Characterization of Flagellum and Toxin Phase Variation in *Clostridioides difficile* Ribotype 012 Isolates

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ABSTRACT *Clostridioides difficile* causes diarrheal diseases mediated in part by the secreted toxins TcdA and TcdB. *C. difficile* produces flagella that also contribute to motility and bacterial adherence to intestinal cells during infection. Flagellum expression and toxin gene expression are linked via the flagellar alternative sigma factor, SigD. Recently, we identified a flagellar switch upstream of the early flagellar biosynthesis operon that mediates phase variation of both flagellum and toxin production in *C. difficile* strain R20291. However, we were unable to detect flagellar switch inversion in *C. difficile* strain 630, a ribotype 012 strain commonly used in research labs, suggesting that the strain is phase locked. To determine whether a phase-locked flagellar switch is limited to 630 or present more broadly in ribotype 012 strains, we assessed the frequency and phenotypic outcomes of flagellar switch inversion in multiple *C. difficile* ribotype 012 isolates. The laboratory-adapted strain JIR8094, a derivative of strain 630, and six clinical and environmental isolates were all found to be phase-off, nonmotile, and attenuated for toxin production. We isolated low-frequency motile derivatives of JIR8094 with partial recovery of motility and toxin production and found that additional changes in JIR8094 impact these processes. The clinical and environmental isolates varied considerably in the frequency by which flagellar phase-on derivatives arose, and these derivatives showed fully restored motility and toxin production. Taken together, these results demonstrate heterogeneity in flagellar and toxin phase variation among *C. difficile* ribotype 012 strains and perhaps other ribotypes, which could impact disease progression and diagnosis.

IMPORTANCE *C. difficile* produces flagella that enhance bacterial motility and secretes toxins that promote diarrheal disease symptoms. Previously, we found that production of flagella and toxins is coregulated via a flippable DNA element termed the flagellar switch, which mediates the phase-variable production of these factors. Here, we evaluate multiple isolates of *C. difficile* ribotype 012 strains and find them to be primarily flagellar phase off (*flg*-off state). Some, but not all, of these isolates showed the ability to switch between *flg*-on and -off states. These findings suggest heterogeneity in the ability of *C. difficile* ribotype 012 strains to phase-vary flagellum and toxin production, which may broadly apply to pathogenic *C. difficile*.

KEYWORDS *Clostridium difficile*, phase variation, bistability, DNA recombination, FliA, topoisomerase, TopA, *Clostridioides*, flagella, motility, toxins

The obligate anaerobe *Clostridioides difficile*, recently renamed from *Clostridium difficile* (1), is a leading cause of nosocomial intestinal infections. *C. difficile*-associated infections (CDIs) are most common in individuals who have undergone antibiotic therapy, which disrupts the usually protective microbiota and creates a niche for *C. difficile* outgrowth (2). Virulence of *C. difficile* is largely mediated by two glucosylating toxins, TcdA and TcdB, which target and inactivate Rho and Rac GTPases in the

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intestinal epithelium, resulting in depolymerization of the actin cytoskeleton and eventually host cell death (3). Toxin-mediated damage to the epithelium leads to disruption of the intestinal barrier, diarrheal symptoms, and a robust inflammatory response (3).

C. difficile produces peritrichous flagella that are essential for swimming motility and contribute to host cell adherence (4–7). As in other bacterial species, the expression of flagellar genes occurs in a hierarchical manner (8). In *C. difficile*, at least four different operons include flagellar genes. The early-stage flagellar genes (*flgB* operon) are transcribed first, and they encode the basal body, motor, and rod of the flagella. The *flgB* operon also encodes the alternative sigma factor SigD (σ^D, also known as FliA or σ^{28}). In addition to activating late-stage flagellar gene expression, SigD positively regulates the expression of *tcdR*, which encodes a sigma factor that activates transcription of *tcdA* and *tcdB* (8–10). Therefore, the regulation of flagellar genes also impacts virulence by affecting toxin gene expression.

The expression of flagellum and toxin genes is subject to complex regulation (7, 8, 11–13). Recently, we demonstrated that flagellum and toxin biosynthesis is phase variable via site-specific recombination that inverts a DNA element termed the flagellar switch (14). The flagellar switch consists of a 154-bp invertible DNA sequence flanked by 21-bp inverted repeats and lies upstream of the *flgB* operon. The orientation of the flagellar switch controls expression of the *flgB* operon and, therefore, *sigD* and the toxin genes through an unidentified mechanism occurring posttranscription initiation (14). Bacteria with the flagellar switch in an orientation resulting in flagellum production, swimming motility, and high toxin production were termed flagellar phase on (*flg*-on state). In contrast, bacteria with the flagellar switch in the opposite orientation resulting in the absence of flagella, sessility, and reduced toxin production were termed flagellar phase off (*flg*-off state). RecV, a site-specific tyrosine recombinase, mediates inversion of the flagellar switch and additional genetic switches in *C. difficile* R20291 (14–17). The phase-variable production of flagella and toxins was proposed to allow *C. difficile* to balance the benefits of swimming motility and toxinogenesis with the cost of producing these immunogenic factors (18–21).

Flagellum and toxin phase variation in *C. difficile* appears to vary across ribotypes (14). For *C. difficile* strains R20291, a ribotype 027 strain, and ATCC 43598, a ribotype 017 strain, both the *flg*-on and -off orientations were apparent under multiple conditions tested. However, only the *flg*-on orientation was detectable in 630, a ribotype 012 strain originally isolated from a patient with *C. difficile* infection (22). However, recent work from Collery et al. suggests that the flagellar switch is capable of inversion in strain 630 (23), suggesting that this strain is phase locked. JIR8094 (also named 630E) was isolated through serial passage of 630 to obtain erythromycin-sensitive isolates amenable to genetic manipulation with tools relying on an erythromycin resistance cassette (24). Comparison of the genome sequence of JIR8094 to that of 630 revealed numerous secondary mutations that arose during and since isolation (23). One polymorphism identified was an inversion of the flagellar switch to the off orientation in JIR8094 compared to the *flg*-on orientation in 630. The JIR8094 genotype explains the nonmotile phenotype and reduced flagellum and toxin gene expression previously reported for JIR8094 (8) and indicates an ability to invert the flagellar switch in this strain lineage. In contrast, the 630Δ*erm* strain was similarly derived from 630 through serial passaging, but it remains motile and toxinogenic (25).

In this study, we examined multiple ribotype 012 strains, including laboratory-adapted, clinical, and environmental isolates, to assess their ability to invert the flagellar switch and to phase-vary flagellum and toxin production. Analysis of these strains and their motile derivatives indicates that flagellum and toxin phase variation is conserved in ribotype 012 *C. difficile* strains and that there is considerable variation in the frequency of phase variation in this and perhaps other ribotypes.

RESULTS

The laboratory-adapted *C. difficile* strain JIR8094 is *flg* off. The *C. difficile* strain JIR8094 is a nonmotile, toxin-attenuated derivative of the ribotype 012 strain 630 (8, 23,

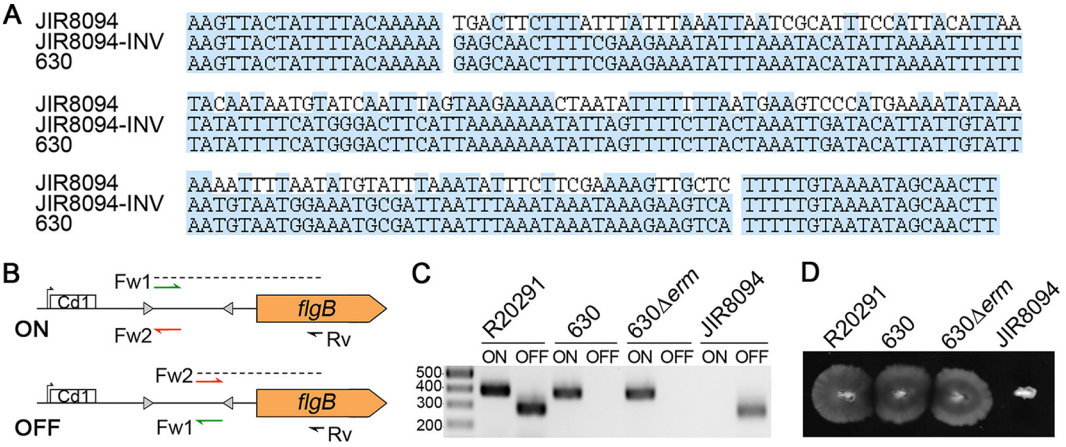

FIG 1 *C. difficile* JIR8094 has the flagellar switch in the off orientation. (A) Multiple-sequence alignment of the flagellar switch sequences from *C. difficile* 630, its JIR8094 derivative, and JIR8094-inv in which the 154-bp sequence between the imperfect inverted repeats was replaced with the reverse complement. Shading indicates identical nucleotides. (B) Schematic for the orientation-specific PCR assay used to determine the orientation of the flagellar switch. Cd1 indicates the c-di-GMP riboswitch. The triangles denote the left and right inverted repeats (LIR and RIR, respectively). The Rv primer (R857) is used with either Fw1/On (R1751 for ribotype 012 strains and R1614 for ribotype 027 strains) or Fw2/Off (R1752 for ribotype 012 strains and R1615 for ribotype 027 strains) to detect the on or off orientation of the flagellar switch, respectively. In the 630 lineage, Fw1/On plus Rv yields a 374-bp product; Fw2/Off plus Rv yields a 250-bp product. For R20291, Fw1/On plus Rv yields a 375-bp product; Fw2/Off plus Rv yields a 281-bp product. The diagram is not drawn to scale. (C) Orientation-specific PCR products using template DNA from *C. difficile* R20291, 630, 630Δ*erm*, and JIR8094. (D) Motility of R20291, 630, 630Δ*erm*, and JIR8094 in motility medium (0.5× BHIS–0.3% agar) after 72 h growth.

24). In *C. difficile*, expression of the *flgB* operon is controlled by multiple mechanisms, including a σ^A-dependent promoter (26), a c-di-GMP riboswitch in the 5′ untranslated region (UTR) (26–28), and the flagellar switch (14). Sequencing of the *flgB* operon regulatory regions from *C. difficile* 630 and JIR8094 revealed no differences in the promoter regions or the c-di-GMP riboswitch sequences. However, while the left inverted repeat (LIR) and right inverted repeat (RIR) were identical in the two strains, the intervening 154 bp was inverted in JIR8094 relative to the sequence of strain 630 (Fig. 1A). Alignment of the flagellar switch sequence using the reverse complement of the sequence obtained for JIR8094 restored sequence identity to 100% with 630 (Fig. 1A). Inversion of the flagellar switch in JIR8094 relative to that of 630 was confirmed by orientation-specific PCR (Fig. 1B). For this assay, a common reverse primer (Rv) is used in combination with a primer (Fw1) that leads to amplification when the template is in the on orientation or with a primer (Fw2) that amplifies when the template is in the off orientation (Fig. 1B) (14). As shown previously, *C. difficile* R20291 cultures contain a mixture of *flg*-on and -off bacteria, whereas 630Δ*erm* cultures contain exclusively *flg*-on bacteria (Fig. 1C) (14). In contrast, only the *flg*-on orientation was detected for strain 630, and only the *flg*-off orientation was detected for JIR8094 (Fig. 1C). In addition, R20291, 630, and 630Δ*erm* demonstrated swimming through motility medium, while JIR8094 was nonmotile after 72 h (Fig. 1D). These observations are consistent with a recent report identifying the sequence inversion in JIR8094 (23) and with phenotypes corresponding to the *flg*-off state (8).

The flagellar switch undergoes inversion at low frequency in JIR8094. Throughout our experimentation, we did not observe motility or motile flares from JIR8094 in standard assays (data not shown), and Collery et al. similarly reported an inability to recover motile bacteria from this strain (23). These findings suggested that JIR8094 is phase locked off or undergoes inversion of the flagellar switch at a low rate. To distinguish between these possibilities, we modified the motility assay to accommodate higher-density inocula. Rather than inoculating motility agar with 2 μl of saturated broth culture, we first concentrated the culture 30-fold, reasoning that this would increase the odds of detecting rare *flg*-on bacteria. Over 48 to 72 h, we were able to detect motility from a subset of JIR8094 inocula (Fig. 2A).

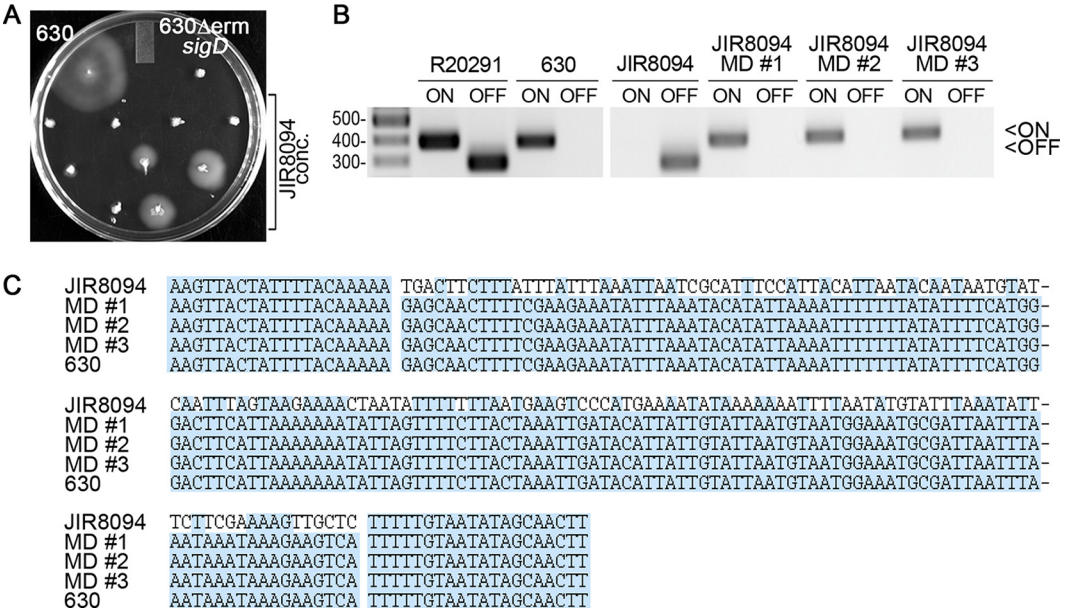

FIG 2 The flagellar switch undergoes inversion at a low frequency in JIR8094. (A) Concentrated liquid cultures of JIR8094 were inoculated into motility medium along with standard (i.e., not concentrated) inocula of 630 (motile) and 630Δ*erm sigD* (nonmotile) as controls. Shown is a representative experiment in which a JIR8094 culture was concentrated 30-fold (9 replicates). After 48 h of growth at 37°C, motility or motile flares were detected for three concentrated inocula with JIR8094. Data are representative of three independent experiments. (B) Orientation-specific PCR products for three motile *flg*-on derivatives of JIR8094 (MD 1 to 3). *C. difficile* R20291, 630, and JIR8094 were included as controls for both PCR primer sets. (C) Multiple-sequence alignment of the flagellar switch of JIR8094, 630, and three *flg*-on derivatives of JIR8094. Shading indicates identical nucleotides.

To determine whether motility was restored as a result of flagellar switch inversion to on or due to extragenic suppressor mutations, we used orientation-specific PCR (Fig. 1B) to assess the orientation of the flagellar switch. Three motile derivatives (MDs) of JIR8094 (MD 1 to 3) were arbitrarily chosen after recovery from the edge of the motile colony for further analysis. All three MDs yielded PCR products indicative of the *flg*-on orientation but not the *flg*-off orientation, as seen in the parental strain 630 (Fig. 2B). Sequencing of the flagellar switch confirmed the *flg*-on orientation of the switch in JIR8094 MD 1 to 3 (Fig. 2C). Given the limited sensitivity of low-abundance targets by PCR, we used quantitative PCR (qPCR) to measure the frequency of bacteria with the flagellar switch in the on and off orientation. *C. difficile* 630, 630Δ*erm*, and JIR8094 MD 1 to 3 were 99.99% *flg* on, whereas JIR8094 was 0.005% *flg* on (Table 1). These results indicate that the flagellar switch remains reversible in JIR8094 though the frequency of inversion appears to be lower than previously observed for R20291 (14).

To estimate the frequency of *flg*-on bacteria in JIR8094, bacteria with a range of densities were assayed for swimming motility. As controls, we used the motile 630 strain, the nonmotile 630Δ*erm sigD* mutant (29), and a *C. difficile* R20291 *flg*-off isolate.

TABLE 1 Frequency of flagellar switch orientations in *C. difficile* 630 and derivatives

Strain	Frequency of orientation[a]	
	% *flg* on	% *flg* off
630Δ*erm*	99.9994 ± 0.0003	0.0006 ± 0.0003
630	99.9996 ± 0.0001	0.0004 ± 0.0001
JIR8094	0.0005 ± 0.0002	99.9995 ± 0.0002
JIR8094 MD 1	99.9992 ± 0.0005	0.0008 ± 0.0005
JIR8094 MD 2	99.9994 ± 0.0003	0.0006 ± 0.0003
JIR8094 MD 3	99.9985 ± 0.0009	0.0015 ± 0.0009

[a]Frequency was calculated as the percentage of the genes in the indicated orientation compared to the total population. Means ± standard deviations from four independent samples are shown.

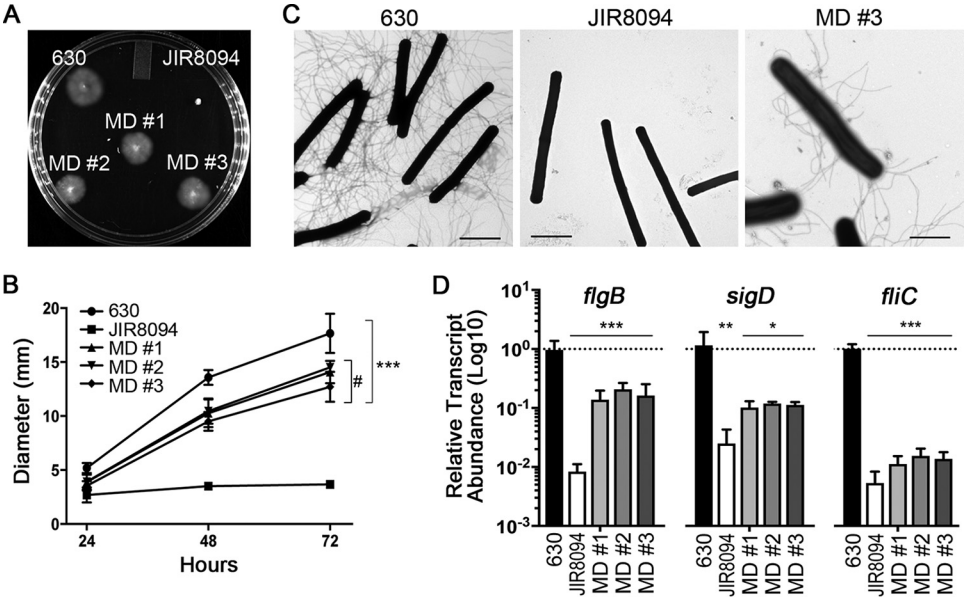

FIG 3 JIR8094 motile derivatives have partial recovery of flagellar gene expression, flagellum biosynthesis, and motility. (A) Motility assay for 630, JIR8094, and JIR8094 MD 1 to 3. Image is representative of three biological replicates. (B) Quantification of motility after 24, 48, and 72 h. Shown are the means and standard deviations of the swim diameters from three independent experiments. At both 48 and 72 h significance was determined as follows: ***, $P < 0.001$ for results compared to those with JIR8094; #, $P < 0.05$ for results compared to those with 630 (one-way analysis of variance and Tukey's multiple-comparison test). The motilities of JIR8094 MD 1 to 3 were not significantly different from each other. (C) Transmission electron microscopy images for 630, JIR8094, and JIR8094 MD 3 showing the presence or absence of flagella. (D) The abundance of flagellar gene transcripts *flgB*, *sigD*, and *fliC* in logarithmic-phase BHIS cultures of 630, JIR8094, and JIR8094 MD 1 to 3 isolates. The data were analyzed using the $\Delta\Delta C_T$ method with *rpoC* as the control gene and 630 as the reference strain. Shown are the means and standard deviations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for results compared to those of the 630 reference by one-way analysis of variance and Tukey's multiple-comparison test. None of the differences between values for JIR8094 and MD 1 to 3 met a P value of <0.05.

The last was chosen because we previously determined that it contains a small (~3%) population of *flg*-on bacteria, which spatially segregates from nonmotile *flg*-off bacteria in motility medium, leading to a motile phenotype that is readily apparent within 24 h (14). Whereas R20291 appeared motile at all inocula tested, motility of JIR8094 was observed only at the highest density, suggesting that flagellar switch inversion occurs at a low frequency in this strain (see Fig. S1 in the supplemental material).

Characterization of JIR8094 motile derivatives. We predicted that restoration of the flagellar switch to the on orientation would restore flagellar motility and toxin production of the JIR8094 MDs to levels observed in the original 630 parental strain. To test this, we assayed swimming motility using standard (not concentrated) inocula, with *C. difficile* 630 and JIR8094 strains included as positive and negative controls, respectively. The JIR8094 MD 1 to 3 were motile at all time points tested, but motility was not fully restored to the level seen for strain 630 (Fig. 3B). By electron microscopy, we also observed that JIR8094 MD strains 1 to 3 were capable of producing peritrichous flagella though fewer and perhaps shorter flagella than those produced by 630 (Fig. 3C). However, the majority of individual bacteria from JIR8094 MD 1 to 3 were aflagellate (data not shown). JIR8094 remained nonmotile and aflagellate (Fig. 3A and C).

The intermediate motility of JIR8094 MD 1 to 3 could be due to several reasons. Motile derivatives of JIR8094 could have partial recovery of flagellar gene transcription and/or biosynthesis, or only a minority of bacteria in the population might produce flagella (Fig. 3C); these phenotypes could be due to additional mutations in JIR8094. Using quantitative reverse transcriptase PCR (qRT-PCR), we measured the transcript abundance for three flagellar genes, *flgB*, *sigD*, and *fliC*. Both *flgB* and *sigD* are in the operon directly controlled by the flagellar switch (early-stage flagellar genes), and *fliC* serves as a representative *sigD*-regulated late-stage flagellar gene. As previously re-

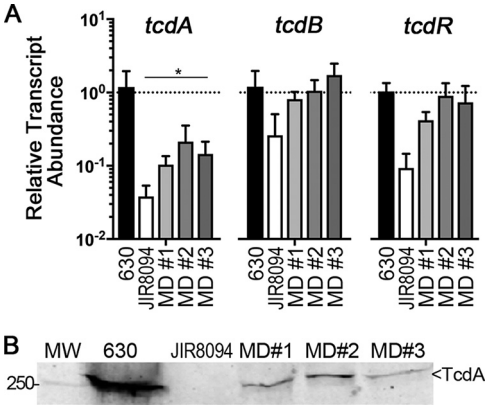

FIG 4 JIR8094 motile derivatives show intermediate toxin gene expression and toxin production. (A) qRT-PCR analysis of *tcdR*, *tcdA*, and *tcdB* transcript abundance in stationary-phase cultures of 630, JIR8094, and JIR8094 MD 1 to 3. The data were analyzed using the $\Delta\Delta C_T$ method with *rpoC* as the control gene and 630 as the reference strain. Shown are the means and standard deviations. *, $P < 0.05$ compared to results for 630 by one-way analysis of variance and Tukey's multiple-comparison test. None of the differences between results for JIR8094 and MD 1 to 3 met a P value of <0.05. (B) Western blot detection of TcdA production by 630, JIR8094, and JIR8094 MD 1 to 3. A representative image from three independent experiments is shown.

ported, the *flgB*, *sigD*, and *fliC* transcripts were significantly lower in JIR8094 than in the 630 strain (Fig. 3D) (8). In MD 1 to 3, *flgB*, *sigD*, and *fliC* transcript levels were higher than in JIR8094 but were not restored to the levels in 630 (Fig. 3D). Nonetheless, the intermediate level of flagellar gene expression and number of flagella on the JIR8094 MDs were sufficient to confer some motility.

Similar intermediate phenotypes were observed with regard to toxin production. Analysis of *tcdR*, *tcdA*, and *tcdB* transcripts in stationary-phase cultures (favoring toxin gene expression) by qRT-PCR showed that all three transcripts were less abundant in JIR8094 than in 630, as seen previously (8), although only *tcdA* was significantly lower (Fig. 4A). The transcripts trended higher in MD 1 to 3 than in their JIR8094 parent, but the differences were not statistically significant (Fig. 4A). Consistent with this, the amounts of TcdA protein produced by MD 1 to 3 appeared more similar to the levels in the JIR8094 parent than to those in 630 (Fig. 4B). Together, these data indicate that JIR8094 MD 1 to 3 have restored the ability to transcribe flagellar and toxin genes and, thus, motility and toxinogenesis but suggest that other mutations in JIR8094 also affect flagellum and toxin gene expression in this strain.

Topoisomerase activity in JIR8094 motile derivatives impacts motility. Comparison of the genome sequences of *C. difficile* 630 and its 630Δ*erm* and JIR8094 derivatives revealed multiple single nucleotide polymorphisms, including 11 found in JIR8094 but not in the other two strains (one of which is the flagellar switch inversion) (23). We speculated that one or more of these polymorphisms are responsible for the difference in flagellar gene expression levels and frequency of flagellated bacteria between the 630 strain and the JIR8094 MD 1 to 3 (all of which are *flg* on). Of interest was a nonsense mutation in a *topA* orthologue that introduced a stop codon at amino acid 386 while the full-length protein is 695 amino acids. Because *topA* encodes DNA topoisomerase I, which controls DNA negative supercoiling, we postulated that the mutation in *topA* could result in changes to the *flgB* operon promoter and/or coding sequence that impair gene transcription in JIR8094 and its derivatives, compared to 630, which encodes the full-length TopA. If so, expressing *topA* in the JIR8094 MD 1 to 3 would be expected to restore motility and toxin production to the levels in 630.

To test this, we ectopically expressed *topA* from an anhydrotetracycline (ATc)-inducible promoter in 630, JIR8094, and the MD 1 to 3 and compared the motilities of these strains to those of the vector controls. A 630Δ*erm sigD* mutant-bearing vector or the *topA* expression plasmid (pTopA) was included as a nonmotile control. After 72 h,

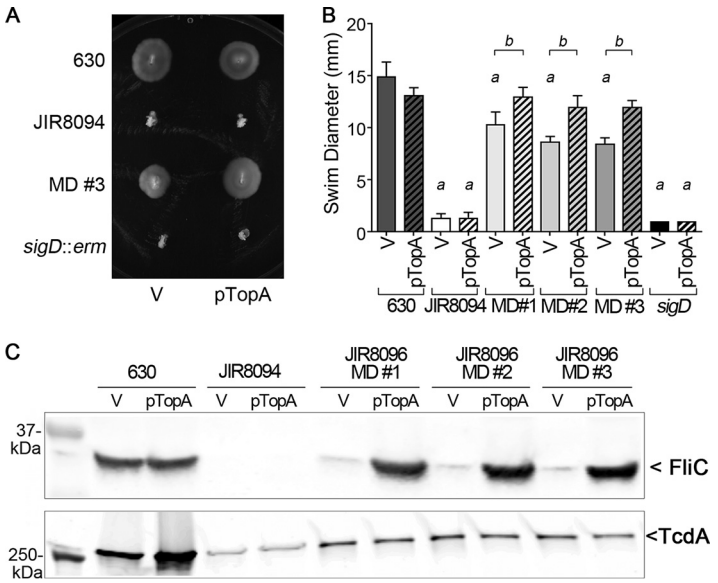

FIG 5 Ectopic expression of *topA* restores motility of JIR8094 motile derivatives to 630 levels. (A and B) Motility of 630, JIR8094, JIR8094, MD 1 to 3, and a *sigD* mutant, each bearing vector (V) or pTopA, after 72 h of growth. (A) Image is representative of two independent experiments with six biological replicates of each strain. Equivalent results were obtained for JIR8094 MD 1 and MD 2. (B) Quantification of motility in the above strains after 72 h. Two independent experiments with six biological replicates were done. Significance was determined at a *P* value of <0.0001 by two-way analysis of variance and Tukey's multiple-comparison test compared to results for 630 with vector (*a*) or for comparisons of the indicated pairs of strains (*b*). (C) Detection of FliC and TcdA by Western blotting. Data are representative of two independent experiments with each strain.

expression of *topA* did not significantly alter the motility of 630 or the *sigD* mutant, as expected (Fig. 5A and B). Expression of *topA* alone is not sufficient to augment motility in *flg*-off bacteria as JIR8094/pTopA remained nonmotile (Fig. 5A and B). However, expression of *topA* in JIR8094 MD 1 to 3 significantly enhanced motility, restoring it to 630 levels (Fig. 5A and B). By Western blotting, expression of *topA* significantly increased FliC production in JIR8094 MD 1 to 3 compared to levels in the vector control and JIR8094 bearing the vector, restoring FliC abundance to 630 levels (Fig. 5C). Presumably, TcdA levels would concomitantly increase upon restoration of TopA activity, but Western blot analysis indicated that *topA* expression in JIR8094 MD 1 to 3 did not restore TcdA production (Fig. 5D). These results suggest that the flagellar switch must be in the on orientation to allow flagellar gene expression and motility, and DNA supercoiling affects flagellar gene expression. Moreover, additional mutations in JIR8094 may interfere with the link between toxin and flagellar gene expression.

Clinical and environmental isolates of *C. difficile* ribotype 012 are *flg* off. Both flagellar switch orientations were readily detected in *C. difficile* strains R20291, a ribotype 027, and ATCC 43598, a ribotype 017, but only the *flg*-on orientation was detected in 630 (14). The 630 strain was isolated in 1982, so passaging within and between labs could have led to accumulation of mutations that reduced the frequency of flagellar phase variation in 630. We thus asked whether the lack of (or very low frequency of) flagellar switch inversion in 630 is unique to this strain and its derivatives or a broader attribute of currently circulating ribotype 012 *C. difficile* isolates (22). To test this, we evaluated flagellar phase variation in six ribotype 012 isolates: SS235, a community environmental isolate; SE838, a hospital environmental isolate; and MAM30, MT4768, MT5065, and MT5066, isolates from patients with CDI (kindly gifted by Kevin Garey). Sequencing of the flagellar switches revealed that all six isolates contain the flagellar switch in the same off orientation as JIR8094 and opposite to that of strain 630 (see Fig. S2 in the supplemental material) (23). The flagellar switches of two isolates also contained polymorphisms within the inverted repeats (Fig. S2). MT4768 had one

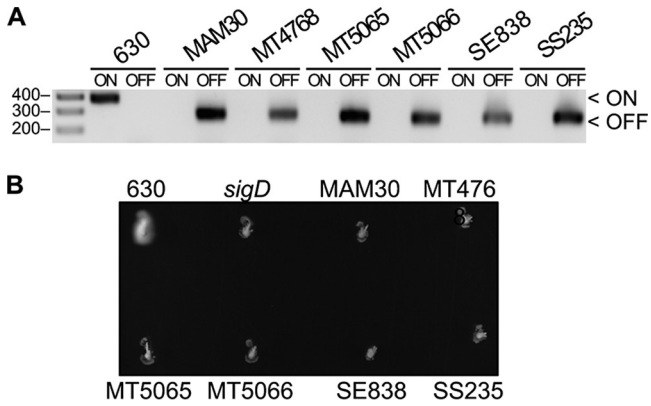

FIG 6 Clinical and environmental 012 ribotype isolates are *flg* off. (A) Orientation-specific PCR products using the indicated strains as templates. Template from 630 is included as a control to detect the *flg*-on orientation. The image is representative of four biological replicates. (B) Swimming motility after 24 h. Data are representative of three independent experiments with eight biological replicates of each strain.

nucleotide substitution in the left inverted repeat (LIR) and an additional nucleotide in the right inverted repeat (RIR). SE838 contained an additional 3 nucleotides (nt) in the LIR and 2 nt in the RIR. In addition, MT4768 possessed three single nucleotide substitutions within the flagellar switch (Fig. S2). SS235, MAM30, MT5065, and MT5066 had flagellar switches identical to the switch in JIR8094.

Sequencing results indicated the conservation of the regulatory flagellar switch element, but they did not reveal if it is functional. It is also possible that the sequencing results reflect the most abundant switch orientation in a population, masking the presence of a lower-abundance orientation. Therefore, we used PCR with orientation-specific primer sets that discriminate between the two DNA orientations (Fig. 6A) (14). As a control we included strain 630, for which only the *flg*-on orientation is detectable (Fig. 6A) (14). For all six isolates only the *flg*-off orientation of the flagellar switch was detectable (Fig. 6A).

Given that *flg*-off bacteria in R20291 are phenotypically aflagellate and nonmotile (14), we predicted that the six isolates with the flagellar switch in the off orientation would be similarly nonmotile. The six ribotype 012 isolates were examined for the ability to swim through motility medium. Strain 630 and a 630Δ*erm sigD* mutant were used as motile and nonmotile controls, respectively (29). After 24 h of incubation, all six ribotype 012 isolates showed a nonmotile phenotype in comparison to the motile 630, which migrated outward from the original inoculation site (Fig. 5B). Collectively, these data indicate that the flagellar switch is conserved in ribotype 012 clinical and environmental isolates and appears to be phase locked in an *flg*-off state or to undergo flagellar switch inversion at a low frequency *in vitro*.

Isolation and phenotypic characterization of *flg*-on derivatives of clinical and environmental 012 ribotype isolates. While examining the motility of the ribotype 012 isolates, we observed that a subset of replicates showed motile flares extending from the stab sites for some isolates (Fig. 7A). This is in contrast with R20291, for which we previously found that clonal *flg*-off populations yielded a motile phenotype within 24 h in 100% of replicates (14). To determine the frequency at which motile flares arise in the ribotype 012 isolates, we increased the number of biological replicates ($n = 32$ over two experiments) and monitored motility every 24 h for 3 days. A small proportion of colonies of three of the isolates developed motile flares by 24 h (MT5066, 6.3%; SE838, 9.4%; SS235, 3.1%), and a fourth isolate showed motile flares by 48 h (MT5065, 18.9%) (Fig. 7B). The number of colonies with motile flares for these four isolates increased and then remained relatively stable between 48 and 72 h (at 48 h, MT5066, 71.9%; SE838, 100%; SS235, 59.4%; MT5065, 18.8%) (Fig. 7B). The remaining two isolates, MAM30 and MT5065, failed to develop motile flares throughout the course of

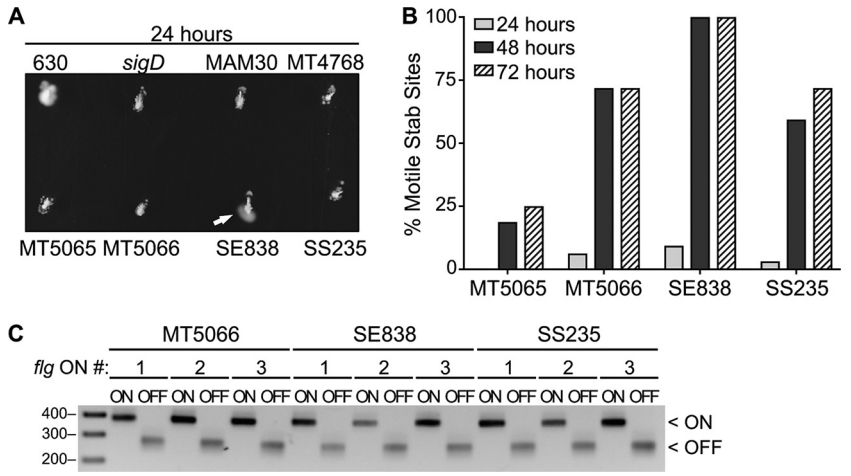

FIG 7 Low-frequency recovery of *flg*-on bacteria from select 012 ribotype isolates. (A) Swimming motility after 24 h. A representative image with a motile flare (white arrow) is shown. (B) The isolates differ in the frequencies of recovery of motile revertants. Shown are pooled data from two independent experiments, each examining 16 biological replicates ($n = 32$). The frequency of motile flares cumulative for each time point is shown, with those detected at 72 h including those from 48 h. (C) Orientation-specific PCR products using the indicated strains as templates. All nine recovered *flg*-on isolates derived from *flg*-off MT5066, SE838, and SS235 yielded PCR products indicating both *flg*-on and *flg*-off populations. Data are representative of three independent experiments.

the experiments with standard and concentrated inocula throughout the course of experiments (data not shown).

To confirm that the bacteria from the edge of the flares regained motility through inversion of the flagellar switch to the on orientation, we arbitrarily chose and isolated bacteria from three motile flares each for MT5066, SE838, and SS235 and subjected them to orientation-specific PCR and sequenced the flagellar switch (Fig. 1B). All nine motile isolates yielded a product for the *flg*-on orientation, indicating that DNA inversion occurred in these strains, yet an *flg*-off product was also observed (Fig. S3) (Fig. 7C). This result is in contrast to results with the JIR8094 MD 1 to 3, for which only the *flg*-on orientation was detected (Fig. 2B). In almost all cases, the intensity of the *flg*-on band was greater than that of the *flg*-off band, suggesting that the majority of the population was *flg* on. Sequencing results confirmed flagellar switch inversion to the on orientation for all nine *flg*-on derivatives and 100% sequence identity with the strain 630 flagellar switch. We noted that in all three *flg*-on derivatives of the parent SE828 strain, the RIR length increased from 22 bp to 23 bp (Fig. S3). By qPCR, we found that >99.99% of MT5066, SE838, and SS235 motile isolates were *flg* on, whereas parent isolates, MAM30, MT4768, and MT5065, were <0.005% *flg* on (Table 2). These results indicate that the flagellar switch remains reversible in MT5065, MT5066, SE838, and SS235 as in JIR8094, though inversion occurs at a lower frequency than in strain R20291 (14). In addition, MAM30 and MT4768 may either contain mutations that abolish swimming motility or require unique conditions for expression and production of flagella for swimming motility.

To determine whether flagellar switch inversion to the on orientation was sufficient to restore *flg*-on phenotypes in these strains, we assayed the original ribotype 012 isolates and their *flg*-on derivatives for swimming motility, FliC production, and toxin production (14). As expected, all three *flg*-on derivatives of each strain showed motility compared to their respective nonmotile parent strains (MT5066, SE838, and SS235) (Fig. 8A). Both FliC and TcdA protein levels were visibly higher in cell lysates of the *flg*-on derivatives relative to levels of the *flg*-off MT5066, SE838, and SS235 strains (Fig. 8B and C). These results demonstrate that the flagellar switch is capable of inversion in some but not all clinical and environmental ribotype 012 isolates and reiterate the regulatory link between toxin and flagellar gene expression via SigD. In addition, the ribotype 012

TABLE 2 Frequency of flagellar switch orientations in *C. difficile* ribotype 012 clinical and environmental isolates

Strain	Frequency of orientation[a]	
	% *flg* on	% *flg* off
MAM30	0.0006 ± 0.0008	99.9994 ± 0.0008
MT4768	0.0010 ± 0.0011	99.9990 ± 0.0011
MT5065	0.0001 ± 0.0001	99.9999 ± 0.0001
MT5066	0.0003 ± 0.0002	99.9997 ± 0.0002
SE838	0.0002 ± 0.0002	99.9998 ± 0.0002
SS235	0.0003 ± 0.0004	99.9997 ± 0.0004
MT5066 *flg*-on 1	99.9989 ± 0.0006	0.0011 ± 0.0006
MT5066 *flg*-on 2	99.9992 ± 0.0003	0.0008 ± 0.0003
MT5066 *flg*-on 3	99.9991 ± 0.0007	0.0009 ± 0.0007
SE838 *flg*-on 1	99.9988 ± 0.0009	0.0012 ± 0.0009
SE838 *flg*-on 2	99.9998 ± 0.0002	0.0002 ± 0.0002
SE838 *flg*-on 3	99.9998 ± 0.0001	0.0002 ± 0.0001
SS235 *flg*-on 1	99.9988 ± 0.0011	0.0012 ± 0.0011
SS235 *flg*-on 2	99.9993 ± 0.0002	0.0007 ± 0.0002
SS235 *flg*-on 3	99.9989 ± 0.0003	0.0011 ± 0.0003

[a]Frequency was calculated as the percentage of the genes in the indicated orientation compared to the total population. Means ± standard deviations from four independent samples are shown.

strains exhibit heterogeneity in their ability to phase vary, which may be attributed to differences in the sequences of the inverted repeats flanking the flagellar switch.

DISCUSSION

Our prior study characterizing flagellum and toxin phase variation in *C. difficile* focused on the epidemic-associated 027 isolate R20291. In this strain, phase variation occurred readily *in vitro*, and the strain consisted of a mixture of *flg*-on and -off bacteria. However, we noted that the ribotype 012 strain 630, which was originally isolated from a patient with *C. difficile* infection, existed primarily as *flg*-on bacteria. Here, we asked whether the 630 lineage is unusual or whether reduced phase variation is a broader attribute of ribotype 012 strains. Strain 630's nonmotile JIR8094 derivative primarily exists as *flg*-off bacteria, and phase variation to *flg* on occurs with low frequency. Our survey of six clinical and environmental ribotype 012 isolates of *C. difficile* indicates that within the 012 ribotype there exists a range of abilities to phase vary flagellum and toxin production, at least from the *flg*-off to -on states. These results are summarized in

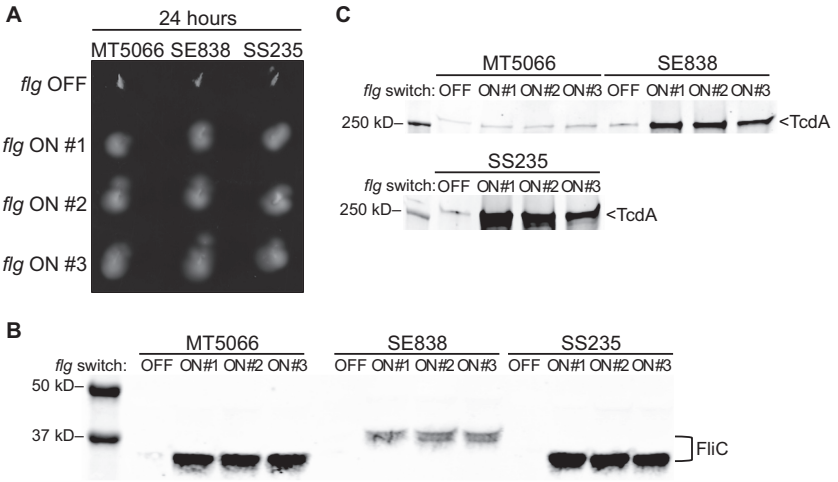

FIG 8 Characterization of select *flg*-on ribotype 012 isolates. (A) Swimming motility assay after 24 h. All nine recovered *flg*-on isolates were motile at 24 h compared to their nonmotile *flg*-off MT5066, SE838, and SS235 parents. Data are representative of three independent experiments with five biological replicates for each strain. (B and C) Detection of FliC or TcdA in the indicated strains by Western blotting. Data are representative of at least two independent experiments.

TABLE 3 Summary of genotypes and phenotypes of strains analyzed in this study

Strain[a]	Flagellar switch			
		Phenotype[c]		
	Genotype[b]	Motility	Toxin	Frequency[d]
R20291	On/off	++/−	++/−	High
630	On	++	++	Low
630Δ*erm*	On	++	++	Low
630E/JIR8094	Off	−	−	Low
JIR8094 MDs	On	+	+	Low
MAM30	Off	−	−	Low
MT4768	Off	−	−	Low
MT5065	Off	−	−	Low
MT5066	Off	−	−	Low
MT5066 MDs	On	++	+	Low
SE838	Off	−	−	Low
SE838 MDs	On	++	++	Low
SS235	Off	−	−	Low
SS235 MDs	On	++	++	Low

[a]All strains except for R20291 were ribotype 012; strain R20291 was ribotype 027.
[b]Flagellar switch orientation in >99 % of the population.
[c]The presence of the phenotype is indicated as follows:− −, full;− , partial;− , absence of the phenotype.
[d]Frequency at which the minority (on or off) orientation is found in the total population sampled (summarized from Tables 1 and 2).

Table 3. This work represents the first examination of flagellar and toxin phase variation within a ribotype family, and our results indicate potentially diverse patterns of phase variation across *C. difficile* strains.

We found that the laboratory-adapted strain JIR8094 contains the flagellar switch in the off orientation, which is consistent with its nonmotile and toxin-attenuated phenotypes. We were able to isolate rare motile derivatives of JIR8094. These were determined to have inverted the flagellar switch to the on orientation, suggesting that the recovery of motility was attributable to flagellar switch inversion. However, phenotypic characterization of these motile derivatives of JIR8094 indicated that motility and toxin production were only partially recovered compared to levels in the original 630 strain (JIR8094 parent). However, JIR8094 contains 11 polymorphisms compared to the sequence of 630 (23), and we found that restoring expression of one of the affected genes, *topA*, was sufficient to fully restore FliC production and motility. This finding indicates that both loss of *topA* and inversion of the flagellar switch from on to off negatively affected flagellar gene expression in JIR8094 and underscores previously stated cautions in using and interpreting results from research done with JIR8094. For example, one study using strain JIR8094 indicated that TcdA is dispensable for virulence in the hamster model (30), while another using 630−*erm* showed that both TcdA and TcdB are sufficient for eliciting disease in hamsters (31). The initial studies suggesting that TcdA was dispensable for infection may have been due to the *flg*-off status of JIR8094. A *tcdA* mutation in the background of a motile derivative of JIR8094 could be attenuated for virulence in the hamster model, supporting a role for TcdA.

The conditions that favor inversion from *flg* on to off in strain 630 may be gleaned from how JIR8094 was generated, specifically through serial passage on solid medium (24). In our studies with R20291, we observed a bias toward the *flg*-off orientation during growth on nutrient-rich solid medium over several days, while switch orientation appeared stable during growth in the same liquid medium (14). In contrast to JIR8094, the 630−*erm* strain was generated by passaging 630 in nonselective liquid medium until a spontaneous erythromycin-sensitive isolate was identified, and the flagellar switch remained in the *flg*-on orientation, unlike JIR8094 (25). The ability to bias a phase-variable genetic switch in one orientation over another is common in other mucosal bacterial pathogens. For example, incubation in human urine biases the type I fimbrial switch to the off orientation in uropathogenic *Escherichia coli* (32). Anoxic conditions bias the fimbrial switch to the on orientation in both uropathogenic *Proteus*

mirabilis and *E. coli* (33). For *C. difficile*, factors that bias the flagellar switch orientation could be host factors sensed by gene products that influence recombination.

All six of the original ribotype 012 isolates, both clinical and environmental, existed primarily, and perhaps exclusively, in an *flg*-off state, both genotypically and phenotypically. Two strains, MAM30 and MT4768, appeared to be phase locked, as we were unable to recover *flg*-on bacteria upon growth in motility medium, which selects for outgrowth of motile bacteria. Although qPCR results suggest a subpopulation of *flg*-on bacteria in MAM30 and MT4768, conditions may be unfavorable for flagellar gene expression in these isolates. The remaining four strains varied in their propensity to switch to *flg* on. A limitation of our study is that it is uncertain whether the isolates were *flg* off at the time of collection or whether the strains switched during outgrowth in the laboratory. However, the fact that all six isolates from different patients and environments were *flg* off argues that our findings are not an artifact of *in vitro* work but a common feature in the *C. difficile* ribotype 012 family. Recent work found that in a patient simultaneously infected with three *C. difficile* isolates, the ribotype isolate 012 was *flg* on, providing evidence that not all ribotype 012 isolates are biased to *flg* off or that the isolate may have arisen from a stage of infection favoring *flg*-on bacteria (34). It is also possible that most ribotypes show a strong *flg*-off bias and lower inversion frequency, similar to the ribotype 012, and the ribotype 027 and 017 strains examined previously are outliers (14). A comprehensive analysis of strains representing a variety of ribotypes both *in vitro* and during infection will help determine the population dynamics of flagellum and toxin phase variation in *C. difficile*.

Inverted repeat length may affect recombination of the flagellar switch in the ribotype 012 strains. Our previous analysis of the flagellar switch with publicly available sequenced genomes of *C. difficile* indicated 21-bp inverted repeats in all strains. Interestingly, all of the clinical and environmental 012 ribotype isolates had both inverted repeats less than or greater than but not equal to 21 bp (see Fig. S2 in the supplemental material). In addition, the SE838 isolate had flagellar switch IRs of 23 and 22 bp, but its *flg*-on derivatives had an RIR of 23 bp, suggesting some flexibility in the ability of the recombinase RecV to interact with these inverted repeats. It is also possible that a recombination directionality factor (RDF) is required for flagellar switch inversion in *C. difficile* strains that harbor nonideal inverted repeats and that the RDF is produced only under certain environmental conditions (35). The involvement of an RDF may allow precise control over when to promote recombination at the flagellar switch, similar to the contribution of an RDF that contributes to the excision of a DNA element and promotes sporulation (36). Alternatively, changes to repeat length could reduce affinity for RecV and perhaps allow recombination by another recombinase. Current work seeks to identify a role for inverted repeat sequence and length in affecting flagellar switch inversion frequency in both directions in all ribotypes.

Flagellar and toxin phase variation in *C. difficile* creates population heterogeneity that has the potential to influence diagnosis, disease progression, and transmission. Characterization of flagellar and toxin phase variation in multiple ribotypes of *C. difficile* could provide insight into factors that influence flagellar switch inversion and find correlations between diarrheal disease symptoms and switch orientation. Identifying surface and exported proteins in *C. difficile* that are consistently produced and not subject to phase-variable expression could serve as better diagnostic and therapeutic targets to ensure targeting of the entire population.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains used in this study are listed in Table S1 in the supplemental material. *C. difficile* strains were cultivated statically at 37°C in brain heart infusion medium (Becton Dickinson [BD]) supplemented with 5% yeast extract (BHIS) or tryptone yeast (TY) medium unless otherwise specified. All *C. difficile* growth was done anaerobically in a Coy anaerobic chamber with an atmosphere of 90% N_2, 5% CO_2, and 5% H_2. *E. coli* was cultured at 37°C under aerobic conditions in Luria-Bertani (LB) medium. For selection of plasmids in *E. coli*, 100 μg/ml ampicillin (Amp) and/or 10 μg/ml chloramphenicol (Cm) was used. Kanamycin (Kan; 100 μg/ml) was used to select against *E. coli* in conjugations. For maintenance of plasmids in *C. difficile*, 10 μg/ml thiamphenicol (Tm) was used.

Characterization of *C. difficile* ribotype 012 clinical and environmental isolates. The clinical and environmental isolates of *C. difficile* used are part of the F ribotype database (37). The strains were ribotyped by fluorescent PCR and sequencing of rRNA genes as previously described (37). The flagellar switch orientation was determined by PCR amplification of the region and sequencing of the products using primers R591 and R857.

Orientation-specific PCR. The flagellar switch orientation was determined by PCR using two primer sets that distinguish the orientation of the switch (14, 38). Primers were designed using the published genomes sequences for strains 630 (NCBI accession number AM180355) and R20291 (NCBI accession number FN545816.1). Primer sequences are listed in Table S2. For the 630 lineage and other ribotype 012 isolates, R1751 and R857 were used to detect the on orientation, and R1752 and R857 were used to detect the off orientation. For R20291, primers R1614 and R857 were used to detect the on orientation, and R1615 and R857 were used to detect the off orientation. PCR products were separated on a 2% agarose gel and stained with ethidium bromide (EtBr) for imaging using a G:Box Chemi imaging system.

Motility assays. Swimming motility was assayed as described previously (14, 27). Briefly, individual colonies were grown in TY broth overnight and then diluted 1:50 in fresh BHIS medium and grown to an optical density at 600 nm (OD_{600}) of ~1.0. In standard assays, 2 μl of culture was inoculated into anaerobic motility medium (0.5× BHIS–0.3% agar, autoclaved for 30 min). In experiments aimed at identifying JIR8094 motile revertants, a range of volumes of overnight bacterial cultures were centrifuged to collect and concentrate bacterial cells. The cells were suspended in 100 μl of phosphate-buffered saline (PBS), and 2 μl was stabbed into soft-agar plates. Swimming motility was assayed by measuring the colony diameter after 24, 48, and 72 h of incubation. Two perpendicular measurements were averaged for each colony. Photographs shown were taken with the G:Box imaging system (Syngene). To measure the frequency of motile recovery in the ribotype 012 isolates, motility assays were performed as described above with 32 independent colonies in two independent experiments. A stab site positive for motility was identified by the presence of a motile flare.

Recovery of motile derivatives of parent JIR8094 and select ribotype 012 isolates. After incubation in motility agar as described above, motile bacteria were collected from the edge of an expanded colony using a sterile loop and passaged onto BHIS agar for recovery. All isolates were confirmed to be motile by independently assaying swimming through motility medium, and the orientation of the flagellar switch was determined by PCR as described above.

Microscopy. For transmission electron microscopy, colonies of *C. difficile* were grown in TY medium overnight, diluted into fresh BHIS medium, and grown to an OD_{600} of ~1.0. Bacteria were washed in Dulbecco's PBS (Sigma) prior to suspension in PBS–4% paraformaldehyde for fixation for 1 h in an anaerobic chamber. Cell suspensions were adsorbed onto Formvar/copper grids, washed with water, and stained for 30 s in two sequential drops of aqueous uranyl acetate (1.5%). Samples were observed with a JEM-1230 transmission electron microscope operating at 80 kV (JEOL USA, Peabody, MA), and digital images were acquired using an Orius SC1000 charge-coupled-device (CCD) camera and Microscopy Suite 3.0 software (both from Gatan, Inc., Pleasanton, CA).

Detection of TcdA production by Western blotting. Overnight cultures were grown in TY broth and then diluted 1:50 in fresh medium. Strains were grown until late stationary phase (OD_{600} of 1.8 to 2.0) for 24 h and normalized to the same density, and then cells were collected by centrifugation. Bacteria were lysed by suspension in SDS-PAGE buffer and heating to 100°C for 10 min. Samples were electrophoresed on 4 to 15% TGX gels (Bio-Rad) and then transferred to nitrocellulose membranes. Membranes were stained with Ponceau S (Sigma) to determine equal loading and imaged using a G:Box Chemi imaging system. TcdA was detected using mouse anti-TcdA primary antibodies (Novus Biologicals), followed by goat anti-mouse IgG secondary antibody conjugated to DyLight 800 4× polyethylene glycol (PEG) (Invitrogen). Blots were imaged using an Odyssey imaging system (Li-Cor). All strains were assayed in at least three independent experiments.

Detection of FliC production by Western blotting. Overnight cultures were grown in TY broth with 10 μg/ml Tm and then diluted 1:50 in fresh BHIS medium with Tm and anhydrotetracycline at 20 ng/ml. Strains were grown for 12 to 16 h and normalized to the same density, and then cells were collected by centrifugation. Bacteria were lysed, electrophoresed, transferred, and evaluated by Ponceau S staining as described above. FliC was detected using serum raised in hamsters against recombinant FliC from *C. difficile* VPI10463 (39), followed by goat anti-hamster IgG secondary antibody conjugated to DyLight 800 (Novus Biologicals). All strains were assayed in two independent experiments.

RNA extraction, cDNA synthesis, and quantitative reverse transcriptase PCR. *C. difficile* was grown in TY medium overnight, diluted in fresh medium, and grown until mid-exponential phase (OD_{600} of ~1.0) or stationary phase (OD_{600} of 1.8 to 2.0), as indicated in Fig. 3 and 4. Cells were collected by centrifugation, and RNA was isolated as described previously (14, 27). RNA was treated with DNase I (Ambion), and cDNA was then synthesized using a high-capacity cDNA reverse transcription kit (Thermo Fisher). qRT-PCR was done using SensiMix SYBR and fluorescein kit reagents (Bioline). The primers used were named following the convention gene-qF and gene-qR (Table S2). Data were normalized using the $\Delta\Delta C_T$ (where C_T is threshold cycle) method, with *rpoC* serving as the reference gene. At least four independent samples were included.

Generation of *topA* expression strains. The *topA* gene was amplified from 630 genomic DNA using primers R2302 and R2303, which introduced SacI and BamHI restriction sites, respectively. After digestion with these enzymes, the *topA* fragment was ligated into similarly digested pRPF185, which allows ATc-inducible expression of the cloned gene (40). Ligation products were transformed into *E. coli* DH5α. Chloramphenicol-resistant clones were collected and screened by PCR with R2302 and R2303. Clones were confirmed by sequencing of the insert. The resulting pRPF185::*topA*

construct was introduced into *C. difficile* 630, JIR8094, and the motile derivatives of JIR8094 by conjugation via HB101/pRK24 as previously described (27, 41, 42). Transconjugants were selected on agar medium with Tm and Kan and confirmed by PCR with plasmid-specific primers R1832 and R1833. To induce expression of *topA*, anhydrotetracycline was added at a final concentration of 20 ng/ml to motility medium to assay swimming motility, and TY medium was used for assaying TcdA production by Western blotting.

Quantitative PCR for flagellar switch orientation. To determine the frequency of the *flg*-on and -off orientations in *C. difficile* ribotype 012 laboratory-adapted, clinical, and environmental isolates, four biological replicates of each were grown in BHIS medium to an OD_{600} of ~1.0, and chromosomal DNA was extracted as previously described (41). In all reaction mixtures, SYBR green real-time qPCR reagents (Thermo Fisher) were used, with primers at a final concentration of 500 nM and an annealing temperature of 55°C. Primers R2412 and R2414 were used for detection of the on orientation, and primers R2413 and R2414 were used for detection of the off orientation. Quantitative PCR was done using 20 ng of DNA from each strain. DNA copy number was calculated using the $\Delta\Delta C_T$ method, with the *rpoC* gene (primers R850 and R851) as the reference gene for DNA copy number as previously described (14, 17).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00056-18.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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