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Deciphering strain differences in CodY regulation of *Clostridioides difficile* sporulation

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Deciphering strain differences in CodY regulation of *Clostridioides difficile* sporulation

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B.S., Eastern Washington University, 2020

Advisor: Shonna M. McBride, Ph.D.

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Abstract

Deciphering strain differences in CodY regulation of *Clostridioides difficile* sporulation

By Marcos Monteiro

Clostridioides difficile is an anaerobic and spore-forming pathogen that causes severe diarrhea, colitis, and even death. *C. difficile* infections are considered a burden to the healthcare and the economic systems of the U.S. Since spores are the only mode of transmission, the formation of spores is crucial for the spread of *C. difficile*. When a host ingests spores, the spores travel through the gastrointestinal tract, reaching the intestines where the spores sense bile salts. By sensing bile salts, spores are triggered to become vegetative cells. Vegetative cells continue traveling the gastrointestinal tract, reaching the colon. In the colon, *C. difficile* can colonize it. In this environment, nutrient availability is limited, prompting intracellular responses to adapt to the conditions. In *C. difficile*, nutrient availability is sensed by various nutritional regulators, including CodY. CodY is a global transcriptional regulator that senses branched-chain amino acids (BCAA) and guanosine-triphosphate (GTP). In rich growth conditions, BCAA and GTP bind to CodY, causing conformational changes that increase its affinity to bind specific sequences of DNA. When there are low concentrations of BCAA and GTP, BCCA and GTP are not bound to CodY, and the affinity of CodY to DNA decreases. In *C. difficile*, CodY is known to repress toxin production and sporulation. However, the direct CodY-regulated factors that control sporulation are not well understood. In this work, we confirm and expand the knowledge that CodY represses the initiation of sporulation in two different strains of *C. difficile* and that CodY continues to have a role in the regulation of sporulation at the stationary phase. Additionally, we identified several direct CodY-regulated factors in both the 630Δ*erm* and UK1 strains that are differentially regulated between the strains and unique in one of the strains. We further determined the effect of many direct CodY-regulated factors on sporulation in strain UK1 and the UK1 *codY* mutant. This work illustrates that CodY has a greater impact on the transcriptome of UK1 and that many factors under CodY regulation impact sporulation in *C. difficile*.

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Chapter 1: Introduction

I: *Clostridioides difficile*

a. *Clostridioides difficile,* a healthcare-associated pathogen

Clostridioides difficile is a pathogen that causes *C. difficile* infection (CDI), the most prevalent healthcare-associated infection in the United States (Guh *et al.* 2020; CDC, 2019; Smits *et al.* 2016). In 2017, more than 500,000 individuals had CDI, and of these individuals, 30,000 died (Guh *et al.* 2020; CDC, 2019). In 2017, the US healthcare costs for CDI were one billion dollars (Feuerstadt *et al*. 2020). Due to its significant healthcare and economic burden, the Centers for Disease Control and Prevention (CDC) has designated *C. difficile* as an urgent threat (CDC, 2019).

There are two categories of acquired CDI designated by the Infectious Diseases Society of America and the Society for Healthcare Epidemiology of America: nosocomial-acquired (NA-CDI) and community-acquired (CA-CDI) CDI (McDonald *et al.* 2018). In recent years, epidemiological studies have shown that CA-CDI cases have increased and NA-CDI cases have decreased (Feuerstadt, Theriault, and Tillotson 2023; Yu *et al.* 2023; Guh *et al.* 2020). It is suggested that the decrease in NA-CDI cases is attributed to the increase in good healthcare practices such as surveillance of NA-CDI cases, adherence to antiseptic techniques, isolation of infected patients, and controlled administration of antibiotics in healthcare settings (Feuerstadt, Theriault, and Tillotson 2023). For both NA-CDI and CA-CDI individuals, the CDI recurrence is about 20%, and each time individuals acquire CDI, it increases their chances of CDI recurrence (Song and Kim 2019).

b. Risk Factors

Individuals who have been treated with antibiotics for a prolonged time are more susceptible to CDI due to the disruption of the gut microbiota, which allows *C. difficile* colonization (Slimings and Riley 2014; Baines *et al.* 2006; Hensgens *et al.* 2012; McFarland 1998). In addition, for those who are treated with antibiotics and have extended stays in

healthcare settings, the risk of CDI increases (Shaughnessy *et al.* 2011; Jullian-Desayes *et al.* 2017). The elderly and immunocompromised are frequent patients in healthcare settings, which increases their exposure to *C. difficile*, which can lead to CDI (Lessa *et al.* 2015; McDonald *et al.* 2018; Dubberke *et al.* 2008; Navaneethan *et al.* 2012).

In a community setting, individuals who have direct contact with farm animals, have poor hygiene and take antiacids have a higher risk of acquiring CDI (Ofori *et al.* 2018; Goorhuis *et al.* 2008; Songer *et al.* 2009; Jhung *et al.* 2008; Bakker *et al.* 2010; Dial 2006; Williams 2001). The increase in cases of CA-CDI, especially in young and non-antibiotic-treated individuals, raises worry about what other risk factors are associated with CA-CDI (Ayada *et al.* 2023). Therefore, it is crucial to conduct more research to identify other risk factors for CA-CDI.

c. CDI Transmission

C. difficile is a strictly anaerobic and spore-forming bacterium, and as such, it cannot survive in the presence of atmospheric oxygen (Edwards, Suarez, and McBride 2013). However, by forming spores, *C. difficile* can persist in the presence of atmospheric oxygen (Nicolas Kint, Morvan, and Martin-Verstraete 2022). Additionally, *C. difficile* spores are resistant to radiation, dehydration, most cleaning products, and heat (Shen *et al.* 2019), making them extremely durable (Lawley *et al.* 2010; Shen 2020). Due to the spores capabilities to survive extreme environments, it is not surprising that spores are the only mode of *C. difficile* transmission (Deakin *et al.* 2012).

When individuals ingest *C. difficile* spores, the spores travel through the gastrointestinal tract, surviving stomach acid and other physical and chemical innate immunity from the host (DuPont 2018). Upon arrival in the small intestine, the spore senses bile acids, activating its germination process (Paredes-Sabja *et al.* 2008; Burns, Heap, and Minton 2010; Giel *et al.* 2010; Francis *et al.* 2013). Germination is a process in which spores develop into vegetative cells (Giel *et al.* 2010; Koenigsknecht *et al.* 2015; Sorg and Sonenshein 2008), which are metabolically active, requiring nutrients to survive within the host (Marshall *et al.* 2023).

Nutrient availability is sensed by regulators such as CodY and CcpA, and upon nutrient deprivation, these regulators decrease their repression of *tcdR, tcdA,* and *tcdB* (Dineen *et al.* 2007; Dineen, McBride, and Sonenshein 2010; Antunes, Martin-Verstraete, and Dupuy 2011; Antunes *et al.* 2012). *tcdR* encodes the toxin sigma factor, whereas *tcdA* and *tcdB* encode toxin A (TcdA) and toxin B (TcdB) (Chandrasekaran and Lacy 2017; Hammond and Johnson 1995; Braun *et al.* 1996; Bouillaut *et al*. 2015; Monot *et al.* 2015; Smits *et al.* 2016). Secretion of TcdA and TcdB by *C. difficile* vegetative cells leads to the hallmark symptoms of CDI (Chumbler *et al.* 2016; Di Bella *et al.* 2016).

Once TcdA and TcdB are released into the host colon, they bind to epithelial receptors, which leads to the internalization of these toxins (Chandrasekaran and Lacy 2017). When internalized, the toxins disrupt the host cells by turning off essential host regulatory proteins (Chandrasekaran and Lacy 2017; Genisyuerek *et al.* 2011; I. Just *et al.* 1995; Just *et al.* 1995). Disruption of the host regulatory proteins leads to cytoskeleton rearrangements in the cell, which disrupts tight junctions and resulting in apoptosis (Chandrasekaran and Lacy 2017; Peritore-Galve *et al.* 2022). Disturbance of tight junctions results in increased intestinal permeability and, consequently, diarrhea (Shen *et al.* 2011; Moonwiriyakit *et al.* 2023).

II. Spore formation

a. Sporulation

Sporulation is one of the most crucial cell processes in spore-forming bacteria, essential for the survival and transmission of the bacterium (Peter Setlow 2014; Martiny *et al.* 2006). Through spores, bacteria can survive harsh environmental stresses and persist for millions of years (Shen *et al.* 2019; Lawley *et al.* 2010; Shen 2020; Setlow 2014; Cano and Borucki 1995; Vreeland, Rosenzweig, and Powers 2000). The process of sporulation is definitive; as such, tight molecular regulation is essential to determine the fate of the cell (Saujet *et al.* 2014; Edwards and McBride 2014). When vegetative cells initiate sporulation and completion of

sporulation occurs, they transform into spores; through process, the bacteria become metabolically active to metabolically dormant (Paredes-Sabja *et al.* 2008; Keijser *et al.* 2007; Gupta *et al.* 2025; Ghosh *et al.* 2015). These metabolically dormant spores can germinate into vegetative cells only under favorable conditions with bile salts acting as germinant (Shen 2020; Baloh and Sorg 2022). Because sporulation is essential for *C. difficile* transmission, the mechanisms of spore formation will be described and discussed.

b. Mechanisms of spore formation

Sporulation, in simple terms, is an asymmetric division of a vegetative cell into a spore (Young and Fitz-James 1959). However, sporulation is a complex process that involves seven stages, from stage 0 (initiation of sporulation) to stage VII (Young and Fitz-James 1959; Murrell 1967b; Hoch 1976). Stage 0 is defined as a transition stage after cell division, where two nucleoids are formed and anchored to mesosomes at the poles (Voitsekhovsky *et al.* 2024; Murrell 1967; Talukdar *et al*. 2015). Then, a conformational change occurs for the two nucleoids, forming an axial filament; this is defined as stage I (Barák, Prepiak, and Schmeisser 1998; Buchanan, Henriques, and Piggot 1994). At stage II, the formation of the septum occurs, giving rise to the asymmetric cell division of the mother cell and the prespore, where the nucleoids are at the opposite poles (Setlow *et al.* 1991). After septation, the mother cell engulfs the prespore, forming the forespore, completing stage III (Higgins and Piggot 1992; Ryter 1965). In stage IV, the inner cell wall and the outer cortex are formed in the forespore by the implementation of peptidoglycan (Freese 1972; Sadoff 1973; Tipper and Linnett 1976). After the formation of the outer cortex, several spore coat proteins are added to the outer cortex, which is designated as stage V (Voitsekhovsky *et al.* 2024; Henriques, Melsen, and Moran 1998). The maturation of the spore continues at stage VI (Setlow 2006), and stage VII is the lysis of the mother cell, releasing the mature spore (Voitsekhovsky *et al.* 2024). After spore release, spores will only germinate when exposed to a germinant, as explained before (Shen 2020; Baloh and

Sorg 2022). All the sporulation stages are necessary for complete spore formation; however, the scope of this work focus on the initiation of sporulation in *C. difficile,* which will be explained.

c. Initiation of sporulation

In all endosporulating bacteria, Spo0A, the master regulator of sporulation, is necessary to initiate sporulation (Brown *et al.* 1994). Upon activation by phosphorylation, Spo0A dimerizes and binds to DNA to regulate the transcription of sporulation genes (DiCandia *et al.* 2022; Rosenbusch *et al.* 2012). In the model organism for sporulating bacteria, *Bacillus subtilis,* the activation and deactivation of Spo0A occur via a phosphorelay system involving several kinases and phosphatases, which are stimulated by various signals (Sonenshein 2000). On the other hand, *C. difficile* does not encode orthologs of the *B. subtilis* phosphorelay (DiCandia *et al.* 2022; Paredes, Alsaker, and Papoutsakis 2005; Underwood *et al.* 2009). To date, the mechanism by which Spo0A is activated in *C. difficile* remains unknown (DiCandia *et al.* 2022). However, several factors play a role in the initiation of sporulation in *C. difficile*. These factors are: SigH, RstA, Spo0E, PtpA, PtpB, PtpC, SigB, Agr, RgaRS, and CD2214-2215 (Rosenbusch *et al.* 2012; Saujet *et al.* 2011; Edwards, Tamayo, and McBride 2016; Edwards, Anjuwon-Foster, and McBride 2019; Edwards, Krall, and McBride 2020; DiCandia *et al.* 2024; Childress *et al.* 2016; Edwards *et al.* 2022; Kint *et al.* 2017; Ahmed *et al.* 2020; Edwards and McBride 2023; Girinathan *et al.* 2018; Ciftci *et al.* 2019). Additionally, other regulators and molecules regulate the initiation of sporulation in *C. difficile* through sensing and acquisition of nutrients; these factors and molecules are OppA, AppA, CD2589, c-di-GMP, CcpA, and CodY (Dineen, McBride, and Sonenshein 2010; Antunes, Martin-Verstraete, and Dupuy 2011; Antunes *et al.* 2012; Edwards, Nawrocki, and McBride 2014; Martins *et al.* 2021; Edwards *et al.* 2021; Dhungel and Govind 2021). In this work, the regulation of sporulation by CodY will be further discussed in detail.

III. CodY

a. Nutritional sensor transcriptional regulator

CodY is a transcriptional regulator that was first characterized in *B. subtilis* as the repressor of the dipeptide permease operon (*dpp*) (Slack *et al.* 1995). CodY was named for its function in the control of *dpp* (Cod). The letter Y comes from its position in the *cod* operon, *codVWXY* (Slack *et al.* 1995). CodY is present in many Gram-positive bacteria with low G-C content and senses intracellularly branched-chain amino acids (BCAA, isoleucine, leucine, and valine) and guanosine triphosphate (GTP) (Dineen *et al.* 2007; Sonenshein 2005). In a nutrient-rich environment, during exponential phase growth, there is an abundance of BCAA and GTP, which binds to CodY, changing its conformation to dimerize CodY and allow binding to DNA (Dineen *et al.* 2007; Dineen, McBride, and Sonenshein 2010; Ratnayake-Lecamwasam *et al.* 2001; Villapakkam *et al.* 2009; Daou *et al.* 2019). When nutrient availability is scarce, during the stationary phase, intracellular concentrations of BCAA and GTP decrease, relieving the binding of CodY to DNA. By sensing BCAA and GTP, CodY primarily represses genes that are not necessary or needed during the exponential phase and derepresses these same genes during the stationary phase.

In *C. difficile,* CodY is predicted to directly regulate more than 100 genes *(Dineen et al.* 2007; Dineen, McBride, and Sonenshein 2010). Of the genes regulated by CodY, the majority are related to metabolic functions, as observed in other bacterial species (Dineen, McBride, and Sonenshein 2010; Slack *et al.* 1995; Daou *et al.* 2019; Hendriksen *et al.* 2008; Lu *et al.* 2015; den Hengst *et al.* 2005; Larsen *et al.* 2006; Geng *et al.* 2018; Malke *et al.* 2006; Majerczyk *et al.* 2010; Kaiser *et al.* 2015; Waters *et al.* 2016; King *et al.* 2018; Batte, Sahukhal, and Elasri, 2018; Bennett *et al.* 2007; Lobel *et al.* 2015; Lobel and Herskovits 2016; Edwards, Nawrocki, and McBride 2014; Brinsmade *et al.* 2010; Belitsky 2011; Kaiser *et al.* 2018; Qi *et al.* 2015; Serror and Sonenshein 1996; Château *et al.* 2011). One of the most significant aspects of CodY regulation in *C. difficile* is the regulation of toxin production. CodY binds directly to the promoter of *tcdR*, an alternative sigma factor for the expression of toxin genes (Dineen et al.

2007). A *codY* mutant expresses much higher concentrations of TcdA and TcdB than its parental strain (Dineen *et al.* 2007; Dineen, McBride, and Sonenshein 2010; Daou *et al.* 2019; Nawrocki *et al.* 2016). The link between nutrient availability and toxin production highlights the importance of nutrient sensing and regulation in virulence.

b. CodY and sporulation

In *B. subtilis*, CodY represses several genes that regulate the initiation of sporulation, such as *spo0A* (Molle *et al.* 2003). Additionally, in *Bacillus anthracis*, CodY was found to bind to the promoter region of *kinB*, which encodes for a major kinase responsible for the phosphorylation of Spo0A (Château *et al.* 2013). Interestingly, overexpression of CodY in *B. anthracis* leads to lower sporulation compared to the parental strain (Gopalani *et al.* 2016). In *Clostridium perfringens*, CodY regulation of sporulation is strain-dependent by differential regulation of *abrB* in different strains. AbrB is a known repressor of initiation of sporulation in *C. perfringens* (Li *et al.* 2013; 2017). However, in *C. difficile*, the mechanism by which CodY impacts sporulation is not well understood. Previously, it was shown that the *codY* mutant in *C. difficile* overexpressed genes involved in sporulation, suggesting that CodY might impact the regulation of sporulation (Dineen, McBride, and Sonenshein 2010). In another study, *codY* mutants of two distinct *C. difficile* strains, UK1 and 630Δ*erm*, sporulated more than their parental strains, which indicates that CodY represses sporulation in *C. difficile*. In this same study, the *codY* mutants of UK1 and 630Δ*erm* had extreme differences in sporulation frequencies, suggesting that CodY-regulation on sporulation is also strain-dependent (Nawrocki *et al.* 2016).

IV. Specific aims

CodY plays a crucial role in *C. difficile* pathogenesis in regulating toxin production and sporulation. The mechanism by which CodY regulates toxin production is well understood. However, it is unknown which direct CodY-regulated factors control sporulation. Additionally,

there appears to be a difference in how CodY regulates sporulation in UK1 and 630Δ*erm* strains. By identifying which direct CodY-regulated factors impact sporulation and determining CodY-regulation differences between UK1 and 630Δ*erm* strains, we can better understand how nutrient availability and sporulation are linked through CodY regulation. To further progress our understanding of CodY regulation of sporulation in *C. difficile,* the goal of my thesis was to identify direct CodY-regulated factors that control sporulation in the UK1 and 630Δ*erm* strains. Here, I investigated the regulation of sporulation by CodY through the following specific aims:

1. Identify direct CodY-regulated factors that differentially control sporulation in the UK1 and 630Δ*erm* strains.
2. Determine the effect on sporulation of direct CodY-regulated factors in UK1

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Chapter 2: Deciphering strain differences in CodY regulation of *Clostridioides difficile* sporulation

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ABSTRACT

Clostridioides difficile is an anaerobic, spore-forming, pathogen that causes diarrhea, colitis, and even death. *C. difficile* grows and replicates in the intestine as a vegetative bacillus, but must transition into a dormant spore to survive and transmit in the environment. The transformation into a spore is a complex developmental process that is regulated in response to conditions within the host, most notably nutrient limitation. Nutrient availability is sensed by *C. difficile* through transcriptional regulators, such as CodY. CodY is a global nutritional gene regulator that controls gene expression in response to branched-chain amino acids (BCAA) and guanosine-triphosphate (GTP). It was previously observed that CodY represses *C. difficile* sporulation, but the impact of CodY on sporulation has differed considerably by strain. Here, we investigated the effects of CodY on gene expression during sporulation in the two common research strains 630Δ*erm* (ribotype 012) and UK1 (ribotype 027). We confirmed that CodY suppressed premature spore formation in both strains through time-elapsed sporulation assays with *codY* mutants. Through transcriptional analyses of *codY* mutant sporulation, we defined the similarities and differences in CodY-dependent gene expression between strains. We also identified differences in putative CodY sites within the 630 and UK1 genomes that may influence CodY regulation. Finally, we performed CRISPRi knockdowns to examine the effects of selected CodY-regulated genes, demonstrating the impact of multiple CodY-dependent factors on sporulation.

IMPORTANCE

C. difficile spore formation is crucial for transmission and survival of the bacterium. Spore formation is triggered by the availability of crucial nutrients, which CodY and other regulators sense. However, the mechanism by which CodY represses sporulation in *C. difficile* is poorly understood. In this study, we identified several CodY-regulated factors that could play a role in sporulation, both in 630Δ*erm* and UK1 strains. Our results show that many factors under the regulation of CodY can impact sporulation.

INTRODUCTION

Clostridioides difficile is an anaerobic and spore-forming nosocomial pathogen that causes severe diarrhea, colitis, and even death (CDC 2019; 2023; 2013). Transmission of *C. difficile* is only possible through spores, which survive environmental threats such as atmospheric oxygen and disinfectants (Sandhu and McBride 2018). After a host ingests *C. difficile* spores, they transit through the gastrointestinal tract, reaching the intestines, where they sense bile salts and germinate into vegetative cells (Wilson 1983; Sorg and Sonenshein 2008; Lee, Rizvi, and McBride 2024). *C. difficile* vegetative cells colonize the host colon, where nutrient availability is limited, leading to toxin production and spore formation (Donnelly *et al.* 2022; Antunes, Martin-Verstraete, and Dupuy 2011; Antunes *et al.* 2012; Dineen *et al.* 2007; Dupuy and Sonenshein 1998; Nawrocki *et al.* 2016; Dineen, McBride, and Sonenshein 2010). Nutrient availability is fundamental for determining whether *C. difficile* grows as a vegetative cell or becomes a spore. Under nutrient-limited conditions, *C. difficile* responds by increasing the expression of factors for nutrient acquisition and biosynthesis of necessary metabolites; when these mechanisms fail to provide for sustained vegetative growth, spore formation is initiated (Lee *et al.* 2022; Neumann-Schaal, Jahn, and Schmidt-Hohagen 2019; Saujet *et al.* 2011).

To sense and control metabolism, *C. difficile* encodes nutritional regulators, such as the global nutrient transcriptional regulator, CodY (Dineen *et al.* 2007; Nawrocki *et al.* 2016; Dineen, McBride, and Sonenshein 2010). CodY was first identified in *Bacillus subtilis* and is present in many Gram-positive bacteria with low G-C genomes *(Slack et al.* 1995; Levdikov *et al.* 2006; Sonenshein 2005; Stenz *et al.* 2011). In a nutrient-rich environment, *C. difficile* senses branched-chain amino acids (BCAAs) and guanosine triphosphate (GTP) through their interactions with CodY (Dineen *et al.* 2007; Nawrocki *et al.* 2016; Dineen, McBride, and Sonenshein 2010; Blagova *et al.* 2003; Brinsmade *et al*. 2010). CodY undergoes a confirmational change when it binds to BCAAs and GTP, which increases its binding affinity to

specific CodY-DNA binding sites, leading to the differential regulation of hundreds of genes (Dineen *et al.* 2007; Nawrocki *et al.* 2016; Dineen, McBride, and Sonenshein 2010; Daou *et al.* 2019). When the intracellular concentrations of BCAAs and GTP decrease, the binding affinity of CodY to DNA is altered, changing gene expression to adapt to nutrient scarcity, including the derepression of toxin production and the initiation of sporulation (Dineen *et al.* 2007; Nawrocki *et al.* 2016; Dineen, McBride, and Sonenshein 2010; Daou *et al.* 2019; Brinsmade *et al.* 2014; Waters *et al.* 2016). While the regulation of specific metabolic genes and toxins by CodY are well-documented, the mechanisms by which CodY affects *C. difficile* sporulation are less clear (Daou *et al.* 2019; Nawrocki, Crispell, and McBride 2014). CodY has varied effects on sporulation in strains 630 (ribotype 012) and UK1 (ribotype 027), as evidenced by a modest increase in sporulation in a 630 *codY* mutant and robust hypersporulation in a UK1 *codY* mutant (Daou *et al.* 2019; Nawrocki *et al.* 2016). The CodY proteins encoded by these strains are identical and similarly expressed, leading us to ask how CodY differentially regulates sporulation outcomes in these strains.

In this study, we examined CodY-dependent gene regulation in the 630 and UK1 backgrounds to identify strain-specific differences in sporulation outcomes. Through transcriptional analysis and mapping of CodY-binding sites, we identified CodY-regulated factors that are differentially expressed in 630Δ*erm* and UK1 and contain a CodY-binding site in at least one strain. In addition, we demonstrated that transcriptional repression of several direct CodY-regulated factors in UK1 or UK1 *codY* impact sporulation. These results illustrate how CodY regulation differs between the 630Δ*erm* and UK1 strains and demonstrate that many CodY-regulated factors can impact sporulation.

RESULTS

The impact of CodY regulation on sporulation is strain-dependent

In previous work, we demonstrated that CodY represses the initiation of sporulation and that CodY regulation of sporulation varies by strain (Nawrocki *et al.* 2016). In the commonly

studied strain 630Δ*erm* (a 630 derivative), CodY was found to modestly repress sporulation, resulting in a two-fold increase in sporulation frequency for the *codY* mutant in sporulation broth cultures. In contrast, the epidemic 027 isolate, the UK1 *codY* mutant, demonstrated more than 1000-fold greater sporulation frequency than the parent strain. To better understand how CodY regulates sporulation dissimilarly in either 630Δ*erm* and UK1, we evaluated sporulation in these strains over time on sporulation agar, which induces more robust sporulation than liquid medium (Fimlaid *et al.* 2013; Edwards, Tamayo, and McBride 2016). Strains UK1, 630Δ*erm*, and their respective *codY* mutants were grown on 70:30 sporulation agar and the formation of ethanol-resistant spores were assessed after 6 h (logarithmic phase), 12 h (stationary phase), and 24 h of growth, to compare the dynamics of spore production. As shown in **Figure 1**, at log phase the 630Δ*erm codY* mutant sporulated ~43-fold more than its parent strain (1.0E-3 +/- 4.3E-4 vs 2.6E-5 +/- 3.3E-5 %). In comparison, at log phase the UK1 *codY* mutant sporulated ~3,150-fold more than its parent strain, UK1. These results support the prior evidence that CodY represses premature sporulation initiation and that CodY repression of sporulation in UK1 is more robust than in 630Δ*erm* (Nawrocki *et al.* 2016). By stationary phase (12 h), the 630 *codY* mutant sporulated ~28-fold less than the parent strain (0.12 +/- 0.07 vs 3.37 +/- 1.02 %), while after 24 h of growth, the 630 *codY* mutant and parent displayed similar sporulation frequencies (**Figure 1**). These results suggest that CodY suppresses early initiation of sporulation in 630, yet this strain requires CodY to reach its full sporulation potential. In contrast, at stationary phase the UK1 *codY* mutant sporulation frequency was ~2000-fold higher than its parent strain (45.4 +/- 16.9 vs 0.02 +/- 0.01 %), and continued at greater frequency than the parent at 24 h (67.9 +/- 4.9 vs 0.33 +/- 0.05). In contrast, in the UK1 strain CodY represses sporulation at all growth stages. These data suggest there are differences in CodY-dependent gene regulation in UK1 and 630 that result in dissimilar sporulation outcomes.

Identifying strain-specific differences in CodY regulation

To understand how CodY regulates sporulation differently in the UK1 and 630 backgrounds, we examined gene expression during growth on sporulation agar in these strains and their *codY* mutants. Since CodY activity is controlled by the availability of BCAA and GTP, we investigated expression at log phase, when nutrients are most abundant and CodY repression is greatest (Dineen *et al.* 2007; Nawrocki *et al.* 2016; Dineen, McBride, and Sonenshein 2010; Slack *et al.* 1995; Blagova *et al.* 2003; Brinsmade *et al.* 2010; Daou *et al.* 2019; Belitsky and Sonenshein 2011; Edwards, Nawrocki, and McBride 2014). Following 6 h of growth on 70:30 agar, samples were processed for RNA-seq analysis to assess the ratio of gene expression in the *codY* mutants relative to their respective parent strain (*codY*/WT) (**Table S1, Table S2**). Transcription was extensively altered in the *codY* mutants of both strains, resulting in 867 genes differentially expressed more than 3-fold in the UK1 *codY* mutant and 449 genes in the 630 *codY* mutant.

Transcripts that were differentially regulated in the UK1 *codY* and 630 *codY* mutants include factors that are directly and indirectly regulated by CodY. To discern which genes may be directly controlled by CodY to influence sporulation, we sought to define genes with known or potential CodY-binding motifs (CodY boxes). Using the CodY binding sites previously identified in *C. difficile* (Nawrocki *et al.* 2016; Girinathan *et al.* 2018; Dineen, McBride, and Sonenshein 2010) and potential CodY boxes identified based on the classical Gram-positive CodY consensus (AATTTTCWGAAAATT) (Bailey *et al.* 2015), we narrowed the list of differentially regulated genes to those most likely to be directly regulated by CodY. The resulting list included 404 genes with prospective CodY-binding sites within the promoter or coding sequence that were 3-fold differentially expressed in at least one of the *codY* mutant strains, relative to the parental control (**Table S3**).

Of the genes listed in **Table S3**, 92 were similarly regulated by CodY in the UK1 and 630Δ*erm* strains, which limits their likelihood for strain-specific, CodY-dependent impacts. While some of these factors may differ in protein similarity or function that result in differences in

sporulation outcomes, such differences were outside the scope of this study. Of the genes in **Table S3** that were dissimilarly CodY regulated between UK1 and 630Δ*erm*, 225 had identical CodY boxes, which suggests that the differences in expression observed were not due to variation in the inherent ability of CodY to bind to these target sequences. We focused further on those CodY-regulated genes with significant differences in expression between the UK1 and 630Δ*erm* strains. **Table 1** includes CodY-regulated genes with associated CodY boxes that differ in expression at least 2-fold between strains, while **Table 2** contains CodY-regulated genes that are unique to the genome of either strain. As expected from the sporulation phenotypes of the *codY* mutants, sporulation-specific transcripts comprised many of the genes differentially expressed in the UK1 *codY* mutant (**Table S3** ~≥ 10%) (Pereira *et al.* 2013; Fimlaid *et al.* 2013), many of which were late-stage sporulation or germination factors. Unfortunately, increased late sporulation gene expression in UK1 *codY* is not helpful for understanding how CodY differentially regulates the initiation of sporulation, which is controlled by the activation of the master sporulation regulator, Spo0A (Fimlaid *et al.* 2013; DiCandia *et al.* 2022). One factor that is directly involved in Spo0A activity and demonstrated reduced expression in UK1 *codY* is *spo0E.* Spo0E interacts with Spo0A to limit Spo0A activation, which prevents sporulation initiation in *C. difficile (*DiCandia *et al.* 2024). However, the putative CodY boxes that potentially impact *spo0E* were identical in UK1 and 630, implying that the CodY-dependent effect on *spo0E* transcription in the UK1 *codY* mutant was not due to strain-specificity in CodY binding. In addition, a large proportion (20%, **Table S3**) of the CodY-regulated transcripts in both strains are genes of unknown function, which limits our understanding of their contribution to CodY-dependent phenotypes.

Though few sporulation initiation-associated genes were identified in these data that would clearly explain the increased spore formation found in the UK1 *codY* mutant, there were notable differences in the expression of genes indirectly associated with greater sporulation. The transcriptional analyses revealed significant changes in CodY regulation between strains,

including increased relative expression of dozens of metabolic genes in UK1 *codY* that are not observed in 630 *codY*. Further, several of the metabolism loci that are upregulated in UK1 *codY* contain differences in their putative CodY boxes compared to 630 *codY* (**Table 1**), while some are only encoded by one strain (**Table 2**). The extensive differences in UK1 *codY* and 630 *codY* metabolic gene expression suggests that these strains have altered responses to nutrient limitation, which may affect the ability to initiate or complete spore formation.

Repression of multiple direct CodY-regulated factors impact sporulation in strain UK1

Given the limited information available on the function of many CodY-regulated factors, we selected an assortment of genes present in both strains that were greatly induced or repressed by CodY for further investigation of their impacts on sporulation (**Table 3**). To determine which directly-regulated, CodY-dependent transcripts may impact spore formation, we employed a CRISPR interference (CRISPRi) approach to suppress transcription of target genes (Müh *et al.* 2019). The UK1 and UK1 *codY* strains were used for these experiments due to the robust CodY-regulated sporulation phenotype in this background. The UK1 strain was used to evaluate the effects of repressing eight CodY-induced factors, while the UK1 *codY* mutant was used to examine repression of six CodY-repressed factors. Strains were transformed with plasmids containing each CRISPRi sgRNA target expressed from a nisin-inducible promoter and grown on 70:30 agar with 1 µg/ml nisin to assess the impact of transcript repression on sporulation (Edwards and McBride 2023; McBride and Sonenshein 2011). The repression of target genes was examined by qRT-PCR during active growth, which confirmed that the targeted transcripts were reduced in all the strains tested (**Figure S1**). The sporulation frequencies of strains carrying each sgRNA target were determined after 24 h, as previously noted, and normalized to the respective parent carrying the vector control (pKD). As shown in **Figure 2**, suppression of two of the eight CodY-induced transcripts in strain UK1 resulted in significant increases in sporulation relative to the control. The repression of *CDIF27147_01510*,

a gene of unknown function, resulted in a ~40-fold increase in sporulation in strain UK1. The expression of *CDIF27147_01510* was reduced approximately 50-fold in the UK1 *codY* mutant and 20-fold 630 *codY* mutant (*CD630_14850*) under sporulation conditions (**Table S3**). The *CD630_14850* gene is controlled by the iron-responsive regulator, Fur, and induced by cysteine, suggesting it is involved in metabolism (Dubois *et al.* 2016; Ho and Ellermeier 2015). Similarly, knockdown of the *CDIF27147_02672* transcript led to ~35-fold greater sporulation in UK1 (**Figure 2**). Expression of *CDIF27147_02672* was decreased 4-fold in the UK1 *codY* mutant and ~3-fold in the 630 *codY* mutant during sporulation (**Table S3**). *CDIF27147_02672* is part of a dicistronic operon encoding a pH-dependent transcriptional regulator and transporter we recently characterized (*smrRT*; *CD630_25050-25060*) that contributes to macrolide and lincosamide resistance (Wetzel *et al.* 2024). SmrR represses expression of the *smrT* transporter, which reduces sporulation and toxin production (Wetzel *et al.* 2024). Expression of SmrRT and CDIF27147_01510 do not appear to directly link to Spo0A activity based on known interactions (DiCandia *et al.* 2024), but more likely support cellular homeostasis through pH or nutritional adaptations, respectively.

The UK1 *codY* mutant was used to assess repression of six CodY-repressed factors by CRISPRi and examine their effects on sporulation, as outlined above. Of the six genes assessed in UK1 *codY*, suppression of *CDIF27147_02081* and *CDIF27147_02803* dramatically reduced spore formation (**Figure 3**). Repression of *CDIF27147_02081* led to a ~150-fold decrease in sporulation, while knockdown of *CDIF27147_02803* resulted in ~35-fold lower spore formation than the control. *CDIF27147_02081* and *CDIF27147_02803* both encode predicted membrane proteins of unknown function that are expressed during sporulation *(Fimlaid et al. 2013; Saujet et al.* 2013; Abhyankar *et al.* 2019; Soutourina *et al.* 2020). *CDIF27147_02081* expression increased 248-fold in the UK1 *codY* mutant during sporulation, but was down 14-fold in the 630 *codY* mutant (*CD630_19280*) (**Table S3**). Similarly, *CDIF27147_02803* expression increased 47-fold in UK1 *codY* and decreased 3-fold in 630

codY (*CD630_26360*) during sporulation. The juxtaposed expression profiles for these genes in the UK1 *codY* and 630 *codY* mutants suggest that both factors support robust spore formation, but further investigation is needed to understand their roles in sporulation.

DISCUSSION

While 630 and UK1 encode identical CodY proteins that can bind to the same target sites, the activity of CodY in these backgrounds may be influenced by many factors that cannot be easily measured. CodY regulation is contingent on the availability of the cofactors GTP and BCAA, which trigger conformational changes in CodY that are necessary for DNA binding (Dineen *et al.* 2007; Nawrocki *et al.* 2016; Dineen, McBride, and Sonenshein 2010; Blagova *et al.* 2003; Brinsmade *et al.* 2010; Daou *et al.* 2019). The availability of GTP and BCAA signal amino acid and energy levels in the cell, which can vary in strains based on their ability to take up nutrients or their capacity to utilize nutrient sources. The UK1 and other 027 isolates grow more poorly than the 630 strain in complete defined minimal media (CDMM) and 027 ribotype isolates demonstrate a narrower metabolic repertoire than 630 and many other strains (Karasawa *et al.* 1995; Woods *et al.* 2018; Rizvi *et al.* 2023; Furtado *et al.* 2024; Nawrocki *et al.* 2018; Scaria *et al.* 2014). The metabolic range of the 027 isolates relative to other strains may contribute to differences in CodY activity. For example, if BCAA are available to bind CodY, even if other growth-limiting nutrients are unavailable, CodY-DNA binding could persist, restricting adaptation to nutrient limitation, and decreasing spore formation (**Figure 1**, UK1 24h). Thus, deletion of *codY* in UK1 could expand metabolite availability through nutrient gene derepression to support sporulation. Our data suggest that at least some of the CodY-regulated genes in UK1 repress sporulation, as indicated by the hypersporulation of the UK1 *codY* mutant, while in the 630Δ*erm* strain, only the timing of sporulation is advanced in the absence of *codY* (**Figure 1**). Overall, the evidence suggests that nutrient availability differs in these strains, leading to differential CodY regulation of sporulation and metabolic processes.

Our data show that the CodY regulons of the 630Δ*erm* and UK1 strains are considerably different (**Table S1, S2, S3**). Additionally, we identified several CodY-dependent genes with putative CodY boxes that differ in these strains (**Table 1**) and unique CodY-regulated factors present only in one strain (**Table 2**). Though we were able to identify several factors that are differentially regulated by CodY that have potential CodY-binding sites, further investigation is needed to determine if CodY is the major regulator of these factors and if CodY binds to these boxes. It is also important to note that by limiting our analysis to factors that were differentially expressed in the *codY* mutants by more than 3-fold, we may have missed some direct CodY-regulated factors that impact sporulation.

Our work demonstrates that multiple factors regulated by CodY can influence sporulation, as illustrated in **Figures 2** and **3**. As CodY regulates hundreds of genes, innumerable effects of global changes in gene expression in the absence of *codY* may contribute to the different sporulation phenotypes in the UK1 and 630Δ*erm* strains. The effects of CodY on sporulation may be an indirect result of altering the nutrients available or cellular functions that are necessary for adapting to post-exponential growth. Many of the CodY-dependent factors that are differentially regulated have no identified function in *C. difficile*, and their roles in sporulation are not known. Further characterization of these CodY regulated factors, especially those that affect sporulation when repressed, could provide targets for preventing spore formation.

MATERIALS AND METHODS

Bacterial strains and growth conditions

C. difficile strains were cultivated in a Coy anaerobic chamber at 37°C with an atmosphere of 10% H_2, 5% CO_2, and 85% N_2 as previously described (Bouillaut, McBride, and Sorg 2011). *C. difficile* strains grew in BHIS broth with addition of 0.1% of taurocholic acid (TA, Sigma-Aldrich) to induce germination and 0.2% of fructose (D-fructose, Fisher Chemical) to

prevent sporulation (Sorg and Dineen 2009). To maintain plasmids in *C. difficile* strains, 2-10 μg/ml of thiamphenicol was added to cultures. For CRISPRi induction, 1μg/ml of nisin was added, as needed. *Escherichia coli* strains were cultivated aerobically at 37°C in LB medium (Lennox) with 20 μg/ml of chloramphenicol and/or 100 μg/ml ampicillin (Sigma-Aldrich) for plasmid maintenance. *E. coli* was counter-selected post-conjugation with 100 μg/ml of kanamycin.

Strain and plasmid construction

The *C. difficile* strain R20291 027 ribotype genome (GenBank accession no. CP_029423.1) was used as a template for primer construction, and UK1 genomic DNA was used for PCR amplification. To generate sgRNAs, the Benchling CRISPR Guide RNA Design tool was used. sgRNAs were amplified by PCR and cloned into pMC1123 (Müh *et al.* 2019; Edwards and McBride 2023). Design details of vector constructions are provided in the supplemental material (**Fig. S2**).

Sporulation assays

Sporulation assays were carried out as previously described (Childress *et al.* 2016; Edwards and McBride 2017). In short, *C. difficile* cultures at mid-exponential phase (OD_{600} ~0.5) were plated on 70:30 agar supplemented with 2 ug/ml of thiamphenicol and 1 ug/ml of nisin as needed. After 6 (H_6), 12 (H_{12}), and 24 hours (H_{24}) of growth, ethanol-resistant sporulation assays were performed as previously described (Edwards and McBride 2017). Sporulation frequencies were calculated by dividing the number of spores by the total quantity of cells (spores + vegetative). A *spo0A* mutant was used as a negative sporulation control. For statistical analysis, GraphPad Prism v10.4.1 was used as stated in the figure legends.

Phase contrast microscopy

Phase-contrast microscopy was performed at H_6, H_{12}, and H_{24}, as specified in the figure legends, using cells grown on 70:30 sporulation agar, as previously described (Edwards and McBride 2023).

RNA sequencing (RNA-seq) analysis

C. difficile strains were grown on 70:30 agar for 6 hours, cells were scraped and suspended into 1:1:2 ethanol-acetone-water solution and stored at -70°C prior to processing. RNA was extracted and treated with DNase I (Ambion), as previously described (Dineen, McBride, and Sonenshein 2010; Edwards, Nawrocki, and McBride 2014). RNA libraries were prepared and processed by the Microbial Genomics Sequencing Center (MiGS; Pittsburgh, PA), as previously described. RNA-seq reads were mapped to the respective reference genome (630; NC_009089.1), and (R20291; CP_029423.1) using Geneious Prime v2022.2.2. Expression levels of transcripts were calculated and compared using DESeq2 (Love, Huber, and Anders 2014). RNA-seq raw sequence reads were deposited to the NCBI Sequence Read Archive (SRA) as BioProject PRJNA1263881.

Identification of CodY-boxes

Potential CodY boxes were found in the 630 and R20291 genomes from previously published sites, in addition to *in silico* identification (Dineen, McBride, and Sonenshein 2010; Girinathan *et al.* 2018). The *C. difficile* strain 630 and R20291 genomes (630, NC_009089.1; R20291, CP_029423.1) were screened for the global CodY AATTTCWGAAAATT consensus sequence containing up to four mismatches using a combination of FIMO MEME and Benchling software(Dineen, McBride, and Sonenshein 2010; Bailey et al. 2015).

Quantitative reverse transcription PCR analysis (qRT-PCR)

C. difficile strains were grown on 70:30 agar for 6 hours, suspended in 1:1:2 Ethanol-Acetone-water solution, and stored at -70°C. RNA extraction, treatment with DNase I (Ambion), and cDNA synthesis using random hexamers (Bioline) were performed as previously described

(Dineen, McBride, and Sonenshein 2010; Edwards, Nawrocki, and McBride 2014). qRT-PCR was conducted on a Roche LigthCycler 96 instrument from 50 ng of cDNA in technical triplicates using Bioline SensiFast SYBR & Fluorescein mix with primers shown in **Table 5**. Expression was normalized to the internal control transcript, *rpoC*, and analyzed using the $\Delta\Delta C_t$ method for relative quantification (Schmittgen and Livak 2008). GraphPad Prism v10.4.1 was used as mentioned in the figure legends, for statistical analysis.

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TABLES

Table 1. Differentially expressed CodY target genes in strains 630Δ*erm* and UK1

UK1				630Δ*erm*					
Genetic Region	**Predicted CodY Box**	**Predicted CodY Target**	**ΔcodY/ WT**	**Genetic Region**	**Predicted CodY Box**	**Predicted CodY Target**	**Δ*codY*/WT**	**Gene names**	**Putative Function**
CDIF27147_00336-00337	AATATTCAAATAATT AACTTTAGGAAAAAT AATTTTTTGAAAAAA	CDIF27147_00336-00337	16.5-18.3	CD02130-02140	AATATTCAAATAATT AACTTTA**A**GAAAAAT AATTTTTTGAAAAAA	CD02130-02140	1.43-1.77		Sporulation
CDIF27147_00351-00353	AATTTTCTGACAAAT	CDIF27147_00352-00353	0.23-0.28	CD02260-02280	A**G**TTTTCTGACAGCT	CD02270-02280	1.57-4.41	*fliN*	Motility
CDIF27147_00374-00397	AACTTTTAGAAAATA AAGTTTATGAAAATT AATTTTGAGAAAAAT	CDIF27147_00382-00397	0.39-0.95	CD02450-02630	AACTTTT**G**GAA**G**ATA AAGTTTATGAAAATT AATTTTGAGAAAAAT	CD02670	1.21-4.13	*flg, fli, mot, flh*	Motility
CDIF27147_00476-00478	CATTTTAGAAAAATT	CDIF27147_00478	1.30-3.28	CD03350-03370	CATTTTA**A**AAAAATT	CD03370	0.59-8.06		Unknown
CDIF27147_00481-00482	AAATATCTGAAAAAA AATTTACTAAAAACT AAGTTTATGAAAAAT GATTTTATGCAAATT	CDIF27147_00481-00482	0.24-0.27	CD03400-03410	AA**T**TATCTGAAAAAA AATTTACTAAAAACT AAGTTTATGAAAAAT GATTTTATGCAAATT	CD03400-03410	1.43-1.62		Unknown
CDIF27147_00526-00530	AAAATTCGGAAAATT	CDIF27147_00526-00530	10.05-23.88	CD04450-04490	AAAATTC**A**GAAAATT	CD04450-04490	0.93-1.34	*oraSE, orr*	Amino Acid
CDIF27147_00566-00567	AGATTTGTGAAAATA AATTTTGAAAATAGT AATATTTTAAAAATC ATCTTTCTCACAATT AAGTTTCAAGAAATA AACTTACTAAAAATC	CDIF27147_00566	1.12-6.02	CD04830-04840	AGATTTGTGAAAATA AATTTTGAAAATAGT AATATTTTAAAAATC ATCTTTCTCACAATT AAGTTTCAAGAAATA AACT**C**ACTAAAAATC	CD04830	1.38-1.45		Transporter
CDIF27147_00618	AACATTCTGAAAAAT AATATAACGAAAATT AAGTTAAAGAAAATT	CDIF27147_00618	8.64	CD05500	AA**T**ATTCTGAAAAAT AATATAACGAAAATT AAGTTAAAGAAAATT	CD05500	1.08		Unknown
CDIF27147_00723-00725	AATATACTTTAAATT AACCTTTATAAAATT AGTTTGAAAAAAATT **AAATATCTGTATATT** AATTATATCAAAATC GATTTTATGGAATTT AATTTACAGATACTG AAAGTTCAGATATTT	CDIF27147_00723-00725	2.10-4.13	CD06490-06510	AATATACTTTAAATT AACCTTTATAAAATT AGTTTGAAAAAAATT AATTATATCAAAATC GATTTTATGGAATTT AATTTACAGATACTG AAAGTTCAGATATTT AGATTTATGAAGATT	CD06490-06510	1.01-1.10		Peptidases

	AGATTTATGAAGATT AATTTGCAGAACTAT CATTACCTGAAAAAT AAATATGTGAAAAAT AACTTTCAGAGATTA				AATTTGCAGAACTAT CATTACCTGAAAAAT AAATATGTGAAAAAT AACTTTCAGAGATTA				
CDIF27147_00739	TATTTTAGGAAAATA	CDIF27147_00739	33.2	CD06640	TATTTT**CCT**AAAATA	CD06640	5.81	*tcdC*	Toxin
CDIF27147_00772	AATTATAAGAAGATT	CDIF27147_00772	10.3	CD06910	A**G**TTATAAGAAGATT	CD06910	0.43		Metabolism
CDIF27147_00939-00943	AATTTGATGAAATTT AATTTTTAAAAAGTT AATTTACGGCAAATG	CDIF27147_00939-00943	0.23-0.53	CD08530-08560	AATTTGATGAAATTT AATTTTTAAAAAGTT AATTTAC**A**GCAAATG	CD08530	0.58-1.12	*oppBCAD*	Metabolite transporter
CDIF27147_00947-00949	TATTATCTGAAAATA AAGTTTTAGAAATTT	CDIF27147_00948-00949	1.23-2.12	CD08610-08630	TATTATCTGAAAATA AAGTTTTAGAAA**C**TT	CD08620-08630	0.27-0.79		Metabolite transporter
CDIF27147_00969-00973	AATTTTATGAAAGCT TATTTTTAGAGAATT AATTTCCTCAAAAGT	CDIF27147_00969-00973	4.92-6.74	CD08820-08860	AATTTTATGAAAGCT TATTTTTAGAGAATT AATTTCCTCAAAA**A**T	CD08820-08860	1.65-2.48	*glgCDAP*	Metabolism
CDIF27147_01044-01045	CTTTTTTAGAAAATT ATTTTTATGAGAATT AATTTTAAGAATATA AAGTTTATTAAAATT AATATTAGTAAAATT	CDIF27147_01044-01045	3.67-20.0	CD10280-10290	CTTTTTTAGAAAATT ATTTTTATGAGAATT AATTTTAAGAATATA AAGT**C**TATTAAAATT AATATTAGTAAA**G**TT	CD10280-10290	0.49-1.06		Signaling
CDIF27147_01075	AATTATTGAAAAATT AAATTTCACAAAATT TATTTCAGGAAAATT	CDIF27147_01075	0.23	CD10540	AATTATTGAAAAATT AAATTTCACAAAATT TATTTCAGGAAAA**C**T	CD10540	0.5	*bcd2*	Metabolism
CDIF27147_01249	-	-	0.89	CD12380	**AATTTTAGGAACATT**	CD12380	4.19		Unknown
CDIF27147_01280-012820	AATTTTCAGCATATT AGATTTCTCAAAATT AATTTTATAAAAAAT	CDIF27147_01280-012820	0.64-0.80	CD12660-12680	AATTTTCAGTATATT **AATTAAAAGAAAATT** **AATTCTCAGAAAATA**	CD12660-12670	2.04-3.21		Transporter
CDIF27147_01285-01288	**CAATTTCAAAAAATT** ATTTTTCTGAAAAAG AATATCCTGAAAATT AATTTGGAGAAGATT AATTTCCATAAATTT	CDIF27147_01285-01288	3.36-8.81	CD12710- 12740	ATTTTTCTGAAAAAG AATATCCTGAAAATT AATTTGGAGAAGGTT AATTTCCATAAATTT	CD1273-12740	0.25-1.10	*topA*	DNA Processing
CDIF27147_01432	CATTTGAAGAAAATT AATTTTAAGTATATT **AATTTTCTTATATTT**	CDIF27147_01432	0.29	CD14120	CATTTGAAGAAAATT AATTTTAAGTATATT	CD14120	1.16		Transcription Regulation
CDIF27147_01665	-	-	49.8	CD15670	**AATATTGATAAAATT**	CD15670	1.01	*cotG*	Sporulation
CDIF27147_01721	ATTTTTCAGACAATT AAATTTTACAAAATT AATTTTGCGTAATTT	CDIF27147_01721	0.30	CD16160	ATTTTTCAGACAATT AAATTTTACAAAATT AATTTTG**T**GTAATTT	CD16160	1.29		Signaling

	AATTTAACAAAAATT AATTTTATTATAATT				AATTTAACAAA**G**ATT AATTTTATTATAATT				
CDIF27147_01737	AATTATTGCAAAATT	CDIF27147_01737	28.1	CD16310	AATT**T**T**GCA**ATAATT	CD16310	0.59	*sodA*	Metabolism
CDIF27147_01805-01806	AATTTTCTTTAAATT	CDIF27147_01805-01806	0.88-1.67	CD16940-16950	**ATTTTTCAAAAACTT** **AATTTTTCAAAAACT** AATTTTCTTTAAATT	CD16940-16950	0.23-0.71		Unknown
CDIF27147_01855-01856	AATTATTGGTAAATT	CDIF27147_01855-01856	6.03-6.48	CD17400-17410	AATTATTG**C**TAAATT	CD17400-17410	0.55	*grdGF*	Metabolism
CDIF27147_01886	AATTTTAAAAAAATT **AATTTTTTGAAAAAA** CATTTTCCTAATATT	CDIF27147_01886	0.02	CD17671-17680	AATTTTAAAAAAATT CATTTTCCTAATATT	CD17671-17680	0.09-0.11		Unknown
CDIF27147_01913	AAATTCCTAAAAATT	CDIF27147_01913	4.77	CD17930	AA**G**T**G**CCTAAAAATT	CD17930	0.52		Unknown
CDIF27147_01965	AATTTTACGATATTT ATTTTCGAGAAAAAT	CDIF27147_01965	8.23	CD18440	AATTTTACGATATTT **AAAATACAGAAAATT** ATTTTTGAGAAAAAT	CD18440	1.02		Unknown
CDIF27147_02022-02023	GATTTTCATAACATT **AATTTTCAAAGATTT** **AAATTTCTAAAAATG**	CDIF27147_02022-02023	4.15-6.48	CD18620-18630	GATTTTCATAACATT	CD18620-18630	0.35-0.58		Conjugative Transposon
CDIF27147_02031	AACTTTCAGACAAAT	CDIF27147_02031	0.11	CD18710	AACT**C**TCAGACAAAT	CD18710	0.55		Conjugative Transposon
CDIF27147_02062-02067	AATTTTTTCTAAATT GATTTGCAGAAAGTT AAGTTTCAGAAGATA	CDIF27147_02062-02067	16.8-22.8	CD19120-19170	AATTTTTTCTAAATT GATTT**A**CAGAAAGTT AAGTTTCAGAAGATA	CD19120-19170	0.18-0.42	*eutABCLME*	Metabolism
CDIF27147_02068	AAATTTATAAAAATA	CDIF27147_02068	67.5	CD19180	AAATTT**C**TAAAAATA	CD19180	0.31	*eutK*	Metabolism
CDIF27147_02170	**ATATTTACGAAAATT**	CDIF27147_02170	321.8	CD20000	-	-	0.12	*ispD*	Metabolism
CDIF27147_02368	AATTTTAAGAATATA AAAATTCTGAAATTT	CDIF27147_02368	33.3	CD22010	AATTTT**G**AGAATATA AAAATTCTGAAATTT	CD22010	7.11		Transporter
CDIF27147_02391-02392	ATTATTCAAAAAATT	CDIF27147_02391-02392	2.25-3.91	CD22310-22330	**T**TTATTCAAAAAATT	CD22310-22330	0.95-1.35	*asrABC*	Redox
CDIF27147_02414	GAATTACTAAAAATA AAGCTTGTGAAAAGT AATATTCATAAATGT AATTTATTGTAATTT AATTTTAATAATCTT	CDIF27147_02414	0.64	CD22520	GAATTACT**G**AAAATA AAGCTTGTGAAAAGT AATATTCATAAATGT AATTTATTGTAATTT AATTTTAATAATCTT	CD22520	5.93	*kamA*	Metabolism
CDIF27147_02424	AATATTCTGAAGATA AAATTACAGATAAAT AATCTTTTGAAAAAG ATTTGACTGAAAAAT AAAATTCAGATAATG	CDIF27147_02424	0.06	CD22630	AATA**C**TCTGAAGATA AAATTACAGATAAAT AATCTTTTGAAAAAG ATTTGACTGAAAAAT AAAATTCAGATAATG	CD22630	1.89	*prsA*	Metabolism

CDIF27147_02479-02480	AATTCTATGAAAATT	CDIF27147_02479-02480	0.63-0.75	CD23260-23270	AATTCTAT**A**AAAATT	CD23260-23270	3.89-4.50	*gatAB*	Metabolism
CDIF27147_02545-02546	ATTTTTTAGAAAGTT AATTTAAAAAAAATT	CDIF27147_02545-02546	0.37-0.36	CD23880-23900	ATTTT**C**TAGAAAGTT **AATTTTATGAAGATA** AA**A**TTAA**G**AAAAAT**A**	CD23880-23890	1.18- 3.24	*blaRI*	Antimicrobial Resistance
CDIF27147_02668	ATTTTTCTGAATATT TATTTTCATAATATT AAATTTCATAAGATT	CDIF27147_02668	2.13	CD25020	ATTTTTCTGAATATT TATTTTCATAATATT AAATTT**T**ATAAGATT	CD25020	6.81		Cofactor synthesis
CDIF27147_02763	-	-	48.6	CD25990	**AATTATATTTAAATT**	CD25990	0.50		Transcriptional regulator
CDIF27147_02961	AGTATTCTGAAAGTT	CDIF27147_02961	0.32	CD27870	AGTATTCTGAAAG**C**T	CD27870	0.82	*cwp84*	Cell surface
CDIF27147_02971	AATATTCAGAAAAAA AGTTAGCAGAAGATT AATTTACTGATAGTA TATTGTCTGAAACTT AATATACACAAAATT	CDIF27147_02971	0.33	CD27970	**G**ATATTCAGAAAAAA A**A**TTAGCAGAAGATT AATTTACTGATA**A**TA TATTGTCTGAAACTT AATATACACAAAATT	CD27970	3.54		Cell surface
CDIF27147_02995	**AATTTTAAGAAAGTT** AATTTTCAATAAGTT	CDIF27147_02995	10.2	CD28181 (partial)	AATTTTCAATAAGTT	CD28181	1.09		Unknown
CDIF27147_03022	GATTTTAGGAAAATT AATTCACTGAGAGTT AATTTTCATTTAATT	CDIF27147_03022	78.5	CD28370	GATTTTAGGAAAATT AATT**T**ACTGAGAGTT AATTTTCATTTAATT	CD28370	5.47		Unknown
CDIF27147_03138	AATTTGACAAAAATT AAGTTTGAAAAAAAT TATTATCAGAAAGTT	CDIF27147_03138	0.51	CD30040	A**T**TTTGACAAAAATT AAGTTTGAAAAAA**T**T TATTATCAGAAAGTT	CD30040	4.40	*kdgT2*	Metabolite Transport
CDIF27147_03140	AATATTCTGTATATG AAATTAGTGAAAATT	CDIF27147_03140	0.41	CD30060	AATATTCTGTATAT**T** AAATTAGTGAAAATT	CD30060	4.60		Metabolism
CDIF27147_03156-03157	AATGTTCCTAAAAAC ATATTTTAGAAAATT	CDIF27147_03156-03157	101.4-174.3	CD30230-30240	AATGTTCCTAAAAA**T** ATATTTTAGAAAATT	CD30230-30240	0.21-0.37		Unknown
CDIF27147_03165	ATTTTTTATAAAATT AATTCTTTGAAAAAT	CDIF27147_03165	37.1	CD30320	ATTTTTTATAA**G**ATT AATTCTTTGAAAAAT	CD30320	0.93		Cofactor synthesis
CDIF27147_03235-03236	AATTTATTTAGAATT	CDIF27147_03235-03236	1.50-2.46	CD30970-30980	AATTTATTTA**A**AATT	CD30970-30980	5.28-6.89	*bglGF*	Metabolite Transport
CDIF27147_03313-03314	AATTATAAGCAAATT	CDIF27147_03313	2.36-9.10	CD31510- 31521	AAT**C**ATAAGCAAATT	CD31510	0.61-1.26		Prophage trancription regulation
CDIF27147_03355	AATATTTATAAAATT	CDIF27147_03355	14.3	CD31840	AA**A**ATTTATAAA**C**TT	CD31840	0.82	*dpaL*	Metabolism
CDIF27147_03396	TATTTTCTAATAATT	CDIF27147_03396	3.83	CD32190	TATTTT**T**TAATAATT	CD32190	0.93	*hslO*	Stress response

CDIF27147_03439-03442	AAAGTACAGGAAATT	CDIF27147_03439-03442	5.71-10.1	CD32600-32630	AAAGTACAGGAAATT**AATTTGATGGAAATA**	CD32600-32630	0.45-1.00	*pstCAB, phoU*	Transporter, Transcription regulation
CDIF27147_03542	GATTTTCTGAAAAGAGAATTTCAAAAAAGT	CDIF27147_03542	0.26	CD33690	GATTTTCTGAAAA**A**A**A**AATTTCAAAAAAGT	CD33690	5.63		Unknown

Table 2. Unique direct CodY-regulated factors present in the 630Δ*erm* or UK1 strains

Genetic Region	Predicted CodY Box	Predicted CodY Target	Δ*codY*/WT	Gene names	Putative Function
		630Δ*erm*			
CD02110-02120	AATTTGATGAAAATA GATTTTCGGAAAAAT	CD02110-02120	2.08-3.89	*licC*	Metabolism
CD02410-02440	ATTTTTTTTAAAATT TATATTCTAAAAATT GATTTTCTGATAATG	CD02410-02440	4.31-8.55		Motility
CD03790-CD03810	AATATACGGAACATT	CD03790-03810	0.34-0.60		Conjugative transposon
CD04090-04120	AAATTTCATAAAAAT	CD04090-04120	0.33-1.11		Conjugative transposon
CD04230	AATTTTCAAAGACTT AAATTACAGAAAAAT AAATTTCTAAAAATG AATATGCTGAAAATC	CD04230	0.32		DNA replication
CD04352	TACTTTCAGAACATT	CD04352	0.27		Conjugative transposon
CD10921-10940	ACTTTACAGAAGATT	CD10921-10940	0.23-0.27		Conjugative transposon, Transcriptional regulator
CD11030	AAGTGTCAGAAAATG	CD11030	0.32		Conjugative transposon
CD18510-18550	GACTTTCTCAAAATT	CD18510-18550	0.30-0.68		Conjugative transposon
CD18840	AATTTTTATAATATT	CD18840	0.07		Unknown
CD18860	AATTTTAGGATTATT AATTTACAGCAACTT	CD18860	4.47		Transcription regulator
CD26170	AATATTCCAAAATTT	CD26170	5.33		Unknown
CD31360-31380	AATTTTATGATGATT ATTTTTATGAAAATT AATTTACTAAAGATT	CD31360-31380	2.95-5.86	*bglA7F5G4*	Metabolism
		UK1			
CDIF27147_ 00347-00350	ATTTTCCTGAAAAAT	CDIF27147_00350	0.23-0.59	*rfbBCAD*	Metabolism
CDIF27147_ 00657-00658	AATTTTCTTAATATT	CDIF27147_00657- 00658	9.18		Signaling
CDIF27147_00757	ACTTAACTGAAAATT	CDIF27147_00757	24.0		Amino acid metabolism
CDIF27147_ 01970-01972	AACTTTTGGAAAAGT	CDIF27147_01972	1.49-7.30		Conjugative transposon
CDIF27147_ 02077-02078	AATTTACTAAAAATA AATATTGAGAAAAAT	CDIF27147_02077- 02078	1.08-3.09		Metabolism
CDIF27147_03267	AATATTCAGGAACTT	CDIF27147_03267	1.56-3.34		Metabolism
CDIF27147_ 03305-03309	AATTTTTAAAATATT GATTTTATGAAAATA AATGTTAGGAAAATT AATTTATGGAAGATT ACTTTTAGGAAAATA AGTTTTTAGAAACTT ATATTTTAGAAAATT	CDIF27147_03305- 03309	0.29-0.50		CRISPR

CDIF27147_03444-03445	AATTTTCTCATAATC	CDIF27147_03444-03445	4.54-5.27	Transporter
CDIF27147_03612	AATTTTCAAAAAGAT AATTTGGAGAAGATT	CDIF27147_03612	0.29	Unknown
CDIF27147_03617	AATTTTCTGATGATG	CDIF27147_03617	4.00	Unknown
CDIF27147_03628	AATTTTTTAAAACTT AATTTTTACAAAAAT	CDIF27147_03628	7.05	Unknown
CDIF27147_03629	AATTTGCAAAAGATT AATTTTTATAAACTT	CDIF27147_03629	10.8	Transposase
CDIF27147_03815-03818	CATTTTTGGAAACTT	CDIF27147_03818	2.31-6.48	Transposon

Table 3. CodY-regulated genes of UK1 selected for knockdown

Direct CodY-induced targets				
Predicted CodY Target	**Predicted CodY Box**	**Δ*codY*/WT**	**Name**	**Putative Function**
CDIF27147_01886	AATTTTAAAAAAATT AATTTTTTGAAAAAA CATTTTCCTAATATT	0.02		Unknown
CDIF27147_01510	CATTATCAGAAAAAT	0.022		Unknown
CDIF27147_02271	AGTTTTTGAAAAATT	0.04-0.04		Transcription regulation
CDIF27147_02499	AAATATCAAAAACTT	0.12		Transcription regulator
CDIF27147_00584	AATATGCAGAAAATG AATTTTCTATAAATA AAAGTTCTGAAAATA AATTATGTGAAAATA	0.18		Transcription antiterminator
CDIF27147_00748	ATATTTCATAAAATT	0.19	*blaI*	Transcription regulator
CDIF27147_03455	AACTTAATGAAAACT AATATTGACAAAATA AATATCCAGAAATAT	0.22-0.24	*spo0E*	Sporulation initation
CDIF27147_02672	ATTTTTCAAAAATTT	0.24-0.30	*smrR*	Transcription regulator
Direct CodY-repressed targets				
CDIF27147_02081	AATCTTCAAAAAATA	248.6-376.2		Unknown
CDIF27147_00252	AATCTTAATAAACTT	267.7		Unknown
CDIF27147_01772	AAATTTATGAATATT	65.9		Unknown
CDIF27147_02803	GATTTTTAGAAGATT	47.4		Unknown
CDIF27147_01821	AAATCTCAGAAAGTT	42.3		Metabolism
CDIF27147_03734	ATTCTTATGAAAATA AATGTTAATAAAGTT AATATTTAGAATAAT	41.4		Unknown

Table 4. Bacterial Strains and plasmids

Plasmid or Strain	Relevant genotype or features	Source, construction or reference
Strains		
E. coli		
DH5α Max Efficiency	F− Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rk−, mk+) *pho*A *sup*E44 λ−thi−1 *gyr*A96 *rel*A1	Invitrogen
HB101	F^- *mcrB mrr hsdS20*(r_B^- m_B^-) *recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20*	B. Dupuy
C. difficile		
630Δ*erm*	Erm^S derivative of strain 630, ribotype 012	N. Minton (Hussain, Roberts, and Mullany 2005)
UK1	Epidemic isolate, ribotype 027	(Sorg and Sonenshein 2010)
LB-CD16	UK1 *codY::ermB*	(Mooyottu et al. 2014)
MC310	630Δ*erm spo0A::ermB*	(A. N. Edwards, Nawrocki, and McBride 2014)
MC364	630Δ*erm codY::ermB*	(K. L. Nawrocki et al. 2016)
MC855	630Δ*erm spo0A::ermB* pMC123	(DiCandia et al. 2022)
MC2186	UK1 pMC1123	(Wetzel et al. 2024)
MC2187	UK1 pMC1170	This study
MC2188	UK1 pMC1171	This study
MC2189	UK1 pMC1172	This study
MC2190	UK1 pMC1173	This study
MC2191	UK1 pMC1174	This study
MC2192	UK1 pMC1175	This study
MC2194	UK1 pMC1177	This study
MC2195	UK1 *codY::ermB* pMC1123	This study
MC2196	UK1 *codY::ermB* pMC1158	This study
MC2197	UK1 *codY::ermB* pMC1160	This study
MC2216	UK1 *codY::ermB* pMC1156	This study
MC2218	UK1 *codY::ermB* pMC1162	This study
MC2219	UK1 *codY::ermB* pMC1163	This study
MC2220	UK1 *codY::ermB* pMC1164	This study
MC2263	UK1 pMC1178	(Wetzel et al. 2024)
Plasmids		
pRK24	Tra^+, Mob^+; *bla, tet*	(Thomas and Smith 1987)
pIA33	P_{xyl}*::dCas9-opt* P_{gdh}::sgRNA-*rfp catP*	(Müh et al. 2019)

pMC123	*E. coli- C. difficile* shuttle vector*, bla, catP*	(McBride and Sonenshein 2011)
pMC404	pMC123 with *catP* replaced by *aad9*	(Purcell et al. 2017)
pMC1123	P_{cprA}*::dCas9-opt* P_{gdh}::sgRNA-*neg catP*; (pKD)	(Adrianne N. Edwards and Shonna M. McBride 2023)
pMC1156	P_{cprA}*::dCas9-opt* P_{gdh}::sgRNA-*CDIF27147_02081 catP*	This study
pMC1158	P_{cprA}*::dCas9-opt* P_{gdh}::sgRNA-*CDIF27147_00252 catP*	This study
pMC1160	P_{cprA}*::dCas9-opt* P_{gdh}::sgRNA-*CDIF27147_01772 catP*	This study
pMC1162	P_{cprA}*::dCas9-opt* P_{gdh}::sgRNA-*CDIF27147_02803 catP*	This study
pMC1163	P_{cprA}*::dCas9-opt* P_{gdh}::sgRNA-*CDIF27147_01821 catP*	This study
pMC1164	P_{cprA}*::dCas9-opt* P_{gdh}::sgRNA-*CDIF27147_03734 catP*	This study
pMC1170	P_{cprA}*::dCas9-opt* P_{gdh}::sgRNA-*CDIF27147_01886 catP*	This study
pMC1171	P_{cprA}*::dCas9-opt* P_{gdh}::sgRNA-*CDIF27147_01510 catP*	This study
pMC1172	P_{cprA}*::dCas9-opt* P_{gdh}::sgRNA-*CDIF27147_02271 catP*	This study
pMC1173	P_{cprA}*::dCas9-opt* P_{gdh}::sgRNA-*CDIF27147_02499 catP*	This study
pMC1174	P_{cprA}*::dCas9-opt* P_{gdh}::sgRNA-*CDIF27147_00584 catP*	This study
pMC1175	P_{cprA}*::dCas9-opt* P_{gdh}::sgRNA-*CDIF27147_00748 catP*	This study
pMC1177	P_{cprA}*::dCas9-opt* P_{gdh}::sgRNA-*CDIF27147_03455 catP*	This study
pMC1178	P_{cprA}*::dCas9-opt* P_{gdh}::sgRNA-*CDIF27147_02672 catP*	(Wetzel et al. 2024)

Table 5. Oligonucleotides

Primer	Sequence (5'→3')	Use/locus tag/reference
oMC44	CTAGCTGCTCCTATGTCTCACATC	Forward primer for *rpoC* qPCR (McBride and Sonenshein 2011)
oMC45	CCAGTCTCTCCTGGATCAACTA	Reverse primer for *rpoC* qPCR (McBride and Sonenshein 2011)
oMC2618	GATTATTATGGCGAACAATGAATTAGAAG	Forward primer for *spo0E* qPCR
oMC2619	AAATATTTCTGGATATTCTATGTATGTATTTATCT	Reverse primer for *spo0E* qPCR
oMC2362	AGTTAAACAGAAAGATAATTGCTGTATGG	Forward primer for *smrR* qPCR (Wetzel et al. 2024)
oMC2363	ACTTGTAGCCTTACGTTGTTCTTC	Reverse primer for *smrR* qPCR (Wetzel et al. 2024)
oMC3088	TTGCAATAAAGTGTGCTATAATTAAACTGTAAATGGCCA	Forward primer to Gibson assemble CRISPRi sgRNAs into pMC1123 (Wetzel et al. 2024; Adrianne N. Edwards and McBride 2023)
oMC3089	CCTTTTTCTATTTAAAGTTTTATTAAAACTTATAGGATCCGCGGCCGC	Reverse primer to Gibson assemble CRISPRi sgRNAs into pMC1123 (Wetzel et al. 2024; Adrianne N. Edwards and McBride 2023)
oMC3101	AATTAAACTGTAAATGGCCAAATAATTCCTCACTATCAAGGTTTTAGAGCTAGAAATAGC	Forward primer for sgRNA-*CDIF27147_02081* amplification
oMC3103	AATTAAACTGTAAATGGCCAGAAGAATTACTAAAACTGAGGTTTTAGAGCTAGAAATAGC	Forward primer for sgRNA-*CDIF27147_00252* amplification
oMC3105	AATTAAACTGTAAATGGCCAAATAGTATATTAAAACATAAGTTTTAGAGCTAGAAATAGC	Forward primer for sgRNA-*CDIF27147_01772* amplification
oMC3108	AATTAAACTGTAAATGGCCAACAACAGTTTCAAGGTCTTGGTTTTAGAGCTAGAAATAGC	Forward primer for sgRNA-*CDIF27147_02803* amplification
oMC3109	AATTAAACTGTAAATGGCCATTGACTTGGATAGTACCAAGGTTTTAGAGCTAGAAATAGC	Forward primer for sgRNA-*CDIF27147_01821* amplification
oMC3110	AATTAAACTGTAAATGGCCAATATTTTTGTAAGGATGCAAGTTTTAGAGCTAGAAATAGC	Forward primer for sgRNA-*CDIF27147_03734* amplification
oMC3131	AATTAAACTGTAAATGGCCATCTTGAAGGTGGTAAAATGGGTTTTAGAGCTAGAAATAGC	Forward primer for sgRNA-*CDIF27147_01886* amplification

oMC3132	AATTAAACTGTAAATGGCCATGGTGACACAAAACAATCCGGTTTTAGAGCTAGAAATAGC	Forward primer for sgRNA-*CDIF27147_01510* amplification
oMC3133	AATTAAACTGTAAATGGCCAGGTATACAAAAGTTTAAGCAGTTTTAGAGCTAGAAATAGC	Forward primer for sgRNA-*CDIF27147_02271* amplification
oMC3134	AATTAAACTGTAAATGGCCAAAAAACGTACCTAAAACTGTGTTTTAGAGCTAGAAATAGC	Forward primer for sgRNA-*CDIF27147_02499* amplification
oMC3135	AATTAAACTGTAAATGGCCAAAAAACGTACCTAAAACTGTGTTTTAGAGCTAGAAATAGC	Forward primer for sgRNA-*CDIF27147_00584* amplification
oMC3136	AATTAAACTGTAAATGGCCAATATCTTACTTATTGAAGAGGTTTTAGAGCTAGAAATAGC	Forward primer for sgRNA-*CDIF27147_00748* amplification
oMC3138	AATTAAACTGTAAATGGCCAAAATGAGATTGAAGCAGTTAGTTTTAGAGCTAGAAATAGC	Forward primer for sgRNA-*CDIF27147_03455* amplification
oMC3139	AATTAAACTGTAAATGGCCAATAAAAAAAATTATACGTCGAGTTTTAGAGCTAGAAATAGC	Forward primer for sgRNA-*CDIF27147_02672* amplification (Wetzel et al. 2024)
oMC3235	TTTCTTAATTATGGCTATGGCAGTT	Forward primer for *CDIF27147_01886* qPCR
oMC3236	ATAAAGGCTTCATAAATACAGCGAA	Reverse primer for *CDIF27147_01886* qPCR
oMC3237	TTGTGTCACCATAAACTTTCCAATA	Forward primer for *CDIF27147_01510* qPCR
oMC3238	AGAGTGATGTTTTTCCTGATGAAAT	Reverse primer for *CDIF27147_01510* qPCR
oMC3239	AACCATCTAAGTTTGGCATCATTAT	Forward primer for *CDIF27147_02271* qPCR
oMC3240	TTTAAGTGCAGAAGGTTATCAAGTT	Reverse primer for *CDIF27147_02271* qPCR
oMC3241	AAATGACTTGGCTTCAACAATATTG	Forward primer for *CDIF27147_02499* qPCR
oMC3242	AGTAATTGCACGTTCTAATGGTATT	Reverse primer for *CDIF27147_02499* qPCR
oMC3243	CAAAGTACGGCCAATTAAATTTTCT	Forward primer for *CDIF27147_00584* qPCR
oMC3244	AATTGCTAACCATTCATCTCTTGAT	Reverse primer for *CDIF27147_00584* qPCR
oMC3245	AAAGTTGCCACAATCAGAATTAAAG	Forward primer for *CDIF27147_00748* qPCR
oMC3246	CGTTTGTTTCCATTGATATTTTTGC	Reverse primer for *CDIF27147_00748* qPCR
oMC3249	AGGTTTGACAAGGCTTTCTAAAATA	Forward primer for *CDIF27147_00252* qPCR

oMC3250	TCAACCATATTTCCAGCATTTGATA	Reverse primer for *CDIF27147_00252* qPCR
oMC3251	CTGCTGTTAATTCAAAATGGAGTTT	Forward primer for *CDIF27147_01772* qPCR
oMC3252	ATCTTATCATTTTTATCCTCTCCATT	Reverse primer for *CDIF27147_01772* qPCR
oMC3394	CACAATAGCTAAAATTGTGCAATGA	Forward primer for *CDIF27147_02803* qPCR
oMC3395	TGCTTATGTTGAAGAAATAGCATCT	Reverse primer for *CDIF27147_02803* qPCR
oMC3396	AATGATTTTATTTGGACTTGGAGGT	Forward primer for *CDIF27147_01821* qPCR
oMC3397	AATTGCTATTGCTGTTAGAGAATCA	Reverse primer for *CDIF27147_01821* qPCR
oMC3398	AGTTGTACCCTCAAAAATATCCATT	Forward primer for *CDIF27147_03734* qPCR
oMC3399	ATTTTGTGTTGGATTTTTGGTTCTT	Reverse primer for *CDIF27147_03734* qPCR
oMC3488	ACGTTACTATTATTGATAATCTTCACTTATATG	Forward primer for *CDIF27147_02081* qPCR
oMC3489	AGATTATAGTACAATAATATAGAAAATTGACACT	Reverse primer for *CDIF27147_02081* qPCR
4084	AACTTATAGGATCCGCGGCCGCTAGTCAGACATCATG CTGATCTAGA	Reverse primer for sgRNAs with NotI site for cloning into pIA33 (Müh et al. 2019)

FIGURES

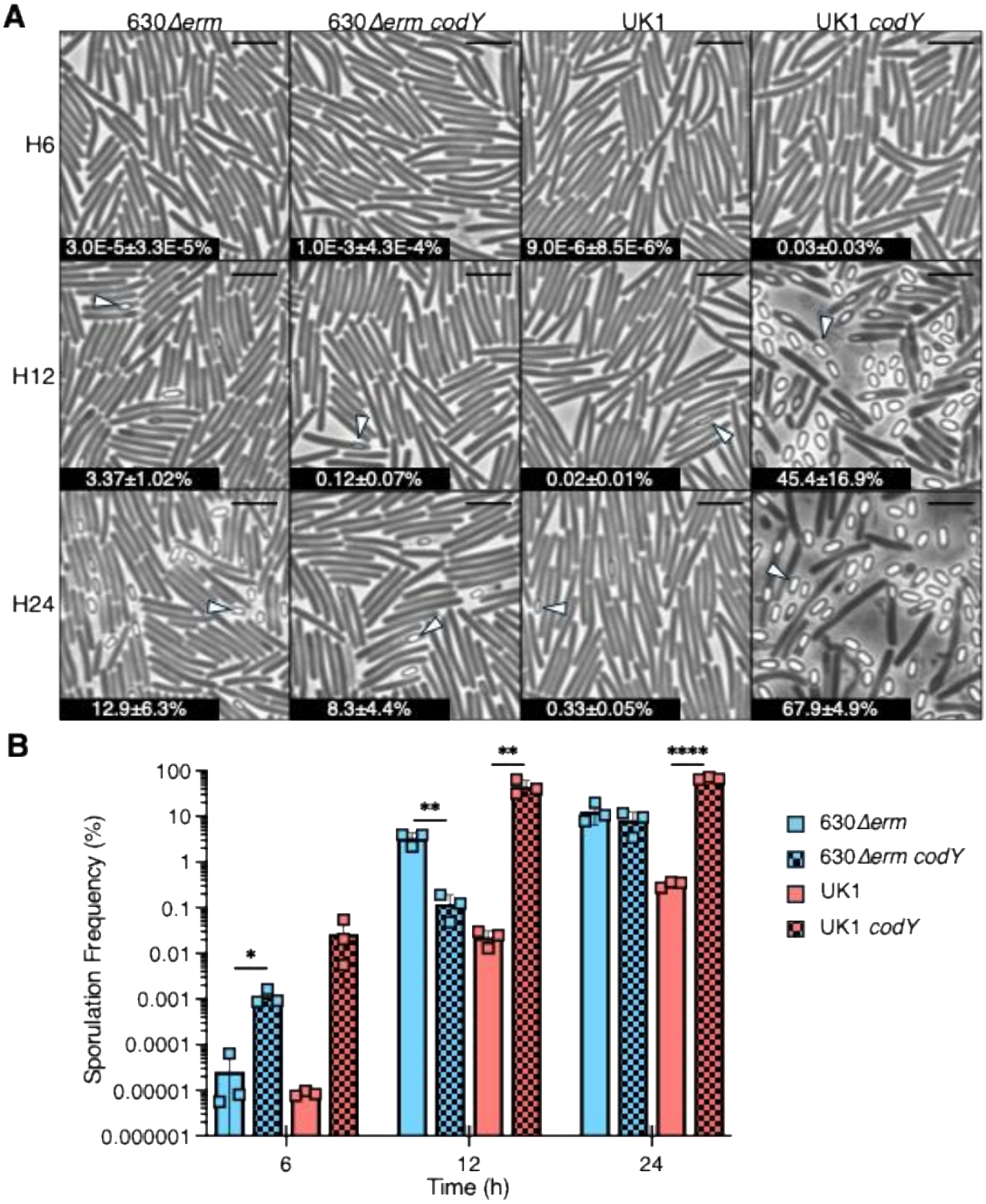
A
630Δerm
630Δerm codY
UK1
UK1 codY
H6
H12
H24
3.0E-5±3.3E-5%
1.0E-3±4.3E-4%
9.0E-6±8.5E-6%
0.03±0.03%
3.37±1.02%
0.12±0.07%
0.02±0.01%
45.4±16.9%
12.9±6.3%
8.3±4.4%
0.33±0.05%
67.9±4.9%
B
Sporulation Frequency (%)
100
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
*
**
**
****
6
12
24
Time (h)
630Δerm
630Δerm codY
UK1
UK1 codY

Figure 1. CodY repression on sporulation is strain-dependent. A) Phase-contrast micrographs of strains 630Δ*erm*, 630Δ*erm codY* (MC364), UK1, and UK1 *codY* (LB-CD16) grown on sporulation agar for 6, 12, or 24 h. White arrowheads indicate bright spores. Scale bar = 5 µm. ‡SD: standard deviation <0.0001. **B)** Ethanol-resistant spore formation for the cultures above. The means and individual values for three biological replicates are shown. Data were analyzed using unpaired Student's *t*-tests comparing the mutants to their respective parent strain. *P<0.05, ** P<0.01, **** P<0.0001.

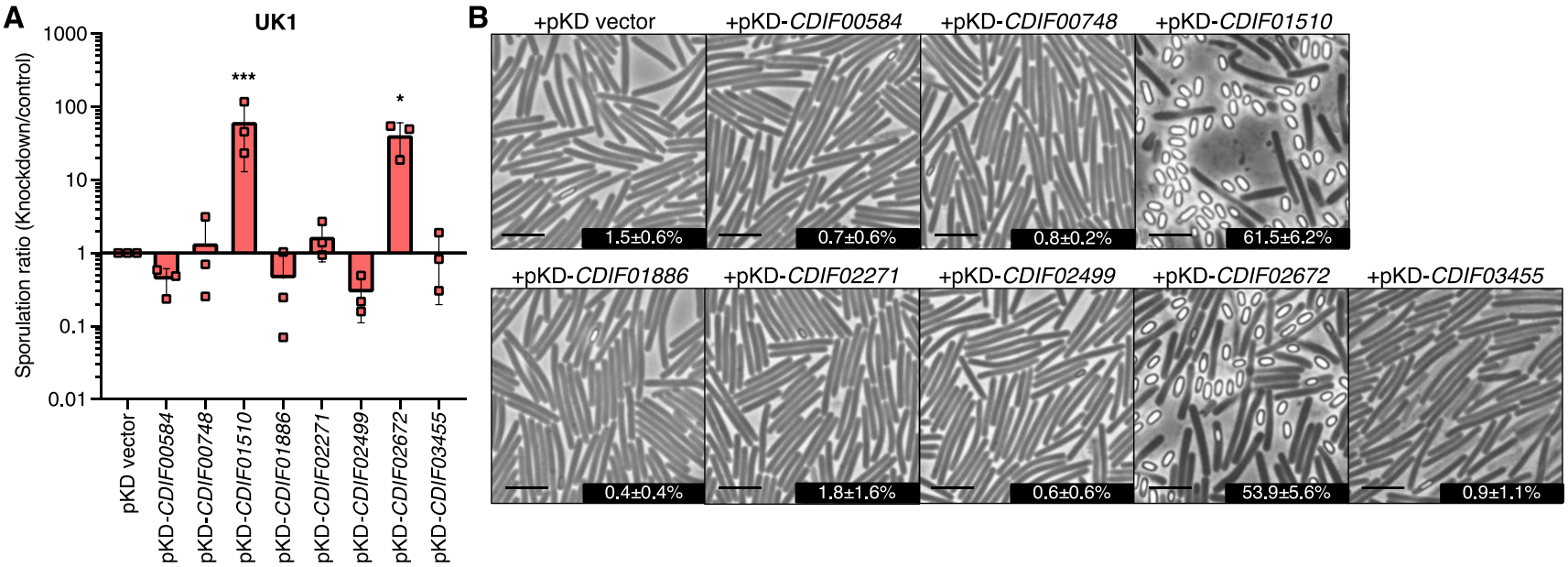

Figure 2. Repression of specific direct CodY-induced factors increases sporulation in UK1. A) Ratio of ethanol-resistant spore formation of strain UK1 expressing CRISPRi knockdown constructs, relative to a vector control. UK1 carrying pKD-*CDIF01886* (MC2187), pKD-*CDIF01510* (MC2188), pKD-*CDIF02271* (MC2189), pKD-*CDIF02499* (MC2190), pKD-*CDIF00584* (MC2191), pKD-*CDIF00748* (MC2192), pKD-*CDIF03455* (MC2194), pKD-*CDIF02672* (MC2263), and the pKD vector (MC2186) were assessed for spore formation after 24 h growth on sporulation agar (70:30 with 2 µg/ml thiamphenicol, 1 µg/ml nisin). The means, individual values, and standard deviations of ratios (Knockdown/control) for at least three biological replicates are shown. **B)** Phase-contrast micrographs of the strains in A with sporulation frequencies. Scale bar = 5 µm. The mean, standard deviations, and SEM are shown for three biological replicates. Data were analyzed using a one-way ANOVA followed by Fisher's LSD. **P*<0.05, *** *P*<0.001.

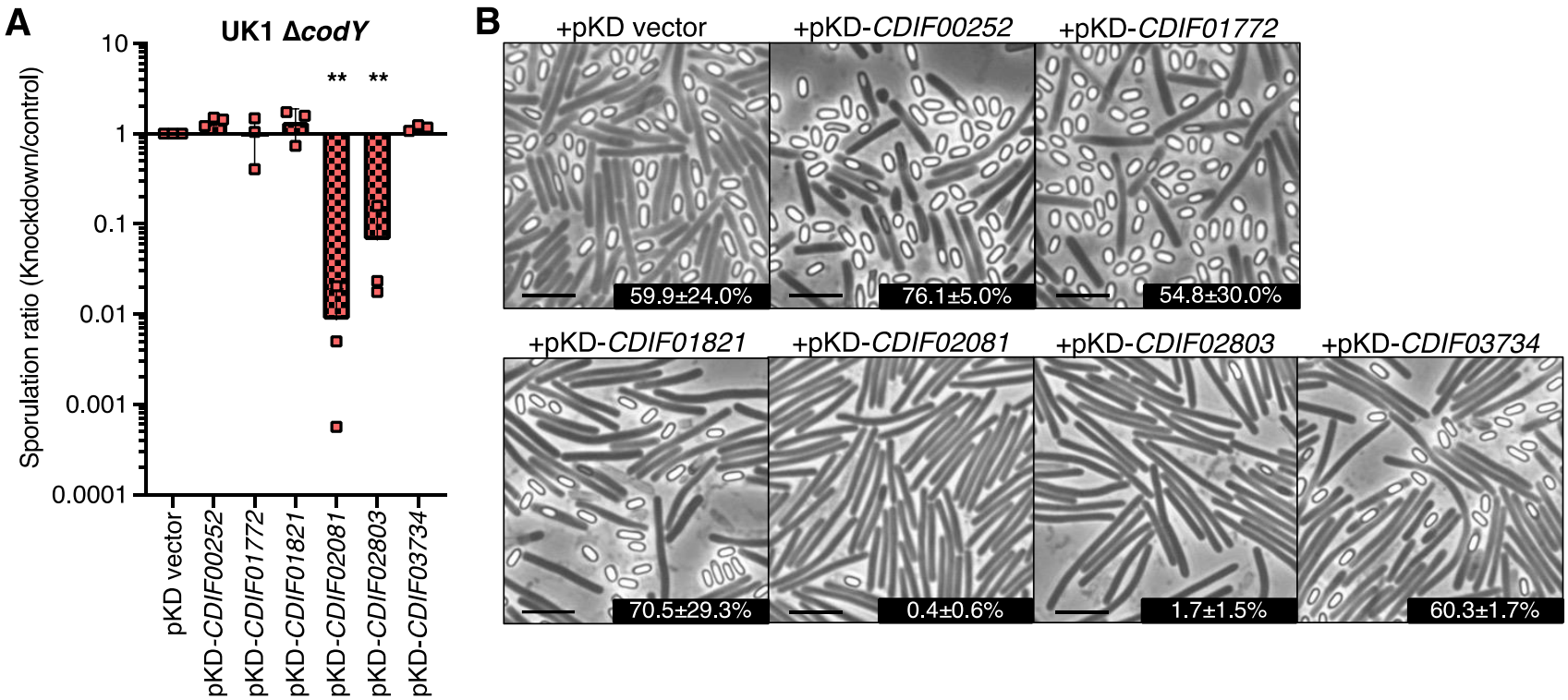

Figure 3. Repression of specific direct CodY-repressed factors reduces sporulation in the UK1 *codY* mutant. A) Ratio of ethanol-resistant spore formation of strain UK1 Δ*codY* mutant expressing CRISPRi knockdown constructs, relative to a vector control. UK1 Δ*codY* carrying pKD-vector (MC2195), pKD-*CDIF00252* (MC2196), pKD-*CDIF01772* (MC2197), pKD-*CDIF01821* (MC2219), pKD-*CDIF02081* (MC2216), pKD-*CDIF02803* (MC2218), and the pKD-*CDIF03734* (MC2220) were assessed for spore formation after 24 h growth on sporulation agar (70:30 with 2 μg/ml thiamphenicol, 1 μg/ml nisin). The means, individual values, and standard deviations of ratios (Knockdown/control) for at least three biological replicates are shown. **B)** Phase-contrast micrographs of

the strains in A with sporulation frequencies. Scale bar = 5 μm. The mean, standard deviations, and SEM are shown for three biological replicates. Data were analyzed using a one-way ANOVA followed by Fisher's LSD. ** $P<0.01$.

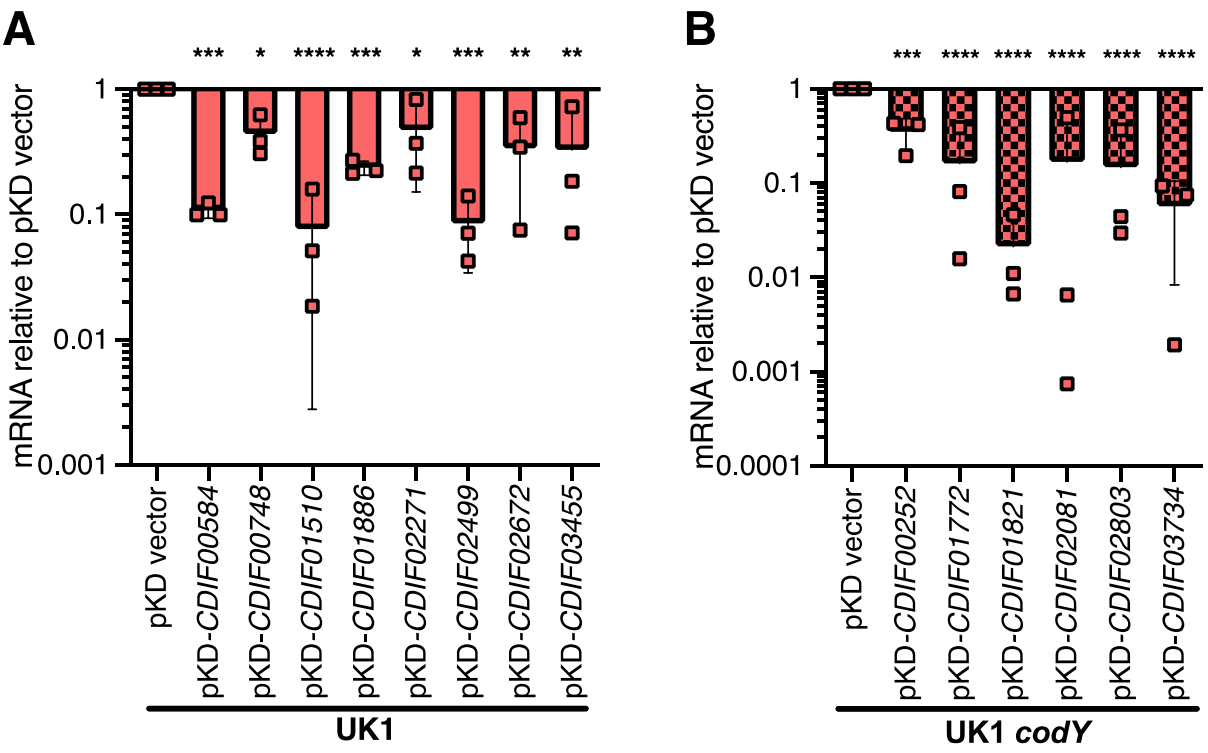

Figure S1. CRISPRi constructs repress expression of target genes. qRT-PCR analysis of gene expression for **A)** UK1 strains expressing CRISPRi knockdown constructs pKD-*CDIF01886* (MC2187), pKD-*CDIF01510* (MC2188), pKD-*CDIF02271* (MC2189), pKD-*CDIF02499* (MC2190), pKD-*CDIF00584* (MC2191), pKD-*CDIF00748* (MC2192), pKD-*CDIF03455* (MC2194), pKD-*CDIF02672* (MC2263), relative to the pKD vector control strain (MC2186) and **B)** UK1 Δ*codY* carrying pKD-*CDIF00252* (MC2196), pKD-*CDIF01772* (MC2197), pKD-*CDIF01821* (MC2219), pKD-*CDIF02081* (MC2216), pKD-*CDIF02803* (MC2218), pKD-*CDIF03734* (MC2220), relative to the pKD-vector control strain (MC2195). Samples were harvested after 6 h of growth on sporulation agar (70:30 with 2 µg/ml thiamphenicol, 1 µg/ml nisin). The means and individual values for three biological replicates are shown. Data were analyzed using a one-way ANOVA followed by Dunnett's multiple comparison test. *P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

Figure S2 – DNA cloning and vector details

pMC1156: A 140 bp PCR product containing sgRNA-*CDIF02081* was created using primers 4084 and oMC3101 and was amplified again using primers oMC3088 and oMC2089, which contain homology to pMC1123. The resulting product was Gibson assembled into pMC1123 via *MscI* and *NotI* sites.

pMC1158: A 140 bp PCR product containing sgRNA-*CDIF00252* was created using primers 4084 and oMC3103 and was amplified again using primers oMC3088 and oMC2089, which contain homology to pMC1123. The resulting product was Gibson assembled into pMC1123 via *MscI* and *NotI* sites.

pMC1160: A 140 bp PCR product containing sgRNA-*CDIF01772* was created using primers 4084 and oMC3105 and was amplified again using primers oMC3088 and oMC2089, which contain homology to pMC1123. The resulting product was Gibson assembled into pMC1123 via *MscI* and *NotI* sites.

pMC1162: A 140 bp PCR product containing sgRNA-*CDIF02803* was created using primers 4084 and oMC3108 and was amplified again using primers oMC3088 and oMC2089, which contain homology to pMC1123. The resulting product was Gibson assembled into pMC1123 via *MscI* and *NotI* sites.

pMC1163: A 140 bp PCR product containing sgRNA-*CDIF01821* was created using primers 4084 and oMC3109 and was amplified again using primers oMC3088 and oMC2089, which contain homology to pMC1123. The resulting product was Gibson assembled into pMC1123 via *MscI* and *NotI* sites.

pMC1164: A 140 bp PCR product containing sgRNA-*CDIF03734* was created using primers 4084 and oMC31110 and was amplified again using primers oMC3088 and oMC2089, which contain homology to pMC1123. The resulting product was Gibson assembled into pMC1123 via *MscI* and *NotI* sites.

pMC1170: A 140 bp PCR product containing sgRNA-*CDIF01886* was created using primers 4084 and oMC3131 and was amplified again using primers oMC3088 and oMC2089, which contain homology to pMC1123. The resulting product was Gibson assembled into pMC1123 via *MscI* and *NotI* sites.

pMC1171: A 140 bp PCR product containing sgRNA-*CDIF01510* was created using primers 4084 and oMC3132 and was amplified again using primers oMC3088 and oMC2089, which contain homology to pMC1123. The resulting product was Gibson assembled into pMC1123 via *MscI* and *NotI* sites.

pMC1172: A 140 bp PCR product containing sgRNA-*CDIF02271* was created using primers 4084 and oMC3133 and was amplified again using primers oMC3088 and oMC2089, which contain homology to pMC1123. The resulting product was Gibson assembled into pMC1123 via *MscI* and *NotI* sites.

pMC1173: A 140 bp PCR product containing sgRNA-*CDIF02499* was created using primers 4084 and oMC3134 and was amplified again using primers oMC3088 and oMC2089, which contain homology to pMC1123. The resulting product was Gibson assembled into pMC1123 via *MscI* and *NotI* sites.

pMC1174: A 140 bp PCR product containing sgRNA-*CDIF0584* was created using primers 4084 and oMC3135 and was amplified again using primers oMC3088 and oMC2089, which contain homology to pMC1123. The resulting product was Gibson assembled into pMC1123 via *MscI* and *NotI* sites.

pMC1175: A 140 bp PCR product containing sgRNA-*CDIF00748* was created using primers 4084 and oMC3136 and was amplified again using primers oMC3088 and oMC2089, which contain homology to pMC1123. The resulting product was Gibson assembled into pMC1123 via *MscI* and *NotI* sites.

pMC1177: A 140 bp PCR product containing sgRNA-*CDIF03455* was created using primers 4084 and oMC3138 and was amplified again using primers oMC3088 and oMC2089, which contain homology to pMC1123. The resulting product was Gibson assembled into pMC1123 via *MscI* and *NotI* sites.

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Chapter 3: Discussion

C. difficile is a nosocomial pathogen that is a burden to the healthcare and economic system in the U.S. (Guh *et al.* 2020; CDC, 2019; Smits *et al.* 2016). Through spore formation, *C. difficile* can survive disinfectants and spread from host to host via fecal-oral transmission (Sandhu and McBride 2018). Since nutrient availability is tightly linked with sporulation, it is crucial to understand better how nutrients regulate sporulation in *C. difficile* to intervene in the important process of dissemination. By comprehending the molecular mechanisms linking nutrient availability and sporulation, we can better illuminate how sporulation works in *C. difficile*.

I. CodY

In *B.* subtilis, CodY represses initiation of sporulation by binding directly to the promoter region of *spo0A* and repressing its transcription (Ratnayake-Lecamwasam *et al.* 2001; Mirouze, Prepiak, and Dubnau 2011). On the other hand, CodY is not a direct repressor of *spo0A* in *C. difficile,* and little is known about the molecular mechanism by which CodY regulates sporulation in *C. difficile* (Dineen, McBride, and Sonenshein 2010; Nawrocki *et al.* 2016; Daou *et al.* 2019). In this study, we increased the understanding of CodY regulation of sporulation in the 630Δ*erm* and UK1 strains, identified several factors directly regulated by CodY in both strains and determined the impact of many directly CodY-regulated factors in the UK1 strain.

Our results demonstrated for the first time using sporulation assays that CodY is repressing initiation of sporulation in both 630Δ*erm* and UK1 strains (**Chap. 2, Fig. 1**). Additionally, our data confirm previous findings that CodY regulation of sporulation is strain-dependent (Nawrocki *et al.* 2016). It would be valuable to investigate if other *C. difficile* ribotypes, such as strains from ribotypes 106, 014, 002, 020, and 076, have a strain-dependent phenotype of CodY regulation of sporulation as observed in 630Δ*erm* and UK1 (**Chap. 2, Fig. 1**) (Kim *et al.* 2022; Guh *et al.* 2020). Investigating other *C. difficile* ribotype strains for CodY regulation of sporulation can lead to a better understanding of how adaptable CodY regulation is

in different strains, as well as identify possible CodY-regulated factors that are shared or unique to each strain, which could serve as targets for drug development.

This work also demonstrated for the first time that after 12 hours on a solid sporulation medium, the 630Δ*erm* strain sporulated more than its *codY* mutant (**Chap. 2, Fig. 1**). These data indicate that CodY is necessary for the advancement of sporulation in the 630Δ*erm* background and not in the UK1 strain. To our knowledge, this work is the first to show that CodY is necessary for the advancement of sporulation in the 630Δ*erm* background. The only other example where CodY can play strain-specific roles (inducer/repressor) as a regulator of initiation of sporulation is in *C. perfringens*, where in one *C. perfringens* strain, CodY represses initiation of sporulation while in another strain of *C. perfringens*, CodY induces initiation of sporulation (Li *et al.* 2013; 2017). However, this is the first evidence that CodY can have both roles of inducer and repressor of sporulation for the same strain in one species of spore-forming bacteria (**Chap. 2, Fig. 1**). Comparing the CodY transcriptome at logarithmic and stationary phase in sporulation medium for the 630Δ*erm* background, could show which direct CodY-induced/repressed factor(s) impact sporulation and if these factors are the same or different at these time points.

By defining the CodY transcriptome of 630Δ*erm* and UK1 under sporulation conditions prior to the initiation of sporulation (for the WT), we identified several transcripts that CodY impacted that might affect the initiation of sporulation (**Chap. 2, Table S1; Chap. 2, Table S2**). We also determined which of these transcripts are potentially direct CodY targets by identifying which factors have a potential CodY box in each genome (**Chap. 2, Table S3**). Several factors listed in **Chap. 2 Table S3**, which are putative CodY-regulated factors, regulate late-stage sporulation and germination processes. These factors were predicted to contain CodY box(es), suggesting that CodY directly regulates later stages of sporulation, as well as initiation. In other sporulating bacteria, CodY is not known to regulate late-stage sporulation and/or germination factors directly, but only initiation of sporulation factors *(*Ratnayake-Lecamwasam *et al.* 2001;

Molle *et al.* 2003; Hilbert and Piggot 2004; Li *et al.* 2013; 2017). Determining if CodY binds to the predicted CodY boxes of the factors that regulate late-stage sporulation and germination would establish the role of CodY in the regulation of these factors and expand the repertoire of CodY regulation of sporulation.

We also identified several genes that are unique in both 630Δ*erm* and UK1 and are putative CodY-regulated factors (**Chap. 2, Table 2**). As explained in Chapter 2, these factors have not yet been characterized in *C. difficile*; characterizing these factors would allow us to understand their roles in sporulation. Another future direction to take from this work is to express the unique factors of 630Δ*erm* in the UK1 strain and vice versa and determine their effects on sporulation.

We also identified unique factors with putative CodY boxes (**Chap. 2, Table 2**); five factors are unique in the UK1 background, and two factors are unique in the 630Δ*erm* background that have metabolism-predicted functions. The presence of unique metabolic factors in 630Δ*erm* and UK1 suggests that these strains adapted to utilize different nutrients within the host (Knight *et al*. 2015; He *et al.* 2010; Kulecka *et al.* 2021). Indeed, it has been shown that different strains of *C. difficile* utilize different nutrients, indicating that some strains can utilize nutrients that other strains cannot (Scaria *et al.* 2014). Because nutrient allocation/utilization is different between 630Δ*erm* and UK1 strains, the CodY regulation of sporulation in these strains would be different, as we showed in this work (**Chap. 2, Fig. 1**). Additionally, it was determined that *C. difficile* exhibits low genome conservation and that strains of ribotype 027 are capable of evolving within a short period of time (Stabler *et al.* 2009; Scaria *et al.* 2010). It can be suggested that changes in the human diet, especially the rise of processed foods over the years, have also pressured *C. difficile* strains to evolve and survive within the host (Castro *et al.* 2025). It was determined that epidemic strains could utilize trehalose, a new additive sugar used in the food industry, and induce toxin production *(Collins et al.* 2018). Most studies investigating the relationship between diet and *C. difficile* infection have

been conducted in murine models rather than in humans, making it challenging to determine which metabolites are impactful in *C. difficile* infection in humans (Jose *et al.* 2021; Mefferd *et al.* 2020; Hazleton *et al.* 2022). However, it is well established that diet has a direct impact on the composition of the intestinal microbiota, which can influence susceptibility to *C. difficile* (Reeves *et al.* 2011; Ross *et al.* 2024). It would be important to determine which metabolites can impact *C. difficile* infection through the direct CodY-regulated factors and how dietary changes in humans can decrease the risk of acquiring *C. difficile* infection.

In addition, we determined that many direct CodY-regulated factors impact sporulation in the UK1 background (**Chap. 2, Fig. 2; Chap. 2, Fig. 3**) (Wetzel *et al.* 2024). These data indicate that CodY regulation of sporulation encompasses several factors controlled by this global nutritional transcription regulator. In *B. subtilis*, CodY directly regulates four genes that participate in the initiation of sporulation (Ratnayake-Lecamwasam *et al.* 2001). However, in *C. difficile,* none of these direct CodY-regulated factors that impact sporulation in UK1 (**Chap. 2, Fig. 2; Chap. 2, Fig. 3**) seem not to have a specific function to sporulation. Even though these targets do not directly regulate sporulation, it is still necessary to understand their roles and how they might impact other important cellular processes that are involved in *C. difficile* pathogenesis, and could be used as targets for future treatments.

Many of the putative CodY-regulated factors identified in this work (**Chap. 2, Table S3**) are also regulated by other transcriptional regulators; these factors are *tcdRBE*, *feoB1, cysKE, ribDBAH, brnQ* and many others (Nawrocki *et al.* 2016; Antunes, Martin-Verstraete, and Dupuy 2011; Antunes *et al.* 2012; Ho and Ellermeier 2015). The transcriptional regulator CcpA is one of the regulators that also regulates some factors, as listed in **Chap. 2, Table S3**. CcpA is another nutritional transcriptional regulator that responds to glucose, repressing toxin production and sporulation (Antunes, Martin-Verstraete, and Dupuy 2011; Antunes *et al.* 2012). CcpA represses sporulation by binding directly to the promoter region of *spo0A* (Antunes *et al.* 2012). Because there is an overlap of the CodY and CcpA regulons, it is important to distinguish if the genes

identified in this work (**Chap. 2, Table 3; Chap. 2 Table S3**) are direct CodY-regulated and if CodY regulation of these genes is the sole cause of the sporulation phenotypes (**Chap. 2, Fig. 1**) (Dineen, McBride, and Sonenshein 2010; Daou *et al.* 2019; Antunes, Martin-Verstraete, and Dupuy 2011; Antunes *et al.* 2012). However, having multiple regulators that sense different signals and regulate the same factors is a way to ensure the tight regulation of cell processes, such as sporulation, to ensure that the cell is ready for spore formation.

II. Final Summary

In this work, we elucidated the differences in CodY regulation of sporulation in two different important strains of *C. difficile*, 630∆erm and UK1. We confirmed and expanded the knowledge of CodY repression of sporulation in both backgrounds. By showing the differences in CodY regulation of sporulation in the 630∆*erm* and UK1 strains, we were able to identify that CodY represses initiation of sporulation in both strains and that CodY is necessary for the advancement of sporulation in the 630 background. Additionally, we identified many factors with putative CodY-boxes with functions on late-sporulation and germination, which we do not see in other sporulating species that encode CodY. Many of the unique factors have been shown to predict function in metabolism, suggesting that these strains have a different metabolic repertoire that affects CodY regulation of sporulation. Also, we determined that four specific direct CodY-regulated factors have an impact on sporulation in the UK1 background.

Because regulation of sporulation is a critical step for *C. difficile* life cycle and necessary for transmission, it is crucial to understand how nutrient-sensing transcriptional regulators, such as CodY, regulate sporulation. In addition, by identifying several putative CodY-regulated factors, this work will serve as a guide to the scientific community on finding possible CodY-regulated factors in 630∆*erm* and UK1, to choose candidate factors regulated by CodY for further characterization in *C. difficile* and to understand that important cell processes such as sporulation, are impacted by many factors under the same regulation.

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