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Regulation of Type IV Pili Contributes to Surface Behaviors of Historical and Epidemic Strains of *Clostridium difficile*

Erin B. Purcell,[a] Robert W. McKee,[a] Eric Bordeleau,[b] Vincent Burrus,[b] Rita Tamayo[a]

Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA[a]; Département de Biologie, Faculté des Sciences, Université de Sherbrooke, Sherbrooke, QC, Canada[b]

ABSTRACT

The intestinal pathogen *Clostridium difficile* is an urgent public health threat that causes antibiotic-associated diarrhea and is a leading cause of fatal nosocomial infections in the United States. *C. difficile* rates of recurrence and mortality have increased in recent years due to the emergence of so-called "hypervirulent" epidemic strains. A great deal of the basic biology of *C. difficile* has not been characterized. Recent findings that flagellar motility, toxin synthesis, and type IV pilus (TFP) formation are regulated by cyclic diguanylate (c-di-GMP) reveal the importance of this second messenger for *C. difficile* gene regulation. However, the function(s) of TFP in *C. difficile* remains largely unknown. Here, we examine TFP-dependent phenotypes and the role of c-di-GMP in controlling TFP production in the historical 630 and epidemic R20291 strains of *C. difficile*. We demonstrate that TFP contribute to *C. difficile* biofilm formation in both strains, but with a more prominent role in R20291. Moreover, we report that R20291 is capable of TFP-dependent surface motility, which has not previously been described in *C. difficile*. The expression and regulation of the *pilA1* pilin gene differs between R20291 and 630, which may underlie the observed differences in TFP-mediated phenotypes. The differences in *pilA1* expression are attributable to greater promoter-driven transcription in R20291. In addition, R20291, but not 630, upregulates c-di-GMP levels during surface-associated growth, suggesting that the bacterium senses its substratum. The differential regulation of surface behaviors in historical and epidemic *C. difficile* strains may contribute to the different infection outcomes presented by these strains.

IMPORTANCE

How *Clostridium difficile* establishes and maintains colonization of the host bowel is poorly understood. Surface behaviors of *C. difficile* are likely relevant during infection, representing possible interactions between the bacterium and the intestinal environment. Pili mediate bacterial interactions with various surfaces and contribute to the virulence of many pathogens. We report that type IV pili (TFP) contribute to biofilm formation by *C. difficile*. TFP are also required for surface motility, which has not previously been demonstrated for *C. difficile*. Furthermore, an epidemic-associated *C. difficile* strain showed higher pilin gene expression and greater dependence on TFP for biofilm production and surface motility. Differences in TFP regulation and their effects on surface behaviors may contribute to increased virulence in recent epidemic strains.

Since the emergence in the early 21st century of so-called "hypervirulent" epidemic strains of *Clostridium difficile*, the intestinal pathogen is now one of the most common causes of nosocomial infections in the developed world (1–3). *C. difficile* is transmitted between patients in the form of dormant spores. During passage through the digestive tract, the spores germinate into actively growing vegetative cells upon exposure to specific nutrients, such as primary bile salts and amino acids, including glycine, cysteine, phenylalanine, and arginine (4–6). These cells do not readily colonize a healthy human colon, but disruption of the native microbiota creates a niche for *C. difficile*, making previous antibiotic therapy the major risk factor for *C. difficile* infection (CDI) (7). Vegetative *C. difficile* secretes glucosylating cytotoxins that inhibit host actin polymerization and disrupt the tight junctions that maintain the integrity of the intestinal epithelium. The toxins result in an inflammatory immune response, with symptoms ranging from diarrhea to pseudomembranous colitis or sepsis (7–10). CDI is recalcitrant to therapy with multiple antibiotics and has a high rate of recurrence, with 10 to 20% of patients experiencing reemergent symptoms after cessation of treatment (9, 11).

Infections caused by some strains associated with epidemics, such as those of ribotype 027, have higher rates of recurrence and death than those caused by historical *C. difficile* strains (3). The exact genetic and biochemical bases of the increased virulence of epidemic strains remain unclear. Differential regulation of toxin production, acquisition of an additional actin-specific binary toxin, higher levels of host inflammation, increased antibiotic resistance, and/or increased spore formation may contribute to the more severe disease caused by epidemic strains (10, 12–15). In addition, some of the genetic differences between historical and epidemic strains are located in genes that encode small RNAs rather than proteins, suggesting that some of the differences ob-

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Address correspondence to Rita Tamayo, rita_tamayo@med.unc.edu.

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served between strains may result from differences in gene regulation (16).

The small nucleotide second messenger cyclic diguanylate (c-di-GMP) has emerged as an important regulator of virulence genes in *C. difficile*. In many Gram-negative and Gram-positive bacteria, c-di-GMP regulates the transition between motile and sessile forms; c-di-GMP generally inhibits cellular motility and promotes biofilm formation (17). c-di-GMP signaling is predicted to play important roles in *C. difficile* adaptation to its environment, as nearly 1% of the historical *C. difficile* 630 (ribotype 012) genome is dedicated to c-di-GMP metabolism. *C. difficile* 630 encodes a total of 37 predicted diguanylate cyclase (DGC) and phosphodiesterase (PDE) enzymes, which synthesize and degrade, respectively, c-di-GMP (17, 18). The catalytic abilities of these enzymes have been independently assessed through heterologous expression in *Vibrio cholerae* and *Bacillus subtilis*, which indicated that the majority (21 and 27 enzymes, respectively) are catalytically active (19, 20). The remaining proteins may be active in *C. difficile* or under conditions not tested. Thirty-one of the 37 predicted DGCs and PDEs are conserved in the epidemic strain R20291 (ribotype 027), suggesting that the historical and epidemic strains employ similar, but not identical, c-di-GMP regulatory networks (19). c-di-GMP repression of flagellum-mediated swimming motility and toxin biosynthesis has been demonstrated in strain 630 and an erythromycin-sensitive derivative, 630Δ*erm*, in which predicted c-di-GMP metabolism genes are completely conserved (18, 21–23). To date, swimming motility is the only c-di-GMP-regulated trait that has been examined in R20291 (21).

In *C. difficile* 630/630Δ*erm*, c-di-GMP promotes the extracellular assembly of type IV pili (TFP) by positively regulating pilus gene expression (24). Regulation occurs via a c-di-GMP-responsive riboswitch that, in the presence of its ligand, promotes transcription elongation of *pilA1*, which encodes the major pilin subunit (24–26). In other bacterial species, TFP mediate surface motility, bacterial adhesion to surfaces and other bacteria, biofilm formation, and/or adherence to host cells (27–31). In *C. difficile*, pilus gene expression promotes autoaggregation, a process that has been proposed to contribute to biofilm formation in 630/630Δ*erm* (21, 24, 25). Consistent with this, *C. difficile* biofilm formation is also stimulated by elevated c-di-GMP (25).

Bacteria employ various mechanisms to move over surfaces, including host tissue. Some bacteria are capable of gliding over surfaces without the use of extracellular appendages, either by forming focal adhesion complexes with the substrate or by passively "sliding" apart during cell division (32). Many flagellated bacteria secrete lubricating molecules known as surfactants and increase flagellar biosynthesis in order to "swarm" across surfaces in multicellular groups (32). Some bacterial species can swarm only on soft, highly elastic surfaces and are immobilized by agar concentrations greater than 1%, while others can swarm readily on up to 3% agar (33). Other species display flagellum-independent twitching or gliding motility, in which cells move across surfaces through cyclic extension, tethering, and retraction of TFP. TFP-mediated motility has been studied almost exclusively in Gram-negative bacteria. Orthologues of TFP biosynthesis genes have been identified in clostridia and other Gram-positive species, and TFP-dependent gliding has been characterized in *Clostridium perfringens* (34–36).

In this study, we investigated the functions of TFP in surface behaviors of *C. difficile*. We uncovered roles for TFP in biofilm formation on plastic and motility on agar surfaces and observed differences in TFP-dependent phenotypes between the historical 630 and the epidemic R20291 strains. Elevated intracellular c-di-GMP promoted biofilm development and surface motility in both strains, but to varying degrees and with differing dependence on TFP. Moreover, R20291 and 630 differ in *pilA1* pilin gene expression. The differences are attributable to higher *pilA1* promoter activity in R20291 and to the ability of R20291 to accumulate c-di-GMP during surface-associated growth. Our results suggest that *C. difficile* surface sensing and behaviors involve c-di-GMP regulation of TFP formation, and these processes differ between strains.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Table S1 in the supplemental material describes the bacterial strains used in this study. *C. difficile* strains were grown at 37°C under anaerobic conditions in tryptone-yeast (TY) medium or in filter-sterilized brain heart infusion (BHI) medium supplemented with 5% yeast extract (BHIS), as indicated (5, 21, 37). *Escherichia coli* strains were grown in Luria-Bertani broth at 37°C with aeration. When appropriate, the following antibiotics were used: 100 μg/ml ampicillin (Amp), 10 μg/ml chloramphenicol (Cm), 10 μg/ml thiamphenicol (Tm), 100 μg/ml kanamycin (Kan), 20 μg/ml lincomycin (Linc), 2.5 μg/ml erythromycin (Erm), 8 μg/ml cefoxitin (Cef), and 250 μg/ml cycloserine (Cyc). Nisin was used for induction of gene expression at final concentrations of 0.01 to 5 μg/ml, as indicated.

Strain construction. Table S2 in the supplemental material lists the plasmids used in this study, and Table S3 in the supplemental material lists the primers. Disruption of the R20291 *pilB1* gene was performed with the ClosTron approach, using a targeted group II intron adapted for clostridia essentially as previous described, resulting in an insertion in the sense orientation at position 795 of the R20291 *pilB1* gene (24, 38, 39). The ClosTron plasmid was mated with *C. difficile* R20291, together with the helper plasmid pR702 (24). ClosTron integrants were selected on BHIS agar plates containing lincomycin. The intron insertion was confirmed by Southern blotting (see Fig. S1A in the supplemental material) and by PCR with primers CD3512-795s-F and LCF439, as previously described (24). Details of the Southern blotting methods are included in Fig. S1. By transmission electron microscopy, the mutant was confirmed to lack TFP production in response to c-di-GMP (for details, see Fig. S1C in the supplemental material).

Insertion of P_{cpr}-*dccA* into the *C. difficile* 630Δ*erm* chromosome to create strain 630Δ*erm*::P_{cpr}-*dccA* was accomplished by amplifying the P_{cpr}-*dccA* fusion from plasmid pDccA with primers SMB47F1 and SMB47R1 (21). The resulting product was digested with SalI and SphI (restriction sites were introduced by the primers) and ligated into similarly digested pSMB47 to create pSMB47::P_{cpr}-*dccA*. pSMB47 is a suicide vector that integrates into the conjugal transposon Tn*916* by homologous recombination. The pSMB47::P_{cpr}-*dccA* plasmid was transformed into *B. subtilis* strain BS49, which contains the transposon Tn*916* (40). pSMB47::P_{cpr}-*dccA* was integrated into Tn*916* by homologous recombination to create the strain BS49 pSMB47::P_{cpr}-*dccA* (41). This strain was mated with *C. difficile* 630Δ*erm* by conjugation under anaerobic conditions on BHIS plates containing 5 mM KNO_3. Transformants in which Tn*916*::P_{cpr}-*dccA* had integrated randomly into the 630Δ*erm* chromosome were selected on BHIS plates containing Erm, Cef, and Cyc (42). Integration was confirmed by PCR with primers PcprF and cd1420R.

To create the *gusA* reporter strains, the *gusA* gene was amplified from *E. coli* JM109 genomic DNA using primers gusAF and gusAR. The *gusA* product was digested with XbaI and SphI, restriction sites for which were introduced by the PCR primers, and ligated into similarly digested pMC123 to create pMC123::*gusA*. Next, the 374-bp region upstream of the 630Δ*erm pilA1* open reading frame, which encompasses the ribosome binding site, promoter (P_{pilA1}), and 5′ untranslated region (UTR) con-

taining the Cdi2_4 riboswitch, were amplified from 630 genomic DNA using primers pilA1sF1 and pilA1-gusA_R, yielding fragment P_{pilA1}-UTR. To obtain an equivalent fragment with a riboswitch blind to c-di-GMP (24), an A70G mutation was introduced into the c-di-GMP binding aptamer of Cdi2_4 by incorporating the nucleotide change into self-complementary primers, pilA1sR1 and pilA1sF2. Primers pilA1sF1 and pilA1R1, and pilA1sF2 and pilA1-gusA_R, were used to amplify two overlapping fragments, which were then spliced together by PCR, creating the fragment P_{pilA1}-UTR^{A70G}. The *gusA* gene was amplified from pMC123::*gusA* using primers pilA1-gusA_F and gusAR, and then, translational fusions to both P_{pilA1}-UTR and P_{pilA1}-UTR^{A70G} were created by splicing by overlapping extension (pilA1-gusA_F and pilA1-gusA_R are self-complementary). The resulting P_{pilA1}-UTR-*gusA* and P_{pilA1}-UTR^{A70G}-*gusA* products were cloned into the EcoRI and SphI sites of pMC123. The plasmids pMC123::P_{pilA1}-UTR-*gusA* and pMC123::P_{pilA1}-UTR^{A70G}-*gusA* were confirmed by PCR using primers pUCmcsF and m13r and by sequencing. The *gluD* promoter was amplified from 630 genomic DNA using primers PgluDF and PgluDR and ligated into the pMC123 vector at the EcoRI and KpnI sites to create pMC-P_{gluD}. The *pilA1* gene with the native 5′ UTR but no promoter was amplified from 630 genomic DNA using primers pilA1sR2 and pilA1R and ligated into pMC-P_{gluD} at the KpnI and PstI sites to create pMC123::P_{gluD}-UTR-*pilA1*. pMC123::P_{gluD}-UTR^{A70G}-*pilA1* was generated by splicing the PCR products of primers pilA1sF1 and pilA1R, and pilA1sF2 and pilA1sR2, and ligating the resulting fragment into pMC-P_{gluD} at the KpnI and PstI sites. Primers PgluDF and pilA1-gusA_R were used to amplify the P_{gluD}-UTR and P_{gluD}-UTR^{A70G} fragments from these vectors. The *gusA* gene was amplified from pMC123::*gusA* using primers pilA1-gusA_F and gusAR and then spliced with both P_{gluD}-UTR and P_{gluD}-UTR^{A70G} by PCR. The resulting translational-fusion products, P_{gluD}-UTR-*gusA* and P_{gluD}-UTR^{A70G}-*gusA*, were cloned into the EcoRI and SphI sites of pMC123. Sequences were confirmed by PCR using primers pUCmcsF and m13r and by sequencing.

Plasmids pMC-P_{cpr}, pDccA, and pDccA^{MUT} and the *gusA* reporter constructs were transformed into *C. difficile* strains 630Δ*erm* and R20291 via conjugation with HB101(pRK24) (21, 42). The *gusA* reporter plasmids were likewise transformed into 630Δ*erm*::P_{cpr}-*dccA*, which allowed simultaneous thiamphenicol-dependent maintenance of the *gusA* plasmids and nisin induction of *dccA*. Transconjugants were selected on BHIS-Tm-Kan agar and appeared after 48 h at 37°C. *C. difficile* strains containing the desired plasmid were identified by PCR using primers pUCmcsF and m13r.

Biofilm assays. Overnight (16- to 18-h) cultures of *C. difficile* were diluted 1:100 in fresh BHIS containing 1% glucose (43). Plasmid-bearing strains were grown in BHIS-Tm with 1% glucose. Where needed to induce gene expression from the P_{cpr} promoter, nisin was included at the indicated concentrations. Biofilms were grown at 37°C for 24 or 48 h, as specified, in untreated tissue culture plates that had been allowed to equilibrate in the anaerobic chamber for at least 72 h. For visualization and quantification of biofilm production, the supernatants were first carefully removed from the wells. The biofilms were then washed once in phosphate-buffered saline (PBS), stained for 30 min in 0.1% filtered crystal violet, and washed twice in PBS. The dye was solubilized in 100% ethanol and quantified at 570 nm. The data were analyzed using a two-way analysis of variance (ANOVA) test.

Surface motility assays. Aliquots (5 μl) of overnight (16- to 18-h) cultures were spotted onto BHIS-1% glucose with the indicated percentages of agar. Plasmid-bearing strains were spotted onto plates also containing 10 μg/ml Tm to maintain the plasmids. When needed to induce gene expression from the P_{cpr} promoter, nisin was added at the indicated concentrations. The spots were allowed to dry, and then, the plates were wrapped in plastic to minimize evaporation over the 5-day incubation. To account for asymmetrical spreading across the plates, two measurements of the diameter of each motility spot were taken and averaged: one measurement of the largest possible diameter and one perpendicular measurement (represented in Fig. S2 in the supplemental material). For each strain, spots derived from five biologically independent cultures were measured at 24-h intervals. The data were analyzed using a one-way analysis of variance test. The plates were photographed using a Syngene G:Box imager.

RNA isolation and real-time PCR. To examine gene transcript levels in liquid culture, overnight (16- to 18-h) cultures were diluted 1:40 in BHIS-Tm and grown to early stationary phase (12 h), as described previously (44). When needed to induce *dccA* expression, nisin was included at a final concentration of 1 μg/ml. To measure transcript abundance in biofilms, supernatants were carefully removed from biofilm samples to serve as the planktonic fractions. The remaining biofilm fractions were washed once with PBS and then manually dispersed by vigorously scraping the cells into PBS with 0.1% Tween (PBS-T) using a pipette tip. The planktonic and biofilm fractions were immediately centrifuged to remove the culture medium or PBS-T and preserved in 1:1 ethanol-acetate. RNA was isolated, treated with DNase I, and reverse transcribed as previously described (21, 45). Quantitative reverse transcriptase (qRT) PCR analyses were done as described previously (21). Primers were designed using the PrimerQuest tool from IDT Technologies, and we verified that the primers chosen are capable of amplifying the same sequences from 630Δ*erm* and R20291 genomic DNA. Control reaction mixtures lacking reverse transcriptase were included for all templates and all primer sets. The data were analyzed by using the $2^{-\Delta\Delta CT}$ method, expressed as the fold change in the transcript level under the test conditions compared to the indicated control and normalized to the reference gene *rpoC* (44). At least three biologically independent samples were used for all analyses. The data were analyzed using the statistical tests specified in the figure legends.

Transcriptional reporter assays. β-Glucuronidase assays were performed as previously described (46, 47). Overnight cultures of strains 630Δ*erm* and R20291 containing the reporter plasmids were diluted 1:50 in BHIS medium and grown to an optical density at 600 nm (OD_{600}) of ~1.0, and then 1.0- and 0.1-ml samples were assayed for hydrolysis of *p*-nitrophenol-β-D-glucuronide. For biofilm β-glucuronidase assays, overnight cultures were diluted 1:50 in fresh BHIS-Tm medium containing 1% glucose in 24-well untreated tissue culture plates and grown anaerobically at 37°C for 24 h. The planktonic fractions were collected, and the remaining biofilms were washed once in anaerobic PBS and then disrupted by vigorous scraping into fresh anaerobic PBS. Sample volumes were normalized to the OD_{600} in order to ensure equivalent cell numbers in the planktonic and biofilm samples. For assays using agar-grown cultures, after 1, 3, or 5 days, cells were scraped from the agar surface into anaerobic PBS and vortexed to homogeneity before the OD_{600} was measured. The β-glucuronidase activities of all samples were normalized to the culture volume assayed and the OD_{600}. At least four biologically independent samples were assayed, and the data were analyzed using two-way ANOVA or the unpaired Student's *t* test as indicated.

c-di-GMP quantification by ultraperformance liquid chromatography coupled to mass spectrometry (UPLC-MS). Nucleotides were extracted from *C. difficile* for quantification as previously described (21). Briefly, overnight cultures of 630Δ*erm* and R20291 strains containing pDccA were diluted 1:50 in BHIS-Tm medium with 0, 0.01, 0.1, or 1 μg/ml nisin and grown to an OD_{600} of ~1.0, and then, 50-ml cultures were centrifuged for 10 min at 6,400 × *g* in sealed 50-ml conical tubes. Samples were returned to the anaerobic chamber, and cell pellets were washed in 1 ml PBS, transferred to clean microcentrifuge tubes, and centrifuged for 3 min at 1,000 × *g*. The pellets were removed from the anaerobic chamber, suspended in 250 μl of extraction buffer (methanol-acetonitrile-distilled H_2O [dH_2O] [40:40:20] plus 0.1 N formic acid), and incubated at −20°C for 30 min. Samples were centrifuged at 12,000 × *g* for 5 min at 4°C, and 200-μl aliquots of the supernatant were transferred to clean tubes and immediately neutralized by adding 8 μl of 15% (wt/vol) NH_4HCO_3. The total protein content of the nonsoluble fraction was quantified using the colorimetric BCA protein assay kit (ThermoScientific). Nucleotide samples were stored at −80°C until they were used. Samples were dried by vacuum centrifugation in a Savant speed-vac and

reconstituted in 100 μl deionized water. Aliquots (10 μl) were injected into a TSQ Quantum Ultra triple-quadrupole mass analyzer (Thermo Scientific, Waltham, MA) coupled to an Acquity UPLC separation system (Waters Corporation, Milford, MA). The analytes were separated on a Waters HSS T3 UPLC column (2.1 by 100 mm; 1.8 μm) by gradient elution from 99.9% solvent A (10 mM ammonium formate in water) to 40% solvent B (10 mM ammonium formate in methanol) for 2 min, followed by column flushing at 90% solvent B for 2.5 min and column reequilibration for 3.5 min. The column effluent was diverted to waste for the first 2 min. Mass spectrometer parameters were as follows: positive ion electrospray mode; spray voltage, 3.5 kV; vaporizer temperature, 250°C; sheath gas, 35 arbitrary units; auxiliary gas, 30 arbitrary units; capillary temperature, 285°C; collision gas pressure, 1.5 mtorr. c-di-GMP was detected by monitoring precursor ion to fragment ion transitions (*m/z* 691 > 152 [collision energy, 35] and *m/z* 691 > 540 [collision energy, 21]) and quantified using a calibration curve of pure c-di-GMP (Biolog Life Science Institute, Bremen, Germany) at known concentrations over a range of 0.1 to 1,000 nM. Cytoplasmic c-di-GMP measurements were normalized to the total protein content of the samples.

RESULTS

c-di-GMP regulates biofilm formation via TFP biosynthesis. We previously reported that manipulation of *C. difficile* c-di-GMP levels is possible through plasmid-based expression of the *C. difficile* diguanylate cyclase gene, *dccA* (21). This system uses a nisin-responsive promoter to induce the production of DccA. Liquid chromatography-mass spectrometry measurements of intracellular c-di-GMP confirmed that in *C. difficile* strain 630, *dccA* induction stimulates c-di-GMP synthesis in proportion to the amount of nisin used (21). We repeated these experiments with *C. difficile* strain R20291 and confirmed that R20291 exhibits the same dose-dependent increase in the intracellular c-di-GMP concentration upon nisin induction of plasmid-borne *dccA* (see Fig. S3 in the supplemental material). Moreover, we determined that in the absence of nisin induction, the 630 derivative strain 630Δ*erm* and R20291 have comparable c-di-GMP levels (see Fig. S3 in the supplemental material). Independent studies have shown that *C. difficile* strains 630 and 630Δ*erm* (which is more amenable to currently available genetic approaches) produce more biofilm upon *dccA* overexpression and that R20291 produces substantially more biofilm than 630/630Δ*erm* even in the absence of c-di-GMP manipulation (25, 43). The mechanisms by which R20291 forms more robust biofilms and the role(s) of c-di-GMP in R20291 biofilm formation have not been reported.

To determine whether c-di-GMP regulation of biofilm formation differs between 630/630Δ*erm* and R20291, we examined biofilm formation by these strains with artificially modified c-di-GMP levels. We assayed biofilm production on plastic for both strains carrying the control vector or the inducible *dccA* expression plasmid for manipulation of c-di-GMP (pDccA). *C. difficile* 630Δ*erm* and R20291 with pDccA^{MUT}, which allows nisin-inducible expression of a *dccA* allele encoding an enzymatically inactive diguanylate cyclase, were included as controls (21). Biofilm formation in strains carrying the vector was indistinguishable from that of the respective parental strains (data not shown). In addition, the presence of nisin did not itself alter biofilm production, as demonstrated by the relatively consistent biofilms, with or without nisin, for the control strains carrying the vector. Consistent with previous reports (43), biofilm formation by R20291 was 9 times higher than that by 630Δ*erm* (Fig. 1A). In 630Δ*erm*, expression of *dccA* dramatically increased biofilm formation in a dose-dependent manner, with measurable effects at 0.01 μg/ml nisin (Fig. 1A). Biofilm accumulation increased with the nisin concentration until 1 μg/ml nisin, for a maximum 10-fold increase in biofilm formation (Fig. 1A). In contrast, R20291 biofilm levels were not measurably affected by nisin concentrations of less

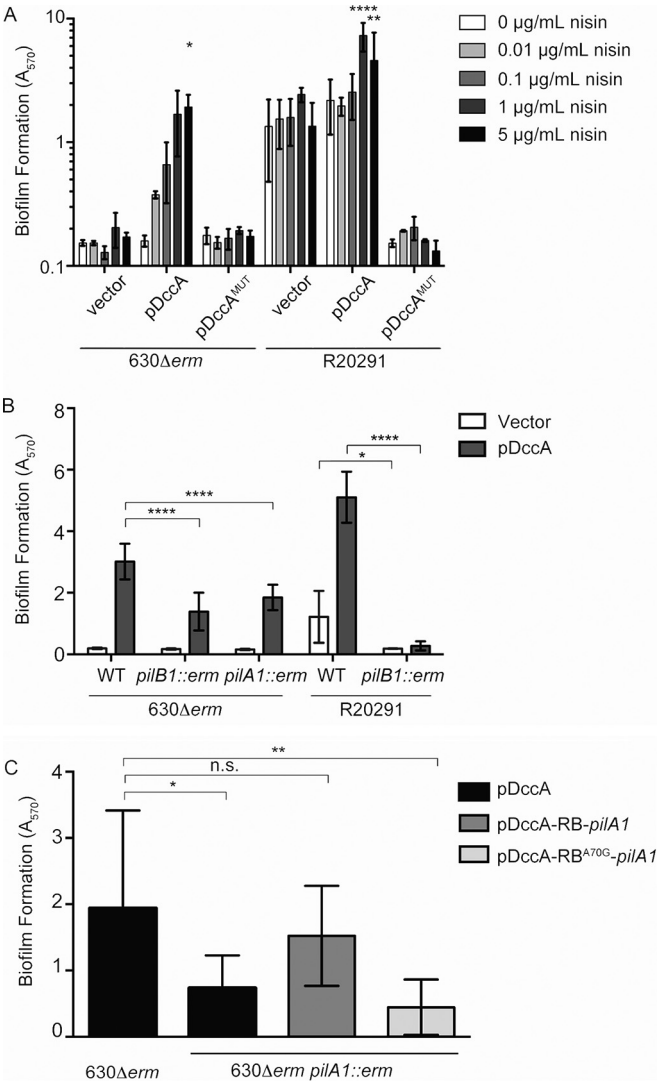

FIG 1 c-di-GMP regulation of TFP synthesis influences biofilm formation in 630 and R20291. Biofilm formation on plastic was measured by crystal violet staining after 24 h of growth in BHIS-Tm. Shown are the means and standard deviations for three biologically independent samples assayed. (A) Biofilm formation by 630Δ*erm* and R20291 carrying vector, pDccA, or pDccA^{MUT} in BHIS-Tm medium containing increasing concentrations of nisin to achieve increasing intracellular c-di-GMP levels. Biofilm measurements at each nisin concentration were compared to biofilms formed by the same strain at 0 μg/ml nisin by two-way ANOVA and Tukey's multiple comparisons. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$. (B) Biofilm formation by wild-type and gene-disrupted 630Δ*erm* and R20291 carrying control vector or pDccA in BHIS-Tm medium with 1 μg/ml nisin. Biofilm measurements of mutant strains were compared to those of their respective wild-type backgrounds. The brackets indicate significantly different comparisons. *, $P < 0.05$; ****, $P < 0.0001$, as determined by two-way ANOVA using Tukey's multiple comparisons. (C) Biofilm formation in the presence of 1 μg/ml nisin by 630Δ*erm* pDccA and the *pilA1* mutant derivative carrying pDccA or pDccA containing *pilA1* expressed in *trans* under the control of the wild-type riboswitch UTR (pDccA-RB-*pilA1*) or the mutant riboswitch (pDccA-RB^{A70G}-*pilA1*). n.s. not significant; *, $P < 0.05$; **, $P < 0.01$, as determined by one-way ANOVA and Tukey's multiple comparisons.

than 1 μg/ml, which increased R20291 biofilm formation 3.7-fold (Fig. 1A). This difference in nisin-induced biofilm formation was not due to strain-specific differences in c-di-GMP production in response to *dccA* expression (see Fig. S3 in the supplemental material). Both strains showed maximal biofilm formation at 1 μg/ml nisin, with no further increase at higher nisin concentrations, suggesting that this condition enables the maximum c-di-GMP-inducible biofilm production in each strain (Fig. 1A). In addition, we observed no increases in biofilm formation for 630Δ*erm* or R20291 with pDccA^{MUT}, regardless of the nisin concentration, indicating that increases in biofilm formation are specifically attributable to elevated c-di-GMP (Fig. 1A). The presence of pDccA^{MUT} actually decreased biofilm formation in R20291, although the effect was independent of nisin. R20291 with vector formed biofilms comparable to those of the parent strain, so the decrease appears to be an effect of the *dccA*^{MUT} gene product rather than of the plasmid. As DGC enzymatic activity is predicted to depend on protein dimerization (48, 49), it is possible that overexpression of *dccA*^{MUT} titrates the enzymatically active gene products of the chromosomally encoded wild-type *dccA* into inactive heterodimers or interferes with signaling pathways affecting biofilm development through some other mechanism.

It was previously shown that c-di-GMP increases the expression of the *pilA1* pilin gene and augments TFP production in *C. difficile* 630Δ*erm* (24). In addition, disruption of either the *pilA1* structural pilin gene or *pilB1*, which is predicted to encode an ATPase essential for pilin export and assembly, produces identical phenotypes, abolishing c-di-GMP-stimulated TFP production in this background (24). TFP in other bacteria participate in surface adhesion and biofilm development. Thus, we hypothesized that TFP contribute to c-di-GMP-induced biofilm formation in *C. difficile* (24, 27–29). To determine the role of TFP in biofilm formation, we assayed 630Δ*erm* and R20291 pilus gene mutants for biofilm formation on plastic. In 630Δ*erm* with unaltered c-di-GMP, neither a *pilA1* nor a *pilB1* mutation affected biofilm production (Fig. 1B). Because wild-type 630Δ*erm* does not produce substantial biofilm under these conditions, we also examined the effect of the *pilA1* and *pilB1* mutations in 630Δ*erm* with elevated c-di-GMP, which produces significantly more biofilm (Fig. 1A). In this case, disruption of *pilA1* or *pilB1* significantly reduced c-di-GMP-induced biofilm formation to 39% and 54% of wild-type levels, respectively, but did not completely abolish it (Fig. 1B). These results indicate that under biofilm-inducing conditions (i.e., high c-di-GMP), TFP contribute to biofilm formation, but additional factors also play a role.

Because the *pilB1* gene is the first gene in a 10.9-kb operon, the ClosTron insertion is likely to have polar effects on the downstream genes. The *pilB1* mutant is thus a *pilB1* operon mutant rather than specifically an assembly ATPase mutant. Genes downstream of *pilB1*, such as the prepilin peptidase gene *pilD* (24), are also important for proper TFP biosynthesis; thus, complementation with the *pilB1* gene alone is unlikely to restore the wild-type phenotype. Due to the size of the operon, we did not attempt to complement the *pilB1* operon mutation. We instead focused on complementation of the mutation of *pilA1*, which is transcribed separately under the control of the c-di-GMP-responsive riboswitch, Cdi2_4 (24). Complementation was done using pDccA as the vector backbone to allow manipulation of intracellular c-di-GMP. As described previously (24), the *pilA1* gene was cloned into this plasmid under the control of the native promoter and 5′ UTR containing Cdi2_4 (pDccA-RB-*pilA1*) or under the control of the native promoter and the 5′ UTR containing an A70G mutation in Cdi2_4 (pDccA-RB^{A70G}-*pilA1*). The A70G mutation greatly reduces Cdi2_4 affinity for c-di-GMP, yielding a riboswitch that is essentially blind to c-di-GMP and prevents the expression of the downstream gene in response to increases in c-di-GMP (24). Upon increasing intracellular c-di-GMP through nisin-induced *dccA* expression, expression of *pilA1* in *trans* under the control of the wild-type riboswitch UTR (pDccA-RB-*pilA1*) restored biofilm formation of the *pilA1* mutant to that of wild-type 630Δerm with high c-di-GMP (Fig. 1C). In contrast, expression of *pilA1* in *trans* under the control of the mutant riboswitch (pDccA-RB^{A70G}-*pilA1*) failed to complement biofilm formation by the *pilA1* mutant. These results support the conclusion that *pilA1* participates in *C. difficile* biofilm formation in a c-di-GMP-dependent manner.

We were unable to obtain a ClosTron insertion in *pilA1* in R20291, but we successfully disrupted *pilB1* (see Fig. S1A in the supplemental material). As in 630Δ*erm*, increasing c-di-GMP through *dccA* expression in R20291 led to the production of surface structures 8 to 9 nm in diameter, consistent in size with TFP (typically 5 to 7 nm) (see Fig. S1B in the supplemental material) (30). The Targetron insertion in *pilB1* eliminated the appearance of the structures (see Fig. S1C in the supplemental material). As the *pilA1* and *pilB1* mutations result in the same phenotypes in 630Δ*erm*, we used the R20291 *pilB1* mutant strain to assess the effects of diminished TFP biosynthesis on biofilm formation in the strain. In contrast to the results seen in 630Δ*erm*, disruption of the *pilB1* gene in R20291 reduced biofilm formation significantly compared to that produced by wild-type R20291 in the absence of c-di-GMP manipulation. Moreover, *pilB1* disruption in R20291 eliminated c-di-GMP-induced increases in biofilm formation (Fig. 1B). These findings indicate that TFP make a greater contribution to biofilm development in R20291 than in 630Δ*erm.*

R20291 and 630Δ*erm* exhibit distinct *pilA1* expression profiles between planktonic and biofilm growth. We next postulated that because R20291 appears to rely more heavily on TFP for biofilm formation than 630Δ*erm*, the two strains might differ in their expression of TFP genes in planktonic and/or biofilm culture. We therefore compared the transcript levels of all the predicted *C. difficile* pilin genes in planktonic and biofilm-grown wild-type *C. difficile* of both strains using qRT-PCR. The genes encoding known or putative pilins are all at least 94% identical between *C. difficile* 630 and R20291 (see Table S4 in the supplemental material) (50). As a control in these studies, the transcript level of the *codY* gene was measured and determined not to differ between biofilm and planktonic conditions in either strain (Fig. 2). Transcripts of *pilA5* and *pilA6*, which are predicted to encode minor pilins, were detectable in strain 630 planktonic and biofilm samples but could not be detected under either condition in R20291. The ability of the primers used to detect these transcripts was confirmed using R20291 genomic DNA (data not shown), so it appears that the expression of multiple pilin genes differs between 630 and R20291 under the conditions examined. In 630, all of the predicted pilin genes, including *pilA1*, showed comparable expression levels under planktonic and biofilm conditions (Fig. 2A). In R20291, however, *pilA1* was upregulated 3-fold by growth in a biofilm compared to planktonic growth (Fig. 2B). This change occurs consistently in wild-type cells with no manipulation of cellular c-di-GMP levels and is likely to represent a biologically

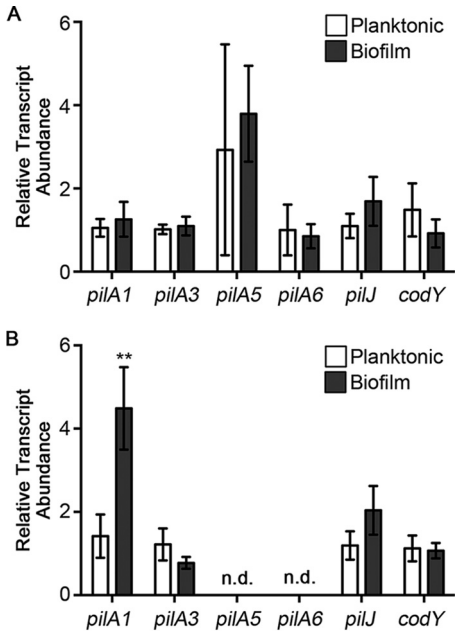

FIG 2 TFP gene transcription in planktonic and biofilm-derived cells. qRT-PCR was used to measure the transcript levels of predicted structural pilin genes in planktonic cells from the broth phase of 48-h biofilm cultures (white bars) and in biofilm-attached cells (gray bars) for *C. difficile* strains 630 (A) and R20291 (B). The data are expressed as the fold change in transcript levels in biofilm-derived cells relative to the planktonic cells, normalized to the reference gene *rpoC*. Shown are the means and standard deviations for four biologically independent samples. The gene names and the corresponding gene numbers in the respective strains are listed in Table S4 in the supplemental material. n.d., not detectable; **, $P < 0.01$, as determined by two-way ANOVA.

meaningful change in TFP production during R20291 biofilm formation, suggesting that differential regulation of *pilA1* transcription contributes to the observed differences in biofilm formation between 630Δ*erm* and R20291.

***C. difficile* R20291 and 630Δ*erm* are capable of surface motility.** In many bacterial species, TFP have been shown to mediate surface motility, often as an early step in biofilm development. Previous work reported that *C. difficile* is capable of motion on agar surfaces (51). We thus explored whether *C. difficile* is capable of motility on solid surfaces and the potential contribution of TFP. To do this, liquid cultures of *C. difficile* 630Δ*erm* and R20291 were spotted and allowed to dry on BHIS-1% glucose plates with a range of agar concentrations, and migration across the surface was monitored over 5 days. On agar concentrations up to 0.9%, neither 630Δ*erm* nor R20291 expanded beyond the boundaries of the initial spot (Fig. 3A). On 1.2% agar, R20291 expanded from the inoculation point in dendritic tendrils. The number, length, and breadth of the tendrils increased with the agar concentration and surface hardness, reaching an apparent maximum on 1.8% agar. 630Δ*erm* also migrated from the initial spot, though over a more limited range of agar concentrations and with no significant expansion on less than 1.8% agar (Fig. 3A). Thus, *C. difficile* is capable of expanding and migrating over agar surfaces in a manner reliant on surface hardness.

TFP are required for surface motility of *C. difficile* R20291. Because TFP participate in surface motility in bacteria such as *Pseudomonas aeruginosa* and *C. perfringens* (36, 52), we hypothesized that TFP contribute to surface motility in *C. difficile*. To test this, we assayed the surface motility of R20291, 630Δ*erm*, and the *pilA1* and *pilB1* mutant derivatives by measuring spreading across 1.2% and 1.8% agar daily for 5 days (Fig. 3B and C). We found that all the strains displayed comparable, limited expansion across

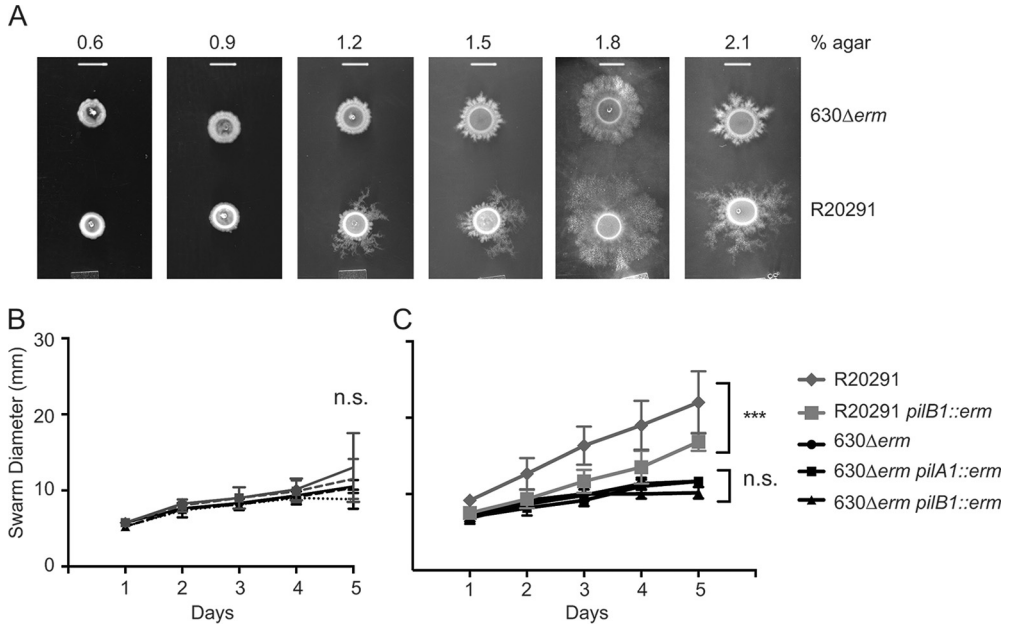

FIG 3 *C. difficile* migrates across agar surfaces in response to surface hardness. (A) Migration of 630Δ*erm* and R20291 on BHIS-1% glucose with varying concentrations of agar (indicated above each panel) after 120 h at 37°C. The scale bars indicate 5 mm. (B and C) Quantification of migration of wild-type and gene-disrupted 630Δ*erm* and R20291 on BHIS-1% glucose with 1.2% agar (B) or 1.8% agar (C) over a 5-day period. Five biologically independent spots were evaluated for each strain at each time point by measuring the diameters in two orthologous directions and using the average value for each spot (see Fig. S3 in the supplemental material). Shown are the means and standard deviations. n.s., $P > 0.05$; ***, $P < 0.001$, as determined by one-way ANOVA.

the softer 1.2% agar. Significant differences between the strains emerged on the harder 1.8% agar. Disruption of *pilA1* or *pilB1* in 630Δ*erm* did not affect motility on 1.8% agar (Fig. 3C). In contrast, disruption of *pilB1* in R20291 significantly diminished surface motility over 1.8% agar (Fig. 3C), indicating that TFP function in *C. difficile* surface motility and play a more prominent role in R20291.

c-di-GMP promotes TFP-based surface motility. Because c-di-GMP promotes *pilA1* gene expression and TFP biosynthesis, we next examined the effect of c-di-GMP on surface motility. This was done by assaying R20291 and 630Δ*erm*, each with vector or pDccA to allow c-di-GMP manipulation, for surface motility on 1.2% agar. This agar concentration was initially selected because R20291 showed some (but not maximal) motility on this surface, allowing increases or decreases to be observable. Nisin was added to the agar medium at a range of concentrations up to 5 μg/ml. For both the 630Δ*erm* and R20291 vector controls, the nisin concentration did not affect surface motility (Fig. 4A). 630Δ*erm* with pDccA also showed a tendency to form small outgrowths from the spot of inoculation, which increased modestly with the addition of nisin (Fig. 4A). In R20291 with pDccA, tendril formation greatly increased with the nisin concentration, indicating that c-di-GMP augments surface motility (Fig. 4A). Notably, the c-di-GMP-induced increase in the surface motility of R20291 is dependent on TFP, as disruption of the *pilB1* gene abolished tendril formation and motility on 1.2% agar, even at elevated c-di-GMP levels (Fig. 4B). On higher (1.5% and 1.8%) concentrations of agar, R20291 and R20291 *pilB1*::*erm* with pDccA exhibited more surface expansion independent of nisin (see Fig. S4 in the supplemental material). However, the addition of nisin to increase c-di-GMP via *dccA* expression further enhanced motility in wild-type R20291 and had minimal effect on R20291 *pilB1* (see Fig. S4 in the supplemental material). Thus, c-di-GMP promotes TFP-dependent surface motility in *C. difficile* on multiple agar concentrations, and this phenomenon is more pronounced in R20291.

***pilA1* transcription and its regulation by c-di-GMP differ between *C. difficile* 630 and R20291.** We hypothesized that TFP-dependent phenotypes differ between 630 and R20291 due to differences in *pilA1* gene expression, potentially in response to c-di-GMP. To test this, we used qRT-PCR to evaluate *pilA1* transcript abundance in 630 and R20291, each with vector, pDccA, or pDccA^{MUT} grown in BHIS-1% glucose medium with 1 μg/ml nisin to early stationary phase. Because the transcript levels of the reference gene *rpoC* did not differ significantly between 630 and R20291 grown under these conditions (see Table S5 in the supplemental material), we used *rpoC* as a reference gene for normalization between strains. Furthermore, *dccA* expression via nisin induction resulted in comparable changes to intracellular c-di-GMP in 630 and R20291 (see Fig. S3 in the supplemental material), so it was possible to directly compare *pilA1* transcript levels upon c-di-GMP stimulation. In vector control strains with no manipulation of c-di-GMP levels, the *pilA1* transcript was 26 times more abundant in R20291 than in 630 (Fig. 5A). In 630, induction of *dccA* expression with 1 μg/ml nisin increased *pilA1* transcript abundance 32-fold; in R20291, *dccA* induction resulted in a modest and statistically insignificant 2-fold increase (Fig. 5A). Induction of *dccA* raises 630 *pilA1* transcript abundance to the level seen in R20291 at native c-di-GMP levels (21). Consistent with the decrease in biofilm formation observed in R20291 with pDccA^{MUT}, expression of *dccA*^{MUT} and production of a catalytically inactive

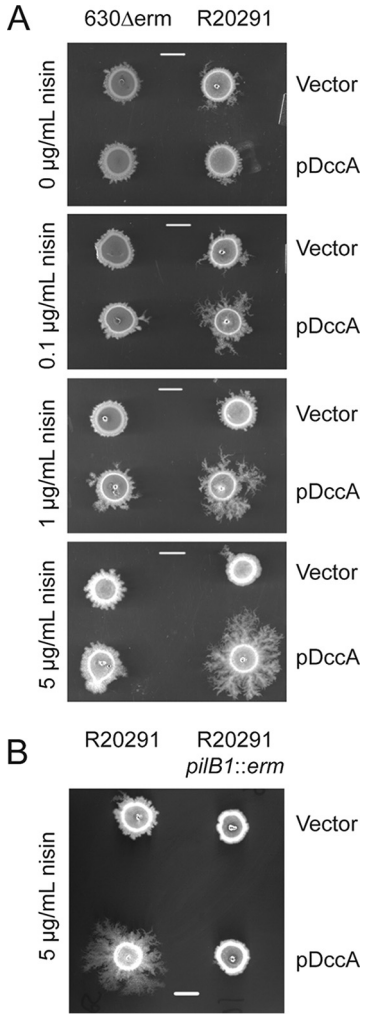

FIG 4 c-di-GMP stimulates R20291 surface motility in a TFP-dependent manner. (A) Surface motility of 630Δ*erm* and R20291, each carrying vector or pDccA, on BHIS-1% glucose-1.2% agar with increasing concentrations of nisin, as indicated on the left. (B) Surface motility of R20291 and the *pilB1* mutant derivative, each bearing vector or pDccA, on BHIS-1% glucose-1.2% agar and 5 μg/ml nisin. Representative images taken after 120 h are shown. The scale bars indicate 5 mm.

diguanylate cyclase reduced *pilA1* transcript abundance in both 630 and R20291 compared to the vector control strains, but the difference was not statistically significant (Fig. 5A). Therefore, c-di-GMP increases *pilA1* transcript abundance, with a greater impact in *C. difficile* 630, in which basal transcription is low compared to R20291.

c-di-GMP regulates *pilA1* expression via the Cdi2_4 riboswitch and does not affect promoter activity. As noted above, we recently reported that *pilA1* expression in 630Δ*erm* is regulated by c-di-GMP through the riboswitch Cdi2_4 (24). The 5′ UTR of *pilA1* is highly conserved between 630/630Δ*erm* and R20291. The promoter regions are identical in the two strains, while the 84 bp of the c-di-GMP binding aptamer contain only two divergent bases, which are not expected to alter stem-loop formation in the riboswitch (see Fig. S5 in the supplemental material). To assess potential differences in promoter and riboswitch activities between the two *C. difficile* strains, we constructed a series of four

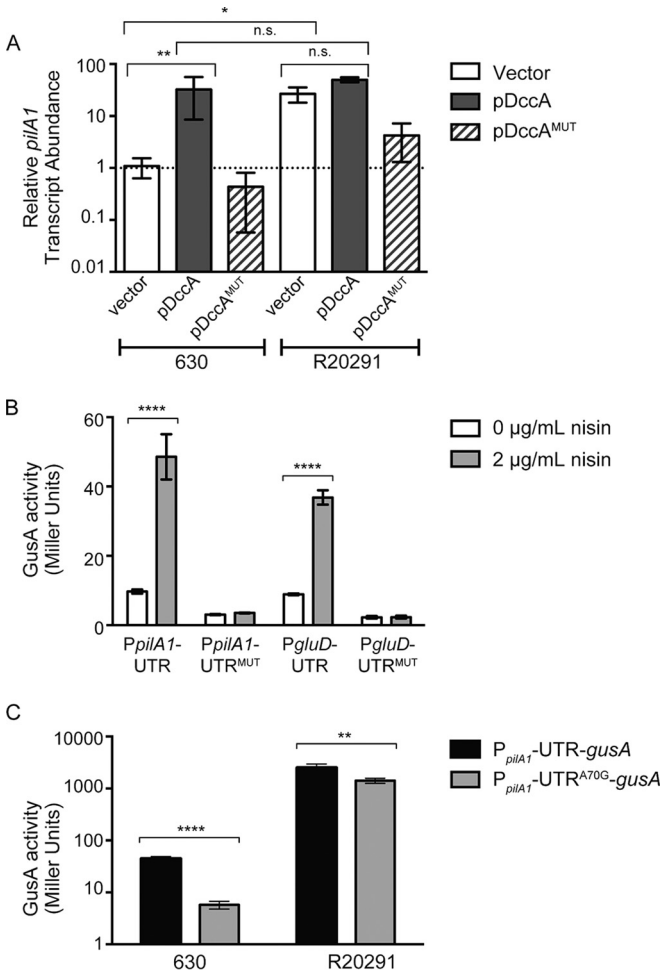

FIG 5 *pilA1* transcription is regulated by c-di-GMP in 630 and R20291, but basal transcription is higher in R20291. (A) qRT-PCR was used to measure the transcript abundances of *pilA1* in *C. difficile* strains 630 and R20291, each with vector, pDccA, or pDccA^{MUT}, grown in BHIS-1% glucose broth with 1 μg/ml nisin to induce *dccA* expression. Samples were collected at early stationary phase. The data were normalized to the *rpoC* reference gene and expressed relative to the mean C_T (threshold cycle) value for 630 containing vector. Shown are the means and standard deviations for three biologically independent samples. n.s., $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$, as determined by two-way ANOVA. (B) Regulation of gene expression by c-di-GMP was assessed using a plasmid-borne *gusA* transcriptional reporter gene controlled by the indicated promoter-Cdi2_4 riboswitch combinations. The experiment was done in 630Δ*erm*::P_{cpr}-*dccA*, which contains the nisin-inducible *dccA* gene at an ectopic chromosomal site. The strains were grown to late exponential phase in BHIS-1% glucose with 0 and 2 μg/ml nisin. Shown are the means and standard deviations for three biologically independent samples. ****, $P < 0.0001$, as determined by two-way ANOVA. (C) Differences in *pilA1* promoter activity between 630Δ*erm* and R20291 were evaluated using a plasmid-borne *gusA* translational fusion to the P_{pilA1} promoter coupled to the 5′ UTR with the wild-type (black) and A70G mutant (gray) Cdi2_4 riboswitch. The strains were grown to late exponential phase in BHIS-1% glucose. Shown are the means and standard deviations for six biologically independent samples. **, $P < 0.01$; ****, $P < 0.0001$, as determined by two-way ANOVA.

translational fusions of the *E. coli gusA* (β-glucuronidase) gene under the control of combinations of the native (*pilA1*) or a heterologous (*gluD*) promoter, with a wild-type or mutant (A70G) Cdi2_4 riboswitch. *C. difficile* 630 served as the template for the four regulatory elements that resulted: P_{pilA1}-UTR, P_{pilA1}-UTR^{A70G}, P_{gluD}-UTR, and P_{gluD}-UTR^{A70G}. The plasmid-borne translational fusions of these regulatory elements to the *gusA* gene served as colorimetric reporters in *C. difficile*.

To evaluate the effects of these regulatory elements on *pilA1* transcription at native and elevated levels of c-di-GMP, the four reporter plasmids were introduced into 630Δ*erm*::P_{cpr}-*dccA*, which bears *dccA* in a single copy under the nisin-inducible P_{cpr} promoter at an ectopic chromosomal site. We then evaluated β-glucuronidase activity in response to unmodified (without nisin induction) and elevated (with nisin induction) levels of c-di-GMP. Compared to the absence of nisin, induction of *dccA* expression with nisin increased β-glucuronidase activity 5-fold in the strain with P_{pilA1}-UTR-*gusA* and 4-fold in the strain with P_{gluD}-UTR-*gusA* (Fig. 5B). Nisin induction of *dccA* did not affect the activities of the strains with either P_{pilA1}-UTR^{A70G}-*gusA* or P_{gluD}-UTR^{A70G}-*gusA*, validating the insensitivity of the mutant riboswitch to c-di-GMP (Fig. 5B). Together, these data indicate that c-di-GMP regulation of *pilA1* transcription occurs via the Cdi2_4 riboswitch and does not involve the *pilA1* promoter.

Riboswitch activity is conserved between *C. difficile* strains, but transcription initiation from the *pilA1* promoter is higher in R20291. We next used the reporters to compare the respective roles of the *pilA1* promoter and the Cdi2_4 riboswitch in activating downstream gene expression in the two *C. difficile* strains. We introduced the P_{pilA1}-UTR-*gusA* and P_{pilA1}-UTR^{A70G}-*gusA* reporter fusions, which were derived from the *C. difficile* 630 sequence, into wild-type *C. difficile* 630Δ*erm* and R20291. We found that during exponential growth, the wild-type riboswitch allowed greater reporter activity than the mutant riboswitch in both 630Δ*erm* and R20291 strain backgrounds (7.6-fold and 1.8-fold, respectively), consistent with an inability of c-di-GMP to promote transcriptional read-through via the mutant Cdi2_4 riboswitch (Fig. 5C). Both P_{pilA1}-UTR-*gusA* and P_{pilA1}-UTR^{A70G}-*gusA* reporters yielded significantly higher activity in the R20291 background than the 630Δ*erm* background (45- and 192-fold, respectively). As the reporter uses the *pilA1* promoter from 630 in both strains, this suggests that the differences in β-glucuronidase activity are caused by greater promoter-driven transcription initiation in R20291.

UPLC-MS indicated that in the absence of experimental manipulation, 630Δ*erm* and R20291 have comparable cytoplasmic levels of c-di-GMP (see Fig. S3 in the supplemental material). To confirm this, we measured β-glucuronidase activity using the P_{gluD}-UTR-*gusA* reporter in both strains as an indirect measure of intracellular c-di-GMP. β-Glucuronidase activity was ~50% higher in the R20291 background during exponential growth in liquid medium, but the difference was not significant, consistent with the UPLC-MS measurements (Table 1). Together, these results suggest that differences in c-di-GMP levels are unlikely to be the sole cause of the observed differences in surface behaviors between 630Δ*erm* and R20291. Thus, differences in rates of promoter-based transcription initiation are likely the primary drivers of the differences in *pilA1* expression between the strains.

R20291, but not 630, accumulates more intracellular c-di-GMP during surface-associated growth than liquid growth. 630Δ*erm* and R20291 exhibit differences in TFP-mediated behaviors after 24 h of biofilm formation or 5 days of growth on agar plates (Fig. 1 and 3). We utilized the P_{gluD}-UTR-*gusA* biosensor as a tool to assess potential differences in c-di-GMP levels between the two *C. difficile* strains under these conditions, where direct

TABLE 1 c-di-GMP measurements in 630Δ*erm* and R20291

C. difficile strain	c-di-GMP level (μmol/mg protein)[a]	β-Glucuronidase activity (Miller units)[b]
630Δ*erm*	1.81 ± 0.37	21.1 ± 2.9
R20291	1.49 ± 0.34	34.4 ± 23.1

[a] Intracellular c-di-GMP determined by UPLC-MS of nucleotide extracts of strains harboring pDccA grown in BHIS-Tm medium with no nisin, normalized to total protein content. The values are the means ± standard deviations for three independent samples; $P = 0.15$ (unpaired Student's *t* test).
[b] β-Glucuronidase activities of strains harboring the P_{gluD}-UTR-*gusA* fusion, grown in BHIS-Tm medium. The values are the means ± standard deviations for six independent samples; $P = 0.06$ (unpaired Student's *t* test).

measurement using liquid chromatography is not feasible due to low bacterial numbers. 630Δ*erm* and R20291 showed no significant differences in β-glucuronidase activity during exponential growth or after 24 h of growth in planktonic culture (Fig. 6A). It was not possible to collect enough biofilm-derived 630Δ*erm* cells to assay β-glucuronidase activity after 24 h, but we were able to measure β-glucuronidase activity in planktonic and biofilm-derived R20291 cells after 24 h of growth. By comparing β-glucuronidase activity in the biofilm-grown cells with that in planktonic cells from the broth phase of the same biofilm cultures, we found that biofilm-associated bacteria had 33-fold-higher c-di-GMP-activated reporter activity than planktonic bacteria (Fig. 6A). At periods exceeding 24 h in liquid- and biofilm-grown cells, the bacterial cultures exhibited a dramatic decrease in viable vegetative cells, suggesting cell lysis and/or sporulation (data not shown). The cell numbers recovered at these times were insufficient for β-glucuronidase activity assays. However, cells remained viable on agar plates for longer periods, allowing us to assess c-di-GMP accumulation on agar surfaces of various hardnesses over the course of 5 days. The β-glucuronidase activities in 630Δ*erm* cells grown on 0.9%, 1.2%, 1.5%, and 1.8% agar did not differ significantly at 1, 3, and 5 days, and none of the conditions resulted in increased β-glucuronidase activity over time (Fig. 6B). In contrast, R20291 cells grown under different agar conditions showed no differences in β-glucuronidase activity at 1 or 3 days, but the 5-day samples showed increased activity compared to the 1- and 3-day samples. The increases were roughly proportional to the agar concentrations of the substrate medium. After 5 days of growth, cells grown on 1.5% or 1.8% agar displayed higher β-glucuronidase activity than cells grown on softer 0.9% or 1.2% agar (Fig. 6C). These data suggest that the intracellular c-di-GMP concentration of strain R20291 increases over time during growth on surfaces, while the c-di-GMP levels of strain 630 are relatively unaffected by surface association.

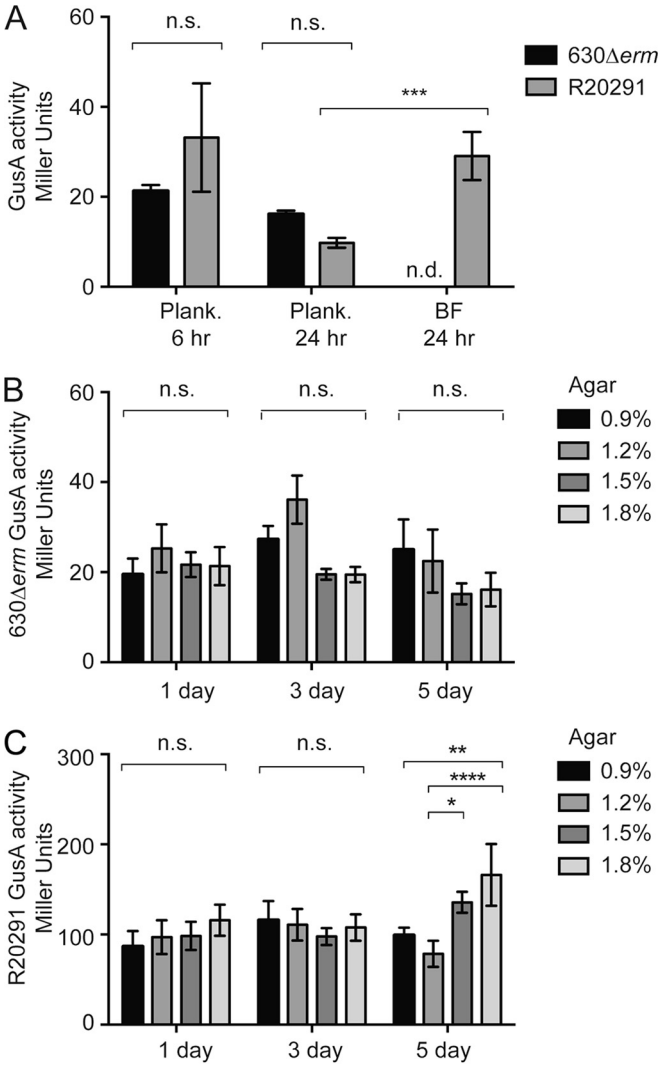

FIG 6 Growth on surfaces increases intracellular c-di-GMP in *C. difficile* R20291. β-Glucuronidase activity was evaluated using the P_{gluD}-UTR-*gusA* reporter in order to assess relative c-di-GMP levels. (A) β-Glucuronidase activity was measured in 630Δ*erm* and R20291 after 6 and 24 h of planktonic growth in BHIS-Tm medium supplemented with 1% glucose and in R20291 after 24 h of growth in a biofilm. β-Glucuronidase activities were compared between planktonic 630Δ*erm* and R20291 and between planktonic (Plank.) and biofilm (BF)-grown R20291 using an unpaired *t* test. n.d., not detected; n.s., $P > 0.05$; ***, $P < 0.001$. (B and C) β-Glucuronidase activity was measured in 630Δ*erm* (B) and R20291 (C) after 1, 3, and 5 days of growth on BHIS-Tm medium supplemented with 1% glucose with the indicated concentrations of agar. β-Glucuronidase activities between same-age samples were compared by two-way ANOVA. n.s., $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

DISCUSSION

The majority of bacterial life in nature is surface associated, and bacteria employ a number of mechanisms to sense and respond to surfaces (53). Some use flagella to sense surfaces, regulating transcription of genes involved in adhesion and motility in response to changes in the mechanical force necessary for flagellar rotation (53–58). Similarly, detection of solid biotic or abiotic surfaces by TFP activates expression of virulence factors and host cell killing in *P. aeruginosa* (59). The surface behaviors of *C. difficile* are poorly characterized, despite the likelihood that the bacterium interacts with the host mucosa and/or epithelium during infection. *C. difficile* adheres *in vitro* to monolayers of multiple cell types, including intestine-derived Caco-2 cells and mucus-secreting HT-29 cells (60–62). *C. difficile* also adheres to intestinal mucus *in vitro* and has been found "intimately associated" with the intestinal mucosa in biopsy specimens taken from a human patient with antibiotic-induced pseudomembranous colitis (63–65). In addition, *C. difficile* can associate with intestinal tissue at sites of inflammatory damage in both hamsters and mice, suggesting that the bacteria interact directly with the intestinal epithelium during infection (66, 67).

Adherent pathogens often employ surface motility to disperse along host tissue to new sites of adherence while remaining in contact with the epithelium and its associated nutrients (68). *C. difficile* strains 630/630Δ*erm* and R20291 both spread symmetrically from the site of inoculation over 1.8% agar, but only R20291 extends in tendrils over a range of more elastic agar surfaces. As R20291 tendril formation is enhanced by increases in the c-di-GMP level and is diminished by disruption of *pilB1*, it appears that surface-induced accumulation of c-di-GMP and subsequent TFP formation result in the greater surface motility observed in R20291. It is important to note that disruption of *pilB1* does not completely eliminate motility in wild-type R20291, but it prevents R20291 with pDccA from increasing its motility in response to increased c-di-GMP. *C. difficile* encodes a second *pilB* orthologue, *pilB2*. Some functional redundancy may permit some surface motility in the absence of *pilB1* (see Table S4 in the supplemental material) (50). As disruption of TFP genes does not affect 630Δ*erm* spreading over high-percentage agar, it is possible that cells on very hard surfaces spread passively by sliding away from sites of cell division.

Our findings suggest that *C. difficile* surface motility is influenced by the hardness of the substratum; the bacterium appears incapable of moving across very soft agar surfaces, although c-di-GMP stimulation of TFP synthesis appears to increase the range of substrata on which the R20291 strain can spread. The minimum surface hardness required for motility by either *C. difficile* strain is 1% agar, which has an elastic modulus, or ability to deform in response to pressure, of roughly 10 kPa (69). R20291 is more adept than 630/630Δ*erm* at spreading upon 1 to 2% agar. Agar percentages between 1 and 2% represent the spectrum between healthy intestinal tissue, which has an elastic modulus of 10 kPa, and intestinal tissue stiffened by inflammation, which has an elastic modulus of 25 kPa (69–71). Notably, the inflammation caused by *C. difficile* infection makes the colon wall harder, causing it to thicken from 6 to 8 mm to 22 to 32 mm and to stiffen significantly (72). We speculate that this inflammatory stiffening of the intestinal epithelium following *C. difficile* attachment promotes bacterial motility and dispersal across the harder surface. Thus, R20291 may spread across host tissue more efficiently, or earlier during the course of an infection, than historical *C. difficile*, potentially contributing to its higher virulence.

Biofilms play a role in the establishment of persistent infection by a number of pathogens (73). *C. difficile* cells removed from biofilms exhibit reduced susceptibility to vancomycin, an antibiotic commonly used to treat *C. difficile* infection in humans, suggesting that biofilm formation within a host could contribute to the difficulty of treating *C. difficile* infections (7, 43, 73–76). Here, we show that c-di-GMP regulates biofilm formation in *C. difficile* strains 630/630Δ*erm* and R20291 via control of TFP formation. Not only does R20291 display greater biofilm formation than 630/630Δ*erm*, TFP (*pilA1*) gene transcription is significantly higher in R20291 than in 630/630Δ*erm*. The Cdi2_4 riboswitch promotes *pilA1* transcript elongation in response to c-di-GMP binding, so we considered the possibility that R20291 has higher basal levels of c-di-GMP, leading to greater expression of *pilA1*. However, direct measurements show that the strains have comparable intracellular concentrations of c-di-GMP during growth in broth culture. This result was corroborated by studies using a c-di-GMP biosensor consisting of the constitutive *gluD* promoter, the Cdi2_4 c-di-GMP riboswitch, and a *gusA* transcriptional reporter, which showed comparable activities in R20291 and 630/630Δ*erm* during growth in broth culture.

Using the P_{gluD}-UTR-*gusA* fusion as a c-di-GMP biosensor, we observed a modest but significant increase in intracellular c-di-GMP during growth of R20291 in a biofilm and on agar surfaces, which was more pronounced on harder agar surfaces. The degree of c-di-GMP fluctuation in *C. difficile* is unknown, so the biological significance of an approximately 2-fold increase in c-di-GMP during growth on harder surfaces is unclear. Nonetheless, this increase coincides with TFP-dependent motility and may reflect the extent of c-di-GMP increases *in vivo*. No evidence of a change in c-di-GMP was observed for 630/630Δ*erm* grown under any of the described conditions. Consistent with this, R20291, but not 630/630Δ*erm*, upregulates *pilA1* transcription during growth in a biofilm. Unfortunately, 630/630Δ*erm* does not yield enough biomass in biofilms to allow estimation of c-di-GMP levels in biofilm-derived cells using either β-glucuronidase assays or UPLC-MS. However, the fact that *pilA1* transcription is upregulated during biofilm formation on a surface in R20291 but not 630/630Δ*erm* suggests that R20291 responds more strongly than 630/630Δ*erm* to the physical properties of its substratum. Together, these findings suggest that a surface-induced increase in intracellular c-di-GMP, which increases read-through transcription of the *pilA1* transcript, promotes R20291 TFP-mediated spread across and attachment to surfaces physically comparable to those it would encounter in an infected host.

Using reporters under the control of the *pilA1* promoter region, which is identical in R20291 and 630/630Δ*erm*, we determined that *pilA1* promoter activity is higher in R20291. This difference in *pilA1* expression between the strains is independent of the c-di-GMP riboswitch. We propose that an unidentified *trans*-acting regulatory factor mediates the higher *pilA1* transcription seen in R20291. Such a regulator may be differentially produced or active in the two strains, and/or the regulator(s) controlling *pilA1* transcription initiation may not be fully conserved. Transcriptional analyses revealed that hundreds of conserved genes are differentially expressed by 630 and R20291, so it is clear that differences in genetic regulation between the two strains will be an important subject for future studies (16). It appears that promoter-driven transcription of *pilA1* differs between the two strains, while control of *pilA1* transcript elongation via the c-di-GMP riboswitch is conserved between 630/630Δ*erm* and R20291. Thus, R20291 may display greater c-di-GMP-induced, TFP-mediated surface behaviors because it produces more *pilA1* transcripts that can respond to increases in intracellular c-di-GMP and, likewise, upregulates c-di-GMP production during growth on surfaces. It is unclear how large a change in the c-di-GMP concentration is required to trigger an increase in pilus gene expression and mediate TFP-dependent processes or if such a threshold differs between the two strains.

c-di-GMP consistently represses flagellum-mediated motility in diverse species, such as *P. aeruginosa* and *B. subtilis*, and appears to repress TFP-based twitching motility in *P. aeruginosa* (20, 77–79). However, we have found that c-di-GMP promotes TFP-dependent surface motility in *C. difficile*. As TFP in Gram-positive bacteria have only recently been identified, it is possible that their regulation and functions differ from those in Gram-negative bacteria (34, 35, 51). c-di-GMP promotes TFP formation and TFP-dependent biofilm formation and surface motility in *C. difficile*, mediating the organism's interactions with surfaces. The discov-

ery that R20291 is more responsive to surfaces than 630/630Δ*erm* makes it clear that the differences between historical and epidemic strains must be evaluated under multiple conditions. As the interactions of *C. difficile* with host epithelial surfaces become better characterized, it may be revealed that surface sensing contributes to *C. difficile* virulence and persistence.

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