**FliW and CsrA Govern Flagellin (FliC) Synthesis and Play Pleiotropic Roles in Virulence and Physiology of Clostridioides difficile R20291**

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**Keywords:** Clostridioides difficile, FliW, FliC, CsrA, R20291, virulence

**INTRODUCTION**

**Clostridioides difficile** (Lawson et al., 2016; Oren and Garrity, 2018) is a Gram-positive, spore-forming, toxin-producing, anaerobic bacterium that is a leading cause of nosocomial antibiotic-associated diarrhea in the developed countries (Sebaihia et al., 2006). *Clostridioides difficile* infection (CDI) can result in a spectrum of symptoms, ranging from mild diarrhea to pseudomembranous colitis and potential death (Lessa et al., 2012). *Clostridioides difficile* has many virulence factors, among which toxin A (TcdA) and toxin B (TcdB) are the major ones...
(Lyras et al., 2009; Kuehne et al., 2010). These toxins can disrupt the actin cytoskeleton of intestinal cells through glucosylation of the Rho family of GTPases, and induce mucosal inflammation and symptoms associated with CDI (Peniche et al., 2013).

CsrA, the carbon storage regulator A, has been reported to control various physiological processes, such as flagella synthesis, virulence, central carbon metabolism, quorum sensing, motility, and biofilm formation in pathogens including Pseudomonas aeruginosa, Pseudomonas syringae, Borrelia burgdorferi, Salmonella typhimurium, and Proteus mirabilis (Sabnis et al., 1995; Pessi et al., 2001; Lawhon et al., 2003; Lucchetti-Miganeh et al., 2008; Timmermans and Van Melderen, 2010; Karn et al., 2011; Morris et al., 2013; Ferreiro et al., 2018). Recently, the role of CsrA on carbon metabolism and virulence-associated processes in C. difficile 630Δerm was analyzed by overexpressing the csrA gene (Gu et al., 2018). Authors showed that the csrA overexpression resulted in flagella defect, poor motility, and induced carbon metabolism change. Oppositely, toxin production and cell adherence increased in the csrA overexpression strain. CsrA is a widely distributed RNA binding protein that post-transcriptionally modulates gene expression through regulating mRNA stability and/or translation initiation of target mRNA (Romeo et al., 1993; Liu et al., 1995; Timmermans and Van Melderen, 2010). It typically binds to multiple specific sites that are located nearby or overlapping the cognate Shine−Dalgarno (SD) sequence in the target transcripts (Sorger-Domenig et al., 2007; Yakhnin et al., 2007).

The roles of CsrA in Bacillus subtilis have been well-studied (Yakhnin et al., 2007; Mukherjee et al., 2011; Oshiro et al., 2019). Flagellin Hag (Flic homolog), a main structure flagellar component, has been reported to be regulated by CsrA in B. subtilis. Yakhnin et al. (2007) first reported that CsrA in B. subtilis can regulate translation initiation of Hag by preventing ribosome binding to the hag transcript. Mukherjee et al. (2011) elucidated that the interaction between CsrA and FliW could govern flagellin homeostasis and checkpoint on flagellar morphogenesis in B. subtilis. FliW, the first protein antagonist of CsrA activity, was also identified and characterized in B. subtilis. They elegantly demonstrated a novel regulation system “a partner-switching mechanism” (Hag-FliW-CsrA) on flagellin synthesis in B. subtilis. Briefly, following the flagellar assembly checkpoint of hook completion, FliW was released from a FliW-Hag complex. Afterward, FliW binds to CsrA which will relieve CsrA-mediated hag translation repression for flagellin synthesis concurrent with filament assembly. Thus, flagellin homeostasis restricts its own expression on the translational level. Results also suggested that CsrA has an ancestral role in flagella assembly and has evolved to coregulate multiple cellular processes with motility. Oshiro et al. (2019) further quantitated the interactions in the Hag-FliW-CsrA system. They found that Hag-FliW-CsrA* functions at nearly 1:1 stoichiometry in B. subtilis. The Hag-FliW-CsrA* system is hypersensitive to the cytoplasmic Hag concentration and is robust to perturbation.

Clostridioides difficile flagellin gene fliC is associated with toxin gene expression, bacterial colonization, and virulence, and is responsible for pleiotropic gene regulation during in vivo infection (Tastreye et al., 2001; Aubry et al., 2012; Baban et al., 2013; Barketi-Klai et al., 2014; Stevenson et al., 2015). The delicate regulations among fliC gene expression, toxin production, bacterial motility, colonization, and pathogenicity in C. difficile are indicated. Though the important roles of CsrA in flagellin synthesis and flagellin homeostasis have been studied in other bacteria (Yakhnin et al., 2007; Mukherjee et al., 2011; Oshiro et al., 2019), the regulation of FliW, CsrA, and FliC and the function of fliW in C. difficile remain unclear.

In this communication, we aimed to study the involvement of FliW and CsrA in fliC expression and C. difficile virulence and physiology by constructing and analyzing fliW and fliW-csrA deletion mutants of C. difficile R20291. We evaluated these mutants in the expression of fliC, motility, adhesion, biofilm formation, toxin production, sporulation, germination, and pathogenicity in a mouse model of CDI.

**MATERIALS AND METHODS**

**Bacteria, Plasmids, and Culture Conditions**

Table 1 lists the strains and plasmids used in this study. *Clostridioides difficile* strains were cultured in BHIS media (brain heart infusion broth supplemented with 0.5% yeast extract and 0.1% L-cysteine, and 1.5% agar for agar plates) at 37°C in an anaerobic chamber (90% N₂, 5% H₂, and 5% CO₂). For spores preparation, *C. difficile* strains were cultured in Clospore media and purified as described earlier (Perez et al., 2011). *Escherichia coli* DH5α and *E. coli* HB101/pRK24 were grown aerobically at 37°C in LB media (1% tryptone, 0.5% yeast extract, and 1% NaCl). *Escherichia coli* DH5α was used as a cloning host, and *E. coli* HB101/pRK24 was used as a conjugation donor host. Antibiotics were added when needed for *E. coli*, 15 µg/ml chloramphenicol; for *C. difficile*, 15 µg/ml thiamphenicol, 250 µg/ml D-cycloserine, 50 µg/ml kanamycin, 8 µg/ml cefoxitin, and 500 ng/ml anhydrotetracycline.

**DNA Manipulations and Chemicals**

DNA manipulations were carried out according to standard techniques (Chong, 2001). Plasmids were conjugated into *C. difficile* as described earlier (Heap et al., 2010). The DNA markers, protein markers, PCR product purification kit, DNA gel extraction kit, restriction enzymes, cDNA synthesis kit, and SYBR Green RT-qPCR kit were purchased from ThermoFisher Scientific (Waltham, United States). PCRs were performed with the high-fidelity DNA polymerase NEB Q5 Master Mix, and PCR products were assembled into *Escherichia coli* HB101/pRK24. Primers (Supplementary Table 1) were purchased from IDT (Corvalle, United States). All chemicals were purchased from Sigma-Aldrich (St. Louis, United States) unless those stated otherwise.
### TABLE 1 | Bacteria and plasmids utilized in this study.

| Strains or plasmids | Genotype or phenotype | Reference |
|---------------------|-----------------------|-----------|
| E. coli DH5α        | Cloning host          | NEB       |
| E. coli HB101/pRK24  | Conjugation donor     | Williams et al., 1990 |
| C. difficile R20291  | Clinical isolate; ribotype 027 | Stabler et al., 2009 |
| R20291ΔW            | R20291 deleted fliW gene | This work |
| R20291ΔWA           | R20291 deleted fliW-csrA genes | This work |
| R20291-E            | R20291 containing blank plasmid pMTL84153 | This work |
| R20291ΔW-E          | R20291ΔW containing blank plasmid pMTL84153 | This work |
| R20291ΔWA-E         | R20291ΔWA containing blank plasmid pMTL84153 | This work |
| R20291ΔWA-W         | R20291ΔW complemented with pMTL84153-fliWA | This work |
| R20291ΔWA-WA        | R20291ΔWA complemented with pMTL84153-fliWA-csrA | This work |
| R20291ΔWA-W         | R20291ΔWA complemented with pMTL84153-fliWA | This work |
| R20291ΔWA-W         | R20291ΔWA complemented with pMTL84153-fliWA | This work |
| R20291ΔWA-A         | R20291ΔWA complemented with pMTL84153-csrA | This work |
| R20291-W            | R20291 containing pMTL84153-fliWA | This work |
| R20291-A            | R20291 containing pMTL84153-csrA | This work |
| R20291-WA           | R20291 containing pMTL84153-fliWA-csrA | This work |

| Plasmids            |                  |           |
|---------------------|------------------|-----------|
| pDL1                | AsCpfI based gene deletion plasmid | This work |
| pUC57-PsRNA         | sRNA promoter template | This work |
| pDL1-fliW           | fliW gene deletion plasmid | This work |
| pDL1-csrA           | csrA gene deletion plasmid | This work |
| pDL1-fliW-csrA      | fliW-csrA gene deletion plasmid | This work |
| pMTL84153           | Complementation plasmid | Heap et al., 2009 |
| pMTL84153-fliW-csrA | pMTL84153 consisting fliW-csrA genes | This work |
| pMTL84153-fliWA     | pMTL84153 containing fliWA gene | This work |
| pMTL84153-csrA      | pMTL84153 containing csrA gene | This work |

### Gene Deletion, Complementation, and Overexpression in R20291

Gene edit plasmid pDL1 containing Cas12a (AsCpfI) under the control of tetracycline-inducing promoter was constructed and used for *C. difficile* gene deletion according to a previous report (Hong et al., 2018). The target sgRNA was designed with an available website tool, and the off-target prediction was analyzed on the Cas-OFFinder website. The sgRNA, up- and down-homologous arms, were assembled into pDL1. Two target sgRNAs for one gene deletion were selected and used for gene deletion plasmid construction in *C. difficile*, respectively.

Briefly, the gene deletion plasmid was constructed in the cloning host *E. coli* DH5α and was transformed into the donor host *E. coli* HB101/pRK24, and subsequently was conjugated into R20291. Potential successful transconjugants were selected with selective antibiotic BHIS-TK plates (15μg/ml thiamphenicol, 50 μg/ml kanamycin, and 8 μg/ml cefoxitin). The transconjugants were cultured in BHIS-Tm broth (15μg/ml thiamphenicol) to log phase, then the subsequent cultures were diluted with PBS serially and plated on the inducing plates (BHIS-Tm: 15 μg/ml thiamphenicol and 500 ng/ml anhydrotetracycline). The plates were incubated at 37°C in the anaerobic chamber for 24–48h, then 20–40 colonies were used as templates for colony PCR test with check primers for correct gene deletion colony isolation. The correct gene deletion colony was sub-cultured into BHIS broth without antibiotics and was passaged several times to cure the deletion plasmid, and then the cultures were plated on BHIS plates and subsequent colonies were replica plated on BHIS-Tm plates to isolate pure gene deletion mutants. The genome of R20291ΔfliW (referred hereafter as R20291ΔW) and R20291ΔfliW-csrA (referred hereafter as R20291ΔWA) were isolated and used as templates for the PCR test with check primers, and the PCR products were sequenced to confirm the correct gene deletion.

The *fliW* (396bp; primers 3-F/R), *csrA* (213bp; primers 4-F/R), and *fliW-csrA* (599bp; primers 5-F/R) genes were amplified and assembled into *SacI-BamHI* digested pMTL84153 plasmid, yielding the complementation plasmid pMTL84153-*fliW*, pMTL84153-*csrA*, and pMTL84153-*fliW-csrA*, and were subsequently conjugated into R20291ΔWA, R20291ΔW, and R20291 yielding complementation strain R20291ΔWA/pMTL84153-*fliW* (referred as R20291ΔWA-*W*), R20291ΔWA/pMTL84153-*csrA* (R20291ΔWA-A), R20291ΔWA/pMTL84153-*fliW-csrA* (R20291ΔWA-WA), and R20291ΔWA/pMTL84153-*fliW* (R20291ΔWA-W), and overexpression strain R20291ΔWA/pMTL84153-*fliW* (R20291-W), R20291/pMTL84153-*csrA* (R20291-A), and R20291/pMTL84153-*fliW-csrA* (R20291-WA).

### Growth Profile, Motility, and Biofilm Assay

*Clostridioides difficile* strains were incubated to an optical density of OD600 of 0.8 in BHIS media and were diluted to an OD600 of 0.2. Then, 1% of the culture was inoculated into fresh BHIS, followed by measuring OD600 for 32h.

To examine the effect of *fliW* and *fliW-csrA* deletion on *C. difficile* motility, R20291, R20291ΔWA, and R20291ΔW were cultured to an OD600 of 0.8. For swimming analysis, 2 μl of *C. difficile* culture was penetrated into soft BHIS agar (0.175%) plates, meanwhile, 2 μl of culture was dropped onto 0.3% BHIS agar plates for swarming analysis. The swimming assay plates were incubated for 24h, and the swarming plates were incubated for 48h, respectively.

For biofilm formation analysis, wild-type and mutant strains were cultured to an OD600 of 0.8, and 1% of *C. difficile* cultures were inoculated into reinforced clostridial medium (RCM) with eight-well repeats in a 96-well plate and incubated in the anaerobic chamber at 37°C for 48h. Biofilm formation was analyzed by crystal violet dye. Briefly, *C. difficile* cultures were...

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[1](http://big.hanyang.ac.kr/cindel/)
[2](http://www.rgenome.net/cas-offinder/)
removed by pipette carefully. Then, 100 μl of 2.5% glutaraldehyde was added into the well to fix the bottom biofilm, and the plate was kept at room temperature for 30 min. Next, the wells were washed with PBS three times and dried with 0.25% (w/v) crystal violet for 10 min. The crystal violet solution was removed, and the wells were washed five times with PBS, followed by the addition of acetone into wells to dissolve the crystal violet of the cells. The dissolved solution was further diluted with ethanol 2–4 times, and biomass was determined at OD570.

**Adherence of C. difficile Vegetative Cells to HCT-8 Cells**

Clostridioides difficile adhesion ability was evaluated with HCT-8 cells (ATCC CCL-244; Janvilisri et al., 2010). Briefly, HCT-8 cells were grown to 95% confluence (2 × 10⁴/well) in a 24-well plate and then moved into the anaerobic chamber, followed by infecting with 6 × 10⁶ of log phase of C. difficile vegetative cells at a multiplicity of infection (MOI) of 30:1. The plate was cultured at 37°C for 30 min. After incubation, the infected cells were washed with 300 μl of PBS three times, and then suspended in RPMI media with trypsin and plated on BHIS agar plates to enumerate the adhered C. difficile cells. The adhesion ability of C. difficile to HCT-8 cells was calculated as follows: CFU of adhered bacteria/total cell numbers.

To visualize the adherence of C. difficile to HCT-8 cells, C. difficile vegetative cells were labeled with the chemical 5(6)-CFDA (-6)-Carboxyfluorescein diacetate (Fuller et al., 2000). Briefly, C. difficile strains were cultured to an OD600 of 0.8, then washed with PBS 3 times and resuspended in fresh BHIS supplemented with 50-mM 5(6)-CFDA, followed by incubation at 37°C for 30 min in the anaerobic chamber. After post-incubation, the labeled C. difficile cells were collected and washed with PBS three times, and then resuspended in RPMI medium. Afterward, the labeled C. difficile cells were used for the infection experiment as described above. After 30-min post-infection, the fluorescence of each well was scanned by the multi-mode reader (excitation, 485 nm; emission, 528 nm), the relative fluorescence unit (RFU) was recorded as F0. Following, the plates were washed with PBS three times to remove unbound C. difficile cells, then the plates were scanned, and the RFU was recorded as F1. The adhesion ratio was calculated as follows: F1/F0. After scanning, the infected cell plates were further detected by the fluorescence microscope.

**fliC Expression Assay**

For fliC transcription analysis, 2 ml of 24-h post-inoculated C. difficile cultures were centrifuged at 4°C, 12,000 × g for 5 min, respectively. Then, the total RNA of different strains was extracted with TRizol reagent. The transcription of fliC was measured by RT-qPCR with primers Q-flic-F/R. All RT-qPCRs were repeated in triplicate, independently. Data were analyzed by the comparative CT (2⁻ΔΔCt) method with 16s rRNA as a control.

To analyze the FliC protein level, C. difficile cell lysates from overnight cultures were used for Western blot analysis. Briefly, overnight C. difficile cultures were collected and washed three times with PBS and then resuspended in 5 ml of distilled water. The suspensions were lysed by TissueLyser LT (Qiagen), followed centrifuged at 4°C, 25,000 × g for 1 h. The final pellets were resuspended in 30 μl of PBS, and the total protein concentration was measured by using a BCA protein assay (Thermo Scientific, Suwanee, GA, United States). Protein extracts were subjected to 10% SDS-PAGE. Sigma A protein (SigA) was used as a loading control protein in SDS-PAGE (Mukherjee et al., 2013). FliC and SigA proteins on the gel were detected with anti-Flic and anti-SigA primary antibody (1:1,000, a generous gift from Dr. Daniel Kearns at Indiana University) and horseradish peroxidase-conjugated secondary antibody goat anti-mouse (Cat: ab97023, IgG, 1:3,000, Abcam, Cambridge, MA, United States) by Western blot, respectively. Anti-Flic antibody used in the Western blot analysis is an anti-FliCD serum, generated in the laboratory. FliCD is a fusion protein containing C. difficile Flic and FliD (Wang et al., 2018). The relative intensity of blot bands was analyzed by ImageJ software, and FliC relative intensity was normalized to SigA control.

**Toxin Expression Assay**

To evaluate toxin expression in C. difficile strains, one single colony from each strain was inoculated into 25 ml of BHIS and incubated in an anaerobic chamber at 37°C, and 10 ml of C. difficile cultures from different strains were collected at 24- and 48-h post-inculation. The cultures were adjusted to the same OD600 value with fresh BHIS. Then, the collected C. difficile cultures were centrifuged at 4°C, 8,000 × g for 15 min, filtered with 0.22 μm filters, and used for ELISA. Anti-TcdA (PCG4.1, Novus Biologicals, United States) and anti-TcdB (AI, Gene Tex, United States) were used as coating antibodies for ELISA, and HRP-Chicken anti-TcdA and HRP-Chicken anti-TcdB (Gallus Immunotech, United States) were used as detection antibodies.

For toxin transcription analysis, 2 ml of 24- and 48-h post-inoculated C. difficile cultures were centrifuged at 4°C, 12,000 × g for 5 min, respectively. Next, the total RNA of different strains was extracted with TRizol reagent. The transcription of tcdA and tcdB was measured by RT-qPCR with primers Q-tcDA-F/R and Q-tcDB-F/R, respectively. All RT-qPCRs were repeated in triplicate, independently. Data were analyzed by using the comparative CT (2⁻ΔΔCt) method with 16s rRNA as a control.

**Germination and Sporulation Assay**

Clostridioides difficile germination and sporulation analysis were conducted as reported earlier (Zhu et al., 2019). Briefly, for C. difficile sporulation analysis, C. difficile strains were cultured in Clospora media for 4 days. Afterward, the CFU of cultures from 48 and 96 h were counted on BHIS plates with 0.1% TA to detect sporulation ratio, respectively. The sporulation ratio was calculated as CFU (65°C heated, 20 min)/CFU (no heated). For C. difficile germination analysis, C. difficile spores were collected from 2-week Clospora media-cultured bacteria and purified with sucrose gradient layer (50, 45, 35, 25, and 10%). The heated purified spores were diluted to an OD600 of 1.0 in the germination buffer [10 mM Tris (pH 7.5), 150 mM NaCl,
100 mM glycine, and 10 mM taurocholic acid (TA) to detect the germination ratio. The value of OD_{600} was monitored immediately (0 min, t₀), and was detected once every 2 min (tₙ) for 20 min at 37°C. The germination ratio was calculated as OD_{600} (tₓ)/OD_{600} (T₀). Spores in germination buffer without TA were used as the negative control.

**R20291, R20291ΔWA, and R20291ΔW Virulence in the Mouse Model of *C. difficile***

C57BL/6 female mice (6 weeks old) were ordered from Charles River Laboratories, Cambridge, MA. All studies were approved by the Institutional Animal Care and Use Committee of University of South Florida. The experimental design and antibiotic administration were conducted as described earlier (Sun et al., 2011). Briefly, 30 mice were divided into three groups in six cages. Group 1 mice were challenged with R20291 spores, group 2 mice with R20291ΔWA spores, and group 3 mice with R20291ΔW spores, respectively. Mice were given an orally administered antibiotic cocktail (kanamycin 0.4 mg/ml, gentamicin 0.035 mg/ml, colistin 0.042 mg/ml, metronidazole 0.215 mg/ml, and vancomycin 0.045 mg/ml) in drinking water for 4 days. After 4 days of antibiotic treatment, all mice were given autoclaved water for 2 days, followed by one dose of clindamycin (10 mg/kg, intraperitoneal route) 24 h before spores challenge (Day 0). After that mice were orally gavaged with 10⁶ spores and monitored daily for a week for changes in weight, diarrhea, and mortality. If body weight loss was equal to or greater than 20%, the mouse was euthanized and counted as a dead one. Mortality also included mice that were succumbed to disease. Diarrhea was defined as soft or watery feces. All survived mice were humanely euthanized on day 7 of post-*C. difficile* challenge.

**Enumeration of *C. difficile* Spores and Determination of Toxin Level in Feces**

Fecal pellets from post-infection day 0 to day 7 were collected from each mouse and stored at −80°C. To enumerate *C. difficile* spores, feces were diluted with PBS at a final concentration of 0.1 g/ml, followed by adding 900 µl of absolute ethanol into 100 µl of the fecal solution, and kept at room temperature for 1 h to inactivate vegetative cells. Afterward, 200 µl of vegetative cells inactivated fecal solution from the same group and the same day was mixed. Then, fecal samples were serially diluted and plated on BHIS-CCT plates (250 µg/ml D-cycloserine, 8 µg/ml cefoxitin, and 0.1% TA). After 48-h incubation, colonies were counted and expressed as CFU/g feces. To evaluate toxin titer in feces, 0.1 g/ml of the fecal solution was diluted two times with PBS, followed by examining TcdA and TcdB ELISA.

**Statistical Analysis**

The reported experiments were conducted in independent biological triplicates, and each sample was additionally taken in technical triplicates. Animal survivals were analyzed by Kaplan–Meier survival analysis and compared by the log-rank test. One-way ANOVA with post hoc Tukey test was used for more than two groups’ comparison. Results were expressed as mean ± SEM. Differences were considered statistically significant if *p* < 0.05 (*).  

### RESULTS

**Highly Conserved fliW and csrA Genes in *C. difficile***

DNA and protein sequences of *fliW* and *csrA* from 10 *C. difficile* strains belonging to different ribotypes (RTs), including RT106, RT027, RT001, RT078, RT009, RT012, RT046, and RT017 were selected and aligned to those of R20291 (Table 2). We found that *fliW* and *csrA* genes are broadly found in *C. difficile* genomes, and both DNA and protein sequences of *fliW* and *csrA* are conserved across different *C. difficile* strains. These results motivated us to investigate the functions of *fliW* and *csrA* in *C. difficile*.

**Construction of fliW and fliW-csrA Deletion Mutants and Complementation Strains**

The *C. difficile* R20291 flagellar gene operon was analyzed through the IMG/M website,¹ and the late-stage flagellar genes (F1) are drawn as Figure 1A (Stevenson et al., 2015). Among them, *fliW* and *csrA* genes have a 10 bp overlap and were demonstrated as cotranscription by RT-PCR (Supplementary Figure 1).

To analyze the role of *fliW* and *csrA* in R20291 (NC_013316.1), CRISPR-AsCpf1-based plasmid pDL1 (pMTL82151-Ptet-Ascpf1)

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¹https://img.jgi.doe.gov/
was constructed for gene deletion in C. difficile (Zhu et al., 2021). pDL1-fliW and pDL1-csrA gene deletion plasmids were constructed, and the fliW gene (288 bp deletion; R20291 ΔW) was deleted successfully. However, after several trials, we could not get the csrA gene deletion mutant possibly due to its small size (213 bp) or particularly unknown roles for R20291. We also tried to use Clostron and pyrE gene edit system to delete csrA gene, but failed to get the correct mutant. Therefore, we constructed fliW-csrA codeletion plasmid pDL1-fliW-csrA. Part of fliW-csrA (445 bp deletion) gene was codeleted, and the plasmid curing mutant R20291 ΔW A was obtained (Figure 1B,C). To study the role of csrA in R20291, the single gene complementation strain R20291ΔWA-W and R20291ΔWA-A were constructed. R20291, R20291-pMTL84153 (R20291-E), R20291ΔW-pMTL84153 (R20291ΔW-E), and R20291ΔWA-pMTL84153 (R20291ΔWA-E) were used as control strains when needed.

The effects of fliW and fliW-csrA deletion on R20291 growth were evaluated. Figure 1D shows that there was no significant difference in bacterial growth between parent strain and mutants in BHIS media.

**Effects of fliW and fliW-csrA Deletions on C. difficile Motility and Biofilm Formation**

To characterize the effects of fliW and fliW-csrA deletions on C. difficile motility, swimming, and swarming motilities of R20291, R20291ΔWA, and R20291ΔW were first analyzed at 24 and 48-h post-inoculation (Figure 2A; Supplementary Figure 2), respectively. The diameter of the swimming halo of R20291ΔWA increased by 27.2% (p < 0.05), while that of R20291ΔW decreased by 58.4% (p < 0.05) compared to that of R20291. Next, we examined the motility of the complementation strains (Figure 2B; Supplementary Figure 2), and similar results were obtained among R20291-E, R20291ΔWA-E (with the swimming halo increased by 74.8%, p < 0.05), and R20291ΔW-E (with the swimming halo decreased by 59.2%, p < 0.05; Figure 2B). No significant difference was detected between complementation strain R20291ΔWA-W, R20291ΔWA-A, and the parent strain R20291-E except R20291ΔWA-A which decreased by 52.0% (p < 0.05) in swimming halo (Figure 2B). The swarming (48 h) and swimming (24 h) motilities analyzed on agar plates are shown in Supplementary Figure 2.

The effects of fliW and fliW-csrA deletions on C. difficile biofilm formation were also analyzed. In comparison with R20291, the biofilm formation of R20291ΔW increased by 49.5% (p < 0.01), and no significant difference in biofilm formation was detected in R20291ΔWA (Figure 2C). The biofilm formation of R20291ΔWA-E increased 112.3% (p < 0.001) and R20291ΔWA-A increased by 79.9% (p < 0.001) compared to R20291-E (Figure 2D). Meanwhile, the biofilm formation...
of R20291ΔWA-WA and R20291ΔWA-W decreased by 42.8% (p < 0.01) and 25.2% (p < 0.05), respectively.

Together, these data indicate that loss of FliW impairs C. difficile motility, and increases biofilm production. The decrease of motility and increase in biofilm production were also detected in R20291ΔWA-A, which was largely restored by coexpressing fliW with csrA in R20291ΔWA (Figures 2B,D), indicating that FliW could antagonize CsrA to regulate bacterial motility and biofilm production.

**Effects of fliW and fliW-csrA Deletions on Bacterial Adherence in vitro**

The ability of C. difficile vegetative cells to adhere to HCT-8 cells in vitro was analyzed. Figure 3A shows that the mean adhesion number of R20291 was 2.40 ± 0.70 bacteria/cell, while that of R20291ΔW was 7.17 ± 0.61, which was 3.0-fold (p < 0.0001) of R20291. No significant difference was detected between R20291ΔWA and R20291. In the complementation strains, we detected a similar result which showed that the mean adhesion number of R20291ΔWA-E (6.17 ± 0.64) was 3.20-fold (p < 0.0001) of R20291-E (1.93 ± 0.25; Figure 3B). The adhesion ability of complementation strains nearly recovered to that of wild-type strain except for R20291ΔWA-A (7.13 ± 0.66, p < 0.0001) which was 3.69-fold of R20291-E in the mean adhesion number (Figure 3B).

To visualize the adhesion of C. difficile to HCT-8 cells, the C. difficile vegetative cells were labeled with the chemical 5(6)-CFDA. Figures 3C,D shows that the fluorescence intensity of R20291ΔW was 3.50-fold (p < 0.0001) of that in R20291, and the fluorescence intensity of R20291ΔWA-E was 2.36-fold (p < 0.001), and R20291ΔWA-A was 4.08-fold (p < 0.0001) of that in R20291-E, respectively, which is consistent with the results shown in Figures 3A,B. Meanwhile, the adherence of C. difficile to HCT-8 cells was also visualized by fluorescence microscopy (Supplementary Figure 3).

Our data showed that FliW negatively affects bacterial adherence. CsrA complementation in R20291ΔWA increased adherence, while the phenotype change can be recovered partially when fliW was coexpressed with csrA in R20291ΔWA, suggesting that FliW could antagonize CsrA to regulate bacterial adherence. The results from bacterial adherence analysis were consistent with biofilm production analysis indicating the close relation between biofilm production and adherence in C. difficile.
Effects of Deletion and Overexpression of *fliW* and *fliW-csrA* on *fliC* Expression

In *B. subtilis*, FliW interacts with CsrA to regulate *hag* (a homolog of *fliC*) translation. We reasoned that FliW and CsrA would also regulate *fliC* expression in *C. difficile*. As shown in Figure 4A, the transcription of *fliC* in R20291ΔWA increased 1.12-fold (*p* < 0.05), while the *fliW* deletion impaired the *fliC* transcription slightly while no significant difference. Figure 4B shows the production of FliC in R20291ΔW dramatically decreased (10.4-fold reduction, *p* < 0.001), while that of R20291ΔWA increased significantly (increased by 27.5%, *p* < 0.05). To further determine the role of the single-gene *csrA* on FliC synthesis, *csrA* and *fliW* were complemented into R20291ΔWA or overexpressed in R20291, respectively. Results showed that the significant difference of *fliC* transcription could only be detected in R20291ΔWA-E (increased by 32.3%, *p* < 0.05; Figure 4C) and R20291-W (increased by 69.8%) compared to R20291-E (Figure 4E). Interestingly, the FliC production of R20291ΔWA-A decreased 3.2-fold (*p* < 0.001) compared to that of R20291-E, while that of R20291ΔWA-WA only decreased by 14.3% (*p* < 0.05), and no significant difference of FliC production in R20291ΔWA-W was detected (Figure 4D). As shown in Figures 4E,F, the *fliC* transcription of R20291-A was not affected compared to R20291-E, but the FliC production in R20291-A decreased 5.3-fold (*p* < 0.0001). The decrease in FliC production in R20291-A can be partially recovered when *fliW* was coexpressed with *csrA* (R20291-WA decreased by 16.2%, *p* < 0.05).

Collectively, our data indicate that CsrA negatively modulates *fliC* expression post-transcriptionally and FliW antagonizes CsrA to regulate *fliC* expression possibly through inhibiting CsrA-mediated negative post-transcriptional regulation.

Effects of *fliW* and *fliW-csrA* Deletions on Toxin Expression

It has been reported that the expression of *csrA* could affect toxin expression in *C. difficile* (Gu et al., 2018). To evaluate the effects of *fliW* and *fliW-csrA* deletions on toxin production, the supernatants of *C. difficile* cultures were collected at 24- and 48-h post-inoculation, and the toxin concentration was...
determined by ELISA. Figure 5A shows that the TcdA concentration of R20291ΔWA decreased by 28.6% \((p < 0.05)\), while R20291ΔW increased by 65.1% \((p < 0.01)\) compared to R20291 at 24-h post-inoculation. However, after 48-h incubation, no significant difference was detected. In Figure 5B, TcdB concentration of R20291ΔWA decreased by 26.4% \((p < 0.05)\) at 24-h post-inoculation, while that of R20291ΔW increased by 93.6% \((p < 0.01)\) at 24h and 33.0% \((p < 0.05)\) at 48h. Similar results were also detected in the complementation strains group (Figures 5C,D). As shown in Figures 5C,D, after 24-h post-inoculation, TcdA (Figure 5C) concentration of R20291ΔWA-E and R20291ΔWA-W decreased by 33.0% (* \((p < 0.05)\) and 47.7% \((p < 0.01)\), and TcdB (Figure 5D) concentration of R20291ΔWA-E and R20291ΔWA-W decreased by 37.9% \((p < 0.05)\) and 31.3% \((p < 0.05)\), respectively, while TcdA concentration of R20291ΔW-E, R20291ΔWA-A, and R20291ΔW-W increased by 83.1% \((p < 0.01)\), 64.7% \((p < 0.05)\), and 56.5% \((p < 0.05)\), respectively. Meanwhile, TcdB concentration of R20291ΔW-E increased by 100.2% \((p < 0.01)\). At 48-h post-inoculation, though no significant difference in TcdA production was detected among different \(C. difficile\) strains, TcdB concentration of R20291ΔWA-A increased by 28.5% \((p < 0.05)\) compared to R20291-E.

To analyze the transcription of tcdA and tcdB in the complementation strains, RT-qPCR was performed. As shown in Figures 5E,F, the transcription of tcdA and tcdB of R20291ΔWA-E and R20291ΔWA-W decreased significantly \((p < 0.05)\), while that of R20291ΔW-E increased significantly \((p < 0.05)\). Interestingly, the tcdA transcription of R20291ΔWA-A also showed a significant increase \((p < 0.05)\) compared to the wild-type strain. Our data indicate that FliW negatively regulates toxin expression, while CsrA plays a positive regulation role in toxin expression.

Effects of \(fliW\) and \(fliW-csrA\) Deletions on Sporulation and Germination

To assay the sporation ratio of \(C. difficile\) strains, R20291, R20291ΔWA, and R20291ΔW were cultured in Clospore media for 48 and 96h, respectively. Results (Supplementary Figure 4A) showed that no significant difference in the sporulation ratio was detected between the wild-type strain and the mutants. The germination ratio of \(C. difficile\) spores was evaluated as well. Purified spores of R20291, R20291ΔWA, and R20291ΔW were incubated in the germination buffer supplemented with taurocholic acid (TA). As shown in Supplementary Figure 4B, there was no significant difference in the germination ratio between the wild-type strain and the mutants.

Evaluation of \(fliW\) and \(fliW-csrA\) Deletions on Bacterial Virulence in the Mouse Model of CDI

To evaluate the effects of \(fliW\) and \(fliW-csrA\) deletions on \(C. difficile\) virulence \(in vivo\), the mouse model of CDI was used. Thirty mice \((n=10\) per group\) were orally challenged with R20291, R20291ΔWA, or R20291ΔW spores \((1 \times 10^9\) spores/mouse\) after antibiotic treatment. As shown in Figure 6A, the R20291ΔW infection group lost more weight at post-challenge days 1 \((p < 0.05)\), and the R20291ΔWA infection group lost less weight at post-challenge days 3 \((p < 0.05)\) compared to the R20291 infection group. Figure 6B shows that 60% of mice succumbed to severe disease within 4 days.
FIGURE 5 | Toxin expression analysis. (A) TcdA concentration in the supernatants of R20291, R20291ΔWA, and R20291ΔW. (B) TcdB concentration in the supernatants of R20291, R20291ΔWA, and R20291ΔW. (C) TcdA concentration in the supernatants of parental and gene complementation strains. (D) TcdB concentration in the supernatants of parental and gene complementation strains. (E) Transcription of tcdA in the supernatants of parental and gene complementation strains. (F) Transcription of tcdB in the supernatants of parental and gene complementation strains. Experiments were independently repeated thrice. Bars stand for mean ± SEM (*p < 0.05, **p < 0.01). One-way ANOVA with post hoc Tukey test was used for statistical significance. ** upon the column directly means the significant difference of experimental strain compared to R20291 or R20291-E.

FIGURE 6 | Effects of fliW and fliW-csrA deletion on C. difficile virulence in mice. (A) Mean relative weight changes. (B) Survival curve. (C) Diarrhea percentage. (D) Clostridioides difficile in feces. (E) TcdA titer of fecal sample. (F) TcdB titer of fecal sample. Bars stand for mean ± SEM (*p < 0.05, **p < 0.01, ***p < 0.001). One-way ANOVA with post hoc Tukey test was used for statistical significance. Animal survivals were analyzed by Kaplan-Meier survival analysis with a log-rank test of significance.
in the R20291ΔW infection group and 20% in the R20291ΔWA infection group compared to 50% mortality in the R20291 infection group (no significant difference with log-rank analysis, \( p = 0.1629 \)). Meanwhile, 100% of mice developed diarrhea in both the R20291ΔW and R20291 infection groups vs. 80% in the R20291ΔWA infection group at post-challenge days 2 (Figure 6C). As shown in Figure 6D, the spores CFU of the R20291ΔW infection group increased in the fecal shedding samples at post-challenge days 1 and 2 (\( p < 0.05 \)), while the spores CFU of the R20291ΔWA infection group decreased at post-challenge days 1, 5, and 6 (\( p < 0.05 \)) compared to the R20291 infection group. Interestingly, while we did not detect significant differences in bacterial growth, germination, and sporulation between the wild-type strain and mutants, the spore numbers from different infection groups were different (Figure 6D). This kind of difference implied that the culture media we used in vitro cannot simulate the complicated intestine environment well, which can lead to the different outcomes in bacterial physiology between in vitro and in vivo analysis. CsrA, as a carbon storage regulator, its regulation on carbon metabolism, and other potential roles in the complicated gut environment in vivo remain to be further studied.

To evaluate the toxin level in the gut, the concentrations of TcdA and TcdB in the feces were measured by ELISA. In comparison with the R20291 infection group, the TcdA of the R20291ΔW infection group increased significantly at post-challenge days 1 (\( p < 0.05 \)), 2 (\( p < 0.05 \)), 3 (\( p < 0.01 \)), and 5 (\( p < 0.05 \); Figure 6E), while the TcdA of the R20291ΔWA infection group decreased significantly at post-challenge days 1 (\( p < 0.05 \)) and 4 (\( p < 0.05 \); Figure 6E). As shown in Figure 6F, the TcdB concentration of the R20291ΔWA infection group decreased significantly at post-challenge days 1 (\( p < 0.05 \)), 2 (\( p < 0.05 \)), and 3 (\( p < 0.05 \)), and that of the R20291ΔW increased significantly at post-challenge days 1 (\( p < 0.05 \)), 2 (\( p < 0.01 \)), and 3 (\( p < 0.01 \)). Taken together, our results indicate that the FlirW defect increases R20291 pathogenicity in vivo, while the flirW-csrA co deletion impairs R20291 pathogenicity.

**DISCUSSION**

In this study, we sought to characterize the impacts of FlirW, CsrA, and FliC on *C. difficile* pathogenicity. Our data suggest that CsrA negatively regulates *fliC* expression post-translationally, and FlirW affects *fliC* expression possibly through inhibiting CsrA-mediated negative post-transcriptional regulation. Our data also indicate that FlirW negatively affects *C. difficile* pathogenicity possibly by antagonizing CsrA in vivo. Based on our current pleiotropic phenotype analysis, a similar partner-switching mechanism “FlirW-CsrA-fliC/FlirC” (FlirC binds FlirW, FlirW binds CsrA, and CsrA regulates *fliC* translation by binding to 5′ untranslated region of *fliC* transcripts) is predicted in *C. difficile*, though more direct experimental data are needed to uncover the molecular interactions of CsrA, FlirW, and FlirC/FlirC in *C. difficile* (Supplementary Figure 5).

It has been reported that overexpression of the *csrA* gene could result in flagella defects, poor motility, and increased toxin production and adhesion in *C. difficile* 630Δerm (Gu et al., 2018). In our study, we found that CsrA and FlirW widely exist in *C. difficile* (Table 2), even in the *C. difficile* strains without flagella like *C. difficile* M120 (Stabler et al., 2009), indicating a potentially important role of FlirW-CsrA in *C. difficile*. Interestingly, while there are no flagella in *C. difficile* M120, six flagellar structure genes (*fliS, fliN, flgK, flgL, fliC, and fliD*) are still found in the genome, which inspired us to explore the potential roles of *fliW, csrA*, and *fliC* in *C. difficile* by deleting or overexpressing *fliW, csrA*, and *fliW-csrA* genes. However, after several trials with different gene edit methods in *C. difficile*, we could not get the *csrA* gene deletion mutant possibly due to its small size. This result motivated us to construct *fliW-csrA* double deletion mutant. While we did not get the single *csrA* gene deletion, we complemented the single *fliW* gene in the *fliW-csrA* double deletion mutant for simulation of the *csrA* deletion effects. The important roles of CsrA in flagellin synthesis and flagellin homeostasis have been reported (Yakhnin et al., 2007; Mukherjee et al., 2011; Gu et al., 2018; Oshiro et al., 2019). A previous study had shown that the overexpression of the *csrA* gene can cause a dramatic motility reduction and a significant Hag decrease in *B. subtilis* (Yakhnin et al., 2007). FlirW (the first protein regulator of CsrA activity) deletion abolished the *B. subtilis* swarming and swimming motility and decreased the number of flagella and flagellar length (Mukherjee et al., 2011, 2016). In this study, we obtained similar results that FlirW defect impaired R20291 motility significantly (Figure 2A) and increased biofilm formation (Figures 2C,D). Interestingly, the *csrA* gene complementation in R20291ΔWA dramatically suppressed bacterial motility and showed a similar result to R20291ΔW, indicating that CsrA can suppress *C. difficile* motility and increase biofilm production, while FlirW antagonizes *csrA* to regulate bacteria motility and biofilm formation indirectly.

The partner-switching mechanism “Hag-FlirW-CsrA” on flagellin synthesis was elucidated in *B. subtilis*, and the intracellular concentration of the flagellar filament protein Hag is restricted tightly by the Hag-FlirW-CsrA system (Mukherjee et al., 2011). To investigate whether FlirW and CsrA coregulate the *fliC* expression in *C. difficile*, we evaluated both the transcriptional and translational expression level of *fliC* gene. Our data (Figure 4) showed that the *fliW* deletion resulted in a 10.4-fold decrease in FlirC accumulation, while the *fliW-csrA* co deletion increased FlirC production, indicating that CsrA could suppress the *fliC* translation and FlirW antagonizes CsrA to regulate FlirC production. In *csrA, fliW*, and *fliW-csrA* overexpression experimental groups, we found that the *csrA* overexpression dramatically decreased FlirC production (5.3-fold reduction) and the reduction in FlirC production in R20291-A can be partially recovered when *fliW-csrA* was coexpressed. The FlirW complementation in R20291ΔWA did not affect FlirC production, but the *fliW* overexpression in R20291 increased FlirC production. Taken together, our data suggest that CsrA negatively modulates *fliC* expression post-transcriptionally and FlirW antagonizes CsrA to regulate *fliC* expression through inhibiting CsrA-mediated negative post-transcriptional regulation, indicating a similar partner-switching mechanism.
"FliW-CsrA-FliC" in *C. difficile*. In *B. subtilis*, two CsrA binding sites (BS1: A51 to A55; BS2: C75 to G82) were identified in the *hag* leader of the mRNA (Yakhnin et al., 2007). Based on the *hag* 5′-UTR sequence and CsrA conserved binding sequence, a 91 bp 5′-UTR structure with two potential CsrA binding sites (BS1: 5′-TGACAAGGATGT-3′; BS2: 5′-CTAAGGAGGG-3′) of *fliC* gene was predicted (Supplementary Figure 6; Dubey et al., 2005). Recently, it was also reported that cytoplasmic Hagg levels play a central role in maintaining proper intracellular architecture, and the Hagg-FliW-CsrA system works at nearly 1:1 stoichiometry in *B. subtilis* (Oshiro et al., 2019). Further studies on the extracellular interactions of CsrA, FliW, and fliC/Flic in *C. difficile* are still needed.

Flagella play multiple roles in bacterial motility, colonization, growth, toxin production, and survival optimization (Harshey, 2003; Duan et al., 2013; Stevenson et al., 2015). Recently, several papers have reported that the flagellar genes can affect toxin expression in *C. difficile*, but results from different research groups were controversial (Aubry et al., 2012; Baban et al., 2013; Stevenson et al., 2015). Aubry et al. (2012) reported that disruption of some early-stage flagellar genes (F3), such as *flif*, *fliG*, and *fliM*, could lead to a significant reduction in *tcdR*, *tcdE*, *tcdA*, and *tcdB* expression in *C. difficile* 630Δerm, but no significant difference of *tcdC* expression was detected. Inversely, disruption of late-stage flagellar genes (F1) such as *fliC* increased toxin expression in *C. difficile* 630Δerm. In 2013, Baban et al. (2013) reported that the mutation of *fliE* (one of the F3 genes) resulted in a tenfold reduction in *tcdA* expression and corroborated that the expression of *tcdA* in a *fliC* mutant increased 44.4-fold compared to the wild-type strain *C. difficile* 630Δerm. Surprisingly, Aubry et al. (2012) found that a glycosylation gene (CD0240, one of F2 region genes) mutation, which can totally abolish *C. difficile* 630 motility, but did not change toxin expression. Meanwhile, cyclic diguanylate (C-di-GMP), a cellular second messenger, was also reported to be involved in bacterial motility, biofilm formation, and toxin production by repressing the expression of flagellar genes in *C. difficile* (Purcell et al., 2012; Mckee et al., 2013). While we did not detect the C-di-GMP concentration in *C. difficile*, it could be perturbed by *fliW* and *csrA* deletion affecting *C. difficile* physiology. It was hypothesized that the regulation of the flagellar genes on toxin expression could be caused by the direct change or loss of flagellar genes (such as *fliC* gene deletion) rather than loss of the functional flagella (Stevenson et al., 2015). Future study about *fliC* deletion in M120 will be very interesting and will further address the *fliC* gene function in *C. difficile* as there are no flagella in RT078 strains. In our study, data indicate that CsrA negatively modulates *fliC* translation and also plays a positive regulation in toxin expression. Inversely, FliW works against CsrA to regulate *fliC* expression, which can negatively regulate toxin production. While studies of flagellar effects on motility and toxin production in *C. difficile* from different groups were controversial, the role of the flagella in *C. difficile* pathogenicity cannot be overlooked. Dingle et al. (2011) and Baban et al. (2013) both showed higher mortality of the *fliC* mutant in the animal model of CDI compared to the wild-type strains. Our study showed results similar to the published data suggesting that R20291ΔW whose FliC production was dramatically suppressed exhibited higher fatality, while R20291ΔWA showed a decreased pathogenicity compared to R20291 (Figure 6). In 2014, Barketi-Klai et al. (2014) examined the pleiotropic roles of the *fliC* gene in R20291 during colonization in mice. Interestingly, the transcription of *fliW* and *csrA* in the *fliC* mutant was 2.03- and 4.36-fold, respectively, of that in R20291 in vivo experiment (Barketi-Klai et al., 2014), which further corroborated that there is a coregulation among *fliC*, *fliW*, and *csrA*. Surprisingly, transcription of *treA*, a trehalose-6-phosphate hydrolase, increased 1776.63-fold in the *fliC* mutant compared to that of R20291 during in vivo infection (Barketi-Klai et al., 2014). Recently, Collins et al. (2018) hypothesized that dietary trehalose can contribute to the virulence of epidemic *C. difficile*. The relationship of FliW, CsrA, FliC, and trehalose metabolism is another interesting question in *C. difficile*, and some other carbon metabolism affected by the *fliC* mutation could also facilitate *C. difficile* pathogenesis in vivo. Previous studies have also highlighted that the flagella of *C. difficile* play an important role in toxin production, biofilm formation, and bacterial adherence to the host (Tasteyre et al., 2001; Dingle et al., 2011; Aubry et al., 2012; Baban et al., 2013; Ethapa et al., 2013). In this study, we showed that the FliW defect led to a significant motility decrease, while the biofilm, adhesion, and toxin production increased significantly. Inversely, R20291ΔWA-W, which can imitate the *csrA* gene deletion, showed an increase in motility and a decrease in biofilm formation, toxin production, and adhesion.

In conclusion, we characterized the function of FliW and CsrA and showed the pleiotropic functions of FliW and CsrA in R20291. Our data suggest that *fliW* and *csrA* play important roles in flagellin (Flic) synthesis, which could contribute to *C. difficile* pathogenicity. Currently, *in vitro* study of the interactions of CsrA, FliW, and fliC/Flic in *C. difficile* is underway in our group.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

**ETHICS STATEMENT**

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of University of South Florida.

**AUTHOR CONTRIBUTIONS**

DZ and XS designed the experiments. DZ wrote the manuscript. DZ and SW performed the experiments. DZ and XS revised the manuscript. All authors contributed to the article and approved the submitted version.
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**ACKNOWLEDGMENTS**

The authors thank Dr. Abraham L. Sonnenshein at Tufts University, Dr. Joseph Sorg at Texas A&M, and Dr. Daniel Kearns at Indiana University for the gifts *C. difficile* R20291, *E.coli* HB101/pRK24, and anti-SigA primary antibody, respectively. We thank Dr. Nigel Minton at the University of Nottingham for the gift plasmids pMTL84151 and pMTL83535. We also thank Jessica Bullock and Dr. Heather Danhof for their mindful revision and comments.

**FUNDING**

This work was supported in part by the National Institutes of Health grants (R01-AI132711 and R01-AI149852).

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.735616/full#supplementary-material
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