, 3.79 ± 0.12 pN, and 2.87 ± 0.15 pN for *mrkD$_{V2}$*, *mrkD$_{V3}$*, and *mrkD$_{V4}$*, respectively. This further supported the result of collagen binding analysis. It has been reported that interacting force between a single type 1 fimbriae and an α-C-mannoside ligand (Liang *et al.*, 2000) was 1.7 pN. The adhesive force was close to our data of the adhesive force

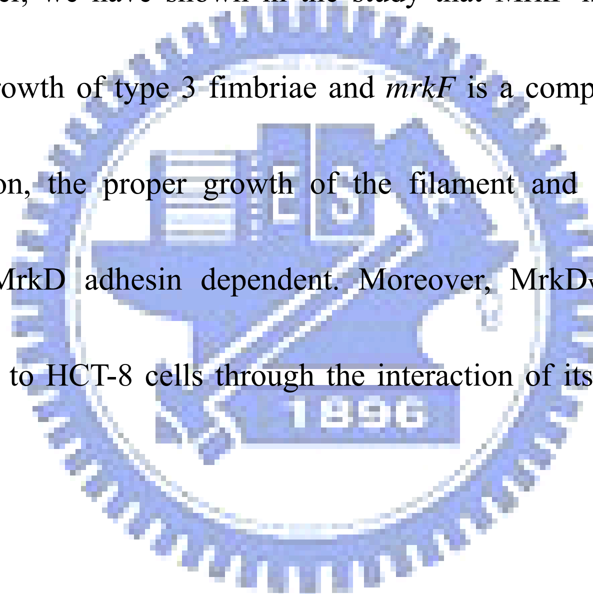
between type 3 fimbriae and collagen IV, suggesting that type 1 and type 3 fimbriae may be expression in the part of body with same fluid flow rate. According to Stokes's Law (F=6(pi)RnVc, R is the radius of the sphere, n is the viscosity, and Vc is the velocity through a continuous fluid), it is calculated that 1 pN could support 1 μm bead to resistant 50 μm/sec flow rate. In addition, the calculated flow rate is about 40 mm/sec in the bladder and about 300 μm/sec in blood capillary. In addition, the data of the adhesive force of a single fimbria of type 3 fimbriae will give more information in the bacterial infection research.

3.2.7 RGD peptide inhibits the adhesion of JM109[pmrkABCD$_{V3}$F] to HCT-8

It has been reported that the RGD sequence in FHA (Filamentous hemagglutinin) of *Bordetella pertussis* is involved in the interaction of the bacteria with macrophage (Relman *et al.*, 1990). To determine if the RGD motif in MrkD$_{V3}$ affects the bacterial adherence to cells, the peptide GRGDSP was added as a competitor in the cell adherence assay. As shown in Table 2, the adhesion of JM109[pmrkABCD$_{V3}$F] to HCT-8 cell was reduced by the addition of GRGDSP and the inhibition was in a dose-dependent manner. In contrast, no inhibition was observed when GRADSP peptide was added. This supported a role of the RGD sequence in affecting the adhesion activity of the fimbriae. RGD tripeptide, which is present in many adhesive

ECM and cell surface proteins, is recognized by integrins on the cell surface (Ruoslahti, 1996). The RGD sequence of *B. pertussis* FHA has been demonstrated to interact specifically with $\alpha5\beta1$ integrin (Ishibashi *et al*., 2001). As shown in Table 4, the anti-$\alpha5\beta1$ integrin monoclonal antibody was able to inhibit the adhesion of JM109[pmrkABCD$_{V3}$F] to HCT-8, indicating the presence of an interaction of MrkD $_{V3}$ with $\alpha5\beta1$ integrin.

Taken together, we have shown in the study that MrkF is able to serve as an initiator for the growth of type 3 fimbriae and *mrkF* is a component of *mrkABCDF* operon. In addition, the proper growth of the filament and fimbrial morphology appeared to be MrkD adhesin dependent. Moreover, MrkD$_{V3}$ may promote the bacterial adhesion to HCT-8 cells through the interaction of its RGD sequence with integrin.



Chapter IV

Regulation of expression of type 3 fimbriae in *Klebsiella pneumoniae* CG43

4.1 ***Abstract***

A lamda phage clone containing type 1- and type 3-fimbriae gene clusters was isolated from a genomic library of *Klebsiella pneumoniae* CG43, a K2 isolate with a high virulence. In-between the two gene clusters, homologues of *Erwinia chrysanthemi* virulence regulatory genes *pecS* and *pecM* were identified and named *phgS* and *phgM* respectively. The expression of type 3 fimbriae in LB or GCAA medium could be readily demonstrated in the bacteria. Whereas, the expression of type 1 fimbriae was evident only in the *mrkA* deletion mutant or in the overexpression mutant of *fimB*, a recombinase-encoding gene. Interestingly, overexpression of *fimB* in turn diminished the expression of type 3 fimbirae. The promoter activity measurement using LacZ as reporter revealed that the deletion of *phgS* or *phgM* reduced the activity of the putative promoter $P_{S\text{-}mrk}$, which suggesting a PhgS/PhgM-dependent expression of type 3 fimbriae. The binding of the recombinant PhgS to $P_{S\text{-}mrk}$ demonstrated by EMSA also supported a transcriptional regulation of PhgS on the expression of type 3 fimbriae. Nevertheless, a PhgS/PhgM independent promoter $P_{L\text{-}mrk}$ was also identified indicating a complicated regulation on the expression of type 3 fimbriae.

4.2 Results and discussion

4.2.1 Type 1 and 3 fimbrial gene clusters are physically linked in K. pneumoniae CG43

The entire gene cluster was isolated from a λDASH genomic library, which was constructed previously in the laboratory (Peng *et al.*, 1992), and the nucleotide sequence determined. Comparative analysis with the corresponding sequences in the genome of *K. pneumoniae* MGH78578 (http://genome.wustl.edu/) revealed 98% nucleotide sequence homology. The *mrkE* and *mrkG* that were reported upstream of the type 3 fimbrial gene cluster *mrkABCDF* (Sebghati *et al.*, 1998) could not be found. Instead, four open reading frames namely *orf1*, *phgS*, *phgM*, and *orf2* were identified (Fig. 9). *Orf1* encodes a protein of 35% amino acid sequence identity with a hypothetical membrane protein of *E. coli* (Dobrindt *et al.*, 2002) and *orf2* shares 42% sequence identity with an ABC-type transporter of *Yersinia frederiksenii*. PhgS and PhgM reveal 36% and 44% amino acid sequence identities respectively with the proteins PecS and PecM of *E. chrysanthemi*. PecS, a transcription regulator with a MarR type of DNA binding domain, and PecM, a putative membrane protein, have been shown to work cooperatively to regulate the virulence of *E. chrysanthemi* (Praillet *et al.*, 1997). PhgM as well as PecM contain 10 membrane-spanning

segments and PhgS carries also a MarR type of DNA binding domain. The finding implies that the regulatory genes, the *phgM* and *phgS*, were recruited to the *mrkABCDF* gene cluster after the gene cluster was organized.

Interestingly, a type 1 fimbriae encoding gene cluster was found next to *orf1* in a divergent transcription orientation (Fig. 9). The close association of the *fim* and *mrk* fimbrial gene clusters is intriguing and has not been reported in any other bacteria. Comparative analysis of the available sequences of the partly completed genome of *K. pneumoniae* MGH78578 also revealed a similar structure, indicating that the physically linked gene organization is not uncommon in *K. pneumoniae*. It has been demonstrated that multiple fimbrial adhesins are required for the establishment of a systemic infection caused by some bacteria and expression of the individual adhesin is known to be conditionally regulated (Schwan *et al.*, 2002). It is therefore reasonable to propose that a coordinate regulation like that between type 1 and the type P fimbriae in *E. coli* is also present in *K. pneumoniae,* between the type 1 and type 3 fimbriae.

In addition, comparing to the type 1 fimbriae operon in *E. coli* (Blattner *et al.*, 1997), the inverted repeats contained in *fim* promoter recognized by the recombinase FimB or FimE were found to be preserved. However, the intergenic regions of *fimA-fimE*, *fimE-fimB*, and *fimB-orf1* shared only 30%, 50%, and 40% nucleotide

sequence homologies, respectively. The transcription factors NagC and NanR binding elements contained upstream of *E. coli fimB* (Sohanpal *et al*., 2004) could not be found, suggesting a different regulation on the expression of type 1 fimbirae in *K. pneumoniae*.

4.2.2 Expression of type 1 and type 3 fimbriae in K. pneumoniae CG43S3

As shown in Fig. 10A, non-specific protein bands were found to cross react with the generated anti-MrkA antiserum in the western blotting analysis. Nevertheless, the detection of specific band with the molecular weight corresponding to MrkA suggested expression of the major pilin of type 3 fimbriae. The expression of type 3 fimbriae in *K. pneumonaie* CG43S3 was further confirmed using TEM detection with an immunogold-labeled anti-MrkA antibody (Fig. 10B). Expression of type 3 fimbriae could be readily detected while *K. pneumonaie* CG43 grown in LB or GCAA medium, suggesting a primary role of type 3 fimbriae in attachment to solid surface. This is consistent with the report that type 3 fimbriae played as a major adherence factor for *K. pneumoniae* to establish infections (Tarkkanen *et al*., 1997).

Although some of the DNA resulted from incomplete digestion was observed, the DNA pattern shown in Fig. 10C type 1 indicated an "OFF" phase promoter of the type 1 fimbriae by using promoter analysis. In *E. coli*, expression of type 1 fimbriae

has been commonly shown to be required for a stable cell-to-surface attachment (Pratt and Kolter, 1998). On the other hand, the expression of type 1 fimbriae were rarely observed in *K. pnemoniae* (Di-Martino *et al*., 2003; Matatov *et al*., 1999). Therefore, it is not surprising to find that the promoter of type 1 fimbriae in *K. pneumoniae* CG43 is in OFF phase.

The mutant with a specific deletion of *mrkA* gene was subsequently generated to investigate if a cross-talk regulation is involved in the expression of the two fimbriae. The specific band corresponding to MrkA, which was not detected in the *mrkA* mutant, reappeared in the bacteria transformed with the *mrkA* expression plasmid pYJA (Fig. 10A). As assesses by TEM analysis, the *mrkA* mutant exhibited fimbriated phenotype suggesting a coordinate regulation for the expression of type 3 fimbriae and an unknown type of fimbriae. The expression of FimA protein, the major pilin of type 1 fimbriae, became evident in the *mrkA* deletion mutant as determined using anti-FimA antibody (Fig. 10D) suggesting the unknown type of fimbriae appeared on the surface of *mrkA* mutant is type 1 fimbriae. As shown in Fig. 10E, an increase of yeast agglutination activity which could be diminished with mannose in the *mrkA* deletion mutant also supported the expression of type 1 fimbriae. Nevertheless, a certain level of the mannose-sensitive yeast agglutination activity could be detected in either the

wild type bacteria or the *mrkA* mutant carrying with pYJA, suggesting a co-expression of type 1 and type 3 fimbriae in the bacteria.

4.2.3 Overexpression of fimB turned on the expression of type 1 fimbriae but repressed type 3 fimbrial expressio

Multiple fimbrial adhesins have been demonstrated to be required for bacteria to establish a systemic infection (Schwan *et al*., 2002). It is generally anticipated that expression of the individual adhesin is cooperatively regulated. In order to demonstrate further a coordinate control of the expression between type 1 and type 3 fimbriae, a construct to overexpress FimB recombinase in *K. pneumoniae* CG43S3 was generated. The analysis of *fim* promoter as shown in Fig. 11A revealed "ON"-phase of type 1 fimbriae in the bacteria carrying the expression plasmid pETQ33-*fimB*. The expression of type 1 fimbriae could be switched on by overproduction of FimB recombinase indicating the *fim* gene cluster is functional in the bacteria. An apparent increase of mannose-sensitive yeast agglutination activity further supported the expression of type 1 fimbriae (Fig. 11B). The overexpression of FimB recombinase was evident by the analysis of protein pattern (Fig. 11C-1). Notably, the *fimB* overexpression turned on the expression of FimA (Fig. 11C-2) but diminished the expression of MrkA (Fig. 11C-3).

The deletion of *mrkA* increased the expression of type 1 fimbriae, while the overproduction of FimB recombinase repressed MrkA expression. Together, both indicated that the expression of type 3 fimbriae and type 1 fimbriae are regulated in a coordinate manner in *K. pneumoniae*. It has been reported that planktonic cells of nonfimbriated and fimbriated cell differ in their OMP patterns (Otto *et al*., 2001), and *ompA* deletion decreased the expression of type 1 fimbriae in *E. coli* K1 (Teng *et al*., 2006). Furthermore, subunit misfolding of P pili was sensed by two parallel pathways: the Cpx two component signaling system and the sigma E modulatory pathway (Jones *et al*., 1997). A report also indicated that CpxR-P competes with Lrp for binding to both promoter proximal and distal *pap* DNA binding sites, inhibiting pap transcription in vitro and pili expression in vivo (Hernday *et al*., 2004). Thus, we proposed that the deletion of *mrkA* or overexpression of *fimB* may result in the envelope stress and activation of the sensing system in the inner-membrane leading to cross-talk regulation.

4.2.4 PhgS/PhgM-dependent or PhgS/PhgM-independent expression of type 3 fimbriae

In *E. chrysanthemi*, PecS has been reported to regulate the expression of cellulase, pectate lyase, and indigoidine, and biosynthesis of flagella (Reverchon *et al*.,

1994). In addition, PecS could positively regulate the expression of polygalacturonase enzyme (Nasser *et al*., 1999). The *phgS* and *phgM* deletion mutants were generated to investigate whether PhgS and PhgM proteins play regulatory role in the expression of the physically linked *fim* and *mrk* gene clusters. The putative promoters of P_{fimB} and P_{fimE} (Fig. 9), containing respectively the intergenic regions of *fimB*-*orf1*and *fimE*-*fimB*, and the putative promoters of *mrk* genes, $P_{L\text{-}mrkA}$ and $P_{S\text{-}mrkA}$ (Fig. 9), containing intergenic region of *orf2*-*mrkA* and approximately 500-bp non-coding region upstream of *mrkA* start codon were also isolated by PCR cloning. Each of the putative promoters was then cloned in front of the promoterless *lacZ* on pLacZ15. The resulting plasmids were transformed into wild type, *phgS* deletion mutant, or *phgM* deletion mutant, respectively, and the β-galactosidase activities measured and compared. Since the promoter activities appeared to be much higher in static cultures in either LB or GCAA, the following measurements were carried out while the bacteria grown in LB in static cultures.

PecM is an inner membrane protein involved in transduction of an extracellular signal (Reverchon *et al*., 1994). Several stimuli including changes of pH, osmolarity, and temperature that have been reported to affect the expression of type 1 fimbriae (Schwan *et al*., 2002) were also applied to evaluate the role of PhgM on expression of type 3 fimbriae. However, the deletion of *phgM* rendered no apparent change of

$P_{S\text{-}mrkA}$ activity under any of the conditions. The effect of *phgM* deletion was only evident while the bacteria grown in static culture, suggesting PhgM senses the low level of oxygen to trigger the expression of type 3 fimbriae.

As shown in Fig. 12A, the deletion of *phgS* or *phgM* appeared to diminish the activity of $P_{S\text{-}mrkA}$ suggesting a PhgS- and PhgM-dependent expression of the promoter. On the other hand, either deletion exerted no effect on the activity of $P_{L\text{-}mrkA}$, P_{fimB}, or P_{fimE}. In addition, activity of $P_{L\text{-}mrkA}$ that carries extra 247 bp at 5'end of $P_{S\text{-}mrkA}$ (Fig. 9), appeared to be lower than the activity of $P_{S\text{-}mrkA}$ implying the presence of a negative regulatory element in the extended region. As shown in the left panel of Fig. 12B, a small amount of soluble PhgS could be obtained after the treatment of the inclusion body with 6M urea and the mixture dialyzed against PBS buffer. The purified PhgS was shown to be able to bind $P_{S\text{-}mrkA}$ DNA and retard the DNA mobility (on the right panel of Fig. 12B). In addition, the formation of binding complex could be inhibited by adding excess amount of unlabelled $P_{S\text{-}mrkA}$ DNA. However, addition of pUC19 DNA also appeared to be able to inhibit the formation of binding complex indicating a non-specific binding of PhgS to the promoter $P_{S\text{-}mrkA}$. It has been reported that a palindromic sequence (CGANWTCGTA)TAT(TACGANNNCG) recognized by PecS could be identified (Rouanet *et al.*, 2004). As well as PecS, PhgS has a MarR type DNA binding domain. Nonetheless, no similar palindromic sequence could be

found on $P_{S\text{-}mrkA}$. It has been suggested that PecS interacts with PecM to exert its regulatory activity (Praillet *et al.*, 1997). In analogy with the regulatory mechanism, the lack of binding specificity of the recombinant PhgS to $P_{S\text{-}mrkA}$ may be compensated by its binding to PhgM. The possibility is currently being investigated.

As shown in Fig. 12A, the $P_{L\text{-}mrkA}$ activity was independent to the control by either PhgS or PhgM. The subsequent RT-PCR analysis revealed that the deletion of either *phgS* or *phgM* exerted no negative effect on the levels of *mrkA* transcript and the amount of MrkA transcript in either mutant appeared to be even higher than that synthesized in wild type bacteria (Fig. 12C). Western blotting analysis shown in Fig. 10D also indicated a PhgS/PhgM-independent expression of MrkA protein.

4.2.5 CRP and LRP probably play a role for the PhgS/PhgM-independent expression of type 3 fimbriae

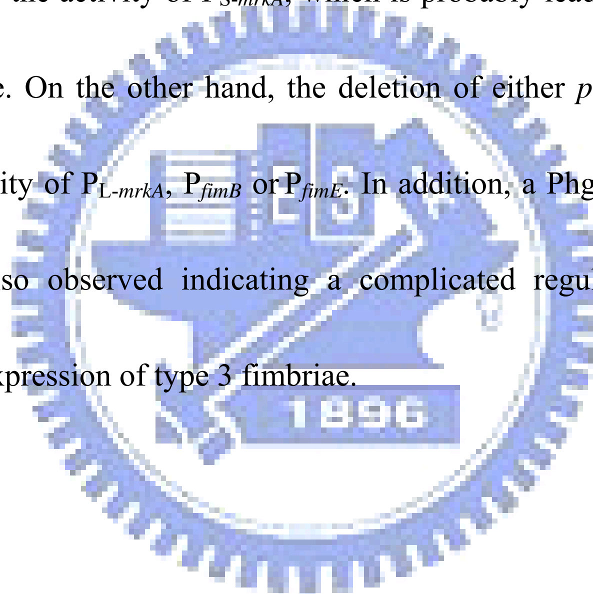
The expression of a certain genes promoted by two separate condition-controlled promoters is not uncommon in bacteria (Heroven *et al.*, 2004). The identification of PhgS/PhgM-dependent and PhgS/PhgM-independent promoters implies different transcripts are produced. In order to map the transcription start site of *mrkA*, RT-PCR analysis using different pairs of primers were carried out. As shown in Fig. 13, the transcripts extended to 5'-end of pA5 or pA6 were detected and confirmed by

Southern blotting hybridization using the PCR product amplified with primer pair pA2 and pA3 as a probe. No transcript was obtained while the primer pA5 replaced with pA4 indicating that the transcription start site of *mrkA* could be assigned to the sequence between pA4 and pA5. Analysis of the sequences upstream of pA4 revealed a conserved LRP and CRP binding elements, 5'-YAGHAWATTWTDCTR-3' (Y=C or T, H≠G, W=A or T, D≠C, R=A or G) (Cui *et al*., 1995) and 5'-TGTGA -N_6-TCATC-3' (Kolb *et al*., 1993), respectively. We speculate that other than the regulation of PhgS/PhgM, LRP or CRP also plays a role on the expression of type 3 fimbriae.

CRP, cAMP receptor protein, has been reported to function both as positive and negative effector to influence many gene expression (Botsford and Harman, 1992). LRP, leucine-responsive regulatory protein, regulates the expression of more than 40 genes and proteins in *E. coli* (Calvo and Matthews, 1994). To determine if CRP and LRP play a regulatory role on the expression of type 3 fimbriae, RT-PCR is being employed to analyze the effect of addition of glucose or leucine on the expression of type 1 and type 3 fimbriae. It is probably that the PhgS/PhgM-independent promoter $P_{L\text{-}mrkA}$ is under control by CRP or/ and LRP, which had been shown to affect the expression of type 1 fimbriae or P pili (Baga *et al*., 1985; Kelly *et al*., 2006) The hypothesis is that, in the presence of certain level of glucose or leucine, CRP or LRP

determines the cross-regulation of the expression of type 1 and type 3 fimbriae. While in the presence of a lot of PhgS and PhgM regulatory proteins, $P_{L\text{-}mrkA}$ is under a negative control and PhgS and PhgM play a major role to positively regulate the expression of type 3 fimbriae. However, the possibility remains to be verified.

Overall, a cross-talk regulation was demonstrated between the expression of type 1 fimbriae and type 3 fimbriae. In addition, PhgS/PhgM appeared to be able to regulate positively the activity of $P_{S\text{-}mrkA}$, which is probably leading to the expression of type 3 fimbriae. On the other hand, the deletion of either *phgS* or *phgM* had no effect on the activity of $P_{L\text{-}mrkA}$, P_{fimB} or P_{fimE}. In addition, a PhgS/PhgM-independent regulation was also observed indicating a complicated regulatory mechanism is involved for the expression of type 3 fimbriae.



Chapter V

Summary

The incidence of *Klebsiella* infection is found to increase in the recent years. Antibiotics are usually used to cure *K. pneumoniae* infections. However, misuse of antibiotics caused the nosocomial *Klebsiella* infections even more severe because of the emergence of muti-drug resistance strains. Therefore, to find new targets for drug intervention to the muti-drug resistance strains is important.

Attachment of pathogens to their host is a prerequisite step of infection and adhesin is one of the major virulence factors involved in *K. pneumoniae* infections. Expression of type 3 fimbriae has been correlated with several *Klebsiella* infections. However, little is known about the roles of the fimbriae in *K. pneumoniae* pathogenesis. In part I, to rule out the possibility that other factors resided in *K. pneumoniae* interfere with the activity of type 3 fimbriae, an *E. coli* type 3 fimbriae display system was established. By using this display system, we have demonstrated that the proper growth of the filament and fimbrial morphology were MrkD adhesin dependent. The changes of fimbrial morphology in turn affected the fimbrial activity as assessed by collagen binding, cell adherence, and biofilm formation. In addition, *E. coli* JM109[pmrkABCD$_{V3}$F] was found to have the highest level of fimbrial activity and the adhesion to HCT-8 cells was probably due to the interaction of RGD sequence on MrkD$_{V3}$ with integrin. Overall, MrkD adhesin appeared to play a major role in determining the activity of type 3 fimbriae and hence the MrkD is a potent drug

target.

Since only one study (Allen *et al*., 1991) of a possible role to stabilize type 3 fimbriae the function, how MrkF stables the type 3 fimbirae and the other function roles of MrkF is not known. As determined previously using the recombinant *E. coli* display system, MrkF was shown to be able to serve as an initiator for the growth of the filament and likely a regulator for the length and the activity of the fimbriae. The demonstration of an operon structure further supported MrkF is a component of the fimbriae. The possibility is investigated by construction of a *mrkF* deletion mutant and the fimbrial morphology and activity of the mutant will be compared to that of wild-type bacteria. The result provide us the information of how MrkF maintains the stability of type 3 fimbirae.

It is generally believed that coordinate expression of multiple fimbriae is required for the establishment of a systemic infection. In part II, a cross regulation of the expression of type 1 and type 3 fimbriae was demonstrated. Both PhgS/PhgM-dependent and PhgS/PhgM-independent promoters of type 3 fimbriae were identified, indicating a complex regulatory system is involved for the expression of type 3 fimbriae. The promoter for *fim* operon transcription is carried on a 314-bp invertible DNA and is catalyzed by FimB and FimE sitespecific recombinases (Abraham *et al*., 1985). In addition to the inversion of the DNA by recombinases,

LRP protein also acts to determine the expression of type 1 fimbriae (Kelly *et al*., 2006). Although the CRP has not been found to affect the expression of type 1 fimbrial, CRP-depedent expression of P fimbriae has been reported (Baga *et al*., 1985). The presence of consensus binding elements of LRP and CRP in the putative promoter $P_{L\text{-}mrk}$ suggested the involvement of the two regulators in modulating the expression of type 3 fimbriae.

References:

呂文鈴，國立清華大學碩士論文，克雷白氏肺炎菌 CG43 第三型纖毛黏附因子的鑑定，中華民國九十年六月。

陳美甄，國立交通大學碩士論文，克雷白氏肺炎菌 CG43 第三型纖毛黏附蛋白特性，中華民國九十一年六月。

廖心瑋，國立交通大學碩士論文，克雷白氏肺炎菌 CG43 第三型纖毛組成蛋白 MrkD 與 MrkF 之功能探討，中華民國九十五年六月。

Abraham J. M., C. Freitag, J. R. Clements, and B. I. Eisenstein. 1985. An invertible element of DNA controls phase variation of type 1 fimbriae of *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. **82:**5724-5727.

Allen, B.L., G. –F. Gerlach, and S. Clegg. 1991. Nucleotide sequence and functions of *mrk* determinants necessary for expression of type 3 fimbriae in *Klebsiella pneumoniae*. J. Bacteriol. **173:**916-920.

Baga, M., M. Goransson, S. Normark, and B. E. Uhlin. 1985. Transcriptional activation of a *pap* pilus virulence operon from uropathogenic *Escherichia coli*. EMBO J. **4:**3887-3893.

Bergogne-Berezin, E. 1995. Treatment and prevention of nosocomial pneumonia. Chest **108:**26S-34S.

Blattner, F. R., G. 3rd. Plunkett, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao. 1997. The complete genome sequence of *Escherichia coli* K-12. Science **277:**1453-1474.

Blomfield, I. C. 2001. The regulation of *pap* and type 1 fimbriation in *Eacherichia coli*. Adv. Microbiol. Physicol. **45:**1-49.

Blumer, C., A. Kleefeld, D. Lehnen, M. Heintz, U. Dobrindt, G. Nagy, K. Michaelis, L. Emody, T. Polen, R. Rachel, V. F. Wendisch, and G. Unden. 2005. Regulation of type 1 fimbriae synthesis and biofilm formation by the transcriptional regulator LrhA of *Escherichia coli*. Microbiology **151:**3287-3298.

Boddicker, J. D., R. A. Anferson, J. Jagnow, and S. Clegg 2006. Signature-tagged mutagenesis of *Klebsiella pneumoniae* to identify genes that influence biofilm formation on extracellular matrix material. Infect. Immun. **74:**4590-4597.

Botsford, J. L., and J. G. Harman. 1992. Cyclic AMP in prokaryotes. Microbiol. Rev. **56:**100-122.

Calvo, J. M., and R. G. Matthews. 1994. The leucine-responsive regulatory protein, a global regulator of metabolism in *Escherichia coli*. Microbiol. Rev. **58:**466-490.

Chang, F. Y., and M. Y. Chou. 1995. Comparison of pyogenic liver abscesses caused by *Klebsiella pneumoniae* and non-*K. pneumoniae* pathogens. J. Formos. Med. Assoc. **94:**232-237.

Chang, H. Y., W. L. Deng, J. H. Lee, T. F. Fu, and H. L. Peng. 1996. Virulence and outer membrane properties of a *Klebsiella pneumoniae galU* mutant. Microb. Pathog. **20:**255-261.

Cheng, H. P., L. K. Siu, and F. Y. Chang. 2003. Extended-spectrum cephalosporin compared to cefazolin for treatment of *Klebsiella pneumoniae*-caused liver abscess. Antimicrob. Agents Chemother. **47:**2088-2092.

Clegg, S., and G. F. Gerlach. 1987. Enterobacterial fimbriae. J. Bacteriol. **169:**934-938.

Cui, Y., Q. Wang, G. D. Stormo, and J. M. Calvo. 1995. A consensus sequence for binding of Lrp to DNA. J. Bacteriol. **177:**4872-4880.

de Lorenzo, V., and K. N. Timmis. 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons. Methods Enzymol. **235:**386-405.

Di Martino, P., Y. Bertin, J. P. Girardeau, V. Livrelli, B. Joly, and A. Darfeuille-Michaud. 1995. Molecular characterization and adhesive properties of CF29K, an adhesin of *Klebsiella pneumoniae* strains involve in nosocomial infections. Infect. Immun. **63:**4336-4344.

Di Martino, P, N. Cafferini, B. Joly, and A. Darfeuille-Michaud. 2003. *Klebsiella pneumoniae* type 3 pili facilitate adherence and biofilm formation on abiotic surfaces.

Res. Microbiol. **154:**9-16.

Di Martino, P., V. Livrelli, D. Sirot, B. Joly, and A. Darfeuille-Michaud. 1996. A new fimbrial antigen harbored by CAZ-5/SHV-4-producing *Klebsiella pneumoniae* strains involved in nosocomial infections. Infect. Immun. **64:**2266-2273.

Dobrindt, U., G. Blum-Oehler, G. Nagy, G. Schneider, A. Johann, G. Gottschalk, and J. Hacker. 2002. Genetic structure and distribution of four pathogenicity islands (PAI I(536) to PAI IV(536)) of uropathogenic *Escherichia coli* strain 536. Infect. Immun. **70:**6365-6372.

Favre-Bonte, S., A. Darfeuille-Michaud, and C. Forestier. 1995. Aggregative adherence of *Klebsiella pneumoniae* to human intestine-407 cells. Infect. Immun. **63:**1318-1328.

Firon, N., S. Ashkenazi, D. Mirelman, I. Ofek, and N. Sharon. 1987. Aromatic alpha-glycosides of mannose are powerful inhibitors of the adherence of type 1 fimbriated *Escherichia coli* to yeast and intestinal epithelial cells. Infect. Immun. **55:**472-476.

Gally, D. L., J. Leathart, and I. C. Blomfield. 1996. Interaction of FimB and FimE with the *fim* switch that controls the phase variation of type 1 fimbriae in *Escherichia coli* K-12. Mol. Microbiol. **21:**725-738.

Gerlach, G. F., B. L. Allen, and S. Clegg. 1989. Type 3 fimbriae among enterobacteria and the ability of spermidine to inhibit MR/K hemagglutination. Infect.

Immun. **57:**219-224.

Gerlach, G. F., and S. Clegg. 1988. Characterization of two genes encoding antigenically distinct type-1 fimbriae of *Klebsiella pneumoniae*. Gene **64:**231-240.

Girardeau, J. P., and Y. Bertin. 1995. Pilins of fimbrial adhesins of different member species of Enterobacteriaceae are structurally similar to the C-terminal half of adhesin proteins. FEBS Lett. **357:**103-108.

Girardeau, J. P., Y. Bertin, and I. Callebaut. 2000. Conserved structural features in class I major fimbrial subunits (Pilin) in gram-negative bacteria. Molecular basis of classification in seven subfamilies and identification of intrasubfamily sequence signature motifs which might be implicated in quaternary structure. J. Mol. Evol. **50**: 424-442.

Han, S. H. 1995. Review of hepatic abscess from *Klebsiella pneumoniae*. An association with diabetes mellitus and septic endophthalmitis. West. J. Med. **162:**220-224.

Heroven, A. K., G. Nagel, H. J. Tran, S. Parr, and P. Dersch. 2004. RovA is autoregulated and antagonizes H-NS-mediated silencing of invasin and *rovA* expression in *Yersinia pseudotuberculosis*. Mol. Microbiol. **53:**871-888.

Holden, N. J., M. Totsika, E. Mahler, A. J. Roe, K. Catherwood, K. Lindner, U. Dobrindt, and D. L. Gally. 2006. Demonstration of regulatory cross-talk between P fimbriae and type 1 fimbriae in uropathogenic *Escherichia coli*. Microbiology

152:1143-1153.

Hornick, D. B., B. L. Allen, M. A. Horn, and S. Clegg. 1992. Adherence to respiratory epithelia by recombinant *Escherichia coli* expressing *Klebsiella pneumoniae* type 3 fimbrial gene products. Infect. Immun. **60:**1577-1588.

Hornick, D. B., J. Thommandru, W. Smits, and S. Clegg. 1995. Adherence properties of an *mrkD*-negative mutant of *Klebsiella pneumoniae*. Infect. Immun. **63:**2026-2032.

Humphries, A. D., M. Raffatellu, S. Winter, E. H. Weening, R. A. Kingsley, R. Droleskey, S. Zhang, J. Figueiredo, S. Khare, J. Nunes, L. G. Adams, R. M. Tsolis, and A. J. Baumler. 2003. The use of flow cytometry to detect expression of subunits encoded by 11 *Salmonella enterica* serotype Typhimurium fimbrial operons. Mol. Microbiol. **48:**1357-1376.

Ishibashi, Y., D. A. Relman, and A. Nishikawa. 2001. Invasion of human respiratory epithelial cells by *Bordetella pertussis*: possible role for a filamentous hemagglutinin Arg-Gly-Asp sequence and alpha5beta1 integrin. Microb. Pathog. **30:**279-288.

Iwahi, T., Y. Abe, M. Nakao, A. Imada, and K. Tsuchiya. 1983. Role of type 1 fimbriae in the pathogenesis of ascending urinary tract infection induced by *Escherichia coli* in mice. Infect. Immun. **39:**1307-1315.

Jacob-Dubuisson, F., J. Heuser, K. Dodson, S. Normark, and S. Hultgren. 1993. Initiation of assembly and association of the structural elements of a bacterial pilus

depend on two specialized tip proteins. EMBO J. **12:**837-847.

Jagnow, J., and S. Clegg. 2003. *Klebsiella pneumoniae* MrkD-mediated biofilm formation on extracellular matrix- and collagen-coated surfaces. Microbiology **149:**2397-2405.

Jones, C. H., P. N. Danese, J. S. Pinkner, T. J. Silhavy, and S. J. Hultgren. 1997. The chaperone-assisted membrane release and folding pathway is sensed by two signal transduction systems. EMBO J. **16:**6394-6406.

Kelly, A., C. Conway, T. O. Croinin, S. G. Smith, and C. J. Dorman. 2006. DNA supercoiling and the Lrp protein determine the directionality of *fim* switch DNA inversion in *Escherichia coli* K-12. J. Bacteriol. **188:**5356-5363.

Klemm, P., and G. Christiansen. 1987. Three *fim* genes required for the regualtion of length and mediation of adhesin of *Escherichia coli* type 1 fimbriae. Mol. Gen. Genet. **208:**439-445.

Kolb, A., S. Busby, H. Buc, S. Garges, and S. Adhya. 1993. Transcriptional regulation by cAMP and its receptor protein. Annu. Rev. Biochem. **62:**749-795.

Krogfelt, K. A., and P. Klemm. 1988. Investigation of minor components of *Escherichia coli* type 1 fimbriae: protein chemical and immunological aspects. Microb. Pathog. **4:**231-238.

Lai, Y. C., H. L. Peng, and H. Y. Chang. 2003. RmpA2, an activator of capsule

biosynthesis in *Klebsiella pneumoniae* CG43, regulates K2 cps gene expression at the transcriptional level. J. Bacteriol. **185:**788-800.

Lai, Y. C., H. L. Peng, and H. Y. Chang. 2001. Identification of genes induced in vivo during *Klebsiella pneumoniae* CG43 infection. Infect. Immun. **69:**7140-7145.

Lee, P. Y., W. N. Chang, C. H. Lu, M. W. Lin, B. C. Cheng, C. C. Chien, C. J. Chang, and H. W. Chang. 2003. Clinical features and in vitro antimicrobial susceptibilities of community-acquired *Klebsiella pneumoniae* meningitis in Taiwan. J. Antimicrob. Chemother. **51:**957-962.

Liang, M. N., S. P. Smith, S. J. Metallo, I. S. Choi, M. Prentiss, and G. M. Whitesides. 2000. Measuring the forces involved in polyvalent adhesion of uropathogenic *Escherichia coli* to mannose-presenting surfaces. Proc. Natl. Acad. Sci. U. S. A. **97:**13092-13096.

Lin, C. T, T. Y. Huang. W. C. Liang, and H. L. Peng. 2006. Homologous response regulators KvgA, KvhA and KvhR regulate the synthesis of capsular polysaccharide in *Klebsiella pneumoniae* CG43 in a coordinated manner. J. Biochem. (Tokyo). **140:**429-438.

Matatov, R, J. Goldhar, E. Skutelsky, I. Sechter, R. Perry, R. Podschun, H. Sahly, K. Thankavel, S. N. Abraham, and I. Ofek. 1999. Inability of encapsulated Klebsiella pneumoniae to assemble functional type 1 fimbriae on their surface. FEMS Microbiol. Lett. **179:**123-130.

Mayhall, C. G., V. A. Lamb, C. M. Bitar, K. B. Miller, E. Y. Furse, B. V. Kirkpatrick, S. M. Jr. Markowitz, J. M. Veazey, and F. L. Marcrina. 1980. Nosocomial *Klebsiella* infection in a neonatal unit: identification of risk factors for gastrointestinal colonization. Infect. Control. **1:**239-246.

Miller, J. H. 1972. Experiments in molecular genetics. New York: Cold Spring Harbor.

Nasser, W, V. E. Shevchik, and N. Hugouvieux-Cotte-Pattat. 1999. Analysis of three clustered polygalacturonase genes in *Erwinia chrysanthemi* 3937 revealed an anti-repressor function for the PecS regulator. Mol. Microbiol. **34:**641-650.

O'Toole, G. A., and R. Kolter. 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signaling pathways: a genetic analysis. Mol. Microbiol. **28:**449-461.

Oelschlaeger, T. A, and B. D. Tall. 1997. Invasion of cultured human epithelial cells by *Klebsiella pneumoniae* isolated from the urinary tract. Infect. Immun. **65:**2950-2958.

Old, D. C. 1972. Inhibition of the interaction between fimbrial haemagglutinin and erythrocytes by D-mannose and other carbohydrates. J. Gen. Microbiol. **71:**149-157.

Old, D. C., and R. A. Adegbola. 1983. Antigenic relationships among type 3 fimbriae of *Enterobacteriaceae* revealed by immunoelectron microscopy. J. Med. Microbiol. **20:**113-121.

Otto, K., J. Norbeck, T. Larsson, K. A. Karlsson, and M. Hermansson. 2001. Adhesion of type 1-fimbriated Escherichia coli to abiotic surfaces leads to altered composition of outer membrane proteins. J. Bacteriol. **183:**2445-2453.

Peng, H. L., P. Y. Wang, C. M. Wu, D. C. Hwang, and H. Y. Chang. 1992. Cloning, sequencing, and heterologous expression of a *Klebsiella pneumoniae* gene encoding an FAD-independent acetolactate synthase. Gene **117:**125-130.

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

Praillet, T., S. Reverchon, J. Robert-Baudouy, and W. Nasser. 1997. The PecM protein is necessary for the DNA-binding capacity of the PecS repressor, one of the regulators of virulence-factor synthesis in *Erwinia chrysanthemi*. FEMS Microbiol. Lett. **154:**265-270.

Pratt, L. A., and R. Kolter. 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol. Microbiol. **30:**285-293.

Relman, D., E. Tuomanen, S. Falkow, D. T. Golenbock, K. Saukkonen, and S. D. Wright. 1990. Recognition of a bacterial adhesion by an integrin: macrophage CR3 (alpha M beta 2, CD11b/CD18) binds filamentous hemagglutinin of *Bordetella pertussis*. Cell **61:**1375-1382.

Reverchon, S., W. Nasser, and J. Robert-Baudouy. 1994. *pecS*: a locus controlling pectinase, cellulase and blue pigment production in *Erwinia chrysanthemi*. Mol. Microbiol. **11:**1127-1139.

Rouanet, C., S. Reverchon, D. A. Rodionov, and W. Nasser. 2004. Definition of a consensus DNA-binding site for PecS, a global regulator of virulence gene expression in *Erwinia chrysanthemi* and identification of new members of the PecS regulon. J. Biol. Chem. **279:**30158-30167.

Ruoslahti, E. 1996. RGD and other recognition sequences for integrins. Annu. Rev. Cell Dev. Biol. **12:**697-715.

Ruoslahti, E., and M. D. Pierschbacher. 1987. New perspectives in cell adhesion: RGD and integrins. Science **238:**491-497.

Sahly, H., R. Podschun, T.A. Oelschlaeger, M. Greiwe, H. Parolis, D. Hasty, J. Kekow, U. Ullmann, I. Ofek, and S. Sela. 2000. Capsule impedes adhesion to and invasion of epithelial cells by *Klebsiella pneumoniae*. Infect. Immun. **68:**6744-6749.

Schaberg, D. R., D. H. Culver, and R. P. Gaynes. 1991. Major trends in the microbial etiology of nosocomial infection. Am. J. Med. **91:**72S-75S.

Schembri, M. A., J. Blom. , K. A. Krogfelt, and P. Klemm. 2005. Capsule and fimbria interaction in *Klebsiella pneumoniae*. Infect. Immun. **73:**4626-4633.

Schembri, M. A., G. Christiansen, and P. Klemm. 2001. FimH-mediated autoaggregation of *Escherichia coli*. Mol. Microbiol. **41:**1419-1430.

Schembri, M. A., D. W. Ussery, C. Workman, H. Hasman, and P. Klemm. 2002. DNA microarray analysis of *fim* mutations in *Escherichia coli*. Mol. Genet. Genomics **267:**721-729.

Schurtz, T. A., D. B. Hornick, T. K. Korhonen, and S. Clegg. 1994. The type 3 fimbrial adhesin gene (*mrkD*) is not conserved among all fimbriate strains. Infect. Immun. **62:**4186-4191.

Schwan, W. R., M. T. Beck, S. J. Hultqren, J. Pinkner, N. L. Woolever, and T. Larson. 2005. Down-regulation of the *kps* region 1 capsular assembly operon following attachment of *Escherichia coli* type 1 fimbriae to D-mannose receptors. Infect. Immun. **73:**1226-1231.

Schwan, W. R., J. L. Lee, F. A. Lenard, B. T. Matthews, and M. Beck. 2002. Osmolarity and pH growth conditions regulate *fim* gene transcription and type 1 pilus expression in uropathogenic *Escherichia coli*. Infect. Immun. **70:**1391-1402.

Sebghati, T. A., and S. Clegg. 1999. Construction and characterization of mutations within the *Klebsiella mrkD1P* gene that affect binding to collagen type V. Infect. Immun. **67:**1672-1676.

Sebghati, T. A., T. K. Korhonen, D. B. Kornich, and S. Clegg. 1998. Characterization of the type 3 fimbrial adhesins of *Klebsiella* strains. Infect. Immun.

66:2887-2894.

Seidler, R. J., M.D. Knittel, and C. Brown. C. 1975. Potential pathogens in the environment: cultural reactions and nucleic acid studies on *Klebsiella pneumoniae* from clinical and environmental sources. Appl. Microbiol. **29:**819-825.

Snyder, J. A., B. J. Haugen, C. V. Lockatell, N. Maroncle, E. C. Hagan, D. E. Johnson, R. A. Welch, and H. L. Mobley. 2005. Coordinate expression of fimbriae in uropathogenic *Escherichia coli*. Infect. Immun. **73:**7588-7596.

Sohanpal, B. K., S. El-Labany, M. Lahooti, J. A. Plumbridge, and I. C. Blomfield. 2004. Integrated regulatory responses of *fimB* to N-acetylneuraminic (sialic) acid and GlcNAc in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. U. S. A. **101:**16322-16327.

Stromberg, N., P. G. Nyholm, I. Pascher, and S. Normark. 1991. Saccharide orientation at the cell surface affects glycolipid receptor function. Proc. Natl. Acad. Sci. U. S. A. **15:**9340-9344.

Struve, C., C. Forestier, and K. A. Krogfelt. 2003. Application of a novel multi-screening signature-tagged mutagenesis assay for identification of *Klebsiella pneumoniae* genes essential in colonization and infection. Microbiology **149:**167-176.

Switalski, L. M., M. Hook, and E. H. Beachey. 1989. Molecular mechanisms of microbial adhesion. New York: Springer-Verlag.

Tarkkanen, A. M., R. Virkola, S. Clegg, and T. K. Korhonen. 1997. Binding of the type 3 fimbriae of *Klebsiella pneumoniae* to human endothelial and urinary bladder cells. Infect. Immun. **65:**1546-1549.

Teng, C. H., Y. Xie, S. Shin, F. Di Cello, M. Paul-Satyaseela, M. Cai, and K. S. Kim. 2006. Effects of *ompA* deletion on expression of type 1 fimbriae in *Escherichia coli* K1 strain RS218 and on the association of *E. coli* with human brain microvascular endothelial cells. Infect. Immun. **74:**5609-5616.

Townsend, S. M., N. E. Kramer, R. Edwards, S. Baker, N. Hamlin, M. Simmonds, K. Stevens, S. Maloy, J. Parkhill, G. Dougan, and A. J. Baumler. 2001. *Salmonella enterica* serovar Typhi possesses a unique repertoire of fimbrial gene sequences. Infect. Immun. **69:**2894-2901.

Verger, D., E. Miller, H. Remaut, G. Waksman, and S. Hultgren. 2006. Molecular mechanism of P pilus termination in uropathogenic *Escherichia coli*. EMBO Rep. **7:**1228-1232.

Wang, J. H., Y. C. Liu, S. S. Lee, M. Y. Yen, Y. S. Chen, J. H. Wang, S. R. Wann, and H. H. Lin. 1998. Primary liver abscess due to *Klebsiella pneumoniae* in Taiwan. Clin. Infect. Dis. **26:**1434-1438.

Westerlund-Wikstrom, B., and T. K. Korhonen. 2005. Molecular structure of adhesin domains in *Escherichia coli* fimbriae. Int. J. Med. Microbiol. **295:**497-486.

Westerlund, B., I. Van Die, C. Kramer, P. Kuusela, H. Holthofer, A. M. Tarkkanen, R. Virkola, N. Riegman, H. Bergmans, W. Hoekstra, and K. Korhonen. 1991. Multifunctional nature of P fimbriae of uropathogenic *Escherichia coli*: mutations in *fsoE* and *fsoF* influence fimbrial binding to renal tubuli and immobilized fibronectin. Mol. Microbiol. **5:**2965-2975.

Xia, Y., D. Gally, K. Forsman-Semb, and B. E. Uhlin. 2000. Regulatiory cross-talk between adhesin operons in *Escherichia coli*: inhibition of type 1 fimbriae expression by the PapB protein. EMBO J. **19:**1450-1457.

Table 1. Bacterial strains and plasmids used in this study

Strains or plasmids	Descriptions	Reference or source
K. pneumoniae		
VHm1~VHm17	Clinical isolates of *K. pneumoniae*, from the Veteran General Hospital, Taipei.	This study
CG43	Clinical isolate of K2 serotype	(Chang *et al*., 1996)
CG43S3	CG43, Sm^{r}	(Lai *et al*., 2003)
CG43S3*mrkA*$^{-}$	CG43S3 with deletion in *mrkA* gene	(呂文鈴，民國九十年)
CG43S3*phgS*$^{-}$	CG43S3 with deletion in *phgS* gene	This study
CG43S3*phgM*$^{-}$	CG43S3 with deletion in *phgM* gene	This study
CG43S3*lacZ*$^{-}$	CG43S3 with deletion in *lacZ* gene	(Lin *et al*., 2006)
CG43S3*lacZ*$^{-}$*phgfS*$^{-}$	CG43S3*lacZ*$^{-}$ with deletion in *phgS* gene	This study
CG43S3*lacZ*$^{-}$*phgM*$^{-}$	CG43S3*lacZ*$^{-}$ with deletion in *lacZ* and *phgM* gene	This study
Plasmids		
pET30a-b	His-tag protein expression vector, Km^{r}	Novagen
pKAS46	Positive selection suicide vector with *rpsL* gene; Km^{r}, Amp^{r} Tc^{r}; suicide vector	(Lai *et al*., 2003)
pACYC184	Cloning vector containing the P15A origin of replication	New England Biolabs
pLacZ15	Containing *K. pneumoniae* CG43S3 *lacZ* as a reporter, Cm^{r}	(Lin *et al*., 2006)
pETQ33	Km^{r}; pET30 was inserted with T5 promoter in front of the muti-cloning site	Our laboratory
pCVD442	Positive selection suicide vector with *sacB* gene; Ap^{r}; suicide vector	Our laboratory
pETQ33-*fimB*	DNA fragment containing *fimB* cloned into pETQ33	This study
pYJA	DNA fragment containing *mrkA*, *mrkB* and partial *mrkC* cloned into pACYC184	This study
pAW01	Km^{r}; pCVD442 was inserted with *km* gene in the *Eco*RI site	Our laboratory
pYJ	DNA fragment conating 2 kb fragment flanking *mrkF*gene cloned into pAW01	This study

Table 2. Primers used in this study

Primers	Sequence (5'→ 3')	Property
A1	CGA GCT CAG CGT GAT GTC TAT CCA G	*mrkA* mutant construction
A2	CGC GGA TCC CGA ATC AAT GAG CAC ACT	*mrkA* mutant construction
A3	CGC GGA TCCACA ATA ATA AAG CGG CAA T	*mrkA* mutant construction
A4	TGC TCT AGA GAC TGC CGA CAA TAA AGC	*mrkA* mutant construction
S1	CGG CGA TAT CTG GCT GCT G	*phgS* mutant construction
S2	CGC GAA CGG ATC CAT GCC	*phgS* mutant construction
S3	AGG ATC CTG GGC GGT ACA TA	*phgS* mutant construction
S4	TTT TGC TCC CGC CGC CTG	*phgS* mutant construction
M1	CCA CGG ATC CTC AGC TGC CA	*phgM* mutant construction
M2	GGC GGA GCT CAG GGC GAC	*phgM* mutant construction
M3	TCT TCG ACT GAC CGG GAG CTC C	*phgM* mutant construction
M4	TTA ATT GGA TCC GCG GCG	*phgM* mutant construction
F1	CGG ATC CGC AGC AAA ATC AG	*mrkF* mutant construction
F2	TTT ACA TTT CCC GCC ATT CCC	*mrkF* mutant construction
F3	CCG TCC GGG ATC CTA ATT GGT C	*mrkF* mutant construction
F4	GTC CTG GAG CTC CTG TAC GCG TC	*mrkF* mutant construction
pB1	GCA GGC AGA TCT TTG GTT TTC C	P_{fimB} construction
pB2	TAG AAG GCC GCC GGA TCC G	P_{fimB} construction
pE1	TGA CGA GCA GCC TGG ATC CT	P_{fimE} construction
pE2	CAG ATC TAA TAG CCT TGA CGC CAC	P_{fimE} construction
pA1	GAG ATC TGG GAG CAT ACC GGC A	$P_{L\text{-}mrkA}$ construction
pA2	GGA GAT CTC ATG GGC TGC CCT	$P_{S\text{-}mrkA}$ construction
pA3	GCG GAT CCC ATT GCT GCA GA	$P_{L\text{-}mrkA}$ $P_{S\text{-}mrkA}$ construction
pA4	TTA AAA ACA ATG GAT TAA TGA TTT GTT	Mapping of the *mrkA* transcription start site
pA5	AAG GGG GGA TCA CAT GG	Mapping of the *mrkA* transcription start site
pA6	TGG CCT TCC GAG GGC TTT	Mapping of the *mrkA* transcription start site

Table 3. Mr/K hemagglutination assay

Recombinant plasmid in *E. coli* JM109	Hemagglutination[a] unit
pGEMT-easy	-[b]
pmrkABC	-
pmrkABCF	-
pmrkABCD$_{V1T}$F	-
pmrkABCD$_{V2}$F	4
pmrkABCD$_{V3}$F	16
pmrkABCD$_{V4}$F	16

[a] expressed as the highest dilution of bacterial suspesion causing visible Mr/K HA.

[b], No hemagglutination.

Table 4. Effect of GRGDSP or anti-α5β1 integrin antibody on binding of JM109[pmrkABCD$_{V3}$F]

Peptide or antibody	Concentration or dilution	HCT-8 binding activity[a]
None		100%
GRGDSP	20 μg/ml	63.76±8.89%
	100 μg/ml	31.23±4.15%
GRADSP	100 μg/ml	102.64±28.6%
Anti-α5β1 integrin antibody	1:200	67.62±15.75%
	1:1000	55.5±28.1%

[a], The JM109[pmrkABCD$_{V3}$F] binding to HCT-8 without peptide or antibody is considered as 100%. Each value represents the means ± SD of triplicate determinations.