Pakistan J. Zool., vol. 54(5), pp 2145-2151, 2022 DOI: https://dx.doi.org/10.17582/journal.pjz/20210112090151

Effect of *hcp1* of the Type VI Secretion System on Biological Characteristics of F18 Shiga Toxin-Producing *Escherichia coli*

Yang Yang[1,2], Hong Zhou[3], Ji Shao[1,2], Xinyi Zhang[1,2], Pengpeng Xia[1,2], Mingxu Zhou[4,5], Qiangde Duan[1,2] and Guoqiang Zhu[1,2]*

[1]*College of Veterinary Medicine, Yangzhou University, Yangzhou 225009, China*
[2]*Jiangsu Co-Innovation Center for Important Animal Infectious Diseases and Zoonoses, Yangzhou 225009, China*
[3]*School of Basic Medical Science, Southwest Medical University, Luzhou 646000, China*
[4]*Institute of Veterinary Immunology and Engineering, National Research Center of Engineering and Technology for Veterinary Biologicals, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, China*
[5]*Jiangsu Key Laboratory for Food Quality and Safety-State Key Laboratory Cultivation Base, Ministry of Science and Technology, Nanjing 210014, China*

Yang Yang and Hong Zhou contributed equally to this work.

Article Information
Received 12 January 2021
Revised 05 December 2021
Accepted 07 January 2022
Available online 27 January 2022 (early access)
Published 01 June 2022

Authors' Contribution
ZG and YY conceived and designed the experiments. ZH, SJ, ZX, XP, ZM, and DQ performed experiments. YY and ZH wrote this manuscript.

Key words
T6SS, *hcp*$_1$, STEC, Shiga toxin-producing *E. coli*, Virulence factor

ABSTRACT

The objective of this study was to investigate the effect of *hcp1*, a main structural gene of type VI secretion system (T6SS), on the biological characteristics related to the virulence factor of F18 Shiga toxin-producing *Escherichia coli* (STEC). In this study, based on the construct of the wild type F18 STEC (F18), *hcp1* deletion mutant (F18ab△*hcp1*) and complement strain (F18ab△*hcp1/phcp1*), their pathogenic differences were compared, and the function of *hcp1* was discussed from the aspects of growth curve, biofilm formation, adherence, invasion, cytotoxicity, and so on. The results showed that *hcp1* of the T6SS did not influence bacterial growth and biofilm formation. In F18ab△*hcp1*, bacterial motility, adherence and invasion towards the intestinal porcine enterocyte cells (IPEC-J2) were reduced significantly, and the cytotoxicity decreased. The results of RT-PCR showed that in F18ab△*hcp1*, the flagella gene *flic* and type I fimbriae *fimA* were significantly down-regulated. In conclusion, *hcp1* gene of F18 STEC is related to flagella expression, bacterial adherence, invasion, and secretion of Stx2e toxin. T6SS *hcp1* is proved to be closely involved in the pathogenesis of F18 STEC.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) expressing F18 fimbriae is one of important pathogens causing diarrhea and edema in weaned piglets, which has brought huge losses to pig industry in China (Yang *et al*., 2013). F18 *E. coli* has many remarkable biological characteristics. It can express F18 fimbriae, type I fimbriae, AIDA and flagella. After adherence and invasion towards pig intestinal epithelial cells, F18 STEC secrete Shiga toxin (Stx2e) and damage pig intestines (Da *et al*., 2001). These virulence factors are closely related to its pathogenicity, and may be regulated by type VI secretory system (T6SS).

Recently, bacterial secretory systems have become one of the frontier research fields of pathogenic microorganisms (Yi *et al*., 2019). Many studies have found that T6SS plays an important role in bacterial pathogenicity, and mediates interaction between bacteria and environment (Zheng *et al*., 2011). It has also been found to influence bacterial interspecific competition. T6SS is encoded by a gene cluster containing 15-20 conservative open reading frames (ORFs) with an average length of more than 20 kb. About 25% Gram-negative bacteria contain T6SS gene cluster (Williams *et al*., 1996; Pukatzki *et al*., 2009).

T6SS has been reported to be closely involved with bacterial pathogenicity (Peng *et al*., 2016). Shrivastava *et al*. (2008) found that the heme carrier protein (HCP), a heme-regulated protein that was encoded by *hcp* gene, can form a hexamer ring structure that can participate in the formation of transport channels connecting the inner

* Corresponding author: yzgqzhu@yzu.edu.cn
0030-9923/2022/0005-2145 $ 9.00/0

and outer membrane of bacteria (Filloux *et al*., 2008). HCP is involved in the pathogenic process of a variety of bacteria. It have been reported to participate in the resistance of *Vibrio cholerae* and *Burkholderia* to the predation of *Amoeba* (Pukatzki *et al*., 2007; Aubert *et al*., 2008), in the inhibition of the phagocytosis of *Aeromonas* by macrophages (Suarez *et al*., 2008), in the increase of *Salmonella* survival ability in host cells (Parsons and Heffron, 2005), in the biofilm formation of *Vibrio cholera* (Yahr, 2006), and in promotion of acute and chronic infection of some pathogens (Yahr, 2006; Filloux *et al*., 2008; Hood *et al*., 2010), etc. *E. coli* contains multiple *hcp* clusters. Zhou *et al*. (2012) found that Hcp1 of *E. coli* could induce cytoskeleton rearrangement, apoptosis, IL-6 and IL-8 release of human brain microvascular endothelial cells. Hcp2 was related to the adherence and invasion of bacteria to host cells. Ding *et al*. (2018) proved that in avian pathogenic *E. coli* (APEC), Hcp1 and Hcp2 were jointly involved in biological characteristics such as adherence and viability. Hcp1 affected Quorum sensing, while as Hcp2 participated in biofilm formation. Hu *et al*. (2021) discovered three *hcp* gene clusters (*hcp1*, *hcp2* and *hcp3*) in porcine extraintestinal pathogenic *E. coli*. All 3 gene clusters were involved into bacterial pathogenicity and participate in the colonization and persistence of bacteria in the host. Song *et al*. (2020) discovered that the deletion of *hcp2a* in APEC could improve biofilm formation, reduce resistance to chicken serum and suppress the expression of IL8 and IL1β in host cells. It was found that *hcp1* and *hcp2* gene clusters also exist in STEC, which may participate in the secretion and assembly of virulence factors and play an important role in bacterial pathogenesis. However, in F18 STEC, exact function of T6SS *hcp1* and its relationship with bacterial virulence have not been explored clearly.

To further analyze the effect of T6SS *hcp1* in bacterial virulence, F18ab STEC 107/86 reference strain was selected, and *hcp1* deletion strain was constructed by λ Red-based recombination system. Function of *hcp1* was further discussed by comparing the differences in growth, biofilm, motility, adherence, Stx2e production between wild type and *hcp1* deletion strain.

MATERIALS AND METHODS

Materials and the construction of F18ab Δhcp1 *and F18ab* Δhcp1/phcp1

The strains and plasmids are listed in Table I. F18ab STEC 107/86 was stored in our laboratory (Yang *et al*., 2014). All strains were grown at 37°C in LB medium with 100μg/mL Ampicillin (Amp) or 34μg/mL chloramphenicol (Cm+) added if requested. The intestinal porcine enterocyte cell line (IPEC-J2) was grown in RPMI 1640-F12 medium supplemented with 10% neonatal bovine serum (37°C, 5% CO_2). Vero cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (37°C, 5% CO_2). Plasmids pKD3, pKD46 and pCP20 used for λ Red-based recombination system were preserved in our laboratory. 4-week-old ICR mice were purchased from the Institute of Comparative Medicine of Yangzhou University.

The deletion strain F18abΔ*hcp1* was constructed by λ Red-based recombination system using △*hcp1-1*/△*hcp1-2* primers (Table II) (Datsenko and Wanner, 2000). Construction of the *hcp1* deletion strain was further verified by DNA sequencing. The ORF of *hcp1* was amplified by PCR by using Hcp1-1/Hcp1-2 primers (Table II). After insertion of *hcp1* into pBR322, the complement plasmid pBR-*hcp1* was constructed. The plasmid was transformed into the deletion strain F18abΔ*hcp1* for the construction of F18abΔ*hcp1*/p*hcp1*.

Growth curves, biofilm formation and mobility assays

The wild type (F18ab), deletion mutant (F18abΔ*hcp1*) and complement strain (F18abΔ*hcp1*/p*hcp1*) were inoculated in LB medium and cultured overnight at 37 °C. OD_{600} of each strain was measured every hour. The experiment was repeated 3 times to obtain the growth curves (Yang *et al*., 2018, 2021).

Table I. Strains and plasmids used in this study.

Items	Details	Provider
Strains		
E. coli F18ab 107/86	Wild-type O139:H1:F18ab, Stx2e; O139:H1:F18ab, Stx2e	Kept and provided by our own lab
E. coli F18abΔhcp1	*Hcp1* deletion mutant	Constructed in this study
E. coli F18abΔhcp1/phcp1	F18abΔ*fliC* carrying pBR-*hcp1*	Constructed in this study
Plasmids		
pBR322	Expression vector, Amp^r	Takara Ltd.
pKD3	Cm^r; Cm cassette template	Kept and provided by our own lab
pKD46	Amp^r, λRed recombinase expression	Kept and provided by our own lab
pCP20	Amp^r,Cm^r; Flp recombinase expression	Kept and provided by our own lab

Table II. The primers for the Real Time PCR.

Primer	Sequences (5'-3')
hcp1-1	5'-GCCGGATCCATGGCGAATTTAATTTAT-TTAAC- 3'
hcp1-2	5'-TAAAGTCGATTAAAAAAGACGATCT-TCCC-3'
Δ*hcp1*-1	5'-ATGCTCCCATATAATTGACTCATAACT-GAAAGAAACTGACTCTTCGGGTTGTG-TAGGCTGGGAGCTGCTTC-3'
Δ*hcp1*-2	5'-TGATAATCAAGGGCTCATTTCAAGTG-GTTGTTCATCACAACCATCCATTGCATAT-GAATATCCTCCTTA -3'
gapA- F	5'- CGTTAAAGGCGCTAACTTCG- 3'
R	5'- ACGGTGGTCATCAGACCTTC- 3'
fedF-F	5' -CCGTTACTCTTGATTTCTTTGTTG- 3'
R	5' -GGCATTTGGGTAGTGTTTGTCTT -3'
fimH-F	5'- GGCTGCGATGTTTCTGCTC -3'
R	5'- CCCCAGGTTTTGGCTTTTC -3'
AIDA-F	5'- CAGTCTACCGCACAAGCAAAAC-3'
R	5'- CAGTCTACCGCACAAGCAAAAC-3'
fliC-F	5'- CAGCAAGCGGTGAAGTGAA -3'
R	5'- AAGCGTAGCCGCAGTAGCA -3'
stx2e-F	5'- CGTCTGCTGTGCCTGTATGG -3'
R	5'- CTGGGCATAAATATCGTTGTCGT -3'

F, forward; R, reverse

The wild type (F18ab), deletion mutant (F18abΔ*hcp1*) and complement strain (F18abΔ*hcp1*/p*hcp1*) were inoculated in biofilm induction medium as previously described (Yang *et al*., 2014; Zhou *et al*., 2014). The OD_{600} values of each well were recorded to measure the amount of biofilm production using a crystal violet staining method. Each strain was tested using six replicates, and the experiment was conducted in triplicate.

The strains were inoculated in LB medium and cultured overnight at 37 °C. 1 μL of each strain was transferred to the middle of semi-solid medium (1% tryptone, 0.25% NaCl, 0.3% agar) respectively, and incubated at 37 °C for 16h. Motility halos were measured as previously described (Duan *et al*., 2013).

Adherence and invasion assay towards IPEC-J2

RPMI 1640 and Ham's F12 medium (1:1) containing 10% newborn bovine serum were used for IPEC-J2. Cells were cultured and transferred into 96 well plate. Briefly, 10^7 CFU of bacteria was added to each well of a 96-well tissue culture plate with a monolayer of approximately 10^5. After incubation for 1h, cells were washed with PBS (pH 7.2) for 3 times, and then lysed with 0.5% Triton X-100 for 20 min. Solutions were diluted to 1:10 in PBS, and were spread on the LB plate for bacterial counting (Yang *et al*., 2021).

Invasion assays were performed as previously described (Yang *et al*., 2013). The monolayer cells were washed with PBS buffer (pH 7.2) for 3 times, and the strains were added with MOI at 1:100 respectively. After incubation for 2h, cells were washed with PBS (pH 7.2) for 3 times, and gentamicin (50 μg/mL) was added. After additional 2h incubation, 0.5% Triton X-100 was used for cell lysis. The number of bacteria that invaded IPEC-J2 cells was enumerated. DH5α was used as negative control.

Bacterial colonization assay in mice intestine

12 ICR mice (4-week-old) were divided into 3 groups: Wild type group, deletion strain group and complement strain group. 48 h before challenge, 5 g/L streptomycin was added to drinking water to eradicate the normal resident flora in the intestine. Fructose (6.7%) was added to increase the amount of drinking water. Cimetidine (50 mg/kg) was administered intraperitoneally 3 h before challenge to reduce gastric acid secretion (van der Velden *et al*., 1998). 2×10^8 CFU bacteria were injected intragastrically into mice. Feces pellets were collected every day after challenge. After dissolving with PBS, 10-fold dilution series were plated on MacConkey agar plates to determine the number of CFU of *E. coli*. Experiment lasted for 5 days.

Vero cell cytotoxicity assay

Wild type (F18ab), deletion strain (F18abΔ*hcp1*), complementation (F18abΔ*hcp1*/p*hcp1*) and DH5α were inoculated in LB medium at 37 °C. Strains were cultured to an OD_{600} of 0.3, then mitomycin C (0.25 μg/mL) was added into each tube. After 12 h induction, culture supernatants were filtered through 0.22 μm filters. 100μL supernatants was added to the Vero cells and incubated for 20 h, with DMEM as negative control (Yang *et al*., 2014). According to the instructions of Cell Proliferation Reagent WST-1 Kit, 100 μL supernatants were added into Vero cells followed by 25 μL Cell Proliferation WST-1 Reagent for 4h incubation. OD_{450} was measured.

RNA extraction and real-time fluorescence quantitative PCR

Each strain was growing to OD_{600} at 2.0. Total RNA of each strain were extracted by TRIzol method (Zhou *et al*., 2014). The primers used for *fliC, fimH, fedf, AIDA* and *stx2e* genes were listed in Table II. *gapA* gene was used as housekeeper gene. SYBR®Premix Ex Taq II (Takara, Shiga, Japan) and ABI 7500 Real Time System (Applied Biosystems) were employed for subsequent experiments.

The obtained data were processed based on the $2^{-\Delta\Delta}$Ct method.

Statistical analyses

All statistical analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Differences in data were analyzed with t-test.

RESULTS

*F18ab*Δhcp1 *and F18ab*Δhcp1/phcp1

The *hcp1* deletion strain F18abΔ*hcp1* was constructed from its wild type F18ab 107/86 by using λ Red-based recombination system. The complete reading frame of *hcp1* was amplified by PCR, inserted into pBR322 plasmid and transformed into F18abΔ*hcp1*. The complement strain F18abΔ*hcp1*/p*hcp1* was also successfully constructed. F18abΔ*hcp1* was verified verified by combined PCR and sequencing data. The results of growth curve and biofilm formation assay showed that there was no significant difference between F18ab, F18abΔ*hcp*1 and F18abΔ*hcp1*/p*hcp1* (Fig. 1).

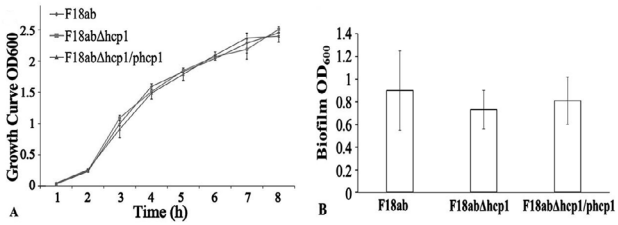

Fig. 1. Results of growth curve (A) and motility assay (B). Notes: **A**, there was no significant difference in growth rate among the three strains. **B**, there was no significant difference in biofilm formation ability among the three strains.

Results of bacterial adhesion and colonization assays

The results showed that the diameter of the motion circle of F18abΔ*hcp*1 decreased by about 18.8%, compared with the wild type. The motility of F18abΔ*hcp1*/p*hcp1* was slightly improved (Fig. 2A), compared with F18abΔ*hcp*1. In the adhesion assay, compared with wild type, the adherence ability of F18abΔ*hcp*1 to IPEC-J2 decreased by about 40% (Fig. 2B).

The invasion ability of each strain was analyzed. Extracellular bacteria which failed to invade IPEC-J2 were killed by antibiotics, and cells were lysed by 0.5% Triton X-100. The intracellular bacteria were released for plate counting. Compared with wild type, the invasion ability of F18abΔ*hcp*1 decreased by 28.3%, and invasion of F18abΔ*hcp1*/p*hcp1* was recovered (Fig. 2C).

MacConkey plates counting assay (Fig. 2D) showed that, after the challenge, the bacterial shedding of wild type strain and F18abΔ*hcp1*/p*hcp1* displayed a gradual upward trend. The excretion of F18abΔ*hcp1* gradually decreased. The knockout of *hcp*1 gene may block the secretion pathway of T6SS and hindered the secretion of protein molecules related to the process of adhesion and colonization.

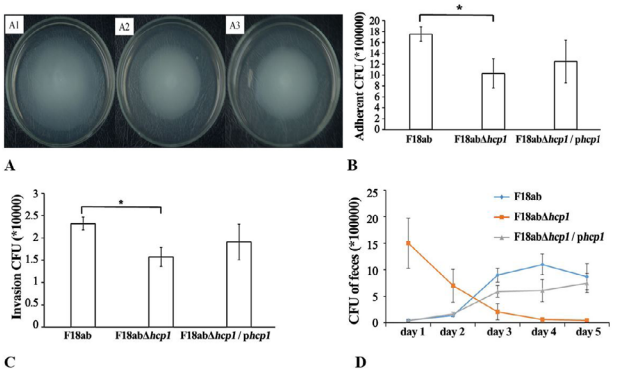

Fig. 2. The motility, adherence and colonization assays of bacterial. **A**, Flagella related motility assays. A1: F18ab; A2: F18abΔ*hcp1*; A3: F18abΔ*hcp1*/p*hcp1*. **B**, Adherence ability towards IPEC-J2 cell line. **C**, Invasion ability towards IPEC-J2 cell line. **D**, Intestinal colonization test. * Significant difference, $p<0.05$.

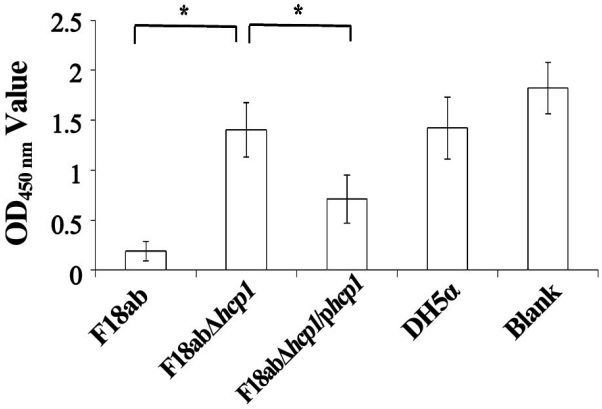

Fig. 3. Vero cell cytotoxicity assay. * Significant difference, $p<0.05$.

Cytotoxicity assay

The results showed that, in *hcp1* deletion strain group, the number of surviving cells is seven times that of wild type group (Fig. 3), which proved that the deletion of *hcp1* inhibited the synthesis or secretion of Stx2e. The cytotoxicity of the deletion strain is similar to that of the negative control group. The cytotoxicity of F18abΔ*hcp1*/p*hcp1* was significantly higher than that of deletion strain.

The transcription levels of virulence genes

Total RNA of F18ab, F18ab∆*hcp1* and F18ab∆*hcp1*/p*hcp1* were extracted, respectively, and were reverse transcribed into cDNA. Housekeeping gene *gapA* was used in this experiment. The expression levels of five important virulence factors *fimH*, *fedF*, *fliC*, *AIDA* and *stx2e* were detected. As shown in Figure 4, the transcription level of *fliC* decreased by about 28%. The expression of *fimH* (type I fimbriae) decreased by 31%, and there was no significant difference in expression of *fedF*, *AIDA* and *stx2e* (Fig. 4).

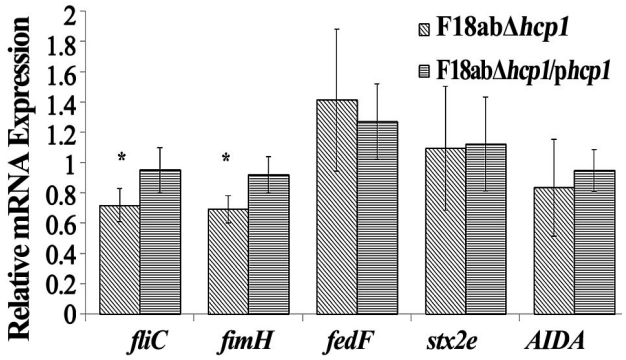

Fig. 4. Transcriptional levels of virulence related genes.
*: The gene transcriptional levels compared with F18ab, $P<0.05$

DISCUSSION

T6SS plays an important role in the pathogenic process of bacterial infection. About 25% of Gram-negative bacteria have the genes encoding T6SS. HCP family plays an important role in the interaction between bacteria and host cells in T6SS. The HCP protein is a landmark component of T6SS. Previous studies have shown that 2-3 *hcp* gene clusters (*hcp1*, *hcp2* and *hcp3*) have been found in meningitis-causing *E. coli*, avian pathogenic *E. coli* and porcine extraintestinal pathogenic *E. coli* (Ding *et al*., 2018; Song *et al*., 2020; Hu *et al*., 2021). Three *hcp* gene clusters exhibit different functions. However, the role of T6SS and HCP in the pathogenesis of F18ab STEC has not been well elucidated.

In this study, the growth trend of wild type, deletion mutant, and complement strains is the same. There is no significant difference in the lag phase, logarithmic growth phase, stable phase and decay phase, indicating that Hcp1 did not affect the growth of F18 *E. coli* (Fig. 1A).

STEC mediates the adherence of bacteria to host cells through F18 fimbriae, flagella and other adhesins (Da *et al*., 2001). After adherence, bacteria would invade into host cells or forms biofilm to colonize in host, and then releases Stx2e and other toxins, causing edema lesions. In this study, the related characteristics of bacterial adhesins influenced by *hcp1* were investigated, and the transcription levels of major adhesins were detected by fluorescence quantitative PCR. F18ab *E. coli* mainly relies on its flagella to achieve its motor function. Most flagella subunits are assembled outside the plasma membrane, thus the protein molecules used for assembly need to be synthesized in the cytoplasm and then transported to the final assembly position through the inner membrane and outer membrane. Flagella not only mediate bacterial motility, but also acts as an important adhesin to mediate the adherence process between bacteria and host cells. The results showed that the motility of *hcp1* deletion strain decreased by 18.8% (Fig. 2A). T6SS may be involved in the transport process of flagella subunit protein, or the secretion of other cofactors involved in the assembly process of flagella subunit protein. The deletion of *hcp1* leads to the decrease of assembly efficiency of flagellum, resulting in the weakening of bacterial motility. In this study, it was found that the adherence ability of ∆*hcp1* deletion strain was reduced by 40% (Fig. 2B), and its invasion ability decreased by 28.3% (Fig. 2C). The transcription levels of several main adhesion factor genes showed that the expression of flagella *fliC*, type I fimbriae *fimH* decreased by 28 and 31%, respectively (Fig. 4). The results indicated that *hcp1* is related to the expression of type I fimbriae and flagella in F18 STEC strain, and deletion of *hcp1* can inhibit the motility, adherence and invasion of F18 STEC.

Biofilm is a bacterial morphological change for adaptability, which can enhance the bacterial resistance to the external environment. Biofilm can greatly improve bacterial colonization in the host (Duan *et al*., 2012). After *hcp1* deletion, there was no significant difference in biofilm formation ability between STEC deletion strain and wild strain (Fig. 1B), which proved that Hcp may not be involved in biofilm formation.

F18 STEC attached to porcine intestinal epithelial cells through adhesins, and then colonizes and secreted Stx2e. Through subunit B, Stx2e binds to Gb5 receptor of intestinal epithelium and can be ingested by the intestinal epithelial cells through endocytosis. Stx2e can induce apoptosis through subunit A, influence the vascular permeability, causes osmotic pressure disorder, and finally leads to edema (Niewerth *et al*., 2001). The expression level of Stx2e toxin gene in deletion strain F18ab∆*hcp1* did not change (Fig. 4), but the toxicity to Vero cells decreased significantly (Fig. 3). The numbers of surviving cells in wild and complement strain group were less than *hcp1* deletion strain, indicating that Stx2e proteins in the supernatant of deletion strain was reduced. T6SS may be an important secretory pathway for Stx2e. The difference in cytotoxicity between the *hcp1*deletion strain and the

wild type did not come from the difference in the synthesis and expression of toxin, but from the release and secretion process.

CONCLUSION

In conclusion, T6SS *hcp1* affects flagella and type I fimbriae in F18 STEC. Deletion of *hcp1* suppress bacterial motility, adherence, invasion ability, and colonization in mice. Moreover, it is closely related to the release and secretion process of Stx2e, and is deeply involved in bacterial cytotoxicity. The exploration of the effect of *hcp1* laid a foundation for further clarifying the complex function of T6SS system in F18 STEC.

ACKNOWLEDGMENTS

This study was supported by grants from the Chinese National Science Foundation Grants (Nos. 31972708, 31502075, 31873010, 31672579), 13th Five-Year National Key Development Program (2016YFD0501000), the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Ethical compliance

The protocols for animal experiments were approved by the Jiangsu Administrative Committee for Laboratory Animals (approval number: SYXK-SU-2007-0005), and complied with the guidelines of Jiangsu laboratory animal welfare and ethics of Jiangsu Administrative Committee of Laboratory Animals.

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Aubert, D.F., Flannagan, R.S. and Valvano, M.A., 2008. A novel sensor kinase-response regulator hybrid controls biofilm formation and type VI secretion system activity in *Burkholderia cenocepacia*. *Infect. Immun.*, **76**: 1979-1991. https://doi.org/10.1128/IAI.01338-07

Da, S.A., Valadares, G.F., Penatti, M.P., Brito, B.G. and Da, S.L.D., 2001. *Escherichia coli* strains from edema disease: O serogroups, and genes for Shiga toxin, enterotoxins, and F18 fimbriae. *Vet. Microbiol.*, **80**: 227-233. https://doi.org/10.1016/S0378-1135(01)00316-9

Datsenko, K.A. and Wanner, B.L., 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. natl. Acad. Sci. U. S. A.*, **97**: 6640-6645. https://doi.org/10.1073/pnas.120163297

Ding, X., Zhang, Q., Wang, H., Quan, G., Zhang, D., Ren, W., Liao, Y., Xia, P. and Zhu, G., 2018. The different roles of *hcp1* and *hcp2* of the type VI secretion system in *Escherichia coli* strain CE129. *J. Basic Microbiol.*, **58**: 938-946. https://doi.org/10.1002/jobm.201800156

Duan, Q., Zhou, M., Zhu, X., Bao, W., Wu, S., Ruan, X., Zhang, W., Yang, Y., Zhu, J. and Zhu, G., 2012. The flagella of F18ab *Escherichia coli* are a virulence factor that contributes to infection in a IPEC-J2 cell model *in vitro*. *Vet. Microbiol.*, **160**: 132-140. https://doi.org/10.1016/j.vetmic.2012.05.015

Duan, Q., Zhou, M., Zhu, X., Yang, Y., Zhu, J., Bao, W., Wu, S., Ruan, X., Zhang, W. and Zhu, G., 2013. Flagella from F18+*Escherichia coli* play a role in adhesion to pig epithelial cell lines. *Microbiol. Pathog.*, **55**: 32-38. https://doi.org/10.1016/j.micpath.2012.09.010

Filloux, A., Hachani, A. and Bleves, S., 2008. The bacterial type VI secretion machine: Yet another player for protein transport across membranes. *Microbiology*, **154**: 1570-1583. https://doi.org/10.1099/mic.0.2008/016840-0

Hood, R.D., Singh, P., Hsu, F., Güvener, T., Carl, M.A., Trinidad, R.R.S., Silverman, J.M., Ohlson, B.B., Hicks, K.G., Plemel, R.L., Li, M., Schwarz, S., Wang, W.Y., Merz, A.J., Goodlett, D.R. and Mougous, J.D., 2010. A type VI secretion system of *Pseudomonas aeruginosa* targets a toxin to bacteria. *Cell Host Microbe.*, **7**: 25-37. https://doi.org/10.1016/j.chom.2009.12.007

Hu, L., Yu, F., Liu, M., Chen, J., Zong, B., Zhang, Y., Chen, T., Wang, C., Zhang, T., Zhang, J., Zhu, Y., Wang, X., Chen, H. and Tan, C., 2021. RcsB-dependent regulation of type VI secretion system in porcine extra-intestinal pathogenic *Escherichia coli*. *Gene*, **768**: 145289. https://doi.org/10.1016/j.gene.2020.145289

Niewerth, U, Frey, A., Voss, T., Le Bouguenec, C., Baljer, G., Franke, S. and Schmidt, M.A., 2001. The AIDA autotransporter system is associated with F18 and stx2e in *Escherichia coli* isolates from pigs diagnosed with edema disease and postweaning diarrhea. *Clin. Diagn. Lab. Immunol.*, **8**: 143-149. https://doi.org/10.1128/CDLI.8.1.143-149.2001

Parsons, D.A. and Heffron, F., 2005. sciS, an icmF homolog in *Salmonella* enterica serovar *Typhimurium*, limits intracellular replication and decreases virulence. *Infect. Immun.*, **73**: 4338-4345. https://doi.org/10.1128/IAI.73.7.4338-4345.2005

Peng, Y., Wang, X., Shou, J., Zong, B., Zhang, Y., Tan, J., Chen, J., Hu, L., Zhu, Y., Chen, H. and Tan, C., 2016. Roles of Hcp family proteins in the pathogenesis of the porcine extraintestinal pathogenic *Escherichia coli* type VI secretion system. *Sci. Rep. U. K.*, **6**: 26816. https://doi.org/10.1038/srep26816

Pukatzki, S., Ma, A.T., Revel, A.T., Sturtevant, D. and Mekalanos, J.J., 2007. Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin. *Proc. natl. Acad. Sci. U. S. A.*, **104**: 15508-15513. https://doi.org/10.1073/pnas.0706532104

Pukatzki, S., McAuley, S.B. and Miyata, S.T., 2009. The type VI secretion system: Translocation of effectors and effector-domains. *Curr. Opin. Microbiol.*, **12**: 11-17. https://doi.org/10.1016/j.mib.2008.11.010

Shrivastava, S. and Mande, S.S., 2008. Identification and functional characterization of gene components of Type VI Secretion system in bacterial genomes. *PLoS One*, **3**: e2955. https://doi.org/10.1371/journal.pone.0002955

Song, X., Hou, M., Jiang, H., Shen, X., Xue, M., Shao, Y., Wang, L., He, Q., Zheng, L., Tu, J and K Qi, 2020. Hcp2a of type VI secretion system contributes to IL8 and IL1β expression of chicken tracheal epithelium by affecting APEC colonization. *Res. Vet. Sci.*, **132**: 279-284. https://doi.org/10.1016/j.rvsc.2020.07.007

Suarez, G., Sierra, J.C., Sha, J., Wang, S., Erova, T.E., Fadl, A.A., Foltz, S.M., Horneman, A.J. and Chopra, A.K., 2008. Molecular characterization of a functional type VI secretion system from a clinical isolate of *Aeromonas hydrophila*. *Microb. Pathog.*, **44**: 344-361. https://doi.org/10.1016/j.micpath.2007.10.005

van der Velden, A.W., Baumler, AJ, Tsolis, R.M. and Heffron, F., 1998. Multiple fimbrial adhesins are required for full virulence of *Salmonella typhimurium* in mice. *Infect. Immun.*, **66**: 2803-2808. https://doi.org/10.1128/IAI.66.6.2803-2808.1998

Williams, S.G., Varcoe, L.T., Attridge, S.R. and Manning, P.A., 1996. *Vibrio cholerae* Hcp, a secreted protein coregulated with HlyA. *Infect. Immun.*, **64**: 283-289. https://doi.org/10.1128/iai.64.1.283-289.1996

Yahr, T.L., 2006. A critical new pathway for toxin secretion? *New Engl. J. Med.*, **355**: 1171-1172. https://doi.org/10.1056/NEJMcibr063931

Yang, Y., Yao, F., Zhou, M., Zhu, J., Zhang, X., Bao, W., Wu, S., Hardwidge, P.R. and Zhu, G., 2013. F18ab *Escherichia coli* flagella expression is regulated by acyl-homoserine lactone and contributes to bacterial virulence. *Vet. Microbiol.*, **165**: 378-383. https://doi.org/10.1016/j.vetmic.2013.04.020

Yang, Y., Zhang, X., Zhang, B., Zhou, M., Duan, Q., Li, Z., Zhang, X. and Zhu, G., 2021. Quorum sensing-1 signaling of N-hexanoyl-l-homoserine lactone contributes to virulence in avian pathogenic *Escherichia coli. Arch. Microbiol.,* Accepted in press. https://doi.org/10.21203/rs.3.rs-674139/v1

Yang, Y., Zhou, M., Hardwidge, P.R., Cui, H. and Zhu, G., 2018. Isolation and characterization of N-acyl homoserine lactone-producing bacteria from cattle rumen and swine intestines. *Front. Cell Infect. MI*, **8**: 155. https://doi.org/10.3389/fcimb.2018.00155

Yang, Y., Zhou, M., Hou, H., Zhu, J., Yao, F., Zhang, X., Zhu, X., Hardwidge, P.R. and Zhu, G., 2014. Quorum-sensing gene luxS regulates flagella expression and Shiga-like toxin production in F18ab *Escherichia coli*. *Can. J. Microbiol.*, **60**: 355-361. https://doi.org/10.1139/cjm-2014-0178

Yi, Z., Wang, D., Xin, S., Zhou, D., Li, T., Tian, M., Qi, J., Ding, C., S Wang, and Yu, S., 2019. The CpxR regulates type VI secretion system 2 expression and facilitates the interbacterial competition activity and virulence of avian pathogenic *E. scherichia coli*. *Vet. Res.*, **50**: 40. https://doi.org/10.1186/s13567-019-0658-7

Zheng, J., Ho, B. and Mekalanos, J.J., 2011. Genetic analysis of anti-amoebae and anti-bacterial activities of the type VI secretion system in vibrio cholerae. *PLoS One*, **6**: e23876. https://doi.org/10.1371/journal.pone.0023876

Zhou, M., Guo, Z., Yang, Y., Duan, Q., Zhang, Q., Yao, F., Zhu, J., Zhang, X., Hardwidge, P.R. and Zhu, G., 2014. Flagellin and F4 fimbriae have opposite effects on biofilm formation and quorum sensing in F4ac+ enterotoxigenic *Escherichia coli*. *Vet. Microbiol.,* **168**: 148-153. https://doi.org/10.1016/j.vetmic.2013.10.014

Zhou, Y., Tao, J., Yu, H., Ni, J., Zeng, L., Teng, Q., Kim, K.S., Zhao, G., Guo, X and Yao, Y., 2012. Hcp family proteins secreted via the type VI secretion system coordinately regulate *Escherichia coli* K1 interaction with human brain microvascular endothelial cells. *Infect. Immun.*, **80**: 1243-1251. https://doi.org/10.1128/IAI.05994-11