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Original Article

A protein containing the DUF1471 domain regulates biofilm formation and capsule production in *Klebsiella pneumoniae*

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Abstract *Background/purpose:* Biofilms formed by *Klebsiella pneumoniae* on medical devices increase infection risk. Fimbriae and capsule polysaccharides (CPSs) are important factors involved in biofilm formation. KP1_4563 in *K. pneumoniae* NTUH-K2044, a small protein containing the DUF1471 domain, was reported to inhibit type 3 fimbriae function. In this study, we aimed to determine whether the KP1_4563 homolog is conserved in each *K. pneumoniae* isolate and what role it has in *Klebsiella* biofilms.
Methods: The genomes of *K. pneumoniae* NTUH-K2044, CG43, MGH78578, KPPR1 and STU1 were compared. The *KP1_4563* homolog in *K. pneumoniae* STU1 was named *orfX*. Biofilms of wild-type and *orfX* mutant strains from *K. pneumoniae* STU1 and one clinical isolate, 83535, were quantified. Transcription levels of the type 3 fimbrial genes, *mrkA* and *mrkH*, were investigated by RT-qPCR. MrkA of the wild-type and *orfX* mutant were observed by Western blotting. The morphology of bacterial cells was observed by transmission electron microscopy (TEM). Bacterial CPSs were quantified.
Results: The gene and upstream region of *orfX* were conserved among the five *K. pneumoniae* isolates. Deletion of *orfX* enhanced *Klebsiella* biofilm formation. However, the amount of mRNA from *mrkA* and *mrkH* and the level of MrkA protein were not different between the wild type and *orfX* mutant. In contrast, the amount of CPS in *orfX* mutants was increased,

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compared to their parental strains, STU1 and 83535.
Conclusion: The role of *orfX* is speculated to be conserved in most *K. pneumoniae* isolates. OrfX negatively controlled biofilm formation by reducing CPS, not type 3 fimbriae, production.

Introduction

Klebsiella pneumoniae is not only ubiquitously distributed in natural surface waters and soils but is also a member of the natural inhabitants of the gastrointestinal tract in humans and animals.[1,2] In addition, *K. pneumoniae* causes opportunistic infections, such as urinary tract infections, pneumonia, bloodstream infections and pyogenic liver abscesses.[3,4]

The biofilm formed by *K. pneumoniae* on the surfaces of medical devices increases the infection risk. An association of *K. pneumoniae* with indwelling medical device infections has been reported, including ventilator-associated pneumonia, catheter-associated urinary tract infections and catheter-related bloodstream infections.[5] Fimbriae, also known as pili, are filamentous structures located at the bacterial surface that mediate bacterial adhesion to surfaces to form biofilms. Type 3 fimbriae are reported to mediate the attachment of *K. pneumoniae* to the extracellular matrix, bind to human endothelial and bladder cells and promote biofilm formation on biotic and abiotic surfaces.[6] The components of type 3 fimbriae are encoded by genes in the *mrkABCDF* operon. The *mrkA* gene encodes the major subunit of the fimbrial shaft.[7] MrkH is a transcriptional activator positively regulating the expression of the *mrkABCDF* operon by either binding to DNA or acting as an anti-repressor of H-NS.[8,9]

Biofilms are complex structures comprised of proteins, polysaccharides and DNA.[10] Capsules, mainly comprised of polysaccharides, surround bacterial cells. Capsule-deficient strains show a dramatically attenuated ability to cause disease in mouse models. Capsules protect bacteria from complement binding and opsonization and from recognition and adhesion by phagocytes and epithelial cells. Such voluminous polysaccharide matrices can reduce the bactericidal effects of antimicrobial peptides.[11] Capsular polysaccharide (CPS) has been reported to be important in biofilm formation. Boddicker et al. identified several mutations in capsule gene cluster that caused defects in *Klebsiella* biofilm formation by signature-tagged mutagenesis.[12] In addition, Wu et al. reported that a mutant strain defective in *treC* that encodes trehalose-6-phosphate hydrolase displayed less CPS and a decreased ability to form biofilms, while the *sugE* mutant displayed more CPS and an increased ability to form biofilms.[13]

Luo et al. reported a novel gene, KP1_4563 in *K. pneumoniae* NTUH-K2044, that inhibited type 3 fimbrial function.[14] KP1_4563 is a small protein containing one DUF1471 domain in the C-terminus. However, Luo et al. did not directly examine type 3 fimbrial proteins or mRNA by Western blotting or quantitative reverse transcription PCR (RT—qPCR). In addition, the role of the *KP1_4563* gene in *Klebsiella* biofilms is unknown, and type 3 fimbriae are important for *Klebsiella* biofilm formation. Therefore, we examined whether type 3 fimbriae are regulated by *KP1_4563* in *K. pneumoniae*. In this study, we found that the homolog of the *KP1_4563* gene is common in several representative isolates of *K. pneumoniae*. We renamed the homolog of the *KP1_4563* gene in two *K. pneumoniae* isolates, STU1 and 83535, *orfX*. We observed that *orfX* controlled biofilm formation in *K. pneumoniae* but did not regulate type 3 fimbriae. The amount of capsular polysaccharide (CPS) was reduced by *orfX*. This finding indicates that the function of *orfX* is conserved in *K. pneumoniae* isolates and it regulates *Klebsiella* biofilm formation.

Methods

Bacterial strains, plasmids, primers and growth conditions

The bacterial strains, plasmids, and primers used in this study are summarized in Table 1 and Table 2 respectively. Unless otherwise indicated, bacteria were routinely in Luria—Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) and appropriate antibiotics were supplemented as described in the previous study.[15] The final concentration of isopropyl β-D-1-thiogalactopyranoside (IPTG) was 50 μg/mL to induce the expression of *orfX* in the pBSK-derivative plasmid.

Construction of *orfX* mutant

The *orfX* gene was deleted using unmarked mutagenesis in *K. pneumoniae* STU1 and 83535 as described in a previous study.[15,16] In brief, the flanking DNA fragments of *orfX* gene which were amplified by PCR using primers (Table 2) and ligated into the suicide vector, followed by plasmid transfer into *K. pneumoniae* by conjugation. The transconjugants were positively selected by antibiotics and negatively selected by sucrose. The mutant strains were confirmed by PCR by several sets of primers (Table 2), followed by sequencing.

Promoter prediction

The −10 and −35 elements of *orfX* promoter are predicted by Softberry-BPROM (http://www.softberry.com/all.htm).

Table 1 Bacterial strains and plasmids used in this study.

Strain	Relevant genotype and phenotype	Reference or source
K. pneumoniae		
STU1	Laboratory-maintained strain, Amp^r	National Taiwan University[16]
STU1ΔorfX	deletion of *orfX* gene in STU1	This work
83535	Clinical strain isolated from blood specimen	Tzu Chi Hospital, Hualien, Taiwan[16]
83535ΔorfX	deletion of *orfX* gene in 83535	This work
Plasmid		
pBSK-Gm	pBSK derivative carrying gentamicin resistance gene at the ScaI site, Gm^r	[29]
pBSK::orfX	pBSK-Gm carrying complete *orfX* gene, Gm^r	This work

Quantification of biofilm formation

The biofilm formation assay was conducted as described in the previous study.[16]

Western blotting

Western Blotting was performed as described previously.[16] Intracellular mannose 6-phosphate isomerase (ManA) was detected as control.

Quantitative reverse transcription PCR (RT-qPCR)

Extraction of RNA, reversed transcription and quantification of cDNA from *mrkA* and *mrkH* using fluorescein-labeled and dual-quenched probes (Integrated DNA Technologies, Coralville, IA, USA) were performed as described previously.[15] Relative gene expression was quantified using 16s rRNA as the endogenous reference gene. cDNA from *recA* was also detected to confirm the consistence of each assay. The primers and probes for 16s rRNA and *recA* analyzed by RT-qPCR were as the same as the previous study.[15] The primers and probes for *mrkH* and *mrkA* were summarized in Table 2.

Transmission electron microscopy (TEM)

The bacterial pellet was suspended in ultra-purified water after 500 μL of overnight bacterial culture was centrifuged and washed with phosphate buffered saline (PBS) once. 10 μl of bacterial suspension was absorbed onto 300-μm-pore-size mesh copper electron microscopy grids coated with carbon and Formvar for 30 s before the grids were rinsed once with ultra-purified water. Then, a drop of 2% (w/v) uranyl acetate was dripped onto the grid and sat for 30 s. After removing the excess liquid, the grid was air dried for 5—10 min at room temperature. Bacterial samples were observed under a Hitachi H-7500 transmission electron microscope (Hitachi, Japan) under standard conditions.

Table 2 Oligonucleotide primers used in this study.

Primer	Sequences (5′→3′)	Target/purpose
EcoRI,SmaI-orfX up-FP	GGGAATTCCCCGGGTCAGGGG ATGCTGATTTATG	PCR amplification for flanking fragments of *orfX* gene/construction of *orfX* mutant
BamHI-orfX up-RP	TGGGATCCCATGTTTGCTTTCG ATGGAC	PCR amplification for flanking fragments of *orfX* gene/construction of *orfX* mutant
BamHI-orfX down-FP	TAGGATCCAATAAGCGAATGG TTGCAGA	PCR amplification for flanking fragments of *orfX* gene/construction of *orfX* mutant
SacI,SmaI-orfX-down-RP	TTGAGCTCCCCGGGATTCTCG ACCTGCTGCTCAC	PCR amplification for flanking fragments of *orfX* gene/construction of *orfX* mutant
BamHI-orfX-FP	TTGGATCCGCAACAGCATGCA GAAAAAG	Confirmation of *orfX* mutant
EcoRI-orfX-RP	TTGAATTCGATGGGATTGATCG TGGTG	Confirmation of *orfX* mutant
KOorfX1HR-FP	TCGCGTAGCTGTTAACCACA	Confirmation of *orfX* mutant
KOorfX1HR-RP	ATTCTCGACCTGCTCAC	Confirmation of *orfX* mutant
MrkA qPCR FP	GCGGTAGCGTTGTCAGTAGA	*mrkA* mRNA/RT- qPCR
MrkA qPCR RP	GCTGCTGATGGCACTAAACA	*mrkA* mRNA/RT- qPCR
MrkA probe	TCCAGTTCA/ZEN/CGCCCAGTT	*mrkA* mRNA/RT- qPCR
MrkH qPCR FP	GCTACGCTTTTTCATTGCC	*mrkH* mRNA/RT- qPCR
MrkH qPCR RP	AACCGCCATCTGAAATGTC	*mrkH* mRNA/RT- qPCR
MrkH probe	CGCTTTCGT/ZEN/TTACGCCAT	*mrkH* mRNA/RT- qPCR

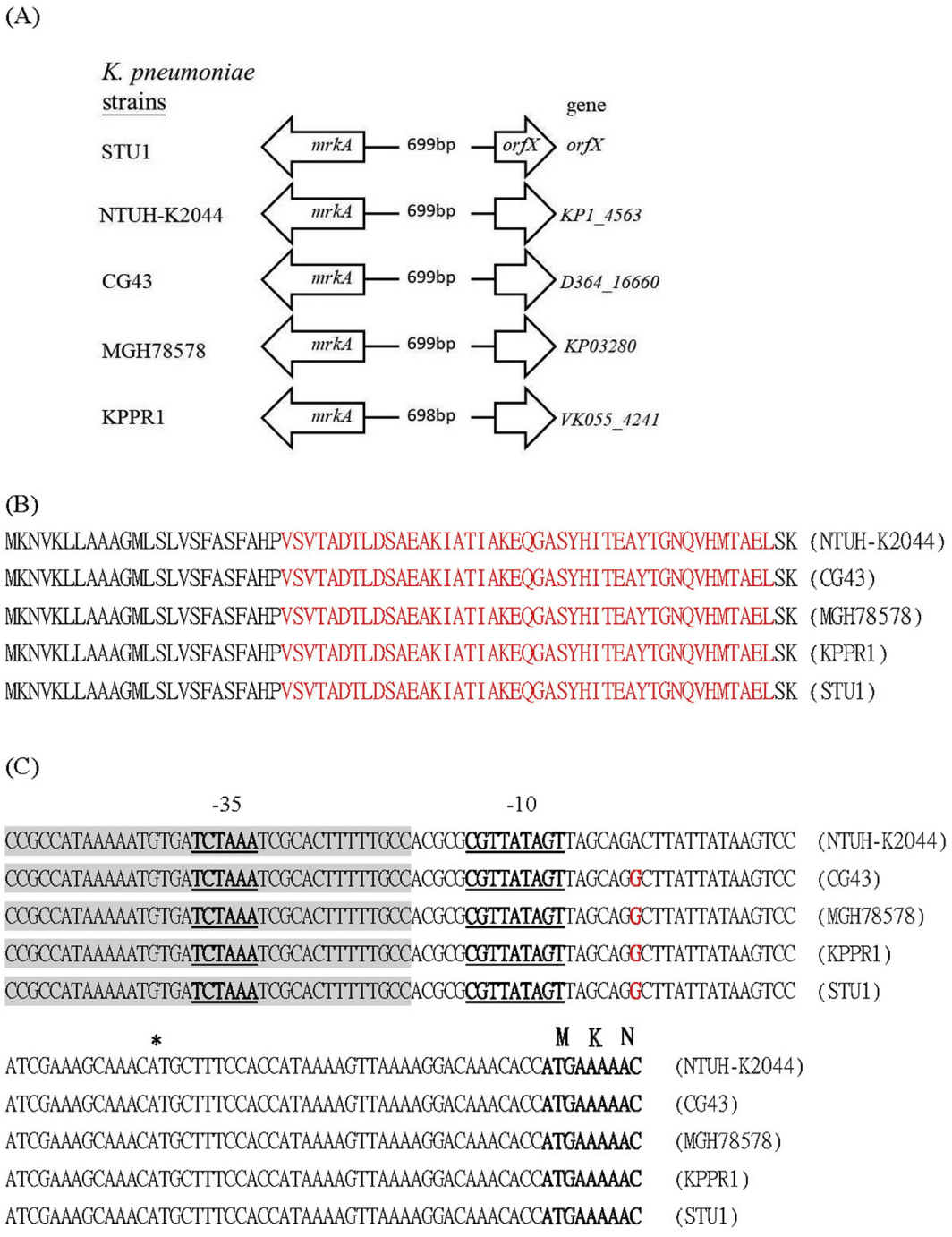

Figure 1. Comparison of *orfX* and its upstream region in different *K. pneumoniae* isolates. (A) Schematic diagram of the genetic organization of *orfX* and *mrkA* in *K. pneumoniae* STU1, NTUH-K2044, CG43, MGH78578 and ATCC 43816 KPPR1. *KP1_4563*, D364_16,660, *KPN03280* and VK055_4241 are homologs of *orfX* in every isolate of *K. pneumoniae*. (B) Alignment of the amino acid sequence of OrfX homologs in different *K. pneumoniae* isolates. The DUF1471 domain is shown as red. (C) Alignment of the upstream region of *orfX* in different *K. pneumoniae* isolates. The putative −10 and −35 in the promoter is shown in bold and underlined. The CRP binding site identified by DNase I footprinting in the previous study is labeled in a gray shade. The start codon of the OrfX homolog speculated in a previous study is labeled with a star.[14] The first three codons (MKN) of the OrfX homolog speculated in this study are labeled in bold. The nucleotides different from that in NTUH-K2044 are shown in red.

Extraction and quantification of capsular polysaccharide (CPS)

CPS was extracted and quantified as described previously.[15]

Statistical analysis

Data from biofilm Formation, RT-qPCR and CPS quantification were shown as the mean ± standard deviation from

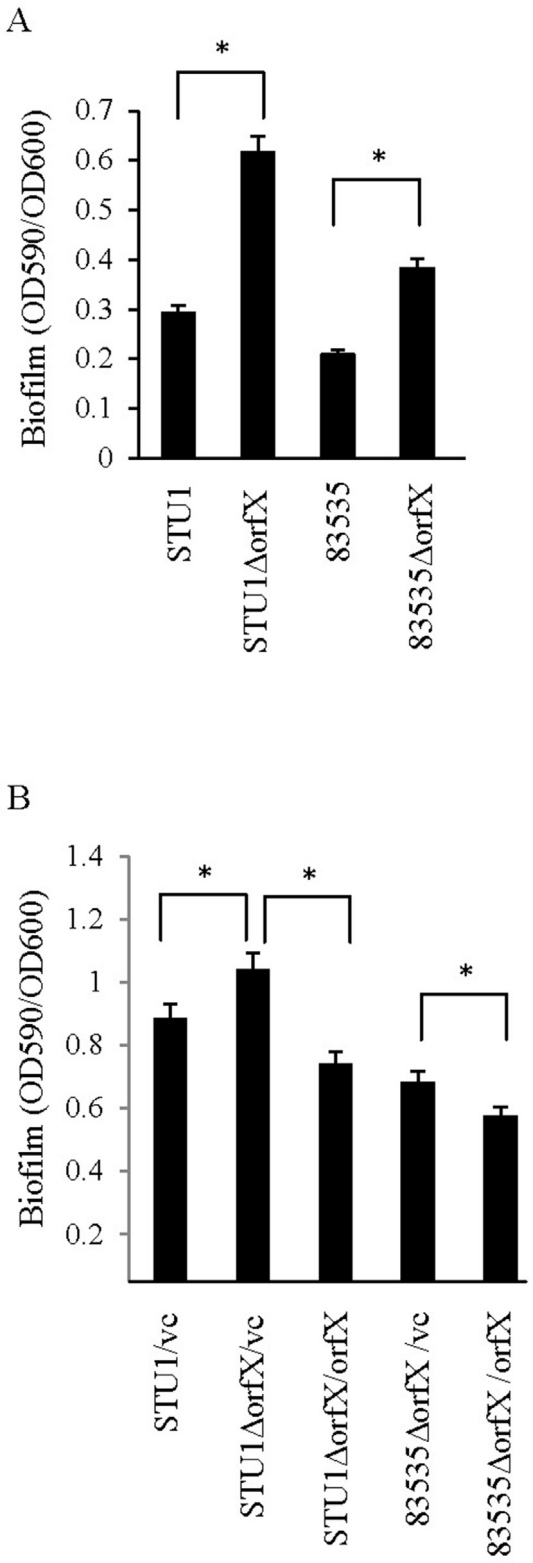

Figure 2. Quantification of biofilm formation by *K. pneumoniae*. (A) The amount of biofilm produced by the two isolates of *K. pneumoniae*, STU1 (STU1) and 83535 (83535), and their corresponding *orfX* mutants (STU1ΔorfX and 83535ΔorfX) was quantified. (B) The amount of biofilm produced by bacteria carrying pBSK-Gm as a vector control (vc) or carrying pBSK:orfX to express OrfX was quantified. *K. pneumoniae* STU1 carrying pBSK-Gm (STU1/vc), and *K. pneumoniae orfX* mutants carrying pBSK-Gm (STU1ΔorfX/vc and 83535ΔorfX/vc). *orfX* mutants carrying pBSK:orfX as the *orfX* complemented strain (STU1-ΔorfX/orfX and 83535ΔorfX/orfX). The error bars indicate standard deviations from three independent experiments. Asterisk (*) represents $p < 0.05$.

three independent bacterial cultures. Data were subjected to one-way analysis of variance (ANOVA) and considered significantly different at $p < 0.05$.

Results

KP1_4563 homologs are conserved in *K. pneumoniae* isolates

To clarify whether the *KP1_4563* homolog is conserved among different isolates of *K. pneumoniae* or is unique to *K. pneumoniae* NTUH-K2044, the genomes of *K. pneumoniae* CG43, MGH78578 and ATCC 43816 KPPR1 were compared by BLAST. The results showed that the *KP1_4563* homolog is D364_16,660 in CG43, KPN03280 in MGH78578 and VK055_4241 in KPPR1. An open reading frame homologous to *KP1_4563* in *K. pneumoniae* STU1 is named *orfX* (Fig. 1A). The amino acid sequences of the *KP1_4563* homologs were identical among the *K. pneumoniae* isolates, although there were some minor differences in the nucleotide sequences of the *KP1_4563* homologs (data not shown). The DUF1474 domain is located in the C-terminal region of OrfX and four other homologs (Fig. 1B), indicating that *KP1_4563* homologs widely exist and are conserved among *K. pneumoniae* isolates. To determine whether the genetic regulation of *KP1_4563* homologs is different or is dependent on the isolates, the upstream region of the *KP1_4563* homolog in each strain was examined. The results showed that the MrkA homolog was located 699 bp or 698 bp upstream of the *KP1_ 4563* homolog and in a head-to-head organization in all five *K. pneumoniae* isolates (Fig. 1A). In addition, the CRP binding site located upstream of *KP1_ 4563* in *K. pneumoniae* NTUH-K2044 was confirmed by DNase I footprinting in a previous study.[14] The 121 bp DNA sequences upstream of the *KP1_4563* homologs, including the CRP binding site and the predicted −10 and −35 elements of the promoter, were conserved in all five *K. pneumoniae* isolates (Fig. 1C), indicating that the transcriptional regulation of *KP1_4563* homologs is predicted to be the same in each *K. pneumoniae* isolate.

OrfX inhibited biofilm formation in *K. pneumoniae* but not via type 3 fimbriae

Luo et al. inferred that the KP1_4563 gene inhibited the function of type 3 fimbriae in *K. pneumoniae* NTUH-K2044.[14] Type 3 fimbriae play a positive role in biofilm formation in *K. pneumoniae*.[17] To determine whether *orfX* inhibited biofilm formation in *K. pneumoniae*, *orfX* was deleted in one laboratory strain, STU1, and one clinical strain, 83535. After incubation for 24 h, both *orfX* mutants showed more biofilms than their parent strains (Fig. 2A).

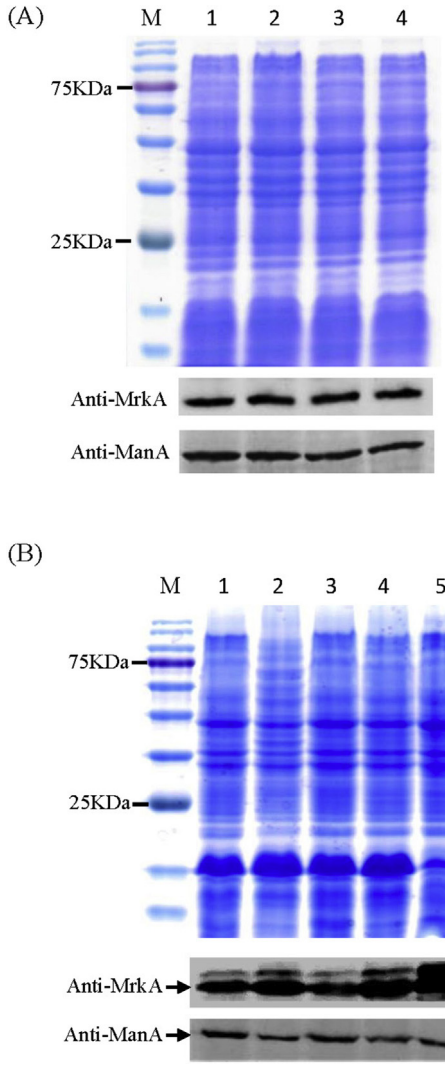

Figure 3. Western blotting analysis of MrkA and ManA expression. Representative Western blotting and SDS-PAGE images from bacteria incubated in LB broth in three independent experiments. (A) Lane 1: *K. pneumoniae* STU1. Lane 2: *K. pneumoniae* STU1 *orfX* mutant (STU1ΔorfX). Lane 3: *K. pneumoniae* 83535. Lane 4: *K. pneumoniae* 83535 *orfX* mutant (83535ΔorfX). (B) Lane 1: *K. pneumoniae* STU1 carrying pBSK-Gm as a vector control (vc). Lane 2: *K. pneumoniae* STU1 carrying pBSK:orfX to overexpress OrfX. Lane 3: STU1ΔorfX carrying pBSK-Gm (vc). Lane 4: STU1ΔorfX carrying pBSK:orfX (*orfX* complemented strain). Lane 5: 83535ΔorfX carrying pBSK-Gm (vc). Lane 6: 83535ΔorfX carrying pBSK:orfX (*orfX* complemented strain). ManA was detected as the control.

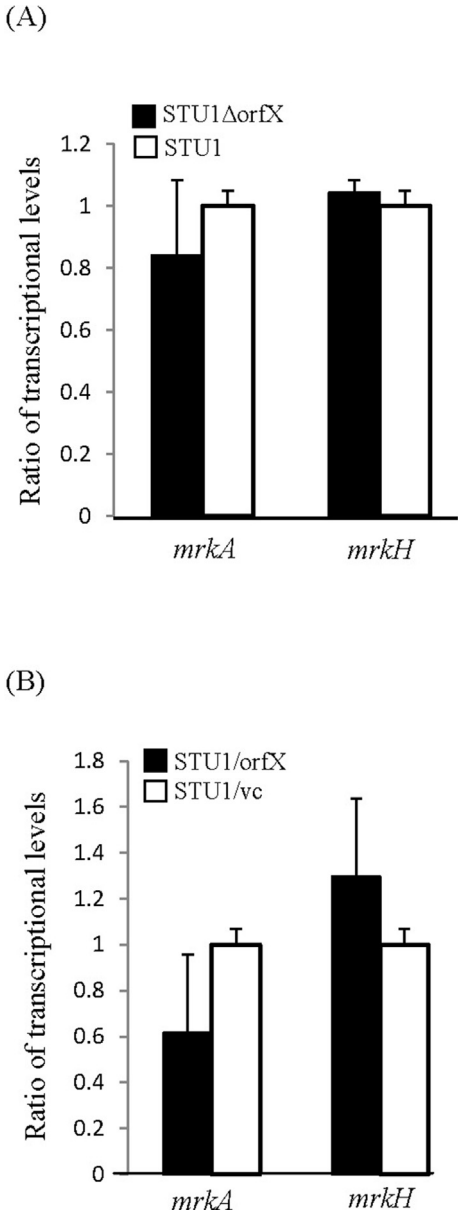

Figure 4. RT-qPCR analysis of *mrkA* and *mrkH* transcription in bacteria grown in LB medium. (A) Transcriptional levels of *mrkA* or *mrkH* in the *K. pneumoniae* STU1 *orfX* mutant (STU1-ΔorfX) were compared to those in *K. pneumoniae* STU1 (STU1). (B) Transcriptional levels of *mrkA* or *mrkH* in *K. pneumoniae* STU1 carrying pBSK:orfX (STU1/orfX) were compared to those in *K. pneumoniae* STU1 carrying pBSK-Gm (STU1/vc). The presented results are the means ± standard deviations of three replicates.

Subsequently, both *orfX* mutants were complemented by transforming plasmids carrying wild-type *orfX* into *orfX* mutants. The biofilms of both *orfX* complemented strains were reduced, compared to their corresponding mutants (Fig. 2B). These results indicated that *orfX* inhibited biofilm formation.

Since MrkA is the major fimbrial subunit that forms a type 3 fimbrial shaft,[18] the MrkA of each strain was quantified by Western blot. However, the results showed that the amount of MrkA in both *orfX* mutants was not increased, compared to their parent strains, STU1 and 83535 (Fig. 3A). The amount of MrkA in both complement mutants was not decreased, compared to their corresponding mutants (Fig. 3B). *mrkH* encode transcriptional activator to control type 3 fimbriae expression.[19] By RT-qPCR, the transcript levels of *mrkA* or *mrkH* in the *K. pneumoniae* STU1 *orfX* mutant and wild-type strain were not different (Fig. 4A). The *mrkA* and *mrkH* transcripts in the *orfX*-overexpressing strain and vector control were also

not significantly different (Fig. 4B). In addition, quantification of FimA (a major fimbrial subunit of type 1 fimbrial shaft) in the wild type and *orfX* mutant by Western blotting did not show a difference (data not shown). Therefore, although *orfX* (*KP1_4563* homologs) plays a negative role in biofilm formation, this effect is not mediated through fimbria.

OrfX reduced the amount of capsular polysaccharide (CPS)

To examine the effects of OrfX on bacterial morphology, the *K. pneumoniae* STU1 and 83535 *orfX* mutants and their parent strains were observed by TEM. The results showed that there was a lot of matrix surrounding the *orfX* mutants, compared to their parental strains (Fig. 5). The matrix was speculated to be CPS. To confirm this, the bacterial CPS was extracted from the *orfX* mutants and their parental strains and the amount of CPS produced by the *orfX* mutants was found to be slightly increased, compared to their parental strains (Fig. 6A). The amount of CPS in the *orfX* complemented strains was lower than that in their corresponding *orfX* mutants (Fig. 6B). Therefore, OrfX plays a negative role in *K. pneumoniae* CPS synthesis. We speculated that OrfX inhibited biofilm formation by reducing CPS production in *K. pneumoniae*.

Discussion

Biofilm formation by *K. pneumoniae* enhances its infection risk, especially for immunocompromised or hospitalized patients. OrfX and the upstream region of *orfX* are conserved among *K. pneumoniae* NTUH-K2044, CG43, MGH78578, KPPR1, STU1 and 83535 (Fig. 1). The role of OrfX in *K. pneumoniae* is thereby speculated to be conserved in most isolates of *K. pneumoniae*. In our study, deletion of *orfX* resulted in increased biofilm formation (Fig. 2). However, synthesis of the major fimbrial subunit (MrkA or FimA) and transcriptional levels of the fimbrial regulator (MrkH) were not affected by *orfX* (Figs. 3 and 4). In contrast, CPS production was partly inhibited by OrfX (Fig. 6). Some reports have shown that CPS has positive effects on biofilms and that mutations in the *cps* gene cluster result in decreased biofilm formation.[12,13] Thus, *orfX* inhibited biofilm formation by repressing CPS production, although overexpression of OrfX in the *orfX* mutant did not completely abolish CPS production (Fig. 6B) or biofilm formation (Fig. 2B). However, some CPS deficiency mutants showed increased biofilm formation in some studies.[20,21] There may be other factors in these CPS-deficient mutants involved in biofilm formation or an unclear relationship between biofilm formation and CPS production.

The OrfX homolog in *K. pneumoniae* NTUH-K2044, KP1_4563, was previously reported to regulate type 3 fimbrial function. However, the authors only performed indirect assays such as mannose-resistant hemagglutination assay, bacterial adhesion to cell line assay and mannan-binding assay without directly examining the MrkA protein or the *mrkA* mRNA transcripts.[14] In contrast, we found that the translational levels of the type 3/type 1 major fimbrial

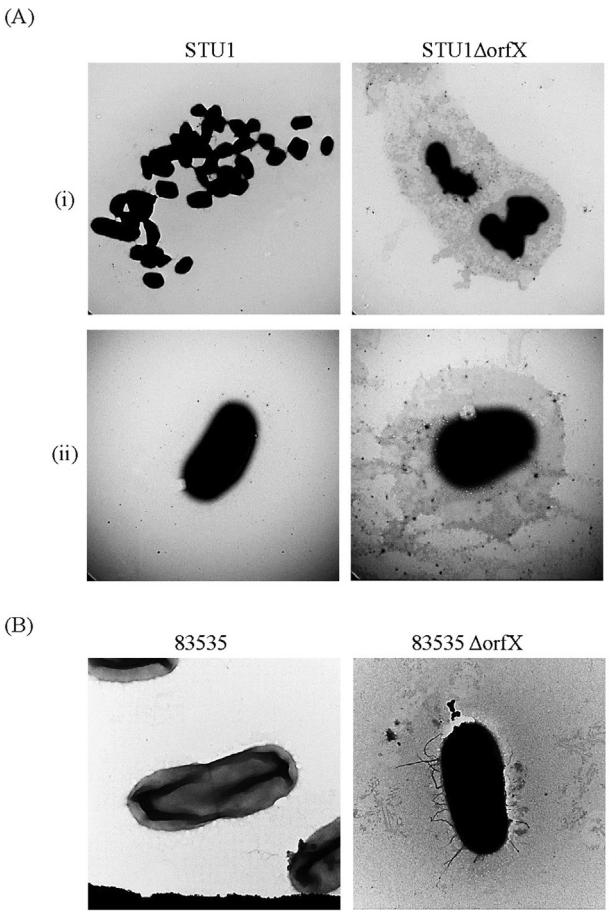

Figure 5. Bacterial morphology was observed by transmission electron microscopy (TEM). Representative TEM images of bacteria incubated in LB broth from three independent experiments. (A) *K. pneumoniae* STU1 (STU1) and the *K. pneumoniae* STU1 *orfX* mutant (STU1ΔorfX) were observed at (i) 10,000 × and (ii) 32,500 × magnification. (B) *K. pneumoniae* 83535 (83535) and the *K. pneumoniae* 83535 *orfX* mutant (83535ΔorfX) were observed at 32,500 × magnification.

subunit MrkA/FimA were not significantly different between the *orfX* mutant and wild type. This phenotype was observed in more than one isolate, in both STU1 and 83535 (Fig. 3 and data not shown), and the mRNA levels of *mrkA* and *mrkH* were not significantly different between the *orfX* mutant and the wild type (Fig. 4). We speculated that the regulation of type 3 fimbriae by OrfX is different in various environments/phenotypes or that OrfX controls unknown factors, not fimbriae, in hemagglutination, bacterial adhesion and mannan-binding assays.

OrfX contains a DUF1471 domain in its C-terminus (Fig. 1). Proteins containing the DUF1471 domain are found exclusively in Enterobacteriaceae. Most proteins containing the DUF1471 domain are small (approximately 90 residues). Some have repeated DUF1471 domains.[22] Many DUF1471 proteins are related to the stress response or biofilm formation, although the mechanism is unknown. For example, *Escherichia coli* DUF1471 protein YbiM (also named McbA) is an 86 aa protein and it showed 30% identity and 44% similarity to OrfX. YbiM is speculated to inhibit biofilm

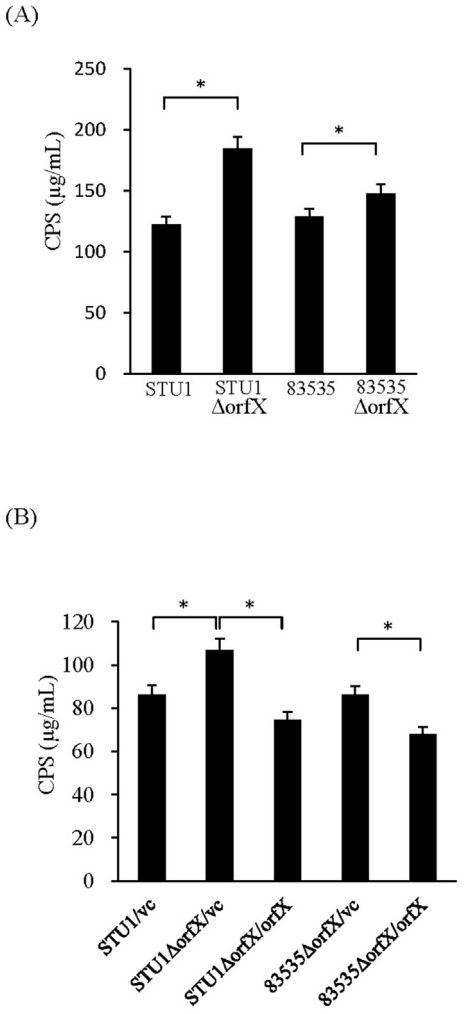

Figure 6. Quantification of capsular polysaccharide (CPS). (A) The amount of CPS produced by *K. pneumoniae* STU1 (STU1) or 83535 (83535) and their corresponding *orfX* mutants (STU1ΔorfX and 83535ΔorfX) was quantified. (B) The amount of CPS produced by bacteria carrying pBSK-Gm as a vector control (vc) or carrying pBSK:orfX to express OrfX was quantified. *K. pneumoniae* STU1 carrying pBSK-Gm (STU1/vc), and *K. pneumoniae orfX* mutants carrying pBSK-Gm (STU1ΔorfX/vc and 83535ΔorfX/vc). *orfX* mutants carrying pBSK:orfX as the *orfX* complemented strain (STU1ΔorfX/orfX and 83535ΔorfX/orfX). Error bars indicate the standard deviations from three independent experiments. The asterisk (*) represents $p < 0.05$.

formation by comparison of the *yncC/ybiM* double mutant and *yncC* mutant (there is no available *ybiM* mutant to confirm the function of YbiM).[23] Another *E. coli* DUF1471 protein, BhsA, formerly named YcfR, that shows 41% identity/50% similarity to OrfX, was demonstrated to inhibit biofilm formation in the presence of glucose and affect bacterial stress resistance.[24] In addition, the identity of 22 residues between the N-terminus of BhsA and OrfX was 73% (data not shown). A predicted transmenbrane segment in the N-terminus (6th-28th residue) of OrfX oriented toward the cytoplasm (predicted by TMpred, data not shown), indicating that DUF1471 domain facing in toward the periplasmic space in the case of OrfX in the inner membrane or oriented toward the extracellular space in the case of OrfX in the outer membrane. Eletsky et al. reported that DUF1471 sequences showed similar structure fold to that of bacterial outermembrane lipoprotein RcsF.[22] RcsF triggers the Rcs envelope stress response pathway via RcsAB that is an atypical two-component system.[25] The gene *galF* that was responsible for the translocation and surface assembly of CPS in *K. pneumoniae* was one of target genes regulated by RcsAB.[26] Adhesion is an initial step in biofilm formation. Perhaps integrity of envelope is disturbed while bacteria adhere to surface.[25] The possible roles of DUF1471 domain in OrfX/BhsA/YbiM may sense the envelop stress and then trigger the response to keep the envelope integrity. Adjustment of CPS amount may be one of responses to envelop stress. Although fimbriae production is important for adherence, the amount of fimbriae is not essential for integrity of envelope. Therefore, effect of *orfX* on CPS, not fimbriae, was observed in *K. pneumoniae*. However, the signaling mechanism through DUF1471 domain may different from RcsF because DUF1471 domain lacks the essential cysteine residues for a phospho-relay that RcsF is involved in.[22]

The Shine Dalgarno (SD) sequence is important for translation initiation. The optimal spacing between the SD sequence and the start codon is 8 nt, not less than 4 nt and not longer than 14 nt.[27,28] The amino acid composition deduced from the nucleotide sequence of *orfX* contains 69 residues (Fig. 1). However, Luo et al. defined the start codon of KP1_4563 as the star sign mentioned in Fig. 1, resulting in a putative 81 amino acid peptide.[14] In this study, the putative SD sequence preceding the start codon we defined in Fig. 1 is similar to the consensus SD sequence, compared to the sequence preceding the codon label with a star sign in Fig. 1. However, the actual start codon of OrfX will be identified in the future. According to the report by Luo et al., the CRP binding site corroborated by DNase I footprinting is shown as a gray background in Fig. 1.[14] This region contains a putative CRP binding site, TGTGATCT, predicted by the Softberry-BPROM program, and it overlaps with the putative promoter in each isolate of *K. pneumoniae* (Fig. 1).

In summary, we demonstrated that OrfX is conserved in *K. pneumoniae* isolates and it inhibits bacterial biofilm formation via regulation of CPS synthesis. We also showed that OrfX does not regulate fimbriae synthesis in *K. pneumoniae*.

Authors' contributions

Conceived and designed the experiments: P.C.S.; Performed the experiments: H.J.C., N.S.D.P. and P.C.S.; Analyzed and interpreted the data: H.Y.C., H.C.Y., Y.H.W., C.C.C. and P.C.S.; Wrote the paper: Y.T.H., P.C.S.; All authors read and approved the final manuscript.

Conflicts of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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