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Pathogenicity of FtsK mutant of avian pathogenic *Escherichia coli*

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Abstract

Introduction: Avian pathogenic *Escherichia coli* (APEC) is a leading cause of extraintestinal infection and heavy economic losses. Imparting immunity after vaccination with live attenuated strain vaccination is an ideal strategy for infection control. This study considers an FtsK knockout mutant strain as a candidate. **Material and Methods:** An FtsK knockout mutant of APEC strain E058 was constructed and the pathogenicity of the mutant and wild-type strains was further evaluated in chickens. **Results:** The 50% lethal doses of each strain for one-day-old specific-pathogen-free (SPF) chickens challenged experimentally *via* trachea were $10^{5.5}$ and $10^{7.0}$ colony-forming units (CFU) respectively. Chickens challenged with the wild-type strain exhibited typical signs and lesions of avian colibacillosis, while those inoculated with the mutant strain showed mild pericarditis and pulmonary congestion. The growth rate of the FtsK mutant strain was much slower than the wild-type strain in the heart, spleen, liver, and lung of infected chickens. **Conclusion:** These results indicated that the APEC FtsK mutant can be attenuated for chickens, and that this mutant has the potential for the development of an APEC vaccine.

Keywords: chickens, *Escherichia coli*, FtsK, mutant.

Introduction

Extraintestinal pathogenic *Escherichia coli* (ExPEC) strains can infect nearly every organ and anatomical site in humans and animals. Avian pathogenic *E. coli* (APEC) strains are one of the leading causes of serious extraintestinal infection of poultry, leading to severe economic losses (11, 14). In immunocompromised hosts, APEC strains cause a variety of severe infections with a complex syndrome characterised by multiple organ lesions like air sacculitis, pericarditis, peritonitis, salpingitis, osteomyelitis, or yolk sac infection. APEC of serogroup O2 is one of the dominant strains, and one which has caused serious economic losses in the poultry industry in China. The ideal strategy for the control of APEC infection could be the establishment of immunity in birds through vaccination with live attenuated strains.

E. coli FtsK is a powerful, fast, double-stranded DNA translocase, which can strip proteins from DNA. FtsK acts in the late stages of chromosome segregation by facilitating sister chromosome unlinking at the division septum (8). To identify the virulence-associated genes of APEC isolates, selective capture of transcribed sequences (SCOTS) was applied to screen the putative virulence genes expressed in APEC E058 strain (serogroup O2) and several genes were selected, including FtsK (3). These resulting data showed that FtsK may be associated with the virulence of APEC. Thus, the knockout mutant of FtsK may attenuate the virulence of APEC, and the mutant may be a potential agent for vaccine development. In the present study, an FtsK knockout APEC mutant was constructed, and its pathogenicity was evaluated.

Material and Methods

Bacterial growth condition, plasmids, and primers. APEC E058 and the mutant strains were

cultured at 37ºC in Luria-Bertani (LB) broth. The mutant strain was selected on LB agar supplemented with kanamycin at 30 μg/mL. Plasmids and primers are listed in Table 1.

Chicken challenge model for FtsK **expression level *in vivo*.** The chicken challenge model was used as previously described (17). Briefly, 10 one-day-old SPF chickens were infected with 10^{10} CFU of wild-type or mutant strains by the air sac route. The chickens were euthanised 4 h later and blood was collected into 15 mL centrifuge tubes containing 1 mL of 0.5% sodium citrate. The blood was centrifuged (5 min, 500 × g, 4ºC) and the bacteria-containing supernatant was transferred into other tubes. Then, the supernatants were centrifuged again for 10 min (2 500 × g, 4ºC), and the final bacterial pellet was stored frozen at −70ºC until RNA extraction.

RNA extraction and cDNA synthesis. RNA was extracted using an RNeasy Mini Kit (QIAGEN, USA) according to the manufacturer's instructions. As for cDNA synthesis, about 20 μg of the extracted RNA was mixed with about 750 ng of random hexamers (Invitrogen, part of Thermo Fisher Scientific, USA). Then, SuperScript II reverse transcriptase (1 500 U; Invitrogen) was added, along with First Strand buffer, deoxyribonucleotides, and dithiothreitol at recommended concentrations according to the manufacturer's protocol. The cDNA synthesis was performed according to the following programme: 37ºC for 60 min, 42ºC for 60 min, and 70ºC for 10 min. The cDNA was further purified using PCR Clean-up NucleoSpin Extract II kits (Macherey-Nagel, Germany).

RT-PCR and quantitative real-time PCR (qRT-PCR). Primer sets for PCR amplification of *irp* (encoding iron receptor protein), *lolA* (encoding outer-membrane lipoprotein carrier protein), and the first 240 amino acids of *ftsK* protein were irpF/irpR, lolA/lolAR, and F1F/FfR respectively. The PCR products were resolved on 1% agarose gels and visualised by ethidium bromide staining.

The qRT-PCR primers of the target gene *ftsK* and the internal standard gene *gapA* (glyceraldehyde 3-phosphate dehydrogenase) were qFtsKF/qFtsKR and qgapAF/qgapAF respectively. About 30 ng of cDNA and 200 *n*M of each primer were mixed with 12.5 μL of 2×SYBR Green I PCR Master Mix (Takara Bio, Japan). The cDNA was then amplified in an ABI Prism model 7300 instrument (Applied Biosystems, part of Thermo Fisher Scientific, USA) with specific primers. The relative expression (ΔCt) was calculated by subtraction of the Ct value between *gapA* and *ftsk*.

Cloning of FtsK and construction of mutant E058FtsK. The fragments of *ftsK1* (nucleotides 14 to 721 of *ftsK*) and part of *ftsK2* (nucleotides 2410 to 3309 of *ftsk*) were amplified by PCR using F1F/F1R and F2F/F2R primer sets respectively, and the two fragments were subcloned into the same pBluescript SK II(-) (Fermentas, part of Thermo Fisher Scientific, Lithuania) plasmid to form pS-*ftsK*. The kanamycin resistance (Kan^r) obtained by PCR was inserted between *ftsK1* and *ftsK2* of pS-*ftsK* to form pS-*ftsK*-kan^r. After verification by sequencing, the disrupted *ftsk* gene containing the inserted Kan^r gene in pS-*ftsK*-kan^r (Fig. 1) was amplified by PCR and purified for the electroporation to E058 competent cells as described previously (12). After 24 h of incubation, the resulting Kan^r colonies were selected for PCR identification using primers F1F and F2R, and the inactivated *ftsk* mutant was verified by sequencing.

***In vitro* and *in vivo* experiments.** The *in vitro* competition tests were conducted as previously described (10). Overnight cultures of the wild-type and mutant bacteria were mixed together at a ratio of approximately 1:1, inoculated into fresh LB, and grown with aeration. At various times, the ratio of Kan^+ and Kan^- colonies was determined and subsequently used to calculate the percentage of each strain. For *in vivo* competition tests, 0.5 mL (2×10^8) of the wild-type and mutant strains were administered intratracheally to 10 chickens at a ratio of 1:1. Five SPF chickens were euthanised 24 and 48 h later and the heart, liver, lungs, and spleen of the infected chickens were collected. Recoveries of the wild-type and mutant strains were determined by serial dilutions of the collected organ homogenate on LB medium with or without kanamycin respectively. The output ratio (mutant/wild-type) and input ratio (mutant/wild-type) were determined. The competitive index (CI) of the mutant strains was calculated by dividing the output ratio by the input ratio. Based on the CI, when the mutant was outcompeted up to 10-fold it was evaluated as slightly attenuated, up to 100-fold as moderately attenuated, and more than 100-fold as highly attenuated (9).

LD_{50} test for wild-type and mutant strains. The virulence of the wild-type and mutant strains were evaluated using the 50% lethal dose (LD_{50}) test using one-day-old SPF chickens as previously described (4). Briefly, the bacteria were grown to OD_{600} of 0.2 in LB, and then pelleted and resuspended in PBS to six different densities (10^8, 10^7, 10^6, 10^5, 10^4, and 10^3 CFU per mL). For each density, five 1-day-old SPF chickens were inoculated into the trachea with 0.1 mL of each suspension. The chickens were observed for one week and the LD_{50} values were calculated as previously described (13).

The animal experimentation in this work adhered to the International Guiding Principles for Biomedical Research Involving Animals, as issued by the Council for the International Organisations of Medical Sciences.

Statistical analysis. Statistical significance was evaluated using Student's *t* test. Differences were considered significant for $P < 0.05$.

Results

FtsK upregulated during the infection in chickens. The qRT-PCR was used to detect the relative expression of *ftsK* of E058 both *in vivo* and *in vitro*. It was demonstrated that *ftsK* was upregulated 23.57 ± 0.83 folds during the infection in chickens *in vivo*, compared with its expression cultured in LB *in vitro* (Fig. 2).

Construction and characterisation of FtsK mutant. The FtsK mutant was constructed by allelic exchange of a 1635 bp deletion within the *ftsK* coding region with a Kan^r cassette (Fig. 1). DNA sequencing also confirmed that the cassette was successfully inserted into the right position of mutant strain E058FtsK. RT-PCR showed that E058FtsK could only express the N-terminal 200-amino-acid domain of FtsK, and that transcription of *irp* and *lolA* were not disrupted (data not shown).

Attenuated virulence of E058FtsK. The growth capacity of E058FtsK to compete with E058 was compared both *in vivo* and *in vitro*. The CIs of mutant in the heart and lungs were 0.08 ± 0.015 and 0.09 ± 0.011 respectively, indicating that the mutant was moderately attenuated in these tissues. Meanwhile, CIs were 0.0051 ± 0.0012 and 0.0035 ± 0.0023 in the liver and spleen respectively, indicating that E058FtsK was strongly out competed by the wild type (Table 2). In contrast, the *in vitro* CI was about 0.30 at 4 h post inoculation. Although the growth rate of the wild strain was higher than the mutants 10–12 h post inoculation, the difference was not significant (Fig. 3).

To imitate the natural infection, E058FtsK and E058 were administered into the trachea of the chickens. The colonising capacities to internal organs of the wild-type and mutant strains were determined by calculating colony numbers in different tissues 12 and 24 h after infection (Fig. 4). Both strains can colonise the heart, liver, lungs, and spleen. Significantly, in E058 after 12 h maximum colonisation of the heart was observed, while E058FtsK exhibited maximum colonisation of the lungs. Minimal colonisation was in the liver and was 6.60 ± 0.23 $\log_{10}$ CFU/g for E058 and 4.09 ± 0.07 $\log_{10}$ CFU/g for E058FtsK. After 24 h, E058 had colonised the lungs maximally, and E058FtsK had colonised the spleen maximally. Colonisation was much lower at 24 h than at 12 h post infection in the heart and lungs for wild-type strains. However, on average the FtsK mutant strain was recovered at 10–1000 times lower levels than that of the wild-type strain from all selected organs at 12 and 24 h post infection (Fig. 4).

E058FtsK and E058 were further evaluated *in vivo* by an LD_{50} test. The LD_{50} of E058FtsK was $10^{7.0}$ CFU and the LD_{50} of E058 was $10^{5.5}$ CFU in one-day-old SPF chickens. Chickens challenged with wild-type strain exhibited signs characteristic for avian colibacillosis, such as inflammation of the air sacs, haemorrhages, and typical fibrinous lesions. In contrast, mutant E058FtsK only induced mild air sacculitis, pericarditis, pulmonary congestion, and slight hypertrophy of the spleen.

Table 1. Plasmids and primers

	Description	Source
Plasmids		
pGEM-T^{R}EasyVector	TA cloning Vector, Ap^r	Promega
pT-*ftsK*	*ftsK* cloned into pGEM-T^R Easy Vector	Present study
pBluescript Ⅱ SK(-)	Cloning Vector	Fermentas
pS-*ftsK*	*ftsK* fragment cloned into SK(-)	Present study
pS-*ftsK*-Kan^r	Kanamycin resistant gene inserted into pS-*ftsK*	Present study
Primers		
qFtsKf	ATGATGCGTCCGATTCCAG	Present study
qFtsKr	ATTCGTCCACCAACACCAC	Present study
qgapAf	CATCGTTTCCAACGCATCCT	Zhao *et al*. (17)
qgapAr	ACCTTCGATGATGCCGAAGTT	Zhao *et al*. (17)
F1F	TAAGCTTTAACCCTTCGGACC	Present study
F1R	GAATTCCTCTTCTTCATCATCCATC	Present study
F2F	GGATCCCTGTTGATGCGTAATG	Present study
F2R	TTCTAGATTCGTCCACCAACACC	Present study
KanF	AATCCGGATCCGTCGACCT	Present study
KanR	AGAATTCCGTCGACCTGCAG	Present study
irpF	CAAGGGTTTATTCAGGGCTAT	Present study
irpR	CCAGACGGCAAACAGGAC	Present study
lolAF	CCGATTGGGAGTGACGT	Present study
lolAR	CTAAACTGATGGATGGATTGTGCC	Present study
FtsKF	ATGGCTGCCTTACTAAGCTT	Present study
FtsKR	TTAGTCAAATGGCGGTGGG	Present study
FfR	ACAACGCAGCGTCTGTTTGC	Present study

Table 2. Competition assay between E058FtsK and E058 was determined using competitive index (CI)

Strains	LD50	CIs				
		in vitro	heart	lung	liver	spleen
WT	$<10^{5.5}$	–	–	–	–	–
FtsK mutant	$>10^{7.0}$	–	–	–	–	–
FtsK mutant/wt	—	0.30 ± 0.05	0.08 ± 0.015	0.09 ± 0.011	0.0051 ± 0.0012	0.0035 ± 0.0023

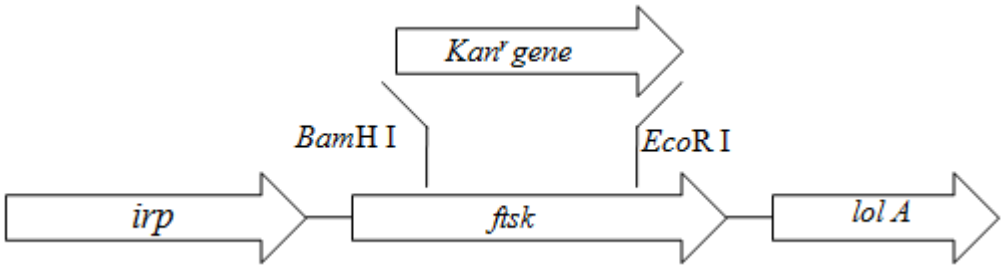

Fig. 1. Genetic organisation of the construction of *ftsk* mutant. The location of the deletion in *ftsk* replaced by a Kan^r gene is between *BamH* I and *EcoR* I cleavage sites introduced by PCR. The genes up- and downstream from *ftsk* are *irp* and *lolA* respectively

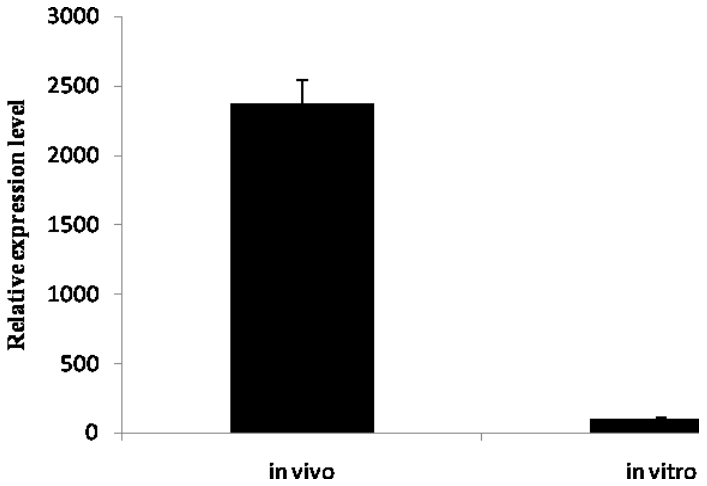

Fig. 2. Quantification of mRNA expression levels of *ftsk*. Relative expression levels of *ftsk* were normalised to internal control (*gapA*)

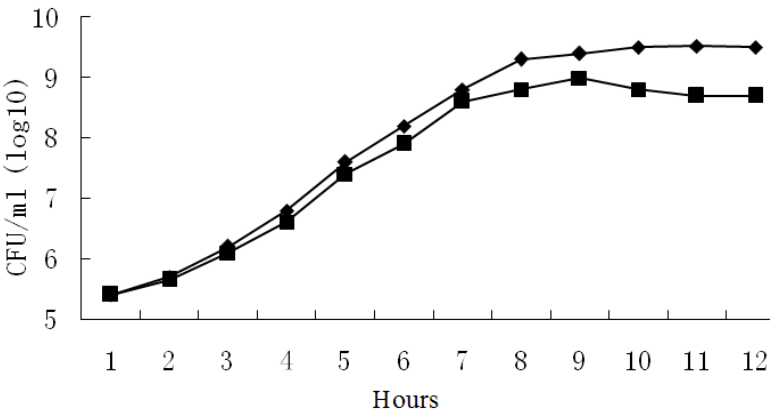

Fig. 3. Growth competition curves of APEC wild-type strain E058 (◆) and E058*FtsK* (■) in LB broth at 37ºC

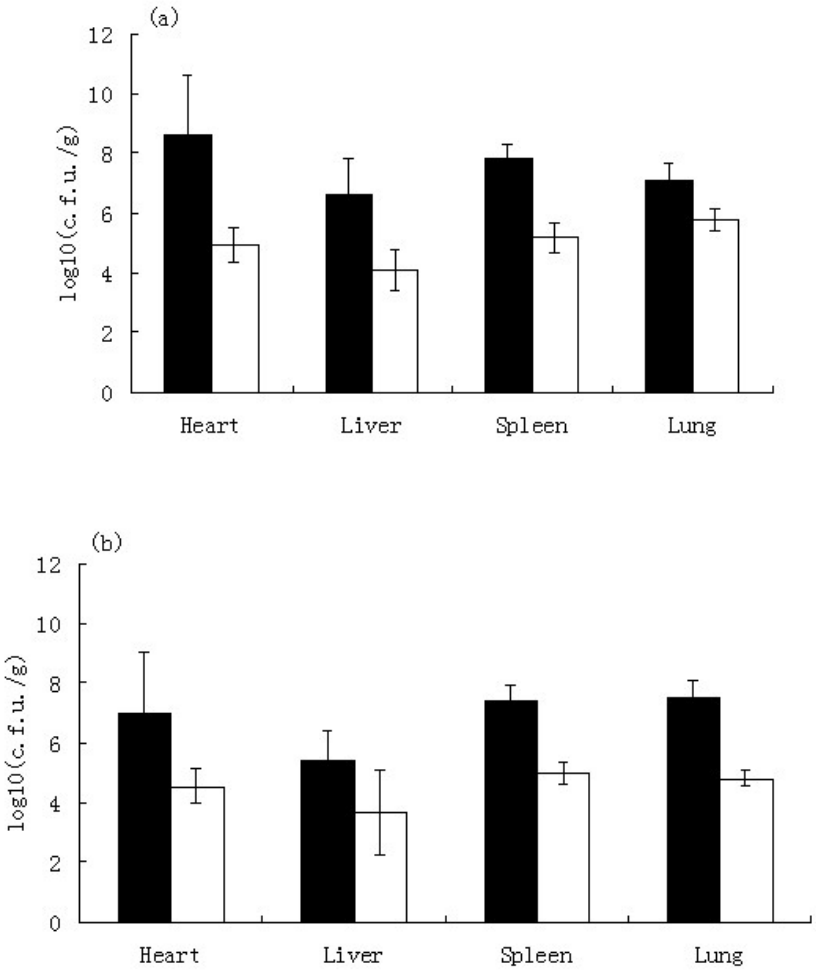

Fig. 4. Bacterial loads in selected tissues from five-week-old chickens 12 h (a) and 24 h (b) after infection with E058 (black bars) and E058*FtsK* (white bars)

Discussion

Up to now, the vast majority of successful anti-bacterial vaccines have been attenuated live vaccines based on the principle that attenuated vaccine strains can confer more effective protection against virulent strains. An ideal way to control APEC infection in birds is vaccination with attenuated strains of dominant serogroups. Therefore, determination of the bacterial genes that are essential for APEC pathogenesis may provide valuable information for the prevention and control of infectious diseases caused by APEC. However, the mechanisms underlying the pathogenicity of APEC are still not fully understood, and lots of virulence-associated genes are also awaiting elucidation. In recent years, new molecular approaches have been applied in the identification of genes involved in APEC pathogenesis, including *in vivo* expression technology, SCOTS, and suppression subtractive hybridisation. Many of these genes have been found, such as genes involved in DNA metabolism and amino acid biosynthesis (5, 16), as well as genes involved in iron regulation and stationary-phase growth (7). These genes are often upregulated during APEC infection (15). Previously, we applied SCOTS to identify putative virulence-associated genes in APEC strain E058 that are expressed in infected chicken tissues, and the *ftsK* gene was selected. In this study, we detected the expression of *ftsK* by qRT-PCR. The results showed that FtsK was upregulated during APEC E058 infection in chickens, compared with its expression in LB, suggesting that FtsK may be involved in the pathogenesis of APEC. However, little is known about the pathogenetic roles of FtsK in APEC. In the present study, an FtsK mutant APEC E058 strain (E058FtsK) was constructed and the virulence of this mutant was analysed.

The *in vitro* competition results indicate that E058FtsK demonstrates a marginally-decreased growth rate compared with the wild strain in LB. This growth rate suggested that the disruption of *ftsk* has a minor impact on bacterial growth *in vitro*. However, *in vivo* virulence is always not as same as *in vitro* assays. So, we tested whether the FtsK mutant strain exhibits decreased virulence as compared with the wild-type strain in the chicken model. Chickens were infected intratracheally with either wild-type or FtsK mutant strains. At specific time points post infection, the number of CFU in selected organs showed that the E058 FtsK mutant remained much less prevalent

than the wild-type strain. These results showed a dramatically-decreased virulence *in vivo* compared to that of the FtsK mutant *in vitro*, suggesting that the deletion of the N-terminal of FtsK is more vital to the proliferation of APEC *in vivo* than *in vitro*. In other words, FtsK may be not only contributing to the cell division, but also to the resistance of the parenteral environment *in vivo*. Therefore, the proliferation rate of the cells was decreased *in vitro* after deletion of the N-terminal of FtsK, and the growth was further delayed *in vivo* as the bacteria grow parenterally.

Following intratracheal administration of the strains, the LD_{50} assays indicated that the virulence of the FtsK mutant strain is much lower than that of the wild-type strain. In addition, the pathological lesions in birds infected with E058FtsK were also milder than those infected with the parent strain E058. These results suggest that the virulence of the E058FtsK mutant was attenuated compared with its parent strain E058.

E. coli FtsK is a large protein that is essential for cell division and resolution of chromosome dimers by site-specific recombination. The FtsK protein contains three distinct domains, the first of which is a 279-residue N-terminal integral membrane domain with a series of transmembrane α-helices, which functions in cytokinesis and localises FtsK to the septum. Next is a proline- and glutamine-rich domain (of about 650 amino acids) which links the N-terminal to the C-terminal DNA translocase domain that acts in the final stages of chromosome unlinking by chromosome dimer resolution and decatenation. The third domain is the C-terminal (of about 469 amino acids) including a nucleotide-binding consensus sequence (1, 2, 6). In this study, the FtsK mutant strain E058FtsK only expresses the N-terminal 240-amino-acid domain of FtsK, and lacks the glutamine-rich domain and the C-terminal domain. *In vitro* and *in vivo* results showed that the E058FtsK could grow in LB and infect chickens. These results support previous experiments demonstrating that only the N-terminal 200-amino-acid domain of FtsK was required for cell division and that deletion of the protein remainder was not lethal to *E. coli* (6). Indeed, the absence of the C-terminal domain of FtsK could be compensated for by the segregation activities of other genes such as MukB. However, the FtsK mutant was attenuated compared with its parent strain both *in vitro* and *in vivo*, suggesting that the proline-and glutamine-rich domain and the C-terminal domain play an important role in the pathogenesis of APEC infection, and that this mutant strain may be a potential agent for the development of APEC vaccine.

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