Role of glycogen and cellobiose PTS operon in *Clostridiodes difficile* virulence and pathogenesis

by

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B.S., Mawlana Bhashani Science and Technology University, 2014
M.S., Mawlana Bhashani Science and Technology University, 2015

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Division of Biology
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2019

Approved by:

Major Professor
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Abstract

Clostridiodes difficile, a Gram-positive, anaerobic bacterium, is the leading cause of antibiotic-associated nosocomial diarrhea in North America. *C. difficile* causes around half a million infections per year and costs about 4.8 billion dollars in healthcare bills. *C. difficile's* major virulence factors are the extracellular toxins A and B. The disease is prevalent in the nosocomial environment and challenging to keep in check because of the highly resistant spores produced by the bacteria. Like many other pathogenic microbes, *C. difficile* virulence factors are strictly regulated in response to the nutrient availability to the cell. Glycogen is a storage carbon that many organisms use as a form of stored energy to use during the starvation condition. *C. difficile* genome harbors a glycogen biosynthesis operon, and we explored the role of glycogen in *C. difficile* growth and virulence by creating a mutant strain with a disrupted *glgC* gene of the operon. The resulting mutant was incapable of glycogen accumulation and produced very few spores, signifying glycogen is required for efficient sporulation in *C. difficile*. In correlation with *glgC* mutant's higher toxin production and faster growth rate compared to its parent counterpart in *in vitro* condition, our animal infection model study showed that glycogen mutants are significantly more virulent in *in vivo* conditions. The second part of the thesis explores the role of cellobiose phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) operon in *C. difficile* virulence. In *C. difficile*, the cellobiose PTS operon is positioned from base 3287617 to 3291739 in the R20291 hypervirulent strain genome. The operon consists of 5 genes with putative functions of a PTS system and cellobiose catabolism. Cellobiose is a complex carbohydrate abundant in human gut originating from dietary cellulose and has documented role in many pathogens' virulence. As such, we hypothesized that cellobiose metabolism plays a significant role in *C. difficile* virulence. CD2781 is a putative GntR class transcriptional regulator. Because of its

immediate vicinity to the Cellobiose PTS operon and putative function we hypothesized that it is a regulator for Cellobiose PTS operon. To test our hypothesis, we created mutant strains R20291::*licB* and R20291:: *cd2781* using ClosTron mutagenesis system. The resulting mutants showed a differential level of virulence factors, which were also corroborated by different molecular techniques. We also identified the CD2781 as a negative transcriptional regulator of cellobiose operon, characterized its target binding attributes, determined its role in virulence, and named it as CelR. Our hamster infection model study demonstrates that cellobiose PTS operon is essential for colonization, pathogenesis, and recurrent infection of *C. difficile* in hamsters. These works, in conclusion, demonstrate that both glycogen and cellobiose metabolism plays a significant role in *C. difficile* virulence.

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Chapter 1 - Introduction

Clostridiodes difficile is a Gram-positive anaerobic bacterium which until very recently belonged to the Clostridium genus and Firmicutes phylum. Because of the significant difference in 16s rRNA sequence, phylogenetic lineage, and other physiological difference such as H_2 gas production, fatty acid production, they have been reclassified as Clostridodes.[1] The firmicutes encompass Gram-positive low GC content bacteria mostly found in the human gut[2]. Firmicutes are responsible for major human physiological functions ranging from nutrient absorption to diabetes and obesity[3].

Among the Firmicutes present in the human gut, clostridia consist of a significant part. Being saprophytic, Clostridias are abundant in many niches, but they are also primarily known for their human pathogenesis. Examples include *Clostridium botulinum*- producing botulinum toxin, *Clostridium perfirenges*, *Clostridium tetani* producing Tetanus neurotoxin, and *Clostridium sordelii* causing myonecrosis. Clostridias are rod-shaped spore-producing anaerobes and are closely related to Bacillus but differ by their inability to grow aerobically and the oblong shaped spores that they produce[4].

Clostridium difficile was first isolated from a stool of a child and named "difficile" because of how difficult it was to culture. At first it was named as *Bacillus difficilis* by Hall and O'Toole [5] and then later to *Clostridium difficile,* and finally *Clostridiodes difficile*. Currently *C. difficile* is responsible for almost half a million infection in North America alone. *C. difficile* is also one of the most prevalent causes of antibiotic-associated diarrhea (15-20%) with the chance of infection

increasing 700-800% with people taking antibiotics [6]. *C. difficile* is also a nosocomial infection because of many patients admitted in the hospitals already carry *C. difficile,* and the spores of *C. difficile* are highly resistant to regular antimicrobial cleaning agents and thus spreads between the patients. Although early diagnosis and treatment can help prevent *C. difficile* mortality significantly, there is a high level of mortality in adult above 65 as one out of every 11 patient dies. The financial burden of *C. difficile* infection (CDI) is also significant, with CDC estimating almost 4.8 billion dollars spent in CDI treatment. One of the most significant emerging risks with CDI is recurrent infections, with 20% of patients having recurrent infections. All these factors accumulate to the severity of the *C. difficile* infection to be classified as urgent healthcare threat by CDC [7] and understanding the underlying molecular mechanism of *C. difficile* pathogenesis factors would lead to better prevention and treatment strategies.

Clostridiodes difficile **Pathogenesis**

C. difficile virulence and pathogenesis is a two-faceted phenomenon. Whereas the produced toxins are the main virulence factors causing diarrhea and colitis, the exospores produced are the primary mode of transmission from host to host. So, when it comes to *C. difficile* pathogenesis, both of these factors are extensively scrutinized.

C. difficile produces two toxins- Toxin A and toxin B from its pathogenicity locus (PaLoc) and are secreted extracellularly. The two toxins are encoded by *tcdA* and *tcdB* and transported extracellularly by a holin like protein encoded by *tcdE* [8]. The toxins are relatively similar in structure and contain four homologous domains [9]. Once released, the toxins target the epithelial cells lining the colon and glycosylate host cells Rho and Rac GTPases leading to the inactivation

of these small GTPases by irreversibly blocking their association with GTP [10]. This causes the epithelial cells to lose their cytoskeleton integrity, causing the loss of epithelial cell viability and loss of colonic epithelial barrier. The toxins also induce the epithelial cells to elicit an inflammatory response. This inflammatory response leads to proinflammatory cytokine release, tissue necrosis, and formation of toxic megacolon[11].

One of the hallmark properties of Clostridium species is the production of exospore. *Clostridiodes difficile* produces exospores that are highly resistant to various chemical and environmental stresses[12]. Initiation of sporulation generally occurs by the end of the stationary phase when the bacteria sense the nutrient deprivation. The spore production is the most significant factor in the transmissibility of the bacteria from host to host as demonstrated by studies with the asporogenic mutants of the bacteria[13]. The spore can withstand dry conditions for months and germinates when ingested by the next host. Once inside, the gastrointestinal tracts, the spore travels to the lower part of the tract and initiates germination. This part of the GI tract is most suitable for spore germination because of the lack of oxygen and the presence of bile acids [14]. Components of bile acids are demonstrated to be very effective in inducing *C. difficile* spore germination in *in vitro* condition. Once established *C. difficile* becomes a part of normal human gut microbiota[15]. The normal gut flora of a healthy human generally keeps the *C. difficile* growth and proliferation in check[16]. Disruption of normal gut microflora causes the proliferation and colonization of *C. difficile* leading to release of a copious amount of Toxin A and B and ultimately causing severe enteric colitis.

Regulation of *C. difficile* Virulence

Regulation of toxin production:

Clostridiodes toxins are large proteins requiring a considerable amount of cellular resources to produce. The toxins are encoded from pathogenicity locus (PaLoc) which contains three additional genes besides *tcdA* and *tcdB*- *tcdC, tdcR* and *tcdE*. TcdR is an RNA polymerase sigma factor responsible for positively regulating the expression of *tcdA* and *tcdB* [17]. Whereas another PaLoc gene - TcdC is experimentally identified as a negative regulator of toxin release since the reduction of its expression leads to increased expression of other PaLoc genes [18]. This is corroborated by the fact that highly pathogenic emerging strains of *C. difficile* were found to harbor mutations in the *tcdC* gene, corresponding to a higher level of toxin production[19]. Toxin production in *C. difficile* is growth phase dependent and thus indicates that the nutritional status of the cell can regulate toxin production via other cellular regulators. These regulators can directly interfere with the PaLoc expression, or indirectly regulate the expression of *tcdR* or *tcdC*. Important sources of nutrient for *C. difficile* are various carbohydrates available in the growth environment. In general, the presence of fast metabolizing sugars reduces toxin production following the classical catabolite repression (CCR) phenomenon,[20] suggesting that the repression is possibly regulated by CcpA, a carbohydrate metabolism master regulator. It has been shown that CcpA a negative regulator of gene expression, strongly binds with the upstream region of *tcdR* and represses its expression along with other PaLoc genes but with a lower affinity [21].

CodY, a global transcriptional repressor in *C. difficile* is a crucial link between the nutrient availability and expression of virulence factors [22]. CodY targets a wide variety of genes ranging from nutrient uptake to mobility[23242526]. CodY binding to its target is mediated by its binding with

Branched Chain Amino Acids (BCAAs) and GTP and subsequent conformational change enabling it to bind to target genes. The intracellular level of BCAAs and GTP changes in accordance with nutrient availability and thus CodY links nutritional status of the cell with the transcriptional regulation of many different physiological pathways. As such, it has been demonstrated that the addition of BCAAs causes reduction of toxin production by *C. difficile* [27]. In response to the carbohydrate availability, CcpA can also mediate the production of the BCCAs, which in turn can modulate the level of activity of CodY and thus provide a synergistic regulation of toxin production.

Regulation of sporulation:

The sporulation pathways in *C. difficile* has some components that are well conserved between other spore-forming bacteria, and some that are unique. The master regulator of sporulation in *C. difficile* is Spo0A- which is highly conserved among the spore formers [28] and Spo0A mutants are of asporogenic phenotype [29]. SpoA is a transcriptional regulator with an N-terminal dimerization domain and a C-terminal DNA binding domain which can recognize and bind to the upstream promoter region of *spo0A* itself, *sigH* and other early genes of sporulation initiation pathways and also with *sinR*, an essential element for sporulation [30]. Along with its role in sporulation, Spo0A is involved in many other physiological events such as metabolism[31], motility [31], biofilm formation [32] and to some extent toxin production [33]. Spo0A works in conjunction with SigH to upregulate the early sporulation genes during the stationary phase. SigH and Spo0A induce each other's expression and SigH has also been demonstrated to upregulate the expression of *sinR*, CD2492-a phosphorylase associated with Spo0A, and genes involved in chromosomal segregation during the initiation of sporulation.[28]

The global master regulators CcpA and CodY play a vital role in the regulation of *C. difficile* sporulation as well as toxin production. CcpA controls genes involved in various carbohydrate metabolism pathways such as carbohydrate uptake, regulation of the PTS systems, and regulation of amino acid metabolism in coordination with CodY. CcpA has been demonstrated to be essential for sporulation in other Clostridias [34]. However, in the case of *C. difficile,* CcpA has been found to be a negative regulator of sporulation [21]. CcpA recognizes and binds to a conserved region of DNA known as catabolite responsive elements situated upstream of its target genes. CcpA targets are found upstream of *spo0A* and repress the expression of *spo0A,* thus inhibiting sporulation. Transcriptome analysis has demonstrated that CcpA also downregulates the expression of *sinR*, a positive regulator of sporulation. CcpA also downregulates CD1579 (a putative histidine kinase of Spo0A responsible for phosphorylating spo0A and increasing its activity) and thus downregulating sporulation [21]. CodY is a global transcription repressor in *C. difficile* and thus plays both direct and indirect role in *C. difficile* sporulation. CodY has been documented to bind many targets in the sporulation pathways such as *opp* operon, CD2492 histidine kinase [35] (a putative Spo0A associated kinase), and CD2123 (a rap phosphatase which indirectly prevents phosphorylation of Spo0A) [36]. Also, CodY is demonstrated to bind directly upstream of *glgC* the first gene of glycogen metabolism operon. As demonstrated by our current study, *glgC* mutant produces significantly less spore than parent strains indicating a possible regulation by CodY and its role in sporulation.

Carbohydrate metabolism and pathogenesis:

The relationship between carbohydrate metabolism and pathogenesis is well documented in many pathogenic bacteria. Bacteria have developed different ways to utilize all the available

nutrients in their environment efficiently, and carbohydrates are one of the most abundant sources of energy available to bacteria. Availability of particular carbohydrate nutrients can also be used as cues for a pathogen to identify the site of infection, competition from other bacteria and overall a signal for fitness. The primary link between carbohydrate metabolism and pathogenicity is maintained through the classical phenomenon of Carbon Catabolite Repression (CCR). CCR ensures that bacteria utilize their most preferred carbohydrate energy source before metabolizing other sugars. CCR enables a bacterium to utilize the carbohydrate source that provides the fastest growth and thus provides a competitive advantage in the host. In its simplest form, CCR controls the expression of other carbohydrate utilization genes and operons in accordance with the availability of the glucose. CCR is exerted in the bacteria by the regulatory role of the PTS system [37]. The components of the PTS system are responsible for both uptake of the target sugar and also phosphorylating its substrate [38]. The phosphate is donated to the substrate by PEP via HPr. Phosphorylated Hpr can interact with CcpA which in turn can bind and repress Catabolite Response Elements (CRE) sites which are present upstream of many other genes and repress their transcription. CcpA is shown to directly represses the PaLoc genes *tcdA, tcdB, tcdR* and *tcdB* in *C. difficile*. Besides, CcpA also represses *spo0A* and *sigF,* the genes regulating the sporulation [21]. In Group A *Streptococcus*, a pathogen responsible many acute diseases in human such as toxic shock syndrome and rheumatic fever, CcpA has been identified as a regulator for *mga*, a major regulator for virulence factors [39]. Recent studies in *Listeria monocytogenes,* a pathogen responsible for severe systemic infections, meningitis, and stillbirth, it has been found that *prfA* activity, a major virulence factor regulator, is regulated by phosphorylation level and expression level of several PTS genes linking carbohydrate metabolism with virulence[40]. In *Vibrio cholera,* the gene *hapR*, responsible for the regulation of motility and chitin utilization, which enables the natural

competence, is regulated by Crp/cAMP CCR mechanism [41]. In *Salmonella typhimurium*, *mlC* is a global regulator of many genes and operons responsible for carbohydrate uptake and metabolism. MlC is a transcriptional repressor which has been experimentally demonstrated to repress the activity of several genes of Salmonella pathogenicity island such as *hilD, hilE*, and *invF*[42]. CcpA mediated regulation is also demonstrated in *Streptococcus mutans*, a pathogen commonly responsible for dental caries. In *S. mutans,* virulence factors such as acid production, and fructanase gene expression is regulated by CcpA dependent CCR [43]. CcpA mediated CCR is also responsible for the repression of several virulence-associated processes like toxin production and motility in *Clostridium perfringens*[44].

Additionally, CcpA has been identified for not only virulence factor regulation, but also antibiotic resistance in several pathogens such as *Staphylococcus aureus* and *Streptococcus gordonii*[4546]. Overall, these studies together demonstrate the importance of studying carbohydrate metabolism not only from the physiological aspect of pathogens but more importantly, also from the virulence standpoints. As such, this thesis work tries to understand the role of complex carbohydrate metabolism on the virulence of *C. difficile*, one of the leading pathogens responsible for antibiotic-associated diarrhea in North America.

Chapter 2 - Role of Glycogen Metabolism in *C. difficile* Virulence

Introduction:

Glycogen is a polysaccharide synthesized from glucose units arranged in a branched structure by a-1,4 glucosyl linkages. Glycogen works as a storage carbohydrate energy source and provides bacteria with extra sugar molecules in case of nutrient deprivation[47]. Glycogen is an excellent storage carbohydrate since it makes a dense granule with minimum effect in the osmolarity of the cell. The synthesis of glycogen is highest in bacteria when the growth media is low in essential nutrients, but excess carbohydrate is available [48]. Glycogen is not necessary for the growth of the bacteria under the normal growth condition as demonstrated by this work and previous studies[49]. Glycogen metabolism has been linked to virulence factors production in different pathogens. Examples include sporulation in *Bacillus subtilis*[50], biofilm formation and virulence in *Salmonella enteritidis*[51], and host dependency in general for many pathogenic bacteria[52]. In *Vibrio cholera*, stored glycogen has been shown to be responsible for increased environmental persistence and transmission[53].

Additionally, several regulators of bacterial glycogen biosynthesis have also been identified as the regulators for various pathogenesis properties in multiple bacterial species, indicating that glycogen metabolism has a significant role in bacterial pathogenesis regulation[54]. In addition to the pathogenic properties, glycogen biosynthesis has significant effects on the physiology of the bacteria too. In *Lactobacillus acidophilus*, glycogen metabolism impacts gut retention and stress tolerance[55]. In an *in vivo* mouse model study of *E. coli* strains with disrupted glycogen biosynthesis genes, the mutants showed a significantly reduced gut colonization capacity [56], indicating glycogen

metabolism may have more considerable significance when it comes to pathogenic commensal bacteria than we assume from *in vitro* studies.

Three primary enzyme contributes to the biochemical pathway of glycogen biosynthesis in bacteria. First, the substrate for glycogen, which in case of bacteria is Glucose -1-Phosphate, gets converted to ADP-Glucose by the enzyme ADP-Glucose Pyrophosphorylase encoded by *glgC* [57]. Using ADPG as sugar donor, glycogen is synthesized by glycogen synthase- encoded by *glgA*. After chain elongation by *glgA*, glycogen branching enzyme (*glgB*) catalyzes the formation of branched oligosaccharide chains having α-1,6-glucosidic linkages. The product of *glgP* is glycogen phosphorylase, which breaks down glycogen to glucose-1-phosphate. In *C. difficile,* the glycogen operon is constituted of 5 genes: *glgC, glgD, glgA, glgP,* and putative alpha-amylase (Figure 1). *glgB*, the gene responsible for glycogen branching is located from base 2,918,803 to 2,920,806 of the *C. difficile* 630 genome (AM180355.1), outside of the operon. *Bacillus subtilis* has a similar assortment of genes in its glycogen operon, albeit in a different organization. Many other gut living and pathogenic bacteria have a comparable assortment of genes in various orders (Figure 2).

Recent studies have shown that *C. difficile* glycogen operon is directly under the transcriptional control of the virulence master regulator CodY[35]. Given the significance of CodY regulation in *C. difficile* virulence in accordance with nutrient availability, it is logical to hypothesize that glycogen synthesis is tightly linked with the bacteria's nutrient signaling and disruption of glycogen accumulation capability could have a significant effect in the virulence properties in *C. difficile*. Additional evidence provided by a recent study on the proteomics analysis of Spo0A mutant

showed that various Glycogen operon proteins are downregulated in the mutant, hints that sporulation, as well as other physiological phenotypes such as autolysis properties can be affected by glycogen metabolism too. [31].

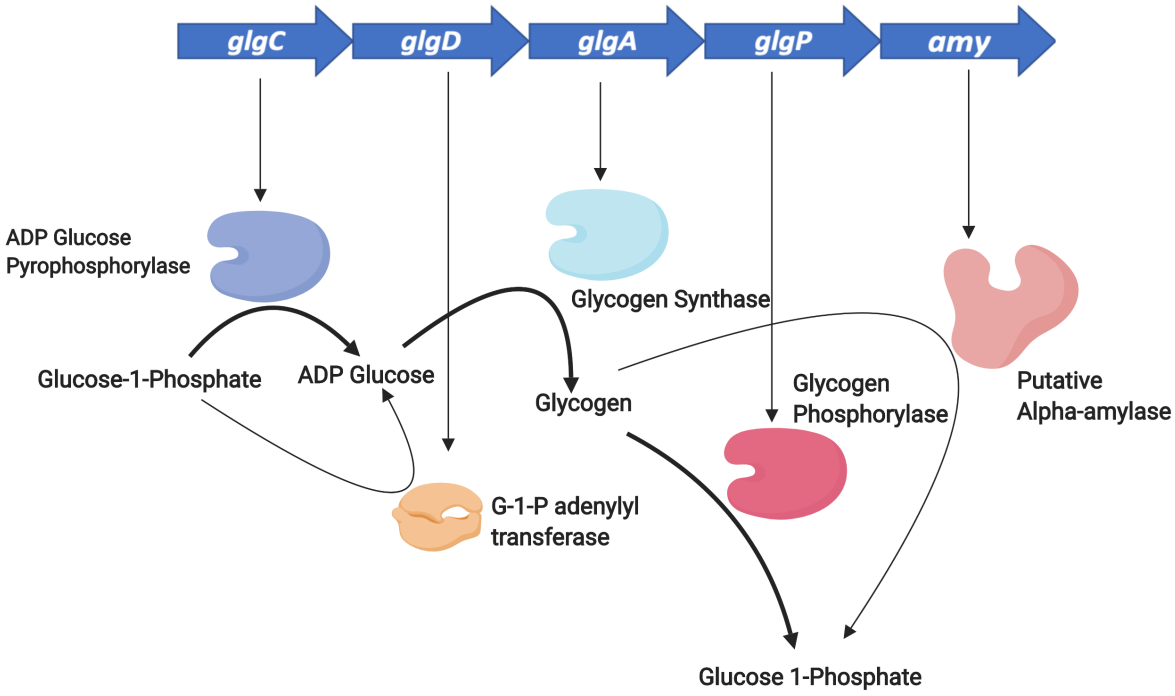


Figure 1 Schematics of glycogen operon of *C. difficile*.
The glycogen metabolism operon consists of 5 genes -*glgC, glgD, glgA, glgP* and a putative alpha amylase.

In this study, we set out to identify the role of glycogen metabolism in *C. difficile* virulence phenotypes and understand the underlying molecular mechanism of these different phenotypes. We created a mutant strain of *C. difficile*, which has disrupted *glgC* gene, responsible for the enzymatic conversion of its substrate glucose to ADP-glucose. Since ClosTron mutagenesis causes insertion of an intron in the first gene of the operon, in effect, all of the genes of the operon will be disrupted. We compared the glycogen mutant strain with the parent for its capacity to produce glycogen by measuring the intracellular glycogen level and imaging for glycogen granules using

Transmission Electron Microscopy techniques. We have also quantified the level of intracellular toxin level and sporulation frequency- the two most important factors for *C. difficile* virulence and pathogenesis, in the *glgC* mutant. To understand the underlying molecular mechanism of differential expression of virulence phenotypes, we used western blot and qRT-PCR techniques to track the differential expression of virulence master regulators of *C. difficile*.

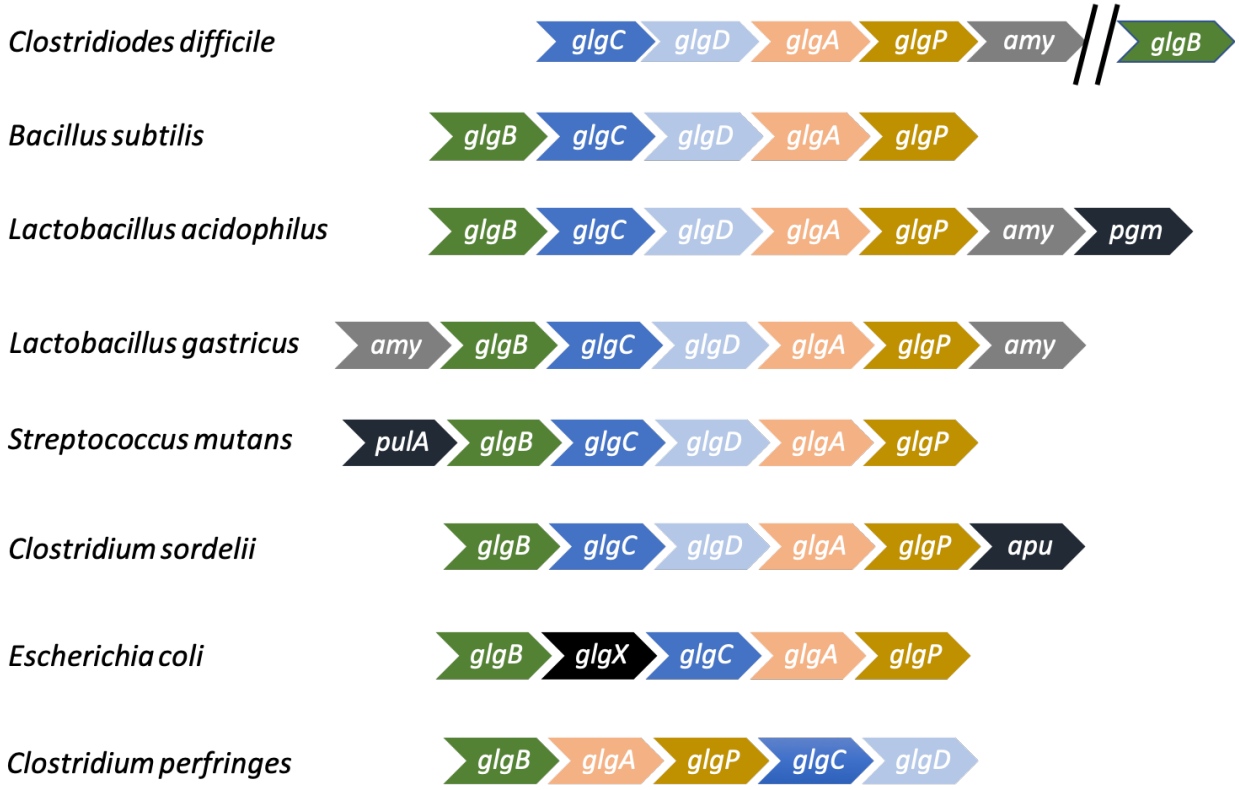


Figure 2 Comparison of glycogen operon between different pathogenic bacteria.

Glycogen biosynthesis operon is present in many pathogenic and non-pathogenic bacteria. Organization of the genes necessary to synthesize and breaking down the glycogen varies greatly.

Materials and Method:

Bacteria strains and growth conditions

Clostridiodes difficile parent and mutant strains were grown anaerobically in TY agar (Tryptose, Yeast Extract), liquid or 70:30 medium as described previously [58,59]. Kanamycin (Kan 50; 50 µg/ml), Thiamphenicol (Thio; 15 µg/ml), and Lincomycin (Lin; 15 µg/ml) were added to *C. difficile* cultures whenever necessary. *Escherichia coli* strains were grown in (LB) broth. *E. coli* strain S17-1 [60], used for conjugation, was supplemented with ampicillin (100 µg/ml) or chloramphenicol (25 µg/ml) when indicated and cultured aerobically in LB broth.

Construction of a JIR:: *glgC* mutant

C. difficile glycogen operon mutant was constructed using a ClosTron mutagenesis system [61]. The group II intron insertion site in the antisense orientation of the *glgC* ORF was selected using the Perutka algorithm, a Web-based design tool available at http://www.clostron.com. The designed retargeted intron was cloned into pMTL007-CE5, and the resulting plasmid, pMTL007-CE5::Cdi-*glgC*, was transferred into JIR by conjugation as described previously [58 62]. The selection of thiamphenicol-resistant transconjugants in 15 $\mu g \cdot ml^{-1}$lincomycin plates confers potential *Lactococcus lactis ltrB* (Ll.ltrB) insertions within the target *glgC* gene in the chromosome of JIR. The presence of a putative *glgC* mutant was identified by PCR using *glgC*-specific primers (Table 1) in combination with the EBS universal primers (Figure 3).

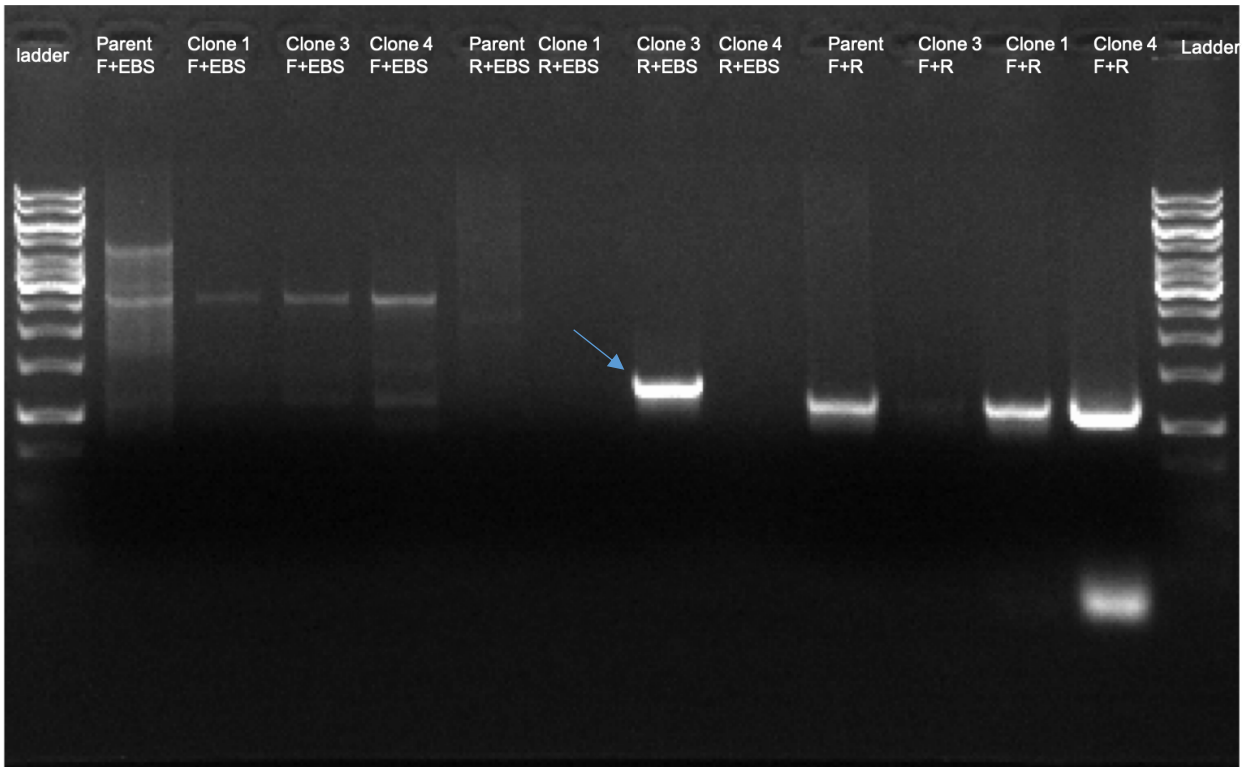


Figure 3 Verification of *glgC* mutant by PCR.

Agarose gel image of the screening and verification of JIR::*glgC* mutant. Clone 3 gives an amplification band (arrowhead) with EBS universal and glgC gene specific reverse primer. No band is seen with gene specific primer pair as the polymerase is unable to amplify now substantially larger glgC gene with the intron inserted.

Growth comparison

Cells were grown for 24 hours or longer in an anaerobic chamber in TY medium. Cell density was measured by taking OD_{600} values at indicated timepoints using spectrophotometric methods. An equal volume of cell was also diluted using fresh TY media and plated in TY plate and grown for 24 hours. The resulting colonies were counted and plotted to get the CFU counts. The experiments were conducted in three replicates.

Toxin assay

Cultures of *Clostridium difficile* JIR and the JIR::*glgC* mutant were centrifuged after 16 h in TY medium, and toxin ELISAs were performed as described previously [58]. Cytosolic toxins from 16h old *C. difficile* cultures grown in TY medium were measured as described previously. In brief, one ml of *C. difficile* culture was harvested and suspended in 200 µl of sterile PBS, sonicated, and centrifuged to harvest the cytosolic protein. 100 µg of cytosolic proteins was used to measure the relative toxin levels using *C. difficile* premier Toxin A &B ELISA kit from Meridian Diagnostics Inc. (Cincinnati, OH).

Sporulation assay (Microscopic analysis)

C. difficile cultures were grown overnight in TY medium. Cells were then diluted in TY medium to an OD_{600} of 0.5, and then 300 µl was used to inoculate 30ml of 70:30 sporulation media. Cultures were incubated at 37°C and monitored for the production of spores. Cells were harvested from the culture after 30 h and were suspended in TY medium for phase-contrast microscopy as described previously [30]. At least six fields per strain were obtained, and the numbers of spores and vegetative cells were counted to calculate the percentage of spores based on the total numbers of spores and vegetative cells.

Quantitative analysis of intracellular glycogen content

The intracellular level of glycogen was measured using a modified version of the assay protocol described by Goh and Klaenhammer[55]. Briefly, *C. difficile* was grown in TY medium, and TY supplemented with 2% glucose or 2% raffinose, known inducers of glycogen biosynthesis. Cells were harvested after 12 hours of growth, washed with PBS and then transferred to a pre-weighed

screw-capped tube. Cells were centrifuged at 16000g and made sure all of the supernatants were removed. The cell pellets weight was measured, and 0.25 ml of 0.25 M Na2CO3 solution was added. The cell suspension was incubated at 95-98 °C for 4 hours. 0.15 ml of 1 M acetic acid and 0.6 ml of 0.2 M sodium acetate (pH 5.2) was added to the cell suspension to bring the pH to ～5.2 and the final volume to 1 ml. 10 µl of amyloglucosidase (0.14 U/µl) was added to the cell suspension and incubated at 57°C overnight with rotation. The next day, the cell suspensions were centrifuged (5,000 x g, 3 min, room temperature) to pellet the cell debris. Measurement of the glucose released from the intracellular glycogen was conducted using a hexokinase/glucose-6-phosphate dehydrogenase-based glucose assay kit according to the manufacturer's instructions (Glucose assay reagent, Sigma-Aldrich, catalog number: G3293).

Transmission electron microscopic imaging of *C. difficile* strains:

Transmission electron microscopic analysis was done to investigate the presence of glycogen granules in the *C. difficile* cells. Parents and mutants were grown in media supplemented with 2% raffinose, cell pellets collected. All steps in sample preparation were performed at room temperature and solutions were prepared in 1X PBS (phosphate-buffered saline) unless indicated otherwise. For transmission electron microscopy, cells were fixed overnight in a solution of 2% glutaraldehyde and 2% paraformaldehyde. The cells were thoroughly rinsed with 1X PBS (5 minutes each) and post-fixed with 1% osmium tetroxide with constant rotation for 1–2 hours. The samples were then washed thrice with 1X PBS (5 minutes each), enblock stained with 2% Uranyl acetate in water for 1hr with light protection, and finally washed three times (5 min each) with distilled water. The cells were further dehydrated in a graded 50% -100% acetone series (vol/vol) for 5 minutes and infiltrated in graded EMBED 812/Araldite resin (Electron Microscopy Sciences)

at RT with constant rotation. Thin sections of polymerized resin were placed on copper grids and stained with 2% alcoholic uranyl acetate and Reynolds' lead citrate respectively. Sections were examined with a transmission electron microscope (Philips CM100) and regions containing the cross-section of the cells were photographed at 80 kV for image analysis.

Western Blot: *C. difficile* cells for western blot analysis were harvested and washed in 1x PBS solution before suspending in sample buffer (Tris 80mM; SDS 2%; and Glycerol 10%) for sonication. Whole cell extracts were then heated at 100°C for 7 min and centrifuged at 17,000 g for 1 min, and the proteins were separated by SDS-PAGE and transferred onto PVDF membrane using semi dry method. Immobilized proteins in the membranes were then probed with specific antibodies at a dilution of 1:10,000. The blot was subsequently probed with HRP-conjugated secondary antibodies at a dilution of 1:10000. Immuno-detection of proteins was performed with ECL Kit (Thermo Scientific) following the manufacturer's recommendations and were developed using Gbox imager. The membranes were probed with Glutamate Dehydrogenase (GDH) as a loading control.

qRT-PCR: *C. difficile* parent and mutant cultures were grown in TY or 70:30 medium and cells were harvested at 20 h by centrifugation at 4 °C for 2 min. Total RNA was extracted from the harvested cells following the protocol described previously [63] and treated with DNase (Turbo; Ambion) for 30 min at 37 °C. 30 µL of final reaction volume comprising of 5 µg of template RNA, 4 µL of deoxynucleoside triphosphates (dNTP; 10mM each), 1 µg of hexamer oligonucleotide primer (5 µg/µL pdN6; Roche), and 6 µL of reverse transcription (RT) buffer was heated at 80 °C for 5 min and cDNA was synthes. ized at 42 °C for 2 hours using avian myeloblastosis virus

(AMV) reverse transcriptase (Promega). Final 20 µL reaction volume 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 real-time PCR instrument (BioRad). Amplification and detection were performed as described previously [63]. Quantity of cDNA of a gene in each sample was normalized to either the quantity of *C. difficile* 16S rRNA gene or DNA polymerase gene and the ratio of normalized target concentrations (threshold cycle [2−ΔΔCt] method) [64] gives the relative change in gene expression.

In vivo animal model study for *C. difficile* pathogenesis

Male and female Syrian golden hamsters (100–120 g) were used for *C. difficile* infection. Upon their arrival, fecal pellets were collected from all hamsters, homogenized in 1 ml saline, and examined for *C. difficile* by plating on CCFA-TA (Cycloserine Cefoxitin Fructose Agar- 0.1% Taurocholate) to ensure that the animals did not harbor endogenous *C. difficile*. After this initial screen, they were housed individually in sterile cages with ad libitum access to food and water for the duration of the study. Hamsters were first gavaged with 30 mg/kg clindamycin[65]. *C. difficile* infection was initiated five days after clindamycin administration by gavage with vegetative cells. We used vegetative *C. difficile* cells because of the test strain produces very few spores if any spores. Bacterial inoculums were standardized and prepared immediately before challenge as described in our earlier study [66]. They were transported in independent 1.5 ml Eppendorf tubes to the vivarium using the Remel AnaeroPack system (one box for each strain) to maintain viability. Immediately before and after infecting the animal, a 10 µL sample of the inoculum was plated onto TY agar with cefoxitin to confirm the bacterial count and viability. There were three groups of animals, including the uninfected control group. Eight animals per group were used for the

infection. Approximately, 2000 *C. difficile* vegetative cells of JIR strain and JIR::*glgC* were used for the animal challenge. In the uninfected control (group 3), only six animals were used, and they received only antibiotics and sterile PBS. Animals were monitored for signs of disease (lethargy, poor fur coat, sunken eyes, hunched posture, and wet tail) every four hours (six times per day) throughout the study period. Hamsters were scored from 1 to 5 for the signs mentioned above (1-normal and 5-severe). Hamsters showing signs of severe disease (a cumulative score of 12 or above) were euthanized by CO2 asphyxiation. Surviving hamsters were euthanized 15 days after *C. difficile* infection. The survival data of the challenged animals were graphed as Kaplan-Meier survival analyses and compared for statistical significance using the log-rank test using GraphPad Prism 7 software (GraphPad Software, San Diego, CA).

Results:

Glycogen is non-essential for *C. difficile* growth in rich media:

To understand the role of glycogen in *C. difficile* growth, we did a growth curve comparison between the parent and the JIR::*glgC* mutant. There was no significant difference in growth observed by OD_{600} method when the cells were grown in TY media (Figure 4A). However, growth analysis by CFU (Colony Forming Units/ mL) count method showed that the glycogen mutant grows to a higher cell density compared to the parent strain (Figure 4B).

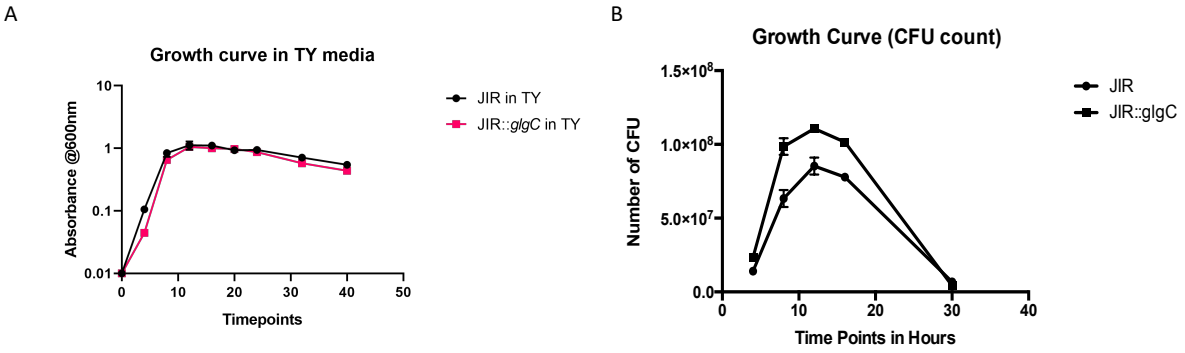


Figure 4 Growth comparison between parent and glycogen mutant strain.

A. *C. difficile* parent and mutant strains were grown in TY and TY supplemented with 5% glucose. OD600 was measured at indicated timepoints. B. Growth comparison by CFU/ml count method. (Data represents three biological replicates)

Phenotypic analysis of glycogen mutant:

To determine the physiological effect of the mutant strains inability to store the extra energy as glycogen, and therefore not being able to utilize a reserved storage carbohydrate in the nutrient-deprived state, we used multiple approaches. First, confocal microscopy was used to image the mutant, which shows that the mutants are significantly shorter compared to the parent (Figure 5A). Indeed, image analysis showed that the mutants are on average 2.5 times shorter than the parent strains (Figure 5B). Combined with our CFU count data, this signifies the mutant cells divides faster compared to the parents.

Figure 5 Morphological difference between parent and glycogen mutant strain.

A. Phase contrast microscopic image showing the difference in cell length between the parent and glycogen mutant (Magnification=100X). B. Quantitative measurement of difference in cell length using imageJ . Length of at least 15 randomly selected parent and mutant cells were measured and compared. P<0.0001 using Student's t-test.

We used qRT-PCR to measure the level of expression of the genes in the glycogen operon and found the operon to be significantly downregulated in the glycogen mutant (Figure 6A). Interestingly, the level of expression of glycogen operon genes was very low in the parent strain itself. We found the Ct values of the *glgC* mutant's glycogen operon genes to be close to non-template control's Ct values. We also measured the intracellular level of glycogen using an amyloglycosidase assay. The assay results showed that the parent strain accumulated about 2.66 mg of glycogen per gram of cell wet weight on average, whereas the glycogen mutant had little to no detectable level of intracellular glycogen (Figure 6B). We used Transmission Electron Microscopy technique to observe the ultrathin section of the parent and glycogen mutant strains of the bacteria to identify any potential difference in cellular structure. We found the parent strains to harbor dark, dense materials resembling glycogen granules in size (30-100 nm) and density (Figure 6C), whereas no such structure was seen in the glycogen mutant (Figure 7).

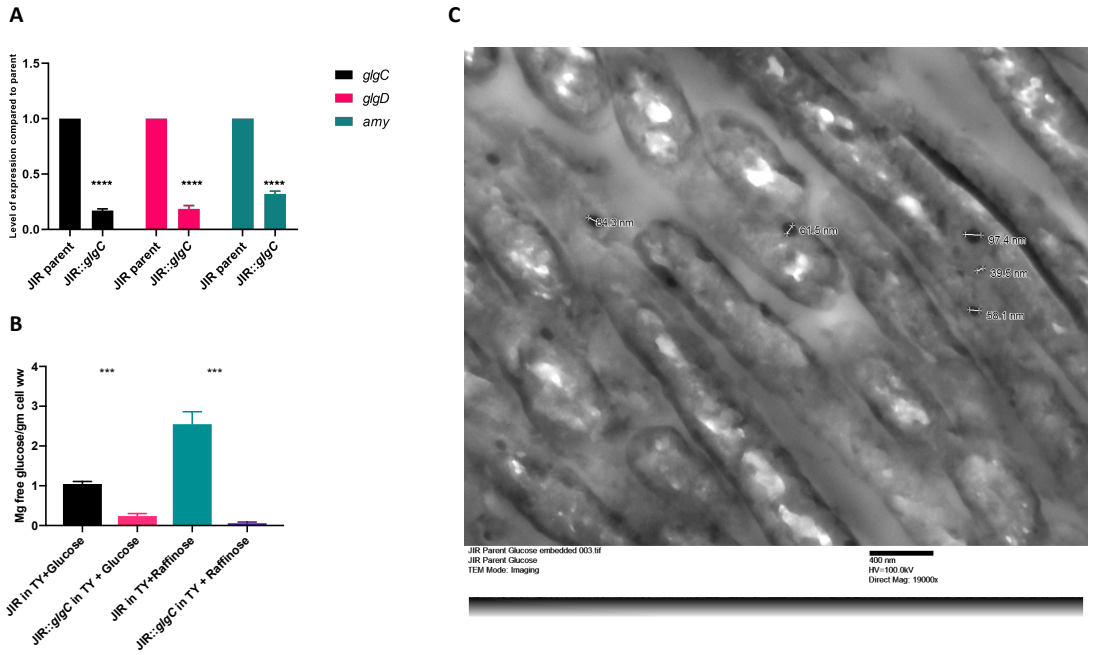
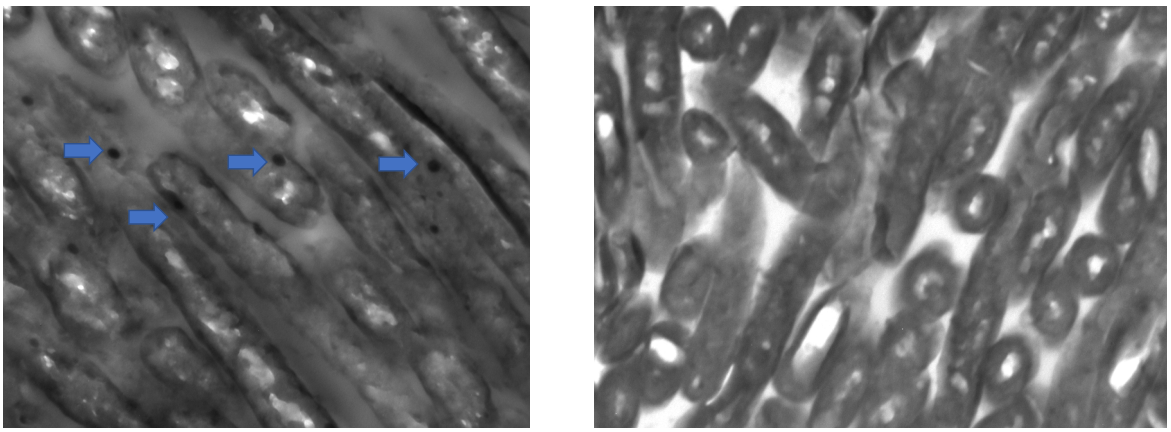


Figure 6 Intracellular glycogen level, glycogen operon expression, and glycogen granules in the parent and glycogen mutant strain.

A. Level of expression of glycogen operon genes in the mutant relative to the parent. ****=P<.0001 using Students t-test B. Intracellular level of glycogen in the parent vs. mutant measured by amylase assay. ***=P<.001 using Students t-test C. Transmission electron microscope image of JIR parent strain. Glycogen granule like structures ranging from 30-100nm in diameter are apparent in the parent strain.



JIR Parent Strain

Glycogen Mutant

Figure 7 No glycogen like granule is visible in the JIR::*glgC* mutant strain.

Visible dark granules present in the parent strain (indicated by arrowheads) in the left panel, and no such structure was present in the JIR::glgC strain right panel.

Glycogen metabolism plays crucial role in *C. difficile* virulence:

To understand the role of glycogen metabolism in *C. difficile* virulence, we conducted an ELISA based toxin assay to measure the intracellular level of toxin. The assay result shows that the glycogen mutant produces a significantly higher level (Approximately three times more in mean absorbance level, $p<0.01$) of toxin at around 16-hour time point when compared to the parent strain (Figure 8A). Additionally, we calculated the sporulation efficiency of the mutant using a microscopic spore count method. Compared to the parent strain, the glycogen mutant shows a significant reduction ($p<0.0001$) in the sporulation efficiency measured after 36 hours of growth in the 70/30 sporulation media (Figure 8B).

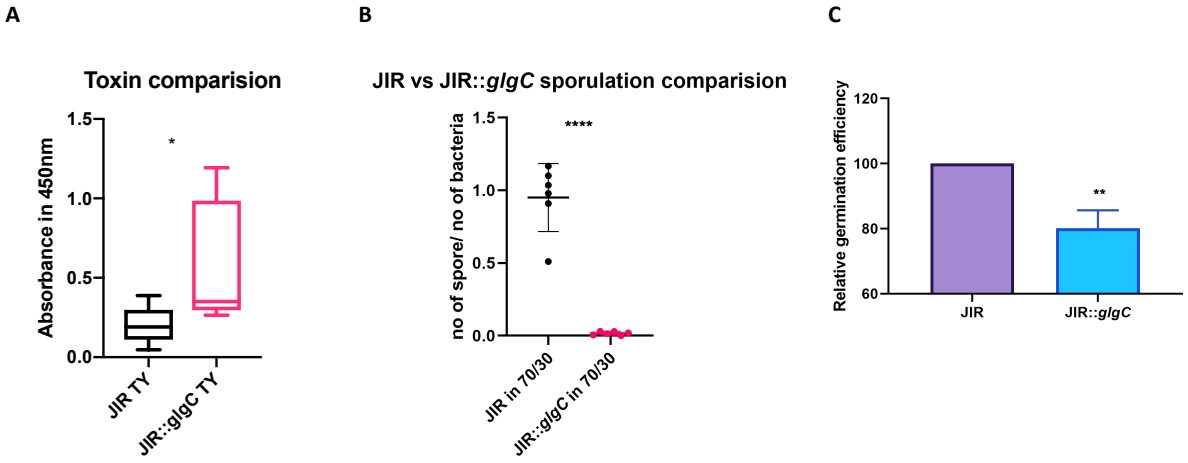


Figure 8 Toxin and sporulation level in the parent and glycogen mutant strain.

A. Measurement of cytotoxic Toxin level by ELISA after 16 hours. *P<0.05 using two tailed t-test for means. B. Spore count after 48 hours (Total bacteria and number of spores counted in 6 Field of View using a phase contrast microscope) ****P<0.0001 using Students t-test. C. Comparison of initial spore viability after spore isolation. **P<0.001 using Students t-test.

***glgC* mutant has significantly lower level of germination capable spores:**

In addition to the sporulation efficiency, we investigated whether glycogen metabolism has any effect on *C. difficile* spore viability. Initial spore stocks were serially diluted and plated in TY supplemented with .1% Taurocholate plate germination capable spores were enumerated by

counting the number of colonies after 24 hours of growth. The optical density of the initial spore stock was measured at $OD_{600,}$ and the spore viability was compared between parent and mutant strains. We observed a significant reduction of spore viability in the mutant, indicating glycogen is not only essential for spore formation but also for spore viability (Figure 8C).

***C. difficile glgC* mutant exerts a differential level of virulence gene regulators:**

C. difficile sporulation and toxin production are regulated by the master regulator CodY, CcpA and the sporulation genes are specifically under control of Spo0A. We investigated the underlying mechanism of observed phenotypes by using western blot and qRT-PCR techniques to quantify the differential expression of the virulence factors. Our results show a higher level of expression of *tcdR*, the sigma factor responsible for the expression of the toxin genes *tcdA* and *tcdB* (Figure 9A). *tcdR* expression level is positively correlated to toxin production in *C. difficile* [67]. Also, *codY*, a global regulator of virulence factors, was significantly overexpressed in the glycogen mutant. Besides, the sporulation initiation factor *spo0A* was significantly downregulated in the glycogen mutant when compared to the parent strain.

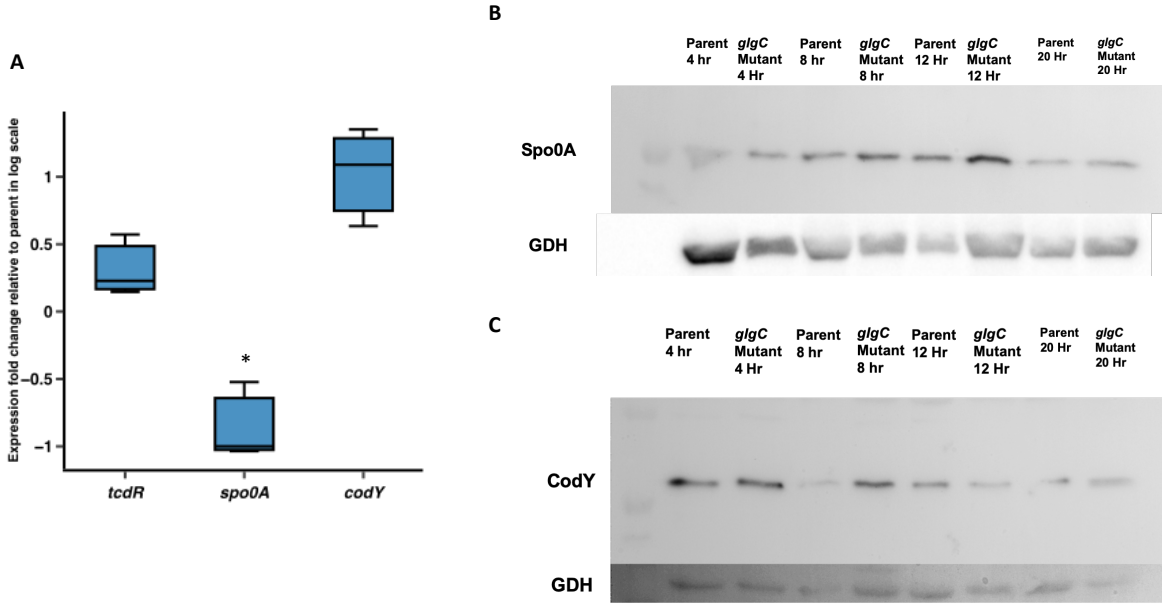


Figure 9 Level of major virulence master regulators in the JIR::*glgC* mutant.

A. Level of expression of tcdR, spo0A, and codY in the mutant strains measured by qRT-PCR. Western blot image for the level of Spo0A (B) and CodY(C) at different timepoints.

Glycogen is non-essential for initial *C. difficile* colonization and pathogenesis in Hamsters:

Since glycogen metabolism was found to be significant in *C. difficile* toxin and sporulation level in *in vitro* condition, we decided to determine the pathogenic significance of the difference in *in vivo* condition. Golden Syrian hamsters were gavaged with 2000 *C. difficile* JIR and JIR::*glgC* and monitored for infections. All but two hamsters of the parent group and one hamster of the mutant animals died by the end of the study period. All surviving hamsters (and uninfected control) hamsters were also sacrificed fifteen days post-infection. Kaplan Meier survival analysis was done (Figure 10), and the results showed that glycogen mutant strain is significantly more pathogenic in the animal model compared to the parent strains. This observed increased virulence could be because of the combination of higher growth rate and increased toxin level produced by the glycogen mutant.

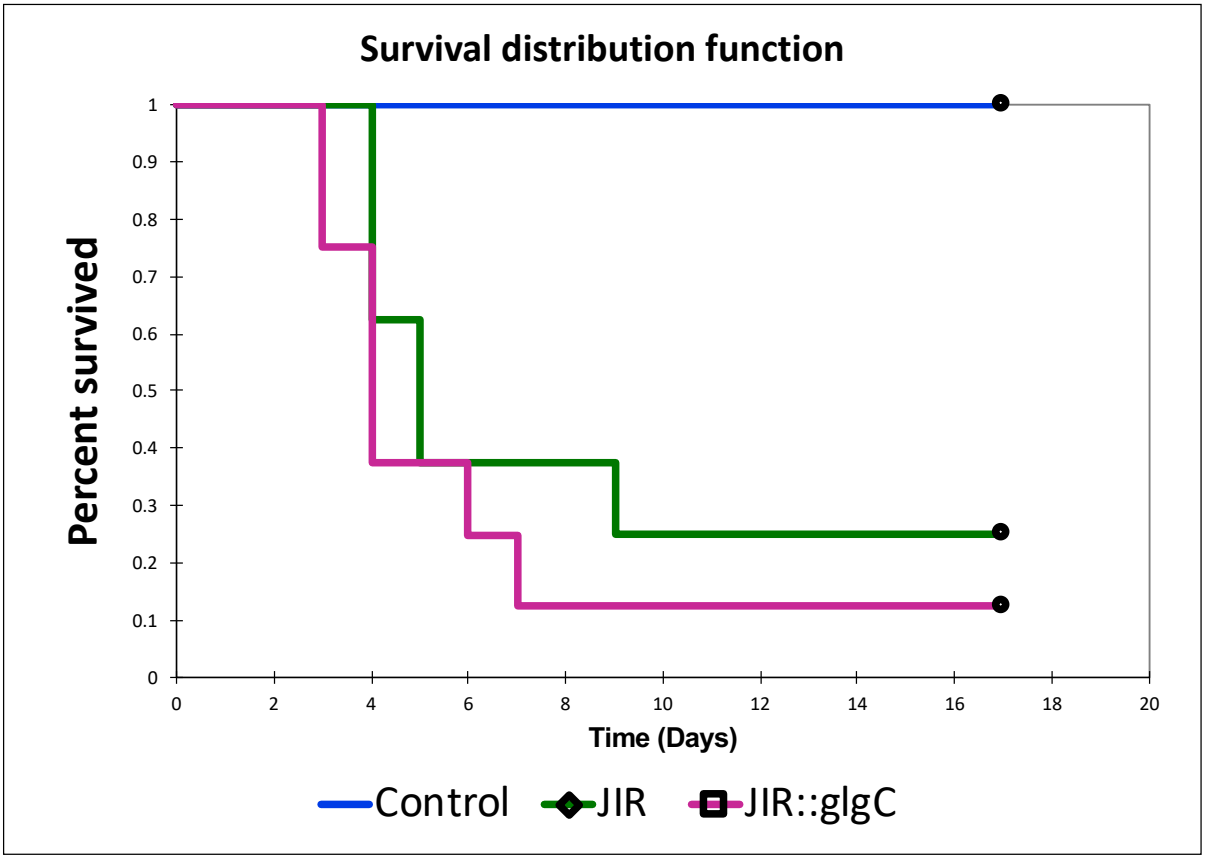


Figure 10 Disrupting glycogen operon in *C. difficile* increases its *in vivo* pathogenesis capacity.

Kaplan-Meier survival curve of clindamycin-treated Syrian golden hamsters inoculated with 2,000 vegetative cells of *C. difficile* JIR (n = 8) or JIR::*glgC* mutant (n = 8). Six animals were used as an uninfected control. Animals were monitored every four hours for the symptoms of lethargy, poor fur coat, wet tail or hunched posture. Moribund animals were euthanized, and log-rank statistical analysis was performed; $p<0.005$.

Discussion:

In the colon, *C. difficile* has to survive through adverse environment imposed primarily by competitive commensals fighting for nutrients. As such, it most probably cycles through nutrient-rich and nutrient-poor conditions. Having a mechanism to store the excess amount of nutrient for future use during the starvation period could give *C. difficile* a better colonization capacity. Besides, glycogen is documented to be a storage carbohydrate for many other bacteria. A recent study of *in vivo* genome analysis with *C. difficile* has shown that expression of glycogen operon is upregulated *in vivo* condition compared to the *in vitro* condition may imply that *in vivo* glycogen metabolism is even more critical than *in vitro*[68]. Given how pathogenesis is tightly regulated with nutrition status in *C. difficile*, the ability to store carbohydrate for use during starvation or other cellular processes can have a significant role in its virulence.

Our results have shown that the glycogen mutant is faster growing and shorter in length. We assume that since the *glgC* mutant strain is not utilizing glucose to synthesize glycogen, the extra energy available from the unutilized glucose leads to faster growth compared to the parent strain. Also, the faster cell division rate could account for the shorter cell lengths.

As our results have indicated, disruption of glycogen biosynthesis capacity causes to *C. difficile* to produce a significantly higher-level of toxin. We hypothesize that the lack of glycogen to buffer the lack of nutrient in the late stationary phage could lead to a cascade of changes in the global metabolic state of the bacteria. The combined effect of these changes in various carbohydrate as well nutrient pathways leads to the differential expression of various virulence master regulators leading to the differential level of virulence factors. This hypothesis is supported by our qRT-PCR

results showing the expression level of *tcdR*, a sigma factor that positively regulates the toxin gene expression, is significantly upregulated in the mutant after 20 hours of growth. Also, the phenomenon of nutrient starvation, leading to increased virulence by CCR mechanism can account for the increased level of toxin production too.

The results of the sporulation study were intriguing. In *C. difficile*, usually toxin level and sporulation have a trend of synergistic upregulation or downregulation in response to the changing nutritional environment as these two virulence factors works together to enable the pathogen to escape the now unfavorable condition and infect a favorable host. Producing higher level of toxin without producing a higher level of competent spores gives the pathogen a lesser chance to spread from host to host. Our hypothesis for the observed result is that sporulation in *C. difficile* is fueled by the energy stored in glycogen. This way, the pathogen can ensure that it has enough stored energy available to manufacture the spore before committing to the sporulation initiation process. In essence, glycogen accumulation could be used as a checkpoint for sporulation. This hypothesis is also supported by our data from the qRT-PCR, which shows reduced expression of *spo0A,* the master regulator of sporulation. Glycogen could also be stored form of energy for the spore to be used during germination, which could explain the lower germination capacity of *glgC* mutant spores.

Our *in vivo* study results show that glycogen is not essential for the initial colonization and pathogenesis in hamster models, which is contrary to the current understanding of the role of glycogen in bacteria[55]. Glycogen is a storage carbohydrate that can provide energy to the bacteria in a starving condition or to drive some specific cellular process. So far, our results indicate that

glycogen in *C. difficile* is essential for producing spores and not much for colonization and pathogenesis. However, the role of glycogen in the bacteria growth and survival in the nutrient limiting environment could not be completely ruled out. Contrary to the hamster model where the commensal flora is wiped out by antibiotics, *C. difficile*, in the normal host, has to compete with a range of gut microbes. Thus *C. difficile* is imposed with a more challenging nutrient condition than when inside an infection model. In those conditions, glycogen could play a significant role in the colonization of the bacteria. Also, the lack of significant difference between the growth dynamics of the parent and mutant strain corroborates to the idea that glycogen in *C. difficile* is more critical for sporulation than survival in nutrient-limiting conditions. In case of a recent study[68] showing an elevated level of glycogen operon expression *in vivo* condition could be because, *C. difficile* in *in vivo* conditions, makes more spores than *in vitro* growth media, which would require the bacteria to synthesize more glycogen *in vivo*. Also, we do not have any data whether the hamsters that are infected with *glgC* mutant can spread the bacteria to next host, which would have provided evidence whether glycogen is essential for spreading of the pathogen.

As for the molecular mechanism by how glycogen synthesis is regulated, we are proposing a model taking into account of findings of various studies in other related Gram-positives and our findings which however needs to be experimentally validated (Figure 11). The genes *glgC* and *glgD* encodes for ADP Glucose Phosphorylase heterotetrameric protein responsible for conversion of Glucose-1-Phosphate to ADP Glucose [69]. The expression of *glgC* is upregulated by the increased availability of Fructose-1-6 Bis Phosphate, which probably works as the sensor nutrient availability for glycogen formulation[69]. Besides, the glycogen operon is also under the control of global repressor CodY[35]. During glycogen synthesis, GlgC provides the substrate for the GlgA,

the Glycogen Synthase. Glycogen Synthase in Gram-positive bacteria can be regulated by phosphorylation and is inactive in its phosphorylated state[70]. On the other hand, its activity is increased by the presence of Glucose 6-Phosphate. GlgP is the enzyme that is responsible for the breakdown of glycogen from the non-reducing end and produces Glucose-1-Phosphate. In Gram-positive bacteria, the GlgP is known to have a high affinity for Hpr, the cytoplasmic component of bacterial phosphoenolpyruvate(PEP):carbohydrate phosphotransferase system (PTS), and is active when it is bound with Hpr [38]. Since there is always a significantly higher amount of HPr present in the bacteria when compared to the GlgP, the enzyme is always bound with Hpr. Hpr is present in the cell in either phosphorylated or non-phosphorylated form. When the GlgP is bound with unphosphorylated HPr, its activity is five times higher compared to when it is bound with phosphorylated Hpr[38]. Thus, glycogen degradation could be regulated by Hpr phosphorylation level. Since the ratio of phosphorylated and unphosphorylated Hpr is dependent on the cellular nutrient level, glycogen degradation could also be regulated by the cell's nutrient availability. This model accounts that since there is always some unphosphorylated Hpr present in the cell, there could be simultaneous low-level degradation of glycogen as it is being accumulated. However, when the ratio between phosphorylated and unphosphorylated Hpr changes, the rate of glycogen degradation could increase significantly.

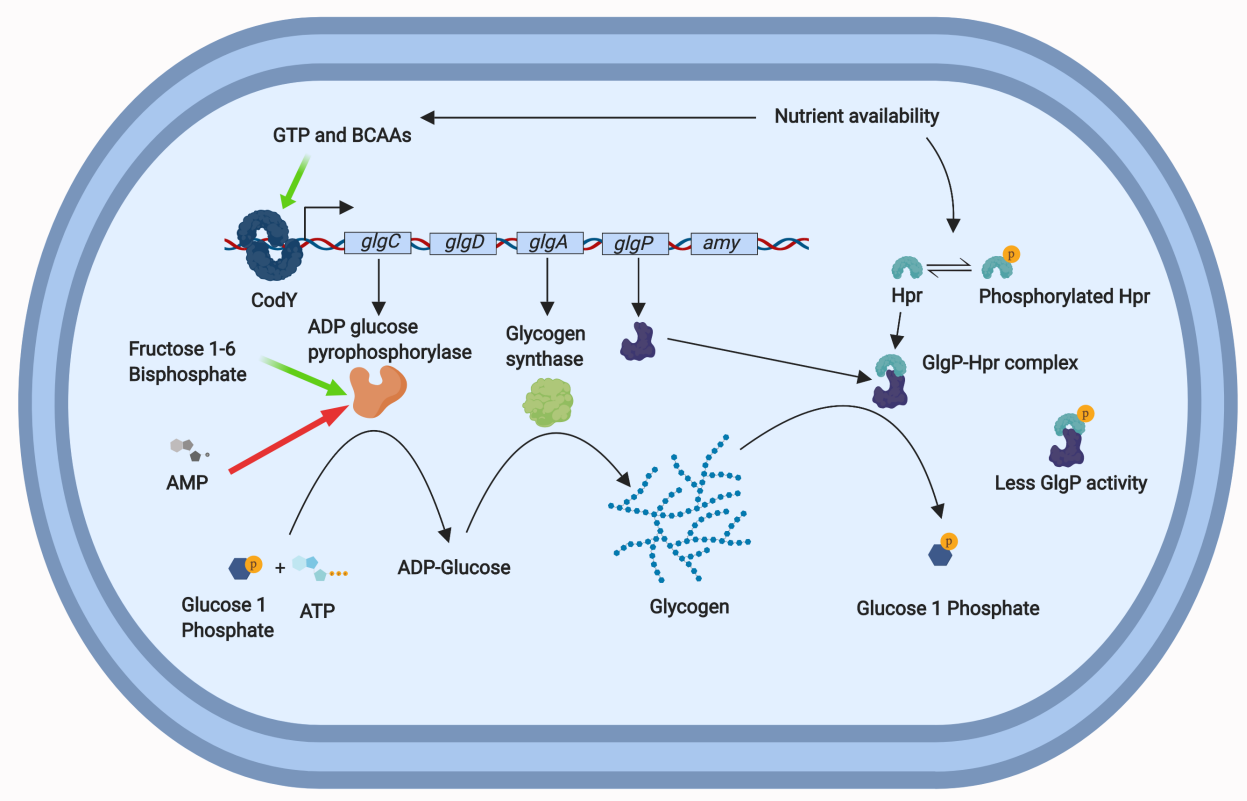


Figure 11 Schematics of a hypothetical model for glycogen metabolism regulation in *C. difficile.*

Glycogen operon in *C. difficile* is under transcriptional repression of CodY. This repression is strongest when the bacteria is in nutrient rich condition because of availability of GTP and Branched Chain Amino Acids (BCAA). When the cell is nutrient starvation condition the repression is relieved and glycogen operon is expressed. GlgC activity is increased with availability of Fructose 1-6 Bis Phosphate and such the glycogen production is highest when the bacteria are in otherwise nutrient limited condition but have excess sugar. GlgA activity is also allosterically regulated and increases with presence Glucose 1 Phosphate. When the cell is nutrient starvation condition and there are no more excess sugars left, the glycogen is degraded by GlgP activity. GlgP activity is dependent on whether its bound with phosphorylated or unphosphorylated Hpr. GlgP bound with unphosphorylated Hpr is five times more active compared to when it is bound with phosphorylated Hpr. When cell is in low nutrients and no sugar is available, Hpr is predominantly unphosphorylated. the unphosphorylated Hpr bound with GlgP increases its catalytic activity, thus increasing glycogen degradation.

As glycogen accumulation requires the utilization of a significant amount of Glucose-1-Phosphate, the *glgC* mutant strain has a relatively higher-level of free Glucose 1 Phosphate than the parent strain which otherwise would have been utilized to make glycogen. This excess Glucose 1 Phosphate in glycogen mutant leads to faster growth as the cell assumes the intracellular nutrient level to be high. However, when the available nutrients are exhausted, the cell starts the sporulation

process. We hypothesize that the sporulation process is driven by the energy provided by glycogen degradation. Since, the *glgC* mutant has no extra energy available from the degradation of glycogen, the bacteria unable to produce spores. In summary, the *glgC* mutant strain of *C. difficile* grows significantly faster compared to the parent strain but possibly has no reserve energy to produce spores when nutrients are exhausted.

Chapter 3 - Characterizing and Identifying the Role of Cellobiose Phosphoenolpyruvate:carbohydrate Phosphotransferase System (PTS) Operon and Its Transcriptional Regulator CelR, in *C. difficile* Virulence and Pathogenesis.

Introduction:

Cellulose is a significant part of the human diet because of its abundance in plant-based foods and vegetables. In the human digestive system, cellulose is broken down by microbial flora. In general digestibility of food derived from cellulose in humans is anywhere between 70-80%[71]. Although the portion of cellulose in total carbohydrate consumed by the average human is just around 20%, nevertheless it provides a significant source of nutrients to the colon microbiota[72]. There have been several studies conducted to find out the gut microbiota population that is responsible for cellulose degradation[73]. Anaerobic cellulose degraders, which account for 5–10% of all cellulose decomposition, uses two different mechanisms for cellulose decomposition. The first is the use of a cellulosome complex of cellulolytic enzymes, first characterized in clostridial species but also shown to occur in the human gut bacterium *Ruminococcus flavefaciens* in a more complex organization [74]. The cellulosome complex is attached to the outer cell envelope of the bacterium and contains proteins with cohesion domains that bind strongly to the dockerin domains of the cellulolytic enzymes. Besides, the scaffoldin often has a separate cellulose-binding domain (CBM) for binding to the cellulosic substrate. The second is the system used by *Fibrobacter succinogenes* in which cellulose is attached to the outer membrane through adhesins, e.g., fibro-slime proteins and possibly type IV pilins[75]. Both of these mechanisms happen outside of the bacteria causing diffusion of its major byproduct in the colon. One of the major byproducts of cellulose is its

repeating units of cellobiose disaccharides. Cellobiose then becomes available to other gut living anaerobes such as *C. difficile*, which can uptake it intracellularly and utilize it as its alternative energy source, use to sense its niche and neighbors. Thus, cellulose degradation and the potential use of its byproducts such as cellobiose is of considerable significance to many enteric pathogenic bacteria.

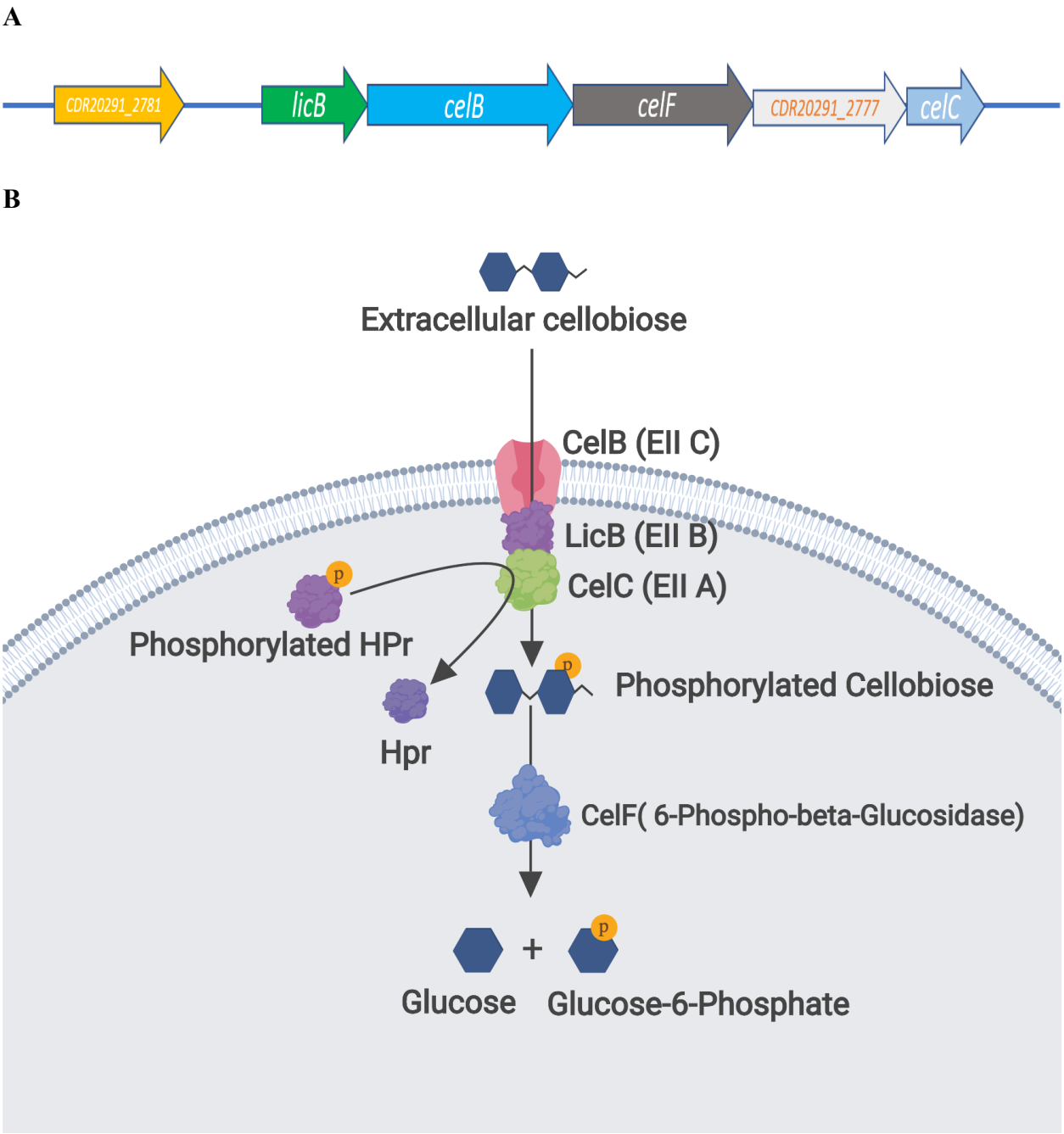


Figure 12 Schematics of Cellobiose Phosphoenolpyruvate:carbohydrate Phosphotransferase System (PTS) operon and the PTS system.

A. The Cellobiose PTS operon of the *C. difficile* R20291 genome. B. The PTS consists of two cytoplasmic energy-coupling proteins (Enzyme I and HPr) and carbohydrate-specific Enzymes II, which catalyze concomitant carbohydrate translocation and phosphorylation of the substrate. In *C. difficile* extracellular Cellobiose is could potentially be uptaken by a PTS system that consists of CelB, LicB, and CelC polypeptides making up the transmembrane EII part of the PTS sytem. The phosphate group could be transferred to the PTS system by the HPr.

Phosphorylated Cellobiose could then be broken down to Glucose and Glucose 6-Phosphate by the enzymatic activity of CelF and incorporated in metabolic pathways.

The bacterial Phosphoenolpyruvate:carbohydrate Phosphotransferase System (PTS) is responsible for the transport and coupled phosphorylation of its substrates. Some of the other PTS functions include involvement in chemotaxis, regulation of gene expression, and other metabolic processes[37] [76]. The PTS is composed of the general proteins enzyme I (EI), HPr, and the substrate-specific enzyme II (EII). The EII complexes represent the sugar-specific permeases, which consist of three or four subunits either fused in single multidomain proteins or built up of individual polypeptides [77]. The proteins of the PTS transfer a phosphate group from PEP to the carbohydrate that is being transported [37].

CCR (Carbon Catabolite Repression) is a common phenomenon in many Gram-positive pathogens responsible for coupling the virulence factors expression with the availability of carbohydrate nutrients. In *C. difficile* the major components of the CCR signal transduction pathway are the PTS proteins (Enzyme I and HPr), the HPr kinase/phosphorylase (HprK/P) and the Catabolite Control Protein A, CcpA[78]. The critical link between the CCR and the PTS sugar transport system is the EI and HPr phosphorylation status.

C. difficile can use a variety of carbohydrates and amino acids as its nutrient source[79]. Availability of nutrients is also crucial for *C. difficile* pathogenesis and disease progression, as *C. difficile* virulence factor gene expression is quite sensitive to nutrient availability [27], [80]. Recent studies have indicated that carbohydrate metabolism (specifically complex carbohydrate metabolism) is a significant factor in many pathogenic bacteria's virulence. *C. difficile* genome

has multiple PTS operons capable of sequestering a host of different carbohydrates [21]. Complex carbohydrates such as cellobiose have been demonstrated to regulate virulence factors in several pathogenic bacteria. For example in *Listeria monocytogenes*, a human pathogen naturally found in soils and decaying food products, cellobiose has been found to reduce the pathogenicity of bacteria by reducing the major virulence factor Listeriolysin and Phosphatidylinositol-specific phospholipase C [81]. On the other hand, a plant pathogen *Streptomyces scabies* has been demonstrated to produce more virulence factor Taxtomin A in response to cellobiose [82]. Later studies identified that the transcriptional repressor for Cellobiose utilization operon CebR was responsible for the increased virulence of the pathogen since CebR has additional DNA binding targets in the promoter regions of virulence genes of this pathogen .[83] In *Klebsiella pneumonia*, a human pathogen capable of causing bronchitis, Cellobiose degradation enzyme CelF has been found to be essential for the biofilm formation and other virulence factors production. [84] Thus, there is a compelling amount of evidence from other bacteria that cellobiose metabolism might have a role in *C. difficile* virulence. Also, a large portion of human diet consists of Cellulose which can be converted into cellobiose by the other commensal bacteria or directly by Cellulosomes on *C. difficile* (which still needs to be experimentally determined), giving a sizable pool of extra nutrients for *C. difficile* to utilize. Inability to utilize cellobiose could lead to changes in the nutrient uptake and metabolism pathways in *C. difficile* in such a way that it might result in significant changes in the virulence properties of the bacteria. As such, in this study, we identified the role of Cellobiose PTS operon in *C. difficile* R20291 (GenBank: FN545816.1) virulence. The operon consists of five genes: *licB, celB, celC, celF,* and a putative cellobiose/chitin deacetylase *CD2777* contributing to the construction of Cellobiose PTS system and encoding for cellobiose catabolism enzyme (Figure 12). In addition to that, we have also identified a novel GntR class transcriptional

regulator, CelR, responsible for negatively regulating the Cellobiose PTS operon expression. We have also explored the role of CelR in *C. difficile* virulence and determined the underlying molecular mechanism of differential virulence phenotypes.

Materials and Methods:

Ethics statement:

All animal procedures were performed with prior approval from the KSU Institutional Animal Care and Use Committee (protocol #3657.3). Animals showing signs of disease were euthanized by CO2 asphyxia followed by thoracotomy as a secondary means of death, in accordance with Panel on Euthanasia of the American Veterinary Medical Association. Kansas State University is accredited by AAALAC International (Unit #000667) and files an Assurance Statement with the NIH Office of Laboratory Animal Welfare (OLAW). KSU Animal Welfare Assurance Number is D16-00369 (A3609-01), and USDA Certificate Number is 48-R-0001. Kansas State University utilizes the United States Government Principles for the utilization and care of vertebrate animals used in testing, research, and training guidelines for appropriate animal use in a research and teaching setting.

Bacterial strains and growth conditions.

Clostridium difficile strains (Table 1) were grown anaerobically in TY agar (tryptose, yeast extract) or 70:30 medium as described previously [58]. Cefoxitin (Cef; 25 μg/ml), thiamphenicol (Thio; 15 μg/ml), and lincomycin (Lin; 15 μg/ml) were added to *C. difficile* cultures whenever necessary. Escherichia coli strains were grown in (LB) broth. E. coli strain S17-1, used for conjugation, was supplemented with ampicillin (100 μg/ml) or chloramphenicol (25 μg/ml) when indicated and cultured aerobically in LB broth.

Construction of R20291::*licB* and R20291::*cd2781* mutant strains.

The mutants were constructed in a *C. difficile* strain using a ClosTron gene knockout system. The group II intron insertion site was selected using the Perutka algorithm, a Web-based design tool available at http://www.clostron.com. The designed retargeted introns were cloned into pMTL007-CE5, and the resulting plasmids, pMTL007-CE5::Cdi-licB-141a and pMTL007-CE5::Cdi-*cd2781*, was transferred into *C. difficile* R20291 by conjugation as described previously[58]. The selection of thiamphenicol-resistant transconjugants in 15 µg·ml−1 lincomycin plates confers potential *Lactococcus lactis* ltrB (Ll.ltrB) insertions within the target genes in the chromosome of *C. difficile* R20291. The presence of putative mutants was identified by PCR using gene-specific primers (Table 1) in combination with the EBS universal primers. The R20291::*cd2781* was complemented by putting pRG381 plasmid harboring *cd2781* gene back into the mutant strain via conjugation.

Growth Comparison between the R20291, R20291::*licB* and R20291::*cd2781*

Cells were grown for 24 hours or longer in an anaerobic chamber in TY medium and cell density was measured by taking OD 600 values at every 4 hours using spectrophotometric methods. The experiments were conducted in three replicates. C. difficile strains were also grown in *C. difficile* Minimum Media where cellobiose or glucose was used as a sole source of carbon.

Measurement of cytosolic toxin level

Cytosolic toxins from 16h old *C. difficile* cultures grown in TY medium were measured as described previously [62]. In brief, one ml of *C. difficile* culture was harvested and suspended in 200 µl of sterile PBS, sonicated, and centrifuged to harvest the cytosolic protein. One hundred

micrograms of cytosolic proteins were used to measure the relative toxin levels using *C. difficile* premier Toxin A &B ELISA kit from Meridian Diagnostics Inc. (Cincinnati, OH).

Sporulation assay (Microscopic analysis)

C. difficile cultures were grown overnight in TY medium. Cells were then diluted in TY medium to an OD_{600} of 0.5, and then 300 µl was used to inoculate 30 ml of 70:30 sporulation media. Cultures were incubated at 37°C and monitored for the production of spores. Cells were harvested from the culture after 30 h and were suspended in TY medium for phase-contrast microscopy as described previously. At least six fields per strain were obtained, and the numbers of spores and vegetative cells were counted to calculate the percentage of spores based on the total numbers of spores and vegetative cells.

Reporter gene fusion assay

Approximately 600 bp of the upstream DNA regions of *licB*, or *cd2781* genes, along with their potential ribosomal-binding sites (RBS), were PCR amplified using specific primers with KpnI and SacI recognition sequences (Table 1) using R20291 chromosomal DNA as a template. Plasmid pRPF185 carries a *gusA* gene for β-glucuronidase under the tetracycline-inducible (*tet*) promoter [85]. Using KpnI and SacI digestion, we removed the *tet* promoter and replaced it with either *licB*, or *cd2781* upstream regions to create plasmids pRG382 and pRG383, respectively. Plasmids pRG382 and pRG383 were introduced into R20291 and R20291::*cd2781* mutant through conjugation as described above. The transconjugants were then grown in TY medium in the presence of appropriate antibiotics overnight. Overnight cultures were used as inocula at a 1:100 dilution to start a new culture. Bacterial cultures were harvested at every 4 hours of growth until 24 hours, and the amount of β-glucuronidase activity was assessed as described elsewhere [86]with

minor modifications. Briefly, the cells were washed, suspended in 0.8 ml of Z buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$ [pH 7.0], 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 50 mM 2-mercaptoethanol), and lysed using an equal amount of silica beads by bead beater method. The enzyme reaction was started by the addition of 0.16 ml of 6 mM *p*-nitrophenyl β-D-glucuronide (Sigma) to the broken cells and stopped by the addition of 0.4 ml of 1.0 M $NaCO_3$. The β-Glucuronidase activity was calculated as described earlier [20].

Western Blot Analysis

C. difficile cells for western blot analysis were harvested and washed in 1x PBS solution before suspending in sample buffer (Tris 80mM; SDS 2%; and Glycerol 10%) for sonication. Whole cell extracts were then heated at 100°C for 7 min and centrifuged at 17,000 g for 1 min, and the proteins were separated by SDS-PAGE and electro-blotted onto PVDF membrane. Immobilized proteins in the membranes were then probed with specific antibodies at a dilution of 1:10,000. The blot was subsequently probed with HRP-conjugated secondary antibodies at a dilution of 1:10000. Immuno-detection of proteins was performed with ECL Kit (Thermo Scientific) following the manufacturer's recommendations and were developed using G-Box.

Cloning, expression, and purification of CelR-6His proteins in *E. coli*

CelR proteins were overexpressed in Rosetta *E. coli* DE3 cells using pET16B expression system. The ORFs for cloning were PCR amplified from R20291 chromosome using gene-specific primers (Table 1), and the amplified gene fragments were then digested with Xho1 and BamH1 to clone into pET16B digested with the same enzymes. The resulting plasmid pRG384 was then transformed into *E. coli* Rosetta DE3 (Novagen) competent cells to obtain recombinant strains. To

overexpress CelR-6His protein, the *E. coli* recombinant strains were grown at 37°C in LB medium containing chloramphenicol (25µg ml-1) and ampicillin (100ug ml-1). Protein expression was achieved by inducing with 1mM IPTG at 17°C overnight. Cells were harvested by centrifugation, and the 6His-tagged proteins were purified by affinity chromatography on Ni++ agarose (Sigma-Aldrich) beads following the manufacturer's recommendations.

Electrophoretic Mobility Shift Assay

CelR binding was performed with radioactively labeled DNA probes. The *cd2781* upstream and the *licB* upstream regions were amplified using primers listed in Table 1, and the products were cloned into a pGEMT cloning vector. The region was then excised from the plasmid construct using *EcoRI* and was radiolabeled using Klenow fragment of DNA polymerase I (NEB. labs) and [α- ^{32} P] dGTP-6000 Ci/mmol (PerkinElmer Life Sciences). Binding experiments with radioactively labelled *cd2781* upstream DNA and *licB* upstream DNA with CelR-6His was performed using reaction buffer containing 10 mM Tris–HCl (pH 8.0), 0.1 mM DTT, 150 mM KCl, 0.5mM EDTA, 0.1% Triton X-100 and 12.5% glycerol. For binding experiments, proteins were mixed in the reaction buffer at a specified concentration and with the probe at last and were incubated at room temperature for 30 minutes before adding loading buffer. Reactions were loaded onto a 6% native polyacrylamide gel in 1XTBE (Tris/Borate/EDTA) and subjected to electrophoresis at 120 V for 90 minutes. Gels were then dried, and the autoradiography was performed with Molecular Dynamics Phosphor-Imager technology.

Hamster model for *C. difficile* pathogenesis

Male and female Syrian Golden hamsters (100–120 g) were used for *C. difficile* infection. Upon their arrival, fecal pellets were collected from all hamsters, homogenized in 1 ml saline, and

examined for *C. difficile* by plating on CCFA-TA (Cycloserine Cefoxitin Fructose Agar- 0.1% Taurocholate) to ensure that the animals did not harbor indigenous *C. difficile*. After this initial screen, they were housed individually in sterile cages with ad libitum access to food and water for the duration of the study. Hamsters were first gavaged with 30 mg/kg clindamycin[65]. *C. difficile* infection was initiated five days after clindamycin administration by gavage with vegetative cells. We used vegetative *C. difficile* cells because of the test strain produces very few spores if any spores. Bacterial inoculums were standardized and prepared immediately before challenge as described in our earlier study [66]. They were transported in independent 1.5 ml Eppendorf tubes to the vivarium using the Remel AnaeroPack system (one box for each strain) to maintain viability. Immediately before and after infecting the animal, a 10 µL sample of the inoculum was plated onto TY agar with cefoxitin to confirm the bacterial count and viability. There were three groups of animals, including the uninfected control group. Eight animals per group were used for the infection. Approximately, 2000 *C. difficile* vegetative cells of JIR strain and JIR::*licB* were used for the animal challenge. In the uninfected control (group 3), only four animals were used, and they received only antibiotics and sterile PBS. For the recurrent infection model study, surviving animals from the initial study ware retreated with clindamycin, to disrupt the natural gut microflora that has been reconstituted by that period and induce an artificial recurrent infection model. Animals were monitored for signs of disease (lethargy, poor fur coat, sunken eyes, hunched posture, and wet tail) every four hours (six times per day) throughout the study period. Hamsters were scored from 1 to 5 for the signs mentioned above (1-normal and 5-severe). Hamsters showing signs of severe disease (a cumulative score of 12 or above) were euthanized by CO2 asphyxiation. Surviving hamsters were euthanized 15 days after *C. difficile* infection. The cecal contents from these hamsters were collected in 15ml Nalgene tubes, secured airtight, and were transported to the

lab using Remel AnaeroPack system. They were then immediately subjected to CFU enumeration. For CFU enumeration, the daily fecal samples or the cecal contents collected post-mortem were resuspended in 1X PBS, serially diluted and plated onto CCFA agar with 0.1% Taurocholate (CCFA-TA). The CFU were counted after 48 h of incubation. The survival data of the challenged animals were graphed as Kaplan-Meier survival analyses and compared for statistical significance using the log-rank test using GraphPad Prism 7 software (GraphPad Software, San Diego, CA).

Results:

Cellobiose metabolism effects on *C. difficile* growth:

We analyzed the impact of Cellobiose PTS operon disruption on the growth of *C. difficile* in TY medium. In TY, *C. difficile* cells grew slightly slower in the exponential stage. At the onset of the stationary phase, the cells grew to a maximum OD600 of 1.4, which is comparable to the parent strain. However, growth impairment was observed during the stationary phase (Figure 13A). R20291::*licB* mutant was also grown in a minimum media where cellobiose was the only carbon source . The growth of the parent and mutant strains were compared using OD_{600} measurement at indicated timepoints. Our results indicated that R20291::*licB* mutants were unable to grow when cellobiose was the sole source of carbon, indicating the Cellobiose PTS operon is responsible for uptake and metabolism of Cellobiose (Figure 13B).

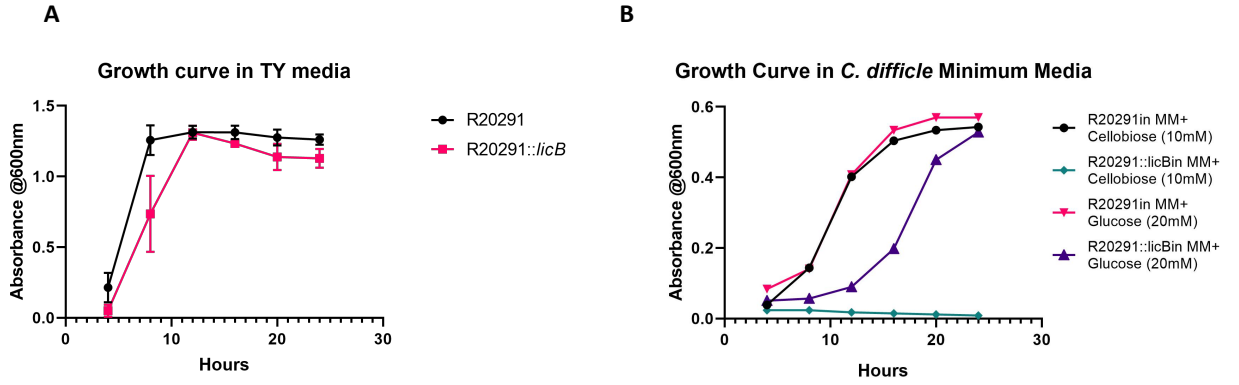


Figure 13 Growth comparison between R20291 and R20291::*licB* mutant.

A. *C. difficile* R20291 parent and R20291::*licB* mutant strains were grown in TY media. Cell growth was measured using spectrophotometer @600nm wavelength. B. *C. difficile* parent and R::*licB* mutant strains were grown in *C. difficile* minimum media with either cellobiose or glucose as sole carbohydrate source.

Cellobiose metabolism plays imporatant role in *C. difficile* virulence:

To understand the role of Cellobiose PTS operon in virulence, toxin and sporulation assays were conducted. We measured the intracellular toxin level using an ELISA based assay kit (Premier

Toxin by Meridian Bioscience, Catalog Number: 616096). The *licB* mutant showed a significant increase (P<0.05) in the intracellular toxin level compared to the parent cells (Figure 14A). A critical aspect of *C. difficile* pathogenesis is its capacity to produce highly resilient exospores that can survive in harsh physical and chemical treatment and causes the spreading of the disease. To evaluate the role of cellobiose metabolism in the spore production of *C. difficile* , we calculated the sporulation efficiency of the *licB* mutant and compared it with the parent strain. In 70/30 sporulation media after 48 hours, *licB* mutant produced a significantly lower number of exospore compared to the parent (P<0.0005)(Figure 14B).

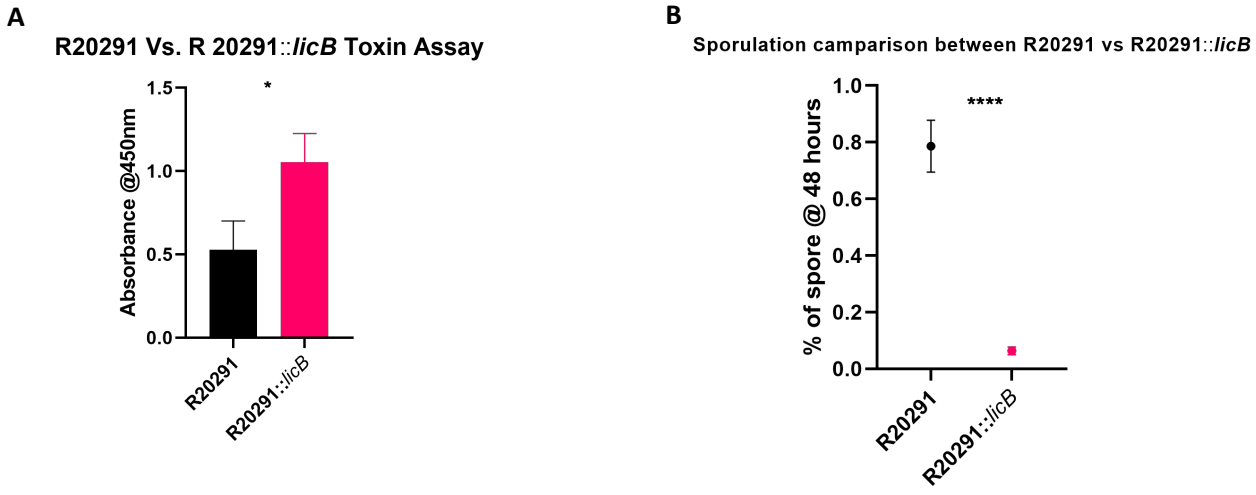


Figure 14 Toxin and sporulation by the cellobiose mutant.

A. Comparison of toxin production (measured using ELISA) in between R20291 parent and R20291::*licB* mutant. Data are expressed as average ± SD of three replicates. *denotes p<0.05; student two t-test. B. Percentage of spores (measured by counting the number of spores producing cells and vegetative cells) in R20291 parent and R20291::*licB* mutant. Data are expressed as average ± SD of four replicates. **** is p<0.00005; students two t-test.

Cellobiose operon transcription is regulated by CelR, a novel GntR class regulator:

Immediately upstream of the cellobiose operon is the putative GntR type regulator CD2781. We hypothesized that because of the immediate vicinity of the cellobiose operon and the fact that it is

a GntR type regulator [87], it has the potential to be a regulator for cellobiose operon transcription. To test our hypothesis, we created a CD2781 disruption mutant in the *C. difficile* R20291 background. We created a promoter fusion in pRPF185 plasmid where the *tet* promoter of *gusA* gene was replaced by the promoter region of cellobiose operon and conducted reporter activity assay (Beta-glucuronidase assay). The results indicated significantly increased level of promoter activity in the R20291::*cd2781* mutant indicating that the cellobiose operon in parent strain is being repressed by CD2781 (P<0.001) (Figure 15). The first gene of the cellobiose operon in R20291 is named as *licB* indicating it as a lichenin uptake operon. However, in the *C. difficile* 630 strain genome (GenBank: AM180355.1) it is annotated as a *celB*, a cellobiose PTS component gene. When we conducted beta glucuronidase assay with the strains grown in the presence of either cellobiose or lichenin, we found that only cellobiose (Figure 16A) but not lichenin (Figure 16B) relieved the repression of the operon, indicating that the PTS operon is indeed a cellobiose PTS operon in the R20291 strain.

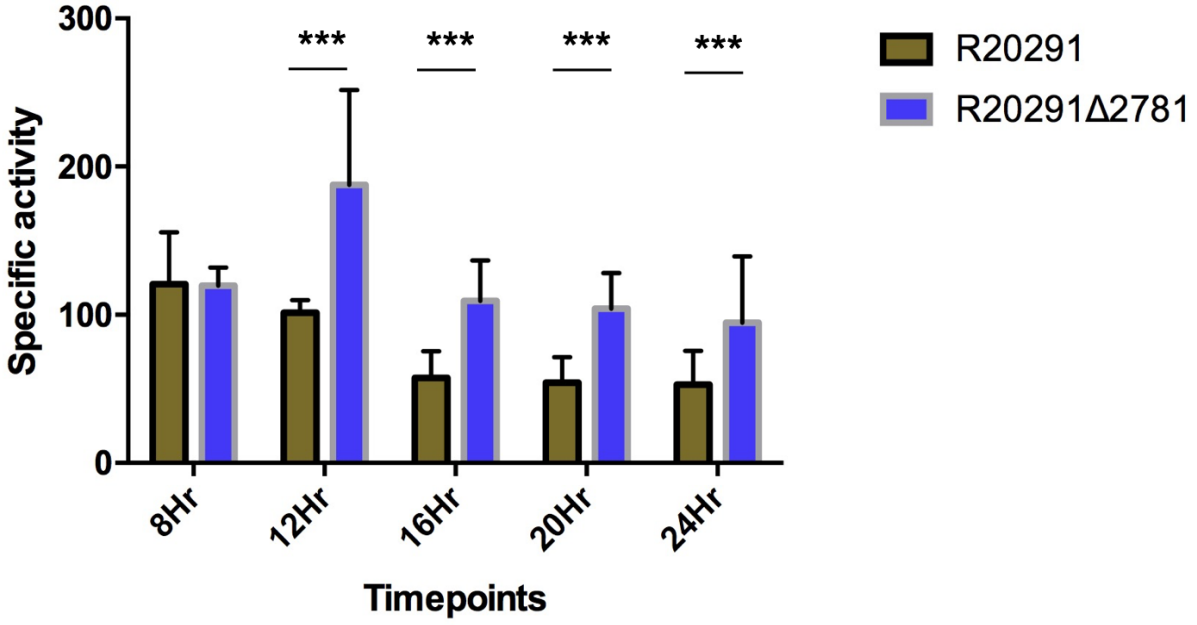


Figure 15 Beta glucuronidase assay of promoter region of cellobiose operon fused with *gusA* of pRPF185 in R20291::*cd2781* mutant.

Reporter fusion assay with the promoter of cellobiose PTS operon. The tet promoter regulating the gusA expression in pRPF185 was replaced by the 200bp upstream region of cellobiose operon. Level of activity was measured by beta glucuronidase assay and calculated as miller units. Significantly higher level of specific activity in mutant indicates that CD2781 is a repressor for cellobiose operons expression (***p<0.001, paired t-Test).

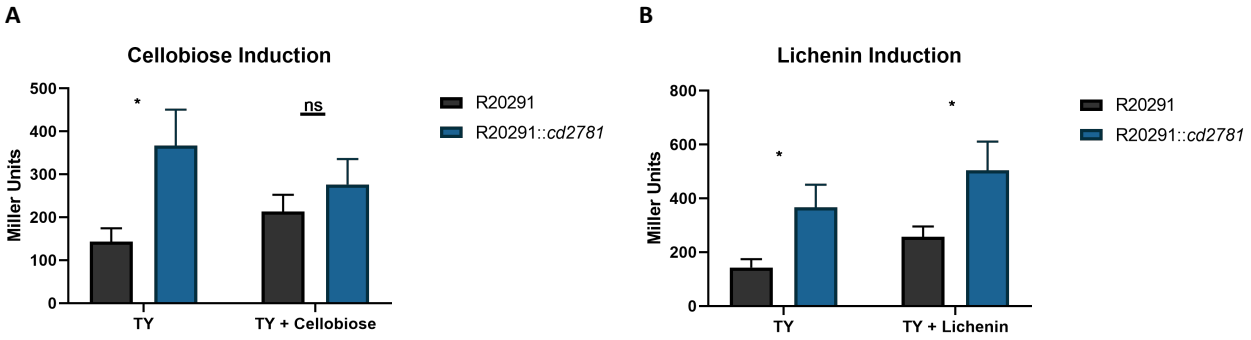


Figure 16 Lichenin and cellobiose induction of the beta glucuronidase assay.

Supplementation of TY growth media by 0.05% cellobiose causes the reporter activity level to be similar between the parent and mutant. Indicating that the repression of the operon is relived when cellobiose is available (A)(p=0.2, paired t-Test). In contrast supplementation of growth media by 0.2% lichenin was unable to relieve the repression (B) (* p<0.05, paired t-Test) . These results indicate that the putative carbohydrate PTS system is used for uptake and utilization of cellobiose.

CelR autoregulates its transcription:

CelR, the transcriptional regulator of the cellobiose PTS operon, also regulates its own expression in a suppressive manner. We constructed a promoter fusion as described in the earlier section with the promoter region of the *celR* gene and conducted a reporter activity assay. Results show a significant increase of activity in the *celR* mutant, which indicates that CelR also represses its own transcription besides the cellobiose operon (Figure 17A). Unlike the cellobiose operon, the repression of CelR on its own transcription could not be relieved by the addition of cellobiose in the growth media (Figure 17B).

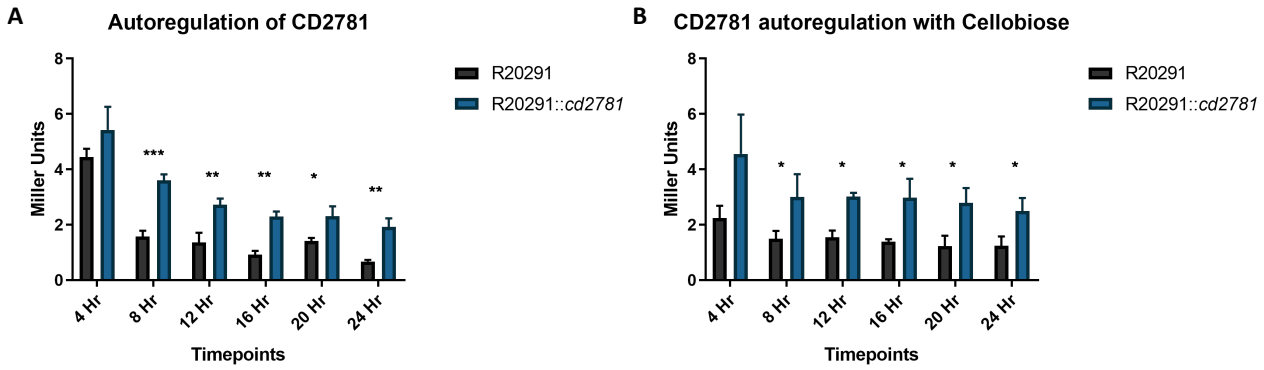


Figure 17 Autoregulation of CD2781 by beta glucuronidase assay.

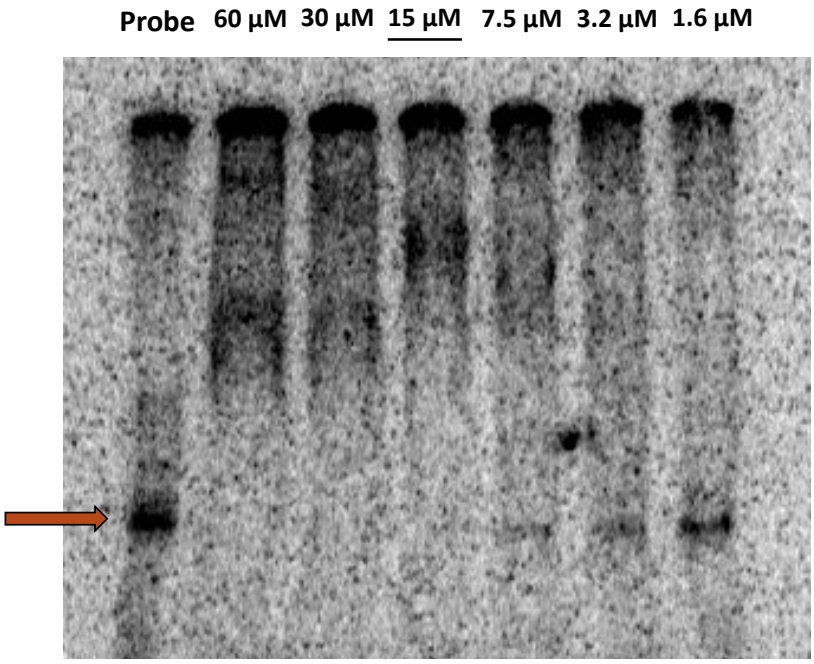
Reporter fusion assay with the promoter region of R20291_*cd2781*.A. Significantly higher level of specific activity (in Miller Unit) in R20291::*cd2781* mutant indicates that CelR represses and thus autoregulates its own expression *=P<0.01,**=P<0.001, ***=P<0.0001 using students t-test. B. Supplementation of growth media by 0.05% cellobiose did not relieve the repression *=P<0.01 using students t-test. However, the CelR promoter activity is extremely low when compared to the cellobiose PTS operon promoter. In such case, any subtle difference induced by the cellobiose could easily be left unidentified.

CelR mediated transcriptional repression of cellobiose operon, and CelR itself is the result of direct binding of CelR with the promoter regions:

We hypothesized that the observed transcriptional repression of cellobiose PTS operon by CelR is the result of direct binding of the CelR protein with the upstream regulatory region of the cellobiose PTS operon. To test the hypothesis, we carried out EMSA (Electrophoretic Mobility Shift Assay) experiments with purified 6-His-tagged CelR protein and radioactively labeled promoter region of the cellobiose operon. The upward shift of the probe band when CelR was added to the reaction mix indicates that CelR directly binds with cellobiose operons promoter region at a concentration equivalent of 7.5 μM (Figure 18A).

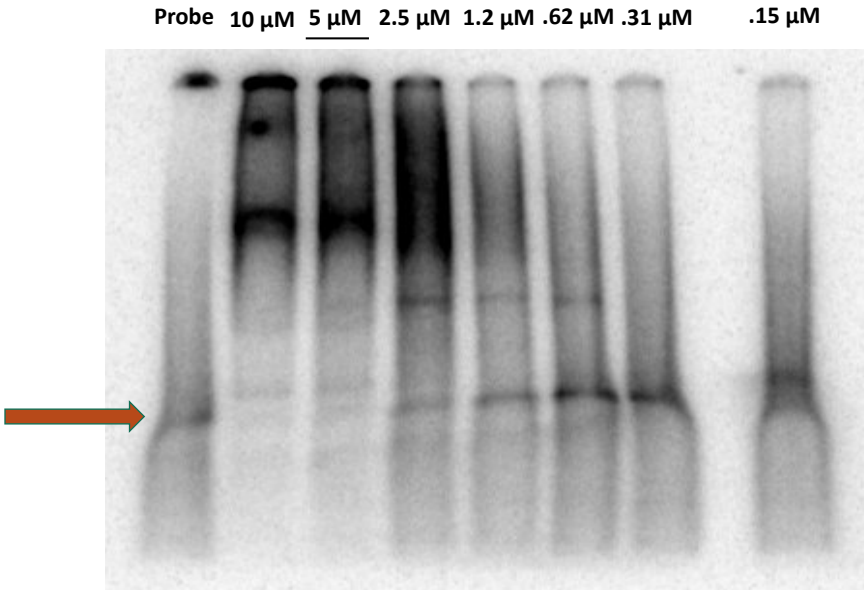
A

Promoter region of Cellobiose PTS operon EMSA with the CelR protein



B

Promoter region of *CelR* EMSA with the CelR protien



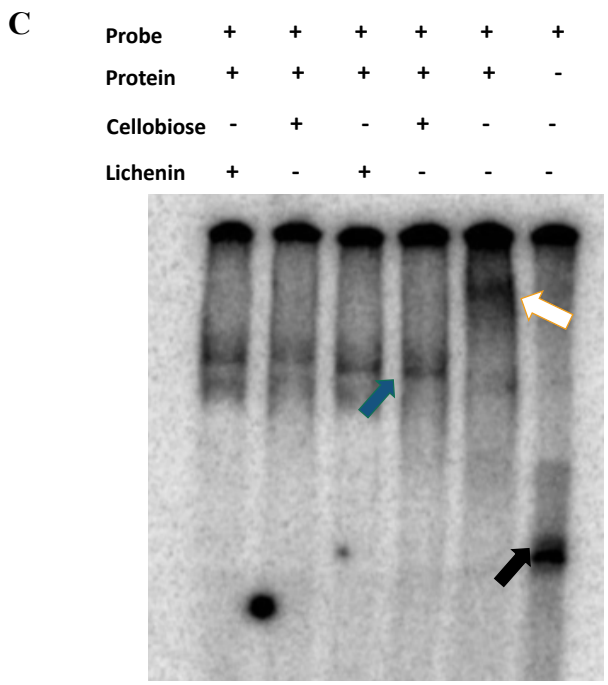


Figure 18 EMSA experiments of CelR protein with promoter region of both cellobiose PTS operon and *celR*.

EMSA (Electrophoretic Mobility Shift Assay) was conducted using p32 radiolabeled probe and purified 6His tagged CelR. Shift of the DNA band was observed for the promoter region of the cellobiose PTS operon (A), and promoter region of R20291_*cd2781* (B). Probe bands are indicated with red arrow and the minimum concentration of protein needed for complete shift is indicated by black underline. (C) Addition of cellobiose (200mM) and lichenin (10mM) to the EMSA binding buffer did not cause a complete release of the protein from probe. But the band shifted (black arrow indicates probe only band) to a lower level (purple arrow) than the original shift (white arrowhead).

CelR also directly binds to the promoter region of the *celR* gene which, suggest that CelR binding with the upstream region could be the mechanism of CelR autoregulation. The complete shift of the band can be observed as low as 5 μM concentrations (Figure 18 B). EMSA was also conducted in the presence of different concentrations of cellobiose and lichenin. However, shifts on the bands were still observed, albeit in a different position (Figure 18C). We hypothesize that since the sugar molecules up taken by PTS system is immediately phosphorylated during entry, the substrate for CelR could be phosphorylated cellobiose, or it could also be any of the byproducts of cellobiose catabolism.

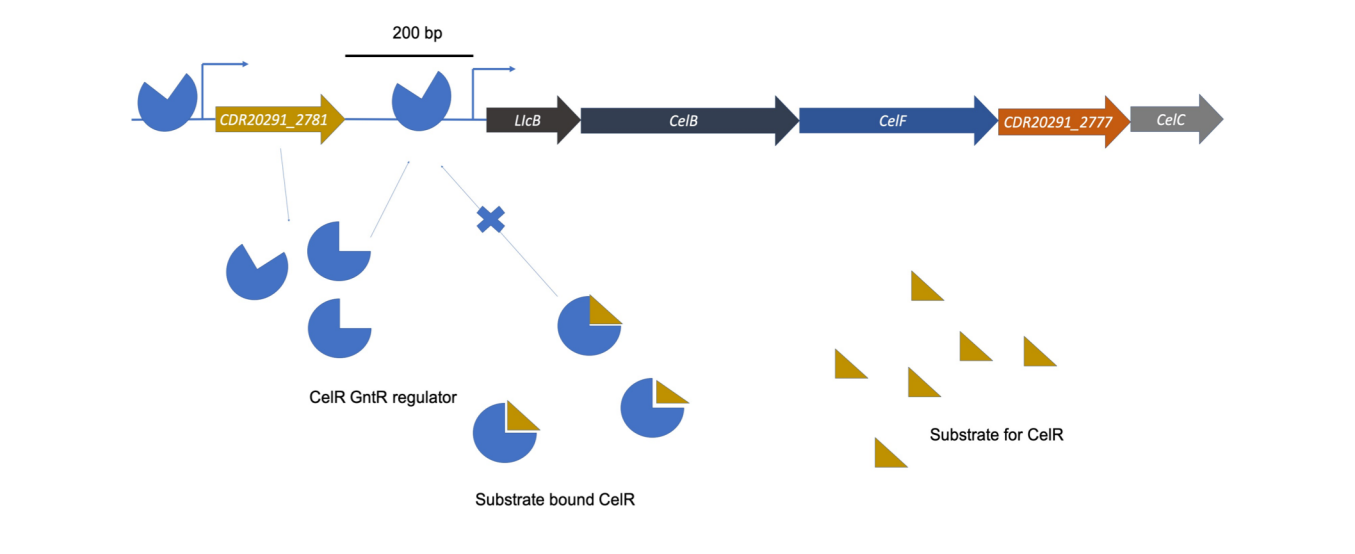


Figure 19 Schematic model of CelR regulation of Cellobiose operon.

A schematic model of cellobiose PTS operon regulation by GntR type regulator CelR (CD2781). CelR binds with the promoter region of Cellobiose PTS operon and represses its expression. Extracellular cellobiose enters the cell and gets phosphorylated. Either the phosphorylated cellobiose or downstream metabolic intermediates binds with the substrate binding domain of CelR. Substrate bound CelR causes the DNA Binding Domain to lose its affinity from the target region and the repression is relieved.

C. difficile growth is unaffected by CelR mutation:

We analyzed the impact of the *celR* mutation in *C. difficile* growth. *C. difficile* strains were grown anaerobically in TY medium, and growth was recorded by measuring OD600 values at the indicated timepoints. There was no significant difference in growth dynamics between the parent and the mutant strain when grown in TY media (Figure 20A). The parent and the mutant strain were also grown in *C. difficile* Minimum Media (CDMM) where either cellobiose or glucose was used as a sole carbon source. Both the parent and *celR* mutant strains grew comparably in CDMM, too (Figure 20B).

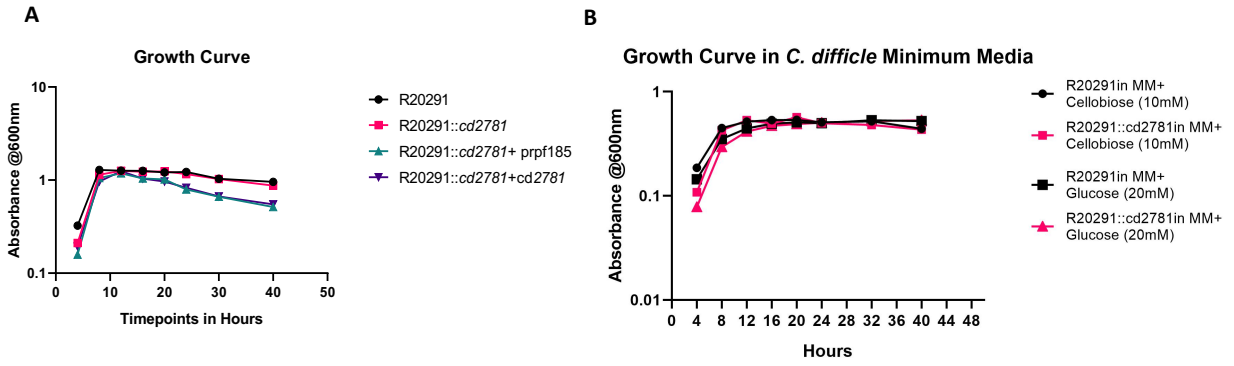


Figure 20 Growth comparison between R20291 parent strain and R20291::*cd2781 mutant.*

A. *C. difficile* R20291 parent and R20291::cd2781 mutant strains were grown in TY media anaerobically. Cell growth was measured at indicated time points using spectrophotometer @600nm wavelength. No significant growth difference is observed between the parent and mutant strain. However, both the strains, when carrying the pRPF185 plasmid showed a slight disadvantage in maintaining stationary phase. B. *C. difficile* R20291 parent and R20291::cd2781 mutant strains were grown in *C. difficile* Minimum Media with either cellobiose or glucose as sole carbohydrate source.

C. difficile celR mutants shows a significant increase in virulence:

Besides regulating the cellobiose PTS operon, CelR has a significant role in the regulation of the virulence properties of the *C. difficile*. We conducted toxin assay to measure the intracellular level of toxin in *C. difficile* and found that the CelR mutant expresses a significantly higher level of extracellular toxin compared to the parent strain (Figure 21C). Production of exospores is a hallmark of *C. difficile* infection and contributes to the challenging nature of controlling *C. difficile* infection. We measured the sporulation efficiency of both the *celR* mutant and complemented strains with the wild type R20291 strain and found that *celR* mutant produces a significantly higher number of spores compared to the parent after 24 hours of growth (Figure 21B). We complemented the CelR mutant by expressing CelR from the inducible *tet* promoter of pRPF185. The complemented mutant's toxin level went down to the level of parent R20291 strain (Figure 21C). More interestingly, the complemented strains sporulation level went down significantly reaching the level of R20291::*licB* (Figure 21B).

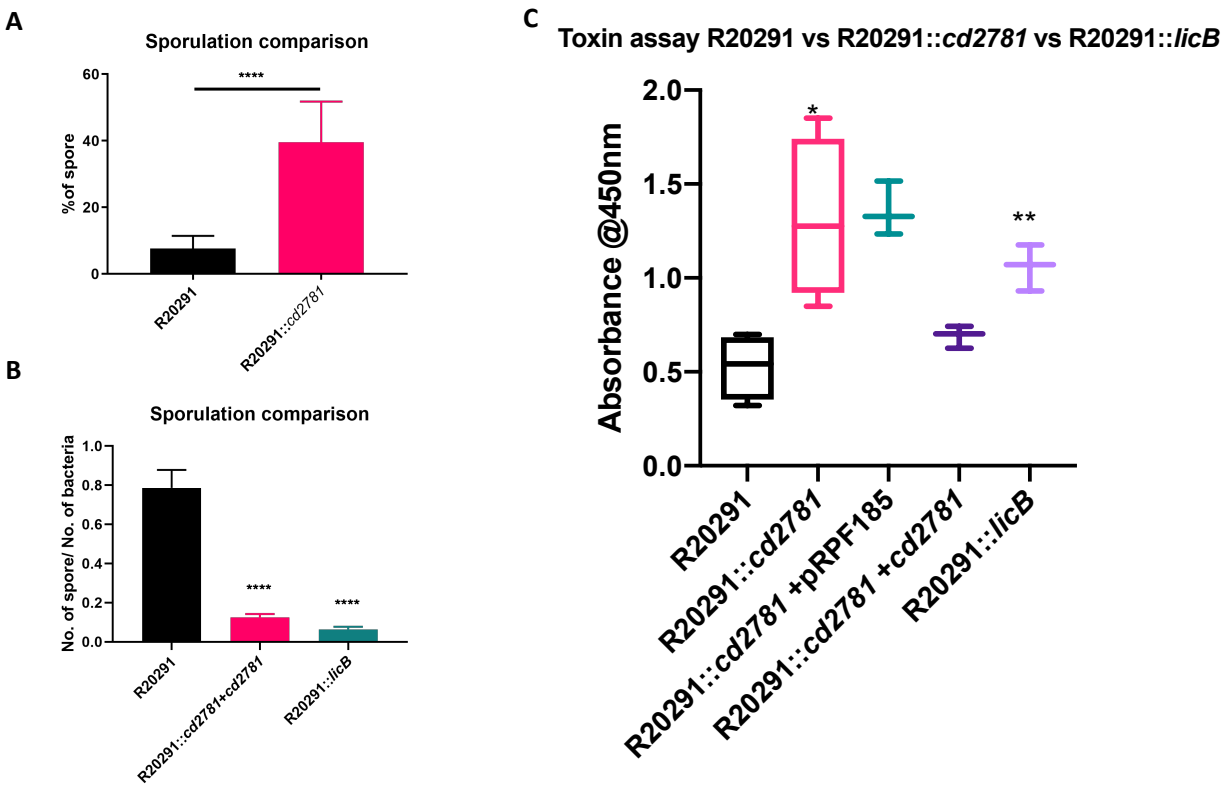


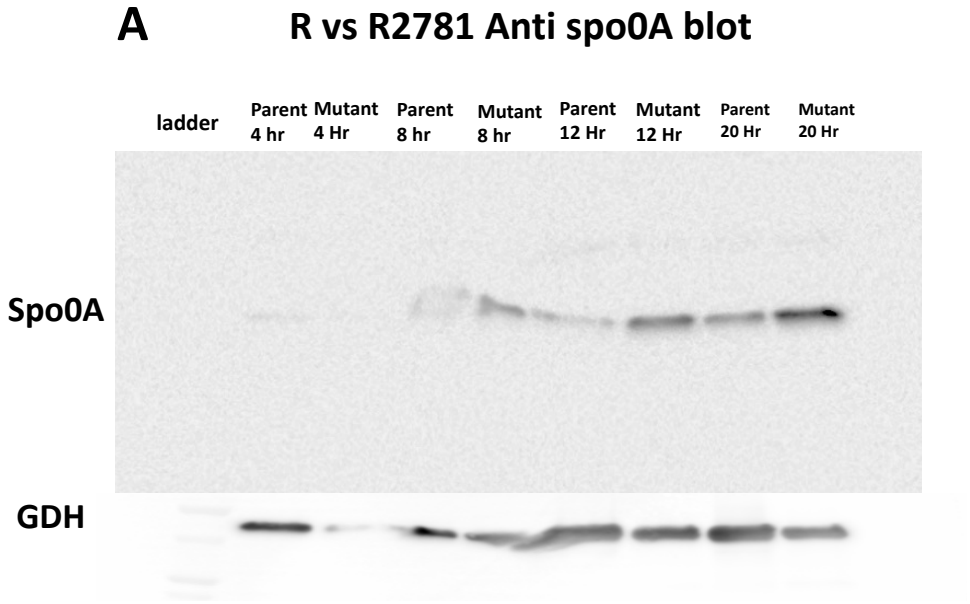
Figure 21 Virulence factor levels of R20291, R20291::*celR*, and R20291::celR+prpF185-celR.

A. Sporulation efficiency is significantly higher in the CelR mutant compared to parent after 24 hours of growth in 70/30 Sporulation medium. ***=<0.0005 students two t-test . B. Complementation of the mutant reduces the sporulation significantly when grown in 70/30 sporulation medium for 48 hours. The level of reduction is close to comparable level of Cellobiose mutant indicating a strong repression of the operon. C. Level of toxin produced by the bacteria by Toxin ELISA indicates that CelR mutants have significantly higher level of toxin compared to the parent R20291 strain. Complementation of the mutant with plasmid derived CelR brings the toxin production down to R20291 level. *P=<0.01, **=P<0.001 using student's t test. The level of toxin produced by the *licB* mutant is also shown for reference.

CelR modulates *C. difficile* virulence by alternating the expression of multiple virulence regulators:

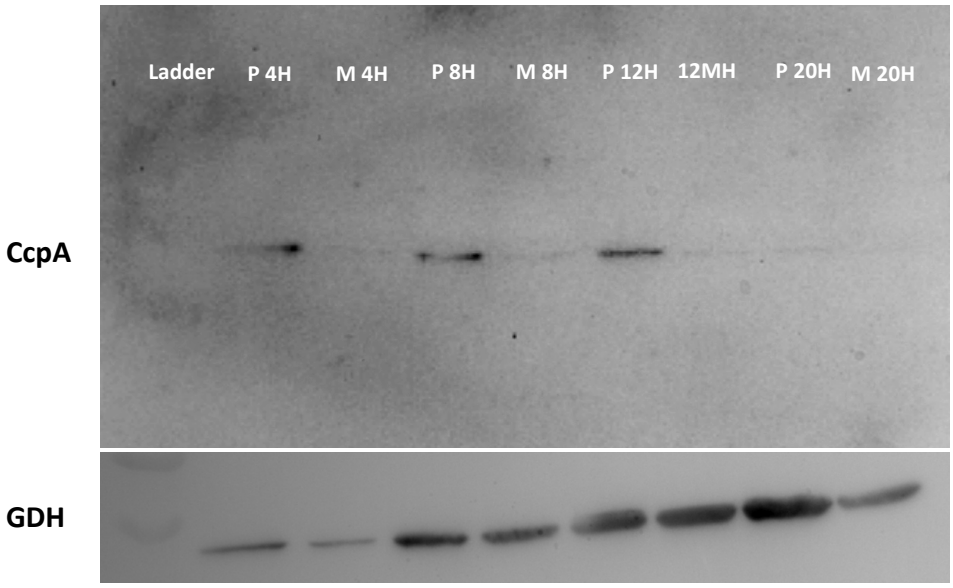
To understand the underlying molecular mechanism of the CelR's role in virulence, the expression level of the virulence master regulators was measured using both qRT-PCR and Western blotting.

Corroborating with our sporulation assay, level of Spo0A, the master regulator of sporulation was higher in the mutant compared to the parent (Figure 22A). Also, the level of CcpA, a global regulator for carbohydrate metabolism and also a negative regulator for virulence factor was lower in the CelR mutant when compared to the parent (Figure 22B). The level of CodY in *C. difficile* cannot be directly linked with the observed phenotype as CodY activity is dependent upon its ligand binding status and such challenging to measure in the western blot (Figure 22C). qRT PCR analysis of the level of expression of different virulence factor shows a significantly higher level of *spo0A*, *tcdR*, and *codY*.



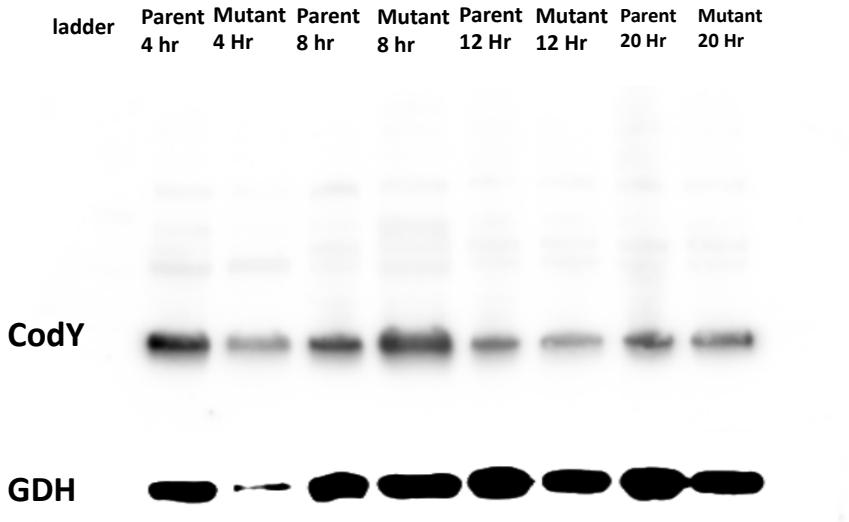
B

Anti-CcpA western blot for R20291 and R20291::*cd2781*



C

R vs R CD2781 Anti codY blot



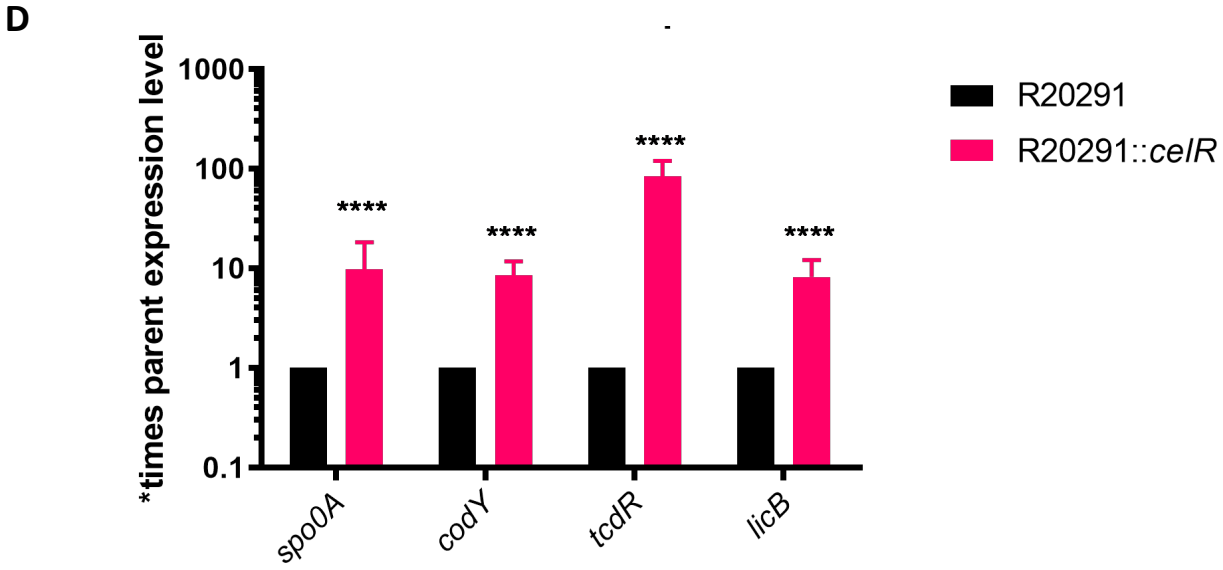


Figure 22 Analysis of major *C. difficile* virulence factor regulators in CelR mutant.

A. Higher level of Spo0A corresponds to the higher sporulation percentage in the mutant. B. In contrast a lower level of CcpA is observed which can account for the hypervirulence since CcpA is also a negative regulator of major virulence factors. C. Level of CodY doesn't have any particular pattern of expression or differences. D. qRT- PCR results of the mutant after 20 Hour growth for spo0A (sporulation regulator), codY (global transcriptional regulator repressor for virulence factor), tcdR (regulator for toxin production) indicates a general trend of overexpression of these genes corroborating with the observed phenotypes. ****=P<.00001 using Student's t-test.

Cellobiose PTS operon is essential for virulence and pathogenesis in both the initial and recurrent infections in hamsters:

Since our *in vitro* assays indicated that Cellobiose PTS operon is responsible for a significant difference in *C. difficile* virulence level, we decided to determine its role in virulence and pathogenesis of *C. difficile in vivo*. Golden Syrian hamsters were gavaged with 2000 *C. difficile* R20291 and R20291::*licB* and monitored for infections for 10 days. Three animals out of eight in the parent group and one animal out of eight of the mutant group died by the end of the study period. Our results showed that *R20291::licB* mutant is less pathogenic in *in vivo* than the parent strain (Figure 23A). The surviving hamsters were given clindamycin again to disrupt the gut microbiota and observed for the occurrence of the second round of infection mimicking a recurrent

infection in humans. None of the animals infected with *licB* mutant strain had a recurrent infection, whereas all of the animals infected with the parent strain got the recurrent infection and had to be euthanized before the end of the study period (Figure 23B). These results demonstrate that cellobiose PTS operon plays a vital role in *C. difficile*'s ability to cause recurrent infection.

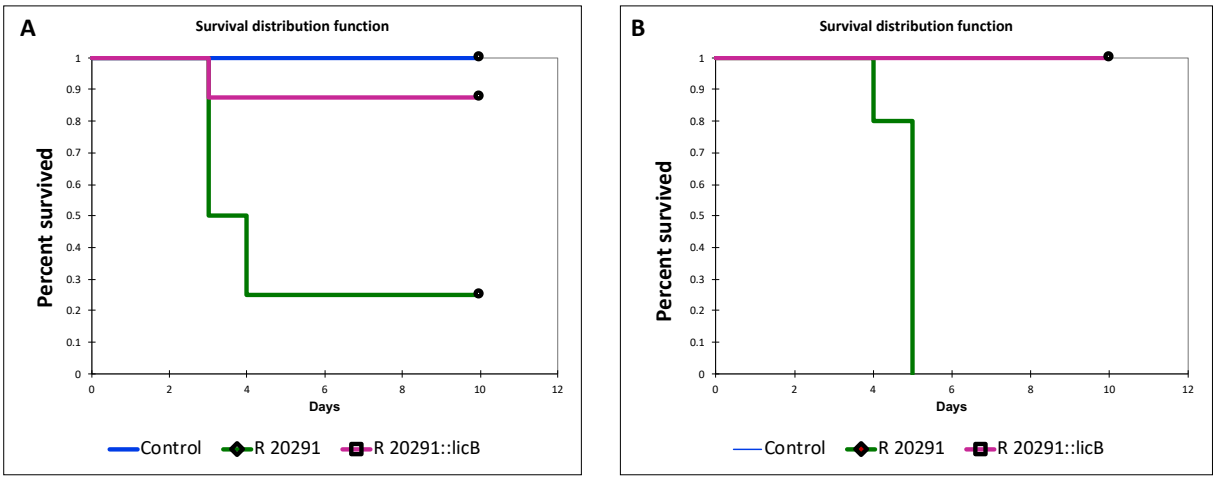


Figure 23 Kaplan-Meier survival analysis of parent and cellobiose mutant strain infected hamsters.

A. Kaplan-Meier survival curve of clindamycin-treated Syrian golden hamsters inoculated with 2,000 vegetative cells of *C. difficile* R20291 (n = 8) or R20291::*licB* mutant (n = 8). Six animals were used as an uninfected control. Animals were monitored every four hours for the symptoms of lethargy, poor fur coat, wet tail or hunched posture. Moribund animals were euthanized, and log-rank statistical analysis was performed. B. Kaplan-Meier survival curve of the Syrian golden hamsters in a recurrent infection study model R20291 (n = 5) or R20291::*licB* mutant (n = 7). six animals were used as an uninfected control. Animals were retreated with clindamycin leading to artificial induction of a recurrent infection. $p<0.001$ in log-rank.

Discussion

Regulation of virulence genes in bacteria is critical since they are generally a highly demanding process in term of energy expenditure. As such they are under intricate control by many regulators. Virulence factor expression is regulated by taking a lot of internal and external factors into consideration. Availability and utilization capability of certain nutrients in the environment can affect a pathogens propensity for upregulating or downregulating its virulence factors.

A healthy human diet contains a large amount of plant-derived parts and vegetables that are rich in Cellulose, which gets converted to cellobiose by different commensal bacteria. Although *C. difficile* primarily relies on Stickland metabolism pathway for nutrient requirements [88], the capability to utilize alternative nutrient source such as cellobiose which is abundant in the colon is undoubtedly beneficial as our results have demonstrated that disruption of the cellobiose PTS operon significantly alters the virulence properties of the bacteria.

When compared to the parent strain, the *C. difficile licB* mutant has a significantly higher level of toxin. This phenomenon can be explained by the principle of nutrient starvation and Carbon Catabolite Repression (CCR). Inability to uptake and utilize a significant source of carbohydrate can simulate a starvation condition in the cell and ultimately lead to increased toxin production. Another potential explanation could be the regulation via PEP-Hpr-Cellobiose-6P, in which the reduced input of cellobiose results in reduced Glucose-6-Phosphate. Reduced quantity of Glucose-6P could lead to a reduced level of fructose bisphosphate (FBP). FBP positively regulates CcpA activity by upregulating the level of phosphorylated Hpr [43]and as such, reduced

FBP causes reduced activity of CcpA in the *licB* mutant, which could result in derepression of TcdR, leading to increased toxin production.

Although the *licB* mutant had an increased level of toxin, we saw a significant decrease in spore production, which like the *glgC* mutant, indicates in *C. difficile*, toxin production and sporulation could be differentially regulated. When compared to the parent strain, the cellobiose mutant also had a slight growth disadvantage in the initial lag phase but eventually reached growth comparable to parent at log phase which again dropped during the stationary phase indicating that cellobiose PTS operon plays a role in maintaining optimum growth of the bacteria. Our animal model study indicates that cellobiose uptake is important for the bacteria to cause pathogenesis in hamsters. In the case of our recurrent infection model study, the cellobiose mutant was significantly attenuated, indicating again that cellobiose uptake is important for inducing recurrent infection also. This could also be because of the significantly lower amount of spore the cellobiose mutant produces which could be cleared out much faster in *in vivo* condition.

Since cellobiose PTS operon plays an important role in *C. difficile* virulence, it is imperative to understand how the cellobiose PTS operon is regulated in *C. difficile*. Previous studies have indicated the importance of these operon regulators in virulence of the pathogen. For example, the recent study on the effect of trehalose in *C. difficile* virulence showed a mutation in the trehalose uptake regulator TreR is responsible for increased virulence in *C. difficile*[80]. Also, another study in *S. scabies* and its cellobiose PTS regulator CebR shows that the regulator has a profound effect on various virulence properties of the bacteria. We identified the CD2781 as a regulator for cellobiose PTS operon and named it as CelR. Our results suggested that when CelR

is disrupted, the expression of cellobiose PTS operon is increased, indicating CelR is a repressor of the operon. Also, our EMSA binding assay shows that CelR can directly bind to the upstream region of Cellobiose PTS operon and potentially repressing its expression by physically blocking the upstream region of the cellobiose PTS operon to transcriptional machinery. We have also found that CelR not only represses the Cellobiose PTS operon but also its expression in a similar manner, and we are suggesting a model of CelR mediated regulation of Cellobiose PTS operon (Figure 19). When additional cellobiose was added to the growth media, the repression of CelR on Cellobiose PTS operon was relieved, but not its expression. This signifies, CelR regulation of its own expression might be independent of direct substrate binding, or a different intracellular energy molecule might be used as its substrate.

In case of the growth of *C. difficile in vitro*, our results suggest that *celR* mutation has no significant effect in *C. difficile* growth, which might indicate that the overexpression of the Cellobiose PTS operon does not provide any additional benefit to the growth. This might be very different in *in vivo* condition because we currently do not have enough data on cellobiose availability and metabolism dynamics in the human gut. However, when it comes to the level of virulence factors in the *celR* mutant, we observed significant upregulation of the virulence factor levels. The *celR* mutant shows hypertoxic and hyper sporulating phenotypes and complementing the mutant with CelR expressed under an inducible promoter reverts its virulence factors levels to the parent strain. Especially in case of the sporulation, the level of spores produced by the complemented *celR* mutant, went down to the level of *licB* mutant, indicating stronger repression than wild type and thus resemblance to the *licB* mutant phenotype. In line with what is seen in the

previous work with trehalose uptake and virulence in *C. difficile*[80], increased Cellobiose PTS operon expression by disruption of its repressor CelR turns the pathogen into a hypervirulent strain.

The exact regulatory pathways by which *celR* mutants increased virulence is exerted still needs to be determined. However, our qRT-PCR and Western blot results indicate that the level of different virulence master regulators in the *celR* mutant corresponds with our observed virulence phenotypes. We observed an increased level of Spo0A –sporulation master regulator, TcdR- a sigma factor positively regulating toxin genes expression, and decreased level of CcpA- a carbohydrate metabolism master regulation with well-documented negative regulation of virulence factors. In the classical CCR phenomenon, the increased uptake of an additional source of carbohydrate should lead to decreased virulence in the pathogen. However, we see a reversal of CCR phenomenon in case of the *celR* mutant. Overall this profound significance of Cellobiose PTS operon in the *C. difficile* virulence factor is puzzling as *C. difficile* majorly uses Stickland metabolism for most of its energy requirement, and the observed phenomenon goes against the traditional CCR mediated virulence suppression. Nevertheless, this work demonstrates that availability and of complex carbohydrate in host gut, and its metabolism inside *C. difficile*, plays a significant role in the virulence and pathogenesis of this bacteria in a rather metabolically unconventional way.

Chapter 4 - Conclusion and Future Studies

Clostridiodes difficile infection is a significant healthcare problem in North America [89]. *C. difficile* generally causes infection when the healthy gut microbiota is disrupted by antibiotic treatment. *C. difficile* is also one of the most common nosocomial infection primarily because of the spores of the bacteria can spread quickly from patient to patient and also because of many patients being treated with antibiotics for various other diseases. Once colonized, *C. difficile* releases exotoxin Toxin A and Toxin B, which target gut epithelial cells and leads to infection that can develop into severe enteric colitis if untreated. Around half a million people get *C. difficile* infection annually, out of which around 30000 die within the first 30 days of infection[6]. Although *C. difficile* infection is treatable using antibiotics such as vancomycin, the global rise of antibiotic resistance has become a significant threat, and some records of increased resistance to antibiotics have already been documented for *C. difficile* [90]. As such, identifying the factors and understanding the mechanisms of virulence and pathogenesis of the bacteria is essential to develop antimicrobial agents, treatment and prevention strategies for *C. difficile* infection.

Microorganisms are the most efficient nutrient harvesters. They can sequester and utilize a vast array of different micro and macromolecules to grow and thrive in different conditions. Carbohydrates are relatively abundant and are high energy low input nutrients. Many pathogenic bacteria rely primarily on carbohydrates for energy requirements. *C. difficile* primarily prefers amino acids as a nutrient source but given the diversity of human diet and also the complexity of gut microbial community, carbohydrate metabolism may play a significant role in *C. difficile* growth, colonization, and potentially virulence and pathogenesis. There is a significant body of

research on other pathogens demonstrating the role of carbohydrate metabolism in the pathogenesis and virulence. In *C. difficile*, the role of complex carbohydrate metabolism in virulence factors such as toxin release and sporulation is mostly unexplored. In this study, we investigated the role of glycogen- a major storage carbohydrate, and Cellobiose- an abundant plant-based carbohydrate in the human gut, in their role on *C. difficile* virulence.

Glycogen is a common form of storage carbohydrate utilized by a host of a different prokaryotic organism and eukaryotic cells. Glycogen is accumulated during the period when there is excess energy present and utilized when the cells are in nutrient-limiting condition and thus works as an energy buffer. Besides its role as a storage carbohydrate, glycogen has also been documented to have a role in virulence factors such as gut retention, stress tolerance, and especially sporulation in different pathogens. Our study shows that *C. difficile* glycogen biosynthesis is not essential for growth in rich media. However, the glycogen mutant grew faster when compared to parent strain in rich media. Accumulation of glycogen probably causes the bacteria to ration a significant portion of available glucose for glycogen production, which could have been used for growth immediately. Also, the energy required for the synthesis of glycogen is saved by the *glgC* mutant. This could explain the faster growth of the mutant strain. Also, the lack of significant difference in viable cells at the late stages of growth curve indicates that in *C. difficile* glycogen is not the primary resource for maintaining cell viability at least in *in vitro* condition.

C. difficile glycogen mutant has significantly shorter cell length signifying the role of glycogen metabolism in cell morphology. TEM imaging demonstrating the presence of potential glycogen granules inside *C. difficile* cells and the amyloglucosidase assay results together provides evidence

of glycogen accumulation inside *C. difficile* cells. *glgC* mutants produced a significantly higher amount of exotoxin compared to the parent strain, providing the first evidence of glycogens role in *C. difficile* virulence. When compared to the parent strain, glycogen mutants were significantly less sporulating, indicating accumulation of glycogen is important for sporulation in *C. difficile*. This phenomenon is also documented in *Bacillus subtilis*, a closely related spore-forming, Gram-positive bacteria. We used qRT-PCR and Western blot techniques to quantify the difference of expression of major virulence factor regulators of *C. difficile*. Corroborating with our ELISA based toxin assay, we saw a higher level of *tcdR* expression in the glycogen mutant. Also, the level of *spo0A*, a positive regulator for sporulation, was significantly downregulated in the *glgC* mutant. CcpA is known to be a repressor of virulence factors such as toxin production and sporulation in *C. difficile*. Our results showed that although the glycogen mutant had a significantly higher level of toxin, the level of *ccpA* was significantly higher in glycogen mutants. This signifies that in *C. difficile*, toxin production and sporulation could be regulated separately from each other, and probably a much more complex interaction between these pathways is present.

In vivo, the glycogen mutants showed a higher level of pathogenicity compared to the parent strains. This could be attributed to two factors. Our *in vitro* results showed that the glycogen mutant is both faster growing and hypertoxic compared to the parent, which in turn can account for the higher level of pathogenicity in the hamsters challenged with the glycogen mutant. This also implies that the glycogen is not essential for colonization and pathogenesis in an *in vivo* condition. We hypothesize that the role of glycogen in colonization might be more pronounced in normal conditions when the bacteria has to compete with other commensals for nutrients. Our animal

models get clindamycin treatment to remove the existing gut bacteria, and thus *C. difficile* had very little competition while colonizing the hamster gut.

All our findings indicate that in *C. difficile,* glycogen might be more relevant as energy storage for sporulation process than growth, and accumulation of glycogen could be a checkpoint before the bacteria commits to sporulation. Our hypothesis for the increased toxin production is, in parent strain, the degradation of glycogen causes the release of glucose 1-P which increases the intracellular level of glucose and thus increases the CcpA mediated repression by the production of excess Fructose Bis Phosphate. Thus, the level of toxin and sporulation is kept in check. In the mutant strain, however, the lack of glycogen leads to lack of glucose 1-P production, which leads to less FBP, which in turn causes reduction of CcpA activity and increase of virulence gene expression. Which, in turn, leads to a higher amount of toxin production. However, since the *glgC* mutant has no glycogen stored to drive the sporulation process, we observe a significantly low level of sporulation.

To test this hypothesis, *C. difficile* double mutants with disrupted glycogen operon in the hyper sporulating background could be used to test whether the bacteria can still produce viable spores or not. The glycogen operon is documented to be regulated by CodY, a global transcriptional repressor of virulence gene. Further studies should be conducted to identify other potential regulators for the operon's expression. Also, the role of CcpA, and how CcpA mediated regulation of virulence is connected to glycogen accumulation needs to be explored to get a complete picture of glycogen operon regulation. Another critical aspect of glycogen metabolism is the dynamics of the accumulation and degradation of glycogen in *C. difficile*. Specifically, identifying the growth

stage at which the glycogen is accumulated and degraded and how is it temporally coordinated with sporulation initiation is necessary to identify the role of glycogen in *C. difficile*.

The human diet is diverse and constitutes of different types of nutrients. Of all the available carbohydrate nutrients, the human digestive system can absorb only a fraction of it directly, and the rest is processed and utilized mainly by the commensals. Commensals play a vital role in harvesting the nutrients available and convert them into micro and macromolecules that are up taken both by the host and the organisms[91–94].

The human digestive system is quite efficient when it comes to absorbing simple carbohydrates like glucose, galactose, and some disaccharides such as sucrose maltose and lactose. Although our digestive system is quite capable of breaking down starch a complex carbohydrate into its monomer glucose, when comes to one of the largest sources of complex carbohydrate- cellulose, our digestive system is not capable of degrading it efficiently. Other plant-based carbohydrates such as pectin, xylan are also largely undigested in human guts. Different anaerobic bacteria digest cellobiose in the large intestine and give rise to a large amount of Cellobiose available for the other bacteria to utilize. Cellobiose has documented role in virulence in many pathogenic bacteria such as Listeria and Streptomyces. Also, a recent study in *C. difficile* has shown that trehalose, another disaccharide similar to Cellobiose, is responsible for significantly increased virulence in clinical strains[80]. Our work shows that cellobiose uptake and metabolism has a role in *C. difficile* virulence. We have shown that cellobiose is not necessary for optimum growth of *C. difficile* in rich media. Our toxin assay demonstrated that the cellobiose mutant produces a significantly higher level of toxin compared to the parents, which could be attributed to the reduced availability of nutrient,

leading to increased virulence by CCR mechanism. We also observed the *C. difficile* cellobiose mutants to have a significantly lower level of spores compared to the parent strain, implying that cellobiose uptake and metabolism is important for sporulation.

To better understand the role of cellobiose uptake and metabolism in *C. difficile* virulence, we identified and characterized a novel regulator for cellobiose PTS operon, CelR. CelR, annotated as CD2781 in *C. difficile* R20291 genome, is a GntR class regulator capable of binding to its target DNA sequence and repress its expression. We identified CelR as a negative regulator of Cellobiose PTS operon by both reporter fusion and DNA binding assay. We also identified that CelR autoregulates its own expression too. To understand the role of CelR regulation of Cellobiose operon, we created a *celR* mutant strain. *celR* mutant is hypertoxic and hyper sporulating. However, when compared to the growth of parent strains, *celR* mutants were not significantly different when grown in rich media. *celR* mutants showed significantly increased level of *tcdR* and *spo0A* when measured by qRT-PCR method. We found a lower level of CcpA in the *celR* mutant compared to the parent strain in western blot. CcpA is a negative regulator of virulence in *C. difficile* and thus lower level of CcpA could be the link between the cellobiose PTS upregulation because of the relieve of CelR repression, and increased level of virulence factors.

Our *In vivo* studies demonstrated that Cellobiose PTS operon is essential for *C. difficile* virulence and pathogenesis. We saw a significantly reduced level of pathogenesis and death in the group of hamsters that were infected with *licB* mutant strains compared to the parent group. This result is in line with our *in vitro* studies. We also did an induced recurrent infection model study by treating the infected hamsters with clindamycin again. Our study results showed that cellobiose PTS

operon is not only essential for establishing primary infection, but also for recurrent infection. We hypothesize that this lack of ability to cause a recurrent infection can be either because the *licB* mutants were unable to colonize in the gut in the first round of infection, or because of the mutant strain being nearly asporogenic. Since the mutant strain produces very few spores, there were not enough spores left to cause recurrent infection.

Further studies are needed to be conducted to fully understand the role of the cellobiose PTS operon on the pathogenesis of *C. difficile*. Especially how cellobiose uptake and metabolism is connected to the virulence factors regulators CcpA, CodY, and Spo0A is a significant piece of the puzzle that is missing. There is also the possibility of CelR to have other DNA targets besides the cellobiose operon, which could help explain how its effects on virulence are so profound. To test the hypothesis, assays like DNAse footprinting could be used to identify the potential CelR binding target DNA sequences. *C. difficile* genome could then be searched for similar sequences, and DNA binding assays could be used to verify the interactions. Thus, additional targets for the CelR could be identified if they exist. Also, to understand the regulatory network of the cellobiose mutant, level of virulence factors regulators in the strain needs to be quantified. To determine the role of Cellobiose in *C. difficile* pathogenesis, animal studies could be conducted with either Cellobiose enriched, and Cellobiose free diets and the effects could be observed.

Overall the body of work done in this thesis indicates that, although *C. difficile* uses Strickland metabolism as its primary method of energy acquisition, carbohydrate metabolism plays a significant role in its virulence. Mainly, as demonstrated by both of our work with glycogen and cellobiose PTS operon, *C. difficile* sporulation is tightly regulated with regards to carbohydrate

metabolism. The role of glycogen metabolism in *C. difficile* has never been investigated before, and our results provide the first evidence that *C. difficile* glycogen metabolism plays a significant role in sporulation. Glycogen storage in *C. difficile* could indeed be a checkpoint for the sporulation initiation. Also, the faster growth, compared to the parent strain indicates that the bacteria might use intracellular level of energy available specifically from carbohydrates for timing the cell division or sporulation. It is indeed valid to hypothesize that the differences between different *C. difficile* strains morphology and potentially the difference in virulence, could be traced back to their subtle differences in carbohydrate metabolism. It could also be possible that regulatory mechanisms could sense the internal ratio of various carbohydrate metabolism intermediates such as glucose 1 P vs. glycogen or glucose 1P vs. glucose 6P, and depending on the ratio, regulate the virulence properties and growth. Besides toxin production and sporulation, *C. difficile* has other virulence factors such as biofilm formation and motility, on which glycogen and cellobiose metabolism could have a significant role too. Unfortunately, due to limited time and scope, we were not able to explore those in this study. We hope what we have learned from this body of work will further signify the importance of studying complex carbohydrate metabolism in *C. difficile*, help unravel the molecular mystery that *C. difficile* virulence regulation is, and ultimately put us one step closer towards designing novel and effective treatment and prevention strategies against this deadly pathogen.

Chapter 5 - References

1. Lawson PA, Citron DM, Tyrrell KL, Finegold SM. Reclassification of Clostridium difficile as Clostridioides difficile (Hall and O'Toole 1935) Prévot 1938. *Anaerobe*. 2016;40:95-99. doi:10.1016/j.anaerobe.2016.06.008

2. Ley RE, Peterson DA, Gordon JI. Ecological and Evolutionary Forces Shaping Microbial Diversity in the Human Intestine. *Cell*. 2006;124(4):837-848. doi:10.1016/j.cell.2006.02.017

3. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Human gut microbes associated with obesity. *Nature*. 2006;444(7122):1022. doi:10.1038/4441022a

4. Maczulak AE. *Encyclopedia of Microbiology*. New York, NY: Facts On File; 2011.

5. HALL IC, O'TOOLE E. INTESTINAL FLORA IN NEW-BORN INFANTS: WITH A DESCRIPTION OF A NEW PATHOGENIC ANAEROBE, BACILLUS DIFFICILIS. *JAMA Pediatr*. 1935;49(2):390-402. doi:10.1001/archpedi.1935.01970020105010

6. CDC. Most cases of C. diff happen when you're on antibiotics…. Centers for Disease Control and Prevention. https://www.cdc.gov/cdiff/risk.html. Published December 17, 2018. Accessed May 26, 2019.

7. CDC. The biggest antibiotic-resistant threats in the U.S. Centers for Disease Control and Prevention. https://www.cdc.gov/drugresistance/biggest_threats.html. Published March 27, 2019. Accessed May 5, 2019.

8. Govind R, Fitzwater L, Nichols R. Observations on the Role of TcdE Isoforms in Clostridium difficile Toxin Secretion. DiRita VJ, ed. *J Bacteriol*. 2015;197(15):2600-2609. doi:10.1128/JB.00224-15

9. Pruitt RN, Chambers MG, Ng KK-S, Ohi MD, Lacy DB. Structural organization of the functional domains of Clostridium difficile toxins A and B. *Proc Natl Acad Sci U S A*. 2010;107(30):13467-13472. doi:10.1073/pnas.1002199107

10. Hunt JJ, Ballard JD. Variations in Virulence and Molecular Biology among Emerging Strains of Clostridium difficile. *Microbiol Mol Biol Rev MMBR*. 2013;77(4):567-581. doi:10.1128/MMBR.00017-13

11. Solomon K. The host immune response to Clostridium difficile infection. *Ther Adv Infect Dis*. 2013;1(1):19-35. doi:10.1177/2049936112472173

12. Rodriguez-Palacios A, LeJeune JT. Moist-Heat Resistance, Spore Aging, and Superdormancy in Clostridium difficile ▿ . *Appl Environ Microbiol*. 2011;77(9):3085-3091. doi:10.1128/AEM.01589-10

13. Deakin LJ, Clare S, Fagan RP, et al. The Clostridium difficile spo0A Gene Is a Persistence and Transmission Factor. *Infect Immun*. 2012;80(8):2704-2711. doi:10.1128/IAI.00147-12

14. Koenigsknecht MJ, Theriot CM, Bergin IL, Schumacher CA, Schloss PD, Young VB. Dynamics and Establishment of Clostridium difficile Infection in the Murine Gastrointestinal Tract. *Infect Immun*. 2015;83(3):934-941. doi:10.1128/IAI.02768-14

15. Iizuka M, Itou H, Konno S, et al. Elemental diet modulates the growth of Clostridium difficile in the gut flora. *Aliment Pharmacol Ther*. 2004;20 Suppl 1:151-157. doi:10.1111/j.1365-2036.2004.01969.x

16. Martz SL, Guzman-Rodriguez M, He S-M, et al. A human gut ecosystem protects against C. difficile disease by targeting TcdA. *J Gastroenterol*. 2017;52(4):452-465. doi:10.1007/s00535-016-1232-y

17. Mani N, Dupuy B. Regulation of toxin synthesis in Clostridium difficile by an alternative RNA polymerase sigma factor. *Proc Natl Acad Sci U S A*. 2001;98(10):5844-5849. doi:10.1073/pnas.101126598

18. Hundsberger T, Braun V, Weidmann M, Leukel P, Sauerborn M, Eichel-Streiber CV. Transcription Analysis of the Genes tcdA-E of the Pathogenicity Locus of Clostridium Difficile. *Eur J Biochem*. 1997;244(3):735-742. doi:10.1111/j.1432-1033.1997.t01-1-00735.x

19. Warny M, Pepin J, Fang A, et al. Toxin production by an emerging strain of Clostridium difficile associated with outbreaks of severe disease in North America and Europe. *The Lancet*. 2005;366(9491):1079-1084. doi:10.1016/S0140-6736(05)67420-X

20. Dupuy B, Sonenshein AL. Regulated transcription of Clostridium difficile toxin genes. *Mol Microbiol*. 1998;27(1):107-120.

21. Antunes A, Camiade E, Monot M, et al. Global transcriptional control by glucose and carbon regulator CcpA in Clostridium difficile. *Nucleic Acids Res*. 2012;40(21):10701-10718. doi:10.1093/nar/gks864

22. Dineen SS, Villapakkam AC, Nordman JT, Sonenshein AL. Repression of Clostridium difficile toxin gene expression by CodY. *Mol Microbiol*. 2007;66(1):206-219. doi:10.1111/j.1365-2958.2007.05906.x

23. Sonenshein AL. CodY, a global regulator of stationary phase and virulence in Gram-positive bacteria. *Curr Opin Microbiol*. 2005;8(2):203-207. doi:10.1016/j.mib.2005.01.001

24. Ratnayake-Lecamwasam M, Serror P, Wong K-W, Sonenshein AL. Bacillus subtilis CodY represses early-stationary-phase genes by sensing GTP levels. *Genes Dev*. 2001;15(9):1093-1103. doi:10.1101/gad.874201

25. Bergara F, Ibarra C, Iwamasa J, Patarroyo JC, Aguilera R, Márquez-Magaña LM. CodY Is a Nutritional Repressor of Flagellar Gene Expression in Bacillus subtilis. *J Bacteriol*. 2003;185(10):3118-3126. doi:10.1128/JB.185.10.3118-3126.2003

26. Molle V, Nakaura Y, Shivers RP, et al. Additional Targets of the Bacillus subtilis Global Regulator CodY Identified by Chromatin Immunoprecipitation and Genome-Wide Transcript Analysis. *J Bacteriol*. 2003;185(6):1911-1922. doi:10.1128/JB.185.6.1911-1922.2003

27. Karlsson S, Burman LG, Akerlund T. Suppression of toxin production in Clostridium difficile VPI 10463 by amino acids. *Microbiology*. 1999;145(7):1683-1693. doi:10.1099/13500872-145-7-1683

28. Saujet L, Monot M, Dupuy B, Soutourina O, Martin-Verstraete I. The Key Sigma Factor of Transition Phase, SigH, Controls Sporulation, Metabolism, and Virulence Factor Expression in Clostridium difficile ▿ . *J Bacteriol*. 2011;193(13):3186-3196. doi:10.1128/JB.00272-11

29. Rosenbusch KE, Bakker D, Kuijper EJ, Smits WK. C. difficile 630Δerm Spo0A Regulates Sporulation, but Does Not Contribute to Toxin Production, by Direct High-Affinity Binding to Target DNA. *PLoS ONE*. 2012;7(10). doi:10.1371/journal.pone.0048608

30. Girinathan BP, Ou J, Dupuy B, Govind R. Pleiotropic roles of Clostridium difficile sin locus. Koehler TM, ed. *PLOS Pathog*. 2018;14(3):e1006940. doi:10.1371/journal.ppat.1006940

31. Pettit LJ, Browne HP, Yu L, et al. Functional genomics reveals that Clostridium difficile Spo0A coordinates sporulation, virulence and metabolism. *BMC Genomics*. 2014;15:160. doi:10.1186/1471-2164-15-160

32. Dapa T, Unnikrishnan M. Biofilm formation by Clostridium difficile. *Gut Microbes*. 2013;4(5):397-402. doi:10.4161/gmic.25862

33. Mackin KE, Carter GP, Howarth P, Rood JI, Lyras D. Spo0A Differentially Regulates Toxin Production in Evolutionarily Diverse Strains of Clostridium difficile. Popoff MR, ed. *PLoS ONE*. 2013;8(11):e79666. doi:10.1371/journal.pone.0079666

34. Varga J, Stirewalt VL, Melville SB. The CcpA Protein Is Necessary for Efficient Sporulation and Enterotoxin Gene (cpe) Regulation in Clostridium perfringens. *J Bacteriol*. 2004;186(16):5221-5229. doi:10.1128/JB.186.16.5221-5229.2004

35. Dineen SS, McBride SM, Sonenshein AL. Integration of Metabolism and Virulence by Clostridium difficile CodY. *J Bacteriol*. 2010;192(20):5350-5362. doi:10.1128/JB.00341-10

36. Edwards AN, Tamayo R, McBride SM. A Novel Regulator Controls Clostridium difficile Sporulation, Motility and Toxin Production. *Mol Microbiol*. 2016;100(6):954-971. doi:10.1111/mmi.13361

37. Postma PW, Lengeler JW, Jacobson GR. Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. *Microbiol Rev*. 1993;57(3):543-594.

38. Deutscher J, Francke C, Postma PW. How Phosphotransferase System-Related Protein Phosphorylation Regulates Carbohydrate Metabolism in Bacteria. *Microbiol Mol Biol Rev*. 2006;70(4):939-1031. doi:10.1128/MMBR.00024-06

39. The catabolite control protein CcpA binds to Pmga and influences expression of the virulence regulator Mga in the Group A streptococcus. - PubMed - NCBI. https://www.ncbi.nlm.nih.gov/pubmed/17905980/. Accessed June 2, 2019.

40. Stoll R, Mertins S, Joseph B, Muller-Altrock S, Goebel W. Modulation of PrfA activity in Listeria monocytogenes upon growth in different culture media. *Microbiology*. 2008;154(12):3856-3876. doi:10.1099/mic.0.2008/018283-0

41. Lo Scrudato M, Borgeaud S, Blokesch M. Regulatory elements involved in the expression of competence genes in naturally transformable Vibrio cholerae. *BMC Microbiol*. 2014;14. doi:10.1186/s12866-014-0327-y

42. Lim S, Yun J, Yoon H, et al. Mlc regulation of Salmonella pathogenicity island I gene expression via hilE repression. *Nucleic Acids Res*. 2007;35(6):1822-1832. doi:10.1093/nar/gkm060

43. Abranches J, Nascimento MM, Zeng L, et al. CcpA Regulates Central Metabolism and Virulence Gene Expression in Streptococcus mutans. *J Bacteriol*. 2008;190(7):2340-2349. doi:10.1128/JB.01237-07

44. Mendez M, Huang I-H, Ohtani K, Grau R, Shimizu T, Sarker MR. Carbon Catabolite Repression of Type IV Pilus-Dependent Gliding Motility in the Anaerobic Pathogen Clostridium perfringens. *J Bacteriol*. 2008;190(1):48-60. doi:10.1128/JB.01407-07

45. Antibiotic resistance as a stress response: complete sequencing of a large number of chromosomal loci in Staphylococcus aureus strain COL that impa... - PubMed - NCBI. https://www.ncbi.nlm.nih.gov/pubmed/10566865?dopt=Abstract&holding=npg. Accessed June 2, 2019.

46. Loss of penicillin tolerance by inactivating the carbon catabolite repression determinant CcpA in Streptococcus gordonii. - PubMed - NCBI. https://www.ncbi.nlm.nih.gov/pubmed/17327292?dopt=Abstract&holding=npg. Accessed June 2, 2019.

47. Eydallin G, Viale AM, Morán-Zorzano MT, et al. Genome-wide screening of genes affecting glycogen metabolism in Escherichia coli K-12. *FEBS Lett*. 2007;581(16):2947-2953. doi:10.1016/j.febslet.2007.05.044

48. Wilson WA, Roach PJ, Montero M, et al. Regulation of glycogen metabolism in yeast and bacteria. *FEMS Microbiol Rev*. 2010;34(6):952-985. doi:10.1111/j.1574-6976.2010.00220.x

49. Preiss J. Bacterial Glycogen Synthesis and Its Regulation. *Annu Rev Microbiol*. 1984;38(1):419-458. doi:10.1146/annurev.mi.38.100184.002223

50. Kiel JAKW, Boels JM, Beldman G, Venema G. Glycogen in Bacillus subtilis: molecular characterization of an operon encoding enzymes involved in glycogen biosynthesis and degradation. *Mol Microbiol*. 1994;11(1):203-218. doi:10.1111/j.1365-2958.1994.tb00301.x

51. Bonafonte MA, Solano C, Sesma B, et al. The relationship between glycogen synthesis, biofilm formation and virulence in *Salmonella enteritidis*. *FEMS Microbiol Lett*. 2000;191(1):31-36. doi:10.1111/j.1574-6968.2000.tb09315.x

52. Henrissat B, Deleury E, Coutinho PM. Glycogen metabolism loss: a common marker of parasitic behaviour in bacteria? *Trends Genet*. 2002;18(9):437-440. doi:10.1016/S0168-9525(02)02734-8

53. Bourassa L, Camilli A. Glycogen contributes to the environmental persistence and transmission of Vibrio cholerae. *Mol Microbiol*. 2009;72(1):124-138. doi:10.1111/j.1365-2958.2009.06629.x

54. Gore AL, Payne SM. CsrA and Cra Influence Shigella flexneri Pathogenesis. *Infect Immun*. 2010;78(11):4674-4682. doi:10.1128/IAI.00589-10

55. Goh YJ, Klaenhammer TR. Insights into glycogen metabolism in Lactobacillus acidophilus: impact on carbohydrate metabolism, stress tolerance and gut retention. *Microb Cell Factories*. 2014;13(1). doi:10.1186/s12934-014-0094-3

56. Jones SA, Jorgensen M, Chowdhury FZ, et al. Glycogen and Maltose Utilization by Escherichia coli O157:H7 in the Mouse Intestine. *Infect Immun*. 2008;76(6):2531-2540. doi:10.1128/IAI.00096-08

57. Ballicora MA, Iglesias AA, Preiss J. ADP-Glucose Pyrophosphorylase, a Regulatory Enzyme for Bacterial Glycogen Synthesis. *Microbiol Mol Biol Rev*. 2003;67(2):213-225. doi:10.1128/MMBR.67.2.213-225.2003

58. Sirigi Reddy AR, Girinathan BP, Zapotocny R, Govind R. Identification and Characterization of Clostridium sordellii Toxin Gene Regulator. *J Bacteriol*. 2013;195(18):4246-4254. doi:10.1128/JB.00711-13

59. Govind R, Vediyappan G, Rolfe RD, Dupuy B, Fralick JA. Bacteriophage-Mediated Toxin Gene Regulation in Clostridium difficile. *J Virol*. 2009;83(23):12037-12045. doi:10.1128/JVI.01256-09

60. Teng F, Murray BE, Weinstock GM. Conjugal transfer of plasmid DNA from Escherichia coli to enterococci: a method to make insertion mutations. *Plasmid*. 1998;39(3):182-186. doi:10.1006/plas.1998.1336

61. Heap JT, Kuehne SA, Ehsaan M, et al. The ClosTron: Mutagenesis in Clostridium refined and streamlined. *J Microbiol Methods*. 2010;80(1):49-55. doi:10.1016/j.mimet.2009.10.018

62. Govind R, Dupuy B. Secretion of Clostridium difficile Toxins A and B Requires the Holin-like Protein TcdE. *PLoS Pathog*. 2012;8(6). doi:10.1371/journal.ppat.1002727

63. El Meouche I, Peltier J, Monot M, et al. Characterization of the SigD Regulon of C. difficile and Its Positive Control of Toxin Production through the Regulation of tcdR. *PLoS ONE*. 2013;8(12). doi:10.1371/journal.pone.0083748

64. Saujet L, Monot M, Dupuy B, Soutourina O, Martin-Verstraete I. The Key Sigma Factor of Transition Phase, SigH, Controls Sporulation, Metabolism, and Virulence Factor Expression in Clostridium difficile. *J Bacteriol*. 2011;193(13):3186-3196. doi:10.1128/JB.00272-11

65. Sambol SP, Tang JK, Merrigan MM, Johnson S, Gerding DN. Infection of hamsters with epidemiologically important strains of Clostridium difficile. *J Infect Dis*. 2001;183(12):1760-1766. doi:10.1086/320736

66. Girinathan BP, Braun S, Sirigireddy AR, Lopez JE, Govind R. Importance of Glutamate Dehydrogenase (GDH) in Clostridium difficile Colonization In Vivo. *PLoS ONE*. 2016;11(7). doi:10.1371/journal.pone.0160107

67. Martin-Verstraete I, Peltier J, Dupuy B. The Regulatory Networks That Control Clostridium difficile Toxin Synthesis. *Toxins*. 2016;8(5). doi:10.3390/toxins8050153

68. Janoir C, Denève C, Bouttier S, et al. Adaptive Strategies and Pathogenesis of Clostridium difficile from In Vivo Transcriptomics. *Infect Immun*. 2013;81(10):3757-3769. doi:10.1128/IAI.00515-13

69. Cereijo AE, Asencion Diez MD, Ballicora MA, Iglesias AA. Regulatory Properties of the ADP-Glucose Pyrophosphorylase from the Clostridial Firmicutes Member Ruminococcus albus. *J Bacteriol*. 2018;200(17). doi:10.1128/JB.00172-18

70. François J, Hers HG. The control of glycogen metabolism in yeast. 2. A kinetic study of the two forms of glycogen synthase and of glycogen phosphorylase and an investigation of their interconversion in a cell-free extract. *Eur J Biochem*. 1988;174(3):561-567. doi:10.1111/j.1432-1033.1988.tb14135.x

71. Cummings JH, Southgate DA, Branch WJ, et al. The digestion of pectin in the human gut and its effect on calcium absorption and large bowel function. *Br J Nutr*. 1979;41(3):477-485.

72. Englyst HN, Anderson V, Cummings JH. Starch and non-starch polysaccharides in some cereal foods. *J Sci Food Agric*. 1983;34(12):1434-1440. doi:10.1002/jsfa.2740341219

73. Flint HJ, Scott KP, Duncan SH, Louis P, Forano E. Microbial degradation of complex carbohydrates in the gut. *Gut Microbes*. 2012;3(4):289-306. doi:10.4161/gmic.19897

74. Chassard, C, Delmas, E, Robert, C, Lawson PA, Bernalier-Donadille A. Ruminococcus champanellensis sp. nov., a cellulose-degrading bacterium from human gut microbiota. *Int J Syst Evol Microbiol*. 2012;62(1):138-143. doi:10.1099/ijs.0.027375-0

75. Gong J, Egbosimba EE, Forsberg CW. Cellulose-binding proteins of *Fibrobacter succinogenes* and the possible role of a 180-kDa cellulose-binding glycoprotein in adhesion to cellulose. *Can J Microbiol*. 1996;42(5):453-460. doi:10.1139/m96-062

76. Saier MH. Protein phosphorylation and allosteric control of inducer exclusion and catabolite repression by the bacterial phosphoenolpyruvate: sugar phosphotransferase system. *Microbiol Rev*. 1989;53(1):109-120.

77. Saier MH, Reizer J. Proposed uniform nomenclature for the proteins and protein domains of the bacterial phosphoenolpyruvate: sugar phosphotransferase system. *J Bacteriol*. 1992;174(5):1433-1438.

78. Antunes A, Martin-Verstraete I, Dupuy B. CcpA-mediated repression of Clostridium difficile toxin gene expression. *Mol Microbiol*. 2011;79(4):882-899. doi:10.1111/j.1365-2958.2010.07495.x

79. Jenior ML, Leslie JL, Young VB, Schloss PD. Clostridium difficile Alters the Structure and Metabolism of Distinct Cecal Microbiomes during Initial Infection To Promote Sustained Colonization. *mSphere*. 2018;3(3). doi:10.1128/mSphere.00261-18

80. Collins J, Robinson C, Danhof H, et al. Dietary trehalose enhances virulence of epidemic Clostridium difficile. *Nature*. 2018;553(7688):291-294. doi:10.1038/nature25178

81. Park SF, Kroll RG. Expression of listeriolysin and phosphatidylinositol-specific phospholipase C is repressed by the plant-derived molecule cellobiose in Listeria monocytogenes. *Mol Microbiol*. 1993;8(4):653-661. doi:10.1111/j.1365-2958.1993.tb01609.x

82. Lerat S, Simao-Beaunoir A-M, Wu R, Beaudoin N, Beaulieu C. Involvement of the Plant Polymer Suberin and the Disaccharide Cellobiose in Triggering Thaxtomin A Biosynthesis, a Phytotoxin Produced by the Pathogenic Agent *Streptomyces scabies*. *Phytopathology*. 2010;100(1):91-96. doi:10.1094/PHYTO-100-1-0091

83. Francis IM, Jourdan S, Fanara S, Loria R, Rigali S. The Cellobiose Sensor CebR Is the Gatekeeper of Streptomyces scabies Pathogenicity. Vidaver AK, ed. *mBio*. 2015;6(2). doi:10.1128/mBio.02018-14

84. Wu M-C, Chen Y-C, Lin T-L, Hsieh P-F, Wang J-T. Cellobiose-Specific Phosphotransferase System of Klebsiella pneumoniae and Its Importance in Biofilm Formation and Virulence. Camilli A, ed. *Infect Immun*. 2012;80(7):2464-2472. doi:10.1128/IAI.06247-11

85. Fagan RP, Fairweather NF. Clostridium difficile Has Two Parallel and Essential Sec Secretion Systems. *J Biol Chem*. 2011;286(31):27483-27493. doi:10.1074/jbc.M111.263889

86. Mani N, Lyras D, Barroso L, et al. Environmental Response and Autoregulation of Clostridium difficile TxeR, a Sigma Factor for Toxin Gene Expression. *J Bacteriol*. 2002;184(21):5971-5978. doi:10.1128/JB.184.21.5971-5978.2002

87. Suvorova IA, Korostelev YD, Gelfand MS. GntR Family of Bacterial Transcription Factors and Their DNA Binding Motifs: Structure, Positioning and Co-Evolution. Rogozin IB, ed. *PLOS ONE*. 2015;10(7):e0132618. doi:10.1371/journal.pone.0132618

88. Neumann-Schaal M, Jahn D, Schmidt-Hohagen K. Metabolism the Difficile Way: The Key to the Success of the Pathogen Clostridioides difficile. *Front Microbiol*. 2019;10. doi:10.3389/fmicb.2019.00219

89. Zhang D, Prabhu VS, Marcella SW. Attributable Healthcare Resource Utilization and Costs for Patients With Primary and Recurrent Clostridium difficile Infection in the United States. *Clin Infect Dis*. 2018;66(9):1326-1332. doi:10.1093/cid/cix1021

90. Peng Z, Jin D, Kim HB, et al. Update on Antimicrobial Resistance in Clostridium difficile: Resistance Mechanisms and Antimicrobial Susceptibility Testing. Kraft CS, ed. *J Clin Microbiol*. 2017;55(7):1998-2008. doi:10.1128/JCM.02250-16

91. den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud D-J, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J Lipid Res*. 2013;54(9):2325-2340. doi:10.1194/jlr.R036012

92. Venema K. Role of gut microbiota in the control of energy and carbohydrate metabolism. *Curr Opin Clin Nutr Metab Care*. 2010;13(4):432-438. doi:10.1097/MCO.0b013e32833a8b60

93. Rowland I, Gibson G, Heinken A, et al. Gut microbiota functions: metabolism of nutrients and other food components. *Eur J Nutr*. 2018;57(1):1-24. doi:10.1007/s00394-017-1445-8

94. Carbohydrate Digestion & Fermentation | The Microbiome. https://sites.tufts.edu/absorption/carbdigestion/. Accessed July 2, 2019.

Appendix A - Strains, Plasmids and Oligonucleotides

Bacteria strains and plasmids	**Relevant features or genotype**	**Source or reference**
Clostridium difficile JIR8094	Erm^s derivative of strain 630	O'Connor *et al.* (2006)
Clostridium difficile R20291	Clinical isolate - NAP1/027 ribotype, isolated in 2006 following an outbreak in Stoke Mandeville Hospital, UK	Stabler *et al.* (2009)
Escherichia coli DH5α	*endA1 recA1 deoR hsdR17* (r_K^- m_K^+)	NEB
Escherichia coli S17-1	Strain with integrated RP4 conjugation transfer function; favors conjugation between *E. coli* and *C. difficile*	Teng *et al.* (1998)
Clostridium difficile JIR8094::*glgC*	JIR8094 with intron insertion within *glg*C	This study
Clostridium difficile R20291::*cd2781*	R20291with intron insertion within *cd2781*	This study
Clostridium difficile R20291::*licB*	R20291with intron insertion within *licB*	This study
pMTL007-CE5	ClosTron plasmid	Heap *et al.* (2010)

pMTL007-CE5:Cdi-*glgC*	pMTL007-CE5 with group II intron targeted to *sinR*	This study
pMTL007-CE5:Cdi-*cd2781*	pMTL007-CE5 with group II intron targeted to *sinR'*	This study
pMTL007-CE5:Cdi-*licB*	pMTL007-CE5 with group II intron targeted to *sigD*	This study
pRPF185	*E. coli*/*C. difficile* shuttle plasmid	Fagan *et al*. (2011)
pRG381	pRPF185 containing *cd2781* under inducible *tet* promoter	This study
pRG382	pRPF185 containing 200bp *upstream region of licB replacing the tet* promoter	This study
pRG383	pRPF185 containing 600bp *upstream region of cd2781-82 replacing the tet* promoter	This study
pRG384	pET16B containing *cd2781* gene with 6 His tag	This study
Oligonucleotides	**Sequence (5'-3')**	
EBS UNIVERSAL	CGAAATTAGAAACTTGCGTTCAGTAAAC	
CD630_glgC forward primer to screen mutants	ATGAAAAAAGAGATGTTAGCTATGATTTTGGCAGGAGG	
CD630_glgC reverse primer to screen mutants	TTAAAGACCTCCTTCTATTAATACGCATCTAGGTTCGATATTTTC	
CDR20291_licB forward primer to screen mutants	CCGAGAGCTCGGAGTGAATTAAATGATAAAAATAATGTTAGCTTG	
CDR20291_licB reverse primer to screen mutants	CCGTGGATCCTTATTTTGAACTATTTAACTCTACAGCTC	
CDR20291_2781 Reverse primer with flag tag and BamH1	CCGAGGATCCTTACTTGTCGTCATCGTCTTTGTAGTCTTCGACAACAGCATT CAAAGTTA	

CDR20291_2781 Forward primer with sac1 to clone in pRPF185	GTTAACAGATCTGAGCTCTAATAAGGAGTGATTTAAATGAAAGAGCCAATTT ATA
Cellobiose PTS operon upstream forward primer with Kpn1 for promoter fusion	GTTAACAGATCTGGTACCGAGTACTCACACACATATCAC
Cellobiose PTS operon upstream reverse primer for promoter fusion	CCGAGAGCTCTTTTTCCCCTCCAAATTAGATTC
CDR20291_2781 forward primers with N terminus His tag	CCGAGAGCTCGGAGTGAATTAAATGCATCATCACCATCACCACAAAGAGCCAATTTA TAAAGTTATAGAAAATCATG
CDR20291_2781 forward to clone into pET16b	CTCGAGATGAAAGAGCCAATTTATAAAGTTATAGAAAAT
CDR20291_2781 reverse to clone into pET16b with 6 His tag	GGATCCTTAGTGGTGATGGTGATGGACTTCGACAACAGCATTCAAAGTTAATGTATA
CDR20291_2781 upstream forward primer for promoter fusion	GTTGTTTTATATATATTATTTATACTTTTTTAGTCTAAATATATATTATATATGTTTTT ATACTGAAATAAGTGCTATTTATTTTTGTA
CDR20291_2781 upstream reverse primer for promoter fusion	TACAAAAATAAATAGCACTTATTTCAGTATAAAAACATATATAATATATATTTAGAC TAAAAAAGTATAAATAATATATATAAAACAAC

Table 1 List of strains, plasmids, and oligonucleotides used in the study.