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Characterization of the type 3 fimbriae with different MrkD adhesins: Possible role of the MrkD containing an RGD motif

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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 most 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-α5β1 integrin monoclonal antibody, indicating that the RGD containing region in $MrkD_{V3}$ is responsible for the binding of type 3 fimbriae to integrin.

Keywords: *Klebsiella pneumoniae*; Type 3 fimbriae; *mrkD* PCR-RFLP; RGD motif; Integrin

Klebsiella pneumoniae is an important opportunistic pathogen that causes urinary tract infection, pneumonia, septicemia, and liver abscess in immune compromised patients, and hence the vast majority of the infections are associated with hospitalization [1]. The bacteria also cause meningitis which is mostly benign with the bacteria acquired from nasopharyngeal colonization, a major reservoir of *K. pneumoniae* infections [2]. The ability of *K. pneumoniae* to colonize respiratory and urinary epithelia has been attributed to the presence of several adhesive molecules, including type 1 and type 3 fimbriae [3], KPF28 [4], and a nonfimbrial adhesin, CF29K [5]. Similar to other fimbriae, type 3 fimbriae is encoded by a multi-gene operon *mrkABCDF*. The *mrkD* gene encodes the adhesin on the tip of the fimbriae, which is responsible for the mannose-resistant *Klebsiella*-like (MR/K) hemagglutination activity [6].

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 [7]. The variation of PapG, the adhesin of P fimbriae, also appeared to alter the fimbrial receptor specificity [8]. 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 [9]. Although a protein receptor has been suggested [10], the identity of MrkD receptor remains unknown.

Herein, we report the identification of additionally four *mrkD* alleles from 17 meningitis-associated *K. pneumoniae* isolates. A type 3 fimbriae display system carrying, respectively, each of the MrkD variants was constructed and the

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influences of the *mrkD* allelic variation on the fimbrial activity investigated.

Materials and methods

Bacterial strains, plasmids, and media. The clinical isolates of *K. pneumoniae*, namely VHm1–VHm17, were recovered from the patients with meningitis during 1998–2000 in Veteran General Hospital, Taipei. The bacteria were grown at 37 °C in GCAA medium, which is composed of minimal medium supplemented with 1% glycerol and 0.3% casamino acids for optimal expression of type 3 fimbriae [11]. The *E. coli* transformants were also grown in GCAA medium supplemented with 100 μg/ml ampicillin.

PCR-RFLP analysis of the mrkD genes. Genomic DNA of the *K. pneumoniae* isolates was prepared as the template and the primers used are corresponding, respectively, to the 5′- and the 3′-ends of $mrkD_{1p}$ coding region [12]. The PCR products were then digested with *Sau3*AI and the restriction fragments resolved on a 2% agarose by gel electrophoresis.

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 [13], the cells were seeded into 24-well plate (TPP industries, France) 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 hexapeptides GRGDSP (Calbiohem 03340035) and GRADSP (Calbiohem 03340052), and anti-integrin monoclonal antibody α5β1 (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.

Antiserum preparation. The coding sequence of *mrkA*, which encodes the major subunit of type 3 fimbriae, was isolated by PCR from *K. pneumoniae* CG43 [14], ligated into pET30a expression vector, and then transformed into *E. coli* Nova-Blue (DE3). The recombinant MrkA was induced by addition of IPTG (isopropyl-β-D-thiogalactopyranoside) and purified by affinity chromatography with a nickel-charged resin (Novagen, Madison, WI). Subsequently, 5-week-old female BALB/c mice, purchased from the animal center of National Taiwan University, were immunized intraperitoneally with 5 μg of the MrkA protein and then boosted with the same amount of the protein 10 days later. Finally, the MrkA antiserum was obtained by intracardic puncture.

Western blotting hybridization. Total cell lysates from each of the bacteria were separated by SDS–PAGE and electrophoretically transferred from the gel onto PVDF membrane (Immobilon™-P, Millipore). The membrane pretreated with 5% non-fat milk was incubated with the anti-MrkA antibody at room temperature for 1 h. After the membrane was washed with PBS, an alkaline phosphatase-conjugated anti-mouse IgG antibody was added and then the membrane was washed with PBS. Finally, the bound antibodies on the membrane were detected using the chromogenic reagents BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (Nitro blue tetrazolium).

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 $pmrkABCD_{V4}$. The gene coding for MrkF, which helps to stabilize type 3 fimbriae [6], 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.

Transmission electron microscopy (TEM). Twenty microliters of bacterial suspension (10^8 cfu/ml) was added to collodion-coated copper grids (300 mesh) and negatively stained by 2% phosphotungstic acid, pH 7.4. The grids were examined under a JEOL JEM 2000EXII transmission electron microscope at an operating voltage of 100 kV [15].

MrlK hemagglutination assay. The hemagglutination assay was performed as described [12]. 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 fourfold 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.

Binding to type IV- and type V-collagen. The binding assay was carried out as described [9]. Essentially, the wells of flat-bottomed microtiter plate (Nunc-Immuno™ plate) 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. 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.

Biofilm formation. The ability of bacteria to form biofilm was analyzed as described with a minor modification [16]. Hundred microliters of the overnight grown bacteria diluted 1/100 in GCAA medium was 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.

Results and discussions

Identification of four novel mrkD alleles

Recently, the incidence of *K. pneumoniae* meningitis in newborns and adult patients has been reported worldwide [2]. Since the role of type 3 fimbriae in determining the tissue tropism has been suggested [10,17], 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 Nos. AY225462, AY225463, AY225464, and AY225465). Notably, 14 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.

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 utmost 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 [18] were identified (Fig. 1). In addition, the residues which have been proposed to facilitate the interaction of MrkD with other fimbrial component [19] were unique in $MrkD_{V3}$ (C102 and R200). The D-R-N (residues 68–70) of $MrkD_{1P}$ that has been shown to affect the fimbrial activity [19] 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.

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 [3,17]. 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. 2, the bacteria VHm5 of $mrkD_{V3}$ allele exerted the highest level of the cell adhesion activity. However, 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 (data not shown). 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 [20], is/are involved in determining the cellular adherence activity.

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 (data not shown). The TEM analysis (data shown in supplemental material) 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. Interestingly, 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

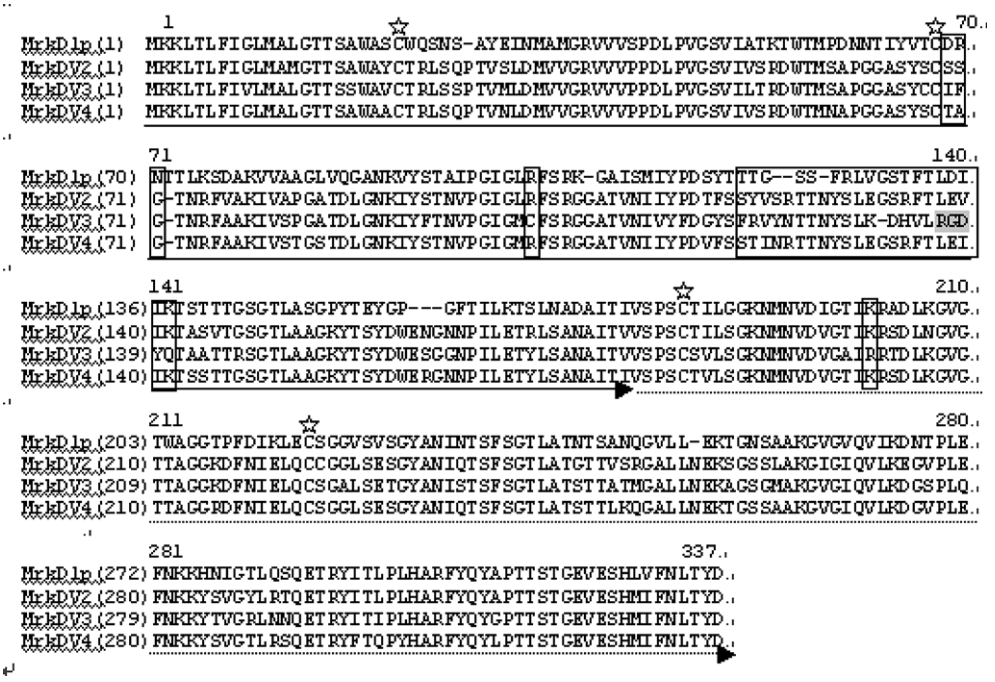

Fig. 1. Amino acid sequence comparison of $MrkD_{1P}$, $MrkD_{V2}$, $MrkD_{V3}$, and $MrkD_{V4}$: the receptor binding (→) and pilin (- ->) domains are shown. The conserved Cys residues are indicated as asterisks. The regions with varied sequences are boxed and the RGD residues are in shadow.

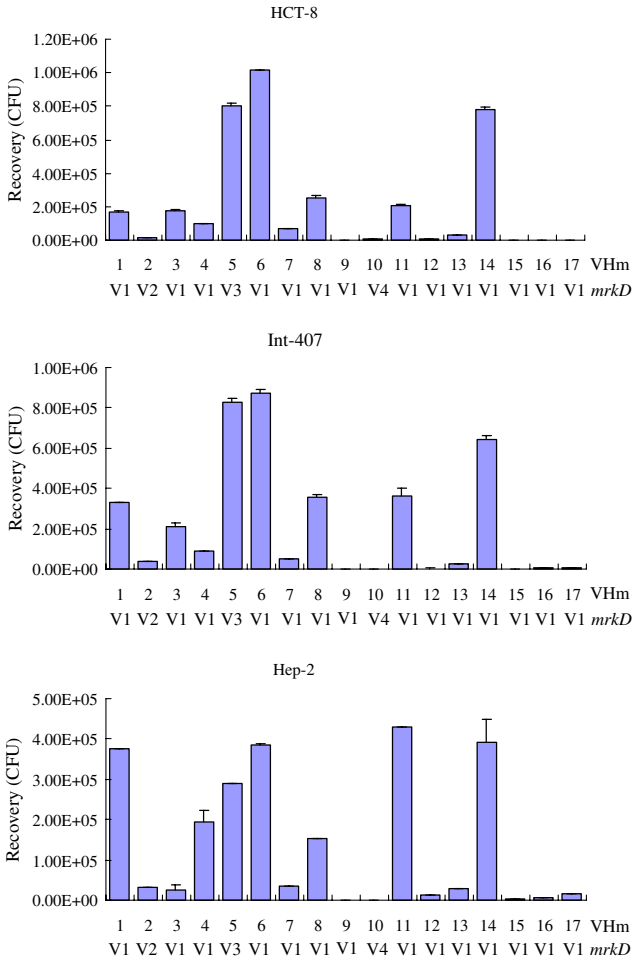

Fig. 2. Cellular adherence activity of the meningitis isolates. Approximately 10^7 bacteria were added to each of the confluent cells of Hep-2, HCT-8, and Int-407. The cell-adhesive bacteria were measured by recovery of the colony formation on LB agar. The results are recorded as recovery of the bacteria and data shown represent mean values and standard deviations.

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.

Activity assessments of the recombinant fimbriae

As shown in Table 1, 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.

Table 1
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 suspension causing visible Mr/K HA.
[b] No hemagglutination.

As shown in Fig. 3A, 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 [17]. Fig. 3B shows 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. 3C). 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 [21]. Interestingly, an autoaggregation phenotype was observed only for JM109[pmrkABCD$_{V3}$F] (data not shown), 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 [22]. It is also likely that the varied sequence in the receptor domain of MrkD$_{V3}$ confers the bacteria an autoaggregation property.

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 [23]. 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 [24]. The RGD sequence of *B. pertussis* FHA has been

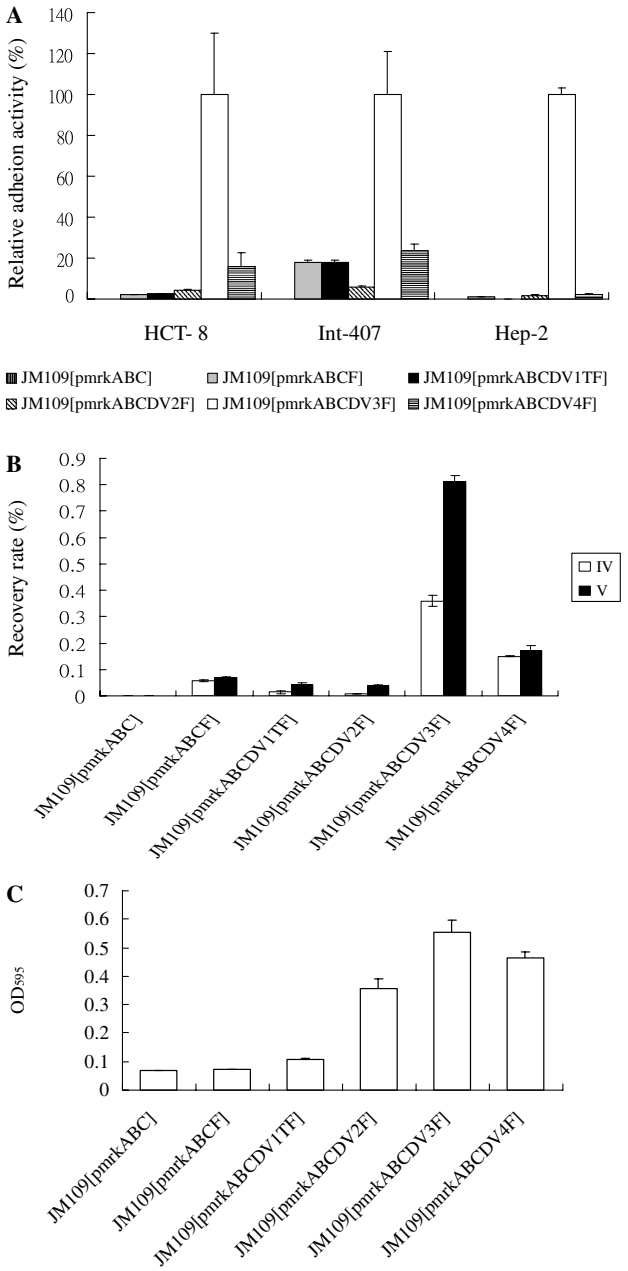

Fig. 3. The activity assessments of the recombinant type 3 fimbriae. (A) Cell adhesion assay. The value is expressed as the percentage of the adherence obtained in comparing with that of *E. coli* JM109[pmrkABCD$_{V3}$F], which has the most binding activity. (B) Collagen binding activity. The binding capabilities are indicated by open columns to type IV collagen, and black solid columns to type V collagen. The results are recorded as percentage derived from the original inoculum and the value is expressed as the means ± standard error. (C) Biofilm formation of the recombinant *E. coli*. The extent of biofilm formation was expressed with the absorbance at 595 nm of the dissolved biofilms stained with 1% crystal violet. For each column, the value is expressed as the mean ± standard error.

demonstrated to interact specifically with α5β1 integrin [25]. As shown in Table 2, the anti-α5β1 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 α5β1 integrin.

Table 2
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

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

Taken together, we have shown in the study that MrkF is able to serve as an initiator for the growth of type 3 fimbriae. 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.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.09.070.

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