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MOLECULAR PATHOGENESIS

Roles of OmpX, an Outer Membrane Protein, on Virulence and Flagellar Expression in Uropathogenic *Escherichia coli*

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ABSTRACT Uropathogenic *Escherichia coli* (UPEC) is a major pathogen that causes urinary tract infection (UTI). This bacterium adheres to and internalizes within urinary tract cells, where it aggregates and subsequently forms biofilm-like multicellular colonies that protect UPEC from antimicrobial agents and the host's immune system. Here, we show that OmpX, an outer membrane protein, plays a role in the pathogenesis of UPEC in renal cells. Deletion of *ompX* decreased bacterial internalization and aggregation within kidney epithelial cells and also impaired the colonization of mouse urinary tracts, but the *ompX* mutant still adhered to the epithelial cells at a level similar to that of the parent strain. FlhD, the master regulator of flagellum-related genes, had a low expression level in the *ompX* mutant compared to the parent strain, and the *ompX* mutant exhibited defective motility due to lower flagellar production than the parent strain. The *fliC* mutant, which lacks flagella, exhibited lower levels of bacterial internalization and aggregation than the parent strain. Additional deletion of *ompX* in the *fliC* mutant did not further decrease bacterial internalization. These combined results suggest that OmpX contributes to flagellar production in UPEC and then sustains UPEC virulence associated with bacterial internalization and aggregation within urinary tract cells and colonization in the urinary tract.

KEYWORDS virulence, pathogenesis, biofilm, motility, urinary tract infection, pyelonephritis, flagella, gene regulation

Urinary tract infection (UTI) is one of the most common infectious diseases. Uropathogenic *Escherichia coli* (UPEC) is a major pathogen that is estimated to cause over 80% of uncomplicated UTIs (1, 2). UPEC is categorized in a pathogenic subgroup termed extraintestinal pathogenic *E. coli* (ExPEC), which causes infectious disease outside the intestines. When UPEC enters the urinary tract, the bacteria adhere to and internalize within bladder epithelial cells, where they aggregate and form biofilm-like microbial colonies, termed intracellular bacterial communities (IBCs) (3). IBCs protect UPEC from antimicrobial agents, which may be associated with the failure of antimicrobial chemotherapy and the recurrence of infection (4, 5). After the bacteria ascend the ureters and reach the kidneys, if chemotherapy fails, this may result in irreversible kidney failure and/or septicemia.

Fimbriae and flagella are major protein structures responsible for the pathogenicity of UPEC. Type 1 fimbriae are required for bacterial adhesion to and internalization within bladder epithelial cells, while P-type fimbriae are proposed to play a role in the pathogenesis of ascending UTIs and pyelonephritis in humans (6–8). Flagella are required for bacterial migration to infection sites as the bacteria colonize the bladder, and flagellum-mediated motility contributes to bacterial fitness and aggregation within bladder epithelial cells (9–11). Flagella also contribute to bacterial entry into renal collecting duct cells, and it has been proposed that they allow the bacteria to ascend from the bladder and initiate kidney infections

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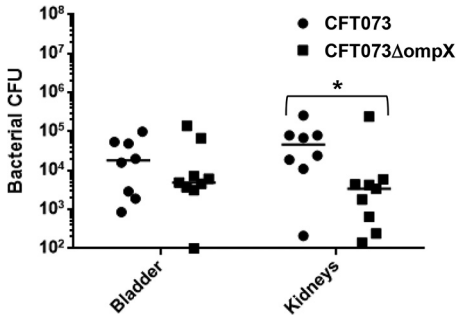

FIG 1 Colonization by the parent strain (CFT073) and *ompX* mutant in the bladders and kidneys of mice with UTIs. The female mice ($n = 3$ for each group) were infected with the parent strain or *ompX* mutant. At 48 h postinfection, cell numbers of bacteria isolated from the bladder and kidneys were determined as CFU. We repeated experiments independently three times, but one mouse infected with the parent strain died within 48 h of infection. Each data point represents a sample from an individual mouse ($n = 8$ for the parent strain and $n = 9$ for the *ompX* mutant). Horizontal bars show median values. *, $P < 0.05$ relative to the value for the parent strain.

(12). It has been demonstrated that antibodies against flagella prevent the dissemination of UPEC into the kidneys (13).

OmpX is an outer membrane protein composed of an eight-stranded β-barrel structure with membrane-spanning regions (14). Although this protein was originally described in *Enterobacter cloacae*, its homolog and paralog proteins have been identified in other Gram-negative pathogens, including *E. coli*, *Salmonella enterica*, *Yersinia pestis*, and *Klebsiella pneumoniae* (15–19). There are some reports that these proteins are implicated in bacterial virulence. In a study on *E. coli*, deletion of *ompX* in an ExPEC strain isolated from the lung of a diseased pig decreased the virulence in systemically infected mice and alveolar basal epithelial cells (20).

We are interested in characterizing proteins that contribute to the pathogenicity of UPEC in the urinary tract. In this study, we show that a UPEC *ompX* mutant colonized the kidneys of UTI mice and internalized and aggregated within human kidney epithelial cells with lower efficiency than the parent strain but still adhered to the cells at a level similar to that of the parent strain. The *ompX* mutant exhibited defective motility due to a low level of flagellin expression compared to the parent strain. We also show that the decreased ability of the UPEC *ompX* mutant to internalize within epithelial cells of the kidney is associated with defective flagellum production and motility.

RESULTS

Deletion of the *ompX* gene reduces UPEC colonization in the kidneys of mice. To characterize the role of OmpX in UPEC pathogenesis, we constructed an in-frame deletion mutant of the *ompX* gene and estimated the abilities of the parent CFT073 strain and its *ompX* mutant to colonize in the mouse urinary tract. C3H/HeN female mice were transurethrally infected with 1×10^8 CFU of either the *ompX* mutant or the parent strain, and the titers of bacteria in the bladder and kidneys were determined at 48 h postinfection. Although no statistical difference was observed in the numbers of bacteria in the bladder between the *ompX* mutant and the parent strain, mice infected with the *ompX* mutant exhibited lower UPEC burdens in the kidneys than those infected with the parent strain (median numbers of CFU for bladder, 1.8×10^4 for the parent strain and 4.9×10^3 for the *ompX* mutant; median numbers for kidneys, 4.7×10^4 for the parent strain and 3.4×10^3 for the *ompX* mutant) (Fig. 1). We confirmed that the reduction of CFU in the mouse kidneys for the *ompX* mutant was not due to a growth defect because the growth rates of the *ompX* mutant were essentially the same as those of the parent strain when cultured in LB medium, artificial urine medium (AUM), and RPMI 1640 media (Fig. S1).

Deletion of the *ompX* gene reduces bacterial internalization in and aggregation within renal epithelial cells. From the result of the mouse experiment, we hypothesized that the *ompX* gene might also contribute to bacterial adhesion to and/or internalization within the kidney cells, and we compared the abilities of the parent strain

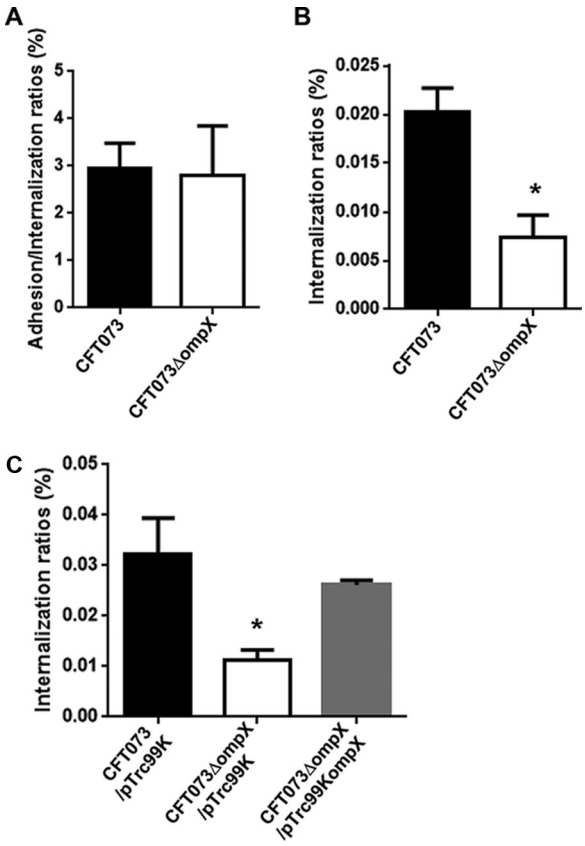

FIG 2 Adhesion to and internalization in kidney epithelial cells (HTB-44) of the parent strain (CFT073) and the *ompX* mutant (A and B) or the parent strain and the *ompX* mutant carrying pTrc99K (empty vector) or pTrc99KompX (*ompX* expression plasmid) (C). Values are percent CFU of adhered/internalized (A) and internalized (B and C) bacteria relative to total bacterial cell numbers. Data are means from three independent experiments; error bars indicate standard deviations. *, $P < 0.05$ relative to values for CFT073 (A and B) or CFT073/pTrc99K (C).

and the *ompX* mutant to adhere to and internalize within HTB-44 kidney epithelial cells using a gentamicin protection assay. No significant difference was seen in the total adherence/internalization values between the parent strain and the *ompX* mutant (2.96 $\pm$ 0.38% for the parent strain and 2.80 $\pm$ 0.74% for the *ompX* mutant) (Fig. 2A). However, the *ompX* mutant exhibited an approximately 3-fold-lower degree of internalization alone than the parent strain (0.020 $\pm$ 0.003% for the parent strain and 0.0073 $\pm$ 0.0016% for the *ompX* mutant) (Fig. 2B). We confirmed that the internalization of the *ompX* mutant was elevated by the introduction of pTrc99KompX, the complementation plasmid for the *ompX* gene (Fig. 2C). We also characterized UPEC colonies within the kidney epithelial cells and then inoculated the green fluorescent protein (GFP)-expressing parent strain or *ompX* mutant into HTB-44 cells. The *ompX* mutant formed smaller and fewer colonies than the parent strain. When the exogenous *ompX* expression plasmid pTH18krompX was introduced, the mutant formed colonies similar to those of the parent strain (Fig. 3A to C). These observations suggest that the *ompX* gene is responsible for optimal internalization and aggregation within the kidney epithelial cells.

The *ompX* mutant exhibits lower motility, due to lower flagellar production, than the parent strain but produces P-type and type 1 fimbriae at the same level as the parent strain. Flagella and flagellum-associated motility contribute to the internalization of UPEC in renal cells, leading to kidney infections (12, 13). Therefore, the reduced bacterial internalization and aggregation within the kidney epithelial cells by *ompX* deletion may be associated with decreased flagellar production and motility. To test this hypothesis, we compared the flagellar production and motility of the *ompX* mutant

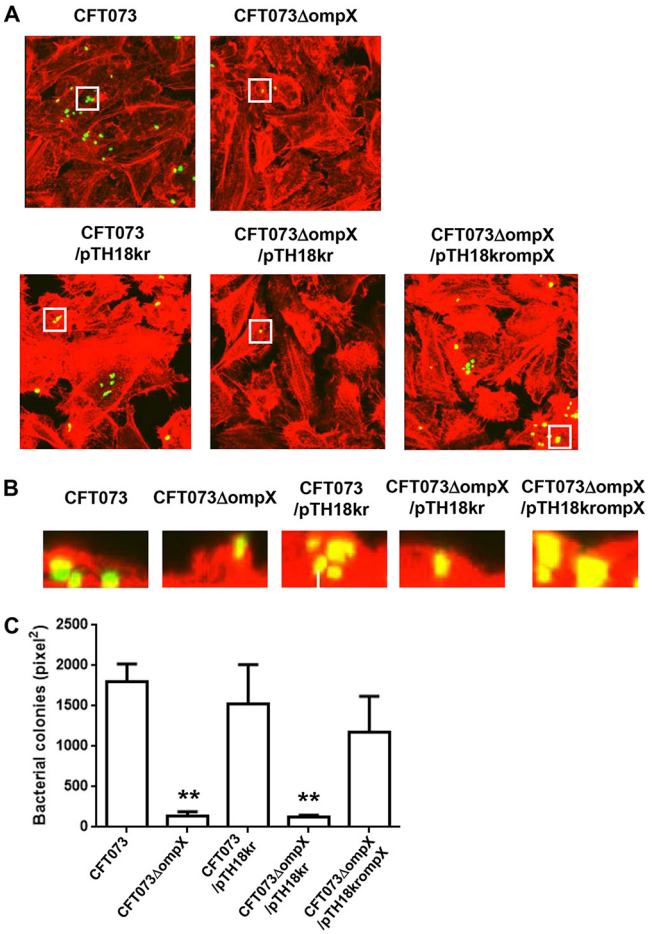

FIG 3 Aggregation within kidney epithelial cells (HTB-44) for the parent strain and the *ompX* mutant or the parent strain and the *ompX* mutant carrying pTH18kr (empty vector) or pTH18krompX (*ompX* expression plasmid). Bacteria carrying a green fluorescence protein (GFP) expression plasmid, pTurboGFP-B, and HTB-44 cells stained with rhodamine-phalloidin were imaged with green and red fluorescence, respectively, using a 100× objective. Images were taken from above (A), and cross-sectional images (B) correspond to the white boxes in panel A. The experiment was repeated twice, and similar results were obtained. (C) Aggregated bacteria within HTB-44 cells were quantified by representing levels of colonized bacteria as areas (in square pixels) of GFP. Microscopy data are means from three fields of view, and error bars indicate standard deviations. **, $P < 0.01$ relative to the value for the parent strain CFT073.

with those of the parent strain. We found that the *ompX* mutant exhibited lower motility than the parent strain, and decreased motility in the *ompX* mutant was restored to the parent level by the introduction of pTrc99KompX (Fig. 4A and B). Flagellar staining showed that the *ompX* mutant produced fewer flagella than the parent strain (Fig. 4C and D).

Flagellin, encoded by *fliC*, is a major component of flagella (21). We assessed the level of flagellin by Western blotting. At the beginning of the experiment, we used a commercial *fliC* antibody, but we were unable to detect FliC in the parent strain. For this reason, we constructed strains carrying pTH18krfliC-VSVG, which produce the recombinant vesicular stomatitis virus G protein (VSVG)-tagged FliC protein under an innate *fliC* promoter on the pTH18kr vector for the parent strain and the *ompX* mutant. We then detected FliC-VSVG in UPEC cell lysates and secreted fractions by Western blotting with the VSVG antibody. The *ompX* mutant produced less FliC-VSVG than the parent strain, and the FliC-VSVG level was elevated when pTrc99AompX, the *ompX* expression plasmid, was introduced (Fig. 5A). We confirmed that the *fliC* mutant internalized within the kidney epithelial cells with low efficiency compared to the parent strain, and deletion of *ompX* in the *fliC* mutant did not further decrease the bacterial

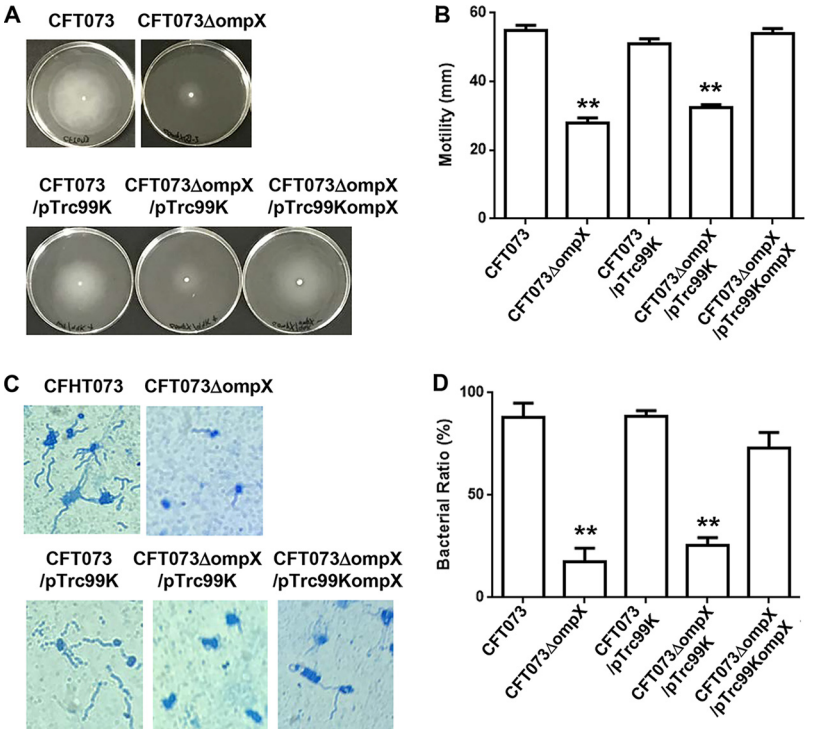

FIG 4 Motilities and flagellar production for the parent strain (CFT073) and the *ompX* mutant or the parent strain and the *ompX* mutant carrying pTrc99K (empty vector) or pTrc99KompX (*ompX* expression plasmid). (A) Bacterial migration on LB medium containing 0.3% agar. (B) Diameters reflecting bacterial migration on the agar. Data are means from three independent experiments; error bars indicate standard deviations. (C) Flagella and bacterial cells were stained with Victoria blue/tannic acid were pictured using a 100× objective. (D) Ratios of bacteria observed with flagella to ~120 to 150 randomly selected bacteria on microscopy, presented as percentages. Data are means, and error bars indicate standard deviations. **, $P < 0.01$ relative to the value for CFT073.

internalization rate within kidney epithelial cells (Fig. 5B and C). We also observed that introduction of the *fliC* expression plasmid elevated the level of internalization to the parent level (Fig. 5D). Like the *ompX* mutant, the *fliC* mutant formed small colonies with a low frequency within kidney epithelial cells compared to the parent strain (Fig. 6A to C).

The P-type fimbria is the second most common fimbria produced by UPEC CFT073 and is thought to be closely associated with pyelonephritis in humans (7, 8). We measured the transcription levels of *papA*, which encodes a P-type fimbrial component, and found no apparent difference in *papA* levels between the parent strain and the *ompX* mutant (Fig. 7A). We also measured the transcription levels of *fimA*, which encodes a major component of the type 1 fimbria, the most common fimbria, and fimbrial activity by determining agglutination titers of guinea pig erythrocytes. However, no apparent difference was observed between the parent strain and the *ompX* mutant in *fimA* transcript levels or agglutination titers (64 for both the parent strain and the *ompX* mutant) (Fig. 7A).

These observations suggest that defective internalization and aggregation in the *ompX* mutant involve lower levels of flagellar production than those in the parent strain, as the *ompX* mutant produces P-type and type 1 fimbriae at the same level as the parent strain.

Deletion of *ompX* decreases transcription of *flhD*, which encodes a master regulator for flagellar expression, and leads to a reduction in flagellar production. To address how the deletion of *ompX* decreases flagellar production, we investigated the mechanism of flagellin expression. *fliC* gene expression is activated by FliA, a flagellar biosynthesis sigma factor, and the *fliA* gene is activated by FlhD, a master regulator of flagellar expression (22). We measured the transcription levels of *fliA* and *flhD* in addition to *fliC* using quantitative PCR (qPCR) and found that *fliA* and *flhD* levels in the *ompX* mutant

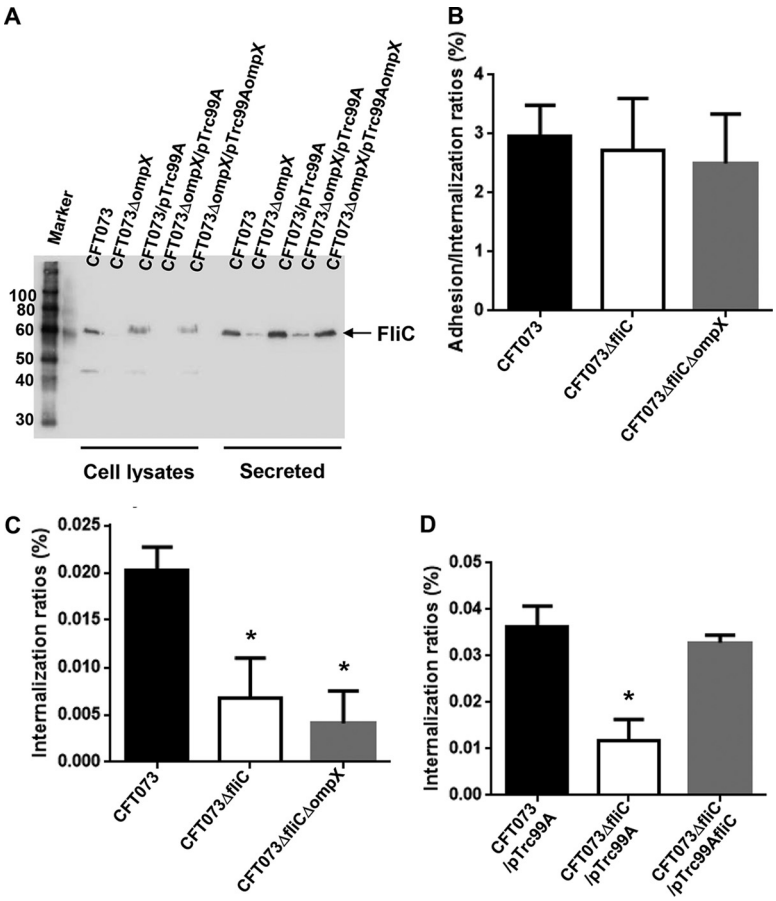

FIG 5 FliC expression in the *ompX* mutant and contribution of *fliC* to bacterial adhesion to and internalization within the kidney epithelial cells. (A) Western blots of cell lysates and secreted proteins from the parent strain (CFT073) and the *ompX* mutant containing a VSVG-tagged FliC expression plasmid (pTH18krfliC-VSVG) and pTrc99A (empty vector) or pTrc99AompX (*ompX* expression plasmid). Locations of molecular mass standards (in kilodaltons) are shown on the left. VSVG-tagged FliC was visualized by probing with a VSVG antibody. Adhesion to and internalization in kidney epithelial cells (HTB-44) of the parent strain, *fliC* mutant, and *fliC*/*ompX* double mutant (B and C) or the parent strain and the *fliC* mutant carrying pTrc99A (empty vector) or pTrc99AfliC (*fliC* expression plasmid) (D). Values are percent CFU of adhered/internalized (B) and internalized (C and D) bacteria relative to total bacterial cell numbers. Data are means from three independent experiments; error bars indicate standard deviations. *, $P < 0.05$ relative to CFT073 (B and C) or CFT073/pTrc99A (D).

were lower than those in the parent strain (Fig. 7A). FlhD also activates the transcription of genes that encode the flagellar components FlgB, FlhB, and FliL and the gene *yecR*, which is not related to flagellar production (23, 24). As with *fliA*, the transcription levels of these genes in the *ompX* mutant were lower than those in the parent strain (Fig. 7A). This observation suggests that the ability of FlhD to activate transcription of *fliA*, followed by induction of FliC expression, is decreased by *ompX* deletion. We also compared the promoter activities of these genes via *lacZ* expression from the reporter plasmids pNNfliC-P, pNNfliA-P, and pNNflhD-P between the *ompX* mutant and the parent strain. The promoter activities of *fliC* and *fliA* corresponding to LacZ expression from pNNfliC-P and pNNfliA-P, respectively, in the *ompX* mutant were lower than those in the parent strain; however, no significant difference in *flhD* levels was observed between the two strains (Fig. 7B).

These combined results suggest that the *ompX* gene contributes to maintaining transcription of *flhD* but does not affect promoter activity.

Deletion of *ompX* does not impair the outer membrane. Since OmpX is an outer membrane protein, its defect may compromise outer membrane integrity, possibly contributing to the attenuated virulence of the *ompX* mutant. We investigated the susceptibility of

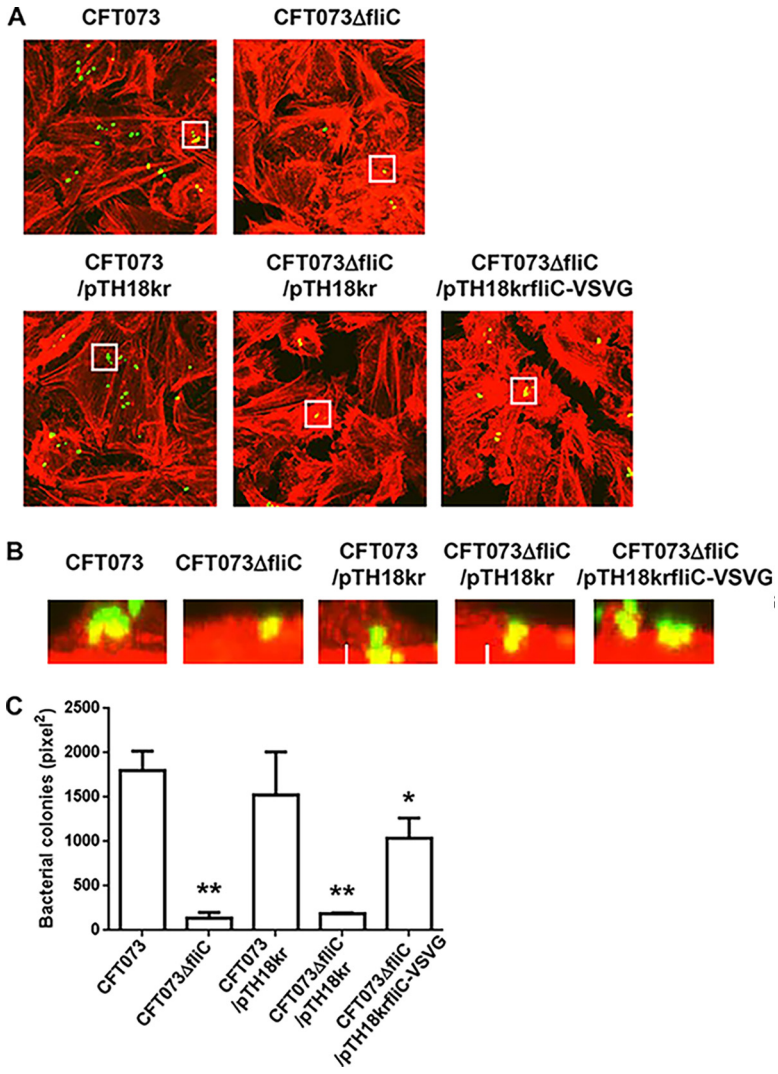

FIG 6 Aggregation within kidney epithelial cells (HTB-44) for the parent strain and the *fliC* mutant or the parent strain and the *fliC* mutant carrying pTH18kr (empty vector) or pTH18krfliC-VSVG (*fliC* expression plasmid). Bacteria carrying a GFP expression plasmid, pTurboGFP-B, and HTB-44 cells stained with rhodamine-phalloidin were imaged with green and red fluorescence, respectively, using a 100× objective. Images were taken from above (A), and cross-sectional images (B) correspond to the white boxes in panel A. The experiment was repeated twice, and similar results were obtained. (C) Aggregated bacteria within HTB-44 cells were quantified by determining levels of colonized bacteria as areas (in square pixels) of GFP. Microscopy data are means from three fields of view, and error bars indicate standard deviations. *, $P < 0.05$, and **, $P < 0.01$, relative to CFT073.

the *ompX* mutant and the parent strain to sodium deoxycholate and SDS by MIC assays to test this hypothesis. There was no significant difference in susceptibility to these reagents between the parent strain and the *ompX* mutant (MICs of sodium deoxycholate were 25,600 μg/ml and MICs of SDS were 32,800 μg/ml for both the parent strain and the *ompX* mutant). Therefore, the outer membrane is unlikely to be impaired by deletion of the *ompX* gene.

DISCUSSION

The roles of OmpX in bacterial pathogenesis have been characterized in some pathogens, such as *S. enterica*, *Y. pestis*, *K. pneumoniae*, and *E. coli* including ExPEC. Deletion of *ompX* in these bacterial species attenuated virulence (15–20). One *E. coli* study suggested that the *ompX* gene contributes to the virulence of pig lung disease-related ExPEC in the respiratory system and during systemic infection, because

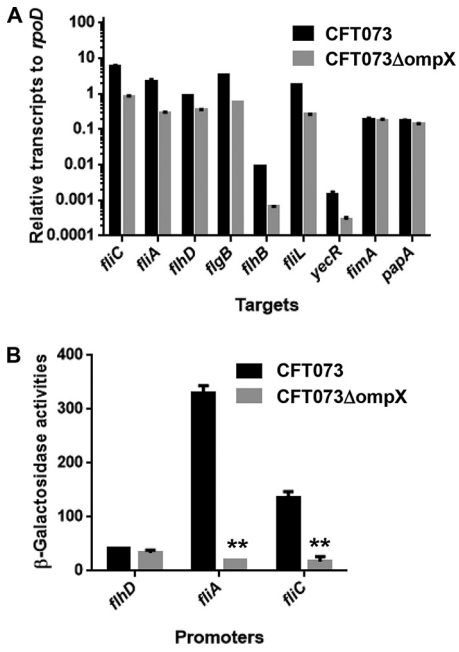

FIG 7 Transcript levels and promoter activities of flagellum-related and fimbrial genes in the parent strain (CFT073) and the *ompX* mutant. (A) Transcript levels were determined relative to that of *rpoD*. Data are means for two biological replicates; error bars indicate the ranges. (B) β-Galactosidase activities corresponding to *flhD*, *fliA*, and *fliC* promoter activities in the parent strain and the *ompX* mutant containing pNNflhD-P, pNNfliA-P, or pNNfliC-P, the *lacZ* reporter plasmid. Data are means from three independent experiments; error bars indicate standard deviations. **, $P < 0.01$ relative to CFT073.

deletion of *ompX* decreased mouse mortality and bacterial distribution in systemically infected mice and decreased adhesion to and invasion of alveolar basal epithelial cells (20).

In this study, we investigated the role of OmpX in the pathogenesis of UPEC. A previous study, which used the UPEC strain J96, reported that the *ompX* gene was not required for bacterial adhesion to host laryngeal epithelial cells (15). Our study, which used the UPEC strain CFT073 and urinary tract cells, suggests that the *ompX* gene is unlikely to contribute to bacterial adhesion. However, we found evidence that an *ompX* gene is required for optimal internalization and aggregation within kidney epithelial cells. In addition, we found that the *ompX* mutant exhibited defective motility due to a low level of flagellar production, which is associated with decreased levels of internalization and aggregation, because a nonflagellar *fliC* mutant also exhibited low levels of internalization and aggregation, and deletion of *ompX* did not further reduce these levels. Thus, we suggest that OmpX is implicated in the flagellum-associated pathogenesis of UPEC in the urinary tract. In another study, a *fliC* mutant was shown to exhibit a growth disadvantage in the urinary tracts of mice when it was inoculated with the parent strain (in which *fliC* is intact); however, the mutant still colonized at a rate similar to that of the parent strain in solo infection experiments (9, 10). P-type fimbriae are thought to contribute to virulence in the kidney (7, 8). However, our qPCR analyses indicated that OmpX was unlikely to be associated with the expression of the *papA* gene. Therefore, the decreased rate of kidney colonization observed in the mice infected with the *ompX* mutant does not seem to be associated with defects in either flagella or P-type fimbriae, implying that there are still unknown factors that participate in OmpX pathogenicity.

Flagellum expression is activated by the transcriptional regulator FlhD (22). The *ompX* mutant exhibited a low level of *flhD* transcription compared to the parent strain; therefore, we concluded that the decrease in flagellum production in the *ompX* mutant was caused by reduced FlhD expression. However, the promoter activity of *flhD* in the

ompX mutant was similar to that of the parent strain. It remains unclear how the deletion of *ompX* reduces the transcription level of *flhD*. The *ompX* gene may contribute to the stability of *flhD* mRNA, perhaps by protecting *flhD* mRNA from RNase activity, either directly or via RNA binding proteins.

Distinctive differences have been observed between some *E. coli* strains regarding the effects of *ompX* deletion on flagellum-associated motility and type 1 fimbrial production (20, 25). The deletion of *ompX* in a pig lung disease-related ExPEC strain attenuated virulence in systemically infected mice and promoted motility (20). We do not know why the effect of *ompX* deletion on motility in this ExPEC strain was inconsistent with results for UPEC. Expression of flagellum-related genes is regulated by many regulatory proteins, including CRP, LrhA, H-NS, QseB, RcsB, OmpR, and CytR, in addition to FlhD (26–32). These proteins activate or repress flagellar gene expression, either directly or via FlhD. Several outer membrane proteins, including OmpX, affect the activity of other proteins, such as RpoE (15, 33). Therefore, the activity of repressor proteins produced by this ExPEC strain, but not UPEC, may be reduced by *ompX* deletion; this reduction may then be overcome by FlhD expression in ExPEC. One study demonstrated that deletion of *ompX* in a nonpathogenic K-12 strain, producing type 1 fimbriae, decreased motility, while it did not decrease motility in a strain that lacked type 1 fimbriae (25). This implies that type 1 fimbria production may affect flagellar expression and motility by deletion of *ompX*. Another study found that type 1 fimbria production was promoted in the *ompX* mutant of the nonpathogenic K-12 strain, while we observed no significant difference in type 1 fimbria production in UPEC (25). Similar to the case of motility, the inconsistent effects of *ompX* deletion on type 1 fimbria production may be due to the different regulatory mechanisms of the *fim* genes, which encode type 1 fimbria proteins, in UPEC and nonpathogenic K-12 strains. OmpX has been proposed as a potential target for the treatment of *Yersinia* infections (34). To expand this idea to *E. coli* infections, extensive information related to the regulatory mechanism of type 1 fimbrial and flagellar expression, together with proteins affected by OmpX, would be necessary.

OmpX is a member of the outer membrane protein family having an eight-stranded β-barrel structure, which is closely related to the structure of OmpA (14). However, the roles of OmpA in UPEC pathogenicity differ from those of OmpX. Deleting *ompA* impaired colony formation in UTI in mice; however, the *ompA* mutant retained motility and the ability to aggregate within bladder epithelial cells at a level similar to that of the strain with intact *ompA* (11, 35). Although OmpX and OmpA share highly conserved transmembrane domain structures, OmpX lacks a periplasmic domain, while OmpA contains one. Therefore, this periplasmic domain may participate in determining the roles of these proteins in pathogenicity.

In conclusion, we have characterized the roles of OmpX in *E. coli* pathogenesis and flagellum expression associated with UPEC virulence. This study provides an insight into the molecular mechanisms involved in the virulence of UPEC.

MATERIALS AND METHODS

Bacterial strains, host cells, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1.Bacteria were grown in Luria-Bertani (LB) medium. Optical density at 600 nm (OD_{600}) was measured as an indicator of cell growth. Antibiotics were added to the growth medium for marker selection and maintenance of plasmids at the following concentrations: 45 μg/ml chloramphenicol, 50 μg/ml kanamycin, and 150 μg/ml ampicillin. HTB-44 kidney epithelial cells were cultured as previously described (36).

Cloning and mutant construction. The *ompX* gene deletion was produced by sequence overlap extension PCR, as previously described (37), using the primer pairs ompX-delta1/ompX-delta2 and ompX-delta3/ompX-delta4, as shown in Table 2. The upstream flanking DNA comprised 450 bp and the first four amino acid codons. The downstream flanking DNA comprised the last two amino acid codons, the stop codon, and 450 bp of DNA. This deletion construct was ligated into the temperature-sensitive plasmid pKO3 (37) and introduced into UPEC strains. We selected sucrose-resistant chloramphenicol-sensitive colonies at 30°C. To construct the OmpX expression plasmids pTrc99KompX and pTrc99AompX, we amplified the *ompX* gene by PCR using primer pairs (Table 2) and ligated the product into the pTrc99K and pTrc99A vectors (38). A FliC expression plasmid, pTrc99AfliC, was constructed by PCR amplification of the *fliC* gene and ligation of its product into the pTrc99A vector (38).

TABLE 1 Strains and plasmids used in this study[a]

Strain or plasmid	Relevant genotype/phenotype	Reference
Strains		
CFT073	Parent strain (ATCC 700928)	ATCC 700928
CFT073ΔompX	*ompX* mutant of CFT073	This work
CFT073ΔfliC	*fliC* mutant of CFT073	11
CFT073ΔfliCΔompX	*fliC* and *ompX* double mutant of CFT073	This work
Plasmids		
pKO3	Temperature-sensitive vector for gene targeting, *sacB*, Cm^r	37
pTrc99K	Vector for IPTG-inducible expression; Km^r	38
pTrc99KompX	*ompX* expression plasmid; Km^r	This work
pTrc99A	Vector for IPTG-inducible expression; Ap^r	38
pTrc99AompX	*ompX* expression plasmid; Ap^r	This work
pTrc99AfliC	*fliC* expression plasmid; Ap^r	This work
pTH18kr	Low-copy-no. plasmid; Km^r	39
pTH18krfliC-VSVG	C-terminally VSVG-tagged FliC expression plasmid; Km^r	This work
pTH18krompX	*ompX* expression plasmid; Km^r	This work
pNN387	Single-copy plasmid with promoterless *lacZ*; Cm^r	40
pNNflhD-P	*flhD* promoter reporter; Cm^r	This work
pNNfliA-P	*fliA* promoter reporter; Cm^r	This work
pNNfliC-P	*fliC* promoter reporter; Cm^r	This work
pTurboGFP-B	GFP expression plasmid; Ap^r	Evrogen

[a]Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Ap^r, ampicillin resistance.

To construct a C-terminal vesicular stomatitis virus glycoprotein (VSVG)-tagged FliC expression plasmid, pTH18krfliC-VSVG, the DNA containing the *fliC* coding region and its 300-bp upstream region was amplified with pTHfliC-F and pTHfliC-VSVG-R primers and ligated into the HindIII and BamHI sites in pTH18kr (39), the low-copy-number plasmid. This DNA fragment contains a *cis*-regulatory element for *fliC* gene expression. In addition, although the pTH18kr vector has a *lac* promoter sequence upstream of the HindIII and BamHI sites, the introduced *fliC* gene is oriented in a reverse direction relative to the *lac* promoter. Therefore, we expected that the resulting bacterial construct would produce FliC as a C-terminal VSVG-tagged protein from its native promoter.

The pHT18krompX plasmid was constructed by amplifying *ompX* and the 263-bp upstream region including the promoter and ligating the product into pTH18kr. Since this plasmid is compatible with pTurboGFP-B, these plasmids were introduced together into the *ompX* mutant to characterize bacterial colonies within kidney epithelial cells using fluorescent images. We also constructed pNNflhD-P, pNNfliA-P, and pNNfliC-P, *lacZ* reporter plasmids to measure *flhD*, *fliA*, and *fliC* promoter activities. We PCR amplified the 1,200-bp upstream region of *flhD* and the 300-bp upstream regions of *fliA* and *fliC*, respectively, and ligated these products into pNN387 (40) with a promoterless *lacZ*. All constructs were confirmed by DNA sequencing.

Urinary tract infections in mice. We estimated UPEC virulence using a UTI mouse model as previously described (11). Bacterial suspensions in phosphate-buffered saline (PBS) (1×10^8 CFU) were administered to 8-week-old C3H/HeN female mice via transurethral catheterization. The numbers of CFU in the bladder and kidneys 48 h postinfection were determined by counting colonies grown on XM-G agar. All animal studies were approved by the Animal Research Committee of Gunma University (approval number 19-094).

Infection of kidney epithelial cells. UPEC cell adhesion and internalization within HTB-44 cells were assessed using a gentamicin protection assay as previously described (41). The numbers of adherent and/or internalized bacterial cells were determined as ratios of CFU (as percentages) to total cell CFU. We also imaged the bacteria in HTB-44 cells using confocal microscopy, as previously described (41). A UPEC strain carrying a green fluorescence protein (GFP) expression plasmid, pTurboGFP-B (Evrogen, Moscow, Russia), was inoculated into cultured HTB-44 cells and incubated for 2 h. Noninternalized bacteria were washed out with gentamicin and PBS+ (PBS containing 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$). The HTB-44 cells were stained with rhodamine-phalloidin (Life Technologies, Carlsbad, CA, USA). Fluorescent images were acquired using an Olympus FV1200 IX81 microscope and processed using FV10-ASW software (Olympus Corp., Tokyo, Japan).

Hemagglutination assays. To estimate the activity of type 1 fimbriae, we tested the hemagglutination of guinea pig red blood cells as previously described (41).

Motility assays. To evaluate bacterial motility, the overnight cultures were spotted onto LB medium containing 0.3% agar and grown for 8 h at 37°C under an atmosphere of 5% CO_2.

Flagellum staining. Bacteria were cultured for 24 h at 30°C in heart Infusion medium containing 1.5% agar. Flagella were stained with Victoria blue/tannic acid solution as previously described (11).

RNA extraction and quantitative real-time PCR. We grew bacteria to late logarithmic growth phase (OD_{600}, ~0.5). Total-RNA extraction, cDNA synthesis, and real-time PCR were carried out as previously described (11). The constitutively expressed *rrsA* and *rpoD* genes were used as internal controls.

TABLE 2 Primers used in this study

Primer	DNA sequence (5′-3′)	Use
ompX-delta1	GCGGGATCCGACTTAGCTAACGAGGCTCC	*ompX* mutant construction
ompX-delta2	ATATCACCGAAGTGATTAGAAGCGAATTTTTTTCATAACCACCTC	*ompX* mutant construction
ompX-delta3	TTTGAGGTGGTTATGAAAAAAATTCGCTTCTAATCACTTCGGTG	*ompX* mutant construction
ompX-delta4	GCGGTCGACAAACAGACGATTTACTGCGC	*ompX* mutant construction
pTrcompX-F	GCGCCATGGAAAAAATTGCATGTCTTTC	pTrc99K/pTrc99AompX constructions
pTrcompX-R	GCGGGATCCTTAGAAGCGGTAACCAACACC	pTrc99K/pTrc99AompX constructions
pTrcfliC-F	GCGCCATGGCACAAGTCATTAATACC	pTrc99AfliC construction
pTrcfliC-R	GCGGTCGACTTAACCCTGCAGCAGAGAC	pTrc99AfliC construction
pTHfliC-F	GCGAAGCTTCCTGACCCGACTCCCAGCG	pTH18krfliC-VSVG construction
pTHfliC-VSVG-R	GCGGGATCCTTATTTTCCTAATCTATTCATTTCAATATCTGTATAA CCCTGCAGCAGAGACAG	pTH18krfliC-VSVG construction
pTHompX-F	GCGGGATCCCCCGTTTTGACTAAAATGCG	pTH18krompX construction
pTHompX-R	GCGAAGCTTTTAGAAGCGGTAACCAACACC	pTH18krompX construction
flhD-PF	GCGGCGGCCGCTGCGGTTAATCTCCCGTAAG	pNNflhD-P construction
flhD-PR	GCGAGTACTTATTCCCACCCAGAATAACC	pNNflhD-P construction
fliA-PF	GCGGCGGCCGCCTGAGACTGACGGCAACGCC	pNNfliA-P construction
fliA-PR	GCGAAGCTTCACGATAAACAGCCCTGCG	pNNfliA-P construction
fliC-PF	GCGGCGGCCGCCCTGACCCGACTCCCAGCG	pNNfliC-P construction
fliC-PR	GCGAAGCTTGATTCGTTATCCTATATTGC	pNNfliC-P construction
rrsA-qPCR-F	CGGTGGAGCATGTGGTTTAA	Quantitative real-time PCR
rrsA-qPCR-R	GAAAACTTCCGTGGATGTCAAGA	Quantitative real-time PCR
rpoD-qPCR-F	CAAGCCGTGGTCGGAAAA	Quantitative real-time PCR
rpoD-qPCR-R	GGGCGCGATGCACTTCT	Quantitative real-time PCR
fimA-qPCR-F	TGCGGGTAGCGCAACAA	Quantitative real-time PCR
fimA-qPCR-R	ACGCAGTCCCTGTTTTATCCA	Quantitative real-time PCR
papA-qPCR-F	TTTTTCGGGTGTCCCAAGTG	Quantitative real-time PCR
papA-qPCR-R	TGTTGCACCGACGGTCTGT	Quantitative real-time PCR
flhD-qPCR-F	GACAACGTTAGCGGCACTGA	Quantitative real-time PCR
flhD-qPCR-R	TTGATTGGTTTCTGCCAGCTT	Quantitative real-time PCR
fliA-qPCR-F	CGAGCGTGGAACTTGACGAT	Quantitative real-time PCR
fliA-qPCR-R	CGACGGCATTAAGTAACCCAAT	Quantitative real-time PCR
fliC-qPCR-F	TCCACTGAAAGCTCTGGATGAA	Quantitative real-time PCR
fliC-qPCR-R	CCCAGGGATGAACGGAATT	Quantitative real-time PCR
flgB-qPCR-F	TCAGGCTCGCGATATCGATT	Quantitative real-time PCR
flgB-qPCR-R	CCGTCCACGTTGCATGACT	Quantitative real-time PCR
flhB-qPCR-F	TCGCGCTGCGCATTC	Quantitative real-time PCR
flhB-qPCR-R	TTCAAGCGTCGGGACGTTA	Quantitative real-time PCR
fliL-qPCR-F	ACTGGCATTCGCATCAGGTT	Quantitative real-time PCR
fliL-qPCR-R	GGCACGACGCGTTGCT	Quantitative real-time PCR
yecR-qPCR-F	GACGCGGCAACAGGTATTGT	Quantitative real-time PCR
yecR-qPCR-R	CGGGCATGCTGCAAAAA	Quantitative real-time PCR

Western blotting. To detect VSVG-tagged FliC from UPEC, bacteria were grown to early stationary phase and separated by centrifugation. The cell pellets were resuspended in 50 mM phosphate buffer containing 8 M urea and then lysed by sonication. Secreted proteins were precipitated from the supernatants with 10% trichloroacetic acid (TCA) and dissolved in Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA). Cell lysates (7.5 μg) and secreted proteins were separated on duplicate 10% acrylamide Tris-glycine SDS-PAGE gels. One gel was stained with Coomassie brilliant blue stain (CBB), and the other was electroblotted onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA). VSVG-tagged FliC was detected with VSVG antibody (Sigma Chemical) and an anti-rabbit horseradish peroxidase-conjugated immunoglobulin G (IgG) secondary antibody (Sigma-Aldrich Co. LLC., St. Louis, MO) using a SuperSignal West Pico kit (Thermo Fisher Scientific, Waltham, MA). VSVG-tagged FliC protein bands were visualized using a LAS-4010 luminescent image analyzer (GE Healthcare Japan, Tokyo).

Promoter assay. UPEC strains carrying pNNflhD-P, pNNfliA-P, or pNNfliC-P, the LacZ reporter plasmid, were grown at 37°C in LB medium. β-Galactosidase activities from *lacZ* expression in cell lysates were determined by Miller's method (42).

Deoxycholate and SDS susceptibility assays. Susceptibility of bacteria to deoxycholate and SDS was estimated by the MIC assay using a serial agar dilution method. Five microliters of 100-fold-diluted overnight cultures (~50,000 cells) was inoculated onto a LB agar plate containing sodium deoxycholate and SDS and incubated for 16 h at 37°C. The MICs were determined as the lowest concentrations at which growth was inhibited.

Statistical analysis. The *P* value in each assay except UTI mouse experiments was determined by the unpaired *t* test with GraphPad Prism version 6.00. To determine *P* value in the UTI mouse experiment, we used the Mann-Whitney test data with GraphPad Prism version 6.00.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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