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MrkF is a component of type 3 fimbriae in *Klebsiella pneumoniae*

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Abstract

Klebsiella pneumoniae type 3 fimbriae are encoded by *mrkABCDF* genes which produce the major pilin subunit MrkA, chaperone MrkB, outer membrane usher MrkC, adhesin MrkD and MrkF of unknown function, respectively. RT-PCR analysis demonstrated that the *mrkF* gene is contained within the *mrk* operon. Deletion of *mrkF* in *K. pneumoniae* CG43 was found to reduce biofilm formation. A higher level of biofilm formation activity was also observed in recombinant *Escherichia coli* JM109[p*mrkABCDF*] compared to that observed for JM109[p*mrkABCD*]. Immunoelectron microscopy analysis of recombinant type 3 fimbriae using anti-MrkA and anti-MrkF antibody-labeled gold particles revealed that MrkF intermittently inserted into the MrkA filament. An interaction between recombinant MrkA and MrkF was demonstrated by co-immunoprecipitation analysis, further supporting the notion that MrkF is a structural component of the fimbriae. Intriguingly, the incorporation of MrkF appeared to decrease fimbrial length but increased activity of autoaggregation and biofilm formation in the bacteria JM109[p*mrkABCDF*]. This suggested that MrkF may play a role in assembly of the filament.
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Keywords: Type 3 fimbriae; MrkF; *Klebsiella pneumoniae*

1. Introduction

Klebsiella pneumoniae is an opportunistic pathogen that infects immunocompromised patients who are hospitalized or suffering from severe underlying diseases, such as chronic pulmonary obstruction or diabetes mellitus [1]. These bacterial infections can lead to complications, including urinary tract infections, septicemia and pneumonia in the elderly or in patients with predisposing factors [2]. The identified virulence factors include polysaccharide capsule, lipopolysaccharides, iron acquisition systems and distinct types of adherence factors [3]. The adhesive structures, including type 1 and type 3 fimbriae [4], KPF28 [5] and a nonfimbrial adhesin CF29K [6] have been reported to be responsible for its colonization of respiratory and urinary epithelia.

Type 3 fimbriae, characterized as 2–4 nm wide and 0.5–2 μm long appendages, are produced by most of the *Klebsiella* isolates associated with human urinary or respiratory tract infections [7]. Evidence indicates that type 3 fimbriae mediate the bacterial attachment to the basolateral surfaces of several types of cells such as tracheal epithelial cells [8], renal tubular cells [4], extracellular matrix proteins [9] and basement membrane of human lung tissue [8]. A role in facilitating colonization on denuded and damaged epithelial surfaces of debilitated patients has also been proposed [10].

Type 3 fimbriae have been shown to influence the development of biofilms on plastic [11,12]. Bacterial growth on abiotic surfaces is facilitated in part by the major fimbrial subunit MrkA protein, whereas growth on surfaces coated with a human extracellular matrix (HECM) requires the presence of the MrkD adhesin [11,12]. Expression of type 3 fimbriae could be demonstrated by agglutination of tannic-acid-treated-erythrocytes in the presence or absence of D-mannose [13]. Spermidine was able to block MrkD-mediated adherence and henceforth, a protein receptor of the adhesin was proposed [8,14]. Specific attachment of MrkD to collagen IV or V has also been demonstrated [9], which further supports the possibility of a protein receptor.

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Located downstream of *mrkD*, the *mrkF* gene is proposed to encode a protein that affects the stability of type 3 fimbriae and hence may be a component of the fimbriae [15]. In the study herein, using immunoelectron microscopy, co-immunoprecipitation and western blotting hybridization, we demonstrate that MrkF is a component of type 3 fimbriae. In addition, the incorporation of MrkF into the recombinant fimbriae was found to affect the length of fimbrial polymer.

2. Materials and methods

2.1. Bacteria and plasmids

Recombinant plasmids p*mrkABCD* and p*mrkABCDF* for expression of type 3 fimbriae were derived from p$mrkABCD_{V1T}F$ that was constructed and tested previously [16]. The *mrkD* sequence amplified from *K. pneumoniae* NTUH K-2044 [17] was used to replace $mrkD_{V1T}$ that encodes a truncation protein [16]. The recombinant *Escherichia coli* JM109[pGEMT-easy], JM109[p*mrkABCD*] or JM109[p*mrkABCDF*] was grown in LB (Luria Broth) supplemented with 100 μg ampicillin ml^{-1} at 37 °C. *K. pneumoniae* CG43, a highly virulent clinical isolate [18], was grown statically in LB medium at 37 °C.

2.2. RNA extraction and RT-PCR analysis

Total RNA was isolated from *K. pneumoniae* CG43 cultures, statically grown in LB medium for 20 h, by extraction with TRI reagent (Molecular Research Center, Cincinnati, OH, USA) and residual DNA was eliminated with RQ1 RNase-free DNase (Promega, Madison, WI, USA). The cDNAs used for PCR amplification were each synthesized from 1 μg of the total RNA obtained using a random hexamer primer from an RT-PCR kit (Stratagene, La Jolla, CA, USA). The primer pairs used to amplify MrkA, MrkB, MrkC, MrkD, and MrkF are MrkA-RT-1 (5′-CTCTGACAAGGAAATGG CAATG-3′) and MrkA-RT-2 (5′-GGTAAGTAATT TCGTAA GTCGCGT-3′), MrkB-RT-1 (5′-AGGCCTGGCTGGATAAC GG-3′) and MrkB-RT-2 (5′-AATGTAGAACGGCGTCGGGT-3′), MrkC-RT-1 (5′-GCCCGGG AAGATTAAGCACC-3′) and MrkC-RT-2 (5′-CTTCAGCAGCCAGCCGTCC-3′), MrkD-RT-1 (5′-ATGTCGCTGAGGAAATTACTAAC G-3′) and MrkD-RT-2 (5′-GCTGAAACGCATGCCGAT-3′), and MrkF-RT-1 (5′-ATGAAGGGATTGC CGAAAAA-3′) and MrkF-RT-2 (5′-GCTCCATCCGG CAAGGTA-3′), respectively. The primers specific for the intergenic region of *mrkA* and *mrkB*, *mrkB* and *mrkC*, *mrkC* and *mrkD*, and *mrkD* and *mrkF* are MrkAB-RT-1 (5′-GACAGCACTAGCCGAAAGC-3′) and MrkAB-RT-2 (5′-ATAATATTGTTGG CATGGGC-3′), MrkBC-RT-1 (5′-GCCATCAACAACCCGACGC-3′) and MrkBC-RT-2 (GGTGCTTAATCTTCCCGGGC), MrkCD-RT-1 (5′-GGGC AGTG TTCGGTGGATCT-3′) and MrkCD-RT-2 (5′-AGAG ACGCGTACAGGAGGCC-3′), and MrkDF-RT-1 (5′-AGGAG ACCCGCTACATCACC-3′) and MrkF-RT-2, respectively. 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 a final 10 min of elongation at 72 °C. The amplified products were resolved on 1% (w/v) agarose gels.

2.3. Construction of an mrkF deletion mutant

DNA fragments of 1 kb in length flanking *mrkF* gene were amplified by PCR with the primer sets of 5′-CCGTCCGG GATCCTAATTGGTC-3′ and 5′-GTCCT GGAGCTCCTGT ACGCGTC-3′ for the flanking fragment of the left junction, and 5′-TTTACATTTCCCGCCATTCCC-3′ and 5′-CGGATCC GCAGCAAAATCAG-3′ for the flanking fragment of the right junction. The amplified DNA fragments were cloned in order to the suicide vector pKAS46 [19]. The plasmid was then transformed into *E. coli* S17-1λ*pir* [20] and subsequently mobilized to the streptomycin-resistant strain *K. pneumoniae* CG43S3 [19] by conjugation. Several kanamycin-resistant transconjugants were selected and propagated in 2 ml LB overnight and a small aliquot of the culture was plated on LB agar containing 500 μg streptomycin ml^{-1}. The streptomycin-resistant colonies were screened further for their susceptibility to kanamycin, a property reflecting loss of the vector sequence. Occurrence of a double recombination event was verified by PCR with the primers flanking *mrkF* (MrkFC1: 5′-ATACACCGTCGGCCGCTT-3′ and MrkFC2: 5′-AGAG TCCGGCCGCCGC-3′).

2.4. Construction of the mrkF complement strain

DNA fragments of 822 bp of the *mrkF* coding region with its upstream non-coding sequence were amplified by PCR with the primer sets of 5′-CAGGAGACCCGCTACATCACC-3′ and 5′-GGTCGCCGATGATATTGCCA-3′. The amplified DNA fragments were cloned into vector pRK415 [21], and the resulting plasmid was then transformed into *E. coli* S17-1λ*pir* and subsequently mobilized to the *mrkF* mutant strain by conjugation.

2.5. Biofilm formation

The ability of bacteria to form biofilm was analyzed as described with a minor modification [22,23]. The bacteria diluted 1/100 in LB were inoculated into each well of a 96-well microtiter dish and incubated at 37 °C. After removal of the bacteria, 150 μl of 1% (w/v) crystal violet was added to each well and the plate incubated for 30 min at room temperature. The plate was then washed, the dye was solubilized in 1% (w/v) SDS, and absorbance at 595 nm was determined. The data presented were derived from a single experiment which is representative of three independent experiments. Each sample was assayed in triplicate and the average activity and standard deviation were presented.

2.6. Preparation of the recombinant proteins

The *mrkA* and *mrkF* coding sequences were isolated by PCR cloning from *K. pneumoniae* CG43S3 and ligated into a pET30 expression vector. The recombinant plasmid was then

transformed into *E. coli* NovaBlue(DE3) and overexpression of the recombinant protein was induced by the addition of 0.5 mM IPTG. Most of the recombinant proteins were insoluble; hence, the total proteins were denatured using 6 M urea. The urea-containing proteins were then purified using a nickel column which had been saturated with urea (Novagen, Madison, WI, USA). Finally, urea in the purified proteins was removed by dialyzing against dialysis phosphate buffer saline (PBS).

2.7. MrkA and MrkF 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 MrkF. Ten days later, the mice were immunized again with 5 μg of the protein and the antisera obtained by intracardiac puncture. MrkA antiserum was prepared by immunizing a New Zealand white rabbit with 0.5 mg of the purified recombinant MrkA protein and the immunized rabbit was exsanguinated on day 42. Specificity of the obtained antiobodies was confirmed using western blotting analysis (Supplementary data 1).

2.8. Immunoelectron microscopy

Twenty microliters of bacterial suspension (10^8 CFU ml^{-1}) were added to collodion-coated copper grids (300 mesh) and negatively stained by 2% (w/v) phosphotungstic acid, pH 7.2. For immunogold labeling, the bacteria coated on the grids were incubated with the raised anti-MrkA polyclonal antibody from rabbit (1:50 dilution) and anti-rabbit IgG-gold conjugate of 1:65 dilution (10-nm diameter), or anti-MrkF polyclonal antibody from mouse (1:50 dilution) and anti-mouse IgG-gold conjugate of 1:65 dilution (5-nm diameter) previously stained with 2% (w/v) phosphotungstic acid. The grids were then examined under a JEOL JEM 2000EXII transmission electron microscope at an operating voltage of 100 kV [7].

2.9. Purification of type 3 fimbriae

The purification was carried out with a minor modification of the described method [24]. Briefly, recombinant bacteria *E. coli* JM109[p*mrkABCD*] and JM109[p*mrkABCDF*] were cultured for 25 h in 1.5 L LB broth. The bacteria were then collected by centrifugation at 11,000 × *g* for 30 min at 4 °C and suspended in 100 ml phosphate-buffered saline (PBS). The suspensions were heated at 65 °C for 3 h and then homogenized in a blender for 20 min at ambient temperature. After centrifugation to remove the pellets, sodium azide and sodium deoxycholate were added to the supernatant to a final concentration of 0.1% (w/v) and the suspension was kept at 4 °C overnight. After centrifugation for 3 h (48,000 × *g*, 4 °C), the pellets were resuspended in PBS and the resuspension was subjected to further centrifugation at 16,000 × *g* for 5 min and the type 3 fimbriae containing supernatant was stored at 4 °C before use. Finally, the purified fimbriae were resolved in 12.5% (w/v) SDS-polyacrylamide gels by electrophoresis.

2.10. Immunofluorescence microscopy analysis

The overnight-grown bacteria were suspended in PBS (10^8 CFU ml^{-1}) and 10 μl of the suspension was applied to glass slide. After air-drying, 40 μl of the 1:100-diluted anti-MrkA or anti-MrkF serum was added and the slide incubated at 25 °C for 1 h. After washing with PBS, the slide was incubated with 40 μl 1:100 dilution of a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody or a phodamine red-conjugated goat anti-mouse antibody (Molecular Probes) in PBS for 1 h at 25 °C. Finally, the slide was washed and examined by fluorescence microscopy.

2.11. Co-immunoprecipitation analysis

Co-immunoprecipitation was performed according to the procedures described [25]. Briefly, 200 μl of the purified fimbriae (approximately 100 μg) were incubated with bovine serum albumin (5 μg ml^{-1}), and then anti-MrkA antibody or anti MrkF antibody was added and the mixture incubated with gentle rocking overnight at 4 °C. Protein A-Sepharose beads (50 μL) (Amersham, Piscataway, NJ, USA) were then added and the incubation continued for 3 h at 4 °C. Subsequently, the beads were collected by centrifugation at 6000 × *g* for 2 min, washed three times with 1 ml of 0.1% (w/v) DOC and resuspended in 40 μl of protein lysis buffer (50 mM Tris–HCl pH 8.0, 1 mM EDTA, 100 mM NaCl). Samples were incubated at 95 °C for 10 min, and 20 μl of the immunoprecipitate was detected using anti-MrkA or anti-MrkF antibody.

2.12. Western blot analysis

The recombinant proteins resolved by SDS-polyacrylamide gel were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After incubation with 5% (w/v) skim milk at room temperature for 1 h, the membrane was washed 3 times with 1× PBS. Subsequently, the membrane was incubated at room temperature for 1 h with diluted antibody (anti-MrkA, or -MrkF antibody). After 3 washes with 1× PBS, 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 bound antibodies were detected using the chromogenic reagents BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitro-blue tetrazolium).

2.13. Analysis of the polymerization pattern of the filament

Total proteins isolated from the recombinant bacteria *E. coli* JM109[p*mrkABCDF*] or JM109[p*mrkABCD*], which had been cultured for 20 h in LB broth, were mixed with loading buffer, placed at different temperatures for 10 min and then resolved by electrophoresis through 12.5% (w/v) SDS-PAGE. After SDS-PAGE, the filament polymer was assessed using western blotting analysis against anti-MrkA polyclonal antibody.

2.14. Mannose-resistant Klebsiella-like (Mr/K) hemagglutination assay

Essentially as described [26], bacteria which had been grown overnight were collected and suspended in PBS to approximately 10^9 CFU ml^{-1}. 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% (w/v) D-mannose were mixed with an equal volume of 3% (v/v) tannic acid-treated-erythrocytes in PBS. The mixture was incubated at room temperature for 30 min to allow erythrocytes to settle to the bottom of the glass tube.

2.15. Autoaggregation assay

As described [27], an aliquot (10 µl) of overnight culture grown in GCAA broth at 37 °C was collected and spread onto a glass slide. The bacteria-containing slide was then stained with 1% (w/v) crystal violet and observed with a light microscope under a 100× lens.

3. Results

3.1. The gene cluster mrkABCDF is organized as an operon

The intergenic sequence of 16 bp between *mrkD* and *mrkF* implied that *mrkF* is a component of the *mrk* operon. As shown in Fig. 1, RT-PCR analysis of the RNA from *K. pneumoniae* CG43 that had been confirmed as having no DNA contamination revealed that the transcript could be detected with primers specific to *mrkA*, *mrkB*, *mrkC*, *mrkD* and *mrkF*, and the intergenic region of *mrkA* and *mrkB*, *mrkB* and *mrkC*, *mrkC* and *mrkD*, and *mrkD* and *mrkF*, respectively. This indicated that *mrkA*, *mrkB*, *mrkC*, *mrkD* and *mrkF* are contained within an operon, which is consistent with the recent finding that MrkF is a component of the *mrk* operon in *E. coli* [28].

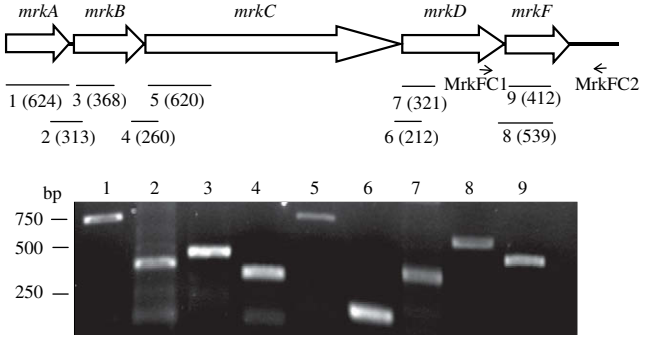

Fig. 1. RT-PCR assessment of the *mrkABCDF* operon structure. The locations of the primers are indicated and results of RT-PCR are shown. PCR amplification was performed on cDNA synthesized from 1 µg of total RNA isolated from *K. pneumoniae* CG43. Primer pairs are as follows: pMrkA-RT-1/pMrkA-RT-2, lane 1; pMrkAB-RT-1/pMrkAB-RT-2, lane 2; pMrkB-RT-1/pMrkB-RT-2, lane 3; pMrkBC-RT-1/pMrkBC-RT-2, lane 4; pMrkC-RT-1/pMrkC-RT-2, lane 5; pMrkCD-RT-1/pMrkCD-RT-2, lane 6; pMrkD-RT-1/pMrkD-RT-2, lane 7; pMrkDF-RT-1/pMrkF-RT-2, lane 8, and pMrkF-RT-1/pMrkF-RT-2, lane 9. The primers MrkFC1 and MrkFC2 for detection of *mrkF* mutant are shown with arrows.

3.2. MrkF possesses all the conserved motifs of a pilin subunit

The common structural characteristics of a pilin subunit include (i) two cysteine residues; (ii) a conserved pattern of alternating hydrophobic residues at positions 4, 6, and 7 from the carboxyl terminus; (iii) a penultimate tyrosine; and (iv) a Gly at position 14 from the C-terminus [29]. MrkF as well as the major pilin MrkA possesses all the above-mentioned sequence signatures and a signal peptide sequence predicted using LipoP in ExPASy proteomic tools (http://www.cbs.dtu.dk/services/LipoP/).

3.3. Deletion of mrkF reduced biofilm formation ability

The capacity for biofilm formation has been reported to be type 3 fimbriae-dependent in *K. pneumoniae*. MrkA pilin or MrkD adhesin, under different conditions, plays a major role in biofilm formation [11,12]. In order to determine whether MrkF as a component affects fimbrial activity, an *mrkF* deletion mutant was constructed and the deletion verified by PCR analysis. Biofilm formation capability and the extent of biofilm formation of the mutant were then determined. As shown in Fig. 2, the *mrkF* mutant demonstrated decreased activity for biofilm formation, and the deletion could be complemented by introducing an *mrkF* expression plasmid, a derivative of pRK415 [21] containing 822 bp of the *mrkF* coding region with its upstream non-coding sequence. This indicated that MrkF has a direct role in type 3 fimbrial activity.

To avoid interference with the thick capsule of *K. pneumoniae*, the recombinant type 3 fimbriae expressed on the surface of *E. coli* was used to assess the structural role of MrkF. As previously demonstrated [16], no fimbriae was observed on the surface of JM109[pGEMT-easy], while expression of the recombinant fimbriae could be found on the

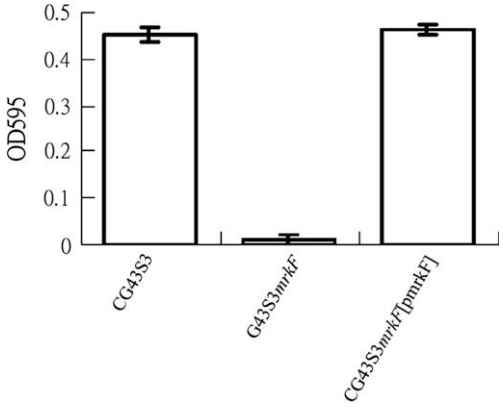

Fig. 2. Biofilm formation capacity of *K. pneumoniae* CG43S3, CG43S3*mrkF*⁻, and CG43S3*mrkF*⁻[pmrkF]. After 36 h of incubation, the amounts of biofilm of *K. pneumoniae* CG43S3, CG43S3*mrkF*⁻, and CG43S3*mrkF*⁻[pmrkF] were qualitatively determined as described in Section 2.

surface of both JM109[p*mrkABCD*] and JM109 [p*mrkABCDF*], as shown in Fig. 3(a). In contrast to the uniform fimbrial morphology of JM109[p*mrkABCD*], JM109[p*mrkABCDF*] exhibited relatively shorter and entangled fimbriae. Whether or not MrkF affects the biofilm formation capacity of the recombinant type 3 fimbriae was also investigated. As shown in Fig. 3(b), the bacteria JM109[p*mrkABCDF*] exerted a higher level of biofilm formation activity than that observed for JM109[p*mrkABCD*] after 24 h of incubation, which supported a positive role of MrkF for type 3 fimbriae in biofilm formation.

3.4. MrkF was located intermittently on the recombinant type 3 fimbriae

When labeling the fimbriae with the anti-MrkA antibody, the antibody tagged-gold particles appeared to be evenly distributed along the expressed filaments of *E. coli* JM109[p*mrkABCD*] and JM109[p*mrkABCDF*], both demonstrating the expression of type 3 fimbriae (Fig. 4(a)). In contrast to the even distribution of anti-MrkA-gold particles, MrkF antibody-tagged gold particles were observed to be dispersed along the filaments in an irregular fashion (Fig. 4(a)).

To confirm that MrkF is co-localized with MrkA on the surface of *K. pneumoniae*, immunofluorescence microscopy analysis was carried out. As shown in Fig. S2, the surface of *K. pneumoniae* CG43S3 and CG43S3*mrkF*[pmrkF] could be stained by fluorescent anti-MrkA antibody and anti-MrkF antibody. On the surface of the *mrkF* mutant, fluorescences were found only using anti-MrkA antibody, but not anti-MrkF antibody.

3.5. Interaction occurred between recombinant MrkF and MrkA

Co-immunoprecipitation analysis was subsequently carried out to determine if a direct interaction occurred between MrkA and MrkF. As shown in Fig. 4(b), the MrkA antibody pull-down lysate of the purified fimbriae of *E. coli* JM109[p*mrkABCD*] could be hybridized with anti-MrkA antibody, but not with anti-MrkF antibody, while the MrkF pull-down lysate from the purified fimbriae of *E. coli* JM109[p*mrkABCDF*] could be detected by both anti-MrkA and anti-MrkF antibodies. These data support the assertion that MrkA interacts with MrkF to form the fimbrial filament.

3.6. The incorporation of MrkF reduced MrkA polymer formation

It has been reported that MrkF plays a role in stabilizing type 3 fimbrial filaments upon heat treatment [15]. In order to determine if MrkF affects the stability of the fimbriae, the cell

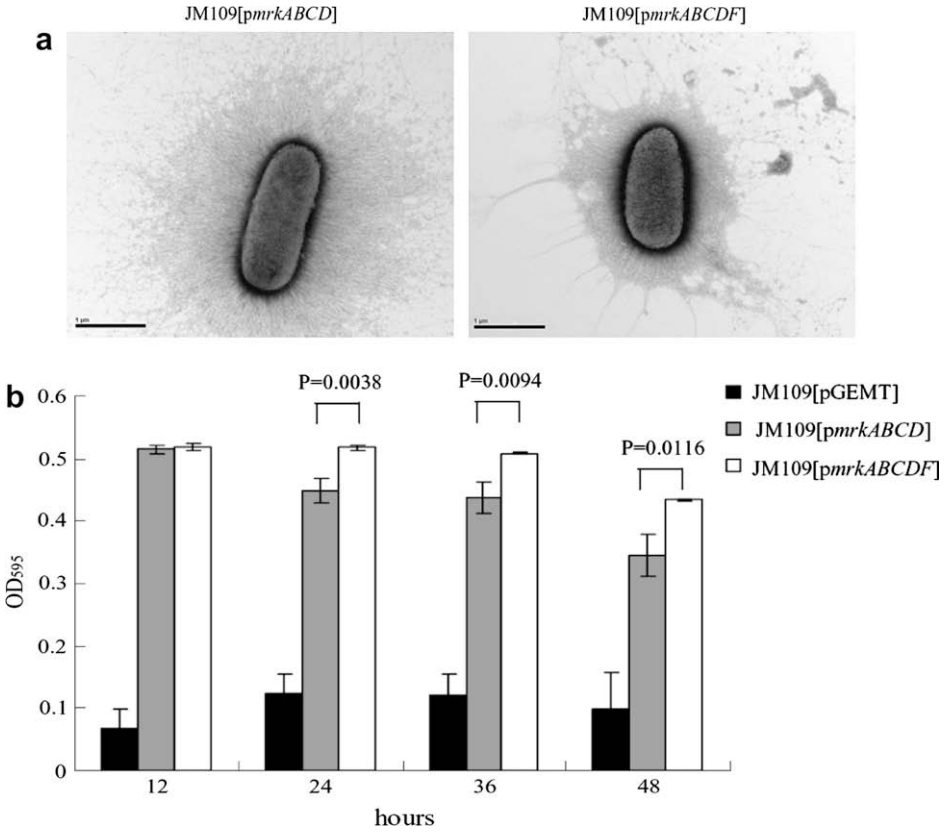

Fig. 3. (a) Transmission electron micrographs of type 3 fimbriae displayed on recombinant bacteria. One drop of bacterial suspension prepared from overnight culture was placed on a carbon-coated copper grid and negatively stained with 2% (w/v) phosphotungstic acid. (b) Biofilm formation of recombinant bacteria. The data are expressed as the average of three independent experiments. Activity of biofilm formation after 12 h, 24 h, 36 h and 48 h of incubation was qualitatively determined as described in Section 2.

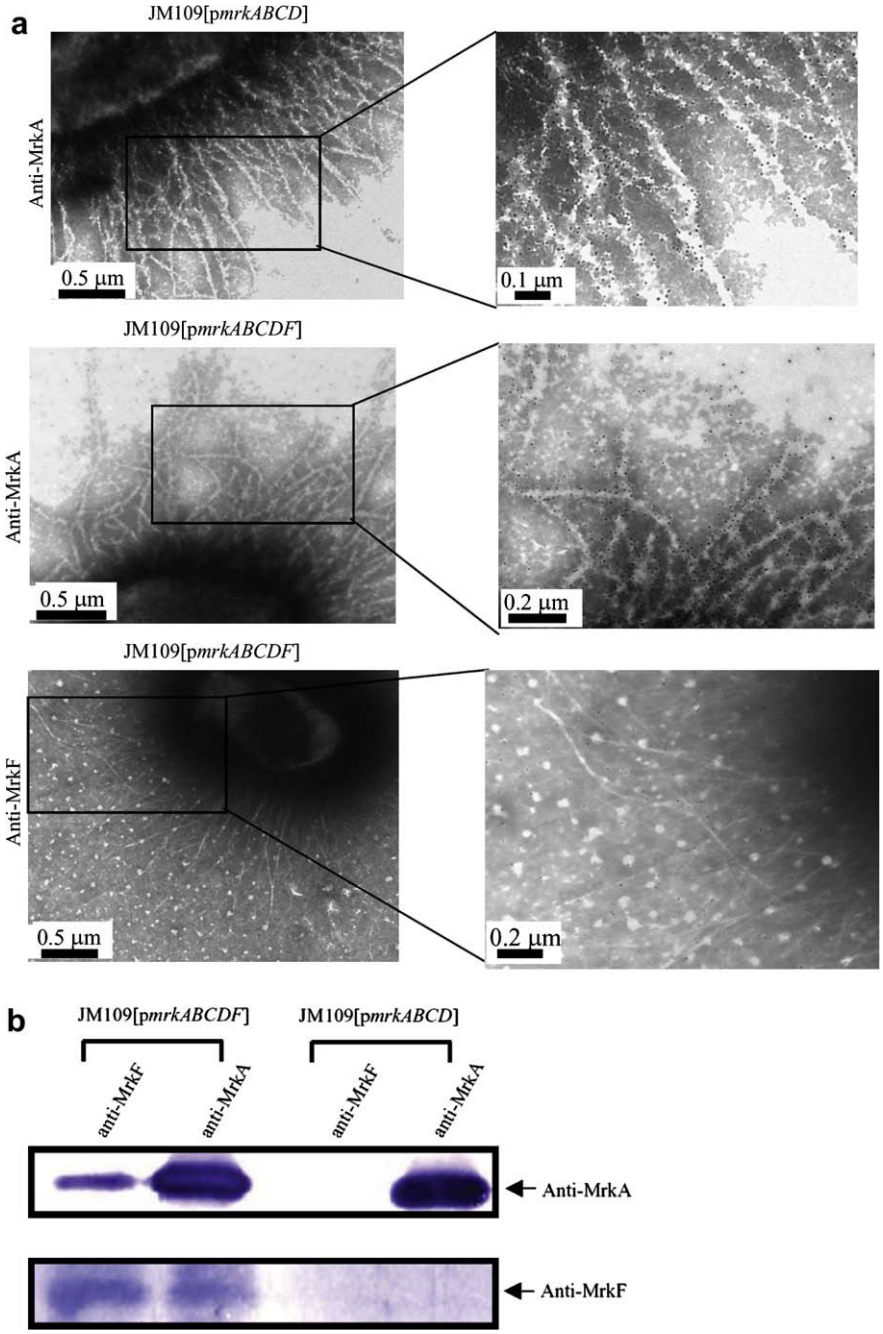

Fig. 4. (a) Immunoelectron microscopic analysis of recombinant fimbriae. A 1:50 dilution of the polyclonal anti-MrkA antiserum and 5 nm gold particle conjugated with anti-rabbit immunoglobulin were used to detect the filament of *E. coli* JM109[p*mrkABCD*] or *E. coli* JM109[p*mrkABCDF*]. A 1:50 dilution of the polyclonal anti-MrkF antiserum and 10 nm gold-particle-conjugated anti-mouse immunoglobulin were used to determine the location of MrkF. (b) Co-immunoprecipitation study with anti-MrkA and anti-MrkF antisera. The purified fimbriae of *E. coli* JM109[p*mrkABCDF*] and JM109[*pmrkABCD*] were precipitated with anti-MrkF antibody (odd lane) or anti-MrkA antibody (even lane), respectively. The precipitates were then subjected to western blotting analysis against the antiserum of MrkA (upper panel) or MrkF (lower panel).

lysates of recombinant *E. coli* were isolated and analyzed after treatment under different temperatures. A protein band with a size corresponding to the MrkA pilin monomer could be detected only when the temperature was raised to 95 °C for the lysates from *E. coli* JM109[p*mrkABCD*] or JM109-[p*mrkABCDF*]. The subsequent western blot analysis using anti-MrkA antibody confirmed the identity of the monomeric MrkA pilin (Fig. 5). The lysate from JM109[p*mrkABCDF*] which was recognized by anti-MrkA antibody appeared to have a smaller molecular weight (<100 kDa) of the polymeric pattern than that of JM109[p*mrkABCD*], suggesting a negative role for MrkF in influencing the length of the fimbriae. This is supported by the fact that a small amount of monomeric MrkA without signal peptide was detected in each of the lysates from JM109[p*mrkABCDF*], but not in that from JM109[p*mrkABCD*] before the temperature was brought to 95 °C. On the other

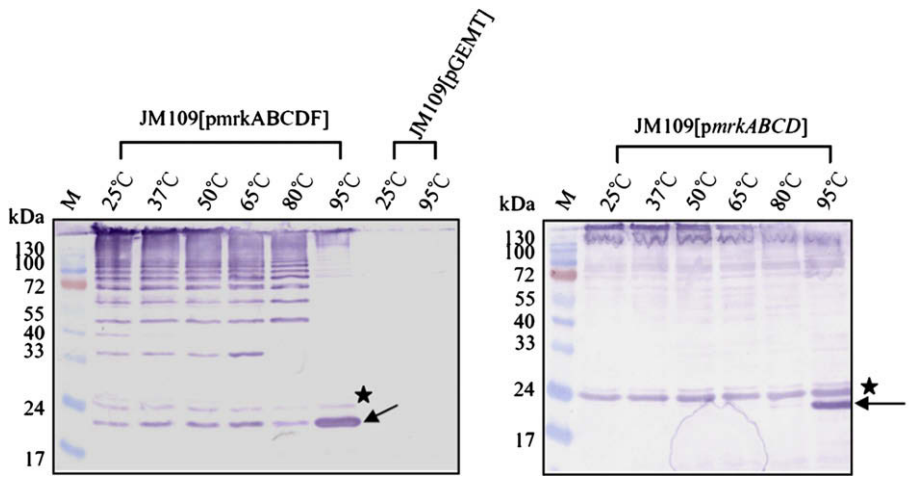

Fig. 5. Temperature effect on filament formation. Total cell lysates from *E. coli* [p*mrkABCD*] or *E. coli* [p*mrkABCDF*] grown in LB medium for 20 h were isolated and aliquoted, and then maintained at different temperatures for 10 min prior to electrophoresis. Coomassie brilliant blue was used to stain the gel and the gel was then subjected to western blotting analysis using anti-MrkA antiserum. The mature MrkA pilin monomer is marked by an arrow, and that carried with signal peptide is marked by a star. M: molecular weight marker.

hand, a somewhat higher amount of the monomeric form of MrkA carried with the signal peptide was obtained from each lysate of JM109[p*mrkABCD*] than that of JM109-[p*mrkABCDF*]. This indicated that MrkF very likely plays an important role in mature MrkA pilin monomer formation.

3.7. Incorporation of MrkF modulated the adhesion activity of the fimbriae

The adhesin MrkD located on the tip of the fimbriae is responsible for mannose-resistant *Klebsiella*-like (Mr/K) hemagglutination activity [15]. To investigate whether incorporation of MrkF influences the presentation of the MrkD adhesin, Mr/K hemagglutination activity (HA) of the recombinant bacteria was determined and compared. While the bacteria JM109[pGEMT-easy] presented no Mr/K HA, bacteria JM109[p*mrkABCD*] and JM109[p*mrkABCDF*], respectively, gave rise to 16 Mr/K HA units and 4 Mr/K HA units. This indicates that incorporation of MrkF affected MrkD adhesion activity.

Autoaggregation, whether mediated by surface self-recognizing adhesins or autoaggregative fimbriae, has been considered to be a determinant of colonization of mammalian hosts by pathogenic bacteria [30–32]. *E. coli* JM109[p*mrkABCDF*] revealed a conspicuous level of autoaggregation. In contrast, no autoaggregative appearance was observed for JM109-[p*mrkABCD*], suggesting a role for MrkF in promoting bacterial autoaggregation (Supplementary data 3).

4. Discussion

The minor subunits FimF and FimG of type 1 fimbriae have been reported to be required for integration of FimH adhesin into the tip of the fimbriae [33]. Their roles in determining morphology and assembly of type 1 fimbriae have also been demonstrated [34]. The minor subunits of P pilus, i.e. PapF, PapK and PapH, play essential roles as adaptor, initiator and terminator, respectively, in growth of the filament [35,36]. Another minor subunit, PapE, was also shown to be responsible for binding to fibronectin [37].

A role for MrkF in stabilization of type 3 fimbriae was proposed almost 15 years ago [15]. However, it is only recently that *mrkF* was shown to be part of the *mrk* operon by Burmolle et al. [28] and by us. As assessed using analysis of immunoelectronmicroscopy, MrkF appeared to intermittently be incorporated into the recombinant type 3 fimbriae. This is different from the location of the minor subunits of type 1 or type P fimbriae, which are located at the very distal end of the fimbrial filaments. However, non-regular incorporation of minor subunits has also been reported for *Pneumococcal* fimbriae [38].

The analysis of the polymeric pattern of the filaments suggested that incorporation of MrkF reduced the overall length of the fimbriae. It has been reported that the point mutations of FaeG, the major subunit of F4 fimbriae, expressed on enterotoxigenic *E. coli*, affected the length of the polymeric filament [39]. Sequence analysis revealed that MrkF possesses all the conserved motifs of a pilin subunit. However, the assembled structure of the MrkA–MrkF complex may be less stable than that of the MrkA–MrkA complex. The mature MrkA monomer with no signal peptide could only be detected after secretion into the periplasm or after assembly onto the cell surface, while more mature MrkA pilin monomers were found in the cell extract of *E. coli* [p*mrkABCDF*] than in that of *E. coli* [p*mrkABCD*] before the temperature was shifted to 95 °C (Fig. 5). This could result from breakdown of unstable filaments on the cell surface.

The shorter filaments might prevent the proper display of the MrkD adhesin on the tip of the fimbriae and hence lower hemagglutination activity. We have previously shown that the *mrkD* gene of *K. pneumoniae* CG43 encodes a truncation MrkD protein with no detectable hemagglutination activity [16]. To test whether incorporation of MrkF affects MrkD adhesion activity, the *mrkF* deletion mutant and the

complement derived from *K. pneumoniae* NTUH K-2044 [17] were then constructed. Interestingly, HA activity measurement revealed that *K. pneumoniae* NTUH K2044S3, NTUHS3*mrkF* and NTUHS3*mrkF*[pmrkF] all exhibited 64 Mr/K HA units. This argues against a role for MrkF in influencing MrkD adhesin activity. Nevertheless, the meshwork-like fimbriae conferred upon recombinant bacteria a higher level of biofilm formation ability and autoaggregation activity.

After initial adhesion, the bacteria tend to recruit additional bacteria by expression of the surface protein for the accretion step of biofilm formation. For example, *E. coli* expressed Ag43 protein to induce autoaggregation of inter- or intraspecies biofilm formation [32]. The observed differences in biofilm formation after 24 h of incubation between *E. coli* [p*mrkABCD*] and *E. coli* [p*mrkABCDF*] in Fig. 3(b) may be due to autoaggregation activity conferred by the minor subunit MrkF, leading to an increase in biofilm formation.

Taken together, we demonstrate that MrkF is a minor pilin subunit of type 3 fimbriae. The intermittent incorporation of MrkF provides a strategy for influencing fimbrial assembly and might thereby modulate the activity of type 3 fimbriae.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.resmic.2008.10.009.

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