THE C-DI-GMP REGULATORY NETWORK IN *CLOSTRIDIOIDES DIFFICILE* AND ITS ROLE IN MODULATING SURFACE ADHERENCE AND PERSISTENCE IN THE MAMMALIAN GUT

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ABSTRACT

Robert Woodrow McKee: The c-di-GMP regulatory network in *Clostridioides difficile* and its role in modulating surface adherence and persistence in the mammalian gut
(Under the direction of Rita Tamayo)

Clostridioides difficile (*Clostridium difficile*) is a spore-forming bacterial pathogen responsible for hundreds of thousands of infections each year in the United States. *C. difficile* outbreaks are common in hospitals because *C. difficile* spores can persist for months on surfaces and are resistant to many disinfectants. Despite the significant disease burden that *C. difficile* represents, we know surprisingly little about the factors necessary for *C. difficile* to colonize and persist in the mammalian intestine. Previous work demonstrated that the signaling molecule cyclic diguanylate (c-di-GMP) regulates a variety of processes in *C. difficile* including production of the toxins that are required for disease symptoms. Using monolayers of human intestinal epithelial cells, we demonstrate that c-di-GMP promotes attachment of *C. difficile* to intestinal epithelial cells. We also demonstrate that regulation of type IV pili (TFP) by c-di-GMP promotes prolonged adherence of *C. difficile* to epithelial cells *in vitro*. *C. difficile* mutants lacking TFP were cleared more quickly than the parental strain during single strain mouse infections and were outcompeted by the parental strain during *in vivo* competition experiments in mice. Thus, our data provides evidence that TFP promote persistence of *C. difficile* in the intestine. To determine what other genes c-di-GMP regulates in *C. difficile*, we performed RNA-sequencing comparing the transcriptome of *C. difficile* with elevated c-di-GMP to that of *C. difficile* with basal levels of c-di-GMP. We demonstrate that c-di-GMP regulates the expression

of 166 genes greatly expanding the known members of the c-di-GMP regulon. We demonstrate that c-di-GMP regulation of several transcripts in *C. difficile* is dependent on c-di-GMP sensing riboswitches present in the 5' untranslated regions of these transcripts. Our results also show that c-di-GMP regulates a number of cell envelope proteins in addition to TFP and flagella. These data suggest a broader role for c-di-GMP in remodeling the *C. difficile* cell surface.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variation
BHIS	Brain heart infusion supplemented with yeast extract
CDI	*Clostridiodes difficile* infection
c-di-GMP	Cyclic diguanylate
CFU	Colony forming unit
CI	Competitive Index
CROP	Combined repetitive oligopeptide
DGC	Diguanylate cyclase
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's modified phosphate buffered saline
DSS	Dextran sodium sulfate
Erm	Erythromycin
FBS	Fetal bovine serum
GTP	Guanosine triphosphate
IBD	Inflammatory bowel disease
MDCK	Madin-Darby canine kidney
mRNA	Messenger RNA
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PTS	Phosphotransferase system

qRT-PCR	Quantitative reverse-transcription polymerase chain reaction
RBS	Ribosomal binding site
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RPKM	Reads per kilobase per million mapped reads
TCCFA	Taurocholate cycloserine cefoxitin fructose agar
TCRS	Two component regulatory system
TFP	Type IV pili
TY	Tryptone yeast extract media
UDP	Uridine diphosphate
UTR	Untranslated region

CHAPTER 1: INTRODUCTION

Clostridioides difficile (formerly *Clostridium difficile*) (1) is a Gram-positive bacterial pathogen that is responsible for approximately half a million infections yearly in the United States and an estimated 4 billion dollars in associated healthcare costs (2). *C. difficile* infections (CDI) are one of the most common hospital-acquired infections. CDI can range in severity from mild diarrhea to potentially life-threatening pseudomembranous colitis and toxic megacolon (3, 4). Disease symptoms are mediated in part due to the secretion of toxin(s), TcdB and/or TcdA, and strains lacking these toxins do not cause disease (5-8). Despite the current prevalence of these infections in the healthcare system, the importance of *C. difficile* as a pathogen has only been appreciated for a few decades.

DISCOVERY OF *CLOSTRIDIOIDES DIFFICILE*

C. difficile was first isolated in 1935 from the stools of healthy infants (9). The researchers named it *Bacillus difficilis* owing to its rod-like shape and the difficulties they experienced when trying to culture the bacterium (9). Despite its prevalence in the stools of healthy infants, they discovered that *C. difficile* was highly virulent in guinea pigs and rabbits, killing nearly all the animals into which bacterial cultures were injected. Similar effects were observed when they injected bacteria-free media from the *C. difficile* cultures into guinea pigs; however, boiling the media rendered it non-toxic. This finding indicated that the bacteria were secreting a heat-labile toxin and that this toxin was responsible for causing disease (9). However,

because the bacterium was isolated from healthy infants with no signs of acute bacterial infection, it was unclear whether *C. difficile* was virulent in humans. In the early 1960s, on the basis of eight *C. difficile* samples isolated from patients, Smith and King concluded that there was no solid evidence that *C. difficile* was "anything other than a secondary invader" and that the bacterium was unlikely to cause significant disease in humans (10). They determined that reports of disease attributed to *C. difficile* were likely coinfections with other more virulent bacteria (10).

By this point, surgeons had noted high rates of pseudomembranous colitis, a severe inflammation of the colon, among patients undergoing invasive surgery, but the cause was assumed to be *Staphylococcus aureus* infection (11, 12), and patients were successfully treated with the antibiotic vancomycin (13). In cases where *S. aureus* was unable to be cultured from patient stools, the source of the pseudomembranous colitis remained mysterious. In 1974, researchers reported high rates of diarrhea (21%) and pseudomembranous colitis (10%) in patients receiving the antibiotic clindamycin (14). Additional reports from other hospitals mirrored the findings associating clindamycin with diarrhea and pseudomembranous colitis, thus renewing interest in determining the cause of antibiotic-induced colitis (15, 16).

For a number of years, scientists had observed that treatment of hamsters with certain antibiotics caused the hamsters to develop colitis that was similar to the pseudomembranous colitis seen in patients (17). A group of scientists at Tufts University tested samples from hospital patients with pseudomembranous colitis and determined that they contained a cytotoxin that was neutralized by antibodies to *Clostridium sordellii* toxin (18, 19). Following up on these findings, a separate set of researchers attempted to isolate the bacterium producing this cytotoxin from the feces of hamsters with clindamycin-associated enterocolitis (20, 21). They successfully isolated *C. difficile* from these hamsters and demonstrated that the bacteria produced toxins that

were neutralized by *C. sordellii* antitoxin (21). These results were quickly corroborated by other groups using the hamster model of antibiotic-induced enterocolitis (22). These experiments finally demonstrated that *C. difficile* was a bona fide pathogen and put an end to the era where CDI was spuriously classified as *S. aureus* enterocolitis.

THE *C. DIFFICILE* LIFECYCLE AND TRANSMISSION

C. difficile is an obligate anaerobic bacterium, so its growth is restricted to environments where oxygen is extremely limited (23). *C. difficile* and a variety of other Clostridia are common members of the gut microbiota in many mammalian species, including humans. Many Clostridia are commensal within the gut and may even provide a variety of benefits to their host (24-26). While the mammalian colon is largely anaerobic, the extracorporeal atmosphere is replete with oxygen, so the bacteria need a way to survive outside the host in order to successfully be transmitted to new hospitable environments (27). *C. difficile*, as well as a variety of other Gram positive bacteria, survive exposure to oxygen and harsh environments by forming endospores (23). When triggered by environmental cues, these bacteria undergo sporulation, which results in the production of metabolically dormant spores that are resistant to a wide variety of environmental stresses (28-30). Many environmental cues that lead to sporulation are known in the model organism *Bacillus subtilis* (31). However, the histidine kinases that control sporulation initiation in *B. subtilis* are not conserved in the genome of *C. difficile*, and the signals regulating sporulation in *C. difficile* are largely unknown (30). *C. difficile* spores are not only resistant to oxygen, but they are also resistant to high temperatures, low pH, antibiotics, and many commonly used disinfectants (32-34). These spores can remain for weeks to months on contaminated surfaces (35, 36). The combination of their resistance to disinfectants and their

potential to contaminate areas for long periods of time makes these spores particularly problematic in healthcare settings (37).

Once the spores are ingested by a susceptible host, the spores are well adapted to survive gastric passage and reach the intestines, where a combination of bile acids, calcium, and amino acids is sensed by the spores and triggers their germination into actively growing "vegetative" *C. difficile* (38, 39). If there is a favorable environment for the growth of *C. difficile*, the bacteria may colonize and grow within the lower GI tract. The factors necessary for *C. difficile* to colonize the colon remain unclear. Toxigenic *C. difficile* strains secrete glucosylating cytotoxins that are critical for disease symptom development (discussed below). In response to poorly-defined environmental cues, a subset of the *C. difficile* population within the intestine undergoes sporulation (40, 41). These spores are expelled in feces with the potential to spread to new animal or human hosts (40, 41). In hospitals and elderly care facilities, the rooms of *C difficile* patients are sometimes quarantined and disinfected with hydrogen peroxide to prevent the spread of *C. difficile* spores to other susceptible patients (34, 42, 43).

Despite its association with disease, *C. difficile* is a relatively frequent constituent of the human gut microbiota, with approximately 2-5% of the adult population asymptomatically colonized with the bacterium at any given point (44). While a portion of these *C. difficile* strains do not encode toxins and are thus nonpathogenic, many seem capable of producing toxins but are nonetheless carried asymptomatically (44, 45). *C. difficile* can also be found as a component of the microbiota in other animals, including pigs, dogs, and birds (45-47). Many of these strains are non-toxigenic, however, toxigenic strains with the potential to cause disease are also isolated from these potential animal reservoirs (45-47). The extent to which these animal reservoirs contribute to *C. difficile* spread to humans is unknown, but there is evidence that transmission

from pigs may have played an important role in the emergence of new strains of *C. difficile* in the Netherlands (46, 48).

RISK FACTORS FOR CDI

Healthy individuals with undisturbed intestinal microbiota are normally resistant to *C. difficile* associated disease, but disruptions of the intestinal microbiota and impairment of the immune system are risk factors for developing CDI (3, 49, 50). Additionally, the elderly have much higher rates of CDI and a worse prognosis upon diagnosis (51). Recent studies have revealed that resistance to CDI is multifactorial and can be affected by the presence of other bacteria in the intestine that promote or inhibit the growth of *C. difficile* (52, 53).

The interactions between *C. difficile* and other members of the gut microbiota are complex, but a few key relationships have been demonstrated over the last few years. *Bacterioides thetaiotaomicron*, a common member of the gut microbiota and a common ingredient in probiotic supplements, encodes a sialidase enzyme that cleaves sialic acid from mucins in the mucus of the colon (54). *C. difficile* is able to import the cleaved sialic acid and use it as an energy source (54). Animal experiments demonstrated that the number of *C. difficile* in the colon was enhanced when mice were colonized with both *C. difficile* and *B. thetaiotaomicron* compared to *C. difficile* alone. These data suggest that *B. thetaiotaomicron* is a poor choice for probiotics in *C. difficile* patients as these bacteria can promote growth of the pathogen in the mammalian colon (54, 55).

An intestinal microbiome rich in bacteria of the class Clostridia is often associated with resistance to *C. difficile* colonization in hosts (53, 56, 57). *Clostridium scindens*, a distant relative of *C. difficile*, is one bacterium that provides resistance to CDI (56). *C. scindens* produces an enzyme that converts primary bile acids in the gut to secondary bile acids. Binding of bile acids

such as cholate and taurocholate to receptors on *C. difficile* spores promotes the germination of spores into actively growing bacteria (39, 58). *C. scindens* converts the primary bile acid cholate into deoxycholate, a secondary bile acid that inhibits the growth of vegetative *C. difficile*. Members of the bacterial families Lachnospiraceae and Ruminococcaceae are also associated with enhanced resistance to *C. difficile* colonization, but the exact mechanism of this protection has not been determined (57). Some of the species in these families can also convert cholate to deoxycholate, so their presence in the microbiota may inhibit *C. difficile* growth due to increased deoxycholate concentrations in the gut (59).

Treatment with broad spectrum antibiotics such as clindamycin shifts the composition of the gut microbiota by removing many commensal bacteria (53, 60, 61). Levels of Ruminococcacea and Lachnospiraceae (including *C. scindens*) are decreased following antibiotic treatment (53, 62). Accordingly, the intestines of mice treated with clindamycin have much lower levels of *C. difficile*-inhibiting bile acids like deoxycholate and higher levels of germination-promoting taurocholate compared to untreated mice, which are resistant to *C. difficile* colonization (52, 60). The cecal contents of animals with high levels of deoxycholate have been shown to inhibit *C. difficile* growth and have led to the prospect of treating patients by increasing the content of deoxycholate in the colon (52). However, high concentrations of deoxycholate in the gut have been identified as a risk factor for colon cancer indicating that there may be unintended side effects to increasing deoxycholate levels (63, 64).

Age is another factor that can affect the severity of CDI (65, 66). Infants often have very high rates of *C. difficile* recovery in the stools with colonization rates as high as 70% compared to ~3% in adults (67, 68). Although many of the *C. difficile* strains recovered from infants are toxigenic, these infants are usually asymptomatic carriers of the bacterium (44, 67). The reason

for the lack of disease in infants is unclear (44). One current hypothesis is that a combination of toxin antibodies in breast milk and a lower incidence of toxin binding receptors in infants render them relatively insensitive to the effects of the *C. difficile* toxins TcdA and TcdB (44, 69). Infants are not immune to disease, however, and there has been an increase in the number of infants with symptomatic CDI in the past decade, possibly due to an increase in the prevalence of "hypervirulent" *C. difficile* strains such as those of ribotype 027 (44). In contrast to infants, the rates and severity of CDI are much higher among the elderly, with most deaths from CDI occurring in patients over age 65 (65, 66, 70). Some of this increase may be due to the increased likelihood of comorbidities in elderly patients, but other factors are likely at play (70, 71). For example, immune system dysfunction is also associated with higher rates of *C. difficile* infection, and immune dysfunction is much more common in elderly patients (72). Recently, it was shown that high calcium levels in the gut promote the germination of *C. difficile* spores (73). Calcium absorption is reduced with age, so it is possible that calcium levels in the intestines of elderly patients enhance their risk of CDI, but this has not been demonstrated experimentally (74, 75). There are also age-related shifts in the composition of the gut microbiota, with elderly patients showing decreases in the diversity of obligate anaerobic bacteria and increases in clostridial species (49). These shifts in the microbiota may also contribute to the increased susceptibility to CDI among the elderly.

PATHOGENESIS OF CDI

C. difficile pathogenicity is in large part driven by the actions of cytotoxins produced by the vegetative bacteria in the colon (71). All strains known to cause disease produce one or two large glucosylating toxins, TcdA and TcdB (7, 71). These proteins are made up of four domains:

an N-terminal glucosyltransferae domain, an autoprotease domain, a delivery domain, and a CROP domain that is involved in receptor binding (76). The two toxins are 48% identical in their amino acid sequence, and they differ primarily in regions involved in receptor binding (76, 77). These toxins bind to receptors on the surface of target cells and are internalized via receptor-mediated endocytosis. Once inside the cell, the toxins are cleaved by the autoprotease domain, releasing the glucosyltransferase domain into the cytoplasm (76, 78). The glucosyltransferase domain uses UDP-glucose as a substrate to glucosylate Rho family GTPases in the host cell (76, 77). The glucosylation of Rho proteins renders them unable to bind to their targets and has a variety of consequences for the host cell (79-81). Because Rho family GTPases are critical for actin cytoskeleton regulation and the maintenance of tight junctions between cells, glucosylation of Rho proteins leads to disruption of the tight junctions between target cells (76, 82). Additionally, the glucosylation of these Rho proteins triggers controlled cell death pathways in a cell-type and toxin-type dependent manner (76). Activation of the inflammasome by glucosylated Rho proteins triggers production of IL-1β and drives inflammation in a mouse model of CDI (83, 84). The combination of cell death, tight junction breakdown, and inflammasome activation leads to extensive inflammation in the colonic epithelium, disruption of the epithelial barrier, and the diarrhea associated with CDI (71, 81-84).

REGULATION OF TOXIN PRODUCTION

The genes encoding the *C. difficile* toxins are located on a genetic region known as the pathogenicity locus (PaLoc) (85). The PaLoc also contains genes encoding an alternative sigma factor (TcdR), a putative anti-sigma factor (TcdC), and a holin protein involved in toxin secretion (TcdE) (85-88). Transcription of the toxin genes is positively regulated by TcdR, a

sigma factor that directs RNA polymerase to the promoters of *tcdA* and *tcdB* allowing transcription of the genes (88). TcdC has been reported to be a negative regulator of toxin production in some studies, but mutation of *tcdC* did not alter toxin production in another study, leaving the role of TcdC in toxin regulation unclear (87, 89, 90).

A number of proteins are known to regulate the transcription of the toxin genes. These include the transcription factors CcpA, CodY, Spo0A, SigH, and SigD (40, 91-96). Toxin synthesis has long been known to be repressed by an abundance of nutrients, in particular glucose (97, 98). CcpA is a global transcriptional repressor that represses transcription of genes related to the transport and catabolism of other carbon sources when glucose (a preferred carbon source) is present (99, 100). In most bacteria, when glucose levels are high inside the cell, the HPr kinase phosphorylates CcpA and activates it, however HPr is not sufficient for CcpA activation in *C. difficile* (91, 99). Activated CcpA then binds to sequences known as *cre* sites and inhibits transcription of downstream genes (99). In *C. difficile*, CcpA binds to *cre* sites 5'of *tcdR*, inhibiting *tcdR* transcription and ultimately repressing expression of *tcdA* and *tcdB* (91, 100). CodY also controls expression of *tcdR* in response to nutrient availability (101). When activated by high intracellular concentrations of branched chain amino acids and GTP, CodY binds with high affinity to regions 5' of the target genes (including *tcdR*) and represses their expression (102). When nutrient levels become limiting, CodY repression is removed and expression of the toxin genes resumes (101). Transcriptome analyses of other major transcriptional regulators in *C. difficile*, namely SigH and Spo0A, suggest additional pathways control the expression of the toxin genes. Mutation of *sigH* results in increased toxin levels (40, 93). However, the effect of SigH on toxin production is likely indirect because no SigH promoter is present upstream of *tcdA*, *tcdB,* or *tcdR* (93, 103, 104). There are conflicting reports on the effect of *spo0A* mutation

on toxin production (103, 105). One group demonstrated that a *spo0A* mutant produced less TcdA than the parental strain, and they also showed that the supernatant of the *spo0A* mutant was less cytopathic to Vero cells (105). However, another group showed that Spo0A was bound to the promoter upstream of *tcdB,* but they did not observe any difference in the cytopathic effect of this strain relative to the parental strain (103).

The last of the demonstrated transcriptional regulators of toxin production is the flagellar sigma factor, SigD (95, 96). The link between toxin production and flagellar biosynthesis in *C. difficile* was first demonstrated when mutations in flagellar genes led to large changes in transcription of the genes within the PaLoc (106). Our group demonstrated that the link between flagella and toxin production is the sigma factor, SigD (95). SigD is responsible for the transcription of the genes involved in later stages of flagellum assembly (96, 107). SigD also regulates a number of genes in *C. difficile* that are unrelated to flagellar function (96). Work from our lab and others showed that SigD regulates the transcription of the toxin genes by directly promoting transcription of the toxin transcriptional regulator, TcdR (95, 96).

COLONIZATION FACTORS OF *C. DIFFICILE*

Much of the research on *C. difficile* has focused on the toxins because they are sufficient for disease in animal models, and their functions can be studied in detail without the need for manipulation of *C. difficile*. However, the factors that *C. difficile* uses to colonize and persist within the gut are largely unknown, despite the fact that colonization of the intestinal mucosa is a requisite step in disease development (108). Several *C. difficile* proteins have been implicated in binding to host cells *in vitro*. These include SlpA, FbpA, and Cwp66. SlpA, the major component of the *C. difficile* S-layer, binds to host cells, and antibodies raised against SlpA

decrease *C. difficile* binding to Hep2 and Caco-2 cells, suggesting a role for SlpA in promoting host cell attachment (109, 110). In the *C. difficile* strain 630Δerm, a mutation in *fbpA*, which encodes a putative fibronectin binding protein, resulted in increased adherence to HT-29 cells but displayed a modest decrease in cecum colonization relative to the parent strain in monoxenic mouse infections (111). Finally, antibodies raised against a *C. difficile* cell wall protein, Cwp66, reduced binding to Vero cells *in vitro*, suggesting that Cwp66 is involved in host cell attachment (112). With the exception of the slight defect in cecum colonization in the *fbpA* mutant, none of these putative colonization factors have been shown to affect colonization in animal models of CDI (111).

While not required for initial colonization of the intestine, the sporulation master regulator Spo0A was shown to be important for persistence in mouse infections (40). In mouse co-infections, the *spo0A* mutant bacteria were recovered in equal numbers to the parental strain at early time points, but were outcompeted by the WT bacteria at later times during infection (40). Spo0A is a transcription factor that controls the expression of a large number of genes in *C. difficile*, so it is unclear whether this persistence defect is due to effects on sporulation or other pleiotropic effects from the loss of *spo0A* (113).

Perhaps the most well-studied colonization factor in *C. difficile* is the flagellum. Flagella are used for motility, but they can also function as adhesins to promote binding to a surface in a number of bacteria (114). *C. difficile* produces peritrichous flagella that are used for motility, and flagellum production is also necessary for optimal colonization of animal models in certain *C. difficile* strains (106, 115, 116). In strain 630Δerm (an erythromycin-sensitive derivative of the historical CD630 strain), mutation of *fliC* and *fliD* resulted in increased adherence to Caco-2 intestinal epithelial strains *in vitro* and increased virulence in hamsters (116). In strain R20291, a

more recently isolated epidemic isolate of *C. difficile*, bacteria lacking flagella were attenuated for colonization in single-strain infections of mice (116). A mutant with paralyzed flagella was able to colonize to nearly the same levels as the parental strain indicating that the flagellum itself is more important than flagellar motility for colonization in the R20291 strain (116). Differences in the role of flagella in colonization in these two strains may be due to differences both in the flagellin protein and in flagellar glycosylation (117). Alternatively, the differences in these experiments could be explained by the differing ability of these two strains to phase vary production of the flagellum (118). Flagella are additionally important in *C. difficile* pathogenesis due to the co-regulation of toxin gene expression with flagellar gene expression via the sigma factor, SigD (95, 96). Thus, factors that regulate flagella are also likely to regulate toxin production. One such factor is the signaling molecule c-di-GMP (95, 119).

C-DI-GMP SIGNALING

Cyclic bis-(3′ 5′) diguanylate (cyclic diguanylate, c-di-GMP) is a second messenger that is produced by nearly all bacteria (120). It was first described in 1987 as a negative regulator of cellulose synthesis in *Komagataeibacter xylinus*. c-di-GMP is a cyclic dinucleotide consisting of a 3' to 5' linkage of two guanosine monophosphate moieties (121). The c-di-GMP concentration within bacterial cells is modulated by enzymes controlling its synthesis and degradation. c-di-GMP is synthesized from two guanosine triphosphate (GTP) molecules by enzymes containing a GGDEF domain known as diguanylate cyclases (120). c-di-GMP is broken down by two distinct classes of c-di-GMP phosphodiesterases containing either an EAL or HD-GYP domain (See Figure 1.1). Processes regulated by c-di-GMP in bacteria include flagellar motility, polysaccharide production, biofilm formation, and virulence factor production (120). c-di-GMP

can regulate such processes by binding to protein and RNA-based receptors in the bacterial cell. Receptors that have been demonstrated to bind c-di-GMP include a subset of PilZ domain containing proteins, certain transcriptional regulators, type IV pilus assembly ATPase proteins, and structured RNA regions called c-di-GMP riboswitches (120, 122).

PilZ domains are widespread among bacteria and represent the best studied class of proteins that bind c-di-GMP (123). The BcsA protein, which controls cellulose synthesis in *Komagataeibacter xylinus*, and the YcgR protein, which controls flagellar motility in *Escherichia coli*, were the first PilZ domain proteins shown to bind c-di-GMP (124). When bound to c-di-GMP, YcgR interacts with the FliG and FliM flagellar switch proteins and serves to decouple these switch proteins from the motor protein, MotA (125, 126). This has the effect of slowing flagellar rotation and biasing the rotation in a single direction, resulting in greatly reduced swimming motility by *E. coli* (126, 127). PilZ domain proteins are widespread among bacteria, with many species encoding multiple PilZ domains, though not all PilZ domains bind c-di-GMP (120, 128, 129). In addition to PilZ domains and riboswitches, there are number of other c-di-GMP receptors that function in signaling. These include transcriptional regulators like VpsT and VpsR from *Vibrio cholerae*, MshEN domains usually found in type IV pilus (TFP) assembly ATPases, and the recently discovered arginine rich repeat regions of certain CheY family proteins (120, 122, 130, 131).

There are two classes of riboswitches known to bind c-di-GMP: the GEMM or class I c-di-GMP riboswitches, and the class II c-di-GMP riboswitches (132, 133). Riboswitches are structured RNA elements found in the 5’ untranslated region (UTR) of some mRNAs that directly and specifically bind a ligand (134). Riboswitches consist of two parts: an aptamer domain responsible for binding the ligand and an expression platform that controls transcription

or translation of the downstream RNA. Conformational changes that result from the interaction between the ligand and aptamer domain alter the expression platform structure to modulate transcriptional read-through or translation initiation (135). Aptamer and expression platform regions are often modular such that binding of a ligand to the aptamer can positively or negatively regulate gene expression, depending on the linked expression platform (135).

One mechanism by which riboswitches control gene expression is the modulation of transcription termination (132, 134). In an "off" riboswitch of this type, ligand binding promotes the formation of a terminator stem loop leading to decreased transcription of downstream genes (Figure 1.2) (135). When the transcript is not bound to the ligand, an anti-terminator stem loop is formed instead allowing transcription of the downstream genes (134, 135). In an "on" riboswitch that regulates transcription termination, binding of the ligand to the riboswitch promotes the formation of an anti-terminator stem loop, allowing expression of the downstream genes (135, 136). When the ligand is absent, formation of a terminator stem loop is favored and transcription of the downstream genes is reduced (135, 136). In addition to regulating transcription, riboswitches can also regulate translation initiation (135). Regulation of translation typically occurs through occlusion of the ribosomal binding site (RBS) within a stem-loop preventing ribosomes from binding to the RNA. As for riboswitches that regulate translation, ligand binding can either promote or prevent the formation of stem loops that occlude the RBS (134-136).

Our knowledge of c-di-GMP riboswitches largely relies on the results of *in vitro* studies of ligand binding or testing of riboswitch function in heterologous hosts (132, 133, 135). Only two riboswitches have been studied in their native genomic context and organism, both in *Vibrio cholerae* (137, 138). These riboswitches, Vc1 and Vc2, lie upstream of a genes encoding a *V. cholerae* colonization factor, GbpA, and a regulator of the type VI secretion system, TfoY,

respectively (137-140). For Vc1, work in our lab showed that Vc1 binds c-di-GMP and that this binding positively regulates the expression of *gbpA* (141). Looking at the riboswitch in isolation might lead one to conclude that c-di-GMP positively regulates *gbpA* expression. However, *gbpA* expression is inhibited in high c-di-GMP conditions through increased expression of a negative regulator of *gbpA* expression, NagC (141). Additionally, low levels of c-di-GMP lead to increased expression of *gbpA*, indicating that in these conditions, the decreased activity of the *gbpA* promoter in high c-di-GMP conditions overrides the positive regulation of *gbpA* expression through the riboswitch (137, 141). For the Vc2 riboswitch, the riboswitch was determined to negatively regulate *tfoY* expression (138). However, high c-di-GMP levels led to increased expression of *tfoY* that was independent of Vc2 (138). These examples suggest that c-di-GMP regulation of riboswitch-regulated genes can be very complex and that studying riboswitches in their native organism is necessary to properly appreciate the contributions of c-di-GMP riboswitches to the overall c-di-GMP regulation of the downstream genes.

C-DI-GMP SIGNALING IN *C. DIFFICILE*

C. difficile encodes 37 proteins with putative or demonstrated DGC or PDE activity, suggesting a complex c-di-GMP signaling network in *C. difficile* (142). Despite the large number of DGCs and PDEs encoded in the genome, *C. difficile* has few predicted protein receptors. *C. difficile* only encodes a single PilZ domain protein (BcsA) and one MshEN domain protein (PilB1) (120). Other potential protein effectors in *C. difficile* include catalytically inactive DGC proteins such as CD630_10280, which is an orthologue of the regulator of biofilm formation PssE from *Listeria monocytogenes* (143). Supplementing its putative protein receptors, *C. difficile* also encodes 16 putative c-di-GMP binding riboswitches. Of these, 12 are GEMM (class I) riboswitches and 4 are class II riboswitches (133). Intriguingly, many of the riboswitches are

positioned to regulate genes encoding putative cell surface proteins or surface structures (e.g. TFP and flagella). This could indicate an important role for c-di-GMP in modification of the *C. difficile* cell surface in response to extracellular stimuli. In *C. difficile*, c-di-GMP regulates a variety of processes including flagellar motility, type IV pilus (TFP) production, biofilm formation and surface motility (summarized in Figure 1.1) (119, 144-146). High intracellular concentrations of c-di-GMP repress flagellar gene expression and lead to decreased flagellar motility in *C. difficile* (119, 133). The flagellar riboswitch (Cdi-1-3) in *C. difficile* has been shown to bind c-di-GMP and promote transcriptional termination *in vitro*. Binding of c-di-GMP to the aptamer domain of the riboswitch is predicted to induce a conformational change in the mRNA, which results in the formation of a terminator stem loop in the expression platform, thus preventing transcription of the downstream flagellar genes (132). Thus, the flagellar riboswitch is an "off" riboswitch because the ligand-bound conformation promotes termination of transcription.

High levels of c-di-GMP promote the formation of type IV pili (TFP) in *C. difficile* (144). At high intracellular concentrations of c-di-GMP, *C. difficile* forms autoaggregates when grown in liquid culture (144). Increased intracellular c-di-GMP also promotes motility on agar surfaces and the formation of biofilm (119, 146). Autoaggregation, surface motility, and biofilm formation in response to high c-di-GMP are dependent on the production of TFP. A c-di-GMP riboswitch (Cdi-2-4) is encoded upstream of the *pilA1* gene, which encodes a major pilin, serves as a positive regulator of *pilA1* transcription (144, 145). The conformation of the RNA in the absence of c-di-GMP is predicted to favor transcription termination via formation of a terminator stem loop (144). Binding of c-di-GMP to the aptamer domain results in a conformational change in the RNA that precludes the formation of the terminator stem loop and allows transcription of

the *pilA1* gene (144). The *pilA1* riboswitch of *C. difficile* is thus an "on" riboswitch in which binding of the ligand promotes transcription of downstream genes. Using transcriptional reporter fusions of the *pilA1* promoter and 5' UTR to *gusA*, we showed that increasing intracellular c-di-GMP stimulated reporter activity (145). However, mutation of conserved riboswitch nucleotides predicted to be required for c-di-GMP binding led to decreased reporter activity overall and rendered reporter activity unresponsive to increased c-di-GMP. In addition, elevated c-di-GMP concentrations also promoted expression of *gusA* when the *pilA1* promoter was replaced with a constitutive promoter (145). These results indicate that regulation of TFP gene transcription by c-di-GMP requires the riboswitch upstream of *pilA1* in strain 630Δerm (145).

The contributions of TFP, biofilm formation, and surface motility to *C. difficile* pathogenesis are not currently known. Dense communities of bacteria covering the surface of the damaged microvilli have been recovered from mice in one model of *C. difficile* disease, and another study showed the formation of biofilm in mice that were mono-associated with *C. difficile* (147, 148). Another group reported that *C. difficile* binds solely to the mucus layer of the intestinal epithelium and they did not observe *C. difficile* attached to the epithelium in mouse infections (149). They did however observe communities of bacteria in the mucus layer, indicating that interactions with other bacteria may be important during *C. difficile* infection (149). Studies in the hamster model of *C. difficile* infection have shown strain-dependent differences in localization and interaction with the intestinal epithelium (150, 151). Overall these data indicate that *C. difficile* has the capacity to form biofilm and to interact with the intestinal epithelium during infection, but the contributions of these interactions to *C. difficile* pathogenesis is still unclear. The factors that mediate colonization of the intestinal tract by *C. difficile* remain unknown (152). As noted above, c-di-GMP controls the expression of type IV pili and other

surface proteins that could potentially serve as colonization factors. In other bacterial species, TFP are important for host colonization and contribute to host cell attachment, but the contribution of TFP to *C. difficile* colonization was hitherto unknown. The goals of my thesis project were to determine the role of TFP in host cell adherence and colonization of the mammalian gut and to expand the known members of the c-di-GMP signaling network in the important intestinal pathogen, *C. difficile*.

FIGURES

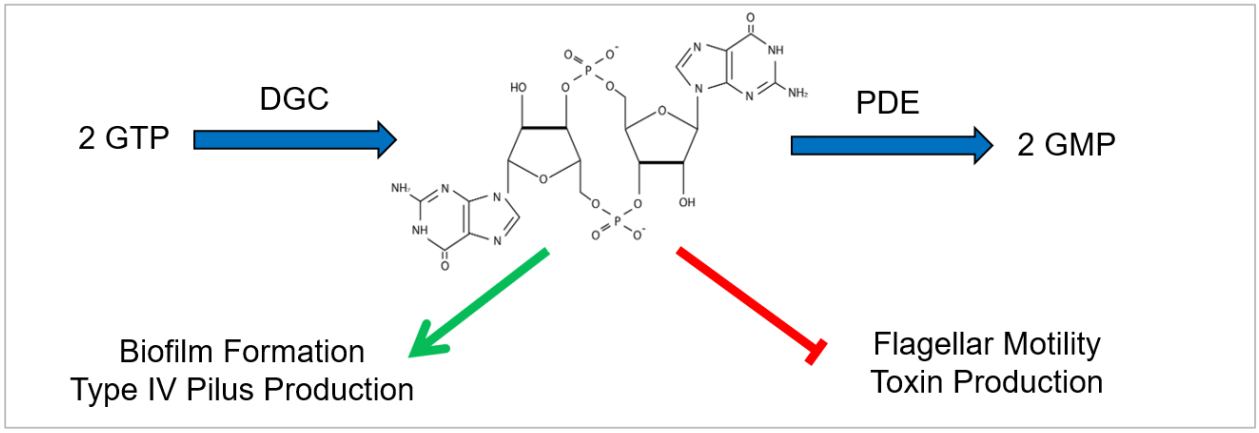

Figure 1.1. c-di-GMP signaling in *C. difficile*. c-di-GMP (pictured in the center) positively regulates TFP production and biofilm formation while negatively regulating flagellar motility and toxin production. DGC = diguanylate cyclase, PDE = phosphodiesterase.

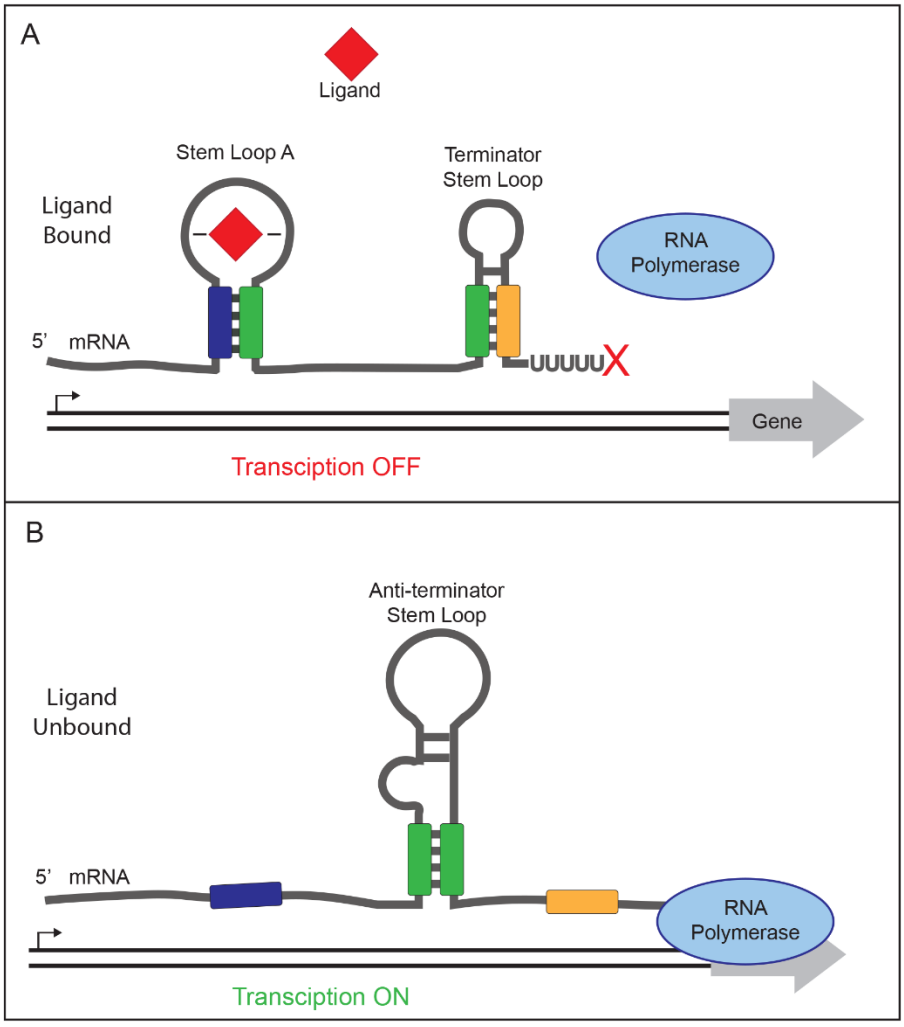

Figure 1.2 Transcriptional termination controlled by an "off" riboswitch. (A) Ligand binding stabilizes stem loop A and promotes formation of a terminator stem loop leading to transcript termination. (B) Ligand is unbound favoring the formation of an anti-terminator stem loop allowing transcript elongation.

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CHAPTER 2: TYPE IV PILI PROMOTE *CLOSTRIDIUM DIFFICILE* ADHERENCE AND PERSISTENCE IN A MOUSE MODEL OF INFECTION[1]

SUMMARY

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* lacking TFP were particularly deficient in association with the cecal mucosa. Together these data indicate that TFP, and their

[1] This chapter previously appeared as an article in the journal *Infection and Immunity*. Figure numbers have been modified. The original citation is as follows: McKee RW, Aleksanyan N, Garrett EM, Tamayo R. *Infect Immun* 2018 Feb 26. pii: IAI.00943-17. doi: 10.1128/IAI.00943-17. I performed all the experiments with the exception of the attachment assays in Figure 2.4, which were performed by Naira Aleksanyan. Liz Garrett assisted me with the animal experiments. I wrote the manuscript with edits from my advisor, Rita Tamayo.

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.

INTRODUCTION

Clostridium difficile, recently reclassified as *Clostridioides difficile* (1), is a spore-forming, obligate anaerobe responsible for diarrheal diseases resulting in substantial morbidity and mortality (1, 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, ~20-30 % 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 the toxin(s), TcdB and/or 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 co-infection experiments (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 630Δerm, 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 co-challenge infections with the parental strain (19, 21, 22). These strain specific-phenotypes may be attributable to differences in the abilities of R20291 and 630Δerm strains to phase vary 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 non-motile 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 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.

MATERIALS AND METHODS

Bacterial growth conditions

Strains and plasmids used in this study are listed in Table S1. 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% H_2, 5% CO_2 and 90% N_2. 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 (Table S2). 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, 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 Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS) at 10% (HT-29) or 20% (Caco-2). Tissue culture-treated 24-well plates (Corning) were seeded with approximately ~10^5 cells per well. Tissue culture cells were grown at 37 °C in 5% CO_2 for 5-7 days until a confluent monolayer was achieved, with fresh media 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 hour attachment assays, Madin-Darby Canine Kidney (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% CO_2 to allow formation of confluent monolayers, with fresh media added as needed.

C. difficile strains grown overnight (~16 hours) 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 µL) was added to 24-well plates seeded with HT-29, Caco-2, or MDCK cells and containing 450 µl DMEM with FBS. The plates were sealed with tape, removed from the anaerobic chamber, and centrifuged for 10 min at 1000 x 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 hour (HT-29 and Caco-2 cells) or 24 hours (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 non-adherent bacteria. After the final wash, the epithelial cells and attached bacteria were scraped from the plate and suspended in 500 µl 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 ~10^5 cells per cm^2 on Thermanox^{Tm} (ThermoFisher) coverslips and grown for 5 days (HT-29) or 3 days (MDCK) in DMEM + 10% FBS. Bacteria were grown, added to the tissue culture cells and incubated as described above for the attachment assays. Following incubation, the monolayers were washed twice with 500 µl

PBS to remove unattached bacteria, then fixed by adding 500 µl of ice cold methanol for 5 minutes. 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 600X magnification.

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 (63) and incubating 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 for release of mature spores. Spores were purified from the suspensions by sucrose gradient as previously described (64). Spores were enumerated by plating serial dilutions on BHIS agar containing the germinant 0.1% sodium taurocholate (65).

Groups of 8-10 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 *ab libitum* for 3 days as described previously (66). The antibiotics were provided at the following concentrations: kanamycin (400 µg/ml), gentamycin (35 µg/ml), colistin (850 units/ml), vancomycin (45 µg/ml), and metronidazole (215 µg/ml) (66). 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 µg/g body weight) was administered either 24 hours prior to infection (single strain infections) or 48 hours prior to infection (competitions). The timing of the clindamycin injection was modified to 48 hours prior to inoculation in the competition experiments to ensure that clindamycin concentrations did not

favor growth of the mutants, which contain the *ermB* gene that provides some resistance to clindamycin (67, 68). Mice were inoculated with 10^5 colony forming units (CFU) of *C. difficile* spores (single strain infections) or 2 x 10^5 total spores (co-infections) by oral gavage. Feces was collected in pre-weighed tubes every 24 or 48 hours for 7-14 days, as indicated.

Fecal samples were weighed following collection, 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 μg/ml cefoxitin, and 250 μg/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 μg/ml erythromycin (for 630Δerm strains) or 20 μg/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 (resistant CFU/[total CFU-resistant CFU])$_{output}$ was divided by the ratio of mutant to parental bacteria in the initial spore inoculum (resistant CFU/[total CFU-resistant CFU])$_{input}$. A CI of 1 indicates no difference in bacterial burden, CI <1 indicates that the parental strain outnumbers the mutant, while a CI of >1 indicates that the mutant bacteria outnumber the parental strain.

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 in 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 does not substantially inhibit growth, particularly at 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 hour under strict anaerobic conditions (required by *C. difficile*), as the monolayers begin to lose integrity by 4 hours (Figure 2.1). Expression of *dccA* in 630Δerm led to a 12- and 96-fold increase in attachment to HT-29 and Caco-2 cell monolayers, respectively, compared to *C. difficile* bearing the control vector grown with nisin induction (Fig 2.2A, 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* 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 (Figure 2.2C-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* 630Δerm 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* 630Δerm with an intron insertion in *sigD*, which encodes the flagellar sigma factor (69); this mutant was previously shown to lack flagella and swimming motility (23, 53, 70). We compared 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 parental strain bearing vector. Consistent with the previous results showing that aflagellate *C. difficile* are more adherent to tissue culture cells, the 630Δerm *sigD* mutant containing vector alone adhered to HT-29 cells at significantly higher levels than the parental vector-bearing strain (Figure 2.3A). Complementation with the *sigD* gene under the control of the nisin-inducible *cpr* promoter restored adherence to parental levels (Figure 2.2A). 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 (Figure 2.3B). 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 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* mutants showed somewhat reduced adherence to HT-29 cells compared to the parental strain, but the differences were not statistically significant (Figure 2.4A). *C. difficile* with basal c-di-GMP levels produce relatively low numbers of TFP during growth in BHIS medium (53). We reasoned that increasing intracellular c-di-GMP by over-expressing *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 (Figure 2.4B).

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 hours (71, 72). However, Caco-2 and HT-29 cell monolayers lose integrity after prolonged incubation under the strict anaerobic conditions required by *C. difficile* (Fig 2.1). 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 (73). Whereas monolayers of either Caco-2 or HT-29 cells were substantially disrupted after as few as 4 hours in the anaerobic chamber, MDCK cells maintained their monolayers after 24 hours of incubation in the anaerobic chamber (Figure 2.1). As with HT-29 and Caco-2 cells, TFP were dispensable for attachment to MDCK cells at 1 hour (Figure 2.5A). After 24 hours incubation, the *C. difficile pilA1* and *pilB1* mutants showed significantly reduced attachment to MDCK cells (Figure 2.5B). Growth of *pilA1* and *pilB1* mutant bacteria

was equivalent to the parental strain in DMEM + 10% FBS (Figure 2.6). The mutations also did not affect flagellum-based swimming motility (Figure 2.7).

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 (Figure 2.5C). 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 (Figure 2.5C, $pPilA1^{mut}$). 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.

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 (74). 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 (66). 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, a medium that allows outgrowth of *C. difficile* spores while inhibiting the growth of other bacteria (75). 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 (Figure 2.8). 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 for the TFP mutant infections were below the limit of detection in all mice, while all but one mouse infected with 630Δerm were still shedding spores above the limit of detection (Fig 2.8). An independent mouse colonization experiment yielded similar results, but with slight changes in the timing of the decline in bacterial burden.

To control for variation between mice and to better capture subtle difference between strains, we performed co-infections with 630Δerm and either the *pilA1* or *pilB1* mutants. The *ermB* gene used to generate the *pilA1* and *pilB1* mutations allowed differentiation between the Erm-resistant mutants and Erm-sensitive parent strain. A competition between the parent strain and a *tcdR* mutant, which expresses the glucosylating toxin genes at significantly lower levels (69, 76) but should be competent for colonization (14, 15, 17), was included as a control. On day 1 post-inoculation, the competition indices (CI) were ~1, indicating an equal ability to establish colonization. Throughout the 14 day experiment, the *pilA1* mutant was usually present in feces at numbers comparable to the parent strain; the exceptions are days 7 and 9, when significantly fewer *pilA1* mutant bacteria were recovered (Figure 2.9A). In contrast, the *pilB1* mutant showed attenuated colonization after day 1, with CI's significantly less than 1 on days 3, 5, 7, 9, and 13 (Figure 2.9B). The control experiment using the *tcdR* mutant yielded CI's equal to or somewhat greater than 1 (Figure 2.9C), 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 630Δerm 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 co-infection with the parental strain, the R20291 *pilB1* mutant was initially recovered from feces in numbers comparable to the parental strain, but was outcompeted by the parental strain on days 3 and 5 post-inoculation (Figure 2.10). 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 co-inoculated with the *pilB1* mutant and 630Δerm strains, and ceca were harvested at day 3, when the mutant first showed decreased recovery from feces (Figure 2.9B). 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 media and the CI's were calculated separately. In the cecal lumen, the *pilB1* mutant 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) (Figure 2.11). 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 interfere 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, could promote early attachment of *C. difficile* to host cells (77, 78).

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 stimulation of c-di-GMP production may occur during interactions with host cells as well. c-di-GMP may also post-translationally regulate TFP biosynthesis in *C. difficile*. Some TFP are associated with PilB ATPases containing a MshEN domain that binds c-di-GMP (79, 80). 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 (81). 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 *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* have been observed associated with the intestinal epithelium of *C. difficile* mono-associated mice (61, 62), and in mucus-associated mixed-species communities in the cecum and colon (82). Given their role in autoaggregation of *C. difficile*, TFP may facilitate or stabilize 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 (83-86). 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 (87), these models may not be not ideal for studying putative colonization factors because these are closed systems. Models using differentiated stem cells grown on scaffolds could allow for co-culture 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 (86). 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).

FIGURES

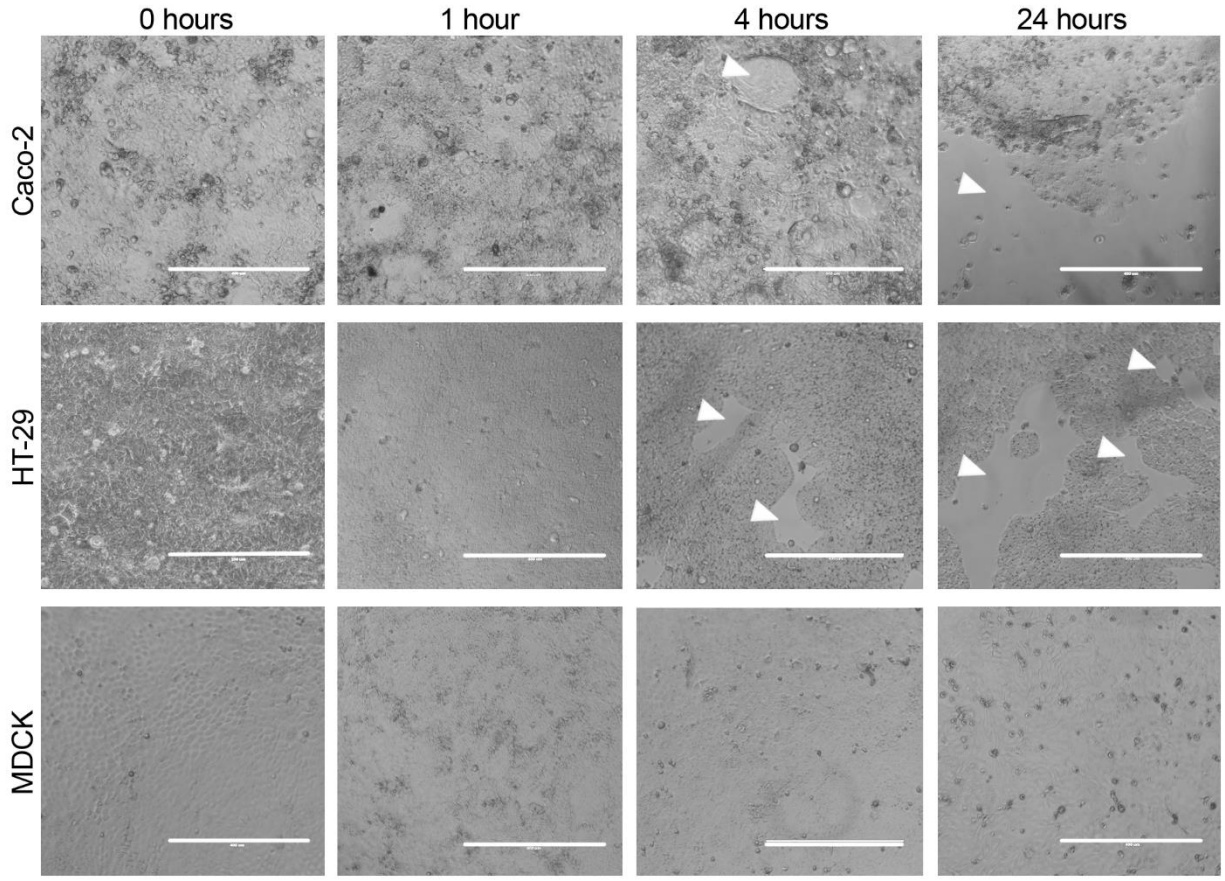

Figure 2.1. Viability of epithelial cell lines under anaerobic conditions. Monolayers of Caco-2, HT-29, and MDCK cells before and after incubation in anaerobic conditions for 1, 4 and 24 hours. Scale bar = 400 μm. White arrows indicate gaps in monolayers.

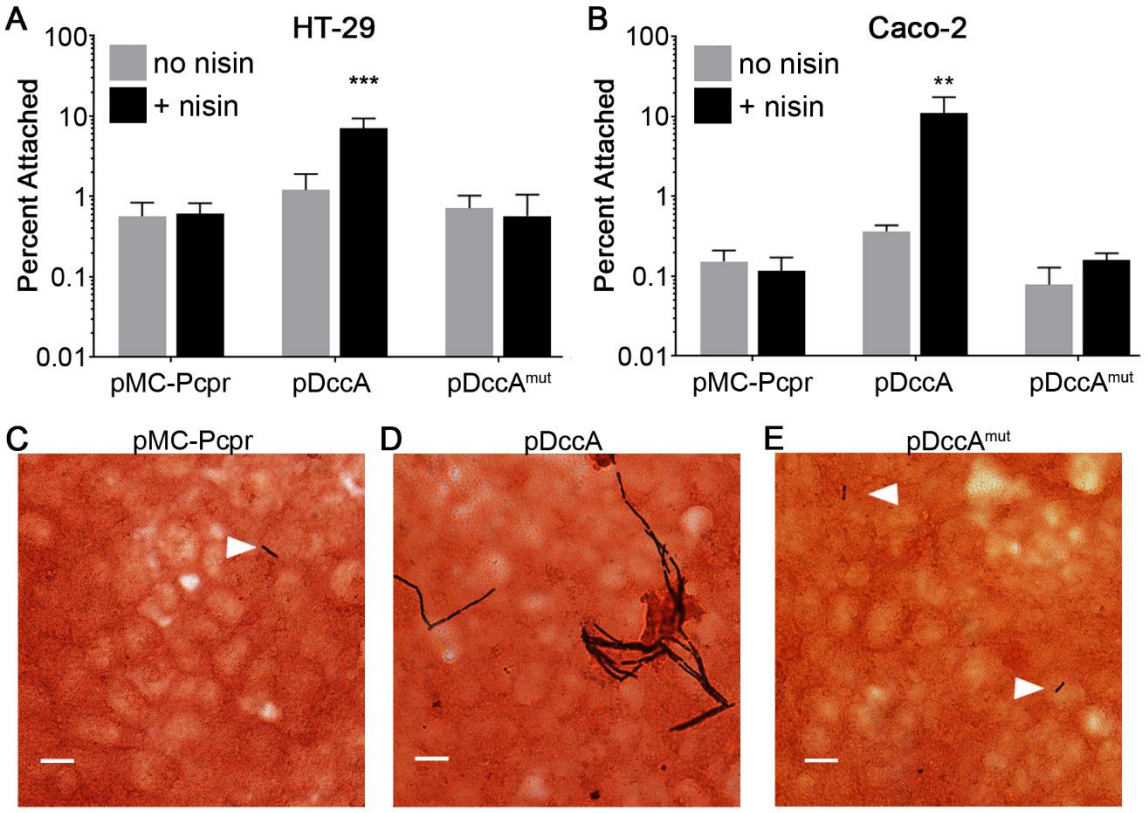

Figure 2.2. c-di-GMP promotes *C. difficile* attachment to epithelial cell monolayers in vitro. *C. difficile* strain 630Δerm containing vector (pMC-Pcpr), pDccA, or pDccA^{mut} were grown to mid-log phase in BHIS with 10 μg/ml thiamphenicol to maintain the plasmids and 1 μg/ml nisin to induce expression as indicated. Bacteria were added to HT-29 (A) or Caco-2 (B) epithelial cell monolayers, centrifuged briefly, and incubated for 1 hour. Serial dilutions of bacterial inoculums and outputs were plated and CFU enumerated to determine the percent of bacteria that remained attached. The means and standard deviations of 3 biological replicates are shown. Data were analyzed using one way analysis of variance (ANOVA) and Tukey's post-test. ** $P < 0.01$, *** $P < 0.001$. (C-E) *C. difficile* containing the indicated plasmids was grown to mid-log phase as above and added to HT-29 cell monolayers grown on coverslips. After 1 hour incubation, coverslips were rinsed with PBS and stained with crystal violet and safranin counterstain. *C. difficile* are stained dark purple (indicated by white arrows), and the HT-29 cell monolayers are stained red. Magnification bars = 10 μm. Images are representative of 4 biological replicates for each strain.

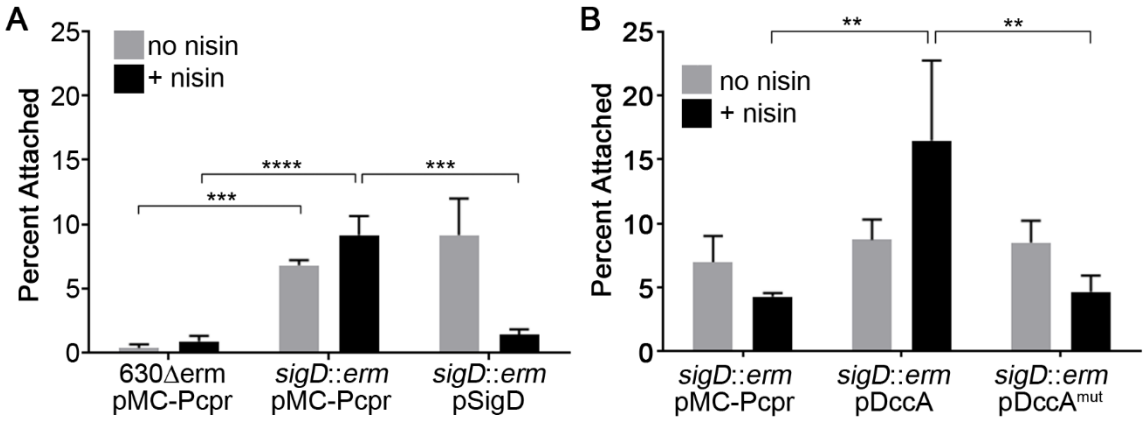

Figure 2.3. Aflagellate *C. difficile* adhere better to HT-29 cell monolayers. *C. difficile* cultures were grown to mid-exponential phase in BHIS with 10 μg/ml thiamphenicol and 1 μg/ml nisin to induce gene expression as indicated. Bacteria were added to HT-29 epithelial cell monolayers, centrifuged briefly, and incubated for 1 hour. Serial dilutions of bacterial inoculums and outputs were plated to enumerate CFU and determine the percent 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 background and plasmids are indicated. The means and standard deviations of 3 biological replicates are shown. Data were analyzed by one way ANOVA and Tukey's post-test. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

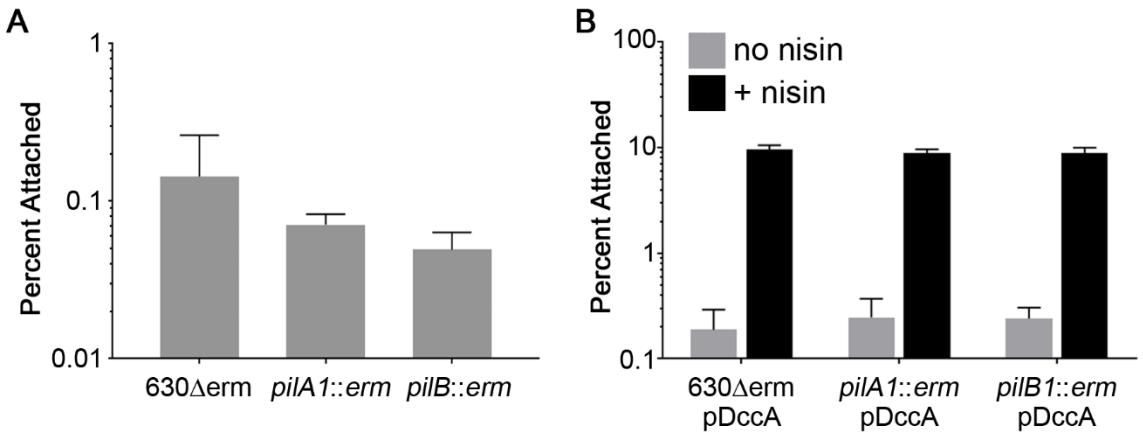

Figure 2.4. Attachment to HT-29 cell monolayers at 1 hour is not dependent on type IV pili. Mid-exponential phase cultures of *C. difficile* 630Δerm, *pilA1* mutant, or *pilB1* mutant were added to HT-29 epithelial cell monolayers, centrifuged briefly, and incubated for 1 hour. Serial dilutions of bacterial inoculums and outputs were plated and enumerated to determine the percent 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.

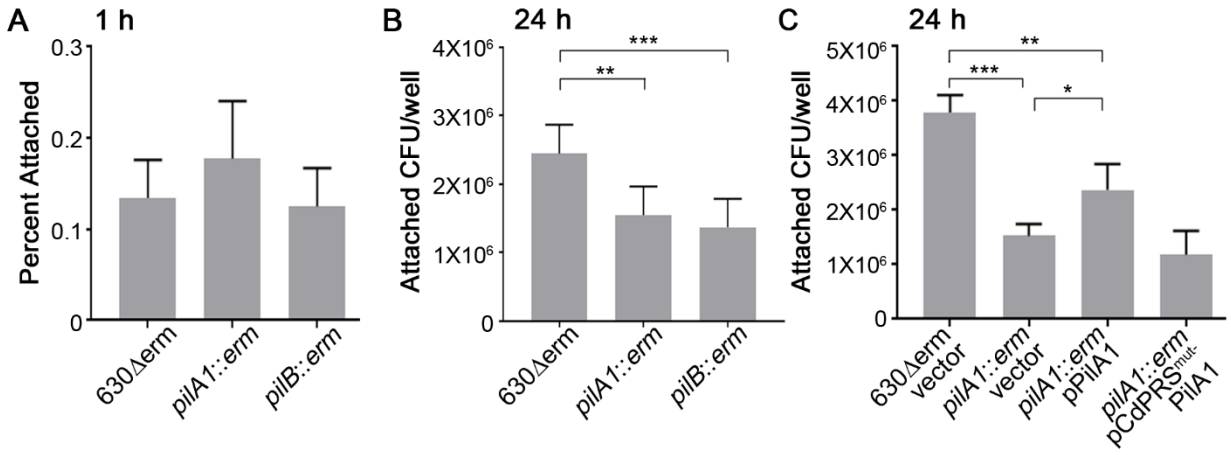

Figure 2.5. Type IV pili promote adherence to MDCK cell monolayers at 24 hrs. Attachment of 630Δerm and TFP-null mutants after 1 hour (A) or 24 hours (B) incubation with MDCK cell monolayers. In (A) data are expressed as the percent of the original inoculum recovered following incubation and PBS washes, with 3 biological replicates for each strain. In (B) data are expressed as the total CFU recovered per well after 24 hours 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 hours 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. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

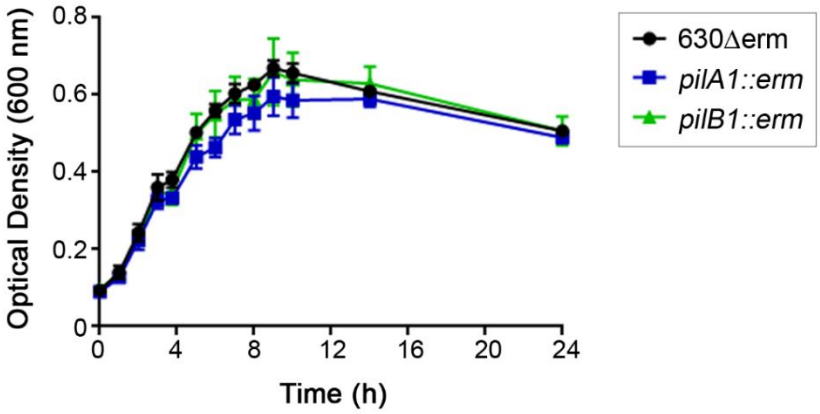

Figure 2.6. Growth of *C. difficile* strains in DMEM culture medium. 630Δerm, the *pilA1* mutant, and *pilB1* mutant were grown in DMEM + 10% FBS without phenol red, with OD_{600} measured over the indicated time frame.

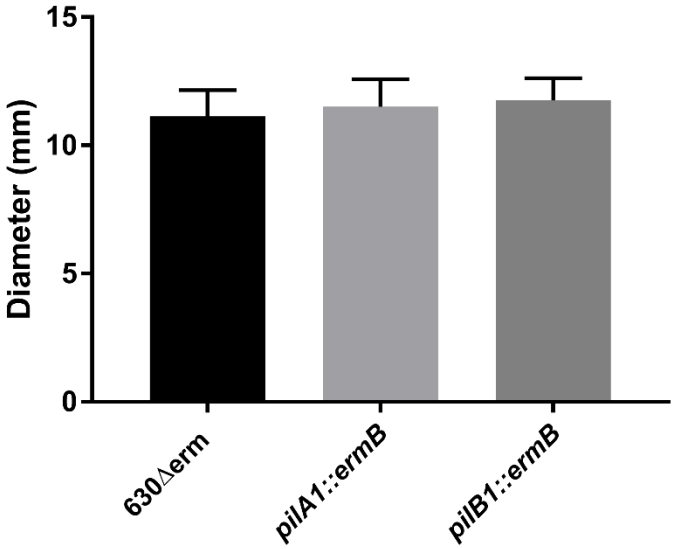

Figure 2.7. Swimming motility of 630Δerm and TFP mutants. Bacteria from individual colonies were inoculated into soft agar plates containing 0.5X BHIS and 0.3% agar and incubated at 37 ºC under anaerobic conditions for 48 hours. Shown are the means and standard deviations of the diameters of the colony swarms from 4 biological replicates. Data were analyzed by one-way ANOVA; no significant differences were found.

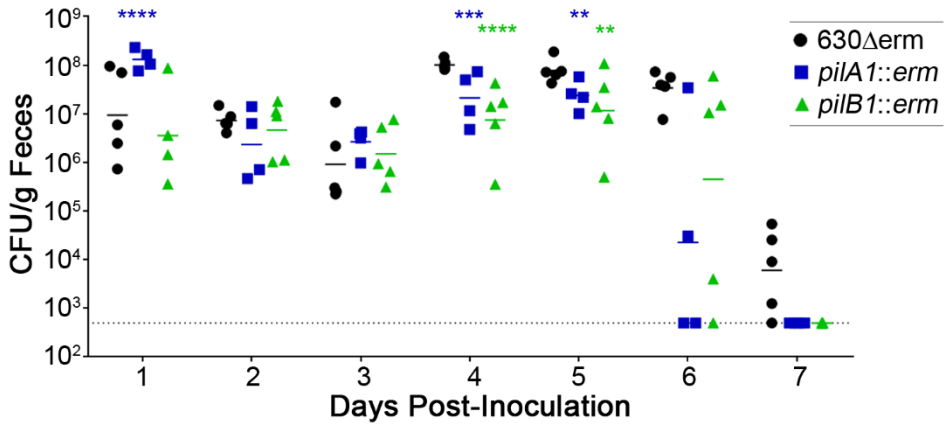

Figure 2.8. Single strain mouse infections of 630Δerm, *pilA1* mutant and *pilB1* mutant. Mice that had been pretreated with antibiotics were inoculated with 10^5 spores. Feces were collected daily and serial dilutions were plated on *C. difficile* selective media with spore germinant (TCCFA) to monitor 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.

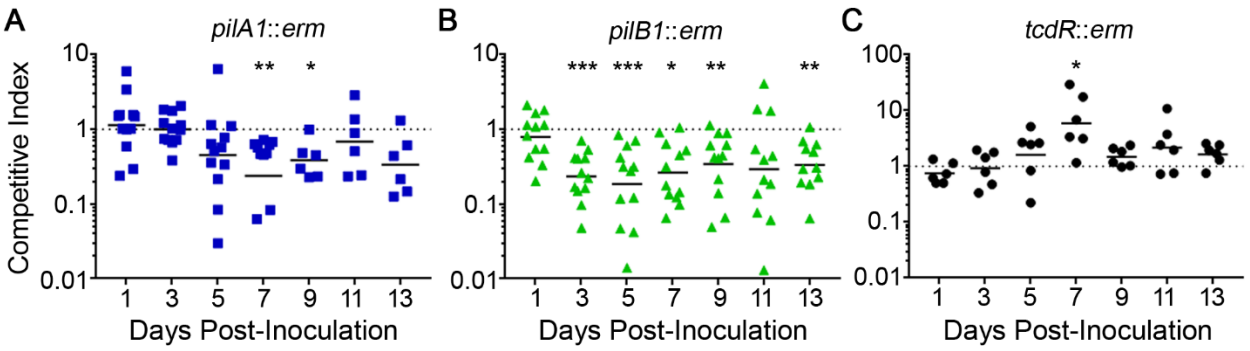

Figure 2.9. *C. difficile* pilus mutants are outcompeted by the parental strain in murine co-infections. Mice were inoculated with mixed inoculums containing ~10^5 spores of each strain. Feces was collected every 2 days starting 1 day post-inoculation and plated on TCCFA (total spores) and TCCFA with erythromycin (mutant spores only) to determine bacterial burden. Competitive indices (mutant:parent) for the competition between (A) 630Δerm and *pilA1* mutant, (B) 630Δerm and *pilB1* mutant, and (C) 630Δerm and *tcdR* mutant. Data were excluded if fewer than 10 total spores were recovered. Symbols represent CI values from individual animals, and error bars indicate the standard deviations. Data were analyzed by Wilcoxon rank sum test comparing values to hypothetical CI of 1 indicating no difference. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

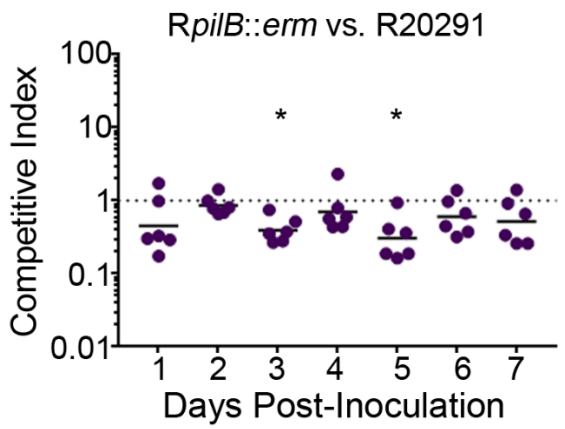

Figure 2.10. Competition of R20291 and R20291 *pilB1*::*ermB* strains in the mouse model. R20291 *pilB1*::*ermB* versus the R20291 parent strain were co-inoculated into antibiotic-treated C57BL/6 mice. CFU recovered from feces were used as a proxy for bacterial load in the intestine. Fecal samples were collected and homogenized at the indicated times post-inoculation and plated on TCCFA and TCCFA supplemented with lincomycin at 20 μg/ml to enumerate total CFU and mutant CFU, respectively. Competitive indices (CI) were calculated as detailed in the Materials and Methods. Symbols reflect CI from individual mice, bars indicate standard deviation. * $P < 0.05$, Wilcoxon matched-pairs signed rank test.

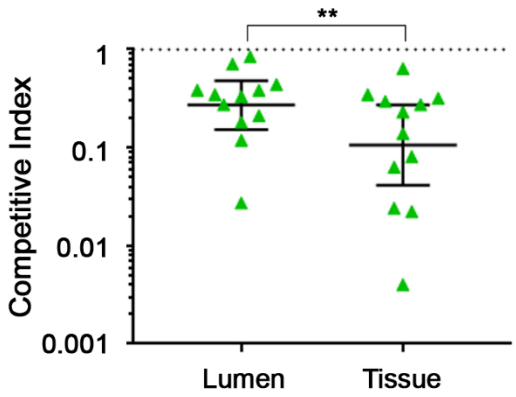

Figure 2.11. TFP promote association of *C. difficile* with the cecal epithelium. Mice were co-inoculated with ~10^5 spores each of the 630Δerm and *pilB1* mutant. Ceca were harvested anaerobically at day 3 post-inoculation. Cecal contents and a 1 ml wash of PBS were combined into the lumenal fraction. The tissue-associated fraction was obtained by homogenizing the remaining cecum. Serial dilutions of the lumenal and tissue-associated fractions were plated on TCCFA (total *C. difficile*) and TCCFA with erythromycin (mutant *C. difficile*) to determine the bacterial burden of each strain. Symbols represent CI (mutant:parent) values from individual animals, and error bars indicate the standard deviations. Data were analyzed by Wilcoxon rank sum test comparing the CI in the lumenal fraction to the CI in the tissue-associated fraction. ** $P < 0.01$.

Table 2.1. Primers used in this study.

Oligonucleotide	Sequence
R978	GACTGCAGCATTCATAATTGAACATTAAAGAAAATAG
R1183	CCGAATTCAAATTCTGTTTCAATATGTAAAAAGTTG
R1184	CAATTTCATAGCCGGCTGCACCACTAACTCAATA
R1185	TATTGAGTTAGTGGTGCAGCCGGCTATGAAATTG

Table 2.2. Strains and plasmids used in this study.

Strain/Plasmid	Description/Purpose	Reference
Plasmids		
pMC123	*E. coli – C. difficile* shuttle vector; Amp^R, Cm^R/Tm^R	[88]
pMC-P_{cpr}	pMC123 with *cpr* promoter in the multiple cloning site	[89]
pDccA	pMC-P_{cpr} ::*dccA* (CD630_14200)	[89]
pDccA^{mut}	pMC-P_{cpr} ::*dccA*^{mut} (AADEF)	[89]
pSigD	pMC-P_{cpr} ::*sigD* (CD630_02660)	[90]
pPilA1	*pilA1* complementation plasmid with native promoter and riboswitch, pMC-P_{pilA1}-RB-*pilA1* (CD630_3513), abbreviated as pPilA1	This study
pPilA1^{mut}	*pilA1* complementation plasmid with native promoter and riboswitch unable to bind c-di-GMP, pMC-P_{pilA1}-RB^{A70G}-*pilA1*, abbreviated as pPilA1^{mut}	This study
***E. coli* strains**		
DH5α	F- φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR*17(rκ -, mκ+) *phoA supE44 thi*-1 *gyrA96 relA1* λ- *tonA*	Invitrogen [91]
HB101	F− *mcrB mrr hsdS20*(rB− mB−) *recA13 leuB6 ara*-14 *proA2 lacY1 galK2 xyl*-5 *mtl*-1 *rpsL20*	[92]
RT1026	HB101(pRK24) pPilA1	This study
RT1027	HB101(pRK24) pPilA1^{mut}	This study
***C. difficile* strains**		
630Δerm	Ribotype 012, erythromycin-sensitive derivative of *C. difficile* 630	[93]
R20291	Ribotype 027, epidemic isolate	[94]
RT761	630Δerm *pilA1*::*ermB*	[90]
RT762	630Δerm pMC-P_{cpr}	[90]
RT763	630Δerm pDccA	[90]
RT764	630Δerm pDccA^{mut}	[90]
RT765	630Δerm *pilB1*::*ermB*, pMC-P_{cpr}	[90]
RT766	630Δerm *pilB1*::*ermB*, pDccA	[90]
RT767	630Δerm *pilB1*::*ermB*, pDccA^{mut}	[90]
RT768	630Δerm *pilA1*::*ermB*, pMC-P_{cpr}	[90]
RT769	630Δerm *pilA1*::*ermB*, pDccA	[90]
RT770	630Δerm *pilA1*::*ermB*, pDccA^{mut}	[90]
RT1075	630Δerm *sigD*::*ermB*	[90]
RT1138	630Δerm *sigD*::*ermB* pSigD	This study
RT1136	630Δerm *sigD*::*ermB* pMC-P_{cpr}	[90]
RT1265	630Δerm *sigD*::*ermB* pDccA	This study
RT1266	630Δerm *sigD*::*ermB* pDccA^{mut}	This study
RT1362	630Δerm *pilA1*::*ermB* pPilA1	This study
RT1366	630Δerm *pilA1*::*ermB* pPilA1^{mut}	This study
Eukaryotic cell lines		
Caco-2	Caco-2 (ATCC® HTB-37) human colorectal adenocarcinoma cells	[95]
HT-29	HT-29 (ATCC® HTB-38) human colorectal adenocarcinoma cells	[96]
MDCK	Madin-Darby Canine Kidney cells (ATCC® CCL-34)	[97]

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CHAPTER 3: TRANSCRIPTIONAL REGULATION IN *C. DIFFICILE* BY THE SECOND MESSENGER C-DI-GMP[2]

SUMMARY

Cyclic diguanylate (c-di-GMP) is a signaling molecule that regulates an array of processes in bacteria, primarily the switch between motile and non-motile lifestyles. The pathogen *C. difficile* encodes 37 proteins known or predicted to be involved in c-di-GMP metabolism and 16 putative c-di-GMP binding riboswitches. Recent work has shown that c-di-GMP signaling controls *C. difficile* swimming motility, biofilm formation, surface motility, and persistence in mouse infections. However, knowledge of the number genes and proteins that are targets of c-di-GMP regulation in this bacterium is limited. Here, we determine the transcriptional regulon of c-di-GMP in *C. difficile* and demonstrate that c-di-GMP controls the production of putative cell surface proteins that promote biofilm formation. We also demonstrate that a number of genes in *C. difficile* are regulated via c-di-GMP sensing riboswitches and that moderate changes in intracellular c-di-GMP can have large effects on their expression.

INTRODUCTION

Cyclic diguanylate (c-di-GMP) is a second messenger that is nearly ubiquitous in bacteria (1). First discovered as a promoter of cellulose production in *Komagataeibacter xylinus*, c-di-

[2] I performed all experiments in this chapter with the exception of the alkaline phosphatase assays, which were performed by Carissa Harvest. I wrote this section with revisions provided my advisor, Rita Tamayo

GMP is now known to regulate a variety of intracellular processes in diverse bacterial species (1-3). These processes include the transition between motile and sessile lifestyles, cell cycle control, and in some cases virulence factor production (4-10). c-di-GMP is synthesized by diguanylate cyclases (DGCs) and broken down by c-di-GMP phosphodiesterases (PDEs). The concentration of c-di-GMP is controlled by regulating the production of these proteins and/or by regulating their enzymatic activity. Many bacteria encode multiple DGCs and PDEs leading to very complex c-di-GMP signaling networks (11).

c-di-GMP acts by binding to targets within the bacterial cell. Proteins that bind c-di-GMP include proteins containing PilZ domains, diguanylate cyclases containing I-sites, MshEN domains and the Cle subfamily of CheY proteins (12-15). c-di-GMP can also bind to certain transcription factors and alter their activity (16-18). In addition to these protein receptors, c-di-GMP can bind to two distinct RNA structures, the GEMM (class I) and class II c-di-GMP riboswitches, to carry out its regulatory function (19, 20). Riboswitches are usually found in the 5' untranslated region (UTR) of transcripts (21). Binding of a ligand to the riboswitch alters the secondary structure of the RNA. These structural changes promote or inhibit transcript termination, mRNA stability, or translation initiation (21, 22). Much of the work on c-di-GMP riboswitches has been performed using purified c-di-GMP and transcripts generated through *in vitro* transcription (19, 23). However, little work has been done to directly test the role of c-di-GMP riboswitches in their native genetic contexts *in vivo*.

The bacterial pathogen *Clostridioides difficile* (formerly *Clostridium difficile*) is an obligate anaerobe that causes hundreds of thousands of infections each year in the United States, resulting in an estimated 44,000 deaths in 2014 (24, 25). Despite the severity of the public health threat, little is known about the factors that allow *C. difficile* to colonize and persist in the

intestine. We recently determined that type IV pili (TFP) promote persistence of *C difficile* in the mammalian intestine (26). The production of TFP is positively regulated by c-di-GMP (27). Additionally, c-di-GMP negatively regulates production of a *C. difficile* colonization factor, the flagellum (28, 29). Because of the importance of c-di-GMP in the regulation of these two surface structures involved in colonization and persistence, we surmised that additional c-di-GMP regulated factors could be important for *C. difficile* pathogenesis.

C. difficile strain 630 encodes 37 proteins with putative or demonstrated DGC or PDE activity (28, 30, 31). Additionally, the *C. difficile* 630 genome encodes 16 predicted GEMM and class II c-di-GMP sensing riboswitches, more than any sequenced bacterial genome outside of a few delta-proteobacteria, suggesting an important role for c-di-GMP signaling through riboswitches in this organism (3, 19, 20). Previous work demonstrated that in *C. difficile* c-di-GMP modulates flagellum biosynthesis and swimming motility, toxin production, type IV pilus (TFP) production, TFP-dependent surface motility, and biofilm formation (27, 28, 32, 33). Transcription of the flagellar genes and the genes for TFP production is under the control of c-di-GMP sensing riboswitches (19, 27, 33). For the flagellar operon, c-di-GMP binding to the Cdi-1-3 riboswitch in the 5' UTR of the *flgB* operon mRNA promotes transcript termination, leading to a decrease in the transcription of *flgB* and other genes in the early-stage flagellar operon (19, 28). Because the early-stage flagellar operon encodes SigD, a positive regulator of *C. difficile* toxin gene transcription, c-di-GMP negatively regulates toxin production as well (10). In contrast, the Cdi-2-4 riboswitch in the 5' UTR of the *pilA1* transcript forms a terminator stem-loop in the absence of c-di-GMP (27). Upon binding to c-di-GMP, the Cdi-2-4 riboswitch undergoes a change in secondary structure resulting in transcription read-through and expression of the

downstream genes (27). Accordingly, c-di-GMP positively regulates pilus gene expression and TFP production *in vivo* (27, 33).

Using transcriptional fusions to a reporter gene, we showed that the Cdi-2-4 riboswitch controls gene expression in response to c-di-GMP in two strains of *C. difficile*, 630Δerm and R20291 (33). *In vivo* studies of GEMM riboswitches in *Vibrio cholerae* showed that c-di-GMP regulates expression of genes adjacent to the two riboswitches, Vc1 and Vc2, but these studies also showed that the promoters of these genes, *gbpA* and *tfoY*, were regulated by c-di-GMP concentrations as well (34, 35). Moreover, for these two genes, transcriptional control of the promoter and post-transcriptional regulation via c-di-GMP binding to the riboswitch were in opposing directions (34, 36). These studies indicate that c-di-GMP can regulate the expression of a gene through multiple, sometimes opposing mechanisms. The observations from the *in vivo* analysis of riboswitches demonstrate that while *in vitro* transcription can be used to measure interactions between c-di-GMP and nascent mRNA, these interactions need to be placed in the appropriate biological context before making conclusions about the regulation of expression *in vivo*.

Some of the phenotypic changes that are induced by high levels of intracellular c-di-GMP can be attributed at least partially to regulation of TFP and flagellar biosynthesis (26-28, 33). Biofilm formation and autoaggregation of *C. difficile*, for example, are both decreased in mutants that lack TFP, yet high intracellular c-di-GMP levels still promote biofilm formation and autoaggregation in these pilus-deficient strains (27, 33). Initial attachment of *C. difficile* to epithelial cells *in vitro* is enhanced in bacteria that lack flagella and also in bacteria with high c-di-GMP (26, 37). These results suggest that negative regulation of flagella contributes to the enhanced attachment of bacteria with high intracellular c-di-GMP levels. However, increasing

the c-di-GMP concentration in a *sigD* mutant, which lacks flagella, further augmented adherence to epithelial cells, indicating that the flagellar regulation alone did not account for the difference in attachment levels in high c-di-GMP conditions (26). These data indicate that c-di-GMP regulates additional factors that contribute to autoaggregation, biofilm formation, and attachment to epithelial cells.

To identify additional factors regulated by c-di-GMP in *C. difficile*, we compared the transcriptomes of *C. difficile* strain 630Δerm with elevated intracellular c-di-GMP concentrations and basal c-di-GMP levels. We identified a large number of genes that are known or predicted to be involved in motility or adherence to surfaces, consistent with a role for c-di-GMP in controlling the switch between motile and non-motile states. We further examined the roles of several c-di-GMP regulated cell envelope proteins in biofilm formation. All eleven genes with c-di-GMP riboswitches encoded upstream were regulated by c-di-GMP. We used transcriptional reporters coupled with qRT-PCR analysis to quantify changes in the transcription of these riboswitch-adjacent genes over a broad range of c-di-GMP levels, and show that moderate changes in c-di-GMP levels can have large effects on the transcription of some of these genes. The c-di-GMP regulation of some of these genes occurs through the promoter in addition to regulation through the riboswitch.

RESULTS

c-di-GMP controls the transcription of a large number of genes in *C. difficile*

Recent work has shown that c-di-GMP controls a number of processes in *C. difficile* including flagellar motility, biofilm formation, surface motility, and adherence to epithelial cells (26-28, 32, 33). Transcriptional regulation of genes encoding flagellar and TFP proteins by c-di-

GMP accounts for only a portion of these phenotypic changes (26, 27, 33). In addition *C. difficile* harbors a large number of proteins involved in c-di-GMP metabolism and 16 predicted c-di-GMP riboswitches. We used RNA-seq to profile the transcriptomes of *C. difficile* 630Δerm with basal or elevated c-di-GMP. To increase intracellular c-di-GMP, we used our previously described approach involving ectopic, nisin-inducible expression of a *C. difficile* diguanylate cyclase gene, *dccA* (28). *C. difficile* 630Δerm bearing the pDccA plasmid or vector control were grown to mid-exponential phase with 1 μg/ml nisin to induce expression of *dccA*. RNA was processed for sequencing using an Illumina Hiseq-2500. Reads were mapped to the CD630 genome (AM180355) and normalized by RPKM (Reads mapped per kilobase per million reads). Genes regulated by c-di-GMP were identified using the following criteria: changes in RPKM >2-fold between the two conditions and a Bonferoni-corrected p-value of <0.05 after differential expression analysis. A complete list of the significantly-regulated genes is found in Table A1.1 in APPENDIX 1. These genes were grouped according to the predicted Riley functional class of their encoded proteins (38, 39) (Fig 3.1A).

A total of 166 genes met the criteria, with 120 genes being negatively regulated and 46 being positively regulated by c-di-GMP (Figure 3.1B). The largest class of genes regulated by c-di-GMP were genes involved in chemotaxis and flagellar motility (37 total genes). These genes account for nearly 25% of the c-di-GMP regulated genes we identified. Consistent with previous work showing that c-di-GMP negatively regulates flagellar motility, all these genes showed decreased expression in the pDccA (high c-di-GMP) strain (Fig 3.1B). The next largest set of c-di-GMP regulated genes consist of genes encoding proteins that are known or predicted to localize to the cell envelope. Among this set of gene products are proteins involved in biosynthesis of TFP as well as three putative surface proteins that are predicted to be controlled

by c-di-GMP riboswitches (CD630_27970, CD630_28310 and CD630_32460). The third major class of c-di-GMP regulated genes encode proteins predicted to be involved in transport/binding. Of these, seven are predicted PTS system proteins that are likely involved in importing sugars or sugar alcohols. Several of the remaining genes are predicted to encode phage proteins, proteins involved in the transfer of mobile genetic elements, and proteins involved in iron regulation (e.g. FeoB1) or oxidative stress (e.g. Rbr1 and TrxA1).

Ectopic expression of c-di-GMP regulated cell envelope proteins promotes biofilm formation

Because several of the genes regulated by c-di-GMP encoded putative cell envelope proteins, we speculated that these proteins play a role in biofilm formation, a process that is promoted by c-di-GMP in *C. difficile* (33, 40). The fold changes in transcript levels for these six genes under elevated c-di-GMP conditions, based on the RNA-seq analysis, are listed in Table 3.1. To determine their effects on biofilm formation, genes encoding the individual cell envelope proteins were ectopically expressed in *C. difficile* from an ATc-inducible promoter (P_{tet}). Four of the six genes (CD630_27950, CD630_27960, CD630_27970, and CD630_3246) significantly increased biofilm formation compared to the vector control indicating a potential role for those proteins in biofilm formation (Fig 3.2).

Genes downstream of predicted c-di-GMP riboswitches are regulated by c-di-GMP in a dose-dependent manner

We observed transcriptional regulation of many genes with predicted c-di-GMP riboswitches encoded in their 5' UTR. For five of the riboswitches, we were unable to detect a

coding sequence downstream of the riboswitch, either based on the genome annotation or analysis of the reads in the regions (Table 3.2, Figures A1.1 to A1.16). For the remaining 11 riboswitches, the transcription abundance of genes downstream of the riboswitch were significantly altered in the pDccA strain (Table 3.2). Transcript abundance was significantly reduced in the high c-di-GMP condition in the RNA-seq dataset for all but one of the genes (Cdi-1-1) downstream of GEMM riboswitches. The expression of CD630_19900, the gene downstream of Cdi-1-1, was increased more than 30-fold. Of the genes downstream of putative class II riboswitches, all five showed increased expression in the high c-di-GMP condition. For many of these riboswitches (11 out of 16), the number of reads mapped to the riboswitches themselves was also significantly affected by increasing c-di-GMP levels (Table 3.2). For example, there were approximately 13-fold more normalized reads mapped to the riboswitch Cdi-1-1 in the high c-di-GMP condition. This observation suggests that c-di-GMP levels alters the activity of the promoters controlling transcription initiation for these genes, in addition to regulating via the riboswitches.

To validate the RNA-seq results and to confirm that regulation of transcription was due to increased c-di-GMP and not due to other potential functions of the DccA protein, we performed qRT-PCR for a subset of genes, focusing on those predicted to be regulated by riboswitches. This was done using *C. difficile* strains bearing vector, pDccA or pDccA^{mut}, which encodes a catalytically inactive form of DccA (28), grown in the presence of nisin to induce *dccA* and *dccA*^{mut} expression. Expression of all four of the genes 3' of the class II c-di-GMP riboswitches was significantly increased in *C. difficile* with pDccA compared to the vector and pDccA^{mut} controls (Table 3.3). Four out of the seven genes adjacent to GEMM riboswitches showed increased transcript levels in *C. difficile* pDccA (Table 3.4). The average transcript levels for the

other three genes was decreased in agreement with the RNA-seq data, but the differences did not meet statistical significance. For one of these genes, CD630_19900, the pDccA samples without inducer had 3.25 times as many normalized reads as the vector control even though the differences in intracellular c-di-GMP in the pDccA samples without inducer was only ~40% higher (680 nM vs 380 nM).

The observation that small increases in c-di-GMP might promote expression of some genes, but not others led us to hypothesize that these might differ in their responsiveness to c-di-GMP. Differences between riboswitches of the same type can have large effects on the binding affinity of the riboswitches for their ligands and thus respond at differing thresholds of c-di-GMP (19, 34). In order to determine how responsive the transcription of these riboswitch-regulated genes were to c-di-GMP levels and whether there were different thresholds for expression of the different riboswitch-adjacent genes, we analyzed the abundance of transcripts of interest and related those results to the c-di-GMP concentration. To achieve a range of intracellular c-di-GMP concentrations, *C. difficile* with pDccA was grown with a range of concentrations of nisin (0 to 2 μg/ml). *C. difficile* with vector and pDccA^{mut} grown with 0 or 1 μg/mL nisin were included as controls (28). RNA was collected for qRT-PCR analysis and nucleotides were extracted for quantification by LC-MS/MS from the same samples. Intracellular concentrations of c-di-GMP were determined by determining the CFU/ml in each sample, and normalizing the determined c-di-GMP concentration to the total cell volume extracted, as described previously (28). The control strains contained low intracellular c-di-GMP concentrations (between 40 and 380 nM) consistent with previously reported data for *C. difficile* (28). Induction of *dccA* with increasing levels of nisin led to a dose-dependent increase in c-di-GMP, ranging from 630 nM with no nisin

to 145 μM at the highest nisin concentration (Figure 3.3 and 3.4). In agreement with the RNA-seq data, the transcript levels were reduced for 6 out of 7 genes predicted to be regulated by GEMM riboswitches, with the exception of the gene encoded downstream of Cdi-1-2, CD630_19900 (Fig 3.3). For the class II c-di-GMP riboswitch-regulated genes, increasing nisin concentrations resulted in a dose-dependent increase in transcript levels (Figure 3.4). In contrast, the transcript levels of the genes downstream of GEMM and class II riboswitches did not change in the presence of nisin in the vector and pDccA^{mut} control strains (Tables 3.3 and 3.4), indicating that regulation occurs in response to increased c-di-GMP. For most of these genes, the differences in transcript levels are significant at a nisin concentration of 0.1, which corresponds to a 19-fold increase in the intracellular c-di-GMP concentration. Increasing nisin concentrations above 0.1 had only a modest additional effect on the expression of these genes. The trends for the genes downstream of the GEMM riboswitches are fairly consistent with the exception of Cdi-1-1. The transcript levels of these genes decreased in a dose-dependent manner up to nisin 0.5 μg/ml with decreases of between 3 and 10-fold. The class II riboswitches all show increased transcript levels as intracellular c-di-GMP increases. There is some variation in the magnitude of the response with CD630_32460 only showing a 5-fold increase over vector control at the highest intracellular c-di-GMP levels vs a 25-fold increase in CD630_28310 transcripts. This suggests similar regulation of these genes by c-di-GMP and is consistent with riboswitch regulation through the riboswitches.

DISCUSSION

In this work, we set out to better define the transcriptional regulon of c-di-GMP in the human pathogen *C. difficile.* We found that c-di-GMP positively regulates 124 genes and

negatively regulates 42 others under the growth conditions tested (Figure 3.1B). These genes consist not only of genes previously demonstrated to be c-di-GMP regulated (e.g. flagellar and TFP genes), but also many others encoding proteins known or predicted to be involved in a wide array of cellular processes (Figure 3.1A).

Several of the proteins identified are predicted to localize to the cell envelope and the expression of a subset of these proteins in *C. difficile* enhanced biofilm formation, a process that is promoted by c-di-GMP in this bacterium (Figure 3.2) (32, 33). Two of the c-di-GMP regulated cell envelope proteins we identified (CD630_28310 and CD630_32460) were recently shown to be regulated by c-di-GMP (41). The cleavage of CD630_28310 (and possibly CD630_3246) from the surface of the cell is cleaved by another protein, ZmpI, which is itself negatively regulated by c-di-GMP (Table 3.2, Figure 3.3) (41). This presents an interesting scenario where increased intracellular c-di-GMP could promote attachment to a surface via these putative adhesins, and decreasing c-di-GMP levels could promote transcription of *zmpI* followed by ZmpI cleavage of the adhesins and dispersal of the bacteria. This model has yet to be tested, however.

For many of the remaining genes, the mechanism of regulation by c-di-GMP is unclear. The only predicted transcriptional regulators that we identified in our RNA-seq datasets were *sigD* and two response regulators (CD630_32670 and CD630_32660). Most of the genes we identified are not in the *sigD* regulon as reported by El Meouche et al (42), so we think *sigD* is unlikely to regulate many of these genes. The targets of the two response regulators have not been identified, so it is possible that they regulate the expression of a subset of the genes we identified. Interestingly, the response regulators are encoded in a potential operon downstream of the Cdi-2-2 riboswitch, so their expression is likely regulated by c-di-GMP directly. It is possible that binding of c-di-GMP to as yet undiscovered transcriptional regulators in *C. difficile* could be

responsible for regulation of some genes on our list. This is a common mechanism of regulation by c-di-GMP (17, 18, 43).

Of the 16 predicted c-di-GMP riboswitches in *C. difficile*, 11 of them are encoded near genes on the same coding strand, positioning them to regulate the expression of these downstream genes (Table 3.2) (32). We demonstrated that expression of all 11 of these genes was altered by increasing c-di-GMP concentration *in vivo* (Figure 3.3, 3.4). The magnitude of the response correlated with increasing c-di-GMP levels, and most of the genes responded to induction with 1ug/ml nisin, which corresponded to an increase in c-di-GMP of ~19-fold. The dynamic range of c-di-GMP in *C. difficile* is unknown, and whether a 19-fold increase in c-di-GMP is commonly attained under physiological conditions is unclear. For one of the riboswitch-adjacent genes (CD630_19990), the uninduced pDccA strain showed a statistically significant increase in transcript levels (3.4 fold) even though this strain only had 40% higher intracellular c-di-GMP (630 nM vs 380 nM) than the uninduced vector control strain. This indicates that even small changes in c-di-GMP can affect the expression of some of these genes.

It can be tempting to assume that regulation by c-di-GMP occurs via the riboswitch for genes that are putatively riboswitch-controlled. However, there are multiple examples of ligands affecting promoter activity of genes downstream of their cognate riboswitch independent of riboswitch binding (34-36, 44). Thus, it is important to consider other possible mechanisms of regulation when determining the effects of riboswitches on transcription. To determine whether promoter regulation by c-di-GMP played a role in altered transcription of the putative riboswitch-controlled genes, we examined the response of the promoters alone to c-di-GMP. We demonstrated via reporter fusions that c-di-GMP levels regulate the expression of CD630_19900 via the promoter. The riboswitch may still contribute to regulation of this gene's transcription

because the fold change in alkaline phosphatase activity (~20) is much lower than the fold change in transcript levels measured by qRT-PCR (~100 fold). Constructing a fusion of this promoter and the WT riboswitch could answer this question. We also observed that for three genes adjacent to riboswitches in *C. difficile* (*pilA1*, CD630_33682, and *zmpI*), the promoter activity was not affected by increased c-di-GMP indicating that the regulation of these genes by c-di-GMP is likely due to the riboswitch.

One limitation of our approach to measure intracellular c-di-GMP is that we can only estimate c-di-GMP levels across the population, so the data may not accurately represent the c-di-GMP concentration in individual cells due to population heterogeneity. However, this population heterogeneity is likely present in many contexts that are commonly regarded as high or low c-di-GMP conditions and may be more representative of responses than single cells or *in vitro* experiments (45-47). We have observed heterogeneity in the expression of flagellar genes using an mCherry reporter, which may also involve cell-to-cell variation in c-di-GMP levels (48).

The characterization of riboswitches *in vivo* allows us to better contextualize the function of these regulatory RNA structures in the larger c-di-GMP signaling networks of bacteria. This work expands the known c-di-GMP signaling network in *C. difficile* and demonstrates that the riboswitch-regulated genes in *C. difficile* are truly part of the c-di-GMP network. Given the importance of other c-di-GMP regulated genes for virulence (*tcdA*, *tcdR*) and persistence of this pathogen (*pilA1*), understanding the c-di-GMP network may lead to a better understanding of *C. difficile* infections (26).

MATERIALS AND METHODS

Bacterial growth conditions

C. difficile cultures were grown at 37 °C in an atmosphere of 5% CO2, 5% H2 and 90% N2 inside an anaerobic chamber (Coy). Overnight cultures of *C. difficile* were grown in 2-5 mL of TY medium (30 g/liter Bacto tryptone, 20 g/liter yeast extract, 1 g/liter thioglycolate) with antibiotics as necessary for plasmid maintenance. Except for overnights, *C. difficile* growth was in BHIS medium (37 g/ liter brain heart infusion, 5 g/liter yeast extract). 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. Nisin was added at 0.01-2.0 μg/ml as indicated to induce expression from the *cpr* promoter.

RNA sequencing

Single colonies of 630Δerm bearing vector or pDccA were inoculated in TY medium and grown for ~16 hours, with 4 independent replicates per strain. Cultures were diluted 1:100 in 5 mL of BHIS with thiamphenicol and grown to OD600 of 0.2. Nisin was then added to each culture for a final concentration of 1 μg/ml. Cultures were then grown to OD600 of 1.0 and then centrifuged for 10 minutes at 3,000 rcf. Supernatants were removed and pellets were suspended in 1 mL of TriSure (Bioline). Cells were added to ~250 μL of 0.1 mm diameter zirconia beads (Biospec), lysed using a bead beater as described previously (28) and 200 μL of chloroform was added. Cells were vortexed for 20 sec, incubated at room temperature for 10 minutes, and then centrifuged for 10 min at 13,000 rpm. The aqueous phase was transferred to a microcentrifuge tube and an equal volume of 100% isopropanol was added to precipitate the RNA. Samples were centrifuged at 13,000 x g for 10 minutes at 4 °C. Supernatants were removed, pellets were

washed with 1 ml of cold 70% ethanol and centrifuged again 13,000 x g for 10 minutes at 4 °C. Supernatants were removed and pellets were washed with 1 ml cold 70% ethanol and centrifuged again. Supernatants were removed, pellets were allowed to air dry, and pellets were suspended in 50 µl of nuclease free water. RNA was treated with Turbo DNA-free (Ambion) to remove DNA and ribosomal RNA was depleted using a Ribozero (bacteria) rRNA removal kit (Illumina). Libraries were prepped, pooled and sequenced on a Hi-Seq 2500 sequencer (Illumina).

RNA sequencing analysis was performed using CLC Genomic Workbench (Qiagen). Transcripts were mapped to the CD630 genome (AM180355) and riboswitches were manually added based on the predictions by Lee et al. (20). Transcript reads for each gene were normalized to the total number of reads (transcripts per million reads or TPM) before calculating the fold change. Fold changes were calculating by dividing the TPM in the pDccA bearing strain by the TPM in vector control strain. Fold decreases are expressed as negative numbers instead of ratios. For example, if a transcript had 40 TPM in the pDccA bearing strain, and 20 TPM in the vector control strain, the fold change would be -2, not 0.5. Genes were considered regulated if the fold change between the means of the pDccA and vector bearing strains was greater than 2 and the p value was less than 0.05 following Bonferroni correction for multiple comparisons.

Quantitative reverse transcription PCR (qRT-PCR)

Single colonies of *C. difficile* 630Δerm bearing vector, pDccA, or pDccA^{mut} were inoculated in TY medium and grown for ~16 hours. Cultures were diluted 1:100 into 25 ml of BHIS with 10 µg/ml thiamphenicol and varying concentrations of nisin. For the vector and pDccA^{mut} strains, two nisin concentrations, 0 µg/ml and 1 µg/ml, were used. For the pDccA strain, 7 concentrations were used: 0, 0.01, 0.1, 0.25, 0.5, 1.0 and 2.0 µg/ml. The cultures were

grown to exponential phase (OD_{600} of 1.0), and 3 ml samples were collected. The remaining 22 ml was saved for quantification of c-di-GMP. Bacteria were centrifuged for 10 min at 2,000 x g, supernatants were removed, and bacteria were suspended in 1 ml TriSure. RNA isolation was carried out as described above for RNA-seq. Following RNA isolation, cDNA was synthesized using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). A total of 10 ng of cDNA template was used per qRT-PCR reaction. SensiMix SYBR Green (Bioline) was used for qRT-PCR. The data were analyzed using the $2^{-\Delta\Delta CT}$ method, with r*poC* as a reference gene normalized to the stated reference condition or strain.

Quantification of intracellular c-di-GMP

Serial dilutions were made of each culture grown for RNA isolation, CFU were enumerated. The remaining 22 ml of culture was centrifuged at 2500 x g for 10 minutes. Supernatants were removed, pellets were suspended in 1 ml PBS and transferred to 1.5 ml microcentrifuge tube. Samples were centrifuged at 10,000 x g and supernatants were removed. Pellets were suspended in 200 µl of extraction buffer (40% acetonitrile, 40% methanol, 0.1 N formic acid) and placed at -20 °C for 30 minutes. 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 c-di-GMP concentration in these samples was determined by UPLC/MS as described previously (49). Intracellular c-di-GMP was calculated using estimates of *C. difficile* cell volume from electron micrographs and the number of CFU recovered from the cultures.

Construction of plasmids for expression of genes encoding cell wall proteins

The *tet* promoter from pRPF185 (50) was amplified by PCR using primers ATc_F + ATc_R. This PCR product was digested with the restriction enzymes EcoRI and SacI, ligated into similar digested pMC123 and transformed into *E. coli* DH5α. This plasmid (pRT1648) served as the vector control. The CD630_19870 gene was amplified from 630Δerm genomic DNA using primers CD630_19870_F and CD630_19870_R, digested with SacI and BamHI and ligated into similarly digested pRT1648. The resultant plasmid is p1987. The rest of the plasmids were constructed similarly to p1987 using primers LOCUS_TAG_F and LOCUS_TAG_R for the given locus tags. These plasmids were conjugated into *C. difficile* 630Δerm as described previously (28).

Biofilm assays of putative cell envelope proteins

Overnight cultures of *C. difficile* grown in 2 ml of TY were diluted 1:100 in 1 mL modified BHIS (BHIS + 1% glucose + 50 mM sodium phosphate pH 7.5) containing 10 µg/ml thiamphenicol and 20 ng/ml ATc in 24 well plates. Bacteria were grown statically for 24 hours at 37 °C anaerobically. After 24 hours, supernatants were removed, wells were gently washed once with PBS and stained with 1 ml of 0.1% crystal violet for 30 min. Crystal violet was removed and wells were washed twice with PBS. One ml of 95% ethanol was added to solubilize the stain and the absorbance at 570 nm was taken. A total of six independent overnight cultures were used for each strain. Absorbance values were normalized to vector control.

Construction of reporter fusions

Promoter regions upstream of each riboswitch were amplified from 630Δerm genomic DNA using primers named cdiXXpromF and cdiXXpromR. For example, for Cdi-1-1, the promoter was amplified using Cdi1-1promF and Cdi1-1promR. Plasmid pRT1346 (pSMB47::phoZ) (48) was digested with SalI and SphI. Amplified promoter regions and digested pRT1346 were added to Gibson assembly reactions. DH5α cells were transformed using 2 µl of the Gibson reactions. Plasmids were isolated from each confirmed strain. Plasmids were used to transform *Bacillus subtilis* BS49 as described previously (10).

Single colonies of the BS49 strains containing the reporter fusions were grown for ~5 hours in BHIS supplemented with 2.5 µg/ml erythromycin and 10 ng/ml tetracycline. Bacillus cultures were then transferred into an anaerobic chamber and 100 µl of culture was spread evenly on the surface of a BHIS agar plate containing 2 mM KNO3 and allowed to dry for 5 min. After the *B. subtilis* had dried, 100 µl of an early stationary phase culture of *C. difficile* was spread evenly over the same BHIS plate. These conjugations were incubated for 24 hours then the bacterial growth was scraped from the plate and suspended in 300 µl of BHIS. After resuspension, 100 µl was spread evenly on BHIS plates supplemented with 2.5 µg/ml erythromycin. Plasmids pMC-P_{cpr} (vector) and pDccA were conjugated into these *C. difficile* strains as previously described (28).

Alkaline phosphatase assay

Single colonies of reporter fusion strains bearing vector and pDccA were inoculated into 2 ml TY medium containing 10 µg/ml thiamphenicol and grown for ~16 hrs. Cultures were then diluted 1:100 into 3 ml of BHIS containing 10 µg/ml thiamphenicol and various concentrations

of nisin (0, 0.01, 0.1, 0.25, 0.5, 1.0 and 2.0 μg/ml) as indicated. At mid-exponential phase (OD_{600} of ~1.0), 1ml of each culture was collected and the bacteria were centrifuged for 1 min at 12,000 rcf to pellet the bacteria. Supernatants were discarded and bacteria were stored at -20 °C. Alkaline phosphatase activity was measured as described by Edwards et al. (51).

FIGURES

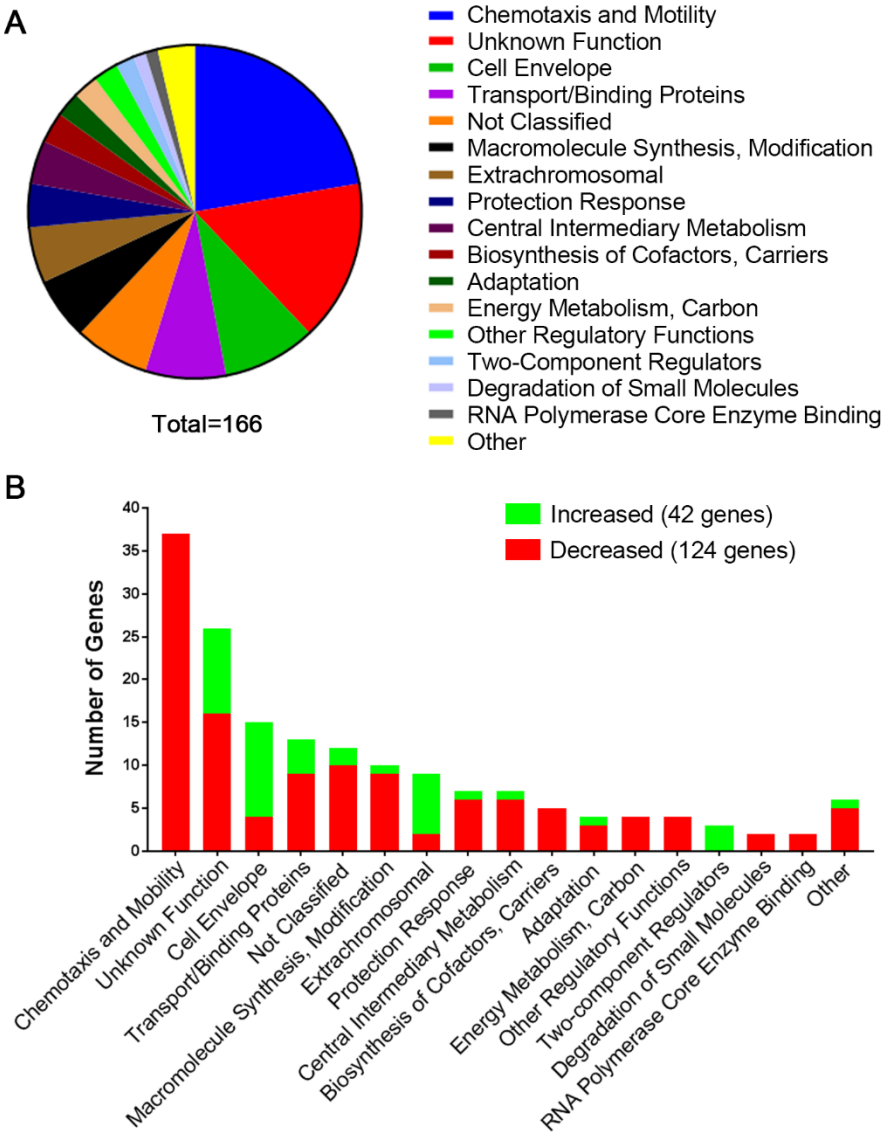

Figure 3.1. *C. difficile* genes regulated by c-di-GMP grouped by Riley classification of predicted gene products. (A) c-di-GMP regulated genes from RNA-seq data grouped by Riley classification. (B) c-di-GMP regulated genes grouped by Riley classification and whether expression was increased or decreased in the pDccA strain (high c-di-GMP). Genes were included if the fold change in expression (differences in RPKM) was greater than 2-fold and $p < 0.05$ after Bonferroni correction for multiple comparisons.

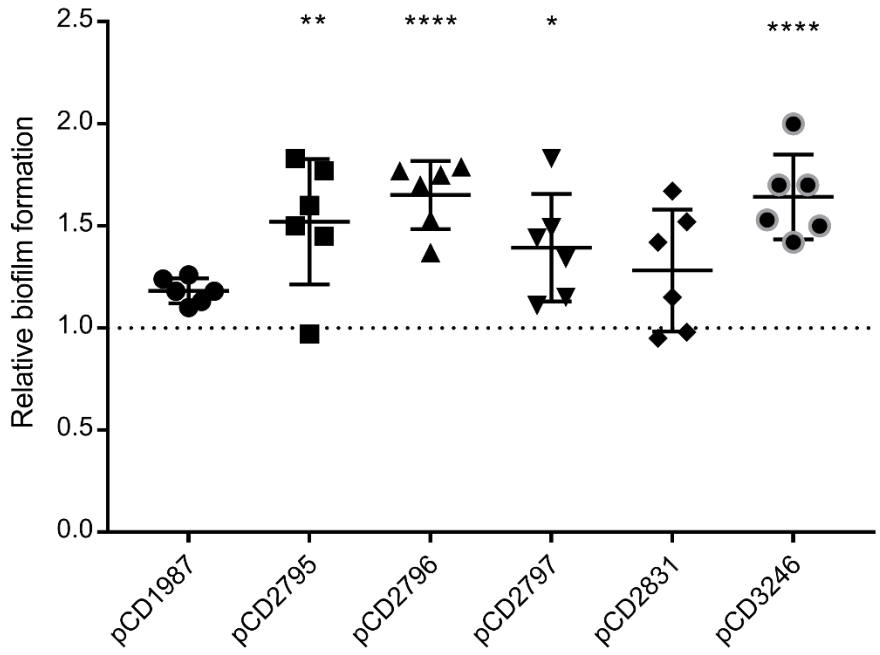

Figure 3.2. Biofilm formation is promoted by ectopic expression of genes encoding cell envelope proteins. Genes encoding putative cell envelope proteins were placed under the control of an ATc-inducible promoter and expressed ectopically from a plasmid in *C. difficile* with 20 ng/ml ATc. Biofilm formation was determined after 24 hours of static growth in 24-well plates by crystal violet staining. Six biological replicates were evaluated for each strain. Values are normalized to induced vector control. Individual symbols indicate a value from a single replicate, bars indicate the means and standard deviations. Data were analyzed by 1-way ANOVA to vector control with Dunnet's post test.* $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$.

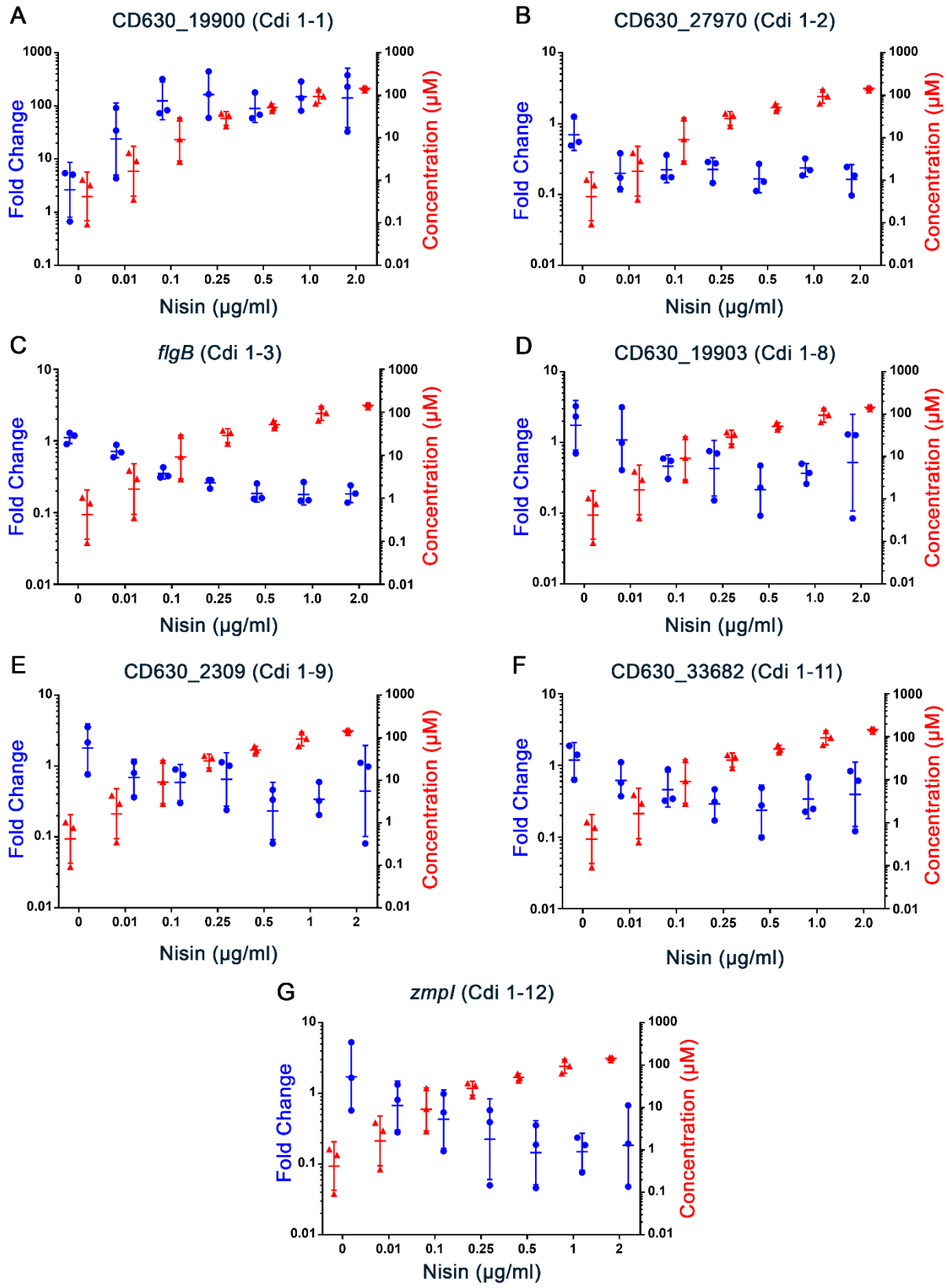
A
CD630_19900 (Cdi 1-1)
B
CD630_27970 (Cdi 1-2)
C
flgB (Cdi 1-3)
D
CD630_19903 (Cdi 1-8)
E
CD630_2309 (Cdi 1-9)
F
CD630_33682 (Cdi 1-11)
G
zmpI (Cdi 1-12)
Fold Change
Concentration (μM)
Nisin (μg/ml)
0
0.01
0.1
0.25
0.5
1.0
2.0
1
2

Figure 3.3. Transcript levels of genes downstream of GEMM riboswitches in *C. difficile* with increasing c-di-GMP. Fold change of transcripts normalized to vector control are shown in blue. Intracellular c-di-GMP levels are shown in red (The same c-di-GMP data is used on each graph. Bars represent the geometric mean and geometric standard deviation of three biologically independent samples.

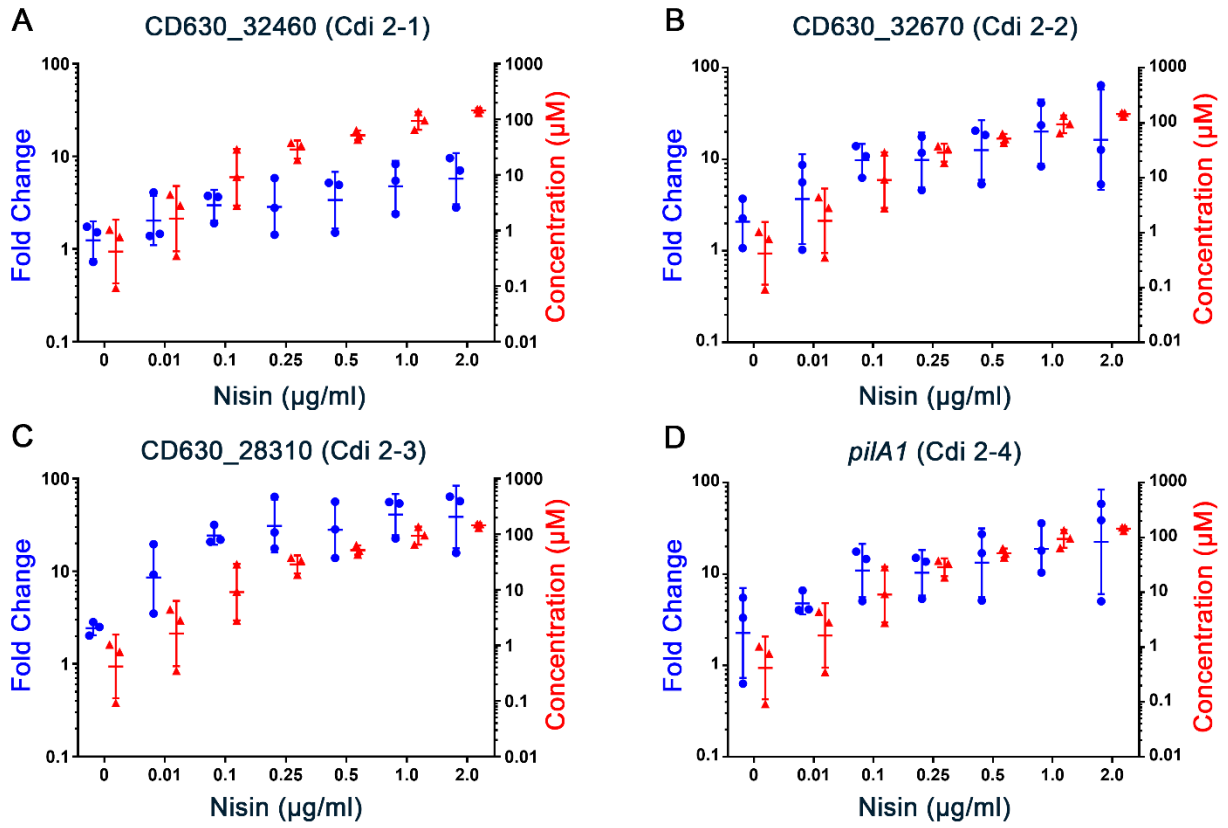

Figure 3.4. Transcript regulation of genes downstream of class II c-di-GMP riboswitches in *C. difficile* with increasing c-di-GMP. Fold change of transcripts normalized to vector control are shown in blue. Intracellular c-di-GMP levels are shown in red. Each data point represents a biological replicate. Lines and bars represent the geometric mean and geometric standard deviation.

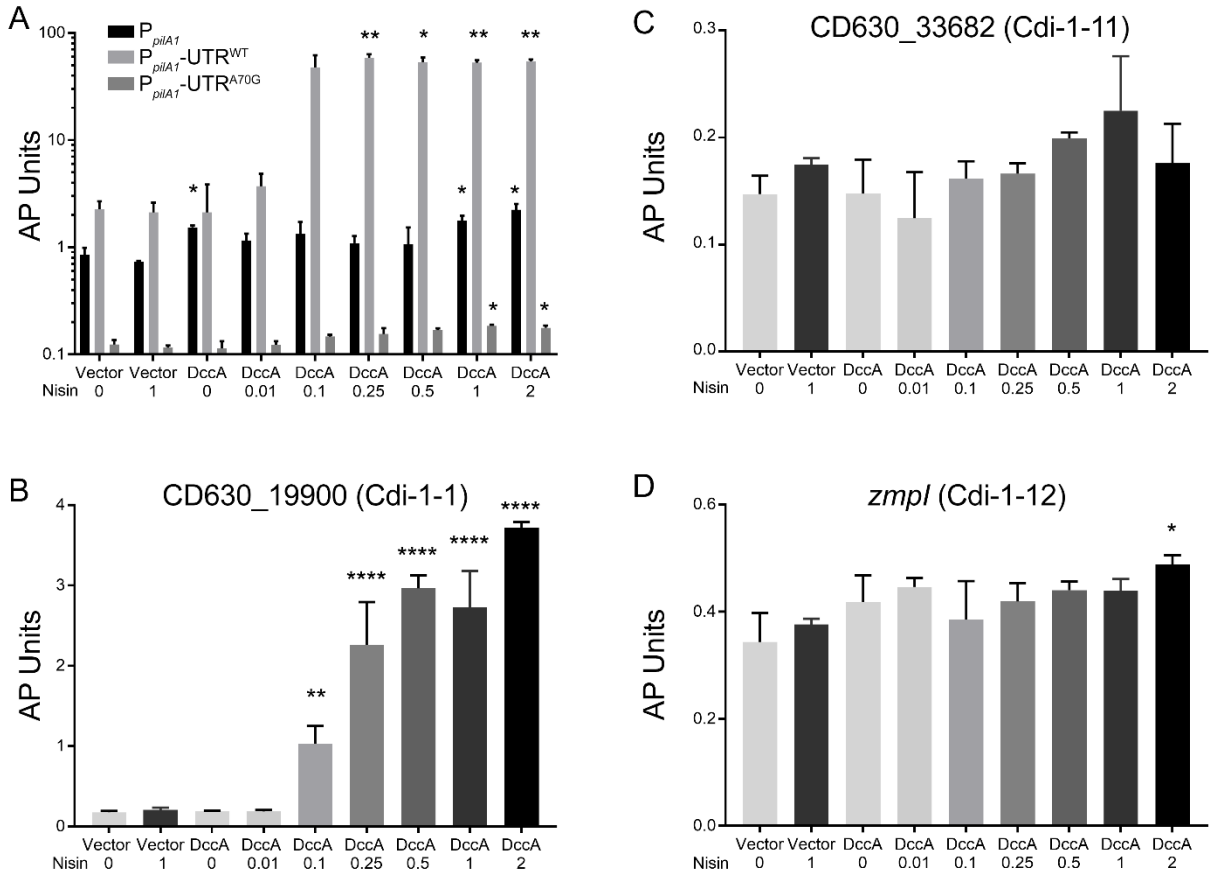

Figure 3.5 Alkaline phosphatase reporter assays of riboswitch-adjacent gene promoters. *C. difficile* 630Δerm bearing either vector or pDccA were grown to mid-exponential phase with the indicated concentration of nisin in μg/ml. (A) Reporter activity of the *pilA1* promoter alone (black), *pilA1* promoter and native riboswitch (light gray), and *pilA1* promoter and c-di-GMP unresponsive riboswitch (dark gray). (B) Reporter activity of the CD630_19900 promoter. (C) Reporter activity of the CD630_33682 promoter. (D) Reporter activity of the *zmpI* (CD630_28300) promoter. The means and standard deviations of 3 biological replicates are shown. Data were analyzed using one-way ANOVA with Dunnett's post test to determine statistical significance compared to induced vector control. * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$.

Table 3.1. Putative cell envelope proteins whose expression is regulated by c-di-GMP.

Locus	Fold Change (pDccA/vector)	Predicted Function	Riboswitch upstream?
CD630_19870	4.32	cell wall protein 28	No
CD630_27950	2.90	cell wall protein 11	No
CD630_27960	-4.06	cell wall protein 10	No
CD630_27970	-7.15	calcium-binding adhesion protein	Yes
CD630_28310	42.51	putative adhesin	Yes
CD630_32460	4.29	surface protein	Yes

Table 3.2. Changes in transcript abundance for c-di-GMP riboswitches and the downstream genes.

Riboswitch[a]	Chromosome region start[a]	Fold Change (pDccA/vector)[b]	Downstream Gene	Chromosome region	Fold Change (pDccA/vector)[c]
Cdi-1-1	(-) 2296134	13.44	CD630_19900	(-) 2295867..2296352	31.90
Cdi-1-2	(-) 3266578	-15.63	CD630_27970	(-) 3260792..3266755	-7.15
Cdi-1-3	(+) 308778	-2.74	CD630_02450 (*flgB*)	(+) 309272..309589	-15.06
Cdi-1-4	(+) 3379981	1.02	ND		
Cdi-1-5	(-) 1142269	1.03	ND		
Cdi-1-6	(+) 2285923	-6.41	ND		
Cdi-1-7	(+) 2907226	1.19	ND		
Cdi-1-8	(+) 2297492	-9.25	CD630_19903	(+) 2297643..2297819	-7.78
Cdi-1-9	(+) 2671809	-4.81	CD630_23090	(+) 2671951..2672127	-4.66
Cdi-1-10	(-) 1653520	-2.07	ND		
Cdi-1-11	(+) 3936240	-8.49	CD630_33682	(+) 3936389..3936565	-8.90
Cdi-1-12	(-) 3303074	-31.30	CD630_28300 (*zmpI*)	(-) 3302613..3303275	-62.61
Cdi-2-1	(-) 3801063	4.48	CD630_32460	(-) 3798299..3800482	4.29
Cdi-2-2	(-) 3826609	2.34	CD630_32670	(-) 3825352..3826029	18.78
Cdi-2-3	(-) 3306681	1.10	CD630_28310	(-) 3303646..3306564	42.51
Cdi-2-4	(-) 4105796	4.10	CD630_35130 (*pilA1*)	(-) 4105120..4105635	11.74

[a] Riboswitch naming and start sites based on predictions by Sudarsan, et al. (2008), and Lee, et al. (2010). (+/-) indicate sense versus antisense strand

[b] Fold change for the riboswitch region only

[c] Fold change for gene 3' of the riboswitch

Table 3.3. Fold changes in transcripts for genes controlled by class II c-di-GMP riboswitches

	CD3267 (Cdi-2-1)	CD3246 (Cdi-2-2)	CD2831 (Cdi-2-3)	*pilA1* (Cdi-2-4)
Vector -	1.00	1.00	1.00	1.00
Vector +	0.87	0.75	0.99	0.82
pDccAmut -	1.14	1.07	1.27	1.12
pDccAmut +	1.16	0.76	0.88	0.60
pDccA -	1.25	1.88	2.43	2.22
pDccA +	4.76*	17.35*	40.86*	18.44*

+/- indicate cultures grown with 1 ug/ml nisin or without nisin, respectively.
* Asterisks indicate values significantly different from vector ($p<0.05$) by 2-way ANOVA.

Table 3.4. Fold changes in transcripts for genes controlled by GEMM riboswitches.

	CD1990 (Cdi-1-1)	CD2797 (Cdi-1-2)	*flgB* (Cdi-1-3)	CD19903 (Cdi-1-8)	CD2309 (Cdi-1-9)	CD33682 (Cdi-1-11)	*zmpI* (Cdi-1-12)
Vector -	1	1	1	1	1	1	1
Vector +	1.09	1.46	1.21	0.81	0.85	0.61	0.85
pDccAmut -	1.28	2.02	1.50	1.61	1.48	1.27	1.44
pDccAmut +	1.27	1.82	1.57	2.04	2.12	1.25	0.90
pDccA -	3.25*	0.70	1.12	1.75	1.58	1.18	1.72
pDccA +	149.50*	0.24*	0.18*	0.59	0.31	0.34	0.15*

+/- indicate cultures grown with 1 ug/ml nisin or without nisin, respectively.
* Asterisks indicate values significantly different ($p<0.05$) from vector by 2-way ANOVA.

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CHAPTER 4: DISCUSSION

SUMMARY OF RESULTS AND SIGNFICANCE

Over the past few decades, *C. difficile* has become an increasingly prevalent pathogen in the developed world causing approximately half a million infections each year in the United States alone (1-3). Despite the importance of this pathogen, we know very little about the factors that *C. difficile* uses to colonize and persist within the mammalian intestine following spore germination. Previous research in our lab and others has shown that high intracellular c-di-GMP concentrations in *C. difficile* lead to a variety of phenotypic changes, including decreased swimming motility, decreased toxin production, enhanced biofilm formation, autoaggregation of the bacteria in broth culture, and increased surface motility on hard agar surfaces (4-8). The transcription of type IV pilus (TFP) genes and the production of TFP are positively regulated by c-di-GMP (7, 8). The increased autoaggregation, biofilm formation and surface motility observed in bacteria with high intracellular c-di-GMP were shown to be at least partially dependent on type IV pili (7, 8).

The goal of my project in Chapter 2 was to determine the relevance of type IV pili and their regulation by c-di-GMP to the colonization of the intestine by *C. difficile*. We initially hypothesized that TFP were colonization factors of *C. difficile* based on our *in vitro* data and on evidence that TFP are used as colonization factors in other bacteria, including some intestinal pathogens (9-14). In the current study, we demonstrated that c-di-GMP promotes attachment of *C. difficile* to a variety of epithelial cells *in vitro*. Through the use of bacterial mutants lacking

TFP, we showed that TFP are dispensable for initial attachment to epithelial cells, but TFP contribute to prolonged adherence to epithelial cell monolayers. Additionally, we showed that the TFP-dependent adherence to epithelial cells requires c-di-GMP signaling through the riboswitch upstream of *pilA1*. To test our hypothesis that TFP are involved in colonization of the intestine, we infected antibiotic-treated mice with *C. difficile* spores from the parental strain (630Δerm) and two TFP-null strains with insertional mutations in the genes encoding the major pilin (*pilA1*) and the pilus assembly ATPase (*pilB1*). Contrary to our hypothesis, we found that the burden of *C. difficile* in the feces was similar for all infection for the first few days of infection. These results indicate that TFP are dispensable for establishing *C. difficile* colonization of the mammalian intestine. However, the pilus mutants were cleared more quickly from mice, and the TFP-null bacteria were recovered in fewer numbers in co-infection experiments after 3 days, suggesting that TFP play a role in persistence of *C. difficile* in the host.

How TFP promote maintenance of *C. difficile* host colonization is still undetermined, but there are a couple mechanisms that could explain the persistence defect in the *pilA1* and *pilB* mutants. Perhaps the most straightforward mechanism for TFP to promote persistence would be to increase the strength of the interactions with the mucosal layer and prevent shedding. The observations that TFP promote prolonged adherence to host cells and that *pilB* mutant bacteria were more likely to be found loosely attached to the cecum than the parental strain are consistent with this mechanism. Interactions with the mucosa are known to be important for the persistence of *Lactobacillus spp.* in the gut (15-18). Interestingly, one of the mucin-binding proteins in *L. johnsonnii* and *L. rhamnosus* is the sortase-dependent pilin, SpaC (16, 19). Mutants lacking *spaC* were defective for mucin binding and persisted for about one week less than the parental strain (19). In *Bifidobacterium breve,* a mutant strain lacking the TadA pilus assembly ATPase was

defective for persistence in mice pre-colonized with other bacteria (20). Expression of the Tad locus of a different *Bifidobacterium* species in *Lactobacilli* promoted attachment of these bacteria to Caco-2 cells and fibrinogen (21). While the Tad and SpaBCA pili are not closely related to the pili produced by *C. difficile*, these data show that promotion of adherence to mucin as a mechanism for prolonged adherence is not without precedent. It is tempting to speculate that these three evolutionarily distinct pili converged on a similar mechanism to promote persistence of the bacteria within the intestine. It is also important to note that we have not directly determined whether type IV pili promote *C. difficile* adherence to mucus or to cells that produce mucus. Co-culture of *C. difficile* with mucus producing cells would provide an ideal environment to test the contributions of TFP to the mucous layer directly, but the lifespan of Caco-2 and HT-29 intestinal epithelial cells in an anaerobic environment is on the order of a few hours. Several labs are developing improved models that would allow us to examine long-term interactions between *C. difficile* and the intestinal mucosa *in vitro*. A recent study developed a system using porous silk scaffolds seeded with primary myofibroblasts and a mix of Caco-2 and HT29 intestinal epithelial cells to construct a 3D architecture and an artificial lumen (22). When grown submerged vertically in media to maintain anaerobiosis, these 3D systems supported growth and toxin production of *C. difficile* over 48 hours. *C. difficile* grown in these systems formed mat-like structures on the surface of the epithelial cell layers on the scaffold, supporting the hypothesis that *C. difficile* can grow in communities on the surface of epithelial cells. Similarly, a recent article on biorxiv reported a system for growing *C. difficile* anaerobically on the apical side of mucus-producing HT-29MTX cells while providing oxygen to the basolateral side. In collaboration with Dr. Nancy Allbritton (UNC-CH), we are developing an improved model using a stem-cell derived epithelium that tolerates anaerobiosis and includes mucus-producing goblet

cells. These systems would allow us to test the contributions of TFP to interactions with the mucus, but also the contributions of other c-di-GMP regulated factors that may play a role in adherence.

In Chapter 2, we demonstrated that a c-di-GMP regulated surface structure, the type IV pilus, is a *C. difficile* persistence factor. We previously demonstrated that c-di-GMP also negatively regulates production of flagella and toxins in *C. difficile* (5, 23). Because c-di-GMP signaling regulates these *C. difficile* virulence factors, characterizing the broader c-di-GMP network in *C. difficile* could lead to a better understanding of *C. difficile* pathogenesis. In Chapter 3, we determined the transcriptional regulon of c-di-GMP in *C. difficile* using RNA-seq analysis of *C. difficile* 630Δerm with basal levels of c-di-GMP and with artificially increased c-di-GMP levels. Among the most interesting classes of differentially expressed genes were those encoding proteins predicted to be localized to the cell envelope. We postulated that these proteins might be adhesins responsible for surface attachment in *C. difficile*, so we expressed them ectopically and found that expression of 4 of the 6 proteins enhanced *C. difficile* biofilm formation. These data point to a broader role for c-di-GMP in the rearrangement of the surface of *C. difficile*. One of the c-di-GMP regulated proteins that enhanced biofilm formation was CD630_32460. This protein is a putative adhesin and its expression was previously shown to be enhanced under high c-di-GMP conditions (24). This protein is also proteolytically cleaved by the ZmpI protease, which is negatively regulated by c-di-GMP (24). ZmpI also cleaves another c-di-GMP regulated protein, CD630_28310, which was previously shown to bind collagen (24-26). The inverse regulation of these putative adhesin proteins and a protease that cleaves them leads to a model in which high levels of c-di-GMP promote adherence through production of the adhesins, and low levels of c-di-GMP promote detachment by inhibiting CD630_28310 and

CD630_32460 expression and increasing ZmpI production to cleave the existing adhesin proteins. Notably, reduced c-di-GMP also promotes flagellum biosynthesis and swimming motility, which would further facilitate *C. difficile* dispersal (27, 28). I tested whether ectopic expression of the cell envelope proteins would enhance binding to HT29 intestinal epithelial cells *in vitro* and did not see an effect, but these short-term binding experiments to one cell type are not a good model for binding to the intestinal mucus or epithelium. A co-culture system of *C. difficile* with mucus-producing cells would enable a straightforward test of this model allowing the manipulation of c-di-GMP levels and appropriate mutants to test the adherence of mutants lacking the adhesins or protease under both high and low c-di-GMP.

In addition to CD630_32460, CD630_28310, and *zmpI*, which are all riboswitch-adjacent, we also determined that the expression of genes 3' of 8 of the remaining 13 riboswitches of *C. difficile* are regulated by c-di-GMP. To determine what intracellular concentration thresholds of c-di-GMP were required for these changes in transcription, we evaluated transcript abundance in *C. difficile* with a broad range of intracellular c-di-GMP. We found that for most genes, a c-di-GMP concentration of between 2 and 50-fold greater than basal c-di-GMP levels was sufficient to observe significant differences in transcription by qRT-PCR. Above the 50-fold threshold over basal c-di-GMP levels there were only small additional increases in transcriptional regulation –such extreme increases in c-di-GMP are unlikely to be physiologically relevant. The observation that small changes in c-di-GMP levels lead to appreciable changes in gene expression indicates that c-di-GMP signaling could be used by *C. difficile* to quickly adapt to changing environmental conditions. Indeed, c-di-GMP signaling networks have been shown to respond rapidly to environmental stimuli (29-31). Work from our lab has demonstrated that growth on a surface results in increased intracellular c-di-GMP levels

through an as yet unknown signal (8). It would make evolutionary sense for *C. difficile* in the intestine to respond to a surface by expressing adhesins that promote the stability of the *C. difficile* population within the gut.

The last section of Chapter 3 was devoted to determining whether the transcriptional changes we observed in riboswitch-adjacent genes were due to riboswitch regulation of transcription or through regulation of promoter activity by c-di-GMP. For three of the promoters we tested, there was weak or no stimulation of promoter activity by increased c-di-GMP levels in the cell, suggesting that c-di-GMP regulation of these genes occurs via the riboswitch or some other mode of regulation. Promoter activity of the CD630_19900 promoter, however, was much higher (~20-fold) in the high c-di-GMP conditions, indicating that at least some of the c-di-GMP-dependent regulation of this gene occurs at the level of the promoter. CD630_19900 is particularly interesting because it is positively regulated by c-di-GMP, while all the other genes controlled by GEMM riboswitches are negatively regulated. The average sequence identity between the Cdi-1-1 (upstream of CD630_19990) and the other GEMM riboswitches is 69% (Figure A1.17), and yet the effects of c-di-GMP on the expression of these genes are completely opposite. This opposite mode of regulation demonstrates the potential versatility of riboswitch regulation and also suggests caution going forward not to assume that similar riboswitches produce similar effects.

Another set of genes we identified consists of a putative operon encoding a predicted two component regulatory system (TCRS) consisting of a histidine kinase (CD630_32660) and two response regulators (CD630_32670 and CD630_32650). In the canonical model of TCRS signaling, a stimulus is sensed by the histidine kinase which undergoes autophosphorylation and transfers the phosphate group to a phosphoreceiver (REC) domain of the response regulator (32,

33). Phosphorylation of the response regulator typically activates the response regulator (16, 17). Because the two response regulators we identified have conserved OmpR-family transcriptional regulator domains (based on the RefSeq database), we hypothesize that expression of a subset of genes that are regulated by c-di-GMP are controlled by these two response regulators. Work underway in our lab is currently testing this hypothesis.

The mechanism by which c-di-GMP regulates transcription of many of the genes we identified is still unclear. The flagellar sigma factor, SigD, is regulated by c-di-GMP and has pleiotropic effects on the expression of other genes unrelated to flagellar biosynthesis (34). Counterintuitively, despite c-di-GMP regulation of *sigD* expression, many of the genes identified in the SigD transcriptome are not statistically different in our RNA-seq data set (4, 5, 34). It is possible that slight differences in growth between the experiments explains this discrepancy, and SigD could affect the transcription of some non-flagellar genes in our dataset. In other bacteria, c-di-GMP has been shown to bind to certain transcriptional regulators and alter the transcription of genes they regulate (35-37). It is possible that c-di-GMP binds to a transcriptional regulator in *C. difficile* that is responsible for altering transcription of a subset of c-di-GMP regulated genes as well. Such a protein could be discovered by pull-down assays of *C difficile* proteins using c-di-GMP as bait. This approach has proven successful in identifying a c-di-AMP receptor in *Staphylococcus aureus* (38)

The functions of most of the riboswitch regulated genes is unknown. CD630_19900 contains two SH3_3 domains, which bind to other proteins to control peptidoglycan remodeling in some bacteria (39-41). One of the effects that increased intracellular c-di-GMP has on *C. difficile* is the elongation of cells into chains (4, 7, 24). Perhaps the CD630_19900 interactions with proteins to cause this chaining effect in *C. difficile* under high c-di-GMP conditions. It

could also be contributing the autoaggregation phenotype seen when intracellular c-di-GMP concentrations are elevated. CD630_27970 is annotated as a calcium binding adhesin, but its most well conserved domain is a peptidase M60 domain that has been shown to bind and cleave mammalian mucin in other bacteria (42, 43). It is tempting to speculate that this protein plays a role in mucin binding or degradation to facilitate colonization by *C. difficile.* The remaining two riboswitch regulated genes (CD630_33682, and CD630_32490) encode small hypothetical proteins of ~50 amino acids with no conserved domains, so their function is completely mysterious.

IMPORTANCE

The binding of *C. difficile* to the mucous layers of the epithelium could also provide a more stable population in the gut, which would be advantageous for the bacterium. The work in Chapter 2 demonstrated the contribution of TFP to *C. difficile* persistence and also hints at the possibility that bacteria closely associated with the intestinal epithelium could serve as reservoirs *C. difficile* infections. The increased duration of *C. difficile* would allow for the production of additional spores and increase the likelihood of transmitting spores to a new susceptible host. If the bacteria are also forming biofilms on the surface of the epithelium as seen in some animal models of diseases (44), these could serve as reservoirs for the bacteria. For *C. difficile* this could be especially important due to the high recurrence rates (~20-30%) in patients treated for *C. difficile* infections (45-47). Persistence of spores within patients has been postulated as one cause of relapse following cessation of antibiotics, but there is debate over what percentage of infections are reinfections with the same strain versus new infections with new strains of *C. difficile* (48). Persistent infections not only cause problems of recurrence, but also of

transmission, particularly in hospital settings where there is an increased percentage of people at high risk for CDI (49).

Another potential role of TFP in mediating intestinal persistence of *C. difficile* could be through the stabilization of interactions with other bacterial species and the formation of multispecies biofilms. The formation of monospecies *C. difficile* biofilms and adherence to epithelial cells were both partially dependent on TFP *in vitro* (8, 23). In the oral cavity where biofilms are common, the pili of *Actinomyces oris* are required for its coaggregation with *Streptococcus oralis*, an interaction that makes up one of the initial steps in biofilm development (50). The mucosa of the intestinal tract is normally free of biofilms perhaps owing to the rapid turnover rate of the mucin layer (estimated at 240 μm/h) and the ability of certain mucins to disrupt bacterial biofilms (51-53). Alterations to the mucus layers may have implications for disease development (54, 55). For example, aberrant mucus layers in the lungs of cystic fibrosis patients allow for the formation of long-lasting pathogenic biofilms (56). The importance of microbial biofilms in patients with inflammatory bowel disease (IBD) and ulcerative colitis (UC) is also being increasingly recognized (54, 57). Bacterial mats have been observed in animal models of *C. difficile* disease that are reminiscent of biofilm, but the importance of these biofilms is still unclear.

In patients with CDI, the mucin composition of the intestinal mucosa is altered (58). The mucus layer in these patients is more acidic, and the mucin composition shifts from the normally protective mucin MUC2 to enhanced production of MUC1 (58, 59). *C. difficile* was found to be more adherent to MUC1-rich mucus than mucus from healthy patients, and injection of *C. difficile* into human intestinal organoids was sufficient to decrease expression of MUC2 (58). *C. difficile* is often found in close association with mucus in patients with CDI as well as in animal

models of the disease (60-62). It is still unclear what role this alteration of mucin plays in the progression of CDI, but it is tempting to speculate that adherence of *C. difficile* to the mucin layer promotes the persistence of the bacterium. If adherence to mucins is important for infection, the increased production of more easily bound mucins coupled with the promotion of adherence by c-di-GMP, TFP, and other c-di-GMP regulated adhesins could be key to maintenance of *C. difficile* in the lower GI tract.

CONCLUSIONS AND FUTURE DIRECTIONS

Overall, the work in this dissertation has expanded the known regulon of c-di-GMP in *C. difficile*. We also demonstrated that c-di-GMP regulates TFP promote adherence to host cells and promote persistence in animal models of these disease. The data from chapter 2 coupled with the RNA-seq data that showed regulation of several putative adhesins c-di-GMP leads to the hypothesis that c-di-GMP promotes colonization of the mucous layer through multiple, possibly redundant mechanisms. Increasing availability of fluorescent reporters that function anaerobically and in *C. difficile*, as well as improved techniques in anaerobic microscopy, could allow for real time monitoring of *C. difficile* responses to surfaces sensing to determine which genes are regulated in response to surface-associated increases in c-di-GMP.It could also allow us to better understand what processes regulate the formation of *C. difficile* surface communities. These results could then be applied to *in vitro* co-culture of *C. difficile* and epithelial cells along with animal models to give us a better understanding of how *C. difficile* colonizes the intestine. This knowledge could be potentially be used to prevent colonization of *C. difficile* in patients at risk for CDI and prevent these infections from occurring.

CONCLUSIONS AND FUTURE DIRECTIONS

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APPENDIX: SUPPLEMENTAL FIGURES FOR CHAPTER 3

Table A1.1 Genes regulated by c-di-GMP in *C. difficile*

Locus tag identifier	Gene name	Gene Product	Fold Change (pDccA/vector)	Riley Functional Class[a]
CD630_01300	metK	S-adenosylmethionine synthetase	-2.15	3.3.16
CD630_01480		uncharacterised protein	-4.40	7.0.0
CD630_01740	cooS	Carbon monoxide dehydrogenase	-8.61	3.5.2
CD630_01750		putative oxidoreductase, Fe-S subunit	-5.15	3.5.3
CD630_01910		putative DNA glycosylase	-2.06	2.2.3
CD630_02110	licC	CTP:phosphocholine cytidylyltransferase	-10.96	4.1.9
CD630_02120		putative sulfatase	-9.03	3.3.19
CD630_02130		putative spore coat protein	-4.79	1.8.1
CD630_02140		uncharacterised protein	-5.77	7.0.0
CD630_02260		putative lytic transglycosylase	-6.05	3.3.21
CD630_02270		conserved hypothetical protein	-5.77	0.0.2
CD630_02290	flgM	Negative regulator of flagellin synthesis (Anti-sigma-d factor)	-5.49	6.2.2
CD630_02300		putative flagellar biosynthesis protein	-5.56	1.1.1
CD630_02310	flgK	Flagellar hook-associated protein FlgK (or HAP1)	-5.53	1.1.1
CD630_02320	flgL	Flagellar hook-associated protein FlgL (or HAP3)	-5.68	1.1.1
CD630_02330	fliW	Flagellar assembly factor FliW	-5.93	1.1.1
CD630_02340	csrA	Carbon storage regulator homolog CsrA	-5.99	6.5.0
CD630_02350	fliS1	Flagellar protein FliS1	-6.01	1.1.1
CD630_02360	fliS2	Flagellar protein FliS2	-6.03	1.1.1
CD630_02370	fliD	Flagellar hook-associated protein 2 FliD (or HAP2)	-6.45	1.1.1
CD630_02380		conserved hypothetical protein	-6.76	0.0.2
CD630_02390	fliC	Flagellin C	-7.85	1.1.1
CD630_02400		Glycosyltransferase	-5.71	2.2.4
CD630_02410		flagella glycosylation phosphoserine phosphatase	-5.88	2.2.4
CD630_02420		putative nucleoside triphosphate transferase	-5.92	2.2.4
CD630_02430		flagella glycosylation methyltransferase domain protein	-5.96	2.2.4
CD630_02440		putative CDP-glycerol:Poly(glycerophosphate) glycerophosphotransferase	-5.89	2.2.4
CD630_02450	flgB	Flagellar basal-body rod protein FlgB	-15.09	1.1.1
CD630_02460	flgC	Flagellar basal-body rod protein FlgC	-14.93	1.1.1

CD630_02470	fliE	Flagellar hook-basal body complex protein FliE	-15.47	1.1.1
CD630_02480	fliF	Flagellar M-ring protein FliF	-13.28	1.1.1
CD630_02490	fliG	Flagellar motor switch protein FliG	-12.07	1.1.1
CD630_02500	fliH	Flagellar assembly protein FliH	-11.00	1.1.1
CD630_02510	fliI	ATP synthase subunit beta FliI	-11.23	1.1.1
CD630_02520	fliJ	Flagellar protein FliJ	-11.73	1.1.1
CD630_02530	fliK	Flagellar hook-length control protein FliK	-11.30	1.1.1
CD630_02540	flgD	Basal-body rod modification protein FlgD	-11.76	1.1.1
CD630_02550	flgE	Flagellar hook protein FlgE (Distal rod protein)	-11.68	1.1.1
CD630_02551	FlbD	Flagellar protein FlbD	-12.10	1.1.1
CD630_02560	motA	Flagellar motor rotation protein MotA	-11.53	1.1.1
CD630_02570	motB	Flagellar motor rotation protein MotB (Chemotaxis protein MotB)	-11.45	1.1.1
CD630_02580	fliL	Flagellar basal body-associated protein FliL	-11.51	1.1.1
CD630_02590	fliZ	Flagellar protein FliZ	-11.37	1.1.1
CD630_02600	fliP	Flagellar biosynthesis protein FliP	-10.76	1.1.1
CD630_02610	fliQ	Flagellar biosynthetic protein FliQ	-11.10	1.1.1
CD630_02620	flhB	Bifunctional flagellar biosynthesis protein FliR/FlhB	-10.25	1.1.1
CD630_02630	flhA	Flagellar biosynthesis protein FlhA	-10.14	1.1.1
CD630_02640	flhF	Flagellar biosynthesis regulator FlhF (Flagella-associated GTP-binding protein)	-9.89	1.1.1
CD630_02650	flhG	Flagellar number regulator FlhG	-9.76	1.1.1
CD630_02660	fliA	RNA polymerase sigma-28factor for flagellar operon	-9.94	6.2.1
CD630_02670		putative flagellar protein	-10.10	1.1.1
CD630_02671		putative flagellar protein	-11.82	1.1.1
CD630_02680	flgG1	Flagellar hook-basal body complex protein FlgG1	-9.59	1.1.1
CD630_02690	flgG	Flagellar basal body rod protein FlgG	-10.09	1.1.1
CD630_02700	fliM	Flagellar motor switch protein FliM	-9.66	1.1.1
CD630_02710	fliN1	Flagellar motor switch phosphatase FliN1	-5.85	1.1.1
CD630_02280	fliN	Flagellar motor switch protein FliN	-10.19	1.1.1
CD630_02720		conserved hypothetical protein	-9.93	0.0.2
CD630_02730	htpG	Heat shock protein 90 (Heat shock protein HtpG)(High temperature protein G)	-4.17	1.3.1
CD630_02731		conserved hypothetical protein	-3.05	0.0.2
CD630_02850		PTS system, mannose/fructose/sorbose IIB component	2.75	1.5.3
CD630_02860		PTS system, mannose/fructose/sorbose IIA component	2.43	1.5.3

CD630_02880		PTS system, mannose/fructose/sorbose IIC component	2.84	1.5.3
CD630_05110	tndX	Recombinase site-specific resolvase family Tn5397, CTn3-Orf3	-12.90	5.1.4
CD630_05290		putative membrane protein	-5.06	4.1.6
CD630_05600	nfo	Endonuclease IV	-5.33	2.2.3
CD630_05800	gapN	Glyceraldehyde-3-phosphate dehydrogenase (NADP(+)) (GADPH)	-11.79	3.5.5
CD630_05870		uncharacterised protein	2.02	7.0.0
CD630_05880		uncharacterised protein	2.06	7.0.0
CD630_06180		Transcriptional regulator, LytR family	-2.59	6.5.0
CD630_06190		conserved hypothetical protein	-7.92	0.0.2
CD630_06200		conserved hypothetical protein	-8.54	0.0.2
CD630_06210		putative membrane protein	-6.87	4.1.6
CD630_06220		conserved hypothetical protein	-7.09	0.0.2
CD630_06720		conserved hypothetical protein	-4.36	0.0.2
CD630_07570		putative c-di-GMP phosphodiesterase	-5.35	6.5.0
CD630_10910	int1	Integrase Tn1549-like, CTn4-Orf34	2.68	5.1.4
CD630_11030		putative conjugative transposon protein Tn1549-like, CTn4-Orf21	4.91	5.1.4
CD630_11031		putative conjugative transposon protein Tn1549-like, CTn4-Orf20	3.90	5.1.4
CD630_11040		putative conjugative transposon protein Tn1549-like, CTn4-Orf19	3.03	5.1.4
CD630_11250		Nitroreductase-family protein	-6.05	7.0.0
CD630_11260		Transcriptional regulator, AraC family	-4.61	6.3.2
CD630_12790	iscS2	Cysteine desulfurase	-5.22	3.3.19
CD630_12800		fe-s iron-sulfur cluster assembly protein, nifu family	-3.54	3.5.3
CD630_14700		putative rhodanese-like domain-containing protein	-19.49	1.4.2
CD630_14740		putative rubrerythrin (Rr)	-8.36	1.4.2
CD630_14790	feoB1	Ferrous iron transport protein B	2.24	1.6.3
CD630_15240		putative rubrerythrin	-2.56	1.4.2
CD630_15680		conserved hypothetical protein	-7.98	0.0.2
CD630_15800	hom2	Homoserine dehydrogenase	2.73	3.1.0
CD630_16970	ribH	6,7-dimethyl-8-ribityllumazine synthase	-3.74	3.2.13
CD630_16980	ribBA	Riboflavin biosynthesis protein ribBA [Includes: 3,4-dihydroxy-2-butanone 4-phosphate synthase; GTP cyclohydrolase-2]	-2.74	3.2.13
CD630_17290		putative sodium:phosphate symporter	-9.01	1.5.2
CD630_17770		putative arsenate reductase	-5.61	1.4.2
CD630_17940		conserved hypothetical protein	5.11	0.0.2
CD630_17950		conserved hypothetical protein	5.76	0.0.2
CD630_18220	bcp	putative thiol peroxidase	-13.15	1.4.2

CD630_18230		conserved hypothetical protein, UPF0246 family	-10.41	0.0.2
CD630_18240		P-type calcium transport ATPase	-3.15	1.5.2
CD630_18820		Site-specific recombinase	-3.84	5.1.4
CD630_18990		putative dCMP deaminase	-13.88	7.0.0
CD630_19870	cwp28	putative cell wall binding protein cwp28	4.31	4.1.8
CD630_19900		putative protein with SH3 domain	35.08	0.0.2
CD630_19903		conserved hypothetical protein	-2.00	0.0.2
CD630_20460		conserved hypothetical protein	-9.50	0.0.2
CD630_21151		uncharacterised protein	-11.51	7.0.0
CD630_21170	trxB2	Thioredoxin reductase	-5.23	3.2.15
CD630_21580	gabT	4-aminobutyrate aminotransferase	2.06	3.3.20
CD630_22090		putative GTP-binding protein, HflX type	-4.09	7.0.0
CD630_23090		conserved hypothetical protein	-2.64	0.0.2
CD630_23270		PTS system, fructose/mannitol family IIA component	5.32	1.5.3
CD630_24760		gnat family acetyltransferase	-3.38	7.0.0
CD630_24770		uncharacterised protein	-4.50	7.0.0
CD630_24860		PTS system, fructose-like IIC component	-2.44	1.5.3
CD630_24870		PTS system, fructose-like IIB component	-2.64	1.5.3
CD630_24880		PTS system, fructose-like IIA component	-3.23	1.5.3
CD630_24890		Transcription antiterminator, PTS operon regulator	-3.91	6.5.0
CD630_27950	selA	L-seryl-tRNA(Sec) selenium transferase (Selenocysteine synthase) (Sec synthase) (Selenocysteinyl-tRNA(Sec) synthase)	2.89	2.2.1
CD630_27960	selD	Selenide, water dikinase (Selenophosphate synthetase) (Selenium donor protein)	-4.07	2.2.1
CD630_27970	comE	Competence protein ComEA	-7.14	4.1.6
CD630_28300		uncharacterised protein	-65.96	7.0.0
CD630_28310		putative adhesin	42.42	4.1.5
CD630_28410		putative amidohydrolase	-11.37	3.4.5
CD630_28450	rbr1	Rubrerythrin	2.50	1.4.2
CD630_28460		conserved hypothetical protein	2.42	0.0.2
CD630_28470		conserved hypothetical protein	2.38	0.0.2
CD630_28480		conserved hypothetical protein	2.37	0.0.2
CD630_28750	fhuC	ABC-type transport system, ferrichrome-specific ATP-binding protein	-3.78	1.6.3
CD630_29950	nrdE	Ribonucleoside-diphosphate reductase subunit alpha (Ribonucleotide reductase large subunit)	-4.39	3.3.11
CD630_30100		putative cytochrome C assembly protein	-2.30	3.2.6
CD630_30330	trxA1	Thioredoxin	-3.78	3.2.15
CD630_30390		putative ATPase	-6.98	7.0.0
CD630_30400		conserved hypothetical protein	-10.44	0.0.2

CD630_30990		putative amidohydrolase, M20D family	-2.08	2.1.4
CD630_31000		putative C4-dicarboxylate anaerobic carrier,DcuC family	-2.09	1.5.3
CD630_32110		conserved hypothetical protein	2.07	0.0.2
CD630_32150		ABC-type transport system, glycine betaine/carnitine/choline ATP-binding protein	-5.74	1.6.2
CD630_32160		ABC-type transport system, glycine betaine/carnitine/choline permease	-8.39	1.6.2
CD630_32460		putative surface protein	4.28	4.1.8
CD630_32640		Fragment of conserved hypothetical protein (C-terminal region)	3.37	0.0.2
CD630_32650		Two-component response regulator	17.57	6.1.2
CD630_32660		Two-component sensor histidine kinase	18.37	6.1.1
CD630_32670		Two-component response regulator	18.74	6.1.2
CD630_33230		conserved hypothetical protein	-2.25	0.0.2
CD630_33250		conserved hypothetical protein	2.10	0.0.2
CD630_33260		Integrase, lambdoid phage family Tn916-like,CTn6-Orf1	2.14	5.1.2
CD630_33270		putative conjugative transposon protein Tn916-like, CTn6-Orf2	2.12	5.1.4
CD630_34770		putative dCMP deaminase	-2.00	3.3.11
CD630_35040		putative type IV prepilin peptidase, A24A family	3.18	4.1.5
CD630_35050		putative twitching motility protein PilT	3.49	4.1.5
CD630_35060		conserved hypothetical protein	4.28	0.0.2
CD630_35070		putative type IV pilin	4.40	4.1.5
CD630_35080		putative type IV pilin	4.69	4.1.5
CD630_35090		putative type IV pilus assembly protein	4.67	4.1.5
CD630_35100		putative membrane protein	5.08	4.1.6
CD630_35110		putative type IV pilus secretion protein	5.28	4.1.5
CD630_35120		putative type IV pilus transporter system,ATP-binding	4.89	4.1.5
CD630_35130		putative pilin protein	11.72	4.1.5
CD630_35250		ABC-type transport system, iron-family extracellular solute-binding protein	-9.35	1.5.5
CD630_35260		ABC-type transport system, iron-family permease	-9.01	1.5.5
CD630_35270		ABC-type transport system, iron-family ATP-binding protein	-9.81	1.5.5
CD630_36100		putative glyoxalase	-11.82	1.4.2
CD630_36400		putative ribokinase family sugar kinase	-2.01	3.4.3
CD630_36410		conserved hypothetical protein	-4.38	0.0.2
CD630_36420		putative selenocysteine synthase	-4.98	2.2.1
CD630_36430		Dihydroorotase	-5.02	3.7.2

[a] Riley Class identification based on Pettit et al. BMC Genomics 2014.

Table A1.2 Plasmids and Strains used in this study

Plasmids	Description
pMC123	*E. coli – C. difficile* shuttle vector; Amp^R, Cm^R/Tm^R
pMC-P_{cpr}	pMC123 with *cpr* promoter in the multiple cloning site
pDccA	pMC-P_{cpr}::*dccA* (CD630_14200)
pDccAmut	pMC-P_{cpr}::$dccA^{mut}$ (AADEF)
pMC-P_{tet}	pMC123 with *cpr* promoter
p1987	pMC-P_{tet}::CD630_19870
p2795	pMC-P_{tet}::CD630_27950
p2796	pMC-P_{tet}::CD630_27960
p2797	pMC-P_{tet}::CD630_27970
p2831	pMC-P_{tet}::CD630_28310
p3246	pMC-P_{tet}::CD630_32460
Strains	
DH5α	F- ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR*17(rκ -, mκ+) *phoA supE44 thi*-1 *gyrA96 relA1* λ- *tonA*
Bacillus subtilis BS49	CU2189::Tn916
C. difficile 630dErm	Ribotype 012, erythromycin-sensitive derivative of *C. difficile* 630
630dErm pSMB47::P_{CD630_19900}-*phoZ*	CD630_19900 promoter fused to *phoZ* and integrated on the *C. difficile* chromosome
630dErm pSMB47::P_{CD630_33682}-*phoZ*	CD630_33682 promoter fused to *phoZ* and integrated on the *C. difficile* chromosome
630dErm pSMB47::P_{zmpI}-*phoZ*	*zmpI* promoter fused to *phoZ* and integrated on the *C. difficile* chromosome
630dErm pSMB47::P_{pilA1}-*phoZ*	*pilA1* promoter fused to *phoZ* and integrated on the *C. difficile* chromosome
630dErm pSMB47::P_{pilA1}-UTR^{WT}-*phoZ*	*pilA1* promoter and native riboswitch fused to *phoZ* and integrated on the *C. difficile* chromosome
630dErm pSMB47::P_{pilA1}-UTR^{A70G}-*phoZ*	*pilA1* promoter with A70G mutation in the riboswitch fused to *phoZ* and integrated on the *C. difficile* chromosome

Table A1.3 Olignucleotides used in this study

Primer name	Sequence
Atc_F	TTAGAATTCCATAAAAATAAGAAGCCTGCATTTG
Atc_R	TTAGAGCTCAGATCTGTTAACGC
CD630_19870_F	TTAGAGCTCGTAAAGGAGAAAATTTTATGAAATTCTATAAAAGAATATTAACATTGAC
CD630_19870_R	TATGGATCCACAATTACATCTATTCATTTTCAATTAAATAAC
CD630_27950_F	AATGGATCCGTAAAGGAGAAAATTTTATGAACAAAAAAATATTATCATTAGGTCTAG
CD630_27950_R	TATCTGCAGTTTATGGAATTTTTTACAAGCTACC
CD630_27960_F	AATGAGCTCGTAAAGGAGAAAATTTTATGAATAAAAGAAAATCTTTTATAAGAACTATAG
CD630_27960_R	TATGGATCCATTTATTTTTCAATTAAACTCTTAATCTTGTC
CD630_27970_F	AATGAGCTCGTAAAGGAGAAAATTTTATGAAAAAGGCAATATCTTGTGTAC
CD630_27970_R	TATGGATCCACCTACTATTTATCTCTAACAATATTACC
CD630_28310_F	AATGAGCTCGTAAAGGAGAAAATTTTATGAAGAAAGGAAATAGAAAGGC
CD630_28310_R	TATGGATCCTAATGTTAGGGTCTAATTTGTATTTTTATTTC
CD630_32460_F	AATGAGCTCGTAAAGGAGAAAATTTTTTGAAAACAAAAATTAAAAAATCAAGTATAATC
CD630_32460_R	TATGGATCCAGATTAATTTCTTTTTATTTTACATTGATACG
Cdi1-1promF	AGGCTCTCAAGGGCATCGGTCGACTGTTATATATTGTAAAATGTTGAAAAATAGCC
Cdi1-1promR	ATTTCCTTGTTTCCTCCTGCATGCTTTGCCTATATCTGAATAATAACATAATTTGAAC
Cdi1-11promF	AGGCTCTCAAGGGCATCGGTCGACTCAATTTATCTCACTTAAATTAATAACAACTG
Cdi1-11promR	ATTTCCTTGTTTCCTCCTGCATGCTGCCGATTTAGTTTTATTCTACAATTAC
Cdi1-12promF	AGGCTCTCAAGGGCATCGGTCGACAAGAAGAAATAAAAATACAAATTAGACCC
Cdi1-12promR	ATTTCCTTGTTTCCTCCTGCATGCCCTAGTTTTGCCTCATTTAATTTTATATC

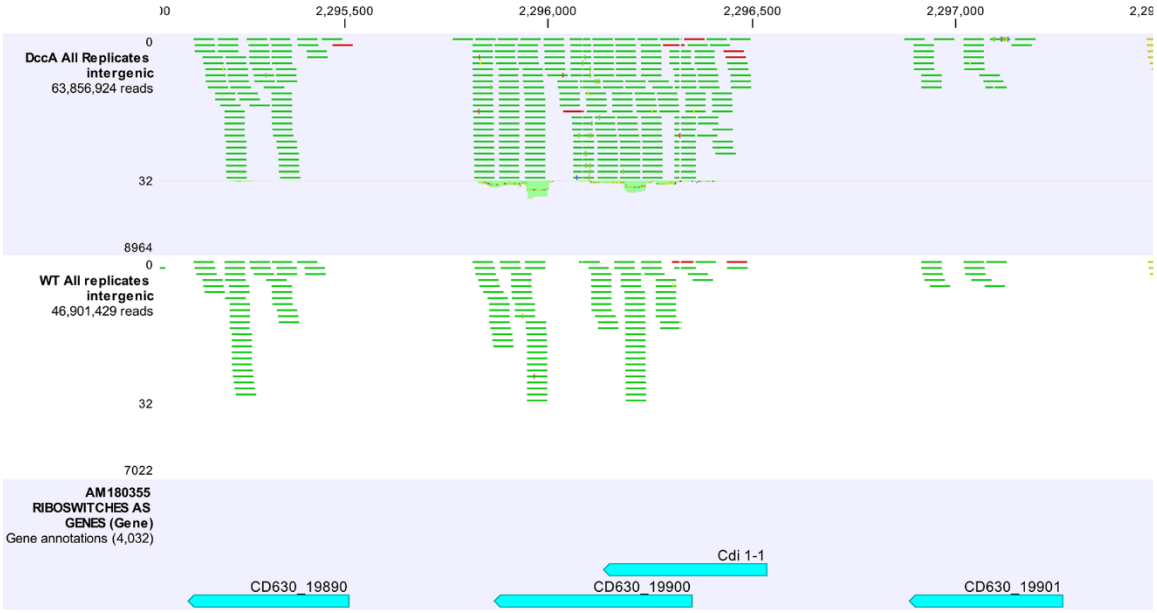

Figure A1.1 RNA-sequencing reads for the region surrounding Cdi-1-1. Reads in red match the sense strand of the genome and reads in green match the antisense strand.

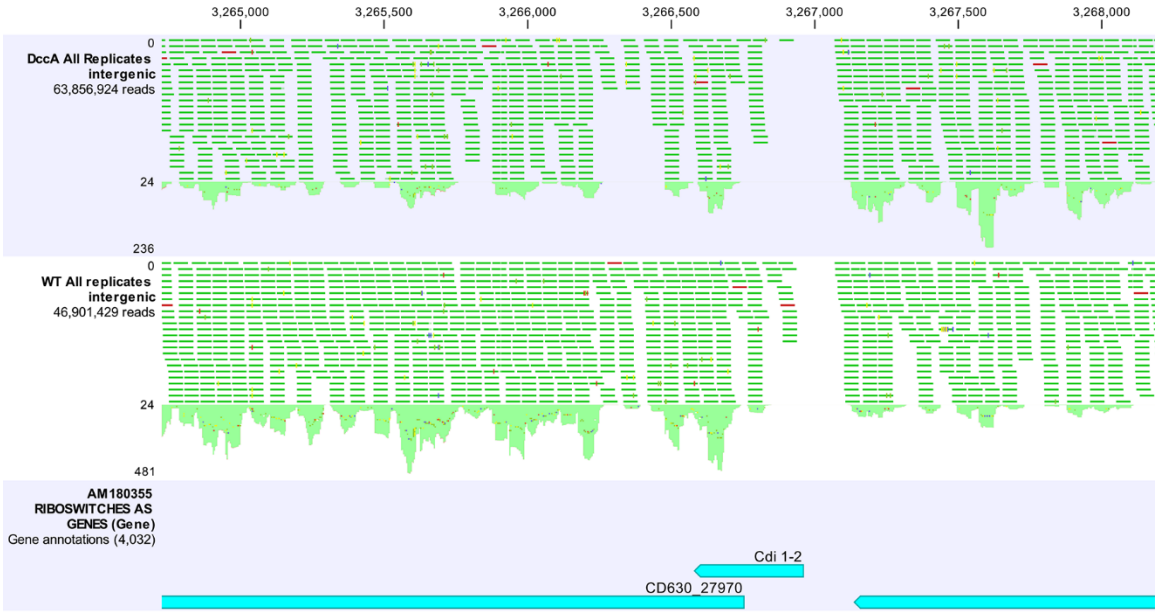

Figure A1.2 RNA-sequencing reads for the region surrounding Cdi-1-2. Reads in red match the sense strand of the genome and reads in green match the antisense strand.

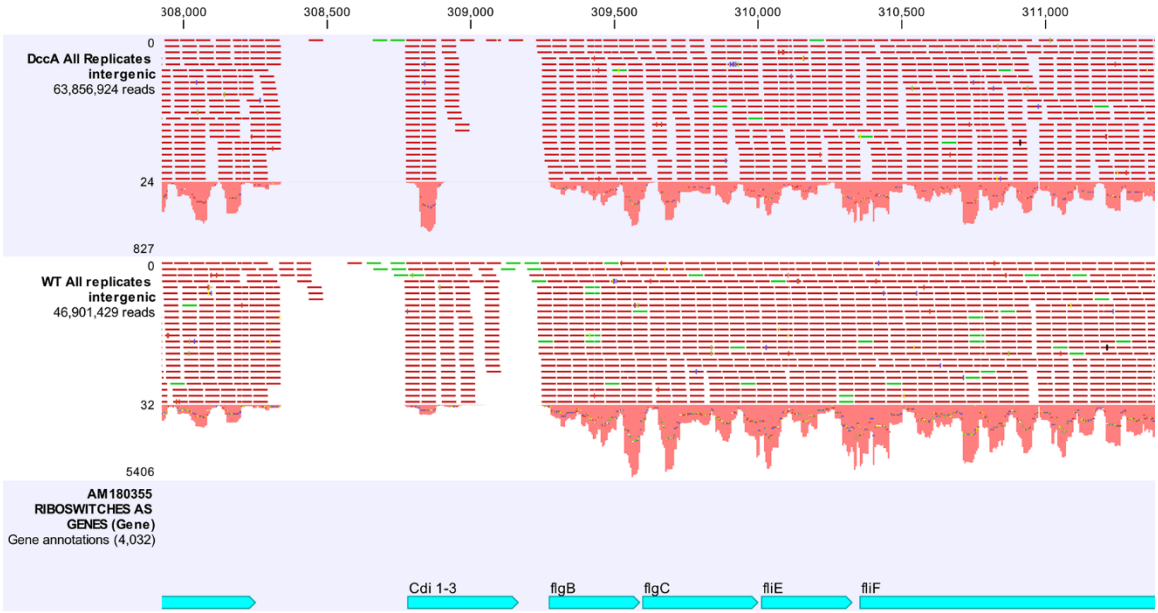

Figure A1.3 RNA-sequencing reads for the region surrounding Cdi-1-3. Reads in red match the sense strand of the genome and reads in green match the antisense strand.

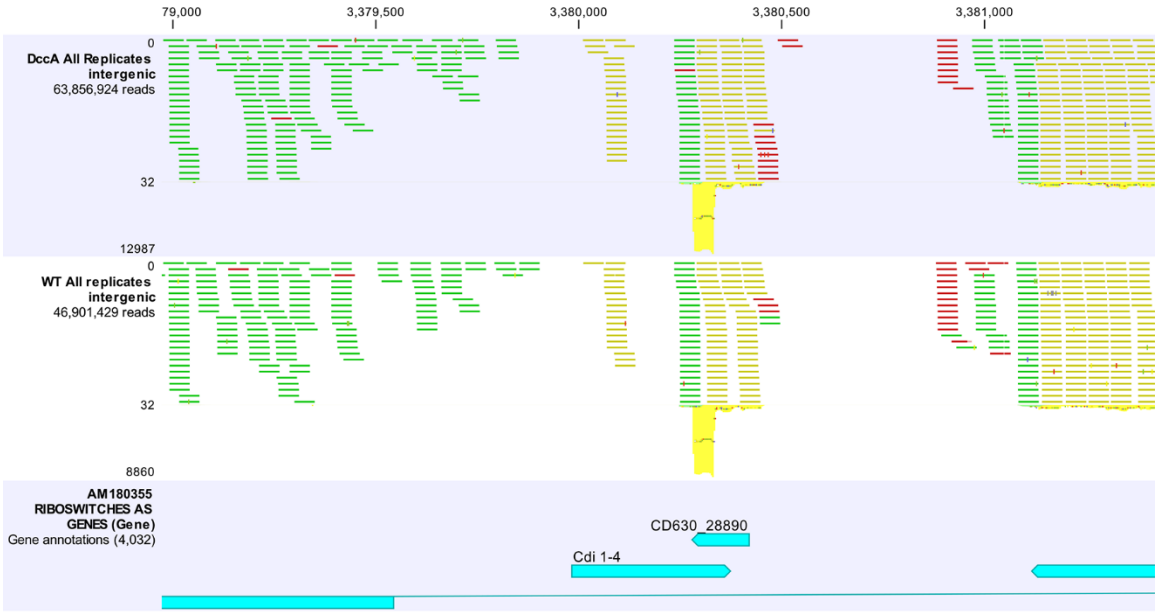

Figure A1.4 RNA-sequencing reads for the region surrounding Cdi-1-4. Reads in red match the sense strand of the genome and reads in green match the antisense strand. Yellow reads represent reads that map to multiple regions of the genome.

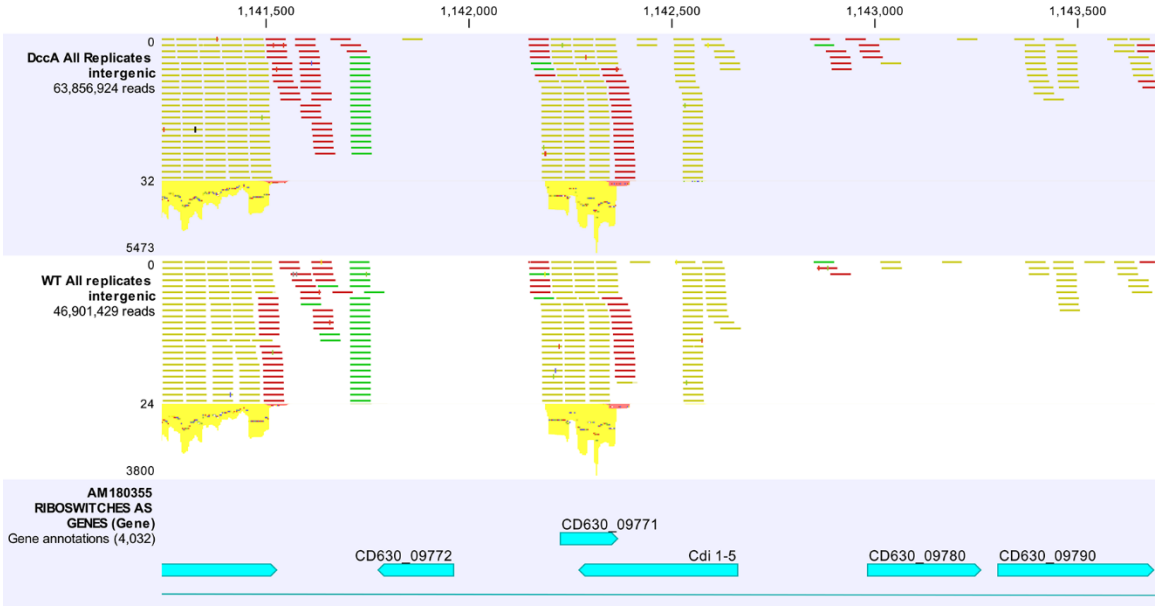

Figure A1.5 RNA-sequencing reads for the region surrounding Cdi-1-5. Reads in red match the sense strand of the genome and reads in green match the antisense strand. Yellow reads represent reads that map to multiple regions of the genome.

Figure A1.6 RNA-sequencing reads for the region surrounding Cdi-1-6. Reads in red match the sense strand of the genome and reads in green match the antisense strand.

Figure A1.7 RNA-sequencing reads for the region surrounding Cdi-1-7. Reads in red match the sense strand of the genome and reads in green match the antisense strand. Yellow reads represent reads that map to multiple regions of the genome.

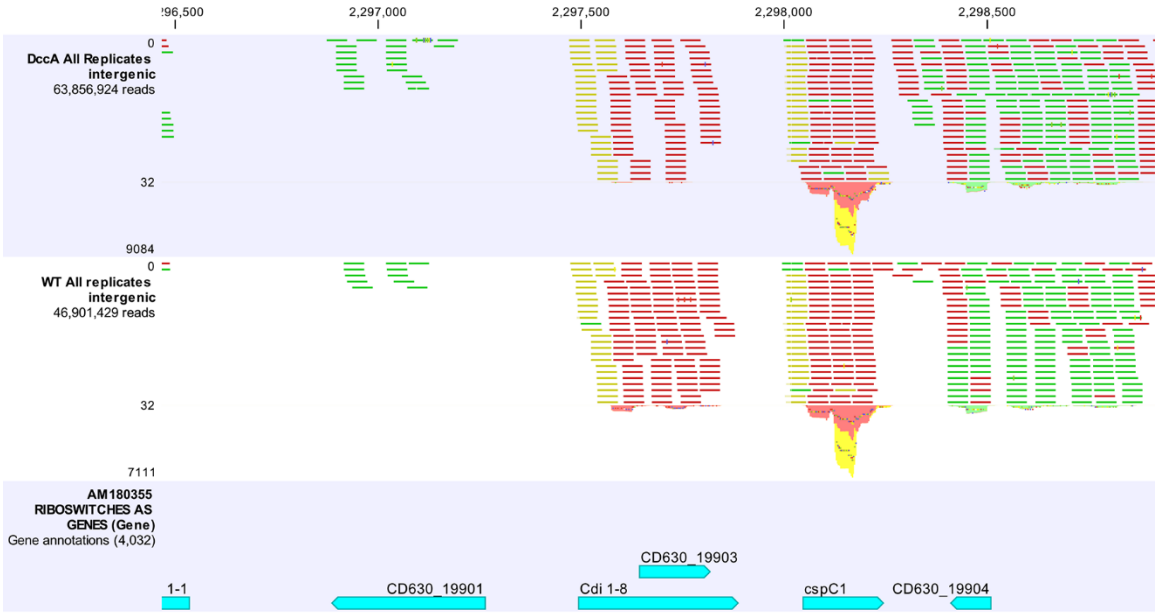

Figure A1.8 RNA-sequencing reads for the region surrounding Cdi-1-8. Reads in red match the sense strand of the genome and reads in green match the antisense strand. Yellow reads represent reads that map to multiple regions of the genome.

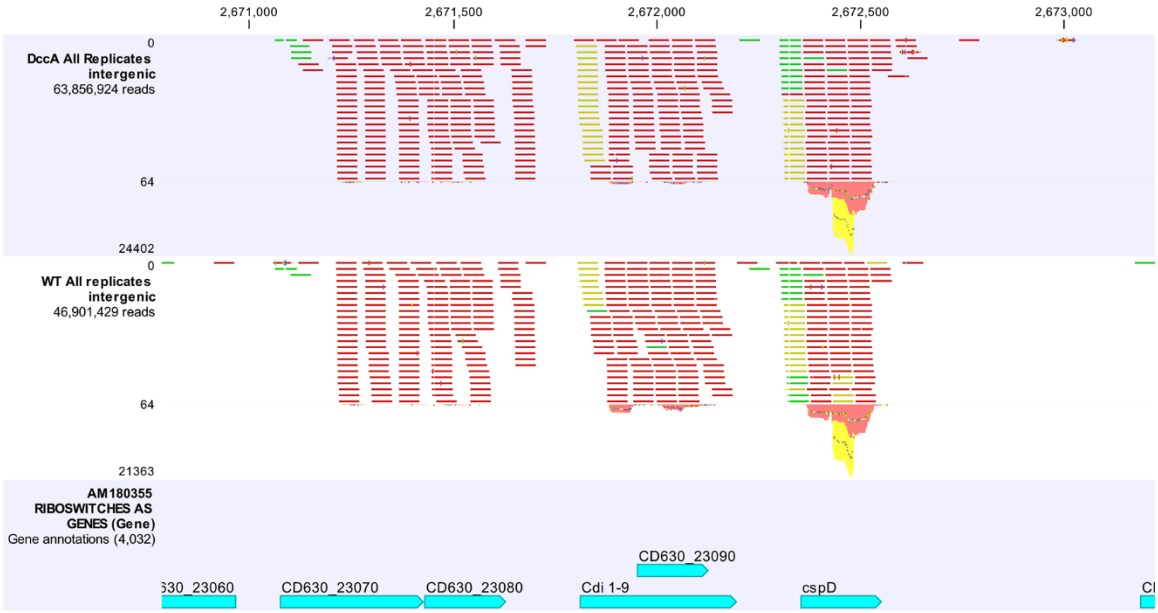

Figure A1.9 RNA-sequencing reads for the region surrounding Cdi-1-9. Reads in red match the sense strand of the genome and reads in green match the antisense strand. Yellow reads represent reads that map to multiple regions of the genome.

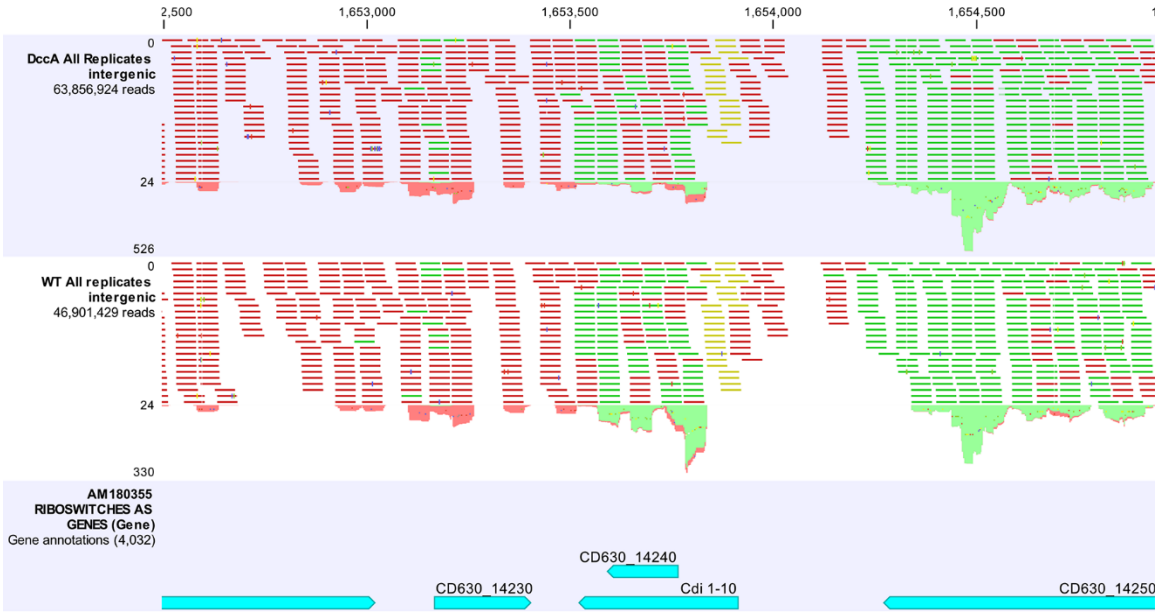

Figure A1.10 RNA-sequencing reads for the region surrounding Cdi-1-10. Reads in red match the sense strand of the genome and reads in green match the antisense strand. Yellow reads represent reads that map to multiple regions of the genome.

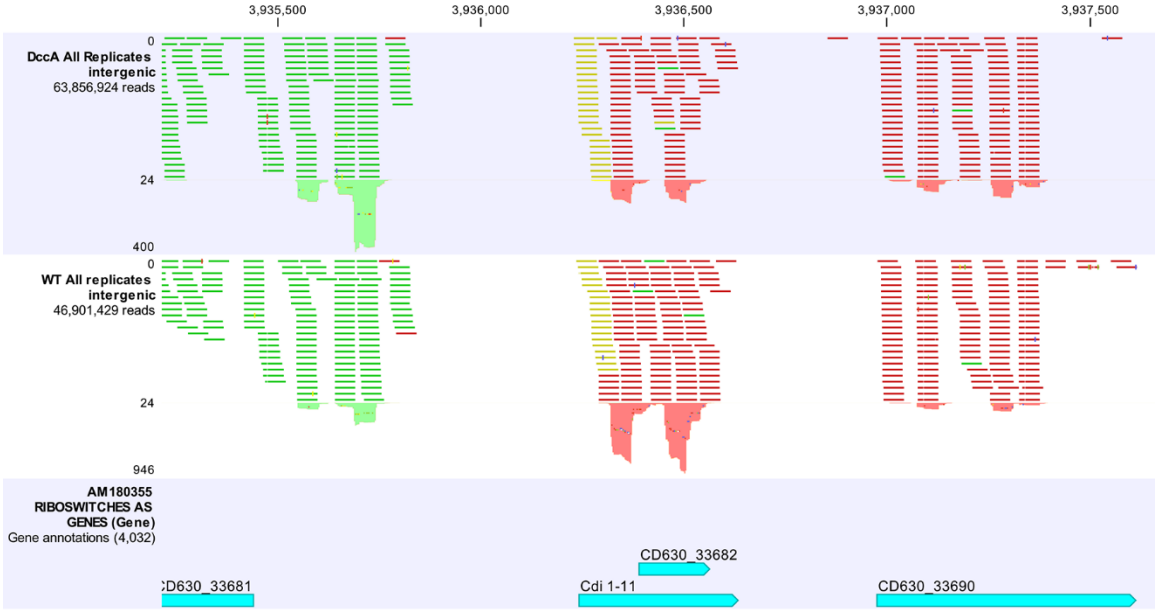

Figure A1.11 RNA-sequencing reads for the region surrounding Cdi-1-11. Reads in red match the sense strand of the genome and reads in green match the antisense strand. Yellow reads represent reads that map to multiple regions of the genome.

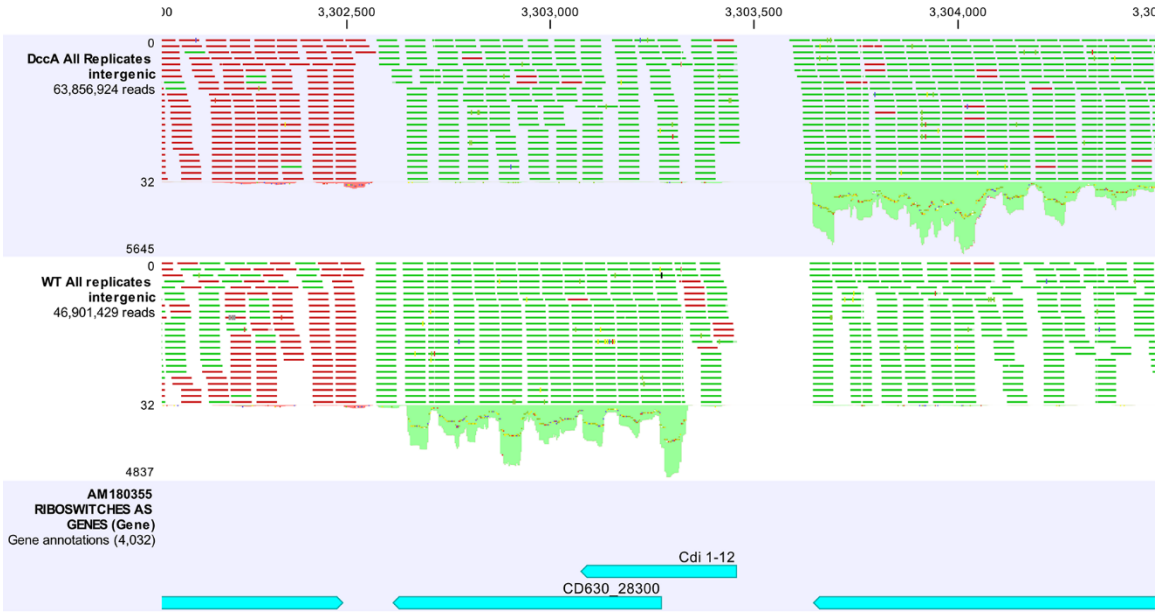

Figure A1.12 RNA-sequencing reads for the region surrounding Cdi-1-12. Reads in red match the sense strand of the genome and reads in green match the antisense strand. Yellow reads represent reads that map to multiple regions of the genome

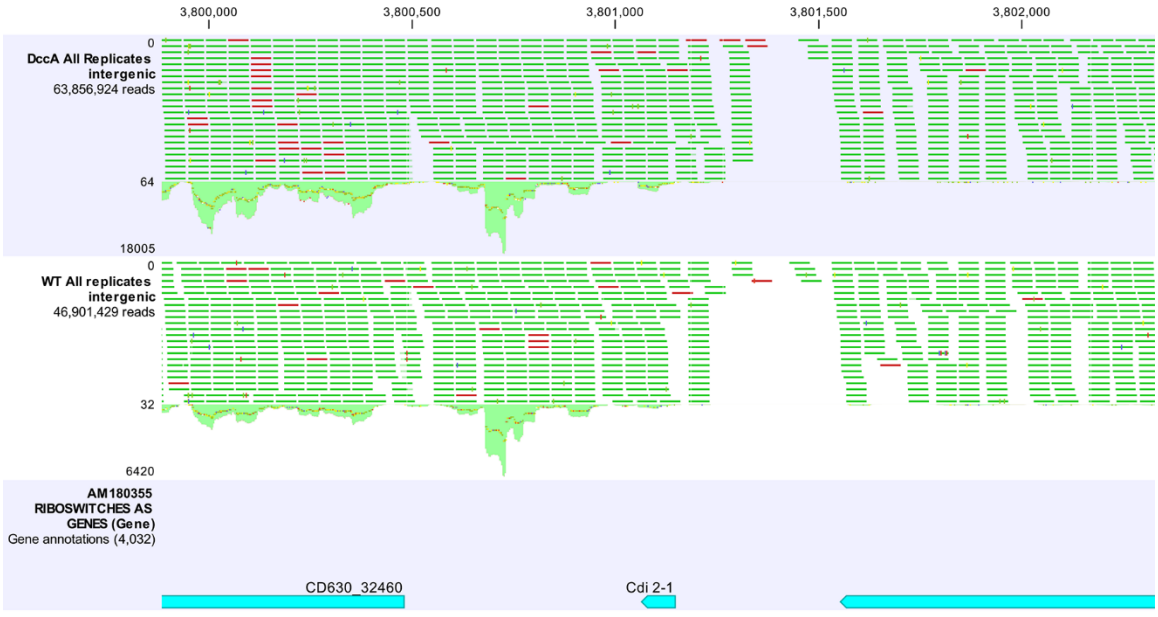

Figure A1.13 RNA-sequencing reads for the region surrounding Cdi-2-1. Reads in red match the sense strand of the genome and reads in green match the antisense strand.

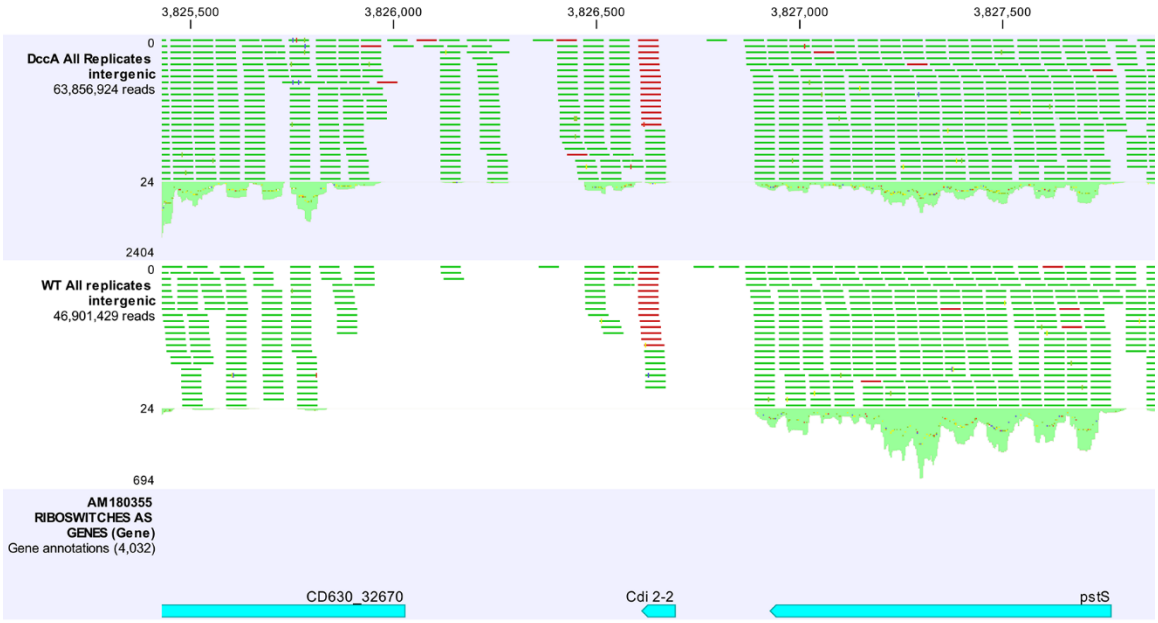

Figure A1.14 RNA-sequencing reads for the region surrounding Cdi-2-2. Reads in red match the sense strand of the genome and reads in green match the antisense strand.

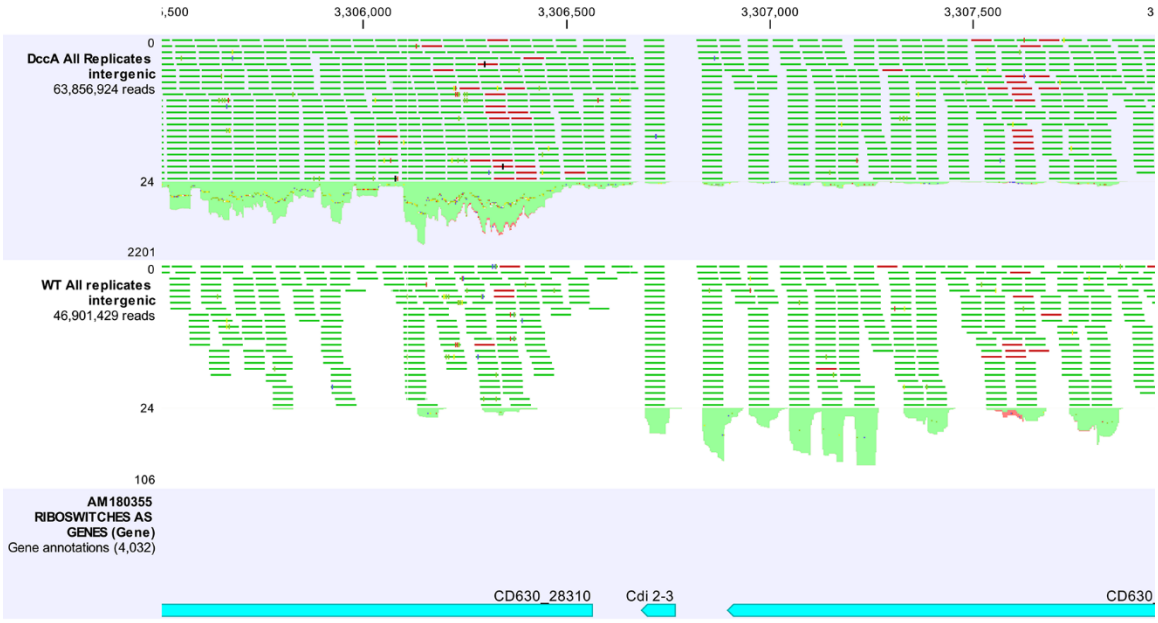

Figure A1.15 RNA-sequencing reads for the region surrounding Cdi-2-3. Reads in red match the sense strand of the genome and reads in green match the antisense strand.

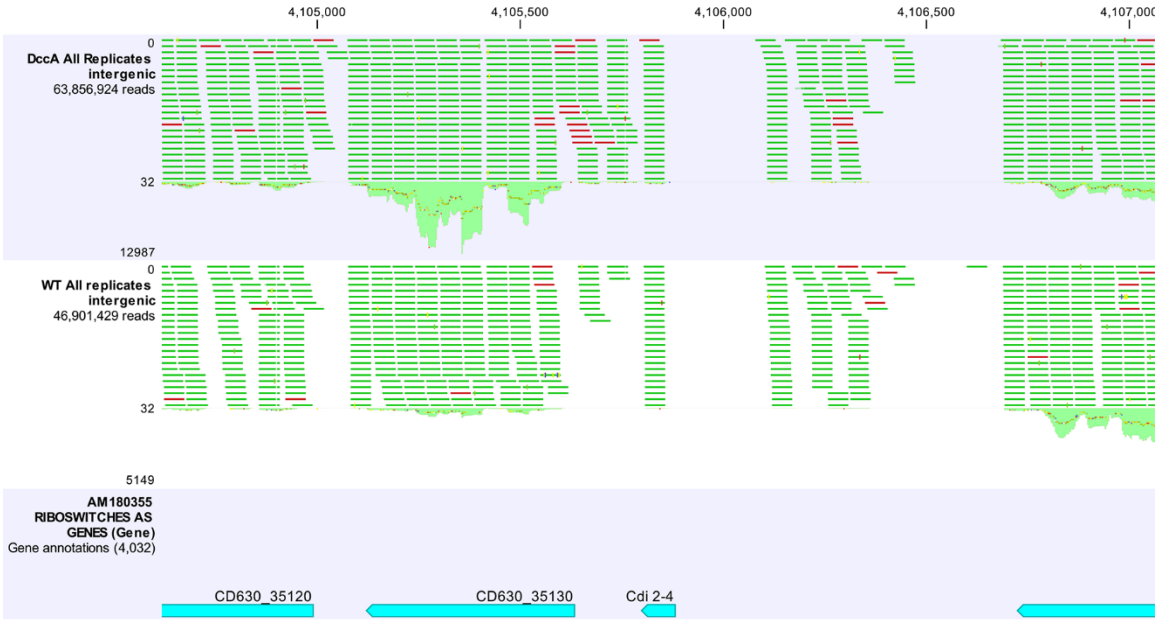

Figure A1.16 RNA-sequencing reads for the region surrounding Cdi-2-4. Reads in red match the sense strand of the genome and reads in green match the antisense strand.

A

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                                    P2                                  P3
Cdi1-1   CAGAUAUAGGCAAUAUUAGAUAAAUCUAAUGACGCAAAACUAUAGGGACUAAAAAUCUUUAUGUAAU----
Cdi1-3   ACUAAAUAGGCAAAUCUAGAGAAAUCUAGUGACGCAAAGCUAUAGGGACUAAGGUUUAUAUACAUAAACU
Cdi1-4   ACUAAAUCGGCAAAACUAGAGAAAAUUAGUGACGCAAAGCUAUAGGGACUAAGAUUUAUA--AAAUUCUU
Cdi1-5   ACUAAAUCGGCAAAACUAGAGAAAAUUAGUGACGCAAAGCUAUAGGGACUAAGAUUUAUA--AAAUUCUU
Cdi1-7   ACUAAAUCGGCAAAACUAGAGAAAUCUAGUGACGCAAAGCUAUAGGGACUAAGACUUAUAUAAAUAUCUU
Cdi1-8   ACUAAAUCGGCAAAACUAGAGAAAUUUAGUGACGCAAAGCUAUAGGGACUAAGACUAAAUAUCUUAUGAG
Cdi1-10  ACUAAAUCGGCAAAACUAGAGAAAUUUAGUGACGCAAAGCUAUAGGGACUAACGCUUAUAAAAU-AAACU
Cdi1-11  ACUAAAUCGGCAAAACUAGAGAAAUUUAGUGACGCAAAGCUAUAGGGACUAACGCUUAUAAAAU-AAACU
Cdi1-6   ACUAAAUAGGCAAAACUAGAGAAAUUUAGUGACGCAAAGCUAUAGGGACUAAGACUUAUGAAAU-GAG--
Cdi1-9   ACUAAAUCGGCAAAACUAGAGAAAUUUAGUGACGCAAAGCUAUAGGGACUAACGCUUAUAAAAU-AAG--
Cdi1-2   AAUUAAAUGGCAAAAUUAGGGAAAUCUAAUGACGCAAAACUAUAGGGAUUAAAGCUUUUAAAGCCAUAU-
Cdi1-12  UUAAAUGAGGCAAAACUAGGGAAACCUAGUCACGCAAAGCUAUAGGGGCUAAAGUUUAGUAAACCAUGC-
                *****   ***  ***  ** * ******* ********  ***    *
                P3
Cdi1-1   AAAGGUCAUGUCAGCCAGUUGCCAAAAAGAUAAUAAUUCUUUUUGUUAAAAGUAUUUUU---
Cdi1-3   -------AUGUCAGCCAGUUGCCAAAAAGAGUCCUAGGUGUAUUGUAUACCUAAGAAAAAGU
Cdi1-4   AUAAAUUAUGCCAGCCAGUUGCCAAAAAGAUAUUGUUCUUUUUGUUUUU-AUGAAAGUUUUUU
Cdi1-5   AUAAAUUAUGCCAGCCAGUUGCCAAAAAGAUAUUGUUCUUUUUGUUUUU-AUGAAAGUUUUUU
Cdi1-7   AUGAGUUAUGUCAGCCAGUUGCCAAAGAGAUAUUGUUCUUUUUGUUUUU-AUGAAAGUUUUUU
Cdi1-8   UAUAUUUAUGUCAGCCAGUUGCCAAAGAGAUAGUGUUCUUUUUGUUUUU-AUGAAAGUUUUUU
Cdi1-10  AUGAGUUAUGUCAGCCAGUUGCCAAAAAGAUAUUGUUCUUUUUGUUUU--AUGAAAGUUUUUU
Cdi1-11  AUGAGUUAUGUCAGCCAGUUGCCAAAAAGAGAUA-UUCUUUUUGUUUUU-AUGAAAGUUUUUU
Cdi1-6   -----UUAUGUCAGCCAGUUGCCAAAGAGAUAUUUU-CUUUUUGUUUUU-AUGAAUGUUUUUU
Cdi1-9   ------UAUGUCAGCCAGUUGCCAAAAAGAUAUUUU-CUUUUUGUUUUUCAUGAAAGUUUUUU
Cdi1-2   ----------UCAGCCAGUUGCUAAAAAGAGUAAAGUCUUUUUAUUUUAUAAUUAAGAUUCU
Cdi1-12  ----------CAGCCAGUUGCCGAAAAGAGUAAAAAUUUCUUUUUGUAUAUACAAAGAUACUCUU
                  ***********  ** ***          *
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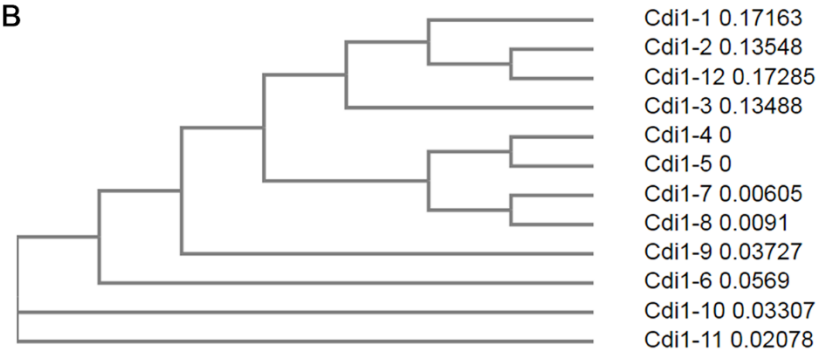
B



C

	Cdi1-1	Cdi1-3	Cdi1-4	Cdi1-5	Cdi1-7	Cdi1-8	Cdi1-10	Cdi1-11	Cdi1-6	Cdi1-9	Cdi1-2	Cdi1-12
Cdi-1-1	100.00	66.95	72.36	72.36	71.20	70.40	69.35	67.74	69.75	70.59	68.42	60.53
Cdi-1-3	66.95	100.00	71.31	71.31	74.19	73.39	76.42	78.05	73.55	74.38	65.55	68.91
Cdi-1-4	72.36	71.31	100.00	100.00	93.08	93.08	85.94	80.47	81.82	87.70	67.80	63.56
Cdi-1-5	72.36	71.31	100.00	100.00	93.08	93.08	85.94	80.47	81.82	87.70	67.80	63.56
Cdi-1-7	71.20	74.19	93.08	93.08	100.00	98.48	88.46	83.08	83.74	88.71	70.00	63.33
Cdi-1-8	70.40	73.39	93.08	93.08	98.48	100.00	88.46	83.08	83.74	88.71	69.17	62.50
Cdi-1-10	69.35	76.42	85.94	85.94	88.46	88.46	100.00	94.62	89.43	90.24	72.88	66.95
Cdi-1-11	67.74	78.05	80.47	80.47	83.08	83.08	94.62	100.00	91.87	92.68	72.03	69.49
Cdi-1-6	69.75	73.55	81.82	81.82	83.74	83.74	89.43	91.87	100.00	89.43	67.52	66.67
Cdi-1-9	70.59	74.38	87.70	87.70	88.71	88.71	90.24	92.68	89.43	100.00	68.64	66.95
Cdi-1-2	68.42	65.55	67.80	67.80	70.00	69.17	72.88	72.03	67.52	68.64	100.00	69.17
Cdi-1-12	60.53	68.91	63.56	63.56	63.33	62.50	66.95	69.49	66.67	66.95	69.17	100.00

Figure A1.17. Sequence analysis of the GEMM riboswitches in *C. difficile*. (A) Alignment of the sequences by Clustal Omega. Asterisks represent bases that are conserved for all four riboswitches. (B) Cladogram representing the relative sequence identity between the riboswitches. (C) Percent sequence identity for each pair of riboswitches.

A

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                P1        P2              P3                pseudoknot      P3         P2
Cdi-2-4 UAAU-AAAAUAGAAACGUUGAUUUAUGUUCUGUAAUGUGGGCACCUU-GGAGCAUAUUGAGUUAGU
Cdi-2-1 -CCUAUUUAUAGAAACUGUGAAGUAUAUCUUAAACC-UGGGCACUUAAAAGAUAUAUGGAGUUAGU
Cdi-2-2 -AAU-AUUUUAGAAACUGAGAAGUAUAUCUUAUUAU-UGGGCAUCUG-GAGAUAUAUGGAGUUAGU
Cdi-2-3 AAAU-AUUAUAGAGAUGUUGAAGUAUAUUCUAUUAU-UGGGCACCUUAUGGAUAUACUGAGUCAGU
           *      **** *     **  *** *  *       ******  *        ***  **** ***

          pseudoknot       P1
Cdi-2-4 GGUGCAACCGGCUAUGAAAUUG
Cdi-2-1 AGUGCAACCUGCUAUAAAUAUA
Cdi-2-2 GGUGCAACCGGCUAUGAAUAU-
Cdi-2-3 GGUGCAACCGGCUAUGAAUAUA
         ******** ***** **  *
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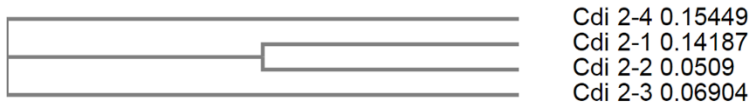
B



C

	Cdi2-4	Cdi2-1	Cdi2-2	Cdi2-3
Cdi-2-4	100.00	64.29	73.49	77.65
Cdi-2-1	64.29	100.00	80.72	72.94
Cdi-2-2	73.49	80.72	100.00	81.93
Cdi-2-3	77.65	72.94	81.93	100.00

Figure A1.18. Sequence analysis of the class II c-di-GMP riboswitches in *C. difficile*.
(A) Alignment of the sequences by Clustal Omega. Asterisks represent bases that are conserved for all four riboswitches. (B) Cladogram representing the relative sequence identity between the riboswitches. (C) Percent sequence identity for each pair of riboswitches.