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KipOTIA detoxifies 5-oxoproline and promotes growth of *Clostridioides difficile*

By

By Cheyenne D. Lee
B.S., University of North Carolina at Pembroke, 2019

Advisor: Shonna M. McBride, Ph.D.

An abstract of
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Abstract

KipOTIA detoxifies 5-oxoproline and promotes growth of *Clostridioides difficile*

By Cheyenne Lee

Clostridioides difficile is a Gram-positive anaerobic pathogen that is the primary cause of antibiotic associated diarrhea. Symptoms of infection range from severe abdominal pain and diarrhea to life-threatening pseudomembranous colitis. There are approximately 55 cases per 100,000 persons with a recurrence rate of 20 – 35%, making *C. difficile* infection a heavy healthcare burden. To be efficiently transmitted and cause infection, *C. difficile* must form a resistant endospore. Endospore formation is tightly regulated since forming a spore is energetically costly and an irreversible commitment. In *Bacillus subtilis*, the Kinase Inhibitory Protein, KipI, binds to the primary sporulation initiating kinase to repress sporulation. KipA antagonizes KipI to prevent KipI from repressing sporulation. Additionally, the KipI and KipA proteins function as part of a complex with KipO to detoxify the metabolic by-product 5-oxoproline. This thesis sought to characterize the *C. difficile* KipOTIA orthologs to determine their impacts on sporulation and 5-oxoproline metabolism. We deleted *kipIA* and *kipOTIA* and discovered that they have no significant impacts on sporulation. We also demonstrate that the *kip* operon encodes a 5-oxoprolinase capable of detoxifying 5-oxoproline. In addition to detoxification, we show that the *C. difficile* 5-oxoprolinase allows 5-oxoproline to be used as a nutrient source to promote growth.

KipOTIA detoxifies 5-oxoproline and promotes growth of *Clostridioides difficile*

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Chapter 1 : Introduction

I : *Clostridioides difficile*

A. Profound Healthcare Burden of *Clostridioides difficile* Infection

The anaerobic spore forming pathogen *Clostridioides difficile* is the primary cause of antibiotic associated diarrhea and was estimated to infect 223,900 hospitalized patients in 2017 (Lessa *et al.*, 2015; CDC, 2019; Feuerstadt *et al.*, 2020; Feuerstadt, Theriault and Tillotson, 2023). The Center for Disease Control and Prevention (CDC) also stated that in 2017 *C. difficile* infection (CDI) was associated with an estimated 12,800 deaths, and $1 billion in attributable healthcare costs, and therefore is considered an urgent threat (CDC, 2019). These data were primarily collected from hospitalized patients or patients in healthcare settings, and largely underestimate the level of infection in community settings. Outside of healthcare settings, CDI has increased from 47% in 2012 to 53% in 2019, particularly among individuals ≥ 65 years old in long-term care facilities (Feuerstadt, Theriault and Tillotson, 2023; Yu *et al.*, 2023). As of 2021, the CDC reports healthcare associated incidence of *C. difficile* to be 54.3 cases per 100,000 persons, and community-associated incidence to be 55.9 cases per 100,000 persons (CDC, 2023). Of all CDC reported incidences in 2021, 12% of these resulted in recurrent infection (CDC, 2023). In general, 20 – 35% of patients will experience a recurrent CDI following an initial infection, resulting in increased mortality and healthcare costs (Feuerstadt, Theriault and Tillotson, 2023).

B. Risk of Contracting *C. difficile*

C. difficile primarily infects individuals ≥ 65 years old, immunocompromised individuals, and especially those who have taken antibiotics (Lessa *et al.*, 2015; McDonald *et al.*, 2018; Feuerstadt, Theriault and Tillotson, 2023; Yu *et al.*, 2023). Individuals ≥ 65 years old make up about 50% of the population that develops CDI, and CDI in these individuals is associated with almost double mortality and total healthcare costs (Dubberke *et al.*, 2008; Lessa *et al.*, 2015; Shorr *et al.*, 2016; McDonald *et al.*, 2018). Increased age and comorbidities such as renal failure, pulmonary obstruction, and cardiovascular disease are associated with the development of severe CDI (Dubberke *et al.*, 2008; Rossen *et al.*, 2022). Recurrent CDI is primarily associated with increased age, prior hospitalization, and previous recurrent

CDI (Rossen *et al.*, 2022). People who have taken corticosteroids or proton-pump inhibitors also increase their risk of recurrent CDI (Tawam *et al.*, 2021; Rossen *et al.*, 2022). Importantly, antibiotic use prior to contracting *C. difficile* remains the predominant risk factor and is associated with both severe and recurrent CDI (Rossen *et al.*, 2022; CDC, 2023; Feuerstadt, Theriault and Tillotson, 2023).

The host gut microbiome acts as a critical barrier against *C. difficile* colonization and infection. Antibiotic use removes this protective barrier, increasing the risk of *C. difficile* infection (Rossen *et al.*, 2022; CDC, 2023; Feuerstadt, Theriault and Tillotson, 2023). In 2021, the CDC stated that antibiotic use within 12 weeks prior to CDI was reported for 61% of cases (CDC, 2023). Antibiotics severely shift the environment and metabolome within the colon and remove the bacteria that compete for nutrients *C. difficile* requires to successfully infect (Theriot *et al.*, 2014; Aguirre *et al.*, 2021). *C. difficile* primarily produces energy through Stickland fermentation, a reaction where one amino acid is oxidized and one is reduced to create NAD+ or ATP depending on the metabolized amino acid (Bouillaut, Self and Sonenshein, 2013; Bouillaut *et al.*, 2015; Aguirre *et al.*, 2021). Proline and glycine are the primary amino acids *C. difficile* metabolizes with Stickland fermentation, and an intact microbiome is capable of resisting *C. difficile* infection by reducing the availability of both glycine and proline (Theriot *et al.*, 2014; Aguirre *et al.*, 2021). Additionally, removal of these bacteria changes the colonic bile acid pool to one more favorable to *C. difficile* colonization and infection (Theriot *et al.*, 2014; Thanissery, Winston and Theriot, 2017; Aguirre *et al.*, 2021; Foley *et al.*, 2023). Normally, bacteria that can perform 7α-dehydroxylation, such as *Clostridium scindens*, inhabit the gut and metabolize primary bile acids, such as cholate, into protective secondary bile acids that repress *C. difficile* growth and sporulation (Sorg and Sonenshein, 2009; Theriot, Bowman and Young, 2016; Usui *et al.*, 2020; Aguirre *et al.*, 2021). Once these bacteria are removed, the amount of primary bile acids that support germination of *C. difficile* spores into disease-causing vegetative cells are increased (Theriot, Bowman and Young, 2016; Aguirre *et al.*, 2021). In summation, antibiotics prime the colon for *C. difficile* infection by removing bacterial competition for nutrients and providing an optimal environment for *C. difficile* spore germination.

C. Clinical Manifestations and Transmission of *C. difficile*

Once *C. difficile* spores reach the large intestine, they encounter a variety of bile acids that support germination into vegetative cells that cause disease. Without an intact microbiome to provide colonization resistance, these vegetative cells proliferate and release the toxins TcdA and TcdB which break down the intestinal epithelium and cause disease symptoms including severe abdominal pain and diarrhea (Dubberke and Olsen, 2012; Finn, Andersson and Madin-Warburton, 2021; CDC, 2023; Feuerstadt, Theriault and Tillotson, 2023) The progression of CDI can also manifest as paralytic ileus, and life-threatening pseudomembranous colitis (Dubberke and Olsen, 2012; Finn, Andersson and Madin-Warburton, 2021; CDC, 2023; Feuerstadt, Theriault and Tillotson, 2023). The TcdA and TcdB toxins function by inactivating Rho-GTPases within epithelium cells through glucosylation (Kordus, Thomas and Lacy, 2022). Once inactivated, Rho-GTPases can no longer maintain the cellular cytoskeleton which results in cell rounding and a removal of tight junctions between epithelial cells (Kordus, Thomas and Lacy, 2022). In some strains a third binary toxin exists called the *C. difficile* transferase, an ADP-ribosyltransferase, that functions to depolymerize actin which further damages the intestinal epithelium (Kordus, Thomas and Lacy, 2022). As the infection progresses and more toxins are made, the colon becomes more inflamed and the symptoms of CDI manifest. Apoptosis of toxin-affected cells also releases nutrients that *C. difficile* can use to bolster growth as basal nutrients become scarce (Fletcher *et al.*, 2021). Specifically, degradation of collagen serves as a major source of amino acids to support *C. difficile* growth in a murine model of infection, suggesting that collagen in human CDI could also promote growth (Fletcher *et al.*, 2021). As infection progresses and vegetative cells release toxins to increase the pool of available nutrients, other cells initiate sporulation prior to being released from the host. These spores are the primary mode of transmission for *C. difficile* and can persist indefinitely in an aerobic environment until they are ingested and infect a new host.

II. Sporulation of *Clostridioides difficile* and *Bacillus subtilis*

A. Sporulation as a survival mechanism

Endospore forming bacteria generally sporulate whenever environmental conditions become unfavorable for vegetative growth. Specific environmental changes differ per organism, but nutrient

deprivation is a common signal to initiate sporulation (Hoch, 1993; Mehdizadeh Gohari *et al.*, 2024). Spores can withstand a wide variety of environmental insults such as antimicrobial exposure, heat stress, desiccation, radiation, and tend to be resilient against most cleaning agents (Ali, Moore and Wilson, 2011; Edwards *et al.*, 2016). These properties make them very difficult to remove from the environment, and for pathogenic spore-forming organisms, this can be an ideal mode of transmission from host to host. Once a metabolically dormant spore is returned to a favorable environment, they can germinate with the help of environmental signals to return to a vegetative state. However, once a cell is committed to sporulation, the process cannot be reverted (Hoch, 1993).

Endospore forming bacteria generally follow a similar pattern of morphological changes as they transition from a vegetative cell to a spore. These changes correspond with different stages of sporulation and consist of stages 0 to VII (Young and Fitz-James, 1959; Hoch, 1976, 1993; Buchanan, Henriques and Piggot, 1994). Stage 0 consists of a binucleated state prior to the chromatin formation of an axial filament (Hoch, 1976). Stage I is signaled by the initial formation of an asymmetric septum which usually appears as two spikes close to one pole of the cell and signals the beginning of sporulation (Hoch, 1976; Buchanan, Henriques and Piggot, 1994). One of the chromosomes is moved into the forming spore prior to full septum formation, and stage II is completed after full assembly of the asymmetric septum which divides the mother cell and the forming pre-spore (Hoch, 1976; Buchanan, Henriques and Piggot, 1994). Stage III consists of the full engulfment of the pre-spore into the mother cell where it becomes surrounded by the inner primordial germ-cell wall and the outer cortex layer (Buchanan, Henriques and Piggot, 1994). Maturation of the primordial germ-cell wall and cortex signals stage IV, while the formation of the spore coat signals stage V (Schaeffer, 1969; Hoch, 1976; Buchanan, Henriques and Piggot, 1994). Thermoresistance is gained in stage VI as the spore cortex matures, and stage VII is characterized by lysis of the mother cell to release a fully formed spore (Schaeffer, 1969; Buchanan, Henriques and Piggot, 1994). Sporulation typically takes between 5.5 and 7 hours to complete, but once finished, the dormant spore can last relatively indefinitely until germination (Schaeffer, 1969; Hoch, 1976).

B. *Bacillus subtilis* sporulation initiation

The process of sporulation is a major energy and time commitment for a cell and cannot be reverted once begun. Therefore, sporulation initiation tends to be heavily regulated so that cells only sporulate when conditions require it. The Bacilli organisms such as *Bacillus cereus* and *Bacillus subtilis* are among the most studied spore forming bacteria, and often serve as model organisms for studying sporulation (Young and Fitz-James, 1959; Schaeffer, 1969; Hoch, 1976, 1993; Burbulys, Trach and Hoch, 1991; Buchanan, Henriques and Piggot, 1994). Sporulation is regulated by the accumulation of the phosphorylated master transcriptional regulator, Spo0A (Ferrari *et al.*, 1985; Burbulys, Trach and Hoch, 1991; Strauch and Hoch, 1993; DiCandia *et al.*, 2022; Mehdizadeh Gohari *et al.*, 2024). Once enough phosphorylated Spo0A has accumulated, Spo0A binds to target genes to initiate sporulation (Burbulys, Trach and Hoch, 1991; Molle *et al.*, 2003; DiCandia *et al.*, 2022; Mehdizadeh Gohari *et al.*, 2024). In *Bacillus subtilis*, this occurs through the phosphorelay beginning with the primary sporulation kinases KinA and KinB, and the secondary kinases KinC, KinD, and KinE, which sense different signals to autophosphorylate (Burbulys, Trach and Hoch, 1991; LeDeaux, Yu and Grossman, 1995; Hoch, 2000; Mehdizadeh Gohari *et al.*, 2024). Once autophosphorylated, these kinases can transfer a phosphoryl group to an aspartate residue on the phosphotransfer protein Spo0F. Spo0F then transfers the phosphoryl group to a histidine on another phosphotransfer protein, Spo0B (Perego *et al.*, 1989; Burbulys, Trach and Hoch, 1991; Strauch and Hoch, 1993; Tzeng and Hoch, 1997). Spo0B finally transfers the phosphoryl group to an aspartate on Spo0A, and as phosphorylated Spo0A increases, sporulation is initiated (Burbulys, Trach and Hoch, 1991; Strauch and Hoch, 1993).

The phosphorelay is regulated at various stages to prevent irreversible early sporulation initiation. At the start of the phosphorelay, the kinase inhibitory protein, KipI, can bind to kinase A (KinA), the primary sporulation kinase, to prevent autophosphorylation at a conserved histidine residue in the dimerization histidine phosphotransfer (DHp) domain (Jacques *et al.*, 2008). KipI binding of KinA represses sporulation by preventing KinA autophosphorylation and transfer of phosphate to Spo0F in *B. subtilis* (Wang *et al.*, 1997). Another protein, KipA, binds to KipI to prevent KipI from suppressing

KinA activation of the phosphorelay (Wang *et al.*, 1997). Additionally, the suppressor of *dnaA* protein, Sda, can bind the DHp domain of KinA and potentially KinB to prevent transfer of phosphate to Spo0F (Burkholder, Kurtser and Grossman, 2001; Rowland *et al.*, 2004; Whitten *et al.*, 2007).

Other proteins responsible for repressing sporulation initiation in *B. subtilis* are the response regulator aspartate phosphatases (Raps) which dephosphorylate Spo0F once it has been phosphorylated by any of the five sporulation kinases. In total, *B. subtilis* encodes eleven chromosomal and five plasmid encoded Raps which are responsible for regulating *B. subtilis* competence and sporulation (Parashar *et al.*, 2011; Gallego del Sol and Marina, 2013). Specifically, the Rap proteins responsible for dephosphorylating Spo0F to repress sporulation initiation are RapA, B, E, H, and RapJ (Perego *et al.*, 1994; Tzeng and Hoch, 1997; Jiang, Grau and Perego, 2000; Parashar *et al.*, 2011; Gallego del Sol and Marina, 2013). RapA, B, E, H and J contain an N-terminal effector domain that is capable of dephosphorylating Spo0F (Gallego del Sol and Marina, 2013). The C-terminal domain contains tetratricopeptide repeats (TPRs) that mediate protein-protein interactions with a regulatory quorum peptide called Phr (Gallego del Sol and Marina, 2013). Interaction of Phr with the TPR of Raps that dephosphorylate Spo0F inactivates them to promote sporulation in response to increasing cell density (Gallego del Sol and Marina, 2013). Other Rap proteins such as RapC, F, G, H, and K regulate via DNA-binding at response regulator promoters (Gallego del Sol and Marina, 2013). Generally, Rap phosphatases repress sporulation by dephosphorylating Spo0F, but they are not the only proteins involved in repressing *B. subtilis* sporulation initiation.

Other sporulation repressing proteins include Spo0E, YnzD, and YisI which prevent phosphorylation of Spo0A. Spo0E was the first discovered sporulation repressor protein specific for Spo0A, with YnzD and YisI being discovered later due to their structural similarities to Spo0E (Perego, 2001). Each of these proteins share an important SQELD motif which contains an aspartate residue important for repressing sporulation (Perego, 2001; Diaz *et al.*, 2008). Originally, a mutant of Spo0E that was incorrectly assumed to be null was characterized, resulting in the original mischaracterization of Spo0E as a protein that promotes sporulation (Perego and Hoch, 1987; Ohlsen, Grimsley and Hoch,

1994). However, a true deletion of Spo0E results in hypersporulation, demonstrating its true function as a repressor of sporulation initiation (Ohlsen, Grimsley and Hoch, 1994). A Δ*spo0E* mutant also has decreased motility in *B. subtilis* which was a phenotype discovered much later, demonstrating that the Spo0E protein is multifunctional (DiCandia *et al.*, 2024). Deletion of both *ynzD* and *yisI* together also resulted in hypersporulation, although single mutants of each had no impact on sporulation (Perego, 2001). Additionally, conditions that do not promote sporulation result in increased expression of *spo0E*, *ynzD*, and *yisI* (Perego, 2001).

As described, the *B. subtilis* phosphorelay is tightly regulated by numerous proteins responding to a variety of signals to balance the amount of phosphorylated Spo0A within a cell. Such tight regulation prevents early sporulation initiation and allows cells to adapt to changes in their environment. The phosphorelay represents one mechanism of sporulation regulation, and initially seemed restricted to Bacilli since most studied Clostridial organisms such as *Clostridium acetobutylicum*, *Clostridium perfringens*, and *Acetivibrio thermocellus* (formerly *Clostridium thermocellum*) have kinases that directly phosphorylate Spo0A (Underwood *et al.*, 2009; Steiner *et al.*, 2011; Mearls and Lynd, 2014; Freedman *et al.*, 2019; Mehdizadeh Gohari *et al.*, 2024). Recent research describes the phosphorelay as having evolved prior to direct phosphorylation of Spo0A by kinases since some Clostridia have components of a phosphorelay (Davidson *et al.*, 2018). As organisms evolved in different environments, aspects of this relay were lost (Davidson *et al.*, 2018). For the purposes of this introduction, only sporulation initiation in *C. difficile* will be described in further detail.

C. *Clostridioides difficile* sporulation initiation

Although some Clostridia class organisms encode proteins like those found in the *B. subtilis* phosphorelay, *C. difficile* does not contain homologs for the Spo0F or Spo0B phosphotransfer proteins (Davidson *et al.*, 2018; Mehdizadeh Gohari *et al.*, 2024). This suggests that *C. difficile* does not contain a classical phosphorelay, and instead may initiate sporulation with direct phosphorylation of Spo0A by histidine kinases like bacteria of the *Clostridium* species. Examples include *C. acetobutylicum*, *C. perfringens*, and *A. thermocellus*, all of which have proteins that directly phosphorylate Spo0A (Steiner

et al., 2011; Mearls and Lynd, 2014; Freedman *et al.*, 2019; Mehdizadeh Gohari *et al.*, 2024). Factors involved in *C. difficile* sporulation initiation are more deeply described in Chapter 3, but key sporulation factors will be briefly covered below.

Currently, the only known protein that promotes *C. difficile* sporulation is the RNPP-family protein RstA (Edwards, Tamayo and McBride, 2016; Edwards, Anjuwon-Foster and McBride, 2019; Mehdizadeh Gohari *et al.*, 2024). Our current model suggests that RstA promotes sporulation by binding to the sporulation repressor Spo0E and preventing Spo0E from blocking Spo0A phosphorylation under certain conditions (DiCandia *et al.*, 2024). In addition to regulating sporulation, we know that RstA binds directly to the *tcdR*, *tcdA*, and *tcdB* toxin gene promoters to repress toxin expression (Edwards, Anjuwon-Foster and McBride, 2019). More work is needed to fully describe the molecular mechanisms behind how RstA promotes sporulation initation.

In *B. subtilis*, sporulation initiation begins when Spo0A is phosphorylated by either of the two primary sporulation kinases KinA or KinB. *C. difficile* encodes three phosphotransfer proteins, PtpA, B, and C, that are significantly similar to KinA and KinB (Edwards *et al.*, 2022; Mehdizadeh Gohari *et al.*, 2024). However, PtpA and PtpB both repress sporulation in *C. difficile*, possibly by forming a hetero-oligomer to directly dephosphorylate Spo0A (Childress *et al.*, 2016; Edwards *et al.*, 2022; Mehdizadeh Gohari *et al.*, 2024). Unlike PtpA and B, PtpC directly phosphorylates Spo0A, and overexpression of PtpC slightly but insignificantly promotes *C. difficile* sporulation (Underwood *et al.*, 2009; Edwards *et al.*, 2022). Additionally, overexpression of a *ptpC-H372A* site-directed mutant of the histidine responsible for phosphotransfer restored sporulation to wild-type levels, supporting the function of PtpC as a kinase (Edwards *et al.*, 2022). In contrast, a Δ*ptpC* mutant has variable sporulation, though it mostly hypersporulates and was complemented (Edwards *et al.*, 2022). Based on these results, PtpC may act as a kinase or phosphatase depending on certain conditions but requires further research to confirm its function. So far, a kinase has not been identified that is solely responsible for phosphorylating Spo0A in *C. difficile*.

In addition to the negative regulators mentioned above, *C. difficile* also encodes a multi-functional Spo0E homolog which represses sporulation (DiCandia *et al.*, 2024). *C. difficile* Spo0E may not be a phosphatase, and instead may repress sporulation by binding to Spo0A and blocking its phosphorylation site (DiCandia *et al.*, 2024). *C. difficile* Spo0E also represses motility and toxin production, likely through RstA, while *B. subtilis* Spo0E promotes motility through an unknown mechanism (DiCandia *et al.*, 2024). Other Spo0E-like homologs exist in *C. difficile*, but their functions currently remain unclear.

Although *C. difficile* does not have any clear KinA orthologs, it does encode homologs for the *B. subtilis* KipI and KipA proteins. Until the work performed in this thesis, the KipI and KipA homologs in *C. difficile* remained uncharacterized. We hypothesized that the KipI and KipA proteins would have similar functions to those in *B. subtilis*, but our work outlined in Chapter 2 demonstrates that KipI and KipA do not play a major role in *C. difficile* sporulation initiation. However, we do demonstrate that they have an additional function as part of a 5-oxoprolinase enzyme which will be described in more detail below.

III. 5-oxoproline and the importance of 5-oxoprolinases

Initially, we hypothesized that the *C. difficile* KipI and KipA proteins would primarily be involved in sporulation. However, we discovered that sporulation regulation was not their primary role. Because of this, we sought potential alternative functions of the KipI and KipA proteins and learned that *B. subtilis* KipI and KipA could bind a third protein, KipO, to form a 5-oxoprolinase (Niehaus *et al.*, 2017). 5-oxoproline, also known as L-pyroglutamic acid, is a compound that has detrimental effects on organisms when it accumulates (Niehaus *et al.*, 2017). In prokaryotes, 5-oxoproline forms from the spontaneous cyclization of glutamine, glutamate, and the γ-glutamyl cycle when present (Seddon, Li and Meister, 1984; Niehaus *et al.*, 2017). Free 5-oxoproline is also created by the pyrrolidone carboxyl peptidase (Pcp) which cleaves N-terminal 5-oxoproline from cyclized glutaminyl or glutamyl on peptides (Doolittle and Armentrout, 1968; Awadé *et al.*, 1992, 1994). Since the presence of 5-oxoproline is

ubiquitous and its accumulation harmful, most organisms encode some way to detoxify this compound. The different methods of 5-oxoproline detoxification are described below.

As mentioned above, 5-oxoproline is a ubiquitous compound found in many different organisms across all domains of life. In humans and other mammals, most 5-oxoproline forms from the metabolism of glutathione through the γ-glutamyl cycle (Werf, Orlowski and Meister, 1971; Sass *et al.*, 2016). The 5-oxoprolinase encoded in the mammalian *OPLAH* gene prevents the accumulation of 5-oxoproline to toxic levels by metabolizing 5-oxoproine to L-glutamate (Werf, Orlowski and Meister, 1971; Sass *et al.*, 2016). Deficiency in glutathione synthetase and mutations within the *OPLAH* 5-oxoprolinase genes can both lead to a buildup of 5-oxoproline, resulting in 5-oxoprolinuria, metabolic acidosis, hemolytic anemia, and central nervous system dysfunction (Njålsson, 2005; Kumar and Bachhawat, 2012; Sass *et al.*, 2016). To detoxify 5-oxoproline, the eukaryotic 5-oxoprolinase forms a homodimer to phosphorylate 5-oxoproline and subsequently converts it to L-glutamate (Griffith and Meister, 1981; Kumar and Bachhawat, 2012). Fungi such as the yeast *Saccharomyces cerevisiae* also encode a 5-oxoprolinase homologous to those found in other eukaryotes which functions as part of a truncated γ-glutamyl pathway (Kumar and Bachhawat, 2010). Archaea also encode a eukaryotic-like 5-oxoprolinase made of 2-domains responsible for metabolizing 5-oxoproline, and one thermoacidophilic crenarchaeon, *Sulfolobus acidocaldarius*, may be able to utilize 5-oxoproline as a carbon source (Park, Lee and Ryu, 2001; Vetter *et al.*, 2019). Since the accumulation of 5-oxoproline is detrimental to both eukaryotic and prokaryotic cells, possessing a 5-oxoprolinase to prevent the toxic accumulation 5-oxoproline is advantageous (Park, Lee and Ryu, 2001; Kumar and Bachhawat, 2010, 2012; Niehaus *et al.*, 2017; Vetter *et al.*, 2019).

Since accumulation of 5-oxoproline is toxic, many bacteria encode some form of 5-oxoprolinase. *Pseudomonas putida* was among the first prokaryotes studied that has a 5-oxoprolinase made up of two reversibly dissociable proteins instead of a single domain protein like those found in eukaryotes (Van Der Werf and Meister, 1974; Seddon, Li and Meister, 1984; Li, Seddon and Meister, 1988). It was later determined that one component of this dissociable protein complex, Component A, phosphorylates bound 5-oxoproline (Seddon and Meister, 1986). Component B binds the phosphorylated 5-oxoproline and

Component A complex to catalyze the decyclization of 5-oxoproline to L-glutamate (Seddon and Meister, 1986). In bacteria, Component A can be translated already fused together, or separated into the KipI and KipA proteins which can bind together to form a complete Component A (Seddon and Meister, 1986; Niehaus *et al.*, 2017). Component B corresponds to the KipO protein which binds to the already formed KipI-KipA-5-oxoproline complex to convert phosphorylated 5-oxoproline to glutamate (Seddon and Meister, 1986; Niehaus *et al.*, 2017). Due to this 5-oxoprolinase function, the KipOIA proteins are also known as the prokaryotic 5-oxoprolinases, or PxpABC (Niehaus *et al.*, 2017).

Many bacteria seem to encode 5-oxoprolinases, but they have only been characterized in *P. putida*, *B. subtilis*, and *Escherichia coli* (Van Der Werf and Meister, 1974; Seddon, Li and Meister, 1984; Niehaus *et al.*, 2017). The primary function of prokaryotic KipOIA seems to be as a damage-controlling 5-oxoprolinase. However, regulation of the *kips* and other possible functions of the Kip proteins differ between organisms. For example, although not looking at 5-oxoprolinase function, KipOIA homologs within the plant pathogen *Ralstonia solanacearum* were important for indirectly regulating virulence (Zhang *et al.*, 2011). In *B. subtilis*, the *kip* operon is differentially regulated by various nitrogen sources, and our own data even demonstrate that the *C. difficile kip* operon is differentially regulated by various bile salts (Wang et al., 1997; Chapter 2). Together, these data suggest that prokaryotic 5-oxoprolinases may have functions other than just detoxifying 5-oxoproline, and that 5-oxoprolinase regulation varies between organisms. Therefore, further characterization of prokaryotic 5-oxoprolinases beyond their primary detoxification function is warranted.

IV. Specific Aims

To be efficiently transmitted and cause disease, the anaerobic pathogen *C. difficile* must be able to form spores to survive in an aerobic environment with a variety of physical insults. Once ingested, *C. difficile* must continue to adapt within the host environment to survive and cause disease. Therefore, understanding both how *C. difficile* initiates sporulation and how it can adapt within the host metabolically is critical for the development of therapeutic strategies. In this dissertation, we initially sought to determine whether the KipI and KipA proteins of *C. difficile* regulated sporulation as was seen

in *B. subtilis*. As we gathered more data, we increased our scope to determine whether the *C. difficile* Kip orthologs possessed a metabolic 5-oxoprolinase function. The main goal of this thesis was to determine whether the Kips functioned as regulators of sporulation and/or if they were critical for the metabolism of 5-oxoproline. I investigated the function of the Kip proteins through the following aims:

1. Determine if Kip proteins regulate growth and sporulation of *C. difficile*
2. Identify how the Kip proteins effect *C. difficile* physiology in response to 5-oxoproline

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Chapter 2: KipOTIA detoxifies 5-oxoproline and promotes growth of *Clostridioides difficile*

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ABBREVIATED SUMMARY

5-oxoproline (OP) is a toxic byproduct of metabolism generated by all organisms. Since OP is ubiquitous, oxoproline detoxification systems are found throughout nature. We demonstrate that the *Clostridioides difficile* KipOTIA system not only detoxifies OP, but also allows OP to be used as a nutrient source to promote *C. difficile* growth.

ABSTRACT

Clostridioides difficile is an anaerobic enteric pathogen that disseminates in the environment as a dormant spore. For *C. difficile* and other sporulating bacteria, the initiation of sporulation is a regulated process that prevents spore formation under favorable growth conditions. In *Bacillus subtilis*, one such mechanism for preventing sporulation is the Kinase Inhibitory Protein, KipI, which impedes activation of the main sporulation kinase. In addition, KipI functions as part of a complex that detoxifies the intermediate metabolite, 5-oxoproline (OP), a harmful by-product of glutamic acid. In this study, we investigate the orthologous Kip proteins in *C. difficile* to determine their roles in the regulation of sporulation and metabolism. Using deletion mutants in *kipIA* and the full *kipOTIA* operon, we show that unlike in *B. subtilis*, the Kip proteins have no significant impact on sporulation. However, we found that the *kip* operon encodes a functional oxoprolinase that facilitates detoxification of OP. Further, our data demonstrate that KipOTIA not only detoxifies OP, but also allows OP to be used as a nutrient source that supports the robust growth of *C. difficile*, thereby facilitating the conversion of a toxic byproduct of metabolism into an effective energy source.

INTRODUCTION

Clostridioides difficile is an anaerobic Gram-positive pathogen that causes illnesses ranging from abdominal pain and diarrhea to life-threatening pseudomembranous colitis (Dubberke and Olsen, 2012; Finn et al., 2021). *C. difficile* infections are easily spread and difficult to contain because the spore form

of the bacterium is resistant to disinfectants and allows the pathogen to survive nearly indefinitely in a dormant state (Ali et al., 2011; Edwards et al., 2016a; Shen, 2020). Although the structure and general form of *C. difficile* endospores are similar to other species, the environmental and nutritional conditions that lead to sporulation vary greatly among spore-forming bacteria (Lee et al., 2022; Mehdizadeh Gohari et al., 2024; Shen et al., 2019). While some basic nutritional requirements and regulatory factors are shared among the sporulating Firmicutes, it is not possible to predict the role of a given factor based on the presence of orthologs with a known function in another spore-former (Edwards et al., 2016b; Edwards et al., 2022, 2014).

Much of what is known about sporulation and nutrition in Gram-positive bacteria is based on extensive studies performed in the model spore-forming species, *Bacillus subtilis* (Errington, 2003; Sonenshein, 2007, 2000). In *B. subtilis*, sporulation initiates with the accumulation of the activated (phosphorylated) master regulator, Spo0A. The activation of Spo0A in *B. subtilis* is subject to a succession of negative and positive regulatory inputs that control the flow of phosphoryl groups to Spo0A via a phosphorelay. A key driver of the sporulation phosphorelay is the sensor kinase, KinA, which can be inhibited by the anti-kinase KipI, resulting in decreased sporulation (Wang et al., 1997). In turn, the co-transcribed factor, KipA, binds to KipI to prevent KipI interference with KinA activation of the phosphorelay (Jacques et al., 2008, 2011). Though the functions of KipI and KipA are known for *B. subtilis*, their roles in the sporulation regulation of other species have not been described.

While sporulation regulation is one function of the KipI and KipA proteins in *B. subtilis*, other studies have demonstrated metabolic functions of these proteins (Niehaus et al., 2017). In *B. subtilis*, *Escherichia coli*, and *Pseudomonas putida*, KipI and KipA bind with KipO (also known as PxpA,B,C) to form an enzyme that metabolizes 5-oxoproline (Li et al., 1988; Niehaus et al., 2017; Seddon et al., 1984; Seddon and Meister, 1986). 5-oxoproline (OP), or L-pyroglutamic acid, is a ubiquitous waste product formed in bacteria from the spontaneous cyclization of glutamate, glutamine, and ɣ-glutamyl phosphate (Niehaus et al., 2017; Seddon et al., 1984). Free 5-oxoproline is also formed from the action of the pyrrolidone carboxyl peptidase (Pcp) which cleaves N-terminal 5-oxoproline from cyclized glutaminyl or glutamyl on

peptides and is found in many prokaryotic organisms (Awadé et al., 1992, 1994; Doolittle and Armentrout, 1968). Accumulation of 5-oxoproline is toxic to eukaryotic and prokaryotic cells and impairs growth, but organisms that encode KipOIA orthologs can convert OP to glutamic acid, thereby preventing OP toxicity (Kumar and Bachhawat, 2010; Park et al., 2001; Vetter et al., 2019).

In this study, we sought to determine whether the *C. difficile* Kip orthologs function as regulators of sporulation and/or metabolism of 5-oxoproline. Our results demonstrate that, unlike *B. subtilis*, KipI and KipA in *C. difficile* do not play a major role in sporulation initiation. We also demonstrate that the *kipOTIA* operon encodes a functional oxoprolinase that allows for growth in OP. Further, our results indicate that 5-oxoproline is not only detoxified by *C. difficile*, it is an effective and valuable nutrient source. Overall, our data indicate that the Kip proteins function primarily as a 5-oxoprolinase that promotes the growth of *C. difficile* in the presence of this toxic metabolite.

RESULTS

Identification and disruption of the KipOTIA orthologs in *C. difficile*

The putative KipI and KipA proteins of *C. difficile* were identified in the strain 630 genome by sequence similarity to the Kip proteins of *B. subtilis* (**Figure S1A**). The *C. difficile* KipI and KipA are encoded immediately downstream of orthologs of *kipO* and *kipT* (CD630_13840-13870), which are also found within the *B. subtilis kip* locus (**Figure S1A**). *C. difficile* does not encode three additional genes (*pxpI, kipR*, and *lipC*) that are encoded within the *B. subtilis kip* region. We examined transcription of the *C. difficile kip* genes in the region to determine if they were encoded as an operon. We performed nested PCR on *C. difficile* cDNA templates and demonstrated that *kipOTIA* are transcribed as a polycistronic unit (**Figure S1B**). Based on the similarities between the shared proteins, we hypothesized that the *C. difficile* Kip proteins may function as regulators of sporulation and metabolism, as observed in *B. subtilis.*

KipOTIA does not inhibit sporulation in *C. difficile*

To assess the Kip protein functions in *C. difficile*, we generated Δ*kipIA* (MC1903) and Δ*kipOTIA* (MC2375) mutants by allelic exchange and replacement with an *ermB* cassette. As controls, both mutants were restored with a knock-in of the *kipOTIA* operon at the *kip* locus (**Table S1, Figure S2**) and all resultant strains were verified by whole-genome sequencing. As KipI is an inhibitor of sporulation in *B. subtilis*, we next examined the impact of deleting the *kip* genes on *C. difficile* sporulation. Sporulation assays were performed on 70:30 sporulation agar, comparing the Δ*kipIA* and Δ*kipOTIA* mutants to the parent strain (**Figure 1**). Deletion of *C. difficile kipIA* resulted in a modest decrease in sporulation, while deletion of the entire *kipOTIA* operon had no apparent effects. The decreased sporulation for Δ*kipIA* mutant, but not the Δ*kipOTIA* mutant, suggests that expression of *kipO* and *kipT* without *kipI* and *kipA*, is detrimental to spore formation. The *kip* deletion phenotypes in *C. difficile* are in contrast to *B. subtilis*, where *kipIA* mutants produce more spores and over-expression of *kipI* reduces sporulation (Wang et al., 1997). These results strongly suggest that unlike in *B. subtilis*, KipI does not inhibit a sporulation-specific kinase in *C. difficile*.

To better understand the underlying basis for the Δ*kipIA* sporulation decrease in *C. difficile*, we examined gene expression in sporulation medium for the Δ*kipIA* mutant relative to the parent strain by RNA-seq (**Table 1**; MC1970, Δ*kipIA* pMC123 and MC324, 630Δ*erm* pMC123). Only 25 genes were differentially regulated more than 2-fold in the Δ*kipIA* mutant, but the greatest transcriptional differences were observed for the three genes representing the oxidative branch of the *C. difficile* TCA cycle (*acnB, aksA*, and *icd*), suggesting changes in metabolism or redox within the mutant (Neumann-Schaal et al., 2019). Of the remaining transcriptional changes, more than half encode proteins of unknown function and two were sporulation-related genes. Aside from the changes in TCA gene expression, no patterns signifying specific regulatory factors, pathways, or processes were evident.

Since the Kip complex did not inhibit sporulation in our standard assay, we considered that the Kip proteins may conditionally influence sporulation, as observed in *B. subtilis*. In *B. subtilis,* the *kip* operon is induced by glucose and repressed by glutamine (Wang et al., 1997). Previous studies in *C. difficile* found that the *kipOTIA* operon is repressed by proline through PrdR, and by glucose, partially through

CcpA, but the impact of these factors on *kip* expression was modest (2-3 fold) (Antunes et al., 2012; Bouillaut et al., 2019). Data from pilot experiments suggested that the secondary bile salt deoxycholate (DCA) induced transcription of the *kipOTIA* genes. We further examined the effects of bile salts on expression of the *kip* operon by assessing *kipI* transcription. To this end, wild-type *C. difficile* was grown in BHIS broth supplemented with physiologically relevant concentrations of deoxycholate (DCA, 0.5 mM), chenodeoxycholate (CDCA, 0.5 mM), lithocholate (LCA, 0.05 mM), cholate (CA, 2 mM), taurocholate (TA, 10 mM), glycocholate (GCA, 10 mM), taurochenodeoxycholate (TCDCA, 10 mM), or glycochenodeoxycholate (GCDCA, 1.5 mM) (Northfield and McColl, 1973) and samples collected for qRT-PCR analysis. The hydrophobic bile salts DCA, CDCA, LCA, and GCDCA significantly increased *kipI* expression, while CA and TA did not (**Figure 2**). To determine if the detergent properties of bile salts played a role in *kipI* expression, *C. difficile* was also exposed to sub-inhibitory concentrations of the detergents Triton X-100 and sodium dodecyl sulfate (SDS) (**Figure 2**). Both detergents induced *kipI* expression, but the differences were not statistically significant. These data demonstrate that *kip* expression is robustly induced by several bile salts that impact growth, and that *kip* induction is not universal to all bile acids or detergents.

Previous studies demonstrated that some bile salts repress *C. difficile* growth and spore formation through undetermined mechanisms (Sorg and Sonenshein, 2009; Theriot et al., 2016; Usui et al., 2020). Examination of growth in the various bile salts in our hands showed that CDCA, DCA, LCA, GCDCA, and GCA repressed growth, similar to previous findings (**Figure S3**). Based on the induction of *kip* expression and the growth impacts of these bile salts, we chose a representative bile acid, CDCA, to test for potential effects on sporulation through the Kip locus (**Figure S4**). The addition of CDCA decreased spore production in the wild-type and *kip* mutant strains proportionally, with similar decreases in the sporulation ratios across strains. Thus, even with induction of *kip* expression by CDCA, sporulation was not differentially impacted in the wild-type or *kip* mutants, further supporting that KipI and KipA do not have the role in *C. difficile* sporulation regulation observed in *B. subtilis.*

***C. difficile* uses the toxic byproduct 5-oxoproline (OP) as a nutrient source**

As mentioned, the KipOIA oxoprolinase complex detoxifies the metabolic byproduct 5-oxoproline to glutamic acid in both Gram-positive and Gram-negative bacteria (Niehaus et al., 2017; Seddon et al., 1984). In other bacteria, Kip oxoprolinase activity has the dual effect of preventing the accumulation of toxic amounts of OP, while also allowing OP use as a nitrogen source (Niehaus et al., 2017; Ratcliffe et al., 1983). To determine if the predicted *C. difficile* KipOTIA orthologs are involved in 5-oxoproline metabolism, we grew wild-type, the Δ*kipIA* and Δ*kipOTIA* mutants, and knock-in strains in Defined Minimal Media (DMM) with and without 30 mM 5-oxoproline or 30 mM glutamic acid (**Figure 3, Figure S5**). In DMM, the Δ*kipIA*, and Δ*kipOTIA* mutants grew more slowly than the parent strain, suggesting an intrinsic growth defect in the absence of the Kip complex (**Figure 3D**). The growth defect of the *kip* mutants was partially restored in the presence of glutamic acid (**Figure 3 B,C,F**), suggesting that additional nutrients help to alleviate the growth delay. However, when grown in the presence of OP, the Δ*kipIA* or Δ*kipOTIA* mutants demonstrated further growth retardation than observed in DMM alone, indicating that the Kip complex is both necessary for the utilization of OP and the prevention of deleterious effects by OP. In contrast, the parent strain 630Δ*erm* experienced markedly improved growth and culture density when OP was added to the medium (**Figure 3 A,E**). Remarkably, the growth of the wild-type strain in OP was more robust than the growth observed with the addition of glutamic acid, suggesting that OP is more readily utilized as a nutrient than the metabolic product, glutamic acid. These data demonstrate that the *kipOTIA* operon encodes proteins responsible for 5-oxoprolinase function, and that utilization of 5-oxoproline promotes *C. difficile* growth.

Considering that the bile acid CDCA increased the expression of the *kip* genes, we examined whether CDCA could further improve *C. difficile* growth in OP. To do so, we added 0.1 mM CDCA with and without 30 mM OP to the DMM cultures and evaluated the effects of each variable on growth (**Figure 4**). No further enhancement of OP-dependent growth was observed when CDCA and OP were both added to the medium. These data suggest that the addition of CDCA does not enhance the ability of *C. difficile* to utilize 5-oxoproline for growth.

As OP has substantial impacts on *C. difficile* growth, we considered that OP utilization may affect sporulation. We extended our sporulation assays to test the impact of exogenous OP on the sporulation frequencies of the *kip* mutants and parent strain, but observed no effect on sporulation outcomes relative to sporulation medium lacking OP (**Figure S4**). Further, we examined the expression of *kipI* in the presence of OP and glutamic acid, and observed only modest changes in *kipI* expression, suggesting that these nutrients do not substantially alter expression of the *kip* operon (**Figure S6**). Thus, OP promotes the growth of *C. difficile* through utilization by the Kip oxoprolinase, but OP does not affect *kip* expression or spore formation.

The *B. subtilis* oxoprolinase enables the use of OP as a nitrogen source, but not a carbon source

In the initial characterization of the *B. subtilis* Kip/Pxp oxoprolinase, it was observed that the oxoprolinase activity facilitated OP utilization as a nitrogen source (Niehaus et al., 2017). However, the OP-dependent enhancement of growth observed with *C. difficile* suggests that the Kip oxoprolinase allows OP to be used as both a carbon and nitrogen source to support growth. These results led us to question whether the Kip oxoprolinases of *C. difficile* and *B. subtilis* share the capacity for growth enhancement by OP and if *B. subtilis* Kips facilitate the use of OP as a carbon source. To test these hypotheses, we obtained previously generated *kipI* and *kipA* deletion mutants and further investigated their growth profiles in the presence of OP with and without the addition of succinate as a carbon source (Koo et al., 2017). Succinate was chosen as the carbon source to avoid the production of glutamic acid and/or 5-oxoproline. The growth of wild-type *B. subtilis,* Δ*kipI,* and Δ*kipA* mutants were compared in Niehaus Defined Medium (NDM) with added succinate, OP, or both (**Figure 5**). When succinate served as the sole carbon source, poor growth was observed for all strains, but the Δ*kipI* and Δ*kipA* mutants demonstrated significantly less growth than the parent strain (**Figure 5A**). Importantly, OP alone did not support the growth of any *B. subtilis* strain and led to decreased cell density for both *kip* mutants (**Figure 5B**). Addition of both succinate and OP to the medium resulted in considerable growth for the parent strain that was not observed for either the Δ*kipI* or Δ*kipA* mutant (**Figure 5C**). These observations

reinforce the previous findings of OP toxicity in the absence of the *kip* genes and demonstrate that OP is not an adequate sole carbon source for *B. subtilis*.

DISCUSSION

In this study, we investigated the role of the KipOTIA factors in the regulation of sporulation and the ability of *C. difficile* to detoxify the metabolite 5-oxoproline. We found that KipI and KipA do not have major effects on *C. difficile* sporulation, in contrast to *B. subtilis*, in which KipI impedes sporulation (Wang et al., 1997). The absence of a sporulation effect by the *C. difficile* Kip proteins is not completely surprising given the considerable differences in the genetic pathways and factors involved in sporulation initiation between *B. subtilis* and *C. difficile* (Edwards et al., 2022; Lee et al., 2022; Mehdizadeh Gohari et al., 2024). In *B. subtilis,* KipI binds to the sporulation activating kinase, KinA to repress spore formation (Jacques et al., 2011, 2008). However, *C. difficile* does not encode a clear KinA ortholog and the phosphotransfer proteins that function in the regulation of sporulation initiation appear to suppress spore formation (Edwards et al., 2022). Moreover, it is not known how the *Bacillus* KinA-KipI-KipA interactions are controlled or if such signals are present or relevant for *C. difficile.* While *C. difficile* Kips have no apparent role in sporulation, there are many sporulating species that encode Kip orthologs, but their functions have not been investigated. Hence, it is possible that other Clostridial KipI proteins function as sporulation kinase inhibitors—most likely those with histidine kinases that activate Spo0A.

The most impactful result of this investigation was the finding that the KipOTIA complex not only functions as a 5-oxoprolinase, but that 5-oxoproline supports robust *C. difficile* growth. For *B. subtilis,* the conversion of OP to glutamic acid provides a detoxification function and supplies an excellent nitrogen source for the bacterium, but glutamic acid cannot serve as a sole carbon source for *B. subtilis* (Belitsky et al., 2000; Commichau et al., 2008). The robust growth observed for wild-type *C. difficile* with OP suggests that KipOTIA facilitates the use of OP as both a carbon and a nitrogen source (**Fig. 3**). However, how KipOTIA metabolizes OP to generate energy is not clear. We observed greater cell growth of *C. difficile* in OP than in equal concentrations of glutamic acid, which is curious. The conversion of OP

to glutamic acid costs energy, so cells would be expected to grow better with glutamic acid than with OP. The growth differences for *C. difficile* in OP and glutamic acid suggest that OP can be utilized by mechanisms that glutamic acid cannot.

It was previously observed by Fletcher et al. that the concentration of OP increases during *C. difficile* colonization in a mouse model of infection, though the cause of the OP increase is not known (Fletcher et al., 2018). The presence of both exogenous and endogenously generated OP necessitates detoxification by both the intestinal microbiota and the host. The fact that *C. difficile* growth is enhanced by a metabolite that is toxic to other species could greatly impact competition with other intestinal microbes and may provide a significant nutritional advantage to support *C. difficile* proliferation during infection. Further, the growth advantages afforded to *C. difficile* by KipOTIA-mediated 5-oxoproline metabolism may be shared by other species that encode Kip orthologs.

MATERIALS AND METHODS

Strain and plasmid construction

See **Table S1** for bacterial strains and plasmids used in this study. The 630 strain genome (GenBank accession no. NC_009089.1) was used to design the primers for *C. difficile* listed in **Table S2**. *C. difficile* 630Δ*erm* genomic DNA, pRT1099, and pJIR1457 were used as templates for PCR reactions and mutant construction. Vector construction details are provided in **Table S3**. All *C. difficile* mutants were made using the pseudo-suicide allele-coupled exchange, as previously described (Edwards et al., 2022; Peltier et al., 2020) All vectors were verified by whole plasmid sequencing using Oxford Nanopore Technology (Plasmidsaurus). *C. difficile* strains were confirmed by PCR analysis and whole-genome sequencing. The *B. subtilis* Δ*kipI* and Δ*kipA* mutants were created by natural competence transformation of strain 1A1 with genomic DNA from strains BKE04080 and BKE04090 respectively (Koo et al., 2017).

Bacterial growth assays and conditions

All *C. difficile* strains were cultured in a Coy anaerobic chamber at 37°C with an atmosphere of 10% H_2, 5% CO_2, and balanced with 85% N_2 as previously described (Edwards et al., 2013; Smith et al., 1981). *C. difficile* strains were grown in brain-heart infusion medium supplemented with yeast extract (Bacto) (Putnam et al., 2013; Sorg and Dineen, 2009). For plasmid maintenance and integrant selection in *C. difficile,* cultures were supplemented with 2-10 µg/mL of thiamphenicol (Sigma-Aldrich) or 500 µg/mL spectinomycin (Thermo Scientific). Counterselection against *E. coli* post-conjugation was achieved using 100 µg/mL of kanamycin. For growth curves and spore assays, strains were grown overnight in BHIS with 0.1% taurocholate (TA; Sigma-Aldrich) and 0.2% fructose (D-fructose, Fisher Chemical) added as needed to induce *C. difficile* germination and prevent sporulation (Putnam et al., 2013; Sorg and Dineen, 2009). 5-oxoproline was neutralized and filter sterilized immediately before use. All bile salts were diluted in dH_2O except for lithocholate, which was solubilized in 95% ethanol.

Escherichia coli strains were grown aerobically at 37°C in LB medium (Lennox) supplemented with 20 µg/mL chloramphenicol, 100 µg/mL spectinomycin, or 100 µg/mL ampicillin (Sigma-Aldrich) as needed for plasmid maintenance.

For *C. difficile* growth curves, strains were grown in a chemically defined minimal medium (DMM), loosely based on modified CDMM (Karasawa et al., 1995; Rizvi et al., 2023); (**Table S4**). Neutralized 5-oxoproline and/or glutamic acid were added to DMM as indicated. Each medium was made fresh and used within 12 h. Cultures were inoculated in BHIS with 0.1% TA and 0.2% fructose, and grown to turbidity. Active cultures were diluted with BHIS, grown to an OD_{600}=0.5, pelleted for 10 min at 3214 x *g*, resuspended to an OD_{600}=0.5 in DMM and 5 ml was inoculated into 45 ml of DMM.

For *B. subtilis* growth curves, cultures were grown to logarithmic phase in LB broth (Lennox), pelleted for 10 min at 3214 x *g*, and washed twice with 5 mL modified Niehaus Defined Media (NDM, **Table S5**) without a carbon or nitrogen source (Niehaus et al., 2017). Cultures were resuspended in NDM to an OD_{600}=0.5 and diluted 1:10 into 1.8 mL of NDM supplemented with 30 mM sodium succinate, 30 mM 5-oxoproline, or 30 mM glutamic acid, as indicated. Cultures were grown in a 24-well suspension cell plate (Sarstedt) for 72 h at 37°C with continuous double orbital shaking at 548 cpm in a BioTek

Synergy H1 microplate reader. The OD_{600} was measured for each culture at 30 minute intervals and visualized using the Gen5 software version 3.11.19. Growth data were analyzed by one-way ANOVA with Dunnett's multiple comparison test or Student's two-tailed *t*-tests as indicated.

Whole Genome Sequencing

C. difficile strains were grown in BHIS, genomic DNA extracted and prepared for whole genome sequencing as previously detailed (Edwards et al., 2016b; Edwards and McBride, 2023; Harju et al., 2004). Library preparation and Illumina sequencing was performed by SeqCenter (Pittsburgh, PA) as previously described (Edwards and McBride, 2023). Demultiplexing, quality control, and adapter trimming was performed with bcl-convert (v3.9.3 for MC2375, v4.1.5 for MC2519 and MC2520; https://support-docs.illumina.com/SW/BCL_Convert/Content/SW/FrontPages/BCL_Convert.htm). Geneious Prime (v2023.1.2 and v2021.2.2) was used to trim reads using the BBDuk plug-in and then mapped to the *C. difficile* reference genome NC_009089. The Bowtie2 plugin was used to find SNPs or InDels under default settings with a minimum variant frequency of 0.9 (Langmead and Salzberg, 2012). Genome sequence files were deposited into NCBI Sequence Read Archive (SRA) BioProject PRJNA1085672.

Sporulation assays

Ethanol-resistant sporulation assays were performed as previously described (Childress et al., 2016; DiCandia et al., 2022; Edwards et al., 2022; Edwards and McBride, 2017, 2016). Briefly, *C. difficile* strains were grown in BHIS broth supplemented with 0.1% TA and 0.2% fructose to germinate spores and prevent spore formation, then diluted in BHIS and grown to log-phase (OD_{600} =0.5). Cultures were plated onto 70:30 sporulation agar, with or without 0.5 mM chenodeoxycholate (Calbiochem), or 30 mM of neutralized 5-oxoproline (L-pyroglutamic acid, Sigma-Aldrich), as indicated. After 24 h, cells were plated for vegetative cell counts or treated with ethanol, diluted, and plated for spore enumeration on BHIS with 0.1% TA. Sporulation frequency was calculated as the number of ethanol-resistant spores

divided by the total number of cells. A *spo0A* mutant (MC310) was used as a negative control to verify vegetative cell death during ethanol treatment. At least four independent experiments were performed, and sporulation frequency data were analyzed by one-way or two-way analysis of variance (ANOVA) with Dunnett's or Šídák's multiple comparison test, as indicated (GraphPad Prism v10.0.2).

Quantitative reverse transcription PCR analysis (qRT-PCR)

C. difficile was grown on 70:30 agar or in BHIS broth, as indicated, and samples processed for RNA extraction. Culture samples were taken at H_{12} (12 h after cultures were applied to the plate) for 70:30 agar samples or at an OD_{600}=0.5 for BHIS growth curve samples. Cultures were resuspended in ethanol:acetone:dH_2O solution and stored at -80°C. RNA was isolated, Dnase-I treated (Ambion), and cDNA was synthesized (Bioline) using random hexamers as previously described (Dineen et al., 2010; Edwards et al., 2014; Suarez et al., 2013). qRT-PCR analysis was performed on 50 ng of cDNA using the SensiFAST SYBR & Fluorescein kit (Bioline) with a Roche Lightcycler 96. qRT-PCR primers are listed in **Table S2** and each qRT-PCR reaction was performed in technical triplicate with at least three biological replicates. The $\Delta\Delta C_t$ method was used to analyze results and normalize expression to the internal control transcript *rpoC* for relative quantification (Schmittgen and Livak, 2008). Statistical analysis of qRT-PCR data was performed using a one-way ANOVA as indicated (GraphPad Prism v10.0.2).

RNA-Seq

C. difficile strains 630Δ*erm* pMC123 (MC324) and 630Δ*erm* Δ*kipIA* pMC123 (MC1970) were grown in 70:30 broth (pH=8.0) with 2 µg/mL thiamphenicol to an OD_{600}=0.5 and samples were taken as described above for qRT-PCR. RNA was isolated and treated with Dnase-I, and samples were sent to Seq-Center for sequencing. Illumina's Stranded Total RNA Prep Ligation with Ribo-Zero Plus kit and 10bp IDK for Illumina indices was used for library preparation. Sequencing was performed on a NextSeq2000 giving 2x50bp reads. Demultiplexing, quality control, and adapter trimming was performed

with bcl-convert (v3.9.3; see reference above for whole genome sequencing). Geneious Prime v2022.2.2 was used to map reads to the 630Δ*erm* reference genome (NC_009089.1) and expression levels were calculated and compared using DESeq2 (Love et al., 2014). DESeq2 performs the Wald test to calculate *P* values which are adjusted by the Benjamini-Hochberg test (Love et al., 2014). Raw RNA sequencing reads were deposited to the NCBI Sequence Read Archive (SRA) BioProject PRJNA1085672.

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TABLES

Table 1. Genes differentially expressed in a Δ*kipIA* mutant

Gene[a]	Fold Change Δ*kipIA*/WT[b]	Product	*P* value
Increased in* Δ*kipIA			
CD630_08330	4.66	AcnB	5.7E-17
CD630_08320	4.57	Re-citrate synthase	2.4E-15
CD630_08340	4.16	Icd	2.6E-13
CD630_06170	2.38	membrane protein	1.6E-07
CD630_04120	2.34	conjugative transfer protein	1.1E-05
CD630_06160	2.18	MerR family regulator	5.4E-05
CD630_05300	2.09	membrane protein	1.1E-06
CD630_36160	2.08	potassium/proton antiporter	7.2E-06
CD630_17170	2.05	hypothetical protein	1.5E-07
CD630_02030	2.02	UvrABC system protein A 1	1.8E-06
CD630_07410	2.02	GlpK1	0.0017
Decreased in* Δ*kipIA			
CD630_13010	0.36	membrane protein	4.1E-06
CD630_14182	0.41	hypothetical protein	7.2E-08
CD630_05720	0.42	sporulation protein	5.1E-10
CD630_07121	0.43	hypothetical protein	1.2E-04
CD630_11990	0.43	SpoIIIAH	3.3E-04
CD630_34570	0.44	hypothetical protein	3.8E-04
CD630_17281	0.44	hypothetical protein	2.4E-04
CD630_17900	0.46	exonuclease	5.3E-05
CD630_24570	0.47	hypothetical protein	2.3E-04
CD630_20250	0.49	membrane protein	3.6E-04
CD630_26030	0.49	CdtR	2.8E-05
CD630_09410	0.50	hypothetical protein	2.2E-05
CD630_12371	0.50	hypothetical protein	0.0028
CD630_19670	0.50	hypothetical protein	5.4E-04

[a]Gene accession numbers for strain 630 (GenBank accession no. NC_009089.1). Listed genes represent those transcripts with a ≥ 2-fold change in expression and a *P* value of ≤ 0.05 as determined by DESeq2 analysis, as described in Methods. Genes representing *kipI*, *kipA*, and the *erm* selectable marker are excluded.

[b]Ratio of expression in Δ*kipIA* pMC123 (MC1970)/630Δ*erm* pMC123 (MC324), as determined by RNA sequencing analysis as described in the Methods.

Table S1. 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* (conjugation)	B. Dupuy
C. difficile		
630Δ*erm*	Erm^S derivative of strain 630	N. Minton (Hussain et al., 2005, p. 630)
MC310	630Δ*erm spo0A*::*erm*	(Edwards et al., 2014)
MC324	630Δ*erm* pMC123	(Edwards et al., 2014)
MC1903	630Δ*erm* Δ*kipIA*::*erm*	This study
MC1970	MC1903 Δ*kipIA* pMC123	This study
MC2375	630Δ*erm* Δ*kipOTIA*::*erm*	This study
MC2519	630Δ*erm* Δ*kipIA::kipOTIA*::*aad9*	This study
MC2520	630Δ*erm* Δ*kipOTIA::kipOTIA*::*aad9*	This study
B. subtilis		
1A1	Wild-type, strain 168 lineage	(Koo et al., 2017)
BKE04080	Δ*kipI*::*erm*	(Koo et al., 2017)
BKK04090	Δ*kipA*::*erm*	(Koo et al., 2017)
MC2624	BKE04080 → 1A1; Δ*kipI*::*erm*	This study
MC2625	BKK04090 → 1A1; Δ*kipA*::*erm*	This study
Plasmids		
pMSR	Pseudo-suicide plasmid used for allelic exchange in *C. difficile* strain 630; P*tet-CD2571.1 catP*	(Peltier et al., 2020)
pMC123	*E. coli-C. difficile* shuttle vector; *bla, catP*	(McBride and Sonenshein, 2011)
pRT1099	pMC123 with *aad9* cassette in place of *cat*	(Purcell et al., 2017)
pJIR1457	shuttle vector with *ermBP* cassette	(Lyras and Rood, 1998)
pMC1076	pMSR with *kipIA* homology regions flanking *ermB*	This study
pMC1233	pMSR with *kipOTIA* homology regions flanking *ermB*	This study
pMC1354	pMSR with homology flanking *kipOTIA* and *aad9*	This study

Table S2. Oligonucleotides

Primer	Sequence (5'→3')[a]	Use/locus tag/reference
oMC44	CTAGCTGCTCCTATGTCTCACATC	*rpoC* (*CD0067*) qPCR; (McBride and Sonenshein, 2011)
oMC45	CCAGTCTCTCCTGGATCAACTA	*rpoC* (*CD0067*) qPCR; (McBride and Sonenshein, 2011)
oMC501	TGAGATTCCAGTATGTTATG	Forward primer in *kipI* (*CD1386*) cds
oMC502	CTCTGCTTGTGTGTATTT	Reverse primer in *kipI* (*CD1386*) cds
oMC504	TCTACAACCCATTCTATCT	Reverse primer in *kipA* (*CD1387*) cds
oMC2802	GTAGAAATACGGTGTTTTTTGTTACCCTAAGTTTAAACGTGGAGCTGCACTTGGT	Forward primer for region 5' of *kipI* for Gibson assembly into pMSR
oMC2803	TAATCTCATGACCAAAATCCCTTAACGAAATGTCACCTCTTCTAAATTAAGCAAATAAAG	Reverse primer for region 5' of *kipI* with homology to *ermB*
oMC2804	CTTTATTTGCTTAATTTAGAAGAGGTGACATTTCGTTAAGGGATTTTGGTCATGAGATTA	Forward primer to amplify *ermB* cassette with homology to region 5' of *kipI*
oMC2805	TTATAATAAAAATTGTATTTGTTAAAATTAAACTCCTTGGAAGCTGTCAGTAGTATACC	Reverse primer to amplify *ermB* cassette with homology to region 3' of *kipA*
oMC2806	GGTATACTACTGACAGCTTCCAAGGAGTTTAATTTTAACAAATACAATTTTTATTATAA	Forward primer to amplify region 3' of *kipA* with homology to *ermB*
oMC2807	GATTTTGGTCATGAGATTATCAAAAAGGAGTTTAAACTGGAGGTTCTTATGCTAGGTGAA	Reverse primer to amplify region 3' of *kipA* Gibson assembly into pMSR
oMC2820	GTAGCTCTTGGAGGATTAGC	Forward primer to screen for *kip* deletions
oMC2823	TCTACTATGTATTCTTATTGCCACTAC	Reverse primer to screen for *kip* deletions
oMC2868	CAACATGTAAAACCACATG	Forward primer in *kipO* cds
oMC2869	TAATCCACTTAATACAGTTCC	Reverse primer in *kipT* cds
oMC2870	GCCACAATGATGAGAG	Forward primer in *kipT* cds
oMC2871	ACGACGGCCAGTGAATTCATATGTACAAATATTGTGCATGTTTTAAAATAATATTTTAAC	Forward primer to amplify 500 bp 5' *kipO* and Gibson assemble into pMC123
oMC3163	AGGTCGACTCTAGAGGATCCTTAAACTGCTATATCATTTAATACTTTTGCCTTTA	Reverse primer in 3' end of *kipA* and Gibson assembly into pMC123
oMC3343	GTAGAAATACGGTGTTTTTTGTTACCCTAAGTTTAAACTACCAGCCAAAACAGAGCG	Forward primer to amplify 5' of *kipO* and Gibson assembly into pMSR
oMC3344	TAATCTCATGACCAAAATCCCTTAACGTTTATGACCCCTCCTCGCTAA	Reverse primer to amplify 5' of *kipO* with homology to *ermB*
oMC3345	TTAGCGAGGAGGGGTCATAAACGTTAAGGGATTTTGGTCATGAGATTA	Forward primer to amplify *ermB* cassette with homology to 5' *kipO* (
oMC3538	CTTTGTCTGGAGATAGTACAATAACA	Forward primer upstream of *kipO* to screen for *kip* deletions
oMC3585	GTAGAAATACGGTGTTTTTTGTTACCCTAAGTTTAAACTTGTCTGGAGATAGTACAATAACACCT	Forward primer to amplify 5' of *kipO* region and Gibson assemble into pMSR
oMC3586	CCAGTCACGTTACGTCGACTTAAACTGCTATATCATTTAATACTTTTGCCTTTAC	Reverse primer to amplify 5' of *kipO* with homology to *aad9* cassette
oMC3587	GTAAAGGCAAAAGTATTAAATGATATAGCAGTTTAAGTCGACGTAACGTGACTGG	Forward primer to amplify *aad9* cassette with homology upstream of *kipO*
oMC3588	CTATTTGTAAGTTATATTATAAATTATAATAAAAATTGTATTTGTTAAAATTAAAGTCGACACCCAAAATTGAAAAAAGTG	Reverse primer to amplify *aad9* cassette with homology 3' of *kipA*
oMC3589	CACTTTTTTCAATTTTGGGTGTCGACTTTAATTTTAACAAATACAATTTTTATTATAATTTATAATATAACTTACAAATAG	Forward primer to amplify 3' of *kipA* with homology to *aad9* cassette
oMC3590	GATTTTGGTCATGAGATTATCAAAAAGGAGTT	Reverse primer to amplify 3' of *kipA* to

	TAAACGCCACTACTTCTATGTTGTGCTATAATTAC	Gibson assemble into pMSR
oMC3966	AGTCACGACGTTGTAAAACGACGGCCAGTGAATTCGTGGAGCTGATATAAGTGGAGC	Forward primer to confirm *B. subtilis* Δ*kipI* and Δ*kipA* mutants
oMC3973	AGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCGCACGCACCAATTCAATTAAGAG	Reverse primer to confirm *B. subtilis* Δ*kipI* and Δ*kipA* mutants

[a]Restriction sites underlined

Table S3. Vector construction

Plasmid	Construction details
pMC1076	A 909 bp homology arm 5' of *kipI* (*CD1386*) and a 796 bp homology arm 3' of *kipA* (*CD1387*) were amplified with primers oMC2802/2803 and oMC2806/2807, respectively. A 1523 bp *ermB* cassette from pJIR1457 was amplified with primers oMC2804/2805, and all three fragments were Gibson assembled into pMSR via the PmeI site.
pMC1233	A 708 bp homology arm 5' of *kipO* (*CD1384*) and a 796 bp homology arm 3' of *kipA* (*CD1387*) were amplified with primers oMC3343/oMC3344 and oMC2806/2807, respectively. A 1523 bp *ermB* cassette from pJIR1457 was amplified with primers oMC3345/2805, and all three fragments were Gibson assembled into pMSR via the PmeI site.
pMC1354	An 817 bp homology arm 5' of *kipO* (*CD1384*) and the 3772 bp *kipOTIA* operon (*CD1384 – 1387*) were amplified with primers oMC3585/3586 for a total fragment size of 4589 bp. An 864 bp homology arm 3' of *kipA* (*CD1387*) was amplified with primers oMC3589/3590. A 1045 bp *aad9* (spectinomycin resistance) cassette from pRT1099 was amplified with primers oMC3587/oMC3588. All three fragments were Gibson assembled into pMSR via the PmeI site.
pMC1181	pMC123 with 500bp 5' of *kipOTIA* to encompass the native promoter for the *kipOTIA* operon and *kipOTIA.* pMC123 was digested with EcoRI and BamHI and the 500bp 5' of *kipOTIA* and *kipOTIA* fragment was amplified with oMC2871 and oMC3163. The two fragments were Gibson assembled into pMC123 via the EcoRI and BamHI sites.

Table S4. Composition of Defined Minimal Media (DMM)

Component	Final Concentration (mg ml^{-1})[b]
Amino Acids	
L-Alanine	0.535
L-Arginine	1.045
L-Aspartic acid	0.799
L-Cysteine	0.727
L-Glycine	0.450
L-Histidine	0.931
L-Isoleucine	0.787
L-Leucine	0.787
L-Lysine	0.877
L-Methionine	0.895
L-Phenylalanine	0.991
L-Proline	0.691
L-Serine	0.631
L-Threonine	0.715
L-Tryptophan	1.225
L-Valine	0.703
L-Tyrosine[a]	1.087
Salts	
Na_2HPO_4	5.0
$NaHCO_3$	5.0
KH_2PO_4	0.9
NaCl	0.9
Trace salts	
$(NH_4)_2SO_4$	0.00800
$CaCl_2 \cdot 2H_2O$	0.0270
$MgCl_2 \cdot 6H_2O$	0.0200
$MnCl_2 \cdot 4H_2O$	0.0100
$CoCl_2 \cdot 6H_2O$	0.00100
Iron	
$FeSO_4 \cdot 7H_2O$	0.00400
Vitamins	
D-Biotin	0.00100
Calcium-D-pantothenate	0.00100
Pyridoxine	0.00100
$ZnCl_2$	0.0100
Sodium selenite	0.000179

[a]Tyrosine dissolved separately in H_2O and HCl, then added after other amino acids
[b]Adjust media pH to 7.4 and filter sterilize

Table S5. Composition of Modified Niehaus Defined Media

Component	Final Concentration (mg ml^{-1})[a]
Amino Acids	
L-Tryptophan	51.06
Salts	
$K_2HPO_4 \cdot 3H_2O$	1.84
KH_2PO_4	0.6
$MgSO_4 \cdot 7H_2O$	0.5
$MgCl_2$	0.0572
Trace salts	
$CaCl_2 \cdot 2H_2O$	0.00735
$MnCl_2 \cdot 4H_2O$	0.00099
$CoCl_2 \cdot 6H_2O$	0.0006
$ZnCl_2$	0.0017
$CuCl_2 \cdot 2H_2O$	0.00043
Na_2MoO_4	0.00052
Iron-Solution	
$FeCl_3 \cdot 6H_2O$	0.02703
Na_3-Citrate$\cdot 2H_2O$	0.088

[a]Adjust media pH to 7.0 and filter sterilize (Niehaus et al., 2017).

FIGURES

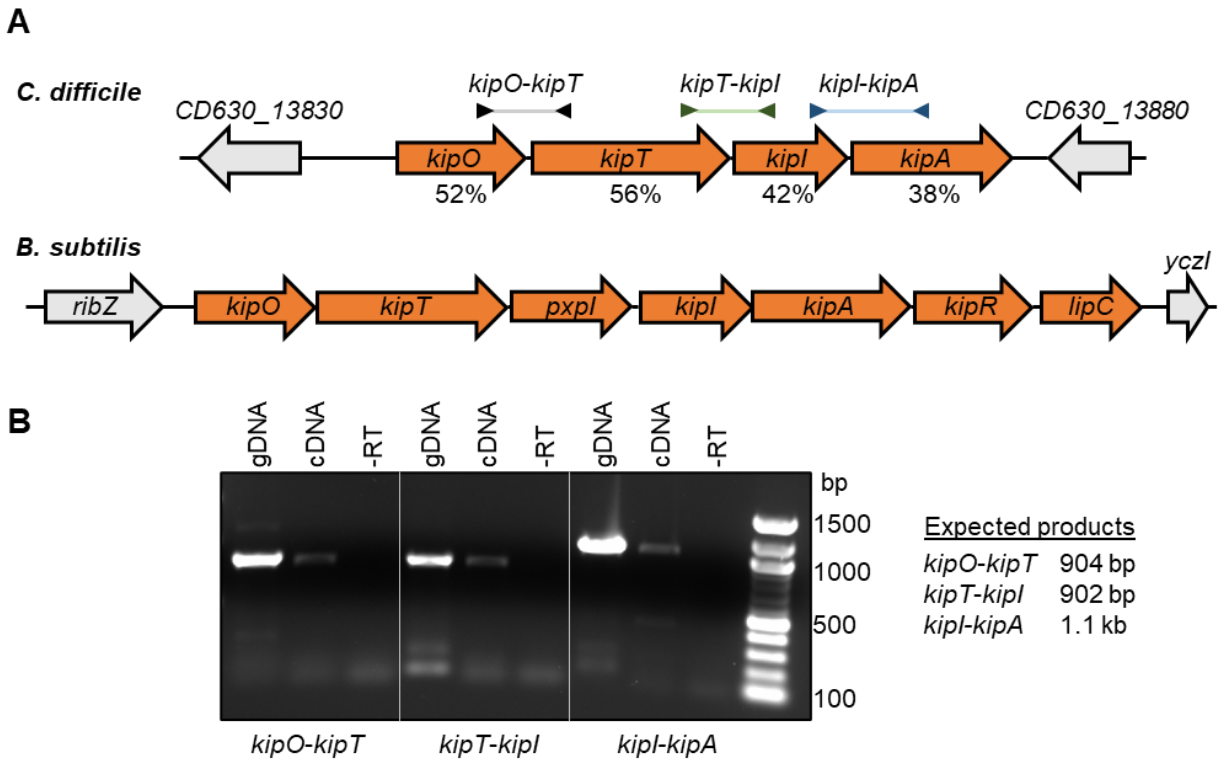

Figure S1. *C. difficile kipOTIA* is transcribed as an operon. A) The organization of the *kip* operons in *C. difficile* (*CD630_13840-CD630_13870*) and *B. subtilis* (*BSU04050-BSU04110*). Arrows above the *C. difficile kipOTIA* operon indicate PCR segments shown in B. Numbers below the *C. difficile kipOTIA* operon indicate percent identity to the corresponding proteins in *B. subtilis*. **B)** Strains were grown on 70:30 agar and samples were collected at H_{12} for RNA and cDNA generation as described in the Materials and Methods. Nested PCR was performed using 50 ng genomic DNA (gDNA, positive control), cDNA, or RNA without reverse transcriptase (-RT, negative control). Primer pairs are as follows: *kipO-kipT*, oMC2868/oMC2869; *kipT-kipI*, oMC2870/oMC502; *kipI-kipA*, oMC501/oMC504. PCR products were visualized on a 0.7% agarose gel.

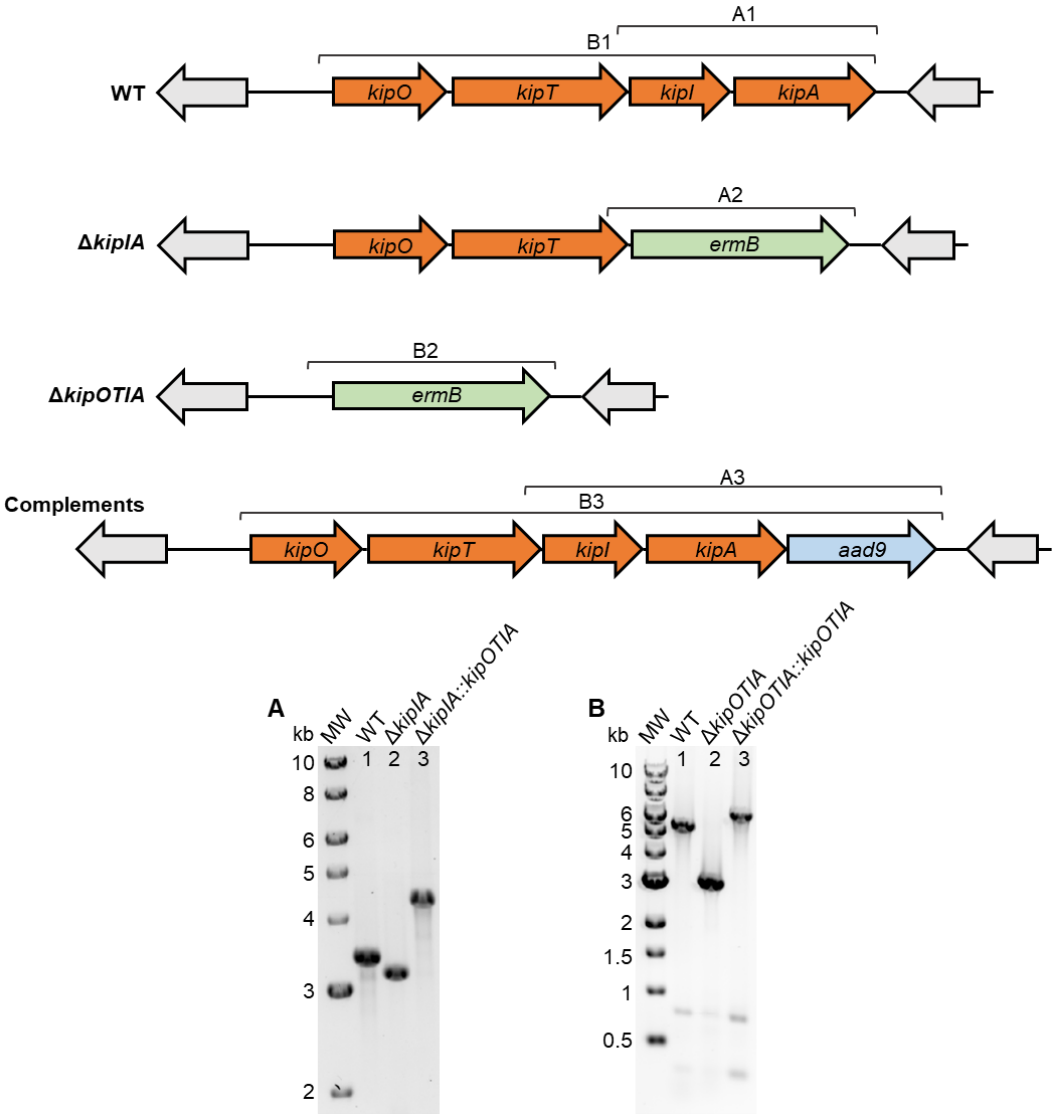

Figure S2. Construction and confirmation of *C. difficile kip* mutants and knock-in strains. **A)** PCR confirmation of *kipIA* deletion and restoration of the *kip* locus with *kipOTIA*::*aad9*. Expected PCR products using primers flanking *kipIA* (oMC2820/2823) are 3,543 bp for WT (630Δ*erm*), 3,354 bp for Δ*kipIA*::*erm* (MC1903), and 4,588 bp for Δ*kipIA*::*kipOTIA*::*aad9* (MC2519). **B)** PCR confirmation of *kipOTIA* deletion and restoration of the *kip* locus with *kipOTIA*::*aad9*. Expected PCR products using primers flanking *kipOTIA* (oMC3538/2823) are 5,473 bp for WT, 3,224 bp for Δ*kipOTIA*::*erm* (MC2375), and 6,518 bp for Δ*kipOTIA*::*kipOTIA*::*aad9* (MC2520).

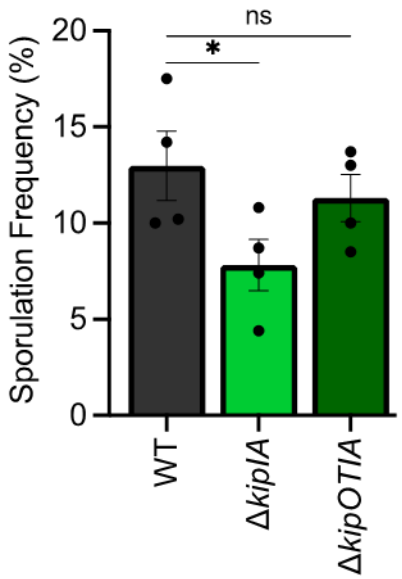

Figure 1. KipOTIA does not inhibit *C. difficile* sporulation. Ethanol-resistant sporulation frequencies of *C. difficile* strain 630Δ*erm* (WT), Δ*kipIA* mutant (MC1903), and the Δ*kipOTIA* mutant (MC2375) grown for 24 h on 70:30 sporulation agar. The means and SEM of four independent experiments are shown. Data were analyzed by one-way ANOVA with Dunnett's multiple comparison test; **P* <0.05.

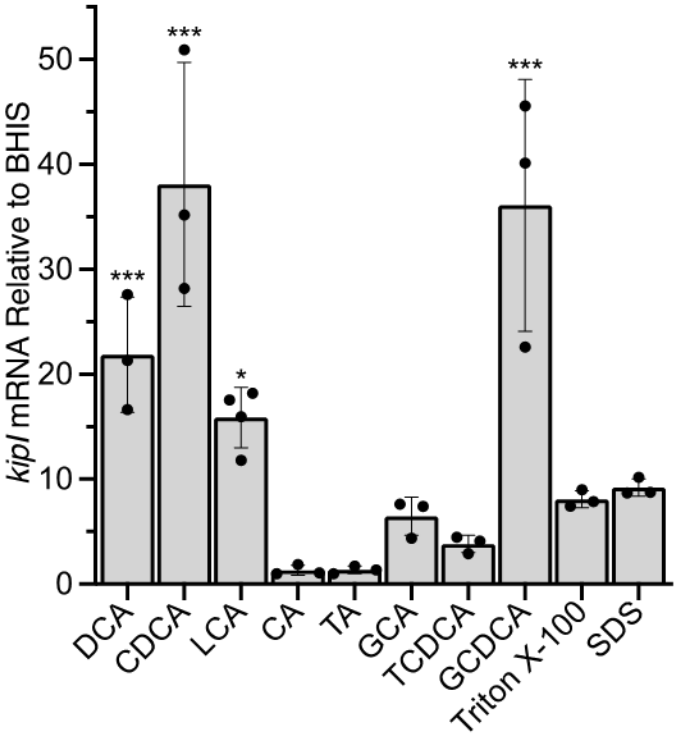

Figure 2. *C. difficile kipI* expression is induced by specific bile salts. WT *C. difficile* (630Δ*erm*) was grown in BHIS broth +/- physiological concentrations of bile salts, or detergents, as follows: deoxycholate (DCA, 0.5 mM), chenodeoxycholate (CDCA, 0.5 mM), lithocholate (LCA, 0.05 mM), cholate (CA, 2 mM), taurocholate (TA, 10 mM), glycocholate (GCA, 10 mM), taurochenodeoxycholate (TCDCA, 10 mM), glycochenodeoxycholate (GCDCA, 1.5 mM), 0.008% triton X-100, or 0.0045% sodium dodecyl sulfate (SDS). Graph shows the mean, individual data points, and standard deviations of *kipI* mRNA levels relative to WT in BHIS alone for a minimum of three independent experiments. Data were analyzed by one-way ANOVA with Dunnett's multiple comparison test comparing treated to untreated samples. **P* <0.01, ****P* <0.001.

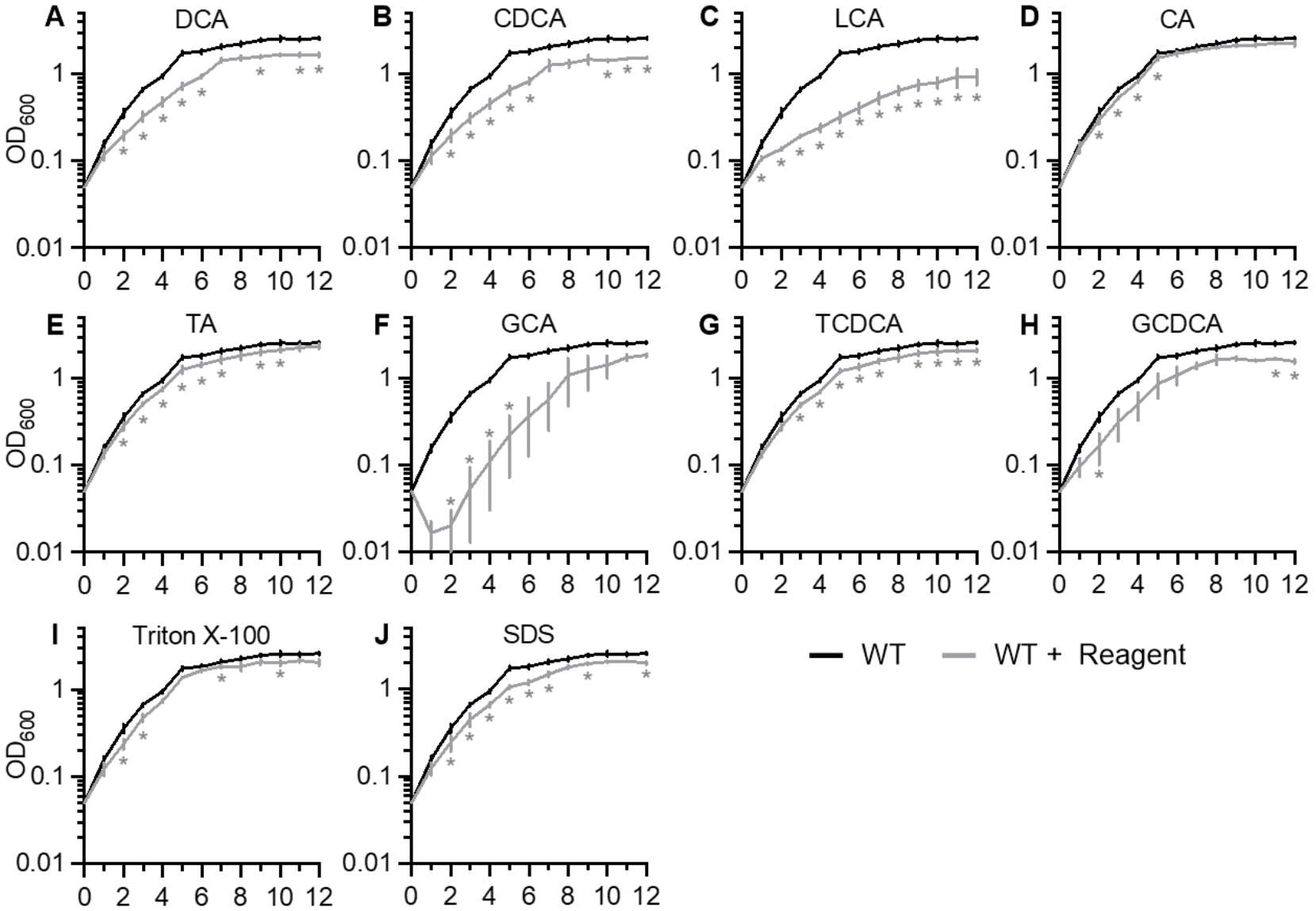
A
DCA
B
CDCA
C
LCA
D
CA
E
TA
F
GCA
G
TCDCA
H
GCDCA
I
Triton X-100
J
SDS
OD600
0.01
0.1
1
0 2 4 6 8 10 12
WT
WT + Reagent

Figure S3. Bile salts variably impact *C. difficile* growth. WT (630Δ*erm*) was grown in BHIS +/- physiological concentrations of bile salts, or detergents, as listed in Figure 2. The means and SD of three independent experiments are shown. All graphs show the same BHIS control (black). Data were analyzed by one-way ANOVA with Dunnett's multiple comparison test at each timepoint to the control. **P* <0.05.

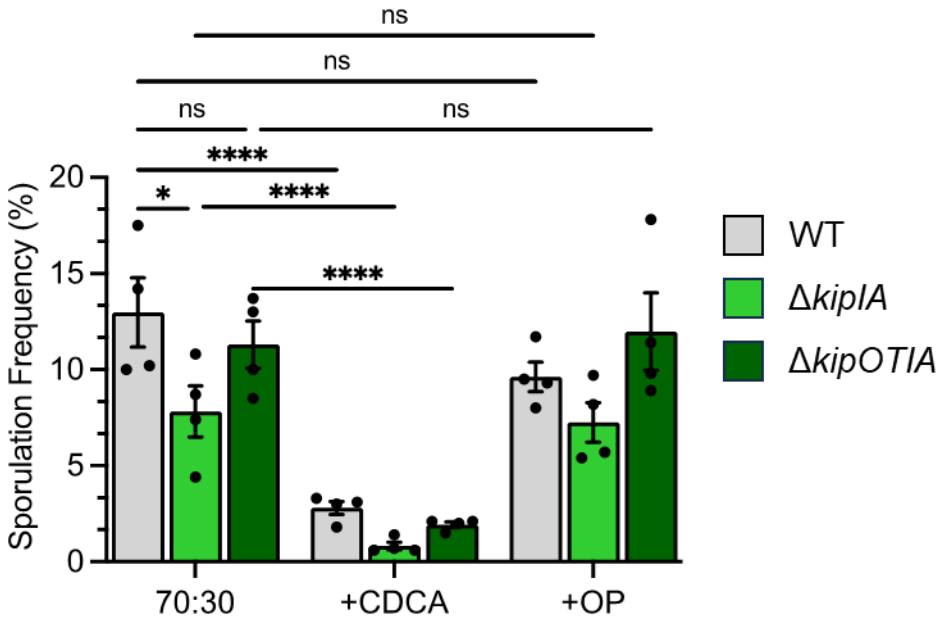

Figure S4. KipOTIA does not inhibit sporulation under conditions associated with its expression or function. Ethanol-resistant sporulation frequencies of *C. difficile* strain 630Δ*erm* (WT), Δ*kipIA* mutant (MC1903), and the Δ*kipOTIA* mutant (MC2375) grown for 24 h on 70:30 sporulation agar +/- 0.5 mM chenodeoxycholate (CDCA) or 30 mM of neutralized 5-oxoproline (OP). The means and SEM of four independent experiments are shown. Data were analyzed by two-way ANOVA with Šídák's multiple comparison test; **P*<0.05, ***P*<0.01, *****P*<0.0001.

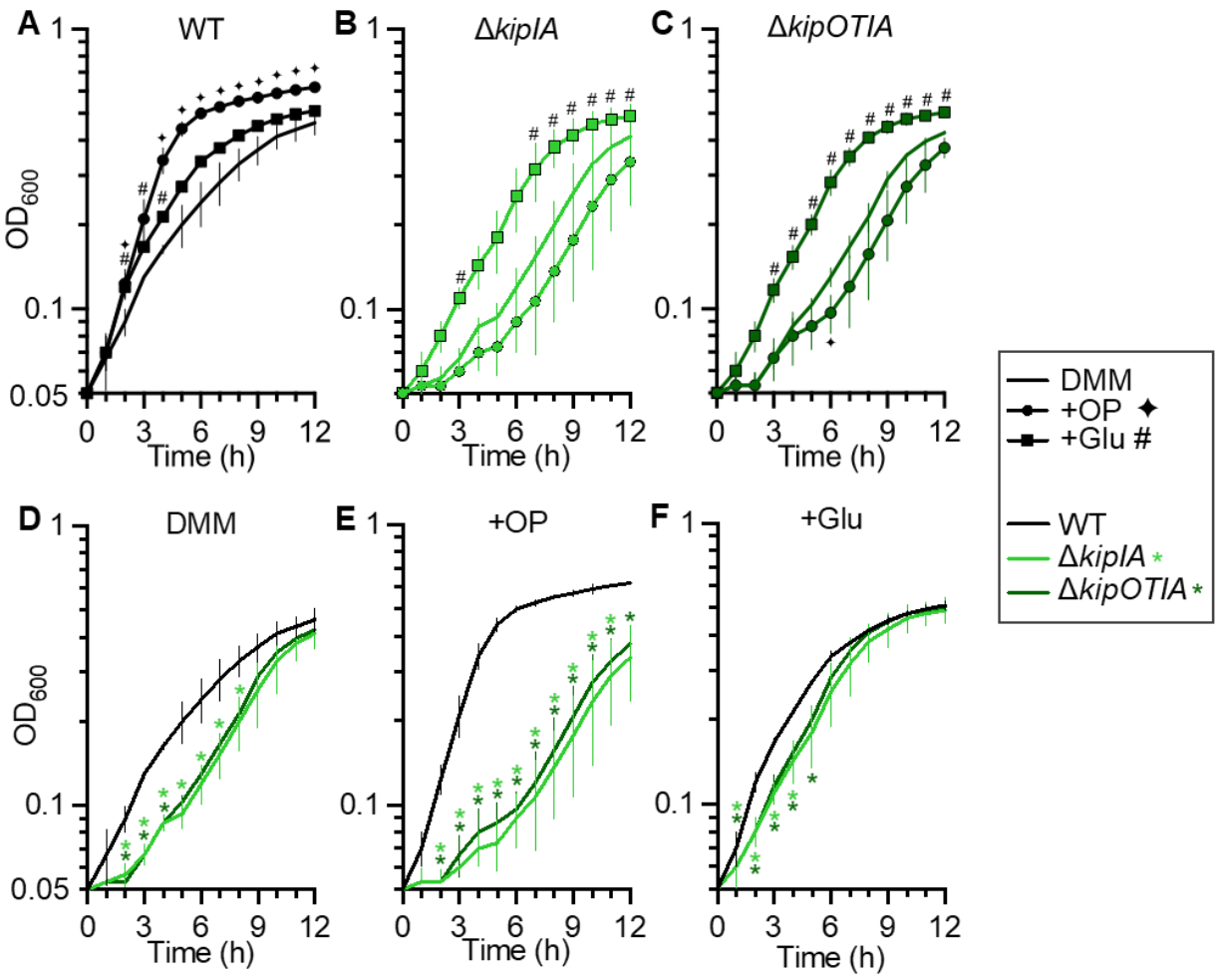

Figure 3. *C. difficile* KipOTIA supports the utilization of 5-oxoproline as a carbon source. **A-C)** Growth of WT (630Δ*erm*), Δ*kipIA* (MC1903), and Δ*kipOTIA* (MC2375) in Defined Minimal Medium (DMM) with and without 30 mM 5-oxoproline (OP) or glutamic acid. **D-F)** Condition-centered display of growth for WT, Δ*kipIA*, and Δ*kipOTIA* in **D)** DMM, **E)** DMM with 30 mM of neutralized 5-oxoproline, and **F)** DMM with 30 mM glutamic acid. The means and SD of three independent experiments are shown. Data in A-C were analyzed by one-way ANOVA with Dunnett's multiple comparison test at each timepoint to growth in DMM. Data in D-F were analyzed by one-way ANOVA with Dunnett's multiple comparison test at each timepoint to WT; *, ✦, # $P < 0.05$.

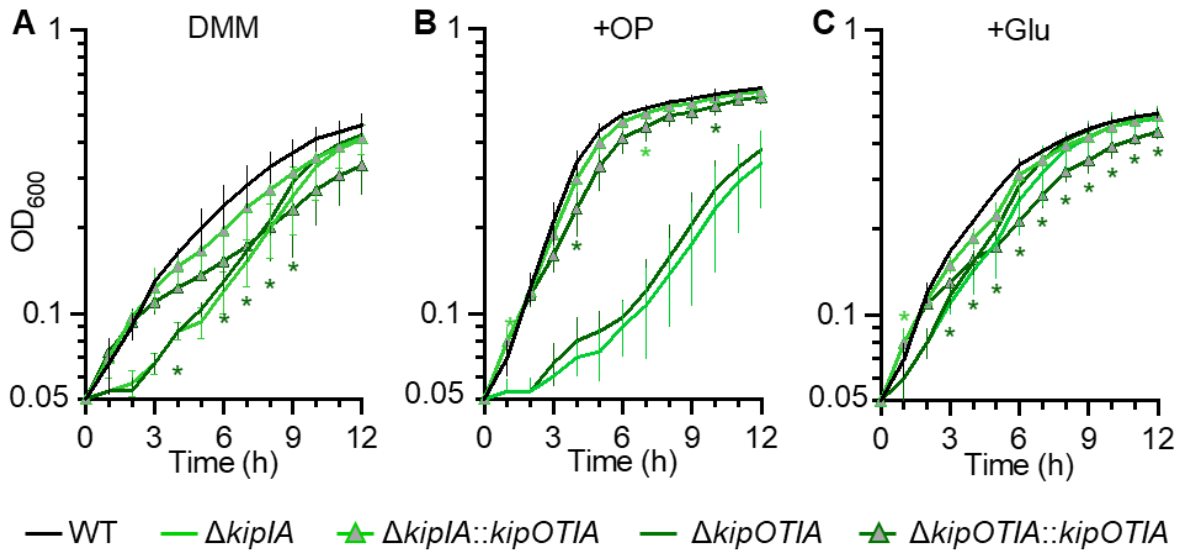

Figure S5. Restoration of the *kip* operon improves the growth of *kip* mutants. Condition-centered display of growth for 630Δ*erm* (WT), Δ*kipIA* (MC1903), Δ*kipOTIA* (MC2375), and *kip* knock-ins Δ*kipIA*::*kipOTIA* (MC2519) and Δ*kipOTIA*::*kipOTIA* (MC2520) in **A)** DMM, **B)** DMM with 30 mM 5-oxoproline (+OP), and **C)** DMM with 30 mM glutamic acid (+Glu). The means and SD of three independent experiments are shown. Data were analyzed by one-way ANOVA with Dunnett's multiple comparison test at each timepoint for the respective *kip* knock-ins to the wild-type. *, P <0.05.

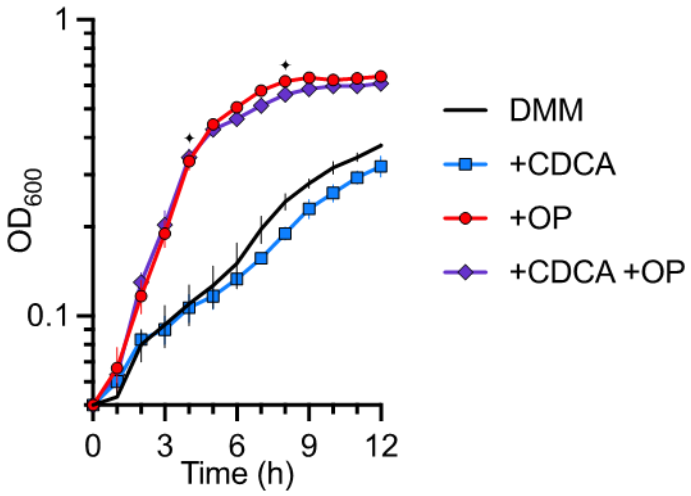

Figure 4. The bile acid CDCA does not enhance *C. difficile* growth in 5-oxoproline. Growth of 630Δ*erm* in Defined Minimal Medium (DMM) with and without 30 mM 5-oxoproline (OP) and/or 0.1 mM of chenodeoxycholate (CDCA). The means and SD of three biological replicates are shown. Data were analyzed by one-way ANOVA with Šídák's multiple comparison test for each timepoint comparing growth in DMM to +CDCA (*, $P<0.05$) or +OP to +CDCA+OP (✦, $P<0.05$).

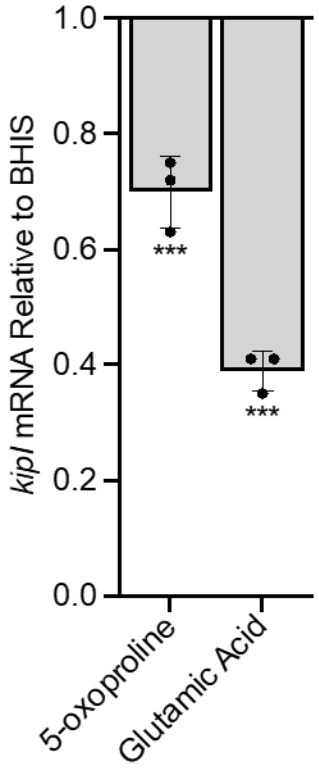

Figure S6. Glutamic acid and 5-oxoproline modestly repress *C. difficile kipI* expression. WT (630Δ*erm*) was grown in BHIS +/- 30 mM neutralized 5-oxoproline or 30 mM glutamic acid. Graph shows the mean, individual data points, and standard deviations of *kipI* mRNA expression levels relative to WT in BHIS alone for three independent experiments. Data were analyzed by one-way ANOVA with Dunnett's multiple comparison test comparing the treated WT with untreated WT. *** P <0.001.

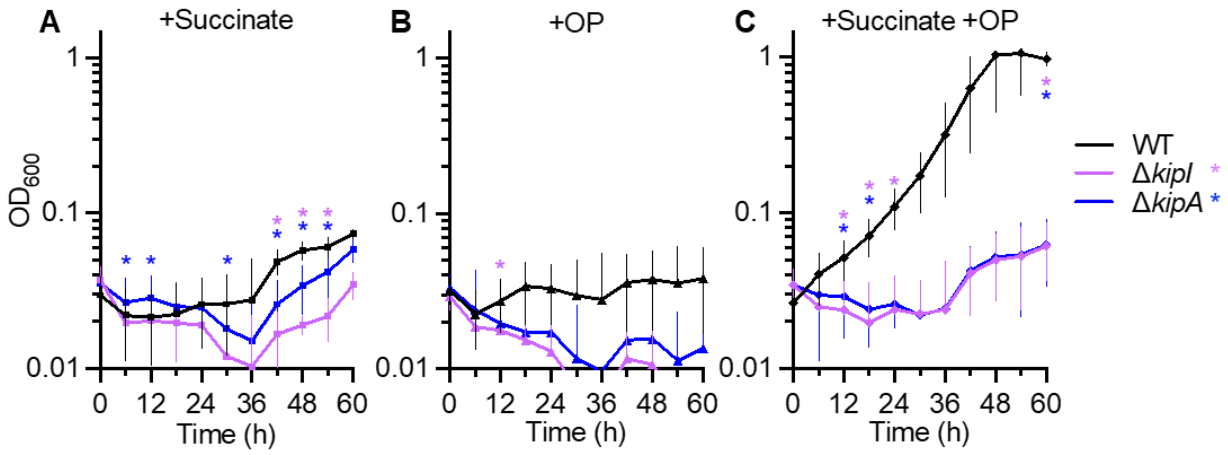

Figure 5. The *B. subtilis* Kip oxoprolinase enables the use of 5-oxoproline as a nitrogen source, but not a carbon source. A-C) Growth of *B. subtilis* 1A1 (wild-type), Δ*kipI* (MC2624), and Δ*kipA* (MC2625) in Niehaus Defined Medium (NDM) with or without 30 mM sodium succinate (SS), 30 mM 5-oxoproline (OP), or 30 mM OP and 30 mM SS. The means and SD of three independent experiments are shown. Data were analyzed by one-way ANOVA with Dunnett's multiple comparison test at each timepoint comparing mutants to wild-type; *, *, $P < 0.05$.

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Chapter 3: Genetic Mechanisms Governing Sporulation Initiation in *Clostridioides difficile*

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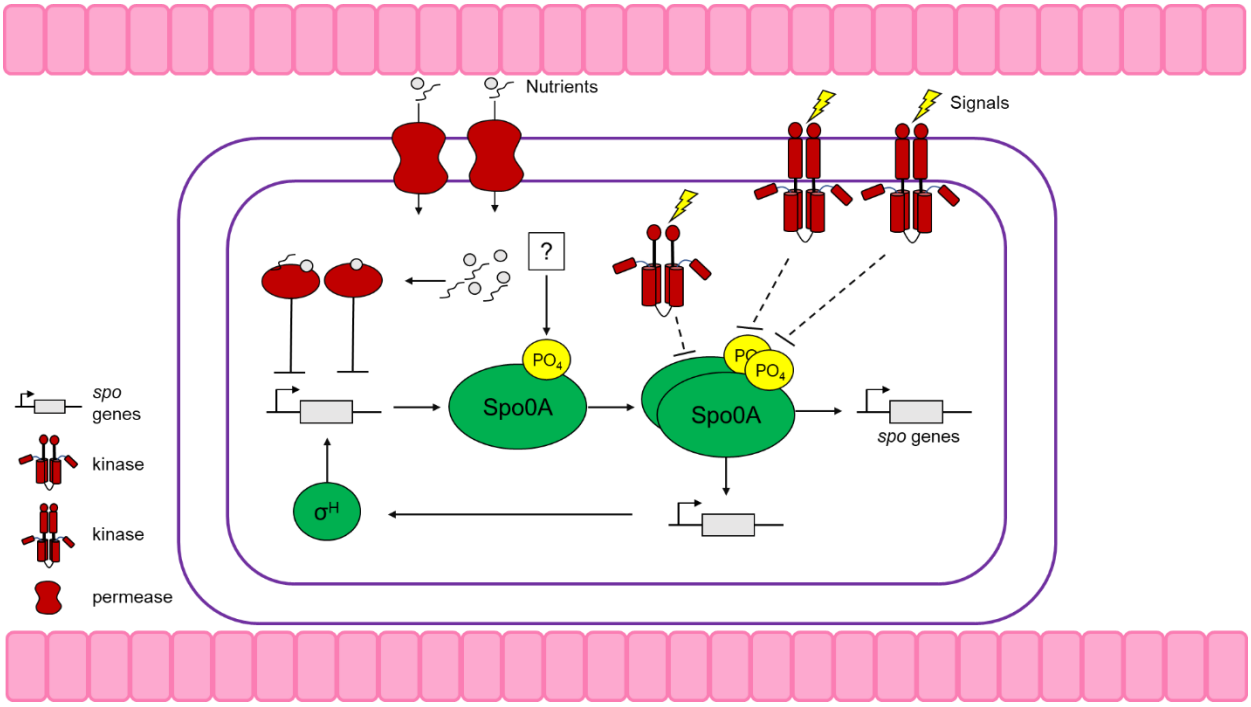
ABSTRACT

As an anaerobe, *Clostridioides difficile* relies on the formation of a dormant spore for survival outside of the mammalian host's gastrointestinal tract. The spore is recalcitrant to desiccation, numerous disinfectants, UV light, and antibiotics, permitting long-term survival against environmental insults and efficient transmission from host to host. Although the morphological stages of spore formation are similar between *C. difficile* and other well-studied endospore-forming bacteria, the *C. difficile* genome does not appear to encode many of the known, conserved regulatory factors that are necessary to initiate sporulation in other spore-forming bacteria. The absence of early sporulation-specific orthologs suggests that *C. difficile* has evolved to control sporulation initiation in response to its unique and specific ecological niche and environmental cues within the host. Here, we review our current understanding and highlight the recent discoveries that have begun to unravel the regulatory pathways and molecular mechanisms by which *C. difficile* induces spore formation.

GRAPHICAL ABSTRACT



HIGHLIGHTS

- The master transcriptional regulator of sporulation in *C. difficile* is Spo0A.
- Spo0A activity depends on phosphorylation.
- Diverse regulators translate environmental signals to impact Spo0A phosphorylation.
- This complex regulatory network ensures sporulation initiates only when required.

INTRODUCTION

Clostridioides difficile is an anaerobic, Gram-positive, spore-forming bacterium that is a foremost cause of antibiotic-associated diarrhea. *C. difficile infection* (CDI) symptoms range from mild diarrhea and abdominal distress to life-threatening pseudomembranous colitis. Antibiotic usage causes gut dysbiosis by altering the gut microbiota and sensitizes individuals to *C. difficile* colonization. A key aspect of *C. difficile* pathogenesis is its ability to form spores, as they are critical for the infection cycle and also resistant to antibiotics, environmental insults, and disinfectants. While much of the general sporulation pathway in *C. difficile* is conserved with other Firmicutes, the molecular mechanisms controlling the initiation of sporulation are not conserved and remain poorly understood. This review summarizes our current understanding of regulation of *C. difficile* sporulation initiation mechanisms and outlines the identified factors that contribute to initiation through the activation of the conserved master regulator of sporulation, Spo0A. Further, we provide a summary of the nutritional and environmental regulations that promote or repress spore formation, as well as an overview of recently characterized factors that contribute to sporulation regulation through undiscovered mechanisms.

Positive Regulators of Sporulation Initiation

<u>Spo0A</u>

Spo0A is the master transcriptional regulator of sporulation in all endospore-forming bacteria [1]. As observed in other spore formers, Spo0A is essential for sporulation in *C. difficile*. A *spo0A* null mutant cannot initiate sporulation and was unable to transmit infection in a mouse model due to the absence of spores [2]. In addition to regulating sporulation in *C. difficile*, Spo0A impacts a myriad of cellular processes, including motility, protein transport, metabolism, cell envelope, and global gene regulation [3].

Once activated by phosphorylation, Spo0A binds to DNA at conserved binding sites known as Spo0A-boxes and induce the expression of genes required for initiating sporulation, including *sigH* and the sigma factors for early spore development, *sigF* and *sigE* [4]. In *B. subtilis*, Spo0A activity is modulated through a phosphorelay composed of a series of phosphotransfer proteins and accessory phosphatases [5]. Spo0A activation is notably different amongst the Clostridia, with most clostridial species lacking apparent orthologs to the phosphorelay kinases [1]. While *C. difficile* lacks orthologs to many of the activating proteins of Spo0A in *B. subtilis*, many of the functional and protein-interaction residues of *B.s.* Spo0A are conserved in *C.d.* Spo0A (DiCandia *et al.*, unpublished). This suggests that while the phosphorelay that controls Spo0A is not conserved in *C. difficile*, there may be factors that perform similar functions to control Spo0A activity. The absence of an obvious phosphorelay in *C. difficile* led to the hypothesis that Spo0A could be directly activated by orphan sensor histidine kinases in the Clostridia [6]. Further contributing to this view, multiple clostridial species directly phosphorylate and dephosphorylate Spo0A via orphan histidine kinases (*i.e.*, kinases that are not encoded with cognate response regulators) [7–9]. Three predicted orphan histidine kinases, PtpA (CD1492), PtpB (CD2492), and PtpC (CD1579), have been implicated in regulating early sporulation events in *C. difficile* [10, 11*, 12]. PtpA, PtpB, and PtpC repress spore production, as evidenced by the hypersporulation phenotype of their respective null mutants [11*–13]. The sporulation-repressing activities of PtpA, PtpB, and PtpC strongly suggest they do not function as Spo0A kinases, thus, the activating kinase for Spo0A in *C. difficile* remains unidentified.

SigH

SigH is an alternative sigma factor and a key regulator of transition phase and sporulation initiation in *C. difficile* and other firmicutes. SigH regulates over 700 genes involved in sporulation, cellular division, motility, virulence, and metabolism [14]. As the primary transition phase sigma factor, SigH allows *C. difficile* to rewire metabolism to adapt to limited availability of nutrients. SigH regulates hundreds of genes involved in energy production, metabolite transport, amino acid synthesis, and carbon fixation, therein linking metabolism with the initiation of sporulation. SigH-RNA polymerase holoenzyme transcribes several genes known to impact sporulation initiation, including *spo0A*, the phosphotransfer protein PtpB, and the *spo0J*, *soj*, and *spoIIP* genes involved in chromosomal segregation and prespore engulfment. SigH is required for both the initiation and progression of spore formation, and consequently, a *sigH* mutant is asporogenic. The transcription of *sigH* and *spo0A* are intertwined, with both factors promoting positive feedback expression of each other [4,14]. In addition, SigH positively regulates genes involved in the later stages of sporulation, including the *spoIIIA* operon, *spoIIID, spoIVA, spoVD, spoVE, spoVG*, and *spoVS* [14].

RstA

Another regulator that modulates Spo0A activity in *C. difficile* is RstA, a highly conserved and multifunctional protein [15]. RstA shares some sequence similarity to the *Bacillus* genus' Rap phosphatases, which directly dephosphorylate Spo0F to inhibit the transfer of phosphate to Spo0A and impede sporulation. RstA belongs to the RRNPP protein family. RRNPP proteins possess an N-terminal DNA-binding domain and/or protein-binding domain, followed by multiple C-terminal tetratricopeptide repeat (TPR) domains that encompass a small quorum-sensing-binding domain. RstA positively influences sporulation and directly represses toxin production and motility through two distinct domains, indicating that RstA employs different molecular mechanisms to regulate these processes [16**]. A null mutation in *rstA* reduces sporulation frequency by ~20-fold and decreases Spo0A phosphorylation through an unknown mechanism [15,17]. RstA does not appear to directly bind Spo0A, but is

hypothesized to interact with PtpA and PtpB to influence Spo0A phosphorylation [13]. Interestingly, there are some strain-dependent effects on RstA regulation between the historical 630Δ*erm* and the epidemic R20291 strains. RstA exhibited stronger regulation of sporulation and toxin production in R02921 compared to 630Δ*erm*, and surprisingly, the loss of *rstA* did not affect R20291 motility [17].

The DNA-binding and/or protein-binding activities of RRNPP proteins are controlled by the interaction of quorum-sensing peptides with the C-terminus of the protein [18]. It is unclear whether RstA is regulated by quorum-sensing peptides. But, evidence suggests that RstA activity is controlled by a cofactor, as purified RstA is unable to bind DNA and substitution of the C-terminal quorum-sensing-binding domain with other species' RstA orthologs abolishes RstA DNA-binding activity [17]. Determining how RstA functions to promote *C. difficile* sporulation may uncover additional sporulation factors and environmental cues that regulate Spo0A phosphorylation and activation.

Negative Regulators of Sporulation Initiation

Spo0E

The Spo0E class of proteins are a family of small aspartyl-phosphate phosphatases that dephosphorylate and inactivate Spo0A in *B. subtilis*, but their functions are poorly understood in *C. difficile* or other anaerobes [19,20]. Comparative genomics identified putative Spo0E orthologs in several Clostridia species by probing candidate genomes for the "SQELD" phosphatase motif identified in *B. subtilis* [21, 22**, 23]. The clostridial Spo0E-like proteins cluster separately from aerobic Spo0E orthologs with no conserved synteny between anaerobic and aerobic Spo0E clusters [22], suggesting that clostridial Spo0E proteins have evolved independently of the *Bacillus* Spo0E orthologs.

C. difficile encodes at least one putative Spo0E ortholog (CD3271) that contains a loosely conserved functional motif (SKKID). Recent work reveals that a *C. difficile spo0E* mutant exhibits increased sporulation, similar to observations in *B. subtilis* (DiCandia *et al*., unpublished). In addition, co-immunoprecipitation of recombinant Spo0A revealed that Spo0E co-purifies with Spo0A, suggesting that

Spo0E in *C. difficile* directly dephosphorylates Spo0A to negatively regulate sporulation initiation (DiCandia *et al*., unpublished). However, the *C. difficile spo0E* mutant has additional phenotypes that were not observed in *B. subtilis*, including hypermotility, increased toxin expression, and mucoid colony morphology, which are not associated with Spo0A regulation. These results suggest that the Spo0E of clostridial species have evolved additional regulatory functions outside of the sporulation pathway, though the additional mechanisms remain to be determined.

PtpA and PtpB

In other sporulating species, phosphorylation of Spo0A occurs through a phosphorelay initiated by the activation of orphan histidine kinases [1,8,24,25]. *C. difficile* encodes five predicted orphan histidine kinases; PtpA (CD1492), PtpB (CD2492), PtpC (CD1579), CprK (CD1352), and CD1949. CprK and CD1949 have no involvement in sporulation initiation [13,26,27]. PtpA and PtpB repress sporulation initiation, though it is not known whether PtpA or PtpB control Spo0A phosphorylation directly, as observed in other Clostridia [7,8]. Deletion of *ptpA* increases sporulation frequency ~2.4-fold (strain 630Δ*erm*), suggesting that PtpA represses sporulation initiation. The hypersporulation phenotype of the *ptpA* mutant suggests that PtpA does not phosphorylate Spo0A, but instead acts as a phosphatase or an indirect regulator of Spo0A function. The *ptpA* mutant also produces less toxin and is less virulent than wild-type, likely due to reduced expression of *sigD*, which induces toxin production, or through reciprocal regulation with RstA [11**].

Results from an early examination of PtpB suggested that this predicted kinase promotes sporulation, since disruption of *ptpB* appeared to reduce sporulation. However, that study did not complement the mutation, and the assay used to examine sporulation was unconventional and did not include important controls [10]. Further examination of a *ptpB* mutant revealed a hypersporulation phenotype, similar to that of a *ptpA* mutant, suggesting that PtpB is also a negative regulator of sporulation initiation [12]. Additionally, a *ptpA ptpB* double mutant results in the same sporulation phenotype as the single mutants, suggesting both proteins function together in the same regulatory

pathway [12]. Surprisingly, the conserved histidine residue necessary for phosphate transfer is required for PtpA function, but not for PtpB function, suggesting that although these proteins function in the same pathway, they may have divergent roles [13]. Finally, the inverse pattern of gene expression and phenotypes for the *ptpA*, *ptpB*, and *rstA* mutants suggests that PtpA, PtpB, and RstA function within the same regulatory pathway to influence sporulation in *C. difficile* [11**]. Although PtpB and PtpA clearly play a role in sporulation regulation, their direct targets and phosphotransfer functions remain to be determined.

PtpC

PtpC (CD1579) is one of the predicted orphan histidine kinases originally hypothesized to promote sporulation by phosphorylating Spo0A [1,10]. PtpC was shown to phosphorylate Spo0A *in vitro*; however, it is unclear whether this is the preferential direction of phosphate transfer between these proteins *in vivo* [10]. Although it was presumed that PtpC would activate sporulation, deletion of *ptpC* results in increased, but variable, sporulation, indicating that PtpC represses sporulation in *C.* difficile [13]. The mechanism of PtpC activation is not known, but *ptpC* transcription is influenced by other sporulation factors, including RstA, PtpA, and PtpB [11**,15]. Further characterization of PtpC is needed to define the role of PtpC in Spo0A phosphoregulation.

SigB

Similar to other Gram-positive species, SigB transcribes factors in *C. difficile* to aid in survival during stress-inducing conditions, including nitrosative and oxidative conditions, acidic environments, and thiol homeostasis [28–31]. In *C. difficile*, a *sigB* mutant displays a 10-fold increase in spore formation relative to wild-type, indicating that SigB has a negative effect on sporulation [29]. However, as a sigma factor, SigB is a direct positive regulator of transcription, thus the negative effects of SigB on sporulation are expected to be indirectly mediated through transcription of genes whose product(s) impedes the initiation process [32]. Candidate SigB-dependent factors that may impede sporulation initiation were identified by

transcriptional analysis of a *sigB* mutant [29]. These factors include the aforementioned phosphotransfer protein PtpA and the Spo0J-soj system, which is involved in chromosome partitioning. Whether through the expression of these or other sporulation suppressing factors, SigB allows *C. difficile* to repress sporulation under conditions of cellular stress that do not support spore formation.

CcpA

The global regulator catabolite control protein, CcpA, is an acI/GalR transcriptional repressor that is responsible for transcriptional carbon catabolite repression (CCR) in Gram-positive bacteria [33]. Antunes *et al.* investigated the role of CcpA in the CCR of *C. difficile* and found that CcpA directly represses expression of genes encoding the key early sporulation regulators *spo0A* and *sigF* [34*]. Relief of CcpA repression in a *ccpA* null mutant resulted in a higher sporulation frequency compared to the parental strain in the absence of glucose, but at later time points, the sporulation frequencies were similar [34*]. The addition of glucose substantially reduced sporulation to a similar level in both the wild-type and *ccpA* mutant, indicating that glucose-mediated CCR of sporulation is independent of CcpA, as observed in *C. perfringens* [35]. However, the mechanism for CcpA-independent glucose-mediated repression of sporulation has not been determined.

CodY

CodY is a nutrient-sensing global regulator that was discovered in *B. subtilis* [36]. CodY acts as a sensor of branched-chain amino acids (BCAAs) and GTP concentrations within the cell and modulates the expression of CodY-dependent genes [37,38**]. CodY acts primarily as a repressor of transcription during exponential phase, when BCAAs and GTP are abundant [37,39]. When GTP and BCAA levels are low in later growth phases, CodY repression is alleviated and the transcription of genes involved in amino acid biosynthesis, virulence, and sporulation increase [39]. CodY represses transcription of the toxin genes, which results in increased toxin production in low nutrient conditions [37]. Deletion of *codY* in the epidemic strain UK1 (RT027) resulted in more than a 1000-fold increase in spore formation [40*]. The

mechanism by which CodY represses sporulation is not clear [38**,40*]. Since CodY represses the expression of several regulatory factors, many genes that are derepressed in a *codY* mutant are not directly regulated by CodY. However, several genes that impact sporulation initiation have predicted CodY binding sites or are enriched in CodY-DNA binding experiments, including the phosphotransfer protein PtpC (CDR20291_1476), *spo0E*, and *sinR/sinR'* [38**]. Although evidence indicates that CodY represses sporulation initiation in *C. difficile*, the specific mechanism(s) through which this is accomplished is not clear.

Other factors that influence sporulation frequency

Agr

The accessory gene regulator (Agr) system is a quorum-sensing system found in most Gram-positive bacteria [41–43]. The Agr system controls many cellular processes, including the expression of genes involved in colonization and virulence. The typical Agr system includes an autoinducer peptide (AIP), AgrD, which is processed and exported by AgrB. The AgrD peptide is sensed exogenously by AgrC, a histidine kinase that activates a response regulator, AgrA, to directly regulate gene expression. There are three identified Agr systems in *C. difficile*: Agr1, Agr2 and Agr3. All sequenced *C. difficile* strains contain a partial Agr1 system, encoding only *agrB1* and *agrD1* in a single operon [44,45]. A complete Agr2 system is found in the R20291 (RT027) [46], while the Agr3 system, lacking the response regulator, is encoded in RT078 strains [47]. Deletion of *agrB1D1* in *C. difficile* strain 630 results in significantly reduced sporulation [48**]. Sporulation of the *agrB1D1* mutant was recovered by providing the supernatant of stationary phase cultures from the parent strain. These data are the first evidence that quorum sensing regulates *C. difficile* sporulation; however, because a cognate histidine kinase (AgrC) and response regulator (AgrA) are missing from strain 630, the signaling pathway that responds to the AgrB1D1 AIP remains unknown.

Opp/App Permeases

Opp and App are oligopeptide permease systems that are hypothesized to import small peptides into *C. difficile*. Loss of Opp and App results in earlier and increased spore formation, increased expression of the SinR orthologs, *CD2214* and *CD2215*, as well as expression of other CodY and CcpA-dependent genes [49]. Loss of Opp and App also results in increased virulence in a hamster model of infection [49]. The data suggest that Opp and App indirectly repress sporulation through importation of small peptides, but the specific cargo that they transport has not been verified. OppA resembles a nickel-uptake receptor, while AppA is structurally similar to the oligopeptide-binding protein CtaP (cysteine transport-associated protein) from *L. monocytogenes*, which binds a restricted set of peptides, suggesting that *C. difficile* AppA may also bind a restricted set of peptides [50–52*].

SinR orthologs

The SinR orthologs (CD2214 and CD2215) impact sporulation, motility, and toxin production in *C. difficile* strain R20291 [53*]. A mutant that does not transcribe either *CD2214* or *CD2215* is asporogenic [53*]. Specifically, CD2214 promotes sporulation, motility, and toxin production, while CD2215 decreases sporulation, motility, and toxin production indirectly by binding and preventing CD2214 from influencing target genes [53*]. CD2214-CD2215 binding is mediated through the multimerization domain of CD2215, which was shown to complement the sporulation, motility, and toxin production phenotypes seen in the R20291 *CD2215 (CDR20291_2122)* mutant [54]. However, the CD2215 helix-turn-helix domain was dispensable for complementation, and its function remains unknown [54]. CD2214 promotes sporulation in *C. difficile* through a currently unknown mechanism, but likely through DNA-binding and regulation of target genes [55].

CsiA (CD2589)

CD2589(0) was identified as a genomic signature for *C. difficile* sporulation in the gastrointestinal tract [56]. Deletion of *CD25890* resulted in increased sporulation in SM broth, but not 70:30 medium, suggesting that CD25890 has a nutritional function or impact [57]. Notably, expression of the *sin* genes was also increased ~five-fold in the *CD25890* mutant (strain 630Δ*erm*). Though the data suggest that CD25890 decreases sporulation in response to nutritional cues, the mechanism is currently unknown.

c-di-GMP

The nucleotide-based second messenger signaling molecule, c-di-GMP, modulates several physiological processes in *C. difficile* important for pathogenesis and colonization. Two recent studies revealed that overexpression of a diguanylate synthase reduces sporulation and the regulated production of a phosphodiesterase increases sporulation in *C. difficile* [12,58], Altogether, these studies support that c-di-GMP inhibits *C. difficile* sporulation, although the molecular mechanisms and regulatory pathways that mediate this response are unknown.

Orthologs of unknown influence

Below is a summary of *C. difficile* orthologs to sporulation initiation proteins from *B. subtilis* that have not been investigated:

The KipI-KipA system in *B. subtilis* regulates sporulation initiation by inhibiting phosphorylation of KinA. Orthologs of KipIA may play a similar role in *C. difficile*, though in the absence of a KinA ortholog, their target kinase is not apparent [59–61].

Soj (ParA) inhibits *B. subtilis* sporulation by preventing early sporulation gene transcription [62]. Spo0J (ParB) is responsible for chromosome segregation in *B. subtilis* and allows Soj to dissociate from DNA, to enable transcription of early sporulation genes [63]. *C. difficile* encodes similar proteins, but they have not been characterized.

Summary

Successful sporulation initiation is critical to *C. difficile* transmission and survival within an aerobic environment. This review highlights our current understanding of sporulation initiation in *C. difficile*; however, sporulation initiation in this species is complex, and many details of the process remain to be determined. It is clear that the sporulation initiation mechanisms of Clostridia, including *C. difficile*, vary considerably from the *Bacillus* paradigm and from each other [1,7–9,64]. Other Clostridia encode predicted kinases that directly phosphorylate or dephosphorylate Spo0A, rather than indirectly via a phosphorelay. But, each of the clostridial initiation pathways that have been characterized appear to have evolved distinct Spo0A regulatory mechanisms with regulators that bear limited similarity to each other [1]. Thus far, the majority of factors that are characterized in the initiation pathway of *C. difficile* repress Spo0A activity and spore formation. Although direct activators of Spo0A have not been identified, the presence of multiple Spo0A inactivating factors in *C. difficile* alludes to the existence of a mechanism for deliberate Spo0A phosphorylation. Most likely, there are unidentified factors involved in Spo0A activation. Identification and characterization of these additional initiation factors will be critical for piecing together the puzzle of the sporulation initiation program.

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Conflict of Interest Statement: Nothing declared.

TABLE 1

Gene name	Locus tag[a]	Known or predicted function	References
spo0A	CD1214	Master transcriptional regulator of sporulation	[2,3]
sigH	CD0057	Transition/stationary-phase sigma factor	[14]
rstA	CD3668	Multifunctional regulator; promotes sporulation	[14, 15, 16**]
spo0E	CD3271	Putative Spo0A phosphatase; sporulation inhibition	
ptpA	CD1492	Sporulation inhibition	[11*]
ptpB	CD2492	Sporulation inhibition	
ptpC	CD1579	Sporulation inhibition	
sigB	CD0011	Alternative sigma factor; stress responses; sporulation inhibition	[29–31]
ccpA	CD1064	Carbon catabolite control, transcriptional regulator; cofactor: fructose-1,6-bisphosphate; sporulation inhibition	[34*]
codY	CD1275	Transcriptional regulator; cofactors: GTP and BCAA; sporulation inhibition	[38**–40*]
agrB1D1	CD27491-50	quorum-sensing related sporulation inhibition	[48**]
oppA-F	CD0853-57	Putative peptide permease; sporulation inhibition	[49]
appA-F	CD2670-74	Putative peptide permease; sporulation inhibition	[49]
sin	CD2214-15	sporulation	[53*,54]
csiA	CD2589	Conditional repression of sporulation	[57]
kipI-kipA	CD1386-87	Putative inhibitor of histidine kinase	
spo0J-soj-spo0J2	CD3671-73	Putative inhibitor of sporulation gene transcription	

[a]Locus tag number based on *C. difficile* 630 reference genome (GenBank: AM180355)

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Chapter 4: Discussion

The anaerobic pathogen *Clostridioides difficile* causes severe diarrhea and colitis in patients with a perturbed gut microbiome. The most predominant risk factor for developing *C. difficile* infection (CDI) is taking antibiotics which disrupt the microbiome and allow for *C. difficile* colonization and infection to occur (Feuerstadt et al., 2023; Rossen et al., 2022). Individuals who develop CDI have higher mortality overall, increased healthcare costs, and 20 – 35% of patients experience recurrent disease following the initial infection (Feuerstadt et al., 2023). Because of the extreme health burden caused by CDI, *C. difficile* is considered an urgent threat to public health by the CDC (CDC, 2023, 2019).

The primary mode of transmission for *C. difficile* is via endospores which persist indefinitely in the environment and are the causative agent for CDI (Ali et al., 2011; Edwards et al., 2016). Once spores reach the large intestine, they encounter bile salts that help initiate *C. difficile* germination into vegetative cells that colonize the large intestine and produce toxins which cause CDI symptoms (Aguirre et al., 2021; Theriot et al., 2016; Wilson et al., 1982). Therefore, understanding *C. difficile* sporulation initiation and the metabolic mechanisms that allow *C. difficile* to survive in the colon is critical to developing future strategies to treat and prevent CDI. This thesis primarily covered the impacts of the *C. difficile* Kip orthologs on both sporulation and metabolism. We demonstrated that the *C. difficile* Kips, unlike the *B. subtilis* Kips, have no major role in sporulation initiation. However, the *C. difficile* Kips do enhance growth in 5-oxoproline through a currently unknown mechanism.

I. Exploring the impact of Kip proteins on *C. difficile* sporulation

Initially, we hypothesized that *C. difficile* KipI and KipA would regulate sporulation as seen in *B. subtilis* with KipI binding to a currently unidentified kinase or phosphatase that regulated sporulation, and KipA acting as an antagonist to KipI (Wang et al., 1997; **Chap. 2**). However, upon deleting *kipIA* and the full operon, *kipOTIA*, we saw no major changes in sporulation frequency. This demonstrated that at least on standard sporulation agar, the Kips do not play a major role in *C. difficile* sporulation (**Chap. 2, Fig. 1**). Since the Δ*kipIA* and Δ*kipOTIA* mutants had minimal impacts on sporulation frequency in our standard conditions, and since the *kip* operon in *B. subtilis* is regulated by a variety of nitrogen sources,

we considered that the Kips may regulate sporulation under specific conditions (Wang et al., 1997). We also had preliminary data showing that the secondary bile salt deoxycholate significantly induced expression of the *kip* operon, so we sought to identify which bile salts induced *kip* expression. We identified multiple bile salts responsible for inducing the *kip* operon and performed future experiments with the primary bile salt chenodeoxycholate (CDCA) since it induced expression the most (**Chap. 2, Fig. 2**). Even when examining *kip* mutant sporulation frequency in the presence of CDCA, we saw no change (**Chap. 2, Fig. S4**). This suggests that either the *kip* operon is not induced by CDCA on sporulation agar, or further confirms that the Kip proteins simply have no major role in regulating sporulation in response to CDCA.

Since the *B. subtilis* KipOIA proteins were previously shown to function as an 5-oxoprolinase, we also considered that the *C. difficile* Kips could regulate sporulation in response to 5-oxoproline (Niehaus et al., 2017). However, even after adding 5-oxoproline to the sporulation agar, wild-type *C. difficile* and the *kip* mutants sporulated as if they were on standard sporulation agar (**Chap. 2, Fig. S4**). This suggested to us that even if *kipOTIA* encoded a 5-oxoprolinase, this role was not important for regulating sporulation in the conditions we tested. Additionally, since wild-type sporulation did not change in the presence of 5-oxoproline, any derivatives from 5-oxoproline metabolism do not impact *C. difficile* sporulation in these conditions (**Chap. 2, Fig. S4**).

Although the Kip proteins do not play a major role in *C. difficile* sporulation initiation, the Δ*kipIA* mutant had slightly less sporulation than the wild-type in every condition for each experiment. This suggests that KipO and KipT expressed without KipI and KipA present may have a repressive effect on sporulation (**Chap. 2, Fig. 1, S4**). We hypothesized that the KipI and KipA proteins specifically regulated sporulation in *C. difficile* based on prior data in *B. subtilis* demonstrating KipI binding to the major sporulation kinase, Kinase A (KinA), to repress sporulation (Wang et al., 1997). However, *C. difficile*, does not encode a clear KinA ortholog and the other phosphotransfer proteins that function in sporulation regulation repress sporulation. Therefore, the *C. difficile* Kips may not be important for regulating sporulation since a KinA ortholog may not be present. For Clostridial organisms that encode KinA

orthologs, the Kips may be important as their KipI could bind these kinases to repress sporulation. Additionally, our sporulation agar may not present the best conditions to test for the specific effects of 5-oxoproline on sporulation since our sporulation medium is rich with many alternate nutrient sources to use in place of 5-oxoproline. It is also possible that KipI could still bind to another kinase or potentially a phosphatase to regulate something other than sporulation, so a co-immunoprecipitation experiment looking at what KipI binds to could confirm currently known KipI binding partners and identify new ones. Regardless, based on our data, the Kip proteins are not critical for regulating sporulation in *C. difficile*. These results led us to characterize the other potential function of the Kip proteins as a 5-oxoprolinase, as was observed in other organisms.

II. Exploring the 5-oxoprolinase function of Kip proteins

Since the Kips did not regulate sporulation, we sought to characterize their potential 5-oxoprolinase function as seen in other organisms (Niehaus et al., 2017; Seddon et al., 1984; Van Der Werf and Meister, 1974). To this end, we grew wild-type *C. difficile* and the Δ*kipIA* and Δ*kipOTIA* mutants in defined minimal media with and without glutamic acid or 5-oxoproline to determine if the Kips were necessary for growth in 5-oxoproline (**Chap. 2, Fig. 3; Chap. 2, Table S4**). We demonstrated that the Kip proteins were necessary for metabolizing 5-oxoproline (**Chap. 2, Fig. 3B, C, E)**, and that when the Kips were present, *C. difficile* exhibited enhanced growth in 5-oxoproline compared to the base media (**Chap. 2, Fig. 3A**). Surprisingly, wild-type *C. difficile* also grew better in the presence of 30 mM 5-oxoproline than in 30 mM glutamic acid, the usual product of 5-oxoproline metabolism (**Chap. 2, Fig. 3A**). We also observed that the Δ*kip* mutants had an intrinsic growth defect compared to wild-type, possibly due to changes in metabolism between the wild-type and Δ*kip* mutant strains (**Chap. 2, Fig. 3D**). These data support that differences in metabolism between the wild-type and the Δ*kip* mutants could account for this intrinsic growth defect. Overall, our data show that the *C. difficile* Kips are responsible for 5-oxoproline metabolism.

Since it takes energy in the form of ATP to metabolize 5-oxoproline to glutamic acid via the Kips in other organisms, we hypothesized that this enhanced growth could be due to 5-oxoproline being

metabolized into proline (Niehaus et al., 2017; Seddon et al., 1984). Structurally, proline is very similar to 5-oxoproline, and perhaps it takes less energy to metabolize 5-oxoproline to proline. Future experiments could analyze the metabolome of *C. difficile* to determine what compound 5-oxoproline is being changed into, if not glutamic acid, and if it enhances growth like 5-oxoproline. Additionally, the way 5-oxoproline is transported into the cell is currently unknown, although KipT has been previously annotated as a 5-oxoproline transporter. If KipT did transport 5-oxoproline, then we might expect to see significantly decreased growth in the Δ*kipIA* mutant compared to Δ*kipOTIA* due to 5-oxoproline accumulation, which we did not (**Chap. 2, Fig 3D**). The KipT protein has also been annotated as a metal cation transporter which makes it more likely that it is transporting a cation for 5-oxoprolinase function. In fact, the *Pseudomonas putida* 5-oxoprolinase requires the cations magnesium and potassium to function properly, which highly suggests KipT transports these cofactors (Seddon et al. 1984). It is possible that 5-oxoproline may be transported through an unknown transporter, and maybe *C. difficile* can more efficiently internalize 5-oxoproline compared to glutamic acid for use as a nutrient source. Regardless, the presence of 5-oxoproline supports robust growth of *C. difficile* through the 5-oxoprolinase function of the Kip proteins in our conditions. To our knowledge, this is the first time an anaerobic bacterium has been shown to have enhanced growth in the presence of 5-oxoproline.

The fact that 5-oxoproline promotes growth of *C. difficile* could be a mechanism for survival in the colon. If other bacteria are only capable of detoxifying 5-oxoproline and are unable to use it as a nutrient source, then the ability to use 5-oxoproline to promote growth could be advantageous. A future experiment could look at the effects of 5-oxoproline utilization *in vivo* by comparing how well wild-type or a Δ*kipOTIA* mutant colonizes in a murine model of infection. A similar experiment was performed with glutamic acid, showing that the ability for *C. difficile* to metabolize glutamic acid was important for *in vivo* infection in a hamster model (Girinathan et al., 2016). At 24 hours post-infection, 5-oxoproline was also associated with increased colonization and infection of *C. difficile* in a murine model, though the cause of increased 5-oxoproline is unknown (Fletcher et al., 2018). Perhaps the effects of *C. difficile*

toxins on the intestinal epithelium results in increased 5-oxoproline production in host cells, and during apoptosis this 5-oxoproline is released to support growth during infection.

Since we observed that 5-oxoproline improved growth of *C. difficile*, and since CDCA increased *kip* operon expression, we hypothesized that the addition of CDCA would further enhance growth. If CDCA induces expression of the *kip* operon and more KipOIA proteins are made, then growth might increase due to the increased presence of KipOIA proteins to metabolize 5-oxoproline. However, the addition of CDCA only slightly decreased growth of *C. difficile* with and without 5-oxoproline present. This suggests that in these conditions the *kip* operon may not be induced by the presence of CDCA, or that the increased expression of the *kip* operon has no effect on the rate of 5-oxoproline metabolism (**Chap. 2, Fig. 4**). Ultimately, through both sporulation and growth experiments, we were unable to discern a connection between bile salts and Kip function. Further experiments to determine how the *kip* operon is regulated would inform how the Kip proteins may respond to the colonic environment during infection.

Our growth data demonstrate that *C. difficile* is potentially using 5-oxoproline as a carbon and nitrogen source to grow better (**Chap. 2, Fig. 3A**). Previous data in *B. subtilis* show that the Kips are responsible for allowing 5-oxoproline to be used as a nitrogen source but did not discern whether *B. subtilis* could use 5-oxoproline as a carbon source (Niehaus et al. 2017). To determine if *B. subtilis* could use 5-oxoproline as a carbon source to enhance growth as observed in *C. difficile*, we grew wild-type *B. subtilis*, Δ*kipI*, and Δ*kipA* in modified Niehaus Defined Media (NDM) with and without sodium succinate and 5-oxoproline. The NDM was the original media used to characterize the 5-oxoprolinase functions of the *B. subtilis* Kip proteins, and we used sodium succinate instead of glucose as a carbon source to prevent the production of glutamic acid or 5-oxoproline (**Chap. 2, Table S5**) (Niehaus et al., 2017). All strains demonstrated poor growth in the presence of sodium succinate as the sole carbon source, and the Δ*kip* mutants grew significantly poorer than wild-type (**Chap. 2, Fig. 5A**). In the presence of 5-oxoproline with no sodium succinate, all strains grew poorly, and the Δ*kip* mutants had decreased cell density over time (**Chap. 2, Fig. 5B**). This suggested that the accumulation of 5-oxoproline was having a

detrimental effect on the cells that were unable to detoxify it. The addition of both 5-oxoproline and sodium succinate resulted in robust growth of the wild-type strain, but not the Δ*kip* mutants, confirming that the *B. subtilis* Kips metabolize 5-oxoproline as a nitrogen source, but not a carbon source (**Chap. 2, Fig. 5C**). Additionally, when glutamic acid was added as a nitrogen source with sodium succinate as a carbon source to the wild-type and *kip* mutants, growth of the *kip* mutants was restored to wild-type levels. These results suggest that 5-oxoproline is being metabolized into glutamic acid in *B. subtilis* to be used as a nitrogen source as previously shown (Niehaus et al., 2017).

III. Final Summary

The work performed in this thesis demonstrates a novel mechanism used by *C. difficile* to promote growth. Normally, accumulation of 5-oxoproline is detrimental to organisms across all domains of life, and many organisms encode 5-oxoprolinases to detoxify this compound. Our work shows that *C. difficile* KipOTIA proteins not only detoxify 5-oxoproline but allow its use as a nutrient source to enhance growth through an unknown mechanism. This function could be advantageous for *C. difficile* during infection by allowing the use of a nutrient source unavailable to other bacteria. In essence, another bacteria's trash is *C. difficile*'s nutritious treasure. Future work could focus on identifying the mechanism behind how the *C. difficile* Kips enhance growth in the presence of 5-oxoproline and how this function impacts *in vivo* infection. Additionally, we demonstrated that the *C. difficile* Kip orthologs do not inhibit sporulation like they do in *B. subtilis*. This function may be important in Clostridial organisms that encode activating kinases, but not for *C. difficile*.

Since spores are the primary mode of *C. difficile* transmission and the causative agent of infection, understanding how sporulation is initiated is critical to developing strategies to prevent transmission and infection. Additionally, understanding the metabolic processes that support growth of *C. difficile* would further inform treatment strategies to prevent infection. Our work has uncovered a metabolic process that supports robust growth of *C. difficile* in the presence of a toxic byproduct. Our data provides a foundation for further metabolic studies characterizing how *C. difficile* uses 5-oxoproline as an effective energy source.

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