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# Phase-variable expression of *pdcB*, a phosphodiesterase, influences sporulation in *Clostridioides difficile*

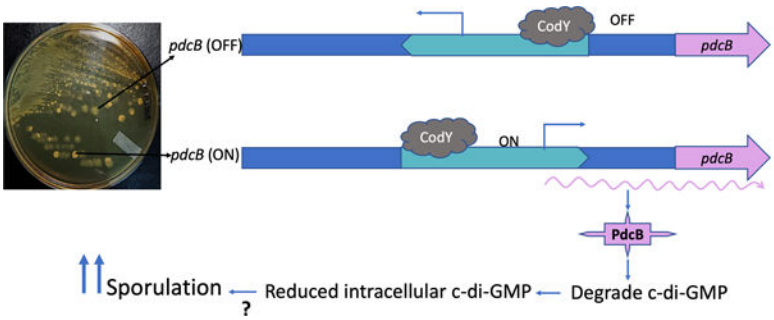
**Babita Adhikari Dhungel**, **Revathi Govind**

Division of Biology, Kansas State University, Manhattan, KS, 66506, USA

## Abstract

*Clostridioides difficile* is the causative agent of antibiotic-associated diarrhea and is the leading cause of nosocomial infection in developed countries. An increasing number of *C. difficile* infections are attributed to epidemic strains that produce more toxins and spores. *C. difficile* spores are the major factor for the transmission and persistence of the organism. Previous studies have identified global regulators that influence sporulation in *C. difficile*. This study discovers that PdcB, a phosphodiesterase, enhances sporulation in *C. difficile* strain UK1. Through genetic and biochemical assays, we show that phase-variable expression of *pdcB* results in hypo- and hyper-sporulation phenotypes. In the "ON" orientation, the identified promotor is in the right orientation to drive the expression of *pdcB*. Production of the PdcB phosphodiesterase reduces the intracellular cyclic-di-GMP concentration, resulting in a hyper-sporulation phenotype. Loss of PdcB due to the *pdcB* promoter being in the OFF orientation or mutation of *pdcB* results in increased cyclic-di-GMP levels and a hypo-sporulation phenotype. Additionally, we demonstrate that CodY binds to the upstream region of *pdcB*. DNA inversion reorients the CodY binding site so that in the OFF orientation, CodY binds a site that is upstream of the *pdcB* promoter and can further repress gene expression.

## Graphic abstract.




In UK_T translucent cells (*pdcB*-OFF), the predicted promoter region of *pdcB* is in the inverted orientation. The binding of CodY downstream further blocks any transcription of the *pdcB*. In

rgovind@ksu.edu .

Data Sharing statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

Conflict of Interests

Authors have no conflicts of interests to declare.

the UK_O opaque cells (*pdcB*-ON), because of the DNA inversion, the predicted promoter is in the right orientation and drives the expression of *pdcB*, and is also not affected by the CodY binding to the upstream. Increased production of PdcB results in decreased c-di-GMP, which in turn influences the sporulation rate.

## Abbreviated Summary

In the *C. difficile* UK1 strain, phase variation resulted in two distinct colony morphologies: transparent UK T cells and opaque UK O cells. Whole-genome sequencing of UK_T and UK_O revealed that they differ in the orientation of the DNA sequence upstream of *pdcB*, which codes for a phosphodiesterase. In UK_T cells, the predicted promoter region of *pdcB* is in the inverted orientation. The binding of CodY downstream further blocks any transcription of *pdcB*. In UK O cells, the predicted promoter is in an orientation that promotes the expression of *pdcB* and is unaffected by CodY binding to the upstream region. Increased production of PdcB results in decreased c-di-GMP, which in turn influences sporulation through a mechanism yet to be discovered.

### Keywords

*Clostridioides difficile* ; *C. difficile* ; phase variation; gene regulation

## Introduction

Phase variation is a phenomenon where two distinct phenotypes are established in a reversible manner within a clonal bacterial population. This phenomenon in bacteria long recognized and has been well studied in various bacterial pathogens. Heterogeneity within a bacterial population is readily visible as colony variation and is often associated with pathogenicity (Silverman and Simon, 1980; Jiang *et al.*, 2019; Li and Zhang, 2019; Reyes Ruiz *et al.*, 2020). Genes that are regulated by phase variation are often involved in pathogenesis; these include genes encoding fimbriae, flagella, cell surface protein, and those involved in the synthesis of capsular polysaccharides. These variable bacterial components are responsible for establishing interactions with the host, giving the bacteria an advantage against the challenging environment of the host, aiding in colonization, establishing an infection, and/or evading from host immune response (Li and Zhang, 2019; Reyes Ruiz *et al.*, 2020). Phase variation has been reported in several Gram-negative bacterial pathogens, including *Salmonella* (Silverman *et al.*, 1979; Kutsukake and Iino, 1980a; Silverman and Simon, 1980; Kutsukake and Iino, 1980b; Scott and Simon, 1982; Smith and Selander, 1990; Salehi *et al.*, 2017), *Escherichia coli* (Klemm, 1986; Eisenstein, 1988; McClain *et al.*, 1991; McClain *et al.*, 1993), *Klebsiella* (Struve *et al.*, 2008), *Neisseria* (van der Ende *et al.*, 2000; Tauseef *et al.*, 2013) and *Campylobacter jejuni* (Bacon *et al.*, 2001; Holst Sørensen *et al.*, 2012) where it has been employed to evade host immune system, increase adhesion, colonization, and virulence. While there are ample reports on phase-variable genes and their role in the pathogenesis of Gram-negative bacteria, little is known about the role of phase variation in Gram-positive pathogens. *Streptococcus pneumoniae* utilizes phase variation to switch colony morphology and to express *pili* (Li and Zhang, 2019). Phase variation has been reported more recently in *C. difficile*, where two distinct colony morphologies:

smooth- circular and rough-filamentous, were observed and were found to be associated with differences in motility and cell shapes (Anjuwon-Foster and Tamayo, 2017; Garrett *et al.*, 2019). In this study, we investigated the molecular mechanisms involved in the phase-variable sporulating (UK1_O) and non-sporulating (UK1_T) colonies observed in the *C. difficile* UK1 strain.

*C. difficile* is an anaerobic pathogen that lives in the mammalian GI tract (Edwards and McBride, 2014). *C. difficile's* growth in the GI tract is kept in check by the commensal gut microbiota. But during antibiotic treatment, a healthy bacterial population in the gut is disrupted, thereby creating an environment that is favorable for *C. difficile* to proliferate and cause infection (Edwards and McBride, 2014). *C. difficile* infection is caused primarily by the toxin produced by the bacteria, which causes inflammation of gut epithelium leading to severe diarrhea, commonly known as antibiotic-associated diarrhea, and ultimately causing fatal pseudomembranous colitis Mullish and Williams, 2018; Prete *et al.*, 2019). Each year >200,000 *C. difficile* infections (CDI) and >12,000 deaths due to CDI are reported in the United States (CDC, 2020). Due to the increased number of infections and a massive cost of health care of ~ 4.2 billion dollars, CDI has been categorized by the CDC as an urgent threat to public health (Lessa *et al.*, 2015). The increasing number of *C. difficile* infections has been partly attributed to the emergence of epidemic *C. difficile* strains that produce more toxins and spores (Denève *et al.*, 2009). *C. difficile* infections often occur as a result of spore transmission. Ingested spores make their way to the intestine, where they germinate with the help of bile salts into active vegetative cells that secrete cytotoxins, causing tissue damage and inflammation (Paredes-Sabja *et al.*, 2014). Since they are the root source of transmission, it is important to understand the regulation of sporulation in *C. difficile*. In this study, by characterizing the phase variation involving sporulating phenotypes, we have uncovered a new link between intracellular cyclic-di-GMP (c-di-GMP) concentrations and sporulation.

C-di-GMP is a common bacterial second messenger found in many bacterial species. Two classes of enzymes are important for c-di-GMP homeostasis in a bacterial cell, diguanylate cyclases (DGCs) that synthesize c-di-GMP from 2 GTP molecules and phosphodiesterases (PDEs) that degrade c-di-GMP. In *C. difficile*, c-di-GMP has been shown to negatively regulate toxin production (McKee *et al.*, 2013; Bordeleau and Burrus, 2015; Purcell *et al.*, 2017; Hall and Lee, 2018; McKee *et al.*, 2018b) and flagella-mediated swimming motility (Purcell *et al.*, 2012; Bordeleau and Burrus, 2015; Hall and Lee, 2018; McKee *et al.*, 2018b), whereas c-di-GMP positively regulates PilA-mediated swarming motility (Bordeleau *et al.*, 2015; Bordeleau and Burrus, 2015; Purcell *et al.*, 2016; Hall and Lee, 2018; McKee *et al.*, 2018b; McKee *et al.*, 2018a), biofilm formation (Purcell *et al.*, 2017; McKee *et al.*, 2018b), cell aggregation (Purcell *et al.*, 2012; Bordeleau *et al.*, 2015) and expression of cell wall anchor structures (Peltier *et al.*, 2015; Arato *et al.*, 2019). While c-di-GMP regulates a plethora of physiological processes, its influence on sporulation has not been investigated. In this study, we discovered a link between low intracellular c-di-GMP levels and a hyper-sporulation phenotype in *C. difficile* UK1. We find that decreasing c-di-GMP levels by over-expressing *pdcB*, which encodes a phosphodiesterase, increases sporulation. We also show that a *pdcB* mutant is hypo-sporulating and overexpression of the *pdcB*-EAL domain complements the sporulation phenotype. Our data shows that *pdcB* is expressed during

the mid-exponential growth phase and a DNA inversion controls its expression. We locate CodY binding sites near the promoter region of *pcdB* gene and show that a DNA inversion re-orients the predicted promoter and moves the CodY binding site to its upstream of the promoter. The rearrangement results in the expression of *pdcB,* which in turn positively influences the sporulation in *C. difficile* UK1 strain.

## Results

### *C. difficile* UK1 strain exhibits phenotypic heterogeneity with two distinguishable colony morphologies

Earlier studies have shown that *C. difficile* strain R20291 belonging to ribotype 027, associated with epidemic infections, have phenotypic heterogeneity, thereby exhibiting two distinct colony morphologies *in vitro* (Anjuwon-Foster and Tamayo, 2017; Garrett *et al.*, 2019). Consistent with these studies, we observed two distinct colony types in the UK1 strain during routine plating on TY agar (Fig. 1A). One colony type has a smooth edge and is round, and is termed “opaque” (UK1_O), while the other is irregular with a rough edge and is termed as “translucent” (UK1_T). Under the phase-contrast microscope (Fig.1B), UK1_T cells were longer with an average length of 9.8 μm while the UK1_O cells are significantly shorter with an average length of 4.9 μm (Fig. 1C). To measure the distribution of UK_O and UK_T in a population, we prepared spores from the UK_O colonies and plated them on TY agar. We did not use any germinant in the TY agar plates, since we did not know the effect of germinants on phase variation. We observed that nearly 78% of the colonies appeared after overnight (~20h) incubation were UK_O phenotype, while the remainder were UK_T type colonies. To check whether each phenotype is heritable, we isolated UK1_T and UK1_O colonies and streaked them on TY agar plates. When a single opaque colony was streaked in TY agar and incubated overnight, 100% of the colonies formed were opaque in nature. Upon prolonged incubation (> 72h) however, nearly 70% of the opaque colonies in the TY plate started exhibiting rough edges with the pale yellowish center over time suggesting that opaque colonies could give rise to translucent colonies. When a single UK1_T colony was streaked on to TY agar and incubated overnight, the majority of the colonies remained translucent, but about 2% of the colonies were opaque (Fig. S1A). Nearly 20% of the UK_T colonies gave rise to UK_O colonies after 72h of incubation. Similar observations were made with the broth cultures. Cells from opaque colonies grown in TY broth overnight stayed shorter, but cells from translucent colonies grown in TY broth overnight (~16–20h) produced primarily long cells with some short cells. Serial dilutions made from broth cultures were plated on TY agar to check for colony morphologies upon overnight incubation. UK_T broth cultures produced ~7% opaque colonies, while UK_O broth culture resulted in 100% opaque colonies (Fig. S1B). After 72h, UK_T broth cultures gave rise to ~70% opaque colonies and UK_O broth culture produced nearly 75% opaque colonies. These results together suggest that the phase variation phenotypes are heritable and are stable at least for 20 hours. It also suggests that bacteria in broth culture favor opaque morphology over the translucent phenotype.

## UK1_T and UK1_O colony morphologies exhibit distinct sporulation and surface motility phenotypes

In order to determine the physiological characteristics of the two colony types, we assayed UK1_T and UK1_O colony types for sporulation, toxin, and motility. To determine the sporulation phenotype, we streaked the isolated UK1_T and UK1_O colonies on solid TY medium, and we found that UK1_O colonies produced significantly more spores compared to UK1_T at 20 h (Fig. 1B, Fig. 2A). We also found that UK1_T and UK1_O produce similar level of cytoplasmic toxins at 16 h of growth (Fig. S2B). While UK1_T and UK1_O exhibited similar swimming motility even after 72h in 0.5% agar TY medium (Fig. S1A), we found that UK1_T exhibited enhanced surface motility compared to the UK1_O when grown in TY medium with 1.8% agar at 72h (Fig. 2B). Similarly, UK1_T formed thick biofilms compared to UK1_O (Fig. 2C). Together, these data demonstrate that UK1_T and UK1_O are phenotypically different in sporulation, swarming motility, and biofilm formation. While the swarming motility and the biofilm formations were previously reported to be associated with phase variation in *C. difficile*, sporulation was not (Garrett *et al.*, 2019).

## UK1_O strain undergoes phase variation by DNA inversion

To determine the genetic basis for the phenotypic heterogeneity that gave rise to translucent and opaque colonies in the UK1 strain, we extracted genomic DNA from UK1_T and UK1_O cells harvested from the overnight cultures grown in TY agar (supplementary methods) and performed whole-genome sequencing at Tufts University Core Facility Genomic core. The Sequenced genome was assembled against the closely related and annotated R20291 genome (Stabler *et al.*, 2009). Contrary to our expectation, the sequencing result did not show mutations in any of the known sporulation-associated genes. However, the intergenic region between *CDR20291_0684* and *CDR20291_0685* genes was listed among unassigned new junction evidence.

The intergenic region between CDR20291_0684 and CDR20291_0685 carried inverted repeat sequences that are known to undergo inversion (Sekulovic *et al.*, 2018). To determine the orientation of this sequence in UK_T and UK_O, we amplified and cloned ~1.5 kb intergenic region in a cloning vector and sent 10 clones from each for sequencing. We aligned the sequences of UK1_T and UK_O to the R20291 genome (Stabler *et al.*, 2009) and analyzed with NCBI BLASTn. The results suggested that all clones from UK1_T perfectly aligned with the published sequence of the R20291. In contrast, all the clones from UK1_O had mismatches in the ~200 bp segment of the intergenic region (Fig. S3), showing DNA inversion in the region.

## DNA inversion regulates the expression of the downstream gene *CDR20291_0685*

DNA inversion is known to regulate the expression of the downstream gene as it gives rise to two orientations where the “ON” orientation enhances, and the “OFF” orientation represses the expression of the downstream gene or operon (Silverman and Simon, 1980; Zieg and Simon, 1980; Van de Putte and Goosen, 1992; Jiang *et al.*, 2019). We performed a reporter fusion assay to determine the effect of DNA inversion on the expression of the downstream *CDR20291_0685* gene. We fused ~1.5 kb of the upstream DNA segment

of *CDR20291_0685,* containing the invertible region from both the UK1_T and UK1_O cells, with a *gusA* reporter gene coding beta-glucuronidase, to create plasmid constructs pBA042 and pBA045 (Table S1). Using site-directed mutagenesis, we mutated the inverted repeat sequences that flanked the invertible region to lock the upstream region either in the translucent (pBA043) or opaque (pBA046) orientation. The plasmid constructs were then introduced into the UK1 undifferentiated parent strain by conjugation. The strains were grown anaerobically in TY broth medium at 37°C and were harvested at different time points to perform the reporter gene expression assay. We observed significantly higher beta-glucuronidase activity at each time point with the reporter fusion that was locked in opaque orientation (Fig. 3A). Very minimal reporter activity was recorded with the reporter fusion that was locked in the translucent orientation. This observation is consistent with our qRT-PCR results, where we detected elevated levels of *CDR20291_0685* transcript in UK1_O when compared to that from UK1_T (Fig. 3B). Taken together, these results suggest that DNA inversion in the upstream region of the *CDR20291_0685* gene resulted in upregulation of its expression in the opaque UK1_O colonies. *CDR20291_0685* is the orthologous gene of *CD630_07570* in strain CD630. CD630_07570 hydrolyzes c-di-GMP to pGpG *in vitro* and therefore is a phosphodiesterase (Bordeleau *et al.*, 2011). Therefore, we named CDR20291_0685 as PdcB (Phosphodiesterase of *C. difficile* B). To emphasize the *pdcB's* ON and OFF orientations, we refer to UK1_O as *pdcB* (ON) and UK1_T as *pdcB* (OFF), here after.

## Promoter of *pdcB* lies within the invertible region

The mechanism by which DNA inversion regulates the expression of the gene depends upon its location. If DNA inversion occurs in the region that contains the promoter, it will disrupt the promoter, and the gene is not expressed (Zieg and Simon, 1980; Eisenstein, 1988). If the promoter is upstream of the switch, then an intrinsic terminator can be formed to terminate the transcription of the gene (Sekulovic *et al.*, 2018). To determine the mechanism by which this inversion regulates the gene expression, we sought to identify the promoter of *pdcB*. We hypothesized that the promoter of *pdcB* is within the invertible region. We extracted total RNA from a 4h culture of *pdcB* (ON) cells and synthesized *pdcB* specific cDNA. We carried out PCR using this cDNA as a template, with several primers spanning the inverted repeat sequence (Fig. 4A). Only the primer pairs ORG921/ORG853, and ORG922/ORG853 gave amplification products, suggesting that the promoter region is located around the region spanned by the primer ORG921 (Fig. 4B). Consistent with this observation, we mapped the transcription start of *pdcB* using 5'RACE. Transcription is initiated 874-bp upstream of the *pdcB* translational initiation codon and is located next to the right repeat sequence (Fig. 4A). Hexamers −10 (TATTT) and −35 (TTGATA) separated by an −17-bp spacer are found upstream of the transcriptional start site.

## Overexpression of *pdcB* results in reduced c-di-GMP levels in UK1

To determine if overexpression of *pdcB* alters the levels of intracellular c-di-GMP in *C. difficile*, we first created UK1::*pdcB* mutant strain using ClosTron mutagenesis (Fig. S4) and extracted and measured the levels of intracellular c-di-GMP from *pdcB* (OFF), *pdcB* (ON) and UK1::*pdcB* strains. Our data suggest that *pdcB* (OFF) and UK1::*pdcB* strains have higher levels of c-di-GMP while *pdcB* (ON) cells has a significantly reduced c-di-GMP

level (Fig. 5B). This result indicates that DNA inversion leads to overexpression of the *pdcB* gene, which encodes an enzyme that cleaves c-di-GMP and reduces the global intracellular concentration of c-di-GMP in the *pdcB* (ON) cells. Similar to *pdcB* (OFF) cells, UK1::*pdcB* mutant also produced translucent colonies and longer cells (Fig 5A), suggesting that these phenotypes are directly related to intracellular levels of c-di-GMP.

## Sporulation in *C. difficile* UK1 is correlated with the intracellular levels of c-di-GMP

Intracellular c-di-GMP homeostasis is maintained by two classes of enzymes, diguanylate cyclases that synthesize c-di-GMP and phosphodiesterases that hydrolyze c-di-GMP. Phosphodiesterases consist of either the EAL domain or an inactive GGDEF and active EAL domain (Hall and Lee, 2018). The PdcB phosphodiesterase contains both the GGDEF and EAL domains, and *in vitro* assays have shown PdcB can cleave c-di-GMP (Bordeleau *et al.*, 2011). Our results so far showed that DNA inversion upstream of *pdcB* regulated its expression and hence controlled the intracellular levels of c-di-GMP in *C. difficile* UK1, which in turn resulted in two phenotypic variants, translucent and opaque colonies. We also observed a significant difference in the frequency of sporulation between the variants. To determine if the intracellular levels of c-di-GMP are associated with a sporulation phenotype, we quantified the percentage sporulation in *pdcB* (OFF), *pdcB* (ON), UK1::*pdcB* mutant, and UK1::*pdcB* mutant complemented with plasmid-encoded *pdcB*-EAL under a tetracycline-inducible promoter. Despite multiple attempts, we were not able to complement the UK1::*pdcB* mutant strain with a full-length plasmid-encoded *pdcB*. Other studies have shown that complementation with just the EAL domain with the phosphodiesterase activity of *pdcA* is sufficient to rescue the associated motility phenotypes (Purcell *et al.*, 2017). Since our objective was to determine the role of intracellular levels of c-di-GMP in regulating sporulation in *C. difficile* UK1 strain, we performed the sporulation assays with the mutant strain complemented with the plasmid that encodes just the PdcB-EAL domain. Percentage sporulation data showed that the hypo-sporulating phenotype in UK1::*pdcB* strain was partly complemented when the PdcB-EAL was produced in the mutant strain (Fig. 5C). To further confirm that the sporulation phenotype is associated with intracellular levels of c-di-GMP, we complemented the UK1::*pdcB* mutant with the plasmid-borne PdcA-EAL domain. PdcA is another well-characterized phosphodiesterase of *C. difficile*, and the conserved GGDEF and EAL domains of PdcB are homologous with those of PdcA (Purcell *et al.*, 2017). We observed that the sporulation phenotype was complemented with the PdcA-EAL domain as well. We also expressed *dccA* coding for the diguanylate cyclase from the *tet* inducible promoter to artificially increase the concentration of c-di-GMP and monitored the sporulation percentage upon the induction of *dccA*. As expected, the production of DccA significantly reduced the sporulation in the UK1 strain (Fig. 5C). Measuring c-di-GMP in strains producing PdcA-EAL or PdcB-EAL or DccA confirmed their enzymatic effect on c-di-GMP concentration (Fig. 5B). The intracellular level of c-di-GMP is known to positively regulate surface motility in *C. difficile* (Bordeleau *et al.*, 2015). Consistent with previous studies, we observed increased surface motility in the *pdcB* (OFF) strain, which has increased intracellular c-di-GMP levels compared with the *pdcB* (ON) strain. So, to further confirm that complementation of the UK1::*pdcB* mutant with the PdcB-EAL and PdcA-EAL domains reduces the intracellular c-di-GMP, we carried out a surface motility test assay with these strains (Fig. S6). As expected, we observed decreased surface motility

in the complemented strains similar to the *pdcB* (ON) strain, which has reduced intracellular c-di-GMP levels. It is also interesting to note that the UK1::*pdcB* mutants producing either PdcB-EAL and PdcA-EAL were more opaque, less translucent and produced fewer longer cells (Fig. 5A). In addition to these experiments, we also silenced the *pdcB* gene using the CRISPRi system ((Müh *et al.*, 2019), where the *pdcB* specific gRNA was expressed from a xylose-inducible promoter and checked its effect on sporulation. Silencing the *pdcB* gene significantly reduced sporulation in the UK1 strain (Fig. S5). While this manuscript is under revision, a preprint also reported that increased concentration of c-di-GMP leading to decreased sporulation in *C. difficile* R20291 and 630erm strains (Edwards *et. al.*, 2021). Taken together, these data strongly suggest that the intracellular levels of c-di-GMP influence sporulation in *C. difficile*.

## CodY binds to the upstream region and represses the expression of *pdcB*

Several studies have shown CodY-mediated regulation of c-di-GMP signaling proteins. Microarray analysis in the CD630 strain has identified two cyclic di-GMP signaling proteins, *CD630_07570* (*pdcB*) and *CD1476*, among the 140 genes that were regulated by CodY (Dineen *et al.*, 2010). The study demonstrated enhanced expression of both of these proteins in the *codY* mutant and identified a consensus CodY binding site in the upstream regions of *CD1476* and *CD630_07570 (pdcB)*. Another study has shown that CodY binds directly to the promoter region and represses the expression of *pdcA*, a phosphodiesterase that cleaves c-di-GMP in the JIR8094 strain (Purcell *et al.*, 2017). To determine if the expression of *pdcB* is controlled by CodY, we first determined whether a CodY binding consensus sequence is present in the upstream region of *pdcB*. The two potential CodY binding sites upstream of *pdcB* are TATTTATTGAAAATTT and ACTTTTCTAAAATTA within the region that undergoes inversion (Fig. 6A). CodY binding site in the opaque orientation is upstream of the predicted promoter region in the *pdcB* (ON) orientation, which can explain the higher expression of *pdcB* in this phenotype. We hypothesized that CodY binding would completely shut down even the low-level expression of *pdcB* in the *pdcB* (OFF) strain. DNA inversion in the *pdcB* (ON) strain would shift the CodY binding site to the upstream region of the predicted promoter, leading to the expression of *pdcB*.

To determine if CodY regulates the expression of *pdcB*, we carried out the reporter fusion assay. We used the same reporter fusions (pBA040, pBA043, pBA046) that were used earlier in this study. Each of the constructs was introduced into the UK1 undifferentiated parent strain and UK1::*codY* mutant strain by conjugation. The strains were grown anaerobically in TY broth medium at 37°C, and cells were harvested at different time points to perform the reporter assay. We observed increased beta-glucuronidase activity at each time point when it was expressed from the *pdcB* upstream locked in opaque orientation, with higher expression at mid-exponential phase from 4–12 h for both UK1 parent and UK1::*codY* mutant strains (Fig. 6B). Minimal reporter activity was observed with the upstream locked in translucent orientation. However, in the absence of CodY, an approximately 1.5-fold increase in the expression could be seen from the promoter locked in the translucent orientation, suggesting the presence of a second but much weaker promoter upstream of the CodY binding site. It is also possible that the purpose of DNA inversion is not only to position the promoter in the right orientation for expression, but also to move the potential CodY binding site

upstream to prevent the CodY-mediated repression. Since, the left inverted repeat (LIR) in *pdcB* upstream is next to the CodY binding site, CodY may also interfere with the binding of the recombinase from recognizing the repeat and can block inversion. This hypothesis, however needs further experimental evidence.

To further confirm the interaction of CodY with *pdcB* upstream DNA, we carried out an Electrophoretic Mobility Shift Assay (EMSA) with purified CodY protein. We synthesized the DNA fragment of 47 bp containing the potential CodY binding site and radio-labeled with $\gamma$- $^{32}$ P. The fragment was bound with increasing concentrations of purified CodY, both in the absence and presence of its cofactors ILV (Branched-chain amino acids isoleucine, leucine, and valine) and GTP (Fig. 6C). The shift in the bands at higher concentrations of CodY protein suggests that purified CodY binds to the DNA fragment, and the binding was enhanced in the presence of its cofactors GTP and ILVs.

## Discussion

The major objective of this study was to determine the role of phase variation in the physiology of *C. difficile* UK1 strain. Genome-wide analysis had previously identified seven different types of invertible sites in the *C. difficile* genome, designated as Cdi1 to Cdi7 (Sekulovic *et al.*, 2018). The Cdi2 site is upstream of *pdcB*, which encodes a c-di-GMP phosphodiesterase (PDE) (Sekulovic *et al.*, 2018). In this work, we have shown the role of Cdi2 in regulating the expression of *pdcB*. Our study identified an “ON” orientation to the *pdcB* promoter region that induces *pdcB* transcription; over-expression of *pdcB* reduces c-di-GMP levels, whereas decreased *pdcB* expression due to the “OFF” promoter orientation increases c-di-GMP levels. Notably, even though *C. difficile* has 18 predicted PDEs (Bordeleau *et al.*, 2011), expression of *pdcB* is sufficient to affect intracellular c-di-GMP levels. While c-di-GMP regulates a plethora of physiological processes in *C. difficile*, its role in sporulation had not been previously described. Only a few studies have investigated the role of c-di-GMP in sporulation. Recently, the relationship between c-di-GMP and sporulation has been explored in *Streptomyces venezuelae*, a Gram-positive soil bacterium, where low levels of c-di-GMP resulted in hyper sporulation phenotype (Hengst *et al.*, 2010; Tschowri *et al.*, 2014; Al-Bassam *et al.*, 2018; Latoscha *et al.*, 2019; Liu *et al.*, 2019; Gallagher *et al.*, 2020) . Gallagher *et al.* reported that c-di-GMP bridges the binding of anti-sigma factor RsiG with sigma factor WhiG, strengthening their interaction and preventing sigma factor WhiG-dependent transcription of late sporulation genes (Gallagher *et al.*, 2020). Similarly, another study demonstrated that c-di-GMP is required to control the transition from vegetative to reproductive cells (Bush *et al.*, 2015). It was reported that c-di-GMP was needed to form the active dimeric form of BldB, the master regulator of cell development, and represses the development of reproductive hyphae and keeps *Streptomyces* in their vegetative state (Hengst *et al.*, 2010; Bush *et al.*, 2015; Al-Bassam *et al.*, 2018; Gallagher *et al.*, 2020). These studies suggest that high c-di-GMP level traps *Streptomyces* in vegetative growth, and low c-di-GMP levels cause precocious hyper sporulation. It is however, important to note that sporulation in *Streptomyces* is different from endospore formation in *Bacillus* and *Clostridia*.

More recently, proteins involved in c-di-GMP turnover were localized as part of spore proteomes and were found to play a role in spore germination in *Bacillus anthracis* (Hermanas *et al.*, 2021). Single-cell microscopy studies carried out in *B. subtilis* have shown a positive correlation of high c-di-GMP levels with sporulation (Weiss *et al.*, 2019). Our current work is the first study to directly correlate intracellular c-di-GMP concentration to sporulation in *C. difficile*. Hyper sporulation was observed when the intracellular c-di-GMP concentration was reduced by ~1.5 fold in *pdcB* (ON) compared to the level in *pdcB* (OFF) cells grown *in vitro*. It is noteworthy that, in our previous study, R20291::*sinRR'* mutant strain, which is asporogenic, had elevated levels of c-di-GMP compared with the R20291 parent strain (Girinathan *et al.*, 2018), corroborating our current observation. By analyzing percentage sporulation in the UK1::*pdcB* mutant strain and PdcB-EAL and PdcA-EAL producing UK1::*pdcB* strains, we have shown that production of a phosphodiesterase that reduces c-di-GMP concentration is positively associated with sporulation phenotype. However, overexpression of EAL domains of both PdcB and PdcA only partially complement the sporulation phenotype. This could be because of the overexpression of the PdcB-EAL domain alone without the associated GGDEF domain is not sufficient. Although the GGDEF domain of PdcB-EAL is catalytically inactive (Sekulovic *et al.*, 2018), it might be necessary to enhance the activity of the EAL domain to fully complement sporulation. In homologous proteins which contain both GGDEF and EAL domains, catalytically inactive GGDEF domains are capable of binding to GTP, thus enhancing the PDE activity of the neighboring EAL domain (Christen *et al.*, 2005).

Regulation of *C. difficile* sporulation can occur either at the transcriptional level by altering the expression of *spo0A* or at the post-translational level by interfering with the phosphorylation of Spo0A (Edwards and McBride, 2014). There are two known mechanisms by which c-di-GMP is known to function. First, c-di-GMP mediated regulation can occur through RNA-based effectors, which includes direct binding of c-di-GMP to riboswitches present in the 5' UTR of the target gene transcript leading to premature termination of the transcript. Second, c-di-GMP mediated regulation can occur through protein effectors containing the PilZ domain, GMP binding domain, diguanylate cyclases containing I-sites, GIL proteins, MshEN domains, and the Cle subfamily of CheY proteins which sense and respond to the changes in the intracellular c-di-GMP concentration (McKee *et al.*, 2018b). Whether c-di-GMP binding riboswitches or c-di-GMP binding domains are present in the histidine kinases that phosphorylate Spo0A, and whether c-di-GMP regulates their expression or activity, thus regulating sporulation needs to be further investigated. On the other hand, c-di-GMP could indirectly influence sporulation by affecting the expression of regulators known to affect the expression of *spo0A*.

Previous studies have shown that the *C. difficile* UK1::*codY* mutant exhibits a hyper-sporulation phenotype suggesting that CodY represses sporulation (Nawrocki *et al.*, 2016). The exact mechanism by which CodY regulates sporulation is not understood. However, studies have shown that CodY directly regulates the expression of *sinRI* (Girinathan *et al.*, 2018) and *opp* and *app* (Edwards *et al.*, 2014; Nawrocki *et al.*, 2016), which are positive regulators of sporulation. Results in this study suggest that phosphodiesterases can play a role in regulating sporulation. This could be indirectly by controlling CodY activity, which is directly regulated by intracellular GTP concentration.

While we were working on characterizing *pdcB* role in UK1 phase variation, another study reported the role of *cmrRST* on phase variation in *C. difficile* R20291 strain (Garrett *et al.*, 2019). Similar to the *pdcB* gene, the *cmrRST* operon carries a DNA inversion element belonging to *cdi6* group. CmrR and CmrS make up a two-component signal transduction system coding for a response regulator and a histidine kinase, respectively. To check any connection between *cmrRST* and phase variation in the UK1 strain, we checked the orientation of the *cmrRST* upstream region in the *pdcB* (ON) and *pdcB* (OFF) cells. In the R20291 strain, the rough colonies (equivalent to the translucent colonies of UK1 where *pdcB* is in the OFF orientation), the *cmrRST* operon is in the ON orientation, and the smooth colonies (equivalent to the opaque colonies of UK1 where *pdcB* is in ON orientation) resulted when the *cmrRST* is in the OFF orientation (Garrett *et al.*, 2019). In the UK1 strain, however, we observed the opposite. The *cmrRST* operon was in the ON orientation in *pdcB* (ON) colonies. In the *pdcB* (OFF) colonies, the PCR product indicating the *cmrRST* OFF orientation amplified at higher frequency compared to the product indicating ON orientation (Fig. S7). Even though the frequency of *cmrRST* being ON/OFF in rough and smooth R20291 cells differs from that of the UK1_O and UK_T cells, there are similarities between the UK1 and R20291 phase-variable colonies. In the *cmrRST* study, the authors reported that the rough colonies in R20291 (equivalent to translucent *pdcB* (OFF)) had the Cdi2 region (i.e. upstream of *pdcB*) in the OFF orientation. Greater levels of intracellular c-di-GMP in the R20291 strain significantly increased cell length and resulted in the formation of rough colonies, similar to what we noticed in *pdcB* (OFF) cells with higher levels of c-di-GMP. Thus, both the *cmrRST* study and ours reveal that c-di-GMP plays a role in creating these phase-variable *C. difficile* colonies.

Site specific recombinases that carry out DNA inversion might be associated with the invertible site or encoded elsewhere in the genome (van de Putte *et al.*, 1980). Prior works with *C. dfficile* R20291 strain has shown that RecV, the tyrosine recombinase, catalyzes the inversion of the Cdi1, Cdi4, Cdi6 switches, partial inversion of Cdi2, Cdi3, Cdi5 and no effect on Cdi7 inversion (Sekulovic *et al.*, 2018). To determine if RecV has a role in inversion of Cdi2 in UK1 strain we constructed a UK1::*recV* mutant using ClostTron mutagenesis (Fig. S8AB). If RecV had a role in DNA inversion of Cdi2, we would expect UK1::*recV* mutant to exist in both the colony UK1_T and UK1_O colony types that are locked in their respective morphotypes in the absence of RecV. However, phase-variable colonies could not be distinguished in UK1::*recV* mutant (Fig. S8C). The orientation PCR using UK1::*recV* colonies detected *pdcB* in both ON and OFF orientation (Fig. S8D). This finding suggested that RecV may be only partially involved in the inversion of the region, which can result in bacterial cells with both ON and OFF orientation to present in a single colony. It is also plausible that a site-specific recombinase other than RecV is involved in the upstream inversion of *pdcB* in the UK1 strain.

In summary, our study has shown phase variation-mediated regulation of intracellular c-di-GMP homeostasis and its effect on *C. difficile* physiology. Although both translucent and opaque cells are shown to produce similar levels of toxins, they differ drastically in cell length and sporulation. Based on the swarming motility and biofilm formation phenotypes, it can be suggested that during initiation of infection, UK1 might prefer to exist as the translucent colony cells so that they can better colonize the host and cause infection.

Under the selective pressure exerted in the intestinal tract and host immune responses, the UK1 opaque colony type might predominate. This might result in the production of more spores allowing *C. difficile* to evade the host immune response and persist in the host, to cause recurrent infection, or in the environment, once it is released outside of the infected individual. Understanding the importance of phase-variable expression of *pdcB* and its associated phenotypes in an *in vivo* infection model is a priority for future investigation and will shed light on the importance of *pdcB* in *C. difficile* pathogenesis.

## Material and Methods

### Bacterial strains and growth conditions

*C. difficile* UK1 strains (Table S1) were grown in TY (tryptose and yeast extract) medium agar or broth culture in an anaerobic chamber maintained at 10% $H_2$, 10% $CO_2$, and 80% $N_2$. Transconjugants were selected and grown in TY agar with lincomycin (Linc 20μg/ml) or thiamphenicol (Thio; 15 μg/ml) or both. An *E. coli* strain optimized for conjugation S17–1 was used for conjugation (Teng *et al.,* 1998), and the *E. coli* DH5α strain was used for cloning. *E. coli* strains were cultured aerobically in LB (Luria-Bertani) supplemented with ampicillin (100 μg/ml) or chloramphenicol (25 μg/ml) when necessary.

### Phase-contrast microscopy

*C. difficile* strains were grown in TY medium as described above. One ml of overnight culture was centrifuged at 17,000g for 1 min and washed with 30μl of sterile PBS. The resulting pellets were fixed for 2 hours at room temperature using 2% paraformaldehyde in 1X PBS. A thin layer of 0.7% agarose was applied to the surface of a microscopic slide, and 2μl of concentrated culture was placed on it. The cells were imaged using Zeiss LSM-5 PASCAL (objective lens 100x/1.4 oil). The LSM 5 service pack was used to acquire the images of at least three fields for each strain.

### Sporulation assay

Cells grown in TY agar overnight were used for sporulation assay. Bacterial strains carrying plasmids were grown in the presence of thiamphenicol (15 μg/ml). ATc at a concentration of 100 ngs/ml was used to induce the expression of genes cloned under the *tet* inducible promoter. To enumerate the number of viable spores, cells were harvested using 1 ml of TY broth, and 500μl of the samples from each culture were mixed 1:1 with 95% ethanol and incubated for 30 minutes at room temperature to kill all the vegetative cells. The ethanol-treated samples and the untreated samples were then serially diluted, plated on TY agar with 0.1% taurocholate, and incubated at 37°C for 24 to 48 hours. The percentage of ethanol-resistant spores was calculated by dividing the number of CFU from spores by the total number of CFU and multiplying the value by 100. The results were based on a minimum of three biological replicates.

### RNA isolation, reverse transcriptase (RT-PCR) and quantitative reverse transcriptase PCR (qRT-PCR).

*C. difficile* cultures were grown in TY medium, and 6 ml of cells were harvested at different time points by centrifugation at 3000g for 30 min at 4°C. Total RNA was extracted from

the harvested cells following the protocol described previously (El Meouche *et al.*, 2013). Total RNA was treated with DNase (Turbo; Ambion) for 2 hours at 37°C. The cDNA was synthesized using 5μg of total RNA at 42°C for 2 h using avian myeloblastosis virus (AMV) reverse transcriptase (Promega). A 20 μL reaction containing 10 ng or 10 pg (for 16S rRNA) of cDNA, 400 nM gene-specific primers, and 12.75 μL of SYBR PCR master mix (BioRad) was used to perform real-time quantitative PCR using iQPCR instrument (BioRad). Amplification and detection were performed as described previously (El Meouche *et al.*, 2013). The quantity of cDNA of a gene in each sample was normalized to the quantity of *C. difficile* 16S rRNA gene, and the ratio of normalized target concentrations (threshold cycle [$2^{-\Delta\Delta Ct}$] method) (Saujet *et al.*, 2011; El Meouche *et al.*, 2013) gives the relative change in gene expression. The cDNA prepared was also used as a template to perform reverse transcriptase PCR to detect *pdcB* transcripts using forward primers ORG925, ORG926, ORG921, ORG922, along with the reverse primer ORG 853 (Table S2).

## 5'RACE

5′RACE (rapid amplification of cDNA ends) assays were performed with total RNA extracted from *pdcB* (ON) using a 5′RACE System kit (Sigma Aldrich), according to the instructions provided by the manufacturer. Gene-specific RT primer (SP1) and PCR primer (SP2) were designed as recommended. The 5'-RACE product was used as a template for a nested PCR reaction using a second primer (SP3). The sequences of primers used in the RACE analysis are shown in Table S2. PCR products were cloned in pGEM-T vector and were sequenced.

## Mutagenesis of *CDR20291_0685* upstream region

Quick Change Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) was used to carry out site-directed mutagenesis whereby the Left Inverted Repeat (LIR) TAGTTGTAAAAGGGTT that flanked the DNA region that undergoes inversion was converted to TACACATGCGAGGGTT. The mutagenic oligonucleotide primers used are listed in Table S2.

## Construction of reporter plasmids and beta-glucuronidase assay

The 1.5 kb upstream DNA regions of *CDR20291_0685* were amplified by PCR using specific primers with KpnI and SacI (Table S2) recognition sequences. UK1_T and UK1_O chromosomal DNA were used as a template to amplify the upstream region in respective orientations. The upstream region was cloned in the pRPF185 shuttle vector using standard cloning procedures. Plasmid pRPF185 carries a *gusA* gene for beta-glucuronidase under the tetracycline-inducible (*tet*) promoter (Fagan and Fairweather, 2011). The *tet* promoter was removed using KpnI and SacI digestion and was replaced with the *CDR20291_0685* upstream region to create plasmids pBA042 and pBA045 (Table S1). The inverted repeats in the upstream region were mutagenized as described earlier to create plasmid pBA043 containing the upstream region locked in translucent orientation and plasmid pBA046 containing the upstream region locked in opaque orientation. The control plasmid pBA040 with promoter-less *gusA* was created by digesting with KpnI and SacI to remove the *tet* promoter and then self-ligating after creating blunt ends. Plasmids were introduced into *C. difficile* strains through conjugation. The transconjugants were grown overnight in TY

medium in the presence of thiamphenicol (15 μg/ml). Overnight cultures were used as inoculum at a 1:100 dilution to start a new culture. Bacterial cultures were harvested at different time points of growth, and the amount of beta-glucuronidase activity was assessed as described elsewhere (Mani *et al.*, 2002). Briefly, the cells were washed and resuspended in 1 ml of Z-buffer (60 mM $Na_2HPO_4.7H_20$ pH 7.0, 40 mM $NaH_2PO_4.H_2O$, 10 mM KCl, 1mM $MgSO_4.7H_2O$, and 50mM β-ME), and lysed by homogenization. The lysate was mixed with 160 μl of 6mM p-nitrophenyl β-D-glucuronide (Sigma) and incubated at 37°C. The reaction was stopped by the addition of 0.4 ml of 1.0 M $NaCO_3$. β-Glucuronidase activity was calculated as described earlier (Dupuy and Sonenshein, 1998; Mani *et al.*, 2002).

## Electrophoretic mobility shift assay (EMSAs)

For the CodY binding experiment, the upstream region of the *pdcB* with the predicted CodY binding sequence 5' CATAGATAATTTTTAGAAAAGTATCTAAATTTTCAATAAATAGTAAC 3' was synthesized. It was labeled with [γ- 32 P] dATP-6000 Ci/mmol (PerkinElmer Life Sciences) using T4 polynucleotide kinase. It was then annealed with the complementary oligo to generate a double-stranded DNA probe. The DNA-protein binding reactions were carried out at room temperature for 30 min in 10μl volume containing 1x binding buffer [10mM Tris pH 7.5, 50mM KCl, 50μg BSA, 0.05% NP40, 10% glycerol, 10 mM GTP and 2mM ILV, 100 μg/ml poly dI-dC and 800nM of DNA probe with varying concentration of purified CodY protein. DNA probe in reaction buffer was incubated for 10 min at room temperature before adding purified CodY-6His protein. The reaction was stopped by adding 5μl of gel loading buffer and electrophoresed at 100V for 1.5 h using 6% 1XTBE gel in 0.5X TBE buffer containing 10 mM ILV. Gels were then dried, and autoradiography was performed with Molecular Dynamics Phosphor-Imager technology.

## Construction and complementation of *C. difficile* UK1::*pdcB* mutant strains

The UK1::*pdcB* mutant in the UK1 strain was created using the ClosTron gene knockout system as described previously (Heap *et al.*, 2010). Briefly, for *pdcB* disruption, the group II intron insertion site between nucleotides 840 and 841 in the *pdcB* gene in the sense orientation was selected using a web-based design tool called the Perutka algorithm. The designed retargeted intron was cloned into pMTL007-CE5 as described previously (Govind and Dupuy, 2012; Girinathan *et al.*, 2016). The resulting plasmid pMTL007-CE5::*pdcB*-840–841s was transferred into *C. difficile* UK1 cells by conjugation. The potential Ll.ltrB insertions within the target genes in the *C. difficile* chromosome were conferred by the selection of lincomycin-resistant transconjugants in 20 μg/ ml lincomycin plates. PCR using gene-specific primers (Table S2) in combination with the EBS-U universal was performed to identify putative *C. difficile* mutants. *C. difficile pdcB* mutants were complemented by introducing pBA048, which contains the PdcB-EAL domain, and pBA050, which contains the PdcA-EAL domain under the *tet* inducible promoter, through conjugation.

### Cyclic-di-GMP measurement

c-di-GMP was measured as described previously (Girinathan *et al.*, 2017), with a few modifications. Briefly, *C. difficile* strains were grown in 50 ml TY medium for 16 h. $OD_{600}$ was recorded and dilution plating was carried out to determine the number of CFU. Cells were centrifuged and pellets were washed once with TE buffer (10 mM Tris [pH 7.5], 1 mM EDTA, pH 8). Pellets were resuspended in extraction solvent consisting of methanol: acetonitrile: distilled water in 40:40:20 ratio plus 0.1 N formic acid. The mixture was incubated in −20°C for 30 min. The extract was harvested by centrifugation. Samples were sent to Kansas University Biochemistry Core facility to be analyzed by high-pressure liquid chromatography (HPLC)-tandem mass spectrometry (MS/MS). To determine the amount of intracellular c-di-GMP, a standard curve was first obtained by analyzing the serial dilution of pure c-di-GMP (Sigma-Aldrich). The peak intensity of each sample was fitted to the standard curve and the value was divided by the total intracellular volume of bacteria extracted. To determine the total intracellular volume of bacteria, the volume of one cell was multiplied by the number of cells extracted, which was based on CFU counts. The volume of one cell was estimated as a cylinder and was determined by measuring the radius and length of the bacterial cell from the electron micrographs.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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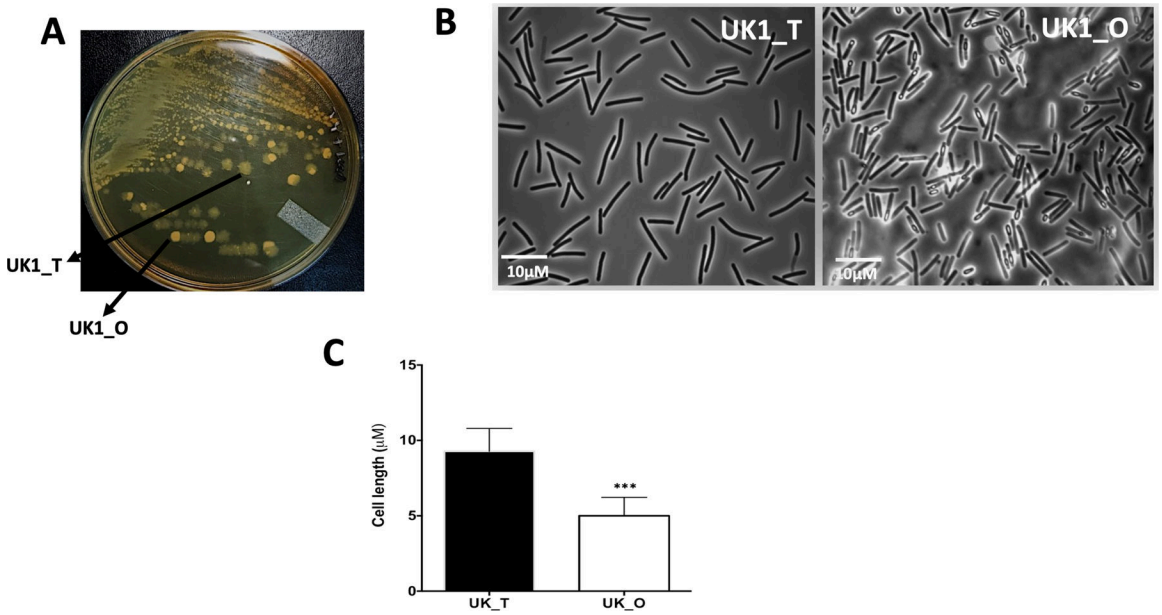


**Figure 1. Colony and cell morphologies of UK1_T and UK1_O strains.**

**A.** UK1 strain when grown in TY medium gives rise to phenotypically distinguishable Translucent (UK1_T) and Opaque (UK1_O) colony morphotypes. **B.** Phase-contrast microscopic images of UK1_T and UK1_O colony morphotypes. **C.** Average cell length showing UK1_T cells are significantly longer than UK1_O cells. Twenty-five individual cells per image were measured using ImageJ software. At least four images from two replicates were used (n=100). Data was analyzed using unpaired *t*-test with Welch's correction where *** indicates $p<0.005$.

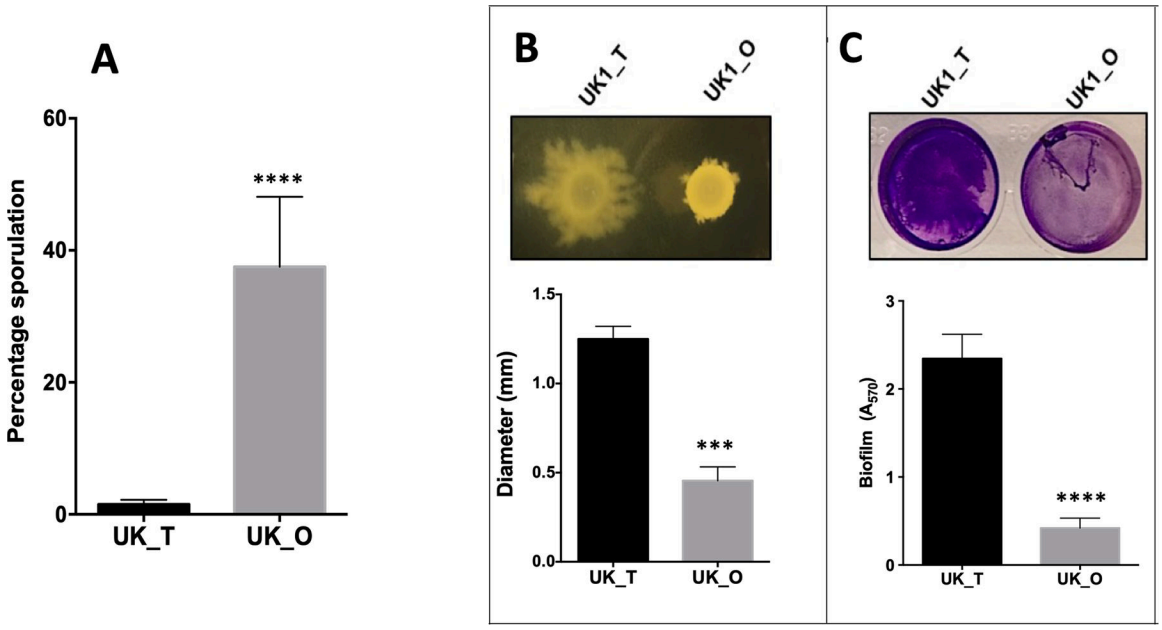


**Figure 2. Phenotypic characterization of UK1_T and UK1_O strains**

**A.** Percentage sporulation of UK1_T and UK1_O cells showing hyper sporulation phenotype in UK1_O morphotype. **B.** Swarming motility exhibited by UK1_T and UK1_O in TY+1.8% agar. **C**. Biofilm formation of UK1_T and UK1_O morphotypes. $OD_{570}$ nm was measured to quantify biofilm formation. Three biological replicates were used to carry out the experiments, and data were analyzed by two-tailed unpaired *t-test* with Welch's correction where **** indicates $p<0.0005$ and *** is $p<0.005$

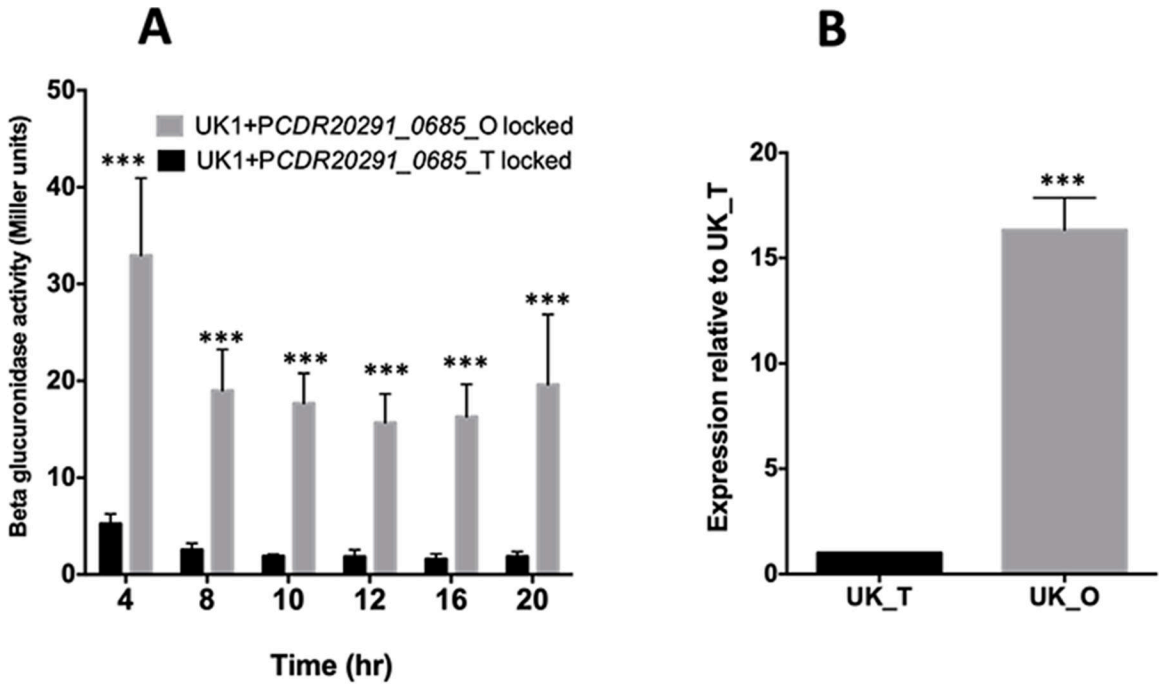


**Figure 3. DNA inversion upregulates the expression of *CDR20291_0685* in UK_O cells.**

**A.** The beta-glucuronidase activity of the P*CDR20291_0685* upstream-*gusA* fusions locked in UK1_T and UK1_O orientation in the UK1 parent strain. The data represent the results from three biological replicates. Data were analyzed using 2-way ANOVA (Sidak's multiple comparisons test) comparing the mean of UK1_T and UK1_O at each time point. *** indicates $p<0.005$. **B.** qRT-PCR results showing overexpression of *CDR20291_0685* in UK1_O strain at 16h time point. The representative results from three independent experiments are shown. Data were analyzed by a two-tailed unpaired t-test with Welch's correction where *** is $p<0.005$.

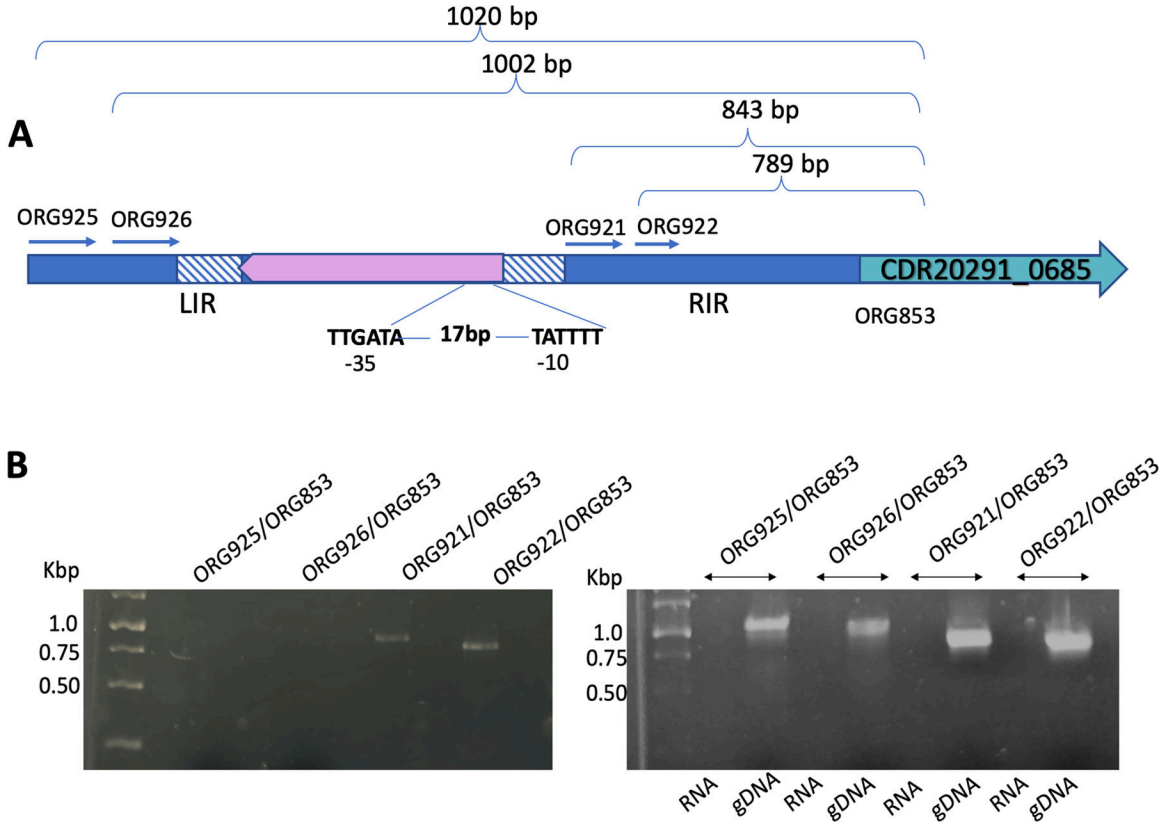


**Figure 4. The promoter of *pdcB* is located within the invertible region.**

**A.** Schematic of the upstream region of *pdcB* from *pdcB* (ON) cells (the orientation in which *pdcB* is expressed) depicting the invertible region flanked by the right and left inverted repeats (RIR and LIR). The forward and the reverse primers designed along the length of the invertible region are shown. The transcription initiation site was found near the RIR, and −35 and −10 sites are marked. **B.** RT PCR detecting the transcripts of *pdcB*. Genomic DNA (gDNA) and RNA were used as positive and negative controls, respectively. Only the primer pairs ORG921/ORG853, and ORG922/ORG853 gave amplification products of size 843 bp and 789 bp, respectively.

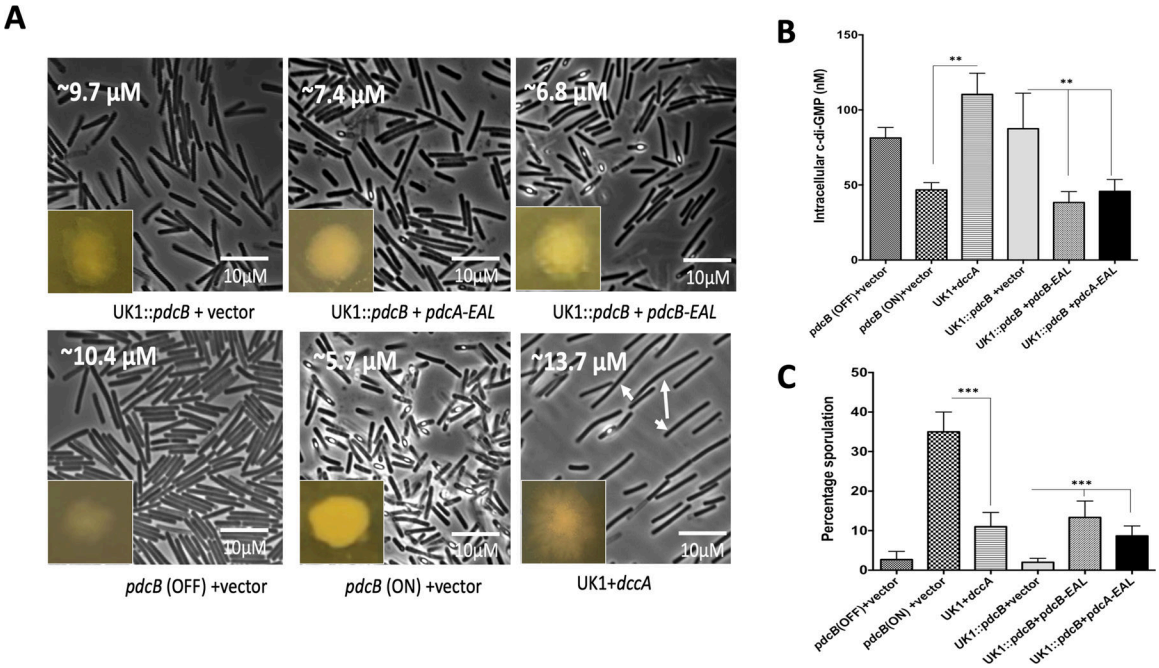


**Figure 5. Intracellular c-di-GMP levels influence sporulation in UK1 strain**

**A.** Phase-contrast microscopic images along with colony morphology of *pdcB* (OFF), *pdcB* (ON), UK1 expressing *dccA*, UK1::*pdcB* mutant complemented with *pdcB-EA*L/ *pdcA-EAL*. Average cell length is marked at the upper right corner of each image. Using ImageJ software, at least three images from two replicates were used for measuring the cell length (n=100). Long cells appeared upon the induction of *dccA* were marked with white arrows. The intracellular levels of c-di-GMP **(B)** and sporulation percentage **(C)** were measured in these strains. To induce the expression of *dccA, pdcB-EAL, pdcA-EAL* 100 ngs/ml of ATc was included in the TY agar plates. Three biological replicates were used and data were analyzed by one-way analysis of variance (ANOVA) where ** indicates $p<0.05$, and *** indicates $p<0.005$.

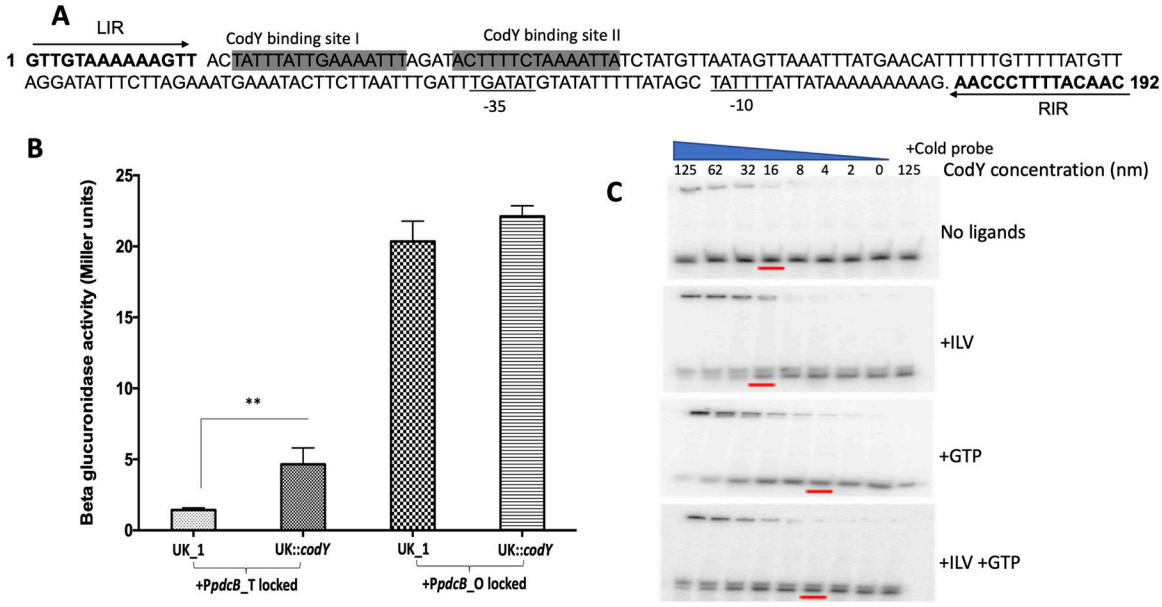


**Figure 6. CodY represses the expression of *pdcB*, which is partially relieved by DNA inversion.**

**A.** Schematic showing the potential CodY binding sites that lie within the invertible region upstream of *pdcB.* LIR and RIR are in bold and marked with arrows **B**. Beta-glucuronidase activity of P*pdcB-gusA* fusions locked in either translucent or opaque orientation in the parent UK1 and UK1::*codY* mutant. The data shown are the standard errors of the mean of three biological replicates. Statistical analysis was performed using two-way ANOVA.** indicates $p<0.05$.**C.** Binding of purified CodY to the upstream region of *pdcB.* Binding was increased with increasing concentration of CodY. The presence of GTP and ILV further enhanced CodY binding. Ten-fold excess of the cold probe was added with 125 ng of purified CodY in a control reaction to rule out non-specific binding.