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The Type VI Secretion System Modulates Flagellar Gene Expression and Secretion in *Citrobacter freundii* and Contributes to Adhesion and Cytotoxicity to Host Cells

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The type VI secretion system (T6SS) as a virulence factor-releasing system contributes to virulence development of various pathogens and is often activated upon contact with target cells. *Citrobacter freundii* strain CF74 has a complete T6SS genomic island (GI) that contains *clpV*, *hcp-2*, and *vgr* T6SS genes. We constructed *clpV*, *hcp-2*, *vgr*, and T6SS GI deletion mutants in CF74 and analyzed their effects on the transcriptome overall and, specifically, on the flagellar system at the levels of transcription and translation. Deletion of the T6SS GI affected the transcription of 84 genes, with 15 and 69 genes exhibiting higher and lower levels of transcription, respectively. Members of the cell motility class of downregulated genes of the CF74ΔT6SS mutant were mainly flagellar genes, including effector proteins, chaperones, and regulators. Moreover, the production and secretion of FliC were also decreased in *clpV*, *hcp-2*, *vgr*, or T6SS GI deletion mutants in CF74 and were restored upon complementation. In swimming motility assays, the mutant strains were found to be less motile than the wild type, and motility was restored by complementation. The mutant strains were defective in adhesion to HEp-2 cells and were restored partially upon complementation. Further, the CF74ΔT6SS, CF74Δ*clpV*, and CF74Δ*hcp-2* mutants induced lower cytotoxicity to HEp-2 cells than the wild type. These results suggested that the T6SS GI in CF74 regulates the flagellar system, enhances motility, is involved in adherence to host cells, and induces cytotoxicity to host cells. Thus, the T6SS plays a wide-ranging role in *C. freundii*.

The type VI secretion system (T6SS) is present in a number of bacterial species, including important pathogens, *Pseudomonas aeruginosa* (1), *Vibrio cholerae* (2), enteroaggregative *Escherichia coli* (3), *Burkholderia thailandensis* (4), *Serratia marcescens* (5), *Burkholderia mallei* (6), and *Salmonella enterica* (7). The T6SS consists of a core structure formed by IAHP (intracellular multiplication protein F [IcmF]-associated homologous protein), the inner membrane proteins IcmF and DotU/IcmH, the lipoprotein SciN, and the ATPase ClpV. IcmF is predicted to be located in the inner membrane and consists of a cytosolic domain and a large periplasmic domain. The cytosolic part harbors a conserved Walker A motif (phosphate-binding loop), implying that IcmF functions as an ATPase during type VI protein secretion. *icmF* mutants have been shown to prevent Hcp secretion (2, 8–10). ClpV is a member of the AAA^+ (ATPases associated with various cellular activities) protein family (8, 11). It forms oligomeric complexes to energize the system for the secretion of effector proteins, which include the secreted VgrG (valine glycine repeat) and Hcp (hemolysin-coregulated protein) proteins (1, 12–17).

It has been reported that the T6SS contributes to the virulence development of various pathogens and is often activated upon contact with target cells for the secretion of effector proteins (1–7, 14–17). The expression and assembly of the T6SS are tightly controlled at both the transcriptional and posttranscriptional levels (12, 13, 18). Furthermore, the IcmF protein of the T6SS has been shown to be involved in flagellar regulation and affects motility and biofilm formation (15). The flagellum is composed of a basal body, a hook, and a filament. FliC, a component of the filament, is transported from the cytoplasm by a number of transport systems in different bacterial species, including the SPI1 type III secretion system (T3SS), the Dot/Icm type IV secretion system (T4SS), and the locus of enterocyte effacement (LEE)-encoded T3SS in *Salmonella enterica* serovar Typhimurium, *Legionella pneumophila*, and enteropathogenic *E. coli*, respectively (19, 20).

Citrobacter freundii is considered a commensal of the intestinal tract in humans and other animals (21). However, *C. freundii* can also cause diarrhea and other infections in humans (22–25). Relatively little is known about the virulence of *C. freundii*. Recently, we identified a *C. freundii* strain, CF74, that showed an aggregative adherence pattern and cytotoxicity to HEp-2 cells. The strain was found to contain a complete T6SS located on a genomic island (GI), and the T6SS gene cluster consists of 16 genes, including key T6SS genes (*clpV*, *hcp-2*, and *vgr*) (26). In this study, we constructed *clpV*, *hcp-2*, *vgr*, and T6SS GI deletion mutants in CF74

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TABLE 1 Strains and plasmids

Strain or plasmid	Characteristics	Source or reference
Strains		
CF74	*C. freundii* isolate from fecal samples from a goat	Laboratory stock
CF74ΔT6SS	CF74 mutant with T6SS core component gene cluster deletion	This study
CF74Δ*clpV*	CF74 mutant with *clpV* gene deletion	This study
CF74Δ*hcp-2*	CF74 mutant with *hcp-2* gene deletion	This study
CF74Δ*vgr*	CF74 mutant with *vgr* gene deletion	This study
E. coli $SM10_{\lambda pir}$	*supE recA*::*rp4-2*-Tc::Mu Km^r λ*pir*	Laboratory stock
E. coli $DH5\alpha_{\lambda pir}$	*RP4-2-tet* Mu-Km::Tn*7* integrant *leu-63*::IS*10 recA1 creC510 hsdR17 endA1 zbf-5 uidA*(Δ*mluI*)::*pir*$^+$ *thi*	Laboratory stock
CF74Δ*clpV*(pBAD24-*clpV*)	Δ*clpV*(pBAD24-*clpV*)	This study
CF74Δ*hcp-2*(pBAD24-*hcp-2*)	Δ*hcp-2*(pBAD24-*hcp-2*)	This study
CF74Δ*vgr*(pBAD24-*vgr*)	Δ*vgr*(pBAD24-*vgr*)	This study
Plasmids		
pBAD24	pMB1 Amp^r; pBAD promoter	Laboratory stock
pBAD24-*clpV*	The *clpV* coding region was cloned into the EcoRI and HindIII sites of pBAD24	This study
pBAD24-*hcp-2*	The *hcp-2* coding region was cloned into the KpnI and HindIII sites of pBAD24	This study
pBAD24-*vgr*	The *vgr* coding region was cloned into the KpnI and HindIII sites of pBAD24	This study
pWM91	Amp^r *f1*$^+$ *ori lacZa* of pBluescript II SK(+); $oriR_{R6K\gamma}$ $oriT_{RP4}$ *sacB* Suc^s	Laboratory stock
pWM91ΔT6SS	pWM91-T6SSup::T6SSdown; Amp^r	This study
pWM91Δ*clpV*	pWM91-*clpV*up::*clpv*down; Amp^r	This study
pWM91Δ*hcp-2*	pWM91-*hcp-2*up::*hcp*down; Amp^r	This study
pWM91Δ*vgr*	pWM91-*vgr*up::*vgr*down; Amp^r	This study

and demonstrate that the T6SS affects the transcription of over 84 genes, and especially the flagellar system, at the levels of transcription and translation and is involved in the secretion of FliC. The T6SS was also found to contribute to adhesion and cytotoxicity to host cells.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. freundii* strain CF74 was isolated from a fecal sample from a goat, as reported previously (26). All strains were grown aerobically at 37°C in Luria-Bertani (LB) medium. Antibiotics were added at the following concentrations: 100 mg/ml for ampicillin and streptomycin and 30 mg/ml for chloramphenicol where appropriate. All strains and plasmids used in this study are listed in Table 1.

Construction of isogenic mutants and plasmids. All in-frame deletion mutants were generated in *C. freundii* strain CF74 via double crossover using the suicide plasmid pWM91 as described previously (27, 28). The upstream and downstream regions of the gene of interest were amplified using the primers shown in Table S1 in the supplemental material. Using fusion PCR of these two fragments, we generated a fragment that was cloned into pWM91, a plasmid containing the counterselectable *sacB* gene. The recombinant plasmids were then purified and introduced into *E. coli* SM10 λ*pir* and conjugally transferred into CF74, and deletion of the genes of interest was selected in LB agar with 10% sucrose and without NaCl. Chromosomal deletion mutants were identified by colony PCR and quantitative reverse transcription (qRT)-PCR.

CF74 deletion mutants were complemented by pBAD24 harboring the genes of interest, allowing arabinose-controlled gene expression. For transformation of plasmids into CF74, electrocompetent cells were prepared as described previously (29).

RNA extraction and qRT-PCR. To prepare cells for RNA extraction, fresh LB medium was inoculated from an overnight culture of CF74, deletion mutants, or their complementation mutants (1:100) and incubated at 37°C with shaking at 220 rpm. The strains were collected at an optical density at 600 nm (OD_{600}) of 1.0. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA samples were further purified using the RNeasy mini-kit (Qiagen, Valencia, CA), followed by treatment with DNase I (Qiagen, Valencia, CA) to eliminate genomic DNA contamination. The RNA size, integrity, and total amount were measured using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA).

To measure gene transcription in different strains, qRT-PCR was performed using specific primers (see Table S1 in the supplemental material) based on the targeted genes. Total RNA (1.0 mg) was reverse transcribed to generate cDNA as the template for qRT-PCR. qRT-PCR was carried out using SYBR Premix Ex Taq II (Perfect Real Time; TaKaRa) using a Rotor-Gene Q thermal cycler (Qiagen, Valencia, CA). Data were analyzed with Rotor-Gene Q series software version 1.7 (Qiagen, Valencia, CA). The data were normalized to the endogenous reference gene *gapA* and analyzed by the cycle threshold method ($2^{-\Delta\Delta CT}$) (30). Three independent technical replicates were carried out for each target.

RNA-Seq and transcriptional data analysis. rRNA was depleted using the MicrobEnrich kit and the Microbexpress bacterial mRNA enrichment kit (both from Ambion, Austin, TX) according to the manufacturer's specifications. Sequencing libraries were prepared using the mRNA-Seq 8-Sample Prep kit (Illumina, San Diego, CA) according to the manufacturer's protocol. The RNA-Seq libraries were sequenced using IlluminaGA IIx and the paired-end sequencing module.

All raw FastQ files were trimmed and sorted by SolexQA (31). To obtain estimates of transcription levels, Burrows-Wheeler alignment (BWA) (32) was used to map the trimmed sequencing reads against the genome sequence of *C. freundii* CF74. Cufflinks (v1.1.0) (33) was then used to estimate gene transcription levels. Reads per kilobase of exon model per million mapped reads (RPKM) (34) was used as a normalized metric to present the gene expression levels.

Swimming motility assays. Motility was evaluated using a medium based on Difco-Tryptone (BD Diagnostic Systems) broth containing 1% (wt/vol) NaCl (Sigma-Aldrich) with Difco Bacto agar at 0.3% (wt/vol) to 1% (wt/vol). For plasmid-based complementation experiments, 0.2% arabinose and 100 μg/ml ampicillin were added to the plates, and all plates were poured the night before use and allowed to air dry on the bench. The plates were stab inoculated and incubated at 30°C for 16 h, and halo diameter measurements were recorded. All strains were tested in tripli-

TABLE 2 Summary of RNA-Seq coverage data

Statistic	Value	
	CF74	CF74ΔT6SS
No. of reads that aligned	22,113,030	20,751,152
No. of reads that did not align	236,198	171,780
Total no. of reads in the sample	22,349,228	20,922,932
% alignments	98.94	99.18
No. of reads that aligned to the 16S rRNA gene	82,443	3,312
No. of reads that aligned to the 23S rRNA gene	144,083	5,112
% of all reads that aligned to 16S and 23S rRNA genes	1.01	0.04

cate, and each experiment was carried out on three separate occasions (35, 36).

Preparation and analysis of whole-cell extracts and culture supernatant proteins. Bacterial culture supernatants were collected at an OD_{600} of 1.0 and filtered through 0.22-μm low-protein-binding filters (Millipore). A 10% (vol/vol) final concentration of trichloroacetic acid (TCA) (Sigma-Aldrich) was used to precipitate the proteins. The supernatants were incubated overnight at 4°C, and the aggregated proteins were precipitated by centrifugation at 4°C, washed with cold acetone, air dried, and stored at −80°C until use.

Whole-cell lysates (WCL) were generated by sonicating the cell pellets. Briefly, the cell pellets were suspended in lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 65 mM dithiothreitol (DTT), and 40 mM Tris with 1/100 (vol/vol) protease inhibitor cocktail (Roche), and the suspension was sonicated.

Western blotting. WCL and culture supernatant proteins were subsequently analyzed by SDS-10% PAGE, and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore) for immunoblotting. The immobilized proteins were incubated with primary antibodies, namely, rabbit anti-FliC (made by Abzome Co., Ltd., China; 1:5,000) and mouse anti-GapA (Thermo Fisher Scientific, Waltham, MA; 1:5,000), followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit (MBL; 1:10,000) and goat anti-mouse IgG (MBL; 1:3,000), respectively, and then detected on ECL chemiluminescence film (Kodak, China) using a Protec automatic film processor (Optimax).

***In vitro* adhesion and cytotoxicity assays.** Comparison of *in vitro* adhesion to host cells was performed using the human epidermoid carcinoma cell line HEp-2 (CCC0068; Beijing Union Medical College cell resource center), as previously described (26). Infections were performed at a multiplicity of infection (MOI) of 100:1. After 3 h of incubation at 37°C with 5% CO_2, the infected monolayers were rinsed with phosphate-buffered saline (PBS), and then the cells were lysed in PBS containing 0.25% Triton X-100. The lysates were plated onto agar plates, and the number of adhesive bacteria was determined by counting the CFU after overnight incubation at 37°C. Each sample determination was performed in triplicate, and experiments were repeated two times.

The lactate dehydrogenase (LDH) released by the HEp-2 cells was determined using the Cytotox96 kit (Promega) according to the manufacturer's instructions. The relative amount of cytotoxicity was expressed as follows: (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100, where the spontaneous release was the amount of LDH activity in the supernatant of uninfected cells and the maximum release was that when cells were lysed with the lysis buffer provided by the manufacturer. All experiments were performed two times in duplicate (26).

Statistical analysis. SPSS software version 13.0 (SPSS Inc., Chicago, IL, USA) was used to conduct all statistical comparisons. A nonparametric test (Mann-Whitney U test) was employed to compare the different groups. A two-tailed *P* value of 0.05 or less was considered to be statistically significant.

RESULTS

Transcriptome comparison of *C. freundii* strain CF74 and T6SS deletion mutant CF74ΔT6SS. A CF74 T6SS deletion mutant (CF74ΔT6SS) was created by allelic exchange, and the deletion from gene CFCDC_0705 to gene CFCDC_0726 was confirmed by PCR sequencing. To analyze differential expression of transcripts between *C. freundii* strain CF74 and the deletion mutant CF74ΔT6SS, an RNA-Seq was performed. Over 20 million reads each were obtained for CF74 and CF74ΔT6SS. Approximately 99% of the reads were mapped to the reference genome, CF74 (Table 2). A total of 84 genes were found to be differentially transcribed between CF74 and CF74ΔT6SS; 15 and 69 genes exhibited higher and lower levels of transcription in CF74ΔT6SS, respectively (see Tables S2 and S3 in the supplemental material).

The genes differentially transcribed were classified into functional categories based on clusters of orthologous groups (COGs) (http://www.ncbi.nlm.nih.gov/COG), and the percentage of significantly upregulated and downregulated genes in CF74ΔT6SS in each COG category was determined. In CF74ΔT6SS, 12 COGs contained more repressed than activated genes. Many of these differentially regulated genes are involved in metabolism, including the utilization of glycerol, ethanolamine, and maltose (see Tables S2 and S3 in the supplemental material). The COG with the largest proportion of T6SS-regulated genes was the cell motility class, in which approximately 31.82% of the genes were downregulated significantly, with no genes in the class upregulated significantly (Fig. 1). The downregulated cell motility genes of CF74ΔT6SS were mainly flagellar genes, including *fliC*, *flgM*, *flgK*, *fliD*, *flgL*, *motA*, and *fliT* (see Fig. S1 in the supplemental material).

The T6SS affects the flagellar system at the transcriptional level. To confirm the RNA-Seq results, we selected six flagellar genes (*fliC*, *flgM*, *flgK*, *fliD*, *flgL*, and *fliT*) for qRT-PCR analysis of the wild-type CF74 and the mutant CF74ΔT6SS under the same culture conditions as for the RNA-Seq experiments. The relative expression levels of flagellar genes were normalized to that of a housekeeping gene, *gapA*. The results corresponded well to the RNA-Seq data (Table 3 and Fig. 2A). These results indicated that the loss of the T6SS resulted in changes in the expression of flagellar genes at the level of transcription.

The T6SS has three key genes, *clpV*, *vgr*, and *hcp-2*. ClpV is an ATPase that forms oligomeric complexes to energize the system for the secretion of effector proteins, while Vgr and Hcp-2 are effector proteins (1, 12–14, 17). To understand which of these proteins affected the expression of the flagellar genes at the level of transcription, we constructed three mutants (CF74Δ*clpV*, CF74Δ*hcp-2*, and CF74Δ*vgr*) and complemented strains [CF74Δ*clpV* (pBAD24-*clpV*), CF74Δ*hcp-2*(pBAD24-*hcp-2*), and CF74Δ*vgr* (pBAD24-*vgr*)], in which the expression of the six flagellar genes were measured by qRT-PCR analysis. It was shown (Fig. 2B to D) that *fliC* and *flgM* were downregulated in all three mutant strains and were restored upon complementation ($P < 0.05$). *flgK* was downregulated in all three mutants but significantly only in CF74Δ*hcp-2* ($P < 0.01$). *fliD*, *flgL*, and *fliT* were downregulated only in CF74Δ*hcp-2* and complemented back in CF74Δ*hcp-2*(pBAD24-*hcp-2*) ($P < 0.01$). Overall, the six flagellar genes were significantly downregulated only in CF74 Δ*hcp-2*, similar to the outcome of the deletion of the entire

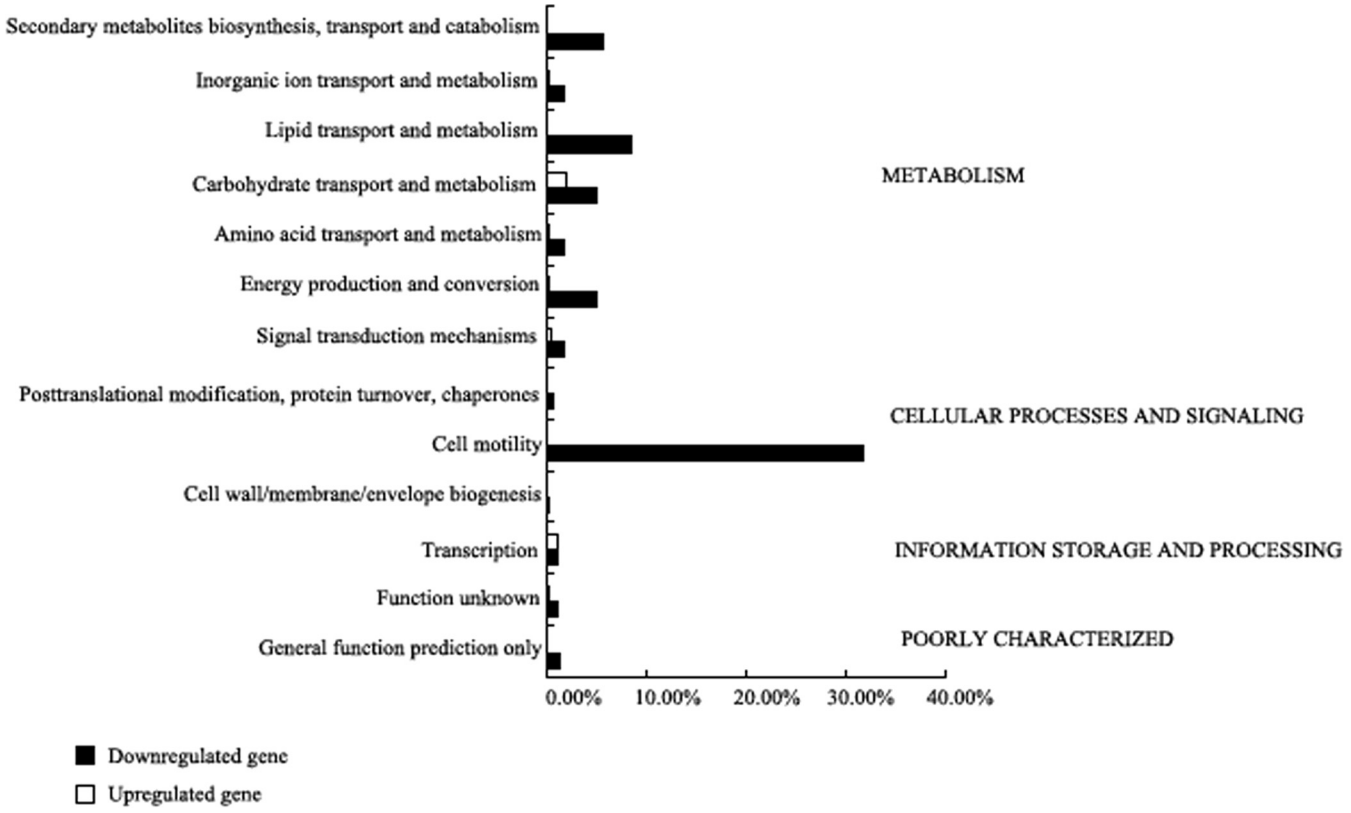

FIG 1 T6SS-regulated genes in CF74. Shown is COG analysis of T6SS-regulated genes in CF74. Major COG categories are indicated on the right, while subcategories are listed on the left. The *x* axis represents the percentage of genes in the corresponding class. Genes that are activated or repressed in CF74ΔT6SS are indicated by white or black bars, respectively.

T6SS in CF74ΔT6SS ($P < 0.01$) (Fig. 2A and B). Therefore, it seems that *hcp-2* is the key gene of the T6SS that affects the expression of flagellar genes at the level of transcription.

The T6SS is involved in the secretion of FliC. We examined the effect of the T6SS on FliC secretion by comparing the wild type, the mutants (CF74ΔT6SS, CF74Δ*clpV*, CF74Δ*vgr*, and CF74Δ*hcp-2*), and the complemented strains [CF74Δ*clpV*(pBAD24-*clpV*), CF74Δ*hcp-2*(pBAD24-*hcp-2*), and CF74Δ*vgr*(pBAD24-*vgr*)]. The strains were grown to an OD_{600} of 1.0 in LB medium, and the proteins from both culture supernatants and WCL were analyzed by Western blotting with a rabbit anti-flagellin antiserum that recognized the FliC protein. As shown in Fig. 3, in the culture supernatant and WCL, the bands of FliC in CF74ΔT6SS were obviously weaker than in the wild type. By densitometric quantification of relative band intensities normalized to GapA, we found that the FliC bands in the culture supernatants of CF74Δ*clpV*, CF74Δ*vgr*, and CF74Δ*hcp-2* were decreased significantly (ranging from 30% to 66%) relative to the wild type and restored partially upon complementation. In the WCL, the FliC bands of CF74Δ*vgr* and CF74Δ*hcp-2* were weaker than those of the wild type, but the FliC band of CF74Δ*clpV* was not clearly weaker. The absence of the cytosolic GapA protein in the culture supernatants indicates that the appearance of FliC in the culture supernatants was not a consequence of bacterial cell lysis. These results showed that the T6SS and its component proteins participated in the production and export of FliC.

TABLE 3 Levels of expression of flagellar genes affected by T6SS

Gene no.	Gene name	Function	Orientation[a]	Expression ratio	
				RNA-Seq[b]	RT-PCR[c]
CFCDC_3602	*fliC*	Bacterial flagellin C-terminal helical region	−	2.940	2.966
CFCDC_3603	*fliD*	Flagellar capping protein	+	2.471	2.745
CFCDC_3605	*fliT*	Flagellar biosynthesis protein	+	2.878	3.772
CFCDC_2668	*flgM*	Anti-sigma28 factor	−	2.127	1.709
CFCDC_2679	*flgK*	Flagellar hook-associated protein	+	2.730	2.042
CFCDC_2680	*flgL*	Flagellar hook-associated protein	+	1.911	4.004

[a] Transcription direction (+, Watson strand; −, Crick strand).
[b] Log_2 of expression ratios (ratio of RPKM in wild-type and T6SS GI deletion mutant samples) obtained from RNA-Seq.
[c] Log_2 of expression ratios (ratio of RPKM in wild-type and T6SS GI deletion mutant samples) obtained from the RT-PCR.

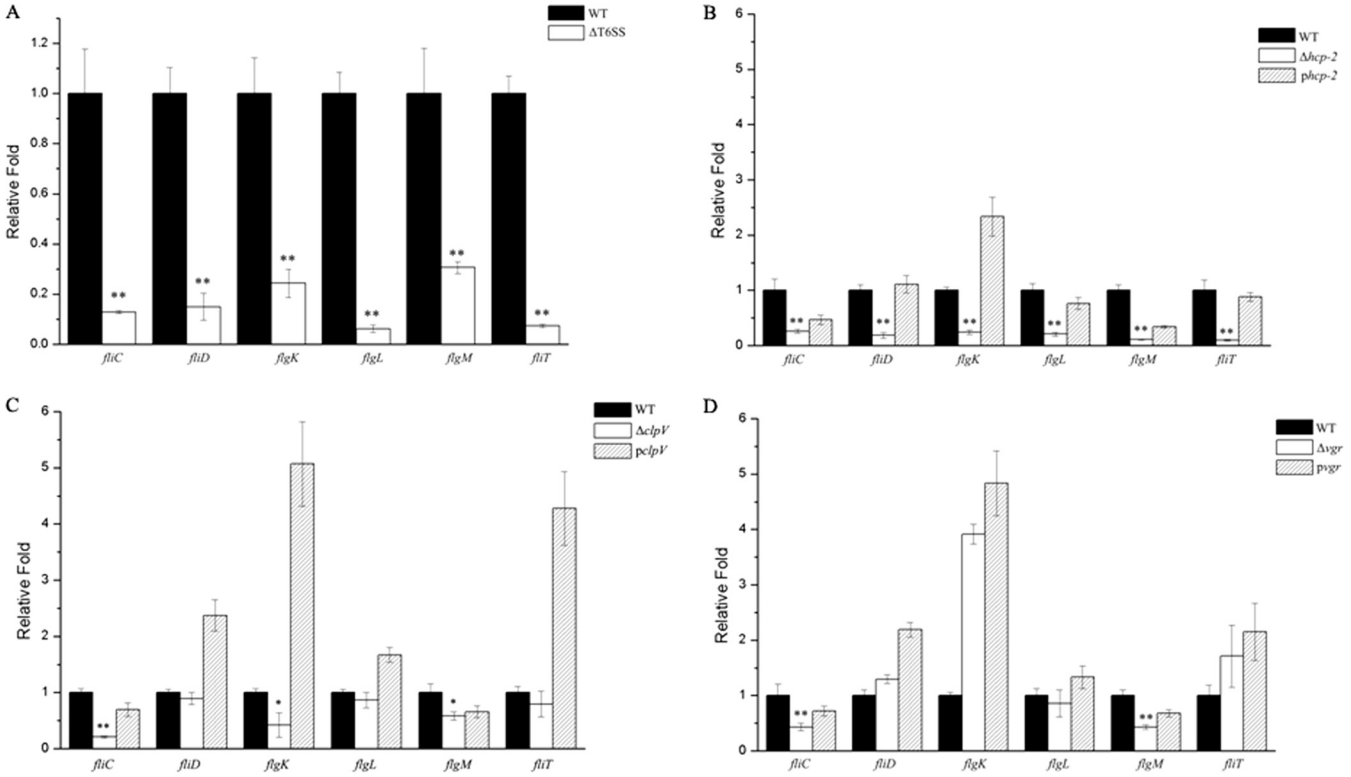

FIG 2 Relative expression levels of six flagellar genes (*fliC*, *flgM*, *flgK*, *fliD*, *flgL*, and *fliT*) at the level of transcription in different mutants in comparison to the wild type. (A) Effect of deletion of the T6SS GI (ΔT6SS) in comparison to the wild type (WT). (B to D) Effects of deletion of the individual T6SS genes *clpV*, *hcp*, and *vgr*. The mutants are marked as Δ*clpV* (CF74Δ*clpV*), Δ*hcp-2* (CF74Δ*hcp-2*), and Δ*vgr* (CF74Δ*vgr*) and the complemented mutants as p*clpV* [CF74Δ*clpV*(pBAD24-*clpV*)], p*hcp-2* [CF74Δ*hcp-2*(pBAD24-*hcp-2*)], and p*vgr* [CF74Δ*vgr*(pBAD24-*vgr*)]. The *y* axis represents the relative fold change among different strains, and the *x* axis represents different flagellar genes. The statistical significance between the wild type and mutants was determined by Mann-Whitney U test. *, $P < 0.05$; **, $P < 0.01$. The error bars indicate 1 standard deviation.

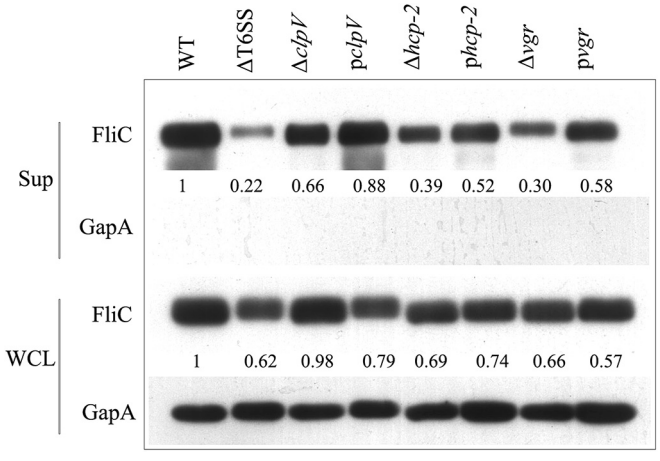

FIG 3 Effect of the T6SS on secretion of FliC. Shown is immunoblot analysis of proteins in the culture supernatant (Sup) and whole-cell lysates (WCL) prepared from mutants of *C. freundii* CF74 grown in LB medium. The WT, CF74ΔT6SS, CF74Δ*clpV*, CF74Δ*vgr*, and CF74Δ*hcp-2* strains were uninduced; CF74Δ*clpV*-(pBAD24-*clpV*), CF74Δ*hcp-2*(pBAD24-*hcp-2*), and CF74Δ*vgr*(pBAD24-*vgr*) were induced with 0.2% arabinose for 30 min. The position of a reactive band corresponding to FliC was detected with anti-FliC antibodies, and GapA was detected with anti-GapA antibodies. GapA was used as a loading control. The numbers represent densitometric quantifications of relative band intensities normalized to GapA and the wild type. p*clpV*, CF74Δ*clpV*(pBAD24-*clpV*); p*hcp-2*, CF74Δ*hcp-2*(pBAD24-*hcp-2*); p*vgr*, CF74Δ*vgr*(pBAD24-*vgr*).

The T6SS and its component proteins affect motility. To determine whether the T6SS affects motility, we used swimming motility assays to compare the wild-type strain, the mutants (CF74ΔT6SS, CF74Δ*clpV*, CF74Δ*vgr*, and CF74Δ*hcp-2*), and the complemented mutants [CF74Δ*clpV*(pBAD24-*clpV*), CF74Δ*hcp-2*(pBAD24-*hcp-2*), and CF74Δ*vgr*(pBAD24-*vgr*)]. The mutant strains were less motile than the wild type, and the motility was restored upon complementation (Fig. 4A). For CF74ΔT6SS, the diameter of the swimming motility halo was 12.99 mm, which was significantly decreased in comparison to a halo of 54.59 mm for the wild type ($P < 0.01$) (Fig. 4B). The halo of CF74Δ*hcp-2* was reduced more than those of the CF74Δ*vgr* and CF74Δ*clpV* mutants ($P < 0.05$). Although the halo of CF74Δ*vgr* was decreased compared with the wild type, it was not statistically significant (Fig. 4C to E). These data suggest that the T6SS, and in particular its effector Hcp, affected motility.

T6SS and its component proteins affect adhesion and cytotoxicity in HEp-2 cells. The capacities to adhere to host cells were compared among the wild type, the deletion mutants, and the complemented strains under the same conditions. The deletion mutants of the entire T6SS and its component genes were defective in adhesion to HEp-2 cells ($P < 0.01$), which was restored partially upon complementation (Fig. 5, left). These results indicated that the T6SS was involved in the adherence of CF74 to host cells.

CF74 showed high cytotoxicity to cultured HEp-2 cells (26).

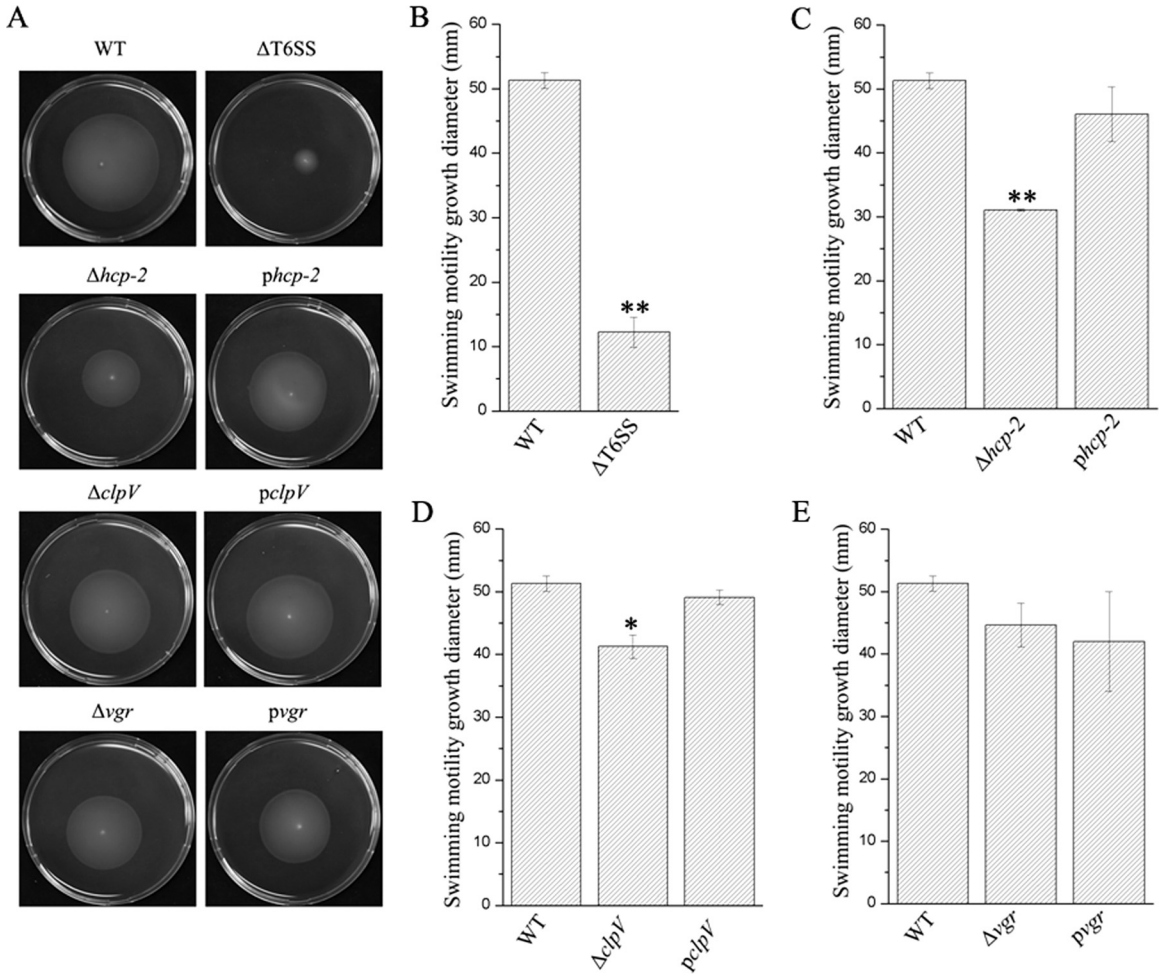

FIG 4 Effect of the T6SS on motility. (A) Representative images of swimming motility of *C. freundii* CF74, CF74ΔT6SS, CF74Δ*clpV*, CF74Δ*vgr*, and CF74Δ*hcp-2* and their complemented mutants [CF74Δ*clpV*(pBAD24-*clpV*), CF74Δ*hcp-2*(pBAD24-*hcp-2*), and CF74Δ*vgr*(pBAD24-*vgr*)]. Cells were inoculated with a toothpick from an overnight LB agar plate onto a swim plate (tryptone broth plus 0.3% agar) and photographed after 16 h of incubation at 30°C. The complemented mutants were induced with swim plates with 0.2% arabinose in the medium. (B to E) Quantification of the inhibition of the swimming motility halo by *C. freundii* CF74 mutants in swim plates. Statistical significance between the wild-type strain and mutants (CF74ΔT6SS, CF74Δ*clpV*, CF74Δ*vgr*, and CF74Δ*hcp-2*) was determined by a Mann-Whitney U test. *, $P < 0.05$; **, $P < 0.01$. p*clpV*, CF74Δ*clpV*(pBAD24-*clpV*); p*hcp-2*, CF74Δ*hcp-2*(pBAD24-*hcp-2*); p*vgr*, CF74Δ*vgr*(pBAD24-*vgr*). The error bars indicate 1 standard deviation.

We hypothesized that the T6SS may play a role in cytotoxicity. Using an LDH release assay, we observed that, unlike CF74Δ*vgr*, CF74ΔT6SS, CF74Δ*clpV*, and CF74Δ*hcp-2* induced lower LDH release by the HEp-2 cells than the wild-type, CF74 ($P < 0.05$) (Fig. 5, right), which was restored partially upon complementation, indicating that the T6SS contributes to the apoptotic death of HEp-2 cells *in vitro*.

DISCUSSION

C. freundii CF74 has a complete T6SS system located on a GI. We analyzed the effect of the T6SS GI on global gene expression at the level of transcription and showed that over 84 genes are affected by the loss of the T6SS in CF74. The expression of 82.14% of these genes was repressed in CF74ΔT6SS, indicating that in CF74, the T6SS or proteins secreted by the T6SS preferentially mediate positive regulation of gene expression. Further work is required to determine why so many genes are regulated by the T6SS or other genes on the GI. One or more of the T6SS genes or other genes on the GI must have affected the expression of such a large number of genes. There are several open reading frames (ORFs) of unknown function in the region deleted in CF74ΔT6SS. There is also an *rhsA* gene and 3 *rhs*-like elements. RhsA, a nuclease, has been shown to affect the expression of genes involved in transcription, RNA processing, nucleotide biosynthesis and metabolism, and amino acid biosynthesis in *E. coli* (37). *rhsA* is also embedded in the T6SS gene cluster in *Dickeya dadantii* and is likely to be exported through the T6SS (38). Therefore, it is possible that RhsA secreted through the T6SS affects gene expression.

Genes significantly downregulated in CF74ΔT6SS include those involved in glycerol and ethanolamine metabolism, while the significantly upregulated genes include those involved in maltose metabolism. It is interesting that the T6SS GI upregulates the ethanolamine utilization genes. Ethanolamine is abundant in the intestines of animals and can serve as both a carbon and a nitrogen source. Ethanolamine utilization has been associated with virulence (39, 40). Ethanolamine utilization genes have been shown to

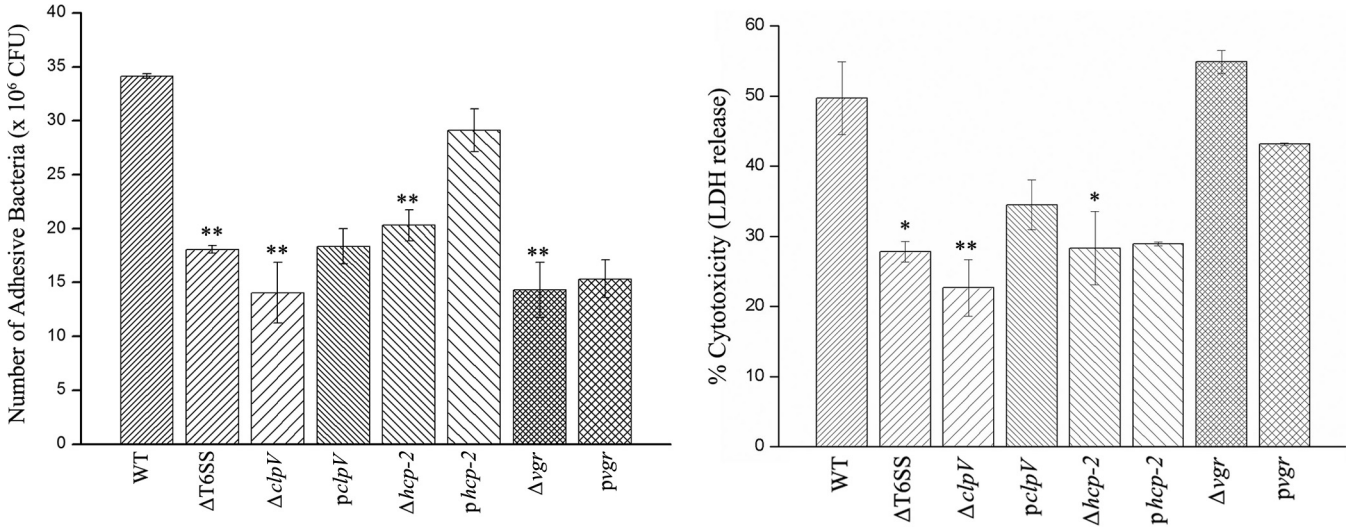

FIG 5 Effects of the T6SS on adhesion and cytotoxicity to HEp-2 cells. (Left) Effects of T6SS on CF74 adherence to HEp-2 cells (MOI, 100). The performances of mutant strains were compared statistically to that of the wild-type strain, CF74. **, $P < 0.01$. (Right) The LDH released from HEp-2 cells was measured after exposure to *C. freundii* CF74, mutants, and their complemented mutants at 10 h using the optical density reading (A_{490}/A_{630}) (vertical axis). Significant differences between the wild type and mutants are indicated with asterisks: **, $P < 0.01$; *, $P < 0.05$. p*clpV*, CF74Δ*clpV*(pBAD24-*clpV*); p*hcp-2*, CF74Δ*hcp-2*(pBAD24-*hcp-2*); p*vgr*, CF74Δ*vgr*(pBAD24-*vgr*). The error bars indicate 1 standard deviation.

be upregulated in the intestines of mice in experimental *Listeria monocytogenes* infection (41) and similarly in the intestines of chickens in experimental *S. enterica* serovar Typhimurium infection (42). Ethanolamine can also induce virulence gene expression in *E. coli* O157:H7 (43). Therefore, it is likely that the upregulation of the ethanolamine utilization genes by the T6SS in *C. freundii* CF74 gives it a competitive advantage in the intestinal or environmental niche, where there is a rich source of ethanolamine. Note that the effect observed with CF74ΔT6SS may have been exerted by non-T6SS genes or regulatory elements on the GI rather than directly by the T6SS, as discussed above, which requires further investigation.

The cell motility COG has the largest proportion of T6SS-regulated genes, with approximately 31.82% of its genes being downregulated significantly in the T6SS deletion mutant, the majority of which are flagellar genes, including those encoding effector proteins, chaperones, and regulators. Mainly *fliC*, *flgM*, *flgK*, *fliD*, *flgL*, *motA*, and *fliT* were regulated by the T6SS in CF74. No flagellar genes were upregulated in the CF74ΔT6SS mutant.

The deletion of *vgr*, *hcp-2*, and *clpV*, key genes of the T6SS, resulted in changes in the expression of flagellar genes at the level of transcription. The expression of flagellar genes was downregulated significantly in CF74Δ*hcp-2*, but not to the same extent as in CF74ΔT6SS. Other T6SS genes are also possibly important, such as *icmF*, which was not examined in this study. IcmF is an ATPase involved in T6SS protein secretion, and IcmF mutants prevent Hcp secretion (2, 8–10). It has been reported that the Δ*icmF* strain decreased the expression of *flhC*, *flhD*, *flgM*, and *fliA* flagellar genes at the level of transcription in avian-pathogenic *E. coli* (APEC) (15). Our results suggested that deletion of T6SS or its key genes was detrimental to the expression of the flagellar regulon at the level of transcription, as was found in *E. coli* (15).

FliC, a component of the flagellum filament, is transported from the cytoplasm by specific transport systems, such as the T3SS and T4SS (19, 20), and is polymerized with the help of the cap protein FliD, producing long helical flagellar filaments (44). In the present study, we found that FliC was reduced, not only at the level of transcription, but also at the level of translation in the T6SS mutant, indicating that the reduction of FliC was due to not only altered expression of *fliC*, but also the inability to transport FliC, possibly via the type VI secretion system. Thus, it is likely that the T6SS is involved in secretion of FliC in *C. freundii*. Further studies are needed to clarify how the T6SS contributes to the production and secretion of FliC.

Swimming motility is dependent on flagella. In the present study, motility tests showed that CF74ΔT6SS was less motile, which corroborates a previous report that deletion of the T6SS component, *icmF*, in *V. cholerae* and APEC produced reduced motility (15, 45). Among the 3 T6SS genes tested—*vgr*, *hcp-2*, and *clpV*—*hcp-2* seems to have the greatest effect, while *clpV* has a less significant effect on motility. Interestingly, both ClpV and IcmF are ATPases, with the latter reported to affect the production and secretion of the FliC protein in both *E. coli* and *V. cholerae* (11, 45). It is likely that IcmF has a similar role in C. *freundii*, which may complement the activity of *clpV* in the *clpV* deletion mutant.

Reduced motility in T6SS deletion mutants is attributed to reduced production and secretion of the FliC protein. We found that the loss of Hcp-*2* resulted in changes in FliC at the level of transcription and translation and affected motility. Thus, Hcp-2 is likely to be one of the important T6SS proteins that play a role in FliC protein production and export, and the mechanisms remain to be determined.

Our previous study showed that CF74 has strong adhesion and high cytotoxicity to HEp-2 cells (26). In this study, we found that the T6SS played a role in the adhesion and cytotoxicity of CF74 to host cells. All 3 T6SS genes tested, *vgr*, *clpV*, and *hcp2*, adversely affected the adhesion of *C. freundii* to HEp-2 cells, and two of the three genes tested, *clpV* and *hcp-2*, contributed to the cytotoxicity of *C. freundii* to HEp-2 cells, while *vgr* seems to have no effect on cytotoxicity. Our results are consistent with findings in other bac-

terial pathogens of the role of T6SS in adherence and cytotoxicity to host cells. It has been reported that the T6SS in APEC is involved in adherence to host cells (46), the *Bordetella bronchiseptica* T6SS mediates cytotoxicity in murine macrophages (47), and the *Campylobacter jejuni* T6SS confers cytotoxicity to red blood cells (48). Since FliC contributes to cytotoxicity (49), it is possible that the T6SS of CF74 contributes to the apoptotic death of host cells *in vitro* indirectly by affecting the production and secretion of FliC.

In conclusion, we show that deletion of the T6SS of *C. freundii* CF74 and its key proteins (ClpV, Hcp-2, and Vgr) affected the production and secretion of the flagellin protein FliC and affected motility. Deletion of the T6SS also affected the adhesion and cytotoxicity of CF74 to HEp-2 cells. Further, the T6SS GI has a much greater effect on the global expression of genes, with more than 84 genes differentially affected, which remains to be further investigated.

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