




Type IV Pili Promote *Clostridium difficile* Adherence and Persistence in a Mouse Model of Infection

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ABSTRACT Cyclic diguanylate (c-di-GMP) is a second messenger that regulates the transition from motile to sessile lifestyles in numerous bacteria and controls virulence factor production in a variety of pathogens. In *Clostridium difficile*, c-di-GMP negatively regulates flagellum biosynthesis and swimming motility and promotes the production of type IV pili (TFP), biofilm formation, and surface motility *in vitro*. Flagella have been identified as colonization factors in *C. difficile*, but the role of TFP in adherence to host cells and in colonization of the mammalian gut is unknown. Here we show that c-di-GMP promotes adherence to epithelial cells *in vitro*, which can be partly attributed to the loss of flagella. Using TFP-null mutants, we demonstrate that adherence to epithelial cells is partially mediated by TFP and that this TFP-mediated adherence requires c-di-GMP regulation. In a mouse model of colonization, the TFP-null mutants initially colonized the intestine as well as the parental strain but were cleared more quickly. Moreover, compared to the parent strain, *C. difficile* strains lacking TFP were particularly deficient in association with the cecal mucosa. Together these data indicate that TFP and their positive regulation by c-di-GMP promote attachment of *C. difficile* to the intestinal epithelium and contribute to persistence of *C. difficile* in the host intestine.

KEYWORDS *Clostridioides difficile*, colonization, adhesin, type IV pili, c-di-GMP, riboswitch, TcdR, *Clostridioides*

Clostridium difficile, recently reclassified as *Clostridioides difficile* (1), is a spore-forming, obligate anaerobe responsible for diarrheal diseases resulting in substantial morbidity and mortality (2). *C. difficile* infections (CDI) usually occur following antibiotic therapy; however, community-associated *C. difficile* infections in patients without recent antibiotic use are increasingly common (3). Moreover, ~20 to 30% of CDI patients experience at least one recurrence of CDI after cessation of treatment, with additional recurrences becoming increasingly likely (4–6). Antibiotic treatment facilitates *C. difficile* infection by disrupting the normally protective microbiota, reducing competition from other bacteria, and altering the bile salt profile in the gut, allowing more-efficient germination and outgrowth of *C. difficile* (7–10). Actively growing *C. difficile* may produce one or both of the toxins TcdB and TcdA, which glucosylate Rho family GTPases in target cells, ultimately resulting in the disruption of the intestinal epithelium and the characteristic inflammation of CDI (11–13). Despite being essential for disease development, TcdA and TcdB are not required for colonization of humans or animal models of CDI (14–17). The factors involved in *C. difficile* colonization and persistence within the gut are largely unknown.

One of the best-studied colonization factors in *C. difficile* is the flagellum. In the epidemic-associated *C. difficile* strain R20291 (18), a mutation in *fliC*, which encodes flagellin, reduced adherence to Caco-2 epithelial cells *in vitro* (19). Additionally, *fliC* mutant bacteria were outcompeted by the parental strain in mouse coinfection exper-

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iments (19). In strain R20291, a point mutation in the flagellar motor gene *motB*, which results in bacteria that produce paralyzed flagella, did not influence colonization or attachment to the cecum in mice, indicating that the flagellum itself functions in adherence (19). However, in strain 630 Δ erm, an erythromycin-sensitive derivative of *C. difficile* strain 630 (20), mutations in *fliC* and *fliD* increased attachment to Caco-2 cells, increased virulence in hamster models of infection, and led to a modest defect in mouse cochallenge infections with the parental strain (19, 21, 22). These strain-specific phenotypes may be attributable to differences in the abilities of strains R20291 and 630 Δ erm to phase vary the production of flagella (23–26).

Other cell surface proteins have been implicated in adherence to epithelial cells *in vitro*, including SlpA, FbpA, and Cwp66 (27–31). While most of these proteins have not been shown to alter colonization of *C. difficile* in animal models, a mutant lacking the putative fructose binding protein FbpA displayed a modest defect in cecal colonization in a mouse model of *C. difficile* infection (30). Given these data and the lack of severe colonization defects reported for putative *C. difficile* colonization factors, it is likely that adhesion of *C. difficile* to the intestinal epithelium involves multiple, potentially redundant factors. Type IV pili (TFP) are involved in a number of bacterial behaviors in other species, including surface attachment, surface-based twitching motility, biofilm formation, and cell-cell interactions (32–34). In many Gram-negative pathogens, TFP contribute to adherence to both primary and transformed host cell lines (35–38). Accordingly, TFP are required for optimal colonization in animal models for a number of pathogens, such as *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Escherichia coli*, and others (39–43). There are a number of mechanisms by which TFP machinery may enhance colonization, including direct adhesion to host cells (44), promotion of microcolony formation (45), and epithelial cell invasion (46). In Gram-positive bacteria, TFP have not been as well studied. Genes encoding TFP are rare in most bacilli but are nearly ubiquitous among clostridia (47). TFP are required for gliding motility in *Clostridium perfringens* (48). Ectopic expression of a pilin gene from *C. perfringens* in *Neisseria gonorrhoeae* increased attachment to mouse myoblasts, suggesting a role in adherence for TFP in *C. perfringens* (49).

TFP genes have been found in every sequenced strain of *C. difficile*, and a comparative phylogenomics analysis of *C. difficile* indicates that TFP genes are among the set of core genes for the species (50, 51). Work from our lab and others revealed that TFP in *C. difficile* are critical for a number of bacterial behaviors, particularly in response to increased levels of the second messenger cyclic diguanylate (c-di-GMP) (52–54). In many bacterial species, c-di-GMP regulates the switch between motile and nonmotile states (55, 56). Likewise in *C. difficile*, c-di-GMP negatively regulates flagellar swimming motility and positively regulates TFP biosynthesis and TFP-dependent behaviors (52, 53, 57). Regulation occurs via c-di-GMP-specific riboswitches upstream of the respective flagellum and TFP biosynthetic operons, where the flagellar riboswitch is an “off switch” and the TFP riboswitch is an “on switch” (53, 57–59). An insertional mutation in the major pilin gene *pilA1* or in *pilB1*, which encodes the pilus assembly ATPase, results in bacteria that lack TFP (52, 53, 60). The *pilA1* and *pilB1* mutants display reduced autoaggregation, surface motility, and biofilm formation under conditions of high c-di-GMP (52, 53). While the contributions of biofilm formation and surface growth to *C. difficile* infection are unclear, bacterial mats have been observed in mouse models of *C. difficile* infection (61, 62).

Based on the known roles of TFP in host colonization by other pathogens and the importance of TFP to autoaggregation and surface behaviors of *C. difficile*, we hypothesized that TFP contribute to host cell attachment and colonization during *C. difficile* infection. Moreover, we postulated that c-di-GMP positively regulates TFP-mediated interactions with host tissues. In this study, we found that high c-di-GMP promotes early attachment to a variety of epithelial cell types *in vitro* and that TFP may be important for maintenance of adherence to epithelial cells. Using an antibiotic-treated mouse model, we further demonstrated that *C. difficile* mutants lacking TFP initially colonize as well as the parent strain but are eliminated more quickly from the intestine.

This study provides evidence that TFP promote adherence of *C. difficile* to epithelial cells and are important for the persistence of *C. difficile* in the mammalian intestinal tract.

RESULTS

c-di-GMP promotes attachment of *C. difficile* to intestinal epithelial cells. Work from our lab and others recently demonstrated that high intracellular c-di-GMP concentrations in *C. difficile* promote autoaggregation, biofilm formation, and surface motility (52, 53, 59). Because c-di-GMP increased both interbacterial interaction and attachment to a surface, we hypothesized that c-di-GMP also promotes attachment to epithelial cells. To test this hypothesis, we performed bacterial attachment assays under anaerobic conditions using monolayers of HT-29 and Caco-2 human intestinal epithelial cells. We used a previously described strategy to artificially increase intracellular concentrations of c-di-GMP in *C. difficile*, using the nisin-inducible expression of *dccA*, which encodes a *C. difficile* diguanylate cyclase (57). This concentration of nisin and level of *dccA* expression did not substantially inhibit growth, particularly at the mid-exponential phase, when the bacteria were collected (53). *C. difficile* strains grown with or without nisin to induce *dccA* expression and stimulate c-di-GMP production were incubated with HT-29 or Caco-2 cells. Incubations were limited to 1 h under strict anaerobic conditions (required by *C. difficile*), as the monolayers begin to lose integrity by 4 h (see Fig. S1 in the supplemental material). Expression of *dccA* in 630 Δ erm led to 12- and 96-fold increases in attachment to HT-29 and Caco-2 cell monolayers, respectively, compared to *C. difficile* bearing the control vector grown with nisin induction (Fig. 1A and B). In contrast, expression of *dccA*^{mut}, which encodes a catalytically inactive variant of DccA, did not significantly alter attachment to either cell line. To confirm that the *C. difficile* isolates were associated with the epithelial cells in these assays, we visualized the bacteria attached to the epithelial cells using a Gram stain. While the bacteria harboring the vector and pDccA^{mut} were sparsely and individually distributed on the surface of the epithelial cell monolayers, the bacteria harboring pDccA were present in much higher numbers and also clustered together (Fig. 1C, D, and E). Together these data indicate that c-di-GMP promotes attachment of *C. difficile* to intestinal epithelial cells.

Negative regulation of flagellum biosynthesis by c-di-GMP contributes to increased attachment to epithelial cells *in vitro*. Dingle et al. previously showed that *C. difficile* 630 Δ erm isolates with mutations in *fliC* or *fliD*, encoding flagellin or flagellar cap, respectively, are more adherent to Caco-2 monolayers (21). We thus tested whether inhibition of flagellum biosynthesis by c-di-GMP is responsible for the increased attachment. We used *C. difficile* 630 Δ erm with an intron insertion in *sigD*, which encodes the flagellar sigma factor (63); this mutant was previously shown to lack flagella and swimming motility (23, 53, 64). We compared the attachment rates of the *sigD* mutant bearing vector alone or complemented with a plasmid containing *sigD* under the control of a nisin-inducible *cpr* promoter to the attachment rates of the parental strain bearing vector. Consistent with the previous results showing that aflagellate *C. difficile* isolates are more adherent to tissue culture cells, the 630 Δ erm *sigD* mutant containing vector alone adhered to HT-29 cells at significantly higher levels than those of the parental vector-bearing strain (Fig. 2A). Complementation with the *sigD* gene under the control of the nisin-inducible *cpr* promoter restored adherence to parental levels (Fig. 2A). We next evaluated the effect of increasing c-di-GMP in the absence of flagella by stimulating c-di-GMP synthesis in the *sigD* mutant background. Expression of *dccA* in the *sigD* mutant strain resulted in a further 3.8-fold increase in attachment over the *sigD* mutant bearing vector or expressing the *dccA*^{mut} allele (Fig. 2B). This result indicates that additional, *sigD*-independent factors mediate increased adherence to epithelial cells in response to c-di-GMP.

Type IV pili promote adherence of *C. difficile* to epithelial cells. Because c-di-GMP promotes TFP production, we hypothesized that TFP contribute to the observed increase in adherence to epithelial cells. To test this, we examined the ability of

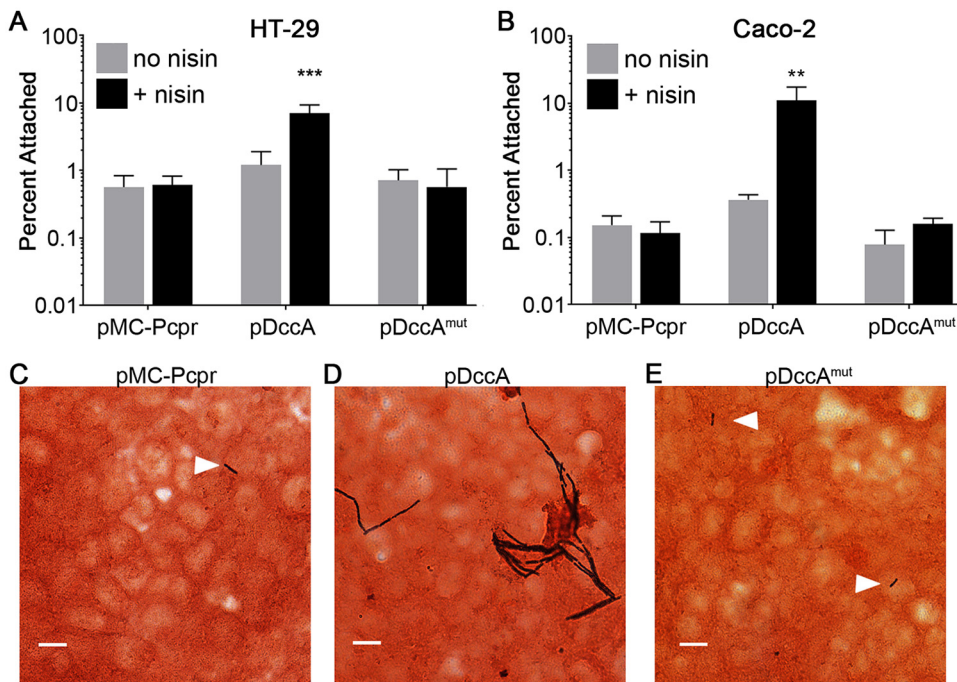


FIG 1 *c*-di-GMP promotes *C. difficile* attachment to epithelial cell monolayers *in vitro*. (A and B) *C. difficile* strain 630Δerm bacteria containing vector (pMC-Pcpr), pDccA, or pDccA^{mut} were grown to mid-log phase in BHIS with 10 μg/ml thiamphenicol to maintain the plasmids and 1 μg/ml nisin to induce expression as indicated. Bacteria were added to HT-29 (A) or Caco-2 (B) epithelial cell monolayers, and the mixture was centrifuged briefly and incubated for 1 h. Serial dilutions of bacterial inoculums and outputs were plated, and CFU were enumerated to determine the percentage of bacteria that remained attached. The means and standard deviations for 3 biological replicates are shown. Data were analyzed using one-way analysis of variance (ANOVA) and Tukey's posttest. **, *P* < 0.01; ***, *P* < 0.001. (C to E) *C. difficile* containing the indicated plasmids was grown to mid-log phase as described for panels A and B and added to HT-29 cell monolayers grown on coverslips. After 1 h of incubation, coverslips were rinsed with PBS and stained with crystal violet and safranin counterstain. *C. difficile* cells are stained dark purple (indicated by white arrows), and the HT-29 cell monolayers are stained red. Bars, 10 μm. Images are representative of 4 biological replicates for each strain.

630Δerm with a mutation in *pilA1* (encoding the major pilin) or *pilB1* (encoding the pilus biosynthesis ATPase) to adhere to HT-29 and Caco-2 cells. These mutants were previously shown to lack the ability to produce TFP under either low or high *c*-di-GMP conditions and to be deficient in TFP-dependent behaviors (52, 53). The *pilA1* and *pilB1*

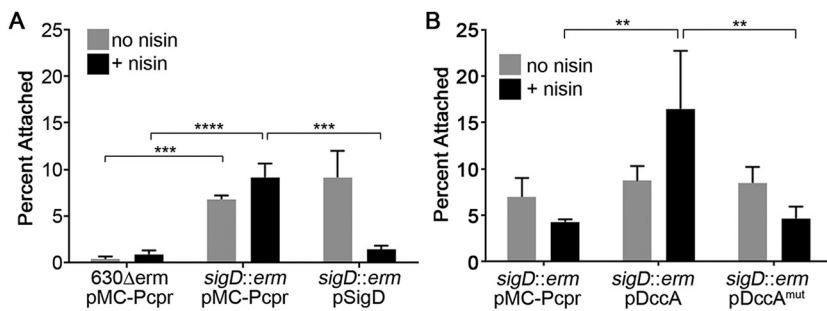


FIG 2 Aflagellate *C. difficile* adheres better than flagellate *C. difficile* to HT-29 cell monolayers. *C. difficile* cultures were grown to mid-exponential phase in BHIS with 10 μg/ml thiamphenicol and 1 μg/ml nisin to induce gene expression as indicated. Bacteria were added to HT-29 epithelial cell monolayers, centrifuged briefly, and incubated for 1 h. Serial dilutions of bacterial inoculums and outputs were plated to enumerate CFU and determine the percentages of bacteria that remained attached. (A) Mutation of *sigD* increases attachment to HT-29 cells; (B) increasing *c*-di-GMP in the *sigD* mutant further promotes attachment to HT-29 cells. Strain backgrounds and plasmids are indicated. The means and standard deviations for 3 biological replicates are shown. Data were analyzed by one-way ANOVA and Tukey's posttest. **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001.

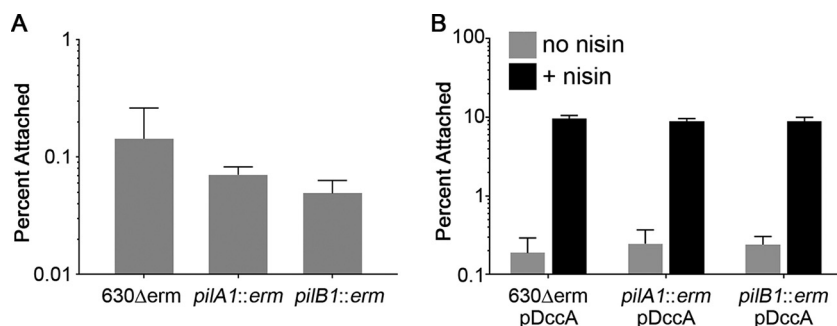


FIG 3 Attachment to HT-29 cell monolayers at 1 h is not dependent on type IV pili. Mid-exponential-phase cultures of *C. difficile* 630Δerm, *pilA1* mutant, or *pilB1* mutant were added to HT-29 epithelial cell monolayers, and the mixture was centrifuged briefly and incubated for 1 h. Serial dilutions of bacterial inoculums and outputs were plated and enumerated to determine the percentages of bacteria that remained attached. (A) Attachment of 630Δerm and TFP-null mutants to HT-29 cells; (B) attachment of 630Δerm and TFP-null mutants containing pDccA, grown in the presence or absence of 1 μg/ml nisin, to HT-29 cells. Shown are the means and standard deviations from 3 biological replicates. No statistically significant differences were observed between the parental strain and either mutant strain grown under the same conditions.

mutants showed somewhat reduced adherence to HT-29 cells compared to the parental strain, but the differences were not statistically significant (Fig. 3A). *C. difficile* with basal c-di-GMP levels produce relatively low numbers of TFP during growth in BHIS medium (37 g/liter Bacto brain heart infusion, 5 g/liter yeast extract) (53). We reasoned that increasing intracellular c-di-GMP by overexpressing *dccA*, which stimulates TFP biosynthesis (53), might reveal a role for TFP. However, elevating c-di-GMP augmented adherence equally in the parental and TFP-null strains (Fig. 3B).

While 1-hour adherence assays effectively measure the initial attachment of the bacteria to the epithelial cells, the relatively short incubation may miss factors that are important for maintained adherence or expansion of the attached bacteria. Indeed, many experiments evaluating bacterial adherence to epithelial cells use longer co-incubation periods, such as 24 h (65, 66). However, Caco-2 and HT-29 cell monolayers lose integrity after prolonged incubation under the strict anaerobic conditions required by *C. difficile* (Fig. S1). To assess adherence of *C. difficile* to epithelial cells over a longer time frame, we decided to use another epithelial cell line that is more tolerant of anaerobic conditions, Madin-Darby canine kidney (MDCK) epithelial cells (67). Whereas monolayers of either Caco-2 or HT-29 cells were substantially disrupted after as few as 4 h in the anaerobic chamber, MDCK cells maintained their monolayers after 24 h of incubation in the anaerobic chamber (Fig. S1). As with HT-29 and Caco-2 cells, TFP were dispensable for attachment to MDCK cells at 1 h (Fig. 4A). After 24 h of incubation, the *C. difficile* *pilA1* and *pilB1* mutants showed significantly reduced attachment to MDCK cells (Fig. 4B). Growth of *pilA1* and *pilB1* mutant bacteria was equivalent to that of the parental strain in Dulbecco's modified Eagle medium (DMEM) plus 10% fetal bovine serum (FBS) (see Fig. S2 in the supplemental material). The mutations also did not affect flagellum-based swimming motility (see Fig. S3 in the supplemental material).

To confirm that the reduced attachment to MDCK cells was due to the lack of TFP, we complemented the *pilA1* mutation by ectopic expression of *pilA1*. For expression of *pilA1*, we used the native promoter and leader sequence (5' untranslated region [UTR]) containing the c-di-GMP-sensing riboswitch. Ectopic expression of *pilA1* from its native promoter (pPilA1) partially restored attachment of the mutant bacteria to near that of the parental strain (Fig. 4C). Incomplete complementation is likely due to polar effects of the intron insertion on the downstream TFP biosynthesis genes (53). However, expression of *pilA1* under the control of the riboswitch containing a mutation (A70G) rendering it unable to bind and sense c-di-GMP (53) did not complement the effect of the *pilA* mutation on adherence to MDCK cells (Fig. 4C, pPilA1^{mut}). Together these data indicate that TFP contribute to host cell colonization at later time points, and sensing of c-di-GMP by the riboswitch is important in this process.

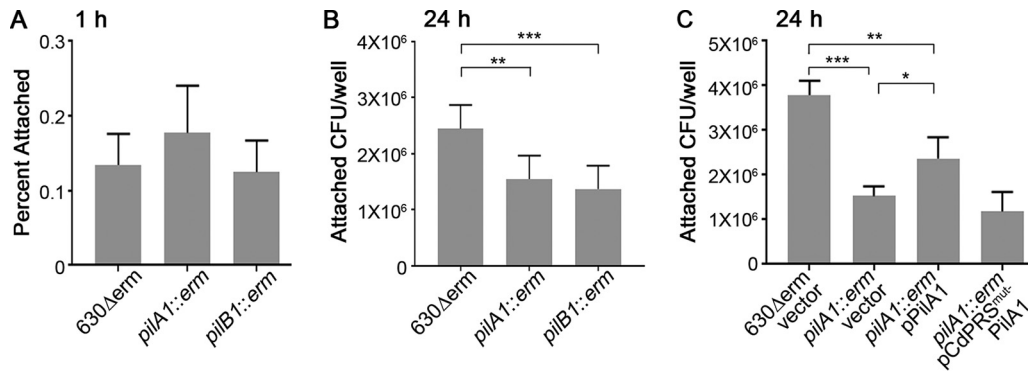


FIG 4 Type IV pili promote adherence to MDCK cell monolayers at 24 h. (A and B) Attachment of 630Δerm and TFP-null mutants after 1 h (A) or 24 h (B) of incubation with MDCK cell monolayers. In panel A, data are expressed as the percentages of the original inoculum recovered following incubation and PBS washes, with 3 biological replicates for each strain. In panel B, data are expressed as the total CFU recovered per well after 24 h of incubation, with 6 biological replicates. Data were analyzed using a one-way ANOVA with Dunnett’s test for multiple comparisons. (C) Complementation of the adherence defect of the *pilA1* mutant after 24 h of incubation with MDCK cell monolayers. Symbols represent values from individual animals, and error bars indicate the standard deviations. Data were analyzed by one-way ANOVA using the Holm-Sidak method to correct for multiple comparisons. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Type IV pili are dispensable at early stages of infection but are important for persistence in the host intestine.

In many bacterial pathogens, TFP play a role in colonization of the host and/or virulence (68). We therefore examined the contribution of TFP to the ability of *C. difficile* to colonize and cause disease in an antibiotic-treated mouse model of CDI (69). After treatment with a cocktail of antibiotics, C57BL/6 mice were inoculated by oral gavage with 10^5 spores of the 630Δerm parental strain, the *pilA1* mutant, or the *pilB1* mutant. The intestinal burden of *C. difficile* was monitored by collecting feces from the mice daily and enumerating colonies on TCCFA (see Materials and Methods), a medium that allows outgrowth of *C. difficile* spores while inhibiting the growth of other bacteria (70). The numbers of spores recovered from the feces of mice infected with a mutant or parent strain were similar for the first 3 days of the infection, suggesting that TFP are dispensable for initial colonization of the mouse large intestine (Fig. 5). By days 4 and 5, mice infected with the *pilB1* mutant showed a modest but statistically significant decline in CFU compared to those infected with 630Δerm bacteria. Both the *pilA1* and *pilB1* mutant-infected animals shed fewer CFU in feces on day 6, although the differences did not reach statistical significance due to high variability among mice infected with the *pilA1* or *pilB1* mutants. On day 7, spore counts

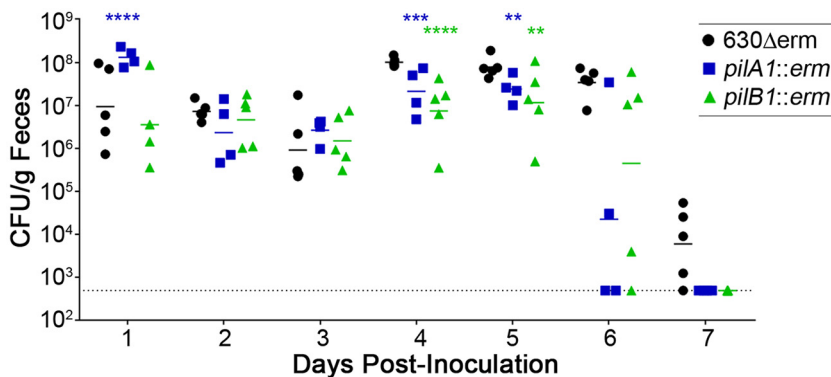


FIG 5 Single-strain infections of mice by 630Δerm, *pilA1* mutant, and *pilB1* mutant. Mice that had been pretreated with antibiotics were inoculated with 10^5 spores. Feces were collected daily, and serial dilutions were plated on *C. difficile* selective medium with spore germinant (TCCFA) to monitor the burden of *C. difficile*. Symbols represent values from individual animals. Data were analyzed by two-way ANOVA using the Holm-Sidak method to correct for multiple comparisons. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

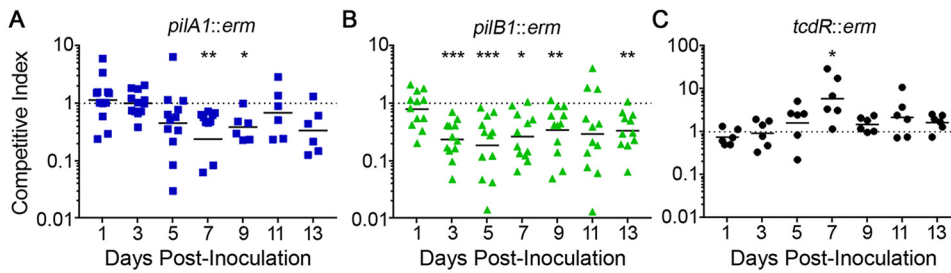


FIG 6 *C. difficile* pilus mutants are outcompeted by the parental strain in murine coinfections. Mice were inoculated with mixed inoculums containing $\sim 10^5$ spores of each strain. Feces was collected every 2 days starting 1 day postinoculation and plated on TCCFA (total spores) and TCCFA with erythromycin (mutant spores only) to determine bacterial burdens. Competitive indices (mutant to parent) for the competition between 630 Δ erm and *pilA1* mutant (A), 630 Δ erm and *pilB1* mutant (B), and 630 Δ erm and *tcdR* mutant (C). Data were excluded if in total fewer than 10 spores were recovered. Symbols represent CI values from individual animals, and error bars indicate the standard deviations. Data were analyzed by the Wilcoxon rank sum test comparing values to a hypothetical CI of 1 indicating no difference. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

for the TFP mutant infections were below the limit of detection in all mice, while all but one mouse infected with 630 Δ erm were still shedding spores above the limit of detection (Fig. 5). An independent mouse colonization experiment yielded similar results, but with slight changes in the timing of the decline in bacterial burden.

To control for variations between mice and to better capture subtle differences between strains, we performed coinfections with 630 Δ erm and either the *pilA1* or *pilB1* mutant. The *ermB* gene used to generate the *pilA1* and *pilB1* mutations allowed differentiation between the Erm-resistant mutants and the Erm-sensitive parent strain. A competition between the parent strain and a *tcdR* mutant, which expresses the glucosylating toxin genes at significantly lower levels (63, 71) but should be competent for colonization (14, 15, 17), was included as a control. On day 1 postinoculation, the competition indices (CI) were ~ 1 , indicating equal abilities to establish colonization. Throughout the 14-day experiment, the *pilA1* mutant was usually present in feces at numbers comparable to those of the parent strain; the exceptions are days 7 and 9, when significantly fewer *pilA1* mutant bacteria were recovered (Fig. 6A). In contrast, the *pilB1* mutant showed attenuated colonization after day 1, with CI significantly less than 1 on days 3, 5, 7, 9, and 13 (Fig. 6B). The control experiment using the *tcdR* mutant yielded CI equal to or somewhat greater than 1 (Fig. 6C), indicating that TcdR is dispensable for colonization in the mouse model and that the presence of the *ermB* cassette is not responsible for the fitness defect of the mutants.

To ensure that the observed defects in colonization for the TFP-null mutants are not specific to the 630 Δ erm background, we also examined the contribution of TFP to colonization and persistence in an epidemic-associated strain of *C. difficile*, the ribotype 027 strain R20291 (18). When inoculated as a coinfection with the parental strain, the R20291 *pilB1* mutant was initially recovered from feces in numbers comparable to those of the parental strain but was outcompeted by the parental strain on days 3 and 5 postinoculation (see Fig. S4 in the supplemental material). These data indicate that TFP are dispensable for initial establishment of colonization by multiple strains of *C. difficile* but support a role for TFP in maintaining colonization of the mouse intestine.

Type IV pili promote association of *C. difficile* with the cecal epithelium. We hypothesized that TFP enhance intestinal colonization by promoting attachment of the bacteria to the intestinal epithelium as observed *in vitro*. To test this, we compared the ratios of TFP-null and parent strain bacteria associated with the cecal mucosa and present in the lumen. Mice were coinoculated with the *pilB1* mutant and 630 Δ erm strains, and ceca were harvested at day 3, when the mutant first showed decreased recovery from feces (Fig. 6B). The luminal fraction consisted of the cecal contents combined with the contents of a single PBS wash. The remaining cecal tissue comprised the tissue-associated fraction. Homogenates of each fraction were plated on selective medium, and the CI were calculated separately. In the cecal lumen, the *pilB1* mutant

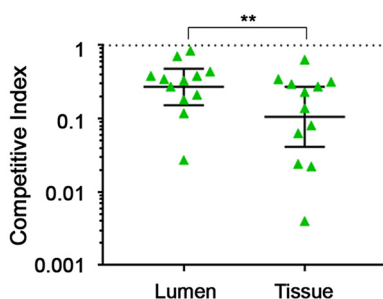


FIG 7 TFP promote association of *C. difficile* with the cecal epithelium. Mice were coinoculated with $\sim 10^5$ spores each of 630 Δ erm and *pilB1* mutant. Ceca were harvested anaerobically at day 3 postinoculation. Cecal contents and a 1-ml wash of PBS were combined into the luminal fraction. The tissue-associated fraction was obtained by homogenizing the remaining cecum. Serial dilutions of the luminal and tissue-associated fractions were plated on TCCFA (all *C. difficile* cells) and TCCFA with erythromycin (mutant *C. difficile*) to determine the bacterial burden of each strain. Symbols represent CI (mutant to parent) values from individual animals, and error bars indicate the standard deviations. Data were analyzed by the Wilcoxon rank sum test comparing the CI in the luminal fraction to the CI in the tissue-associated fraction. **, $P < 0.01$.

bacteria were recovered at a lower rate (geometric mean of CI = 0.27), but in the tissue-associated fraction the CI was significantly lower (geometric mean of CI = 0.12) (Fig. 7). These data indicate that the *pilB1* mutant bacteria are less likely to be found in close association with the epithelium, which may explain the defect in maintenance of colonization in the dual-strain infections and the lack of persistence in single-strain infections.

DISCUSSION

The mechanism by which *C. difficile* colonizes the host intestine is poorly understood, though multiple cell surface proteins and structures have been explored as possible adhesins. In *C. difficile*, c-di-GMP regulates the transition between motile and surface-associated states in part by negatively regulating flagellar gene expression and positively regulating TFP gene expression (52, 53). This study evaluated the contributions of c-di-GMP, flagella, and TFP to the ability of *C. difficile* to adhere to intestinal epithelial cells and the role of TFP in host colonization.

While previous work demonstrated a role for c-di-GMP in biofilm formation and autoaggregation of *C. difficile*, the effects of c-di-GMP signaling on the interaction between *C. difficile* and host cells had not been explored. *In vitro* adherence assays demonstrated that the second messenger c-di-GMP promotes attachment of *C. difficile* to HT-29 and Caco-2 colonic epithelial cells. Inhibition of flagellum biosynthesis by c-di-GMP is partially responsible for increased attachment, consistent with previous studies investigating the roles of flagellar proteins in *C. difficile* adherence (19, 21). It is possible that the presence of peritrichous flagella on the surface of *C. difficile* sterically interferes with the ability of adhesins on the bacterial surface to interact with the epithelial cells. In addition to removing the steric hindrance of the flagella, c-di-GMP may promote the presentation of other adhesins on the bacterial surface. Increasing intracellular c-di-GMP in a *sigD* mutant background led to a further increase in attachment, indicating that c-di-GMP regulates additional factors involved in attachment to epithelial cells. We hypothesized that TFP, which are positively regulated by c-di-GMP, function in this capacity. Our *in vitro* data indicate that TFP are not required for early attachment to epithelial cells but contribute to optimal prolonged adherence. Other putative adhesins regulated by c-di-GMP, such as CD2831 and CD3246, might promote early attachment of *C. difficile* to host cells (72, 73).

Our data also indicate that c-di-GMP signaling is required for promoting adherence by TFP. Restoration of adherence in the *pilA1* mutant required a functional c-di-GMP riboswitch controlling *pilA1* expression. Thus, c-di-GMP levels are high enough to support TFP production in these experiments. There is evidence that the interaction of

C. difficile with a surface leads to increased intracellular c-di-GMP levels (52) and that stimulation of c-di-GMP production may occur during interactions with host cells as well. c-di-GMP may also posttranslationally regulate TFP biosynthesis in *C. difficile*. Some TFP are associated with PilB ATPases containing a MshEN domain, which binds c-di-GMP (74, 75). This c-di-GMP binding motif is conserved in the *C. difficile* MshE orthologue, PilB1, and potentially contributes to the positive regulation of TFP in *C. difficile*, as was recently shown for *C. perfringens* PilB2 (76). Future work will examine the potential roles of two distinct mechanisms of TFP regulation by c-di-GMP.

In a mouse model of CDI, both *pilA1* and *pilB1* mutant bacteria were deficient in long-term colonization of the intestine. The defect was more pronounced for the *pilB1* mutant, suggesting that alternative pilins in addition to PilA1 are important for *C. difficile* persistence. The decreased recovery of the *pilB1* mutant in the tissue-associated fraction of the cecum suggests a role for TFP in promoting close association with the intestinal epithelium. We speculate that *C. difficile* bacteria embedded more deeply in the mucus and/or in direct contact with epithelial cells are less likely to be excreted, serving as a reservoir for persistence and disease recurrence. While the importance of autoaggregation, microcolony formation, and biofilm development in the host for *C. difficile* disease development is unclear, mats of *C. difficile* associated with the intestinal epithelium of *C. difficile*-monoassociated mice (61, 62) and in mucus-associated mixed-species communities in the cecum and colon (77) have been observed. Given their role in autoaggregation, TFP may facilitate or stabilize the formation of single or multispecies microcolonies, and ultimately biofilms, on the epithelium, rather than function as adhesins *per se*.

Cell monolayers in general are not representative of the diverse range of cell types and structures found in the intestinal mucosa. Several groups have been working toward producing more physiologically relevant tissue culture models of the intestine that could improve the understanding of *C. difficile* interactions with host cells (78–81). For example, organoids and enteroids grown from primary stem or progenitor cells preserve some of the structure of intestinal villi and are also made up of a diverse array of cell types, unlike monocultures of transformed cell lines. While valuable for studying the disruption of the epithelial cell barrier by *C. difficile* toxins (82), these models may not be ideal for studying putative colonization factors because these are closed systems. Models using differentiated stem cells grown on scaffolds might enable coculture of *C. difficile* and epithelial cells by allowing oxygenation of the host cells at the basolateral surface while preserving an anaerobic environment in the apical space (81). Improvements to these models will allow for a much better understanding of intestinal colonization and host interaction of *C. difficile* and other anaerobes. Factors that influence *C. difficile* colonization and persistence may prove valuable in improving the treatment and prevention of CDI, especially in light of the high recurrence rate among patients treated for CDI (4–6).

MATERIALS AND METHODS

Bacterial growth conditions. Strains and plasmids used in this study are listed in Table S1 in the supplemental material. Overnight cultures of *C. difficile* were grown in 2 ml of TY broth (30 g/liter Bacto tryptone, 20 g/liter yeast extract, 1 g/liter thioglycolate) in an anaerobic chamber (Coy Lab Products) with an atmosphere of 5% H₂, 5% CO₂, and 90% N₂. For experiments, *C. difficile* was diluted 1:100 in BHIS (37 g/liter Bacto brain heart infusion, 5 g/liter yeast extract) for growth unless otherwise specified. *E. coli* cultures were grown in Luria-Bertani medium (Miller) with appropriate antibiotics as needed. Unless otherwise specified, antibiotics were used at the following concentrations: thiamphenicol (Tm), 10 μg/ml; chloramphenicol (Cm), 10 μg/ml; ampicillin (Amp), 100 μg/ml; and kanamycin (Kn), 100 μg/ml. Where appropriate, nisin was added at a final concentration of 1 μg/ml to induce transcription from the *cpr* promoter.

Construction of *pilA1* complementation plasmids. To generate pPilA1 (pMC123::P_{*pilA1*}-RB-*pilA1*), the promoter, 5' UTR, and coding sequence of *pilA1* (CD630_35130) were amplified by PCR from 630Δerm genomic DNA using primers R1183 and R978 (see Table S2 in the supplemental material). This PCR product was digested with EcoRI and PstI, ligated into similarly digested pMC123, and transformed into DH5α. To generate pPilA1^{mut} (pMC123::P_{*pilA1*}-RB^{A70G}-*pilA1*), we generated a PCR product containing the above fragment with an adenine-to-guanine substitution at position 70 of the riboswitch using splicing by overlap extension (SOE). The upstream region of homology was PCR amplified from 630Δerm genomic DNA using primers R1183 and R1184, and the downstream region was amplified using primers

R1185 and R978. The two fragments were spliced together and amplified using R1183 and R978 and then cloned into pMC123 as above. Clones were confirmed by PCR and sequencing of the inserts. These pPilA1 and pPilA1^{mut} plasmids were introduced into the *pilA1* mutant via conjugation with HB101(pRK24) as described previously (57).

Attachment assays. HT-29 or Caco-2 human intestinal epithelial cells were cultured in DMEM supplemented with FBS at 10% (HT-29) or 20% (Caco-2). Tissue culture-treated 24-well plates (Corning) were seeded with approximately $\sim 10^5$ cells per well. Tissue culture cells were grown at 37°C in 5% CO₂ for 5 to 7 days until a confluent monolayer was achieved, with fresh medium added as needed. To avoid oxygen toxicity to *C. difficile*, prior to inoculation the intestinal cells were transferred to the anaerobic chamber, and the medium was removed and replaced with anaerobic DMEM containing the appropriate concentration of FBS. For 24-h attachment assays, MDCK cells grown in DMEM with 10% FBS were seeded into 24-well plates with 10^5 cells per well. Cells were grown at 37°C for 3 days in 5% CO₂ to allow the formation of confluent monolayers, with fresh medium added as needed.

C. difficile strains grown overnight (~ 16 h) in TY with appropriate antibiotics were diluted 1:50 in filter-sterilized BHIS with antibiotics. Nisin was added if necessary for induction of gene expression. At mid-exponential phase, the bacteria were diluted 1:10 in DMEM with FBS and vortexed. We previously showed that *C. difficile* does not significantly aggregate at this stage of growth (53). Each diluted culture (50 μ l) was added to 24-well plates seeded with HT-29, Caco-2, or MDCK cells and containing 450 μ l DMEM with FBS. The plates were sealed with tape, removed from the anaerobic chamber, and centrifuged for 10 min at 1,000 $\times g$ to sediment the bacteria onto the tissue culture cells. This centrifugation step was performed because c-di-GMP inhibits flagellum biosynthesis and swimming motility, which might influence the ability of some strains tested to reach the cell monolayers. The plates were transferred back into the anaerobic chamber and incubated at 37°C for 1 h (HT-29 and Caco-2 cells) or 24 h (MDCK cells). During the incubation, dilutions of the bacterial inoculums were plated to enumerate the bacteria added to each well. Following incubation, the medium was removed, and the wells were washed 3 times with 1 ml Dulbecco's phosphate-buffered saline (DPBS; Gibco) to remove nonadherent bacteria. After the final wash, the epithelial cells and attached bacteria were scraped from the plate and suspended in 500 μ l of DPBS by pipetting up and down until visible clumps were dispersed. Dilutions were plated on BHIS to enumerate the attached bacteria.

Microscopy. HT-29 or MDCK cells were seeded at $\sim 10^5$ cells per cm² on Thermanox (ThermoFisher) coverslips and grown for 5 days (HT-29) or 3 days (MDCK) in DMEM–10% FBS. Bacteria were grown and added to the tissue culture cells, and the mixtures were incubated as described above for the attachment assays. Following incubation, the monolayers were washed twice with 500 μ l PBS to remove unattached bacteria and then fixed by adding 500 μ l of ice-cold methanol for 5 min. Methanol was removed, and samples were then stained with a Gram stain kit (Becton Dickinson), mounted on slides, and imaged with an Olympus BX61 v2 microscope at a magnification of $\times 600$.

Animal experiments. All animal studies were done in compliance with protocols approved by the UNC-CH Institutional Animal Care and Use Committee.

C. difficile spore inoculums were generated by streaking several colonies of *C. difficile* onto 70:30 agar (83) and incubating them at 37°C for 3 days in an anaerobic chamber. The growth was suspended in 10 ml DPBS, removed from the anaerobic chamber, and left overnight at room temperature to allow the release of mature spores. Spores were purified from the suspensions by sucrose gradient as previously described (84). Spores were enumerated by plating serial dilutions on BHIS agar containing the germinant 0.1% sodium taurocholate (85).

Groups of 8- to 10-week-old female C57BL/6 mice were obtained from Charles River Laboratories. Beginning 7 days prior to inoculation, the mice were given a cocktail of antibiotics in their drinking water, provided *ad libitum* for 3 days as described previously (69). The antibiotics were provided at the following concentrations: kanamycin (400 μ g/ml), gentamicin (35 μ g/ml), colistin (850 units/ml), vancomycin (45 μ g/ml), and metronidazole (215 μ g/ml) (69). Four days prior to inoculation, mice were switched back to regular water for the remainder of the experiment. A single intraperitoneal injection of clindamycin (10 μ g/g body weight) was administered either 24 h prior to infection (single-strain infections) or 48 h prior to infection (competitions). The timing of the clindamycin injection was modified to 48 h prior to inoculation in the competition experiments to ensure that clindamycin concentrations did not favor growth of the mutants, which contain the *ermB* gene, which provides some resistance to clindamycin (86, 87). Mice were inoculated with 10^5 CFU of *C. difficile* spores (single-strain infections) or 2×10^5 total spores (coinfections) by oral gavage. Feces was collected in preweighed tubes every 24 or 48 h for 7 to 14 days, as indicated.

Fecal samples were weighed following collection and then suspended in 1 ml DPBS by vortexing. For single-strain infections, serial dilutions of fecal samples were plated on fructose agar containing 1 mg/ml sodium taurocholate, 16 μ g/ml cefoxitin, and 250 μ g/ml cycloserine (TCCFA). The burden of spores was calculated as CFU per gram of feces. For competition experiments, serial dilutions of fecal samples were plated on TCCFA to enumerate total spore burden, as well as TCCFA containing 2 μ g/ml erythromycin (for 630 Δ erm strains) or 20 μ g/ml lincomycin (for R20291 strains) to enumerate the CFU of mutant bacteria containing the *ermB* resistance gene. To calculate the competitive index (CI), the ratio of mutant to parental bacteria for each fecal sample, obtained with the formula (resistant CFU/[total CFU – resistant CFU])_{output}, was divided by the ratio of mutant to parental bacteria in the initial spore inoculum, obtained with the formula (resistant CFU/[total CFU – resistant CFU])_{input}. A CI of 1 indicates no difference in bacterial burden, and a CI of <1 indicates that the parental strain outnumber the mutant, while a CI of >1 indicates that the mutant bacteria outnumber the parental strain bacteria.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00943-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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