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Genetic Characterization of the Anaerobic β-lactamase of *Clostridioides difficile*

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Abstract

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Clostridioides difficile is an intestinal bacterial pathogen that causes severe antibiotic-associated diarrhea and colitis. *C. difficile* infections are responsible for billions of dollars in healthcare expenses in the United States every year. *C. difficile* is an anaerobic bacterium that is highly resistant to β-lactams, the most commonly prescribed antibiotics. Treatment with β-lactam antibiotics causes microbiome dysbiosis, and the resistance of *C. difficile* to β-lactams allows the pathogen to replicate and cause disease in antibiotic-treated patients. However, the mechanisms of β-lactam resistance in *C. difficile* are not fully understood. We have shown that *C. difficile* produces a β-lactamase, which is a common β-lactam resistance mechanism found in other bacterial species. We have characterized the *C. difficile* operon encoding a lipoprotein of unknown function and a β-lactamase that was greatly induced in response to several classes of β-lactam antibiotics. An in-frame deletion of the operon abolished β-lactamase activity in *C. difficile* strain 630Δ*erm* and resulted in decreased resistance to the β-lactam ampicillin. We found that the activity of the β-lactamase, BlaD, is dependent upon the redox state of the enzyme. In addition, we observed that transport of BlaD out of the cytosol and to the cell surface is facilitated by an N-terminal signal sequence. Our data demonstrate that a co-transcribed lipoprotein, BlaX, aids in BlaD activity. Further, we identified a conserved BlaRI regulatory system and demonstrated that BlaRI controls transcription of the *blaXD* operon in response to β-lactams. These results provide support for the function of a β-lactamase in *C. difficile* antibiotic resistance, and reveal the unique roles of a co-regulated lipoprotein and reducing environment in *C. difficile* β-lactamase activity.

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Chapter 2

Chapter 1: Introduction

I. *Clostridioides difficile*

A. *Clostridioides difficile* poses a significant health risk

Clostrididoides difficile (previously *Clostridium difficile*) is a Gram-positive, obligate anaerobic, spore-forming bacterium, originally discovered in healthy infants, and named *Bacillus difficilis*, by Ivan C. Hall and Elizabeth O'Toole in 1935 (1, 2). *C. difficile* is a pathogen of numerous mammals, including humans (1, 3, 4). *C. difficile* infection, or CDI, causes a range of symptoms, from mild to severe diarrhea, to pseudomembranous colitis, and intestinal rupture. In the most severe cases, CDI causes death.

The Centers for Disease Control and Prevention (CDC) estimates that approximately 453,000 people suffer from a *C. difficile* infection per year in the United States, resulting in 83,000 recurrent infections. This results in 29,000 CDI-associated deaths, with 15,000 of those deaths directly caused by the *C. difficile* infection (5). These infections cost $4.8 billion per year in U.S. healthcare costs (6). Current treatment for CDI includes use of oral vancomycin or oral fidaxomicin for first occurrence or recurrence, and fecal microbiota transplantation for treatment of second recurrences (7). This limited range of treatment options is due to the high resistance *C. difficile* exhibits for a wide array of antibiotics (8-10).

B. Antibiotic-associated diarrhea and CDI

In 1893, J.M.T. Finney published the first report of pseudomembranous lesions in the intestine of a young woman who received gastric surgery, developed diarrhea, and died fifteen days post-operation (11). Another eight decades passed until pseudomembranous colitis and antibiotic-associated diarrhea were directly linked to *C.*

difficile infection (12-15). Although *C. difficile* was originally discovered in healthy infants in 1935, Hall and O'Toole and a subsequent scientist, Marshall Snyder, observed that cell-free supernatants of *C. difficile* cultures were highly lethal to guinea pigs and rabbits (1, 16). In 1943, a guinea pig model was used to test penicillin as a treatment for gas gangrene, an infection of *Clostridium perfringens* (17). Scientists found that the antibiotic was more toxic than the bacteria to the guinea pig, resulting in guinea pigs with large ceca filled with hemorrhagic fluid.

In the 1950s, more reports of pseudomembranous colitis were published, as it became a common complication of antibiotic use. The link between *C. difficile* and antibiotic-associated diarrhea started to solidify two decades later in the 1970s. In 1973, a paper reported that 10% of patients treated with clindamycin developed pseudomembranous colitis, which is life threatening (18). The stool from eight of these patients, tested five years later, were positive for *C. difficile* cells and *C. difficile* toxin. In 1974, scientists reported cytotoxic changes in tissue-cultured cells caused by inocula from intestinal contents from guinea pigs given penicillin (12). The same year, the first Ph.D. thesis on *C. difficile* reported that *C. difficile* was widespread in the environment, that it could be isolated from numerous animals' stools, and that most strains produced lethal toxin (13). In 1977, cytopathic toxin in stool from patients with pseudomembranous colitis was neutralized by *Clostridium sordelli* antitoxin (14, 19). In the 1980s, scientists reported the cause of the cytotoxicity of pseudomembranous colitis infections as toxins A and B from *C. difficile* (20-22). Around the same time, scientists reported that vancomycin had a protective effect on hamsters administered clindamycin (23-25). Almost one century after the first report of pseudomembranous colitis, the

antibiotics vancomycin and metronidazole were administered to patients to successfully treat CDI (25-27).

Scientists now understand that antibiotic treatment is one of the greatest risk factors for contracting CDI. Antibiotic treatment results in gastrointestinal dysbiosis, which eliminates important indigenous anaerobes, thus allowing for *C. difficile* population expansion (28, 29). Other risk factors for CDI include increased age (80% of cases are in people over the age of 65), exposure to healthcare settings, and the use of proton-pump inhibitors, (5, 30, 31).

C. *C. difficile* germination and pathogenesis

As a spore-fomer, *C. difficile* is easily transmissible between hosts through the oral-fecal route (32). Because of its existence as a spore outside of a host, *C. difficile* is difficult to kill in the environment, as the spores are resistant to heat, dryness, starvation, most disinfectants lacking bleach or peroxide, and to many antimicrobials (33, 34). *C. difficile* generally enters the mouth as a dormant spore, travels down the esophagus and enters the stomach, where the bacteria are exposed to gastric acid and digestive enzymes, presumably eliminating most vegetative cells. Because antimicrobials typically target metabolically active cells, *C. difficile* spores are able to survive in the gut even in the presence of antibiotics or the host's antimicrobial compounds (35, 36). The spores travel to the small intestine, and upon exposure to bile acids like taurocholate, *C. difficile* spores begin to degrade the spore cortex and rehydrate, resulting in the outgrowth of vegetative cells in the colon (33).

Production of the toxins TcdA and TcdB occurs in the colon and is induced by exposure to short-chain fatty acids like butyric acid, sub-inhibitory concentrations of antibiotics like ciprofloxacin and co-amoxiclav, and nutrient deprivation (37-41). TcdA

and TcdB, encoded in the Pathogenicity Locus (PaLoc) of *C. difficile*, enter the epithelium of the colon. Both TcdA and TcdB catalyze glucosylation of Rho-GTPases, thus inactivating them and causing disorganization of the cell cytoskeleton (42). This manifests as the disruption of tight junctions, fluid secretion, and epithelial cell death (43).

II. β-lactam antibiotics

A. β-lactams target the bacterial cell wall and lead to cell death

Since one of the greatest risks for CDI is antibiotic treatment, we focused our efforts on identifying the mechanism of resistance of *C. difficile* to the most commonly prescribed class of antibiotics – the β-lactams. The first antibiotic discovered in 1928, penicillin (specifically benzylpenicillin or penicillin G) became mass-produced in the US in the 1940s, around the same time that pseudomembranous colitis became a common complication of antibiotic use (44). Today, β-lactams comprise 62% of all prescribed antibiotics in the United States and are strongly associated with *C. difficile* infections (45-47). β-lactams are inhibitors of bacterial peptidoglycan cell wall synthesis and are characterized by a four-membered core lactam ring (48). β-lactams are further classified into four groups based on adjoining structures: the penicillins (attached thiazolidine ring), cephalosporins (attached six-membered ring), monobactams (single β-lactam ring), and carbapenems (attached five-membered ring) (49).

All β-lactam antibiotics bind and inhibit the activity of the penicillin-binding proteins (PBPs) of bacteria, which help complete the third and final step of cell wall synthesis (50-52). PBPs are membrane-bound enzymes that covalently modify the *N*-acetylglucosamine-*N*-acetylmuramic acid (GlcNAc-MurNAc) glycan chains of the

peptidoglycan cell wall (49). PBPs are divided into classes by molecular weight (53, 54). The low molecular weight PBP enzymes are D-ala-D-ala carboxypeptidases, which release the carboxy-terminal D-alanyl residues from peptidoglycan pentapeptides. High molecular weight PBPs are subdivided into class A or class B enzymes (55). Class A PBPs are bifunctional enzymes with a transpeptidase domain and a transglycosylase domain, and class B PBPs are transpeptidases. The transglycosylase activity links the alternating NAG and NAM sugars to each other, while the transpeptidase cross-links the peptide bridges together. Each species of bacteria has its own set of PBPs, which can range from three to eight enzymes (56). The structure of penicillin resembles the D-alanyl-D-alanine dipeptide of the peptidoglycan chains (50). Thus, the PBPs incorrectly bind β-lactams, which target the active site serine of PBPs and covalently modify them to form an acyl-enzyme, inactivating the peptidase activity (57). Thus, β-lactams target actively dividing cells that are actively generating cell wall cross-links and ultimately result in lysis of cells (58).

The common model for β-lactam-mediated cell death states that β-lactams cause cell wall damage and instability due to the imbalance of peptidoglycan synthases and hydrolases, which are required for cell growth (55, 59-62). Inhibition of various PBPs leads to different outcomes. For example, in *E. coli*, inhibition of PBP1 causes immediate lysis and cell death; inhibition of PBP2 causes cells to become spherical in shape and stop growing; and inhibition of PBP3 leads to production of long filamentous cells (63).

III. Mechanisms of β-lactam resistance

A. β-lactamases

The first discovery of β-lactam resistance was a 1940 report of a penicillin-killing enzyme isolated from *Escherichia coli* (then known as *Bacillus coli*) (64, 65). This enzyme is now known to be a β-lactamase encoded by the chromosomal *ampC* gene of *E. coli*, and is one of the most common antibiotic markers used in laboratories across the globe (65). β-lactamases are enzymes that hydrolyze the β-lactam ring of β-lactams, rendering them inactive against PBPs. More reports of β-lactamase enzymes in numerous other bacteria followed in the subsequent decades, and as of 2019, an NCBI search for β-lactamase proteins yields over 2,770 unique results (65).

Most of the characterized β-lactamases are produced by Gram-negative species. In these bacteria, the β-lactamase is produced constitutively and secreted into the periplasm – the space containing peptidoglycan that is sandwiched between the outer and inner membranes (66). The enzyme is thus concentrated in the periplasm, allowing for high levels of β-lactam resistance (66). β-lactam resistance in Gram-positive bacteria, however, is more commonly conferred by the production of PBPs with low-affinity for β-lactam binding (67). Still, β-lactamases do exist in Gram-positive bacteria, and the seminal biochemical and genetic regulation experiments performed on β-lactamases were actually from Gram-positive bacteria (68-78). Most of the β-lactamases produced by Gram-positive aerobic bacteria are inducible and secreted into the extracellular space (79). Although Gram-positive bacteria lack a periplasmic space for enzyme to concentrate (a thick peptidoglycan cell wall surrounds the membrane), some species do produce membrane-bound β-lactamases (73, 80-83). A few of these enzymes are proteolytically cleaved, producing an exoenzyme that can be released from the

membrane (75, 82, 84). Some β-lactamases in Gram-positive anaerobes are even found mostly secreted into the culture medium (79, 85).

The induction of many β-lactamases in Gram-positive bacteria must be regulated by other proteins. The system of regulation for β-lactamases is a two-component regulatory system defined by a membrane sensor (BlaR, MecR) and a response regulator (BlaI, MecI) (86). In *Bacillus licheniformis* and *Staphylococcus aureus*, the BlaRI system regulates β-lactamase encoding genes, and in *S. aureus*, the MecRI system regulates the MecA, or PBP2a encoding genes (78, 86-90). The membrane sensor binds a β-lactam and activates the protein's protease domain, which then cleaves the dimerized BlaI bound to a palindromic operator region within the β-lactamase gene promoter, derepressing the promoter and activating transcription (88, 91-93).

β-lactamase enzymes are classified into four groups: A, B, C, and D, based on molecular size and homology of their active-site motifs (94-96). Classes A, C, and D are serine hydrolases, while class B β-lactamases are metallohydrolases (97). More recently, the β-lactamase enzymes have been grouped by both molecular and functional characteristics (98). The classes are further divided by their known substrate profiles, their catalytic efficiences of particular substrates, and finally by their inhibitor profile (65). Metallo-beta-lactamases are considered the most dangerous of all β-lactamases because while they are rare, they are active against all classes of β-lactams, spread worldwide, and transferrable. Whereas β-lactamases of all classes have been discovered in Gram-negative bacteria, most Gram-positive β-lactamases belong to classes A or B (76). Class D β-lactamases were identified in Gram-positive bacteria within the last three years, including one that is highly conserved among *C. difficile* isolates (77, 99).

By the 1960s, resistance markers, called R factors, were reported to be transferrable between bacteria via conjugation (100-103). These provided resistance against streptomycin, tetracycline, chloramphenicol, and sulfonamides (104). In 1968, scientists reported the discovery of a β-lactamase on a transferrable plasmid, increasing the potential for this antibiotic resistance mechanism to spread to more bacterial species (104). Within the next 30 years, the number of unique β-lactamases discovered rose from less than 13 to over 200 (65). As discoveries of β-lactamases have increased, so has the clinical use of β-lactamase inhibitors. The first β-lactamase inhibitor, clavulanic acid, was discovered in 1976, as the result of a screen for molecules in *Streptomyces clavuligeris* culture broth that could inhibit β-lactamase activity of *Klebsiella aerogenes* NCTC 418 against penicillin (105). Clavulanic acid has a structure similar to β-lactams, and works by binding its β-lactam ring irreversibly to the active site of β-lactamases (106). By 1981, amoxicillin, with its high oral absorption and broad-spectrum activity, was administered orally with clavulanic acid as Augmentin (107).

The remaining β-lactamase inhibitors in clinical use in the US are the sulfone-inhibitors tazobactam and sulbactam. Tazobactam is used with piperacillin or ticarcillin (penicillins) and sulbactam is used with ampicillin (108, 109). These three inhibitors are active against class A β-lactamases. More recently, two new inhibitors have shown to act against some class A, C, and D β-lactamases: vaborbactam (used with meropenem, a monobactam), and avibactam (used with ceftazidime, a cephalosporin) (110, 111). Tebipenem is a carbapenem that targets PBPs, but also has activity against a class A β-lactamase (112, 113). Currently, tebipenem is only available in Japan. Finally, relebactam is administered with imipenem and is active against classes A and C β-lactamases, and is currently under FDA review (114).

Although β-lactamases were discovered around the same time as β-lactams, phylogenetic analysis suggests that serine β-lactamases have been around for two billion years, pre-dating antibiotic pressures (115). Because of the similar protein structure and ability to form acyl-enzyme complexes with β-lactams, it is commonly believed that β-lactamases evolved from PBPs (54, 116, 117). The PBP5 from *Pseudomonas aeruginosa* even has some β-lactamase activity (118). Interestingly, class D β-lactamases mainly exist in Gram-negative bacteria, with a few orthologs in Gram-positive bacteria, suggesting that an ancestral gene was transferred from a Gram-negative bacterium to a Gram-positive bacterium, approximately 575 to 520 million years ago (115).

B. **Low-affinity PBPs**

As previously stated, although Gram-positive bacteria produce β-lactamases, the production of PBPs with low affinity for β-lactam binding is a much more common mechanism of β-lactam resistance in these bacteria (and is also present in Gram-negative bacteria) (119-121). β-lactam producers, like *Actinobacteria*, use low-affinity PBPs to prevent self-killing (122). The most well defined example of an altered PBP is PBP2a of *S. aureus*, which also happens to produce a β-lactamase (123). PBP2a is encoded by *mecA*, and is found in methicillin-resistant *S. aureus*, or MRSA. *S. aureus* produces four native PBPs (PBP1-4), and has acquired PBP2a from an unknown bacterium (124). Structural and enzymatic analyses of this enzyme have demonstrated that although β-lactams can bind to this PBP, the dissociation constants are high, and the rate of acylation of the active site is slow (123, 125, 126). Since PBPs perform an essential role in cell wall formation, inhibiting these enzymes is not always a viable solution to eliminate a bacterial infection, making altered PBPs particularly powerful.

Furthermore, the potent combination of an altered PBP in addition to a β-lactamase allows a bacterium to resist killing by a broad range of β-lactams.

C. Reduced porin expression

A third mechanism of β-lactam resistance in bacteria is reduced porin expression (127). This is especially common in Gram-negative bacteria, in which hydrophilic β-lactams normally pass through the rich lipid bilayer by way of an outer membrane porin (128, 129). Reduced porin expression is generally a secondary resistance mechanism observed in bacteria that already produce a β-lactamase that contributes to the majority of the β-lactam resistance observed. For example, a *Klebsiella pneumoniae* isolate expresses the β-lactamase KPC-1, which provides the majority of its β-lactam resistance, but also lacks expression of two of the three porin proteins that it encodes (130). Reduced porin expression as a β-lactam resistance mechanism has only been observed in outer membrane porins, and thus, does not contribute to β-lactam resistance in Gram-positive bacteria.

D. Efflux pumps

A fourth mechanism of β-lactam resistance observed in bacteria is the expression of efflux pumps, which prevent the antibiotic from reaching the peptidoglycan cell wall (131, 132). This mechanism of resistance is more common amongst Gram-negative bacteria and has been well characterized in *Pseudomonas aeruginosa* and *E. coli* (133, 134). These pumps are generally not specific to β-lactams, but recognize other antimicrobials as well (135). In *P. aeruginosa*, evidence of β-lactamase activity, downregulation of porins, as well as overexpression of efflux pumps have all been reported (136).

IV. β-lactam resistance in *C. difficile*

A. *C. difficile* utilizes alternate peptidoglycan linkages

Peptidoglyan strands are usually cross-linked by D,D-transpeptidases (PBPs) using 4-3 cross-links (137). In *C. difficile*, however, the cell wall has a high degree (73%) of 3-3 cross-links, which are catalyzed by at least two L,D-transpeptidases (PBPs) (138). In the presence of ampicillin, the percentage of 3-3 cross-links increases to 87%, while the percentage of 4-3 cross-links decreases from 15% to 7%. This suggests that while ampicillin does not target the L,D-transpeptidases of *C. difficile*, 4-3 cross-linking is still important for cell wall synthesis. Because this shift toward more 3-3 cross-links was observed in an ampicillin concentration below the minimum inhibitory concentration, growth was not impaired, thus the presence of 4-3 cross-links suggests that *C. difficile* may enlist another β-lactam resistance mechanism in order to evade killing by ampicillin (138). Other bacteria, outside of mycobacteria, contain mainly or even entirely 4-3 cross-links, therefore, this characteristic is a rare mechanism of resistance against β-lactams (139-141).

B. *C. difficile* expresses altered PBPs

Until recently, *C. difficile* reportedly only carried four high-molecular weight PBPs. In 2018, an imipenem-resistant *C. difficile* strain of ribotype 017 (RT017) isolated from a Portugal hospital was found to have acquired a fifth high-molecular weight PBP, PBP5, encoded on a mobile element (142). This isolate also contains point mutations in the transpeptidase domains of PBP1 and PBP3, which are associated with imipenem resistance.

C. *C. difficile* produces a β-lactamase

A recent study demonstrated that a β-lactamase in *C. difficile* confers resistance to the penicillin, cephalosporin, and monobactam class of β-lactams (99). According to the substrate profile of this enzyme, this β-lactamase belongs to the 2de functional group of β-lactamases, meaning that it is grouped with other class D β-lactamases that hydrolyze penicillins, extended-spectrum cephalosporins, and monobactams (99, 143). The published work on the β-lactamase of *C. difficile* was released during the time of our own investigations into this enzyme, and contained conclusions about expression that contradicted our data (addressed in Chapter 2). While this publication added useful biochemical data of the β-lactamase to the scientific community, more work was to be done regarding the genetic organization and regulation of the gene, as well as the contribution to β-lactam resistance in *C. difficile*.

V. Specific Aims

As the most commonly prescribed antibiotics in the US, β-lactams and thus β-lactam resistance pose a significant health risk to the US population. Treatment with β-lactams creates a dysbiosis in the intestinal tract, which is strongly associated with CDI (144). This dysbiosis and a resistance to any residual antibiotic in the intestine allows for *C. difficile* colonization. *C. difficile* is demonstrably resistant to β-lactams in clinical isolates, yet the mechanism(s) of resistance to β-lactams has not yet been well defined (8). Further understanding of β-lactam resistance in *C. difficile* may expose approaches to prevent or treat β-lactam-associated CDI.

As the production of a β-lactamase is one of the most common mechanisms of β-lactam resistance in bacteria, we decided to investigate the ability of *C. difficile* to

produce and use a β-lactamase for β-lactam resistance. Initial experiments linked a gene, *CD0458*, to inducible β-lactamase activity in *C. difficile*. This gene was found downstream of a putative membrane protein, encoded by *CD0457*, which was also greatly induced in the presence of multiple β-lactams. We were interested in understanding what contribution these genes have toward β-lactam resistance in *C. difficile*, how these genes are transcriptionally organized and regulated, and what role the putative membrane protein has, if any, in the β-lactamase activity observed. The goal of my dissertation was to examine the contributions of the *C. difficile* β-lactamase to β-lactam resistance, and to understand how enzyme production is regulated. I investigated β-lactam resistance in *C. difficile* through the following specific aims:

1. Characterize the transcriptional organization and regulation of the gene, *CD0458,* which encodes the β-lactamase in *C. difficile*.
2. Investigate the localization of the β-lactamase and any contribution to β-lactamase activity by the lipoprotein, *CD0457*, in *C. difficile*.

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Chapter 2: Regulation and anaerobic function of the *Clostridioides difficile* β-lactamase

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S.M.M. wrote and edited the manuscript.

ABSTRACT

Clostridiidioides difficile causes severe antibiotic-associated diarrhea and colitis. *C. difficile* is an anaerobic, Gram-positive spore former that is highly resistant to β-lactams, the most commonly prescribed antibiotics. The resistance of *C. difficile* to β-lactam antibiotics allows the pathogen to replicate and cause disease in antibiotic-treated patients. However, the mechanisms of β-lactam resistance in *C. difficile* are not fully understood. Our data reinforce prior evidence that *C. difficile* produces a β-lactamase, which is a common β-lactam resistance mechanism found in other bacterial species. Herein we characterize the *C. difficile bla* operon that encodes a lipoprotein of unknown function and a β-lactamase that was greatly induced in response to several classes of β-lactam antibiotics. An in-frame deletion of the operon abolished β-lactamase activity in *C. difficile* strain 630Δ*erm* and resulted in decreased resistance to the β-lactam ampicillin. We found that the activity of this β-lactamase, BlaD, is dependent upon the redox state of the enzyme. In addition, we observed that transport of BlaD out of the cytosol and to the cell surface is facilitated by an N-terminal signal sequence. Our data demonstrate that a co-transcribed lipoprotein, BlaX, aids in BlaD activity. Further, we identified a conserved BlaRI regulatory system and demonstrated via insertional disruption that BlaRI controls transcription of the *blaXD* genes in response to β-lactams. These results provide support for the function of a β-lactamase in *C. difficile* antibiotic resistance, and reveal the unique roles of a co-regulated lipoprotein and reducing environment in *C. difficile* β-lactamase activity.

IMPORTANCE

Clostridioides difficile is an anaerobic, gastrointestinal human pathogen. One of the highest risk factors for contracting *C. difficile* infection is antibiotic treatment, which causes microbiome dysbiosis. *C. difficile* is resistant to β-lactam antibiotics, the most commonly prescribed class of antibiotics. *C. difficile* produces a recently discovered β-lactamase, which cleaves and inactivates numerous β-lactams. In this study, we report on the influence of atmospheric oxygen on β-lactamase activity, as well as the transcriptional regulation of the operon by a BlaRI system. In addition, our data demonstrate co-transcription of *blaD* with *blaX*, which encodes a membrane protein of previously unknown function. Furthermore, we provide evidence that BlaX enhances β-lactamase activity in a portion of *C. difficile* strains. This study demonstrates a novel association of a β-lactamase and a membrane protein in a Gram-positive pathogen, and due to the anaerobic nature of the β-lactamase activity, suggests that more β-lactamases are yet to be identified in other anaerobes.

INTRODUCTION

Clostridioides difficile, or *C. difficile*, is an anaerobic, Gram-positive, spore-forming bacterial pathogen that causes antibiotic-associated diarrhea (1-3). *C. difficile* infection, or CDI, can be severe, resulting in psuedomembranous colitis, intestinal rupture, and death. The Center for Disease Control (CDC) estimates that almost half a million people in the U.S. suffer from CDI per year, resulting in approximately 29,000 deaths per year (4). As a result, CDI cases add approximately $4.8 billion per year to U.S. healthcare costs (5). *C. difficile* was first linked to antibiotic-associated diarrhea in 1978, and antibiotic treatment is still one of the highest risk factors for CDI (2, 3). Antibiotic

treatment results in gastrointestinal dysbiosis, eliminating important indigenous anaerobes, thereby allowing for *C. difficile* population expansion (6, 7). Antibiotic treatment of CDI is limited to the use of vancomycin, fidaxomicin, or metronidazole, due to the high resistance *C. difficile* exhibits for a wide array of antibiotics (8-10).

The most commonly prescribed class of antibiotics are the β-lactams, which comprise 62% of all prescribed antibiotics in the United States and are strongly associated with *C. difficile* infections (11-13). β-lactams are inhibitors of bacterial cell wall synthesis and are characterized by a four-membered core lactam ring (14). β-lactams are further classified into four groups based on adjoining structures: the penicillins, cephalosporins, monobactams, and carbapenems (15). All β-lactam antibiotics bind to, and thus disable, cell-wall synthesizers called penicillin-binding proteins (PBPs) of bacteria (16, 17). Since the introduction of β-lactams into modern medicine, multiple mechanisms of resistance to these antibiotics have been discovered in a variety of bacterial species. β-lactam resistance mechanisms include the production of β-lactamases, which hydrolyze the β-lactam ring and render the antibiotic ineffective, mutations acquired in PBPs that prevent binding of the β-lactams, reduced outer membrane permeability due to reduced porin expression, and efflux pumps, which prevent the antibiotic from reaching the cell wall (18-23).

The most common mechanism of β-lactam resistance occurs through the production of β-lactamase enzymes. Most of the characterized β-lactamases have been identified in Gram-negative species; in these bacteria, the β-lactamase is generally secreted into the periplasm, where the enzyme is concentrated, allowing for high levels of β-lactam resistance (24). Less common are the outer membrane-anchored β-lactamases, which may be further packaged into outer membrane vesicles, enabling the

inactivation of nearby β-lactams (25-27). β-lactam resistance in Gram-positive bacteria, however, is more commonly conferred by the modification of the intended targets of the β-lactam, the penicillin-binding proteins (28). Still, β-lactamases do exist in Gram-positive bacteria (29-33). Although Gram-positive bacteria lack a periplasmic space, some species do produce membrane-bound β-lactamases (29, 34-37). A few of these enzymes are proteolytically cleaved, producing an exoenzyme that can be released from the membrane (31, 36, 38).

β-lactamase enzymes are classified into four classes: A, B, C, and D. Classes A, C, and D are serine hydrolases, while class B β-lactamases are metallohydrolases (18). Whereas β-lactamases of all classes have been discovered in Gram-negative bacteria, most Gram-positive β-lactamases belong to classes A or B (32). Class D β-lactamases were recently identified in Gram-positive bacteria, including one that is highly conserved among *C. difficile* isolates (33, 39). A recent study demonstrated that a β-lactamase in *C. difficile* confers resistance to the penicillin, cephalosporin, and monobactam class of β-lactams (39). According to the substrate profile of this enzyme, this β-lactamase belongs to the 2de functional group of β-lactamases (39, 40). The purpose of our study was to characterize the genetic organization, activity, and regulation of the *C. difficile* β-lactamase. To accomplish this, we deleted the genes encoding the β-lactamase and the upstream predicted membrane protein in *C. difficile*, and examined the resulting resistance profiles, biochemical activity, and regulation of this operon. Notably, we observed that the *C. difficile* β-lactamase is inactivated by oxygen, which has not been described for other class D β-lactamases. We also examined how this β-lactamase enzyme is transported, and detail its mechanism of regulation. We demonstrate that unlike other described β-lactamases, the *C. difficile* β-lactamase is co-

transcribed with a membrane protein that facilitates β-lactamase processing and function. These results further our understanding of β-lactam resistance in *C. difficile*, which may expose approaches to prevent or treat β-lactam-associated CDI.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in **Table 1**. *Escherichia coli* was grown at 37°C in LB medium with 100 μg/mL ampicillin (Sigma-Aldrich) and 20 μg/mL chloramphenicol (Sigma-Aldrich) when necessary (41). *C. difficile* was grown anaerobically at 37°C as previously described (42) in brain heart infusion medium supplemented with 2% yeast extract (BHIS; Becton Dickinson Company) or Mueller Hinton Broth (MHB; Difco) with 2 μg/mL thiamphenicol (Sigma-Aldrich), 3.125 – 60 μg/mL cefoperazone (Sigma-Aldrich), 0.25 – 2 μg/mL ampicillin, 0.125 – 1.5 μg/mL imipenem (US Pharmacopeia), 0.75 μg/mL vancomycin (Sigma-Aldrich), 75 μg/mL polymyxin B (Sigma-Aldrich), 1 mg/mL lysozyme (Fisher Scientific), 7.5 μg/mL nisin (MP Biomedicals), 2 μg/mL LL-37 (Anaspec), or 250 μg/mL kanamycin (Sigma-Aldrich) when specified.

Strain and plasmid construction

The oligonucleotide primers used in this study are listed in **Table 2**. Primer design and the template for PCR reactions were based on *C. difficile* strain 630 (GenBank accession NC_009089.1), except for pMC896, which was based on strain M120 (GenBank accession FN665653.1). Accession numbers for BlaD in different *C. difficile* strains are as follows: YP_001086931 (630), CBE02158 (R20291), WP_003417462 (M120), and

WP_009901927 (VPI 10463 / ATCC 43255).

The *blaX*::*erm* and *blaI*::*erm* mutant strains were created by retargeting the Group II intron from pCE240 with the primers listed in **Table 2**, as previously described (43). To generate insertional disruptions, transconjugants were selected on 5 μg/mL erythromycin (Sigma-Aldrich), and 50 μg/mL kanamycin (Sigma-Aldrich) to select against *E. coli*.

The Δ*blaXD* mutant strain was created using a pseudo-suicide plasmid technique, as described previously, with slight variation (44). Briefly, 500 bp regions homologous to the 5' and 3' ends of the *bla* operon were amplified and Gibson assembled into the *Pme*I site of plasmid pMTLSC7215 to create plasmid pMC822. The plasmid was purified using a miniprep kit (Zymo Research), transformed into *E. coli* strain HB101 pRK24, and introduced into *C. difficile* by conjugation. *C. difficile* harboring the plasmid were selected on BHIS agar containing 15 μg/mL thiamphenicol, streaked onto BHIS agar, and subsequently on BHIS agar with 15 μg/mL thiamphenicol and 100 μg/mL kanamycin to force plasmid integration and counterselect against *E. coli*. A clone that screened positive for two crossover events was streaked to purity on BHIS agar for three more passages and the loss of plasmid was confirmed via sensitivity to 5 μg/mL thiamphenicol on BHIS agar.

Detailed construction of plasmids can be found in **Figure S1**. Plasmids were transferred to *C. difficile* as previously described, with slight variation (45, 46). Briefly, plasmids were chemically transformed into *E. coli* strain HB101 pRK24 and mated with *C. difficile* on agar plates for 48 h. Transconjugants were selected on BHIS agar containing 10 μg/mL thiamphenicol for plasmid selection and 100 μg/mL kanamycin to counterselect against *E. coli*.

Nitrocefin hydrolysis disk assays

β-lactamase activity was assessed by hydrolysis of nitrocefin, a chromogenic cephalosporin (Sigma-Aldrich). Briefly, *C. difficile* was grown overnight in BHIS to log phase, then diluted to an OD_{600} of 0.05 in BHIS medium with or without 2 μg/mL ampicillin. Cultures were grown to an OD_{600} of 0.45-0.55, and 1 mL of culture was collected and centrifuged for 5 minutes at 21,130 rcf. For wild-type anaerobic vs. aerobic assays, pellets were resuspended in 1 mL non-reduced or reduced BHIS and incubated at 37°C for 15 min, either aerobically or anaerobically. Cells were then centrifuged for 5 minutes at 21,130 rcf. For all disk assays, all but approximately 30 μL of the supernatant was decanted, the pellets were resuspended, and the cells were spotted onto a nitrocefin disk. The disks were incubated aerobically or anaerobically for 20 min – 2 h at 37°C, as noted.

Nitrocefin liquid hydrolysis assays

β-lactamase activity was determined for wild-type or complemented strains via anaerobic liquid nitrocefin assays, as previously reported, with some modifications (47). Briefly, *C. difficile* was grown overnight in BHIS +/- 2 μg/mL thiamphenicol, as noted, to log phase, then diluted to an OD_{600} of 0.05 in BHIS medium +/- 2 μg/mL thiamphenicol and +/- 2 μg/mL ampicillin, as noted. Cultures were grown to an OD_{600} of 0.45 – 0.55, 1 mL of culture was collected (in duplicate), and cells centrifuged for 5 min at 21,130 rcf. For wild-type strains, pelleted cells were frozen at -20°C until use. Pellets were resuspended either anaerobically or aerobically in BHIS medium and samples were incubated anaerobically or aerobically at 37°C for 15 minutes. Nitrocefin was added at a final concentration of 50 μM to bring the sample volume to 1 mL and

samples were incubated anaerobically or aerobically at 37°C for up to 15 minutes. For whole cell reactions in complemented strains, supernatant was transferred to a fresh tube, and nitrocefin (BioVision) was added to supernatant or whole cell suspensions at a final concentration of 50 μM. For lysed cell reactions, pelleted cells were frozen at -20°C until use. Pellets were resuspended in lysis buffer (100 mM sodium phosphate + 50 mM sodium bicarbonate, pH 7.4), and DTT (Fisher Scientific) was added to each sample for a final concentration of 0.2 mM. Lysed samples were subjected to six freeze-thaw cycles (2 min in dry ice/Ethanol bath, 3 min at 37°C). 0.2 mL of the lysate was transferred to a fresh tube (designated 'lysate'). The remaining volumes of samples were pelleted by centrifugation for 30 min at 21,130 rcf at 4°C, and then filtered via 0.22 μM syringe filters (BD Biosciences). 0.2 mL of this solution (designated 'lysate filtrate') was transferred to a fresh tube. Equal volumes of lysis buffer were added to each sample. Nitrocefin was added at a final concentration of 50 μM to bring the sample volume to 1 mL and samples were incubated anaerobically at 37°C for up to 7 minutes. All reactions were quenched by adding 100 μL of 1 M NaCl and immediately placed on ice. Samples were centrifuged for 5 min at 21,130 rcf to clear cell debris. The entire assay was performed anaerobically until this point, unless previously noted. 300 μL of each supernatant was applied to a 96-well flat-bottom plate, and the OD_{490} was recorded with a BioTek microplate reader. β-lactamase units were calculated by the following equation: $(OD_{490} * 1000) / (OD_{600} * \text{time in min} * \text{vol of cells in mL})$, where OD_{600} is the value at the time of collection and the time is the number of minutes between the addition of nitrocefin and adding 1 M NaCl. Lysate results were normalized to the amount of lysate supernatant used. Time course experiments were run to confirm the

linearity of the reaction. Results reported are the mean of at least three independent experiments.

Minimal Inhibitory Concentration determination (MIC)

β-lactam susceptibility of *C. difficile* was determined as described previously (48). Briefly, active *C. difficile* cultures were diluted in Mueller Hinton Broth (MHB; BD Difco) to an OD_{600} of 0.1, which were grown to an OD_{600} of 0.45, and further diluted 1:10 in MHB. 15 µL of this diluted culture (~$5x10^5$ CFU/mL) was plated in a pre-reduced 96-well round bottom polystyrene plate that contained 135 µL of MHB with appropriate β-lactams in each well. The MIC was determined as the concentration at which there was no visible growth after 24 hours of anaerobic incubation at 37°C.

Alkaline phosphatase activity assays

Alkaline phosphatase activity assays in *C. difficile* were performed as described previously, with minor modifications to the original published assay (49, 50). Briefly, *C. difficile* cultures were grown anaerobically at 37 °C overnight in BHIS with thiamphenicol (2 µg/mL) to log phase, then diluted to an OD_{600} of 0.05 in 10 mL BHIS with thiamphenicol. 1 mL of cells was collected in duplicate when the OD_{600} reached 0.5. Cells were centrifuged at 21,130 rcf for 3 min and the pellets were stored in -20°C at least overnight. For the assay, cell pellets were thawed and resuspended in 500 µL of cold wash buffer (10 mM Tris pH 8.0 + 10 mM $MgSO_4$) and pelleted for 3 min at 21,130 rcf. Alkaline phosphatase assays were performed as previously described (50) without the addition of chloroform (51). The OD_{550} (cell debris) and OD_{420} (pNP cleavage) were measured in a BioTek microplate reader. Values were averaged between the triplicate

wells, and then between duplicate technical samples. AP units were calculated as $((OD_{420} – (1.75* OD_{550})) * 1000) / (OD_{600} * time)$, where OD_{600} is the value at the time of collection. Results reported are the average between three independent experiments.

Quantitative reverse transcription PCR analysis (qRT-PCR)

Actively growing *C. difficile* were diluted to an OD_{600} of 0.02 in 10 – 25 mL BHIS with appropriate antibiotic and grown to log phase. RNA was isolated as described previously (45, 52). Briefly, 3 mL samples were taken at an OD_{600} of 0.45 – 0.55, mixed with 3 mL ice-cold 1:1 acetone:ethanol, and stored immediately in -80°C. RNA was isolated (Qiagen RNeasy kit), treated for contaminating DNA (Invitrogen TURBO DNA-free kit), and RNA was reverse-transcribed into cDNA (Bioline Tetro cDNA synthesis kit). cDNA samples were used for qPCR (Bioline SensiFAST SYBR and Flourescein kit) in technical triplicates on a Roche Lightcycler 96 as described previously (53). Results are presented as the means and standard errors of the means for three biological replicates. Statistical significance was determined using a one-way ANOVA, followed by Dunnett's multiple-comparison test (GraphPad Prism v6.0).

RESULTS

***C. difficile* produces an inducible, anaerobic β-lactamase.**

C. difficile was recently reported to produce a β-lactamase that can cleave β-lactam antibiotics (39). We further investigated the regulation and potential inducibility of *C. difficile* β-lactamase activity and examined the environmental conditions required for its function. Four diverse strains of *C. difficile*, 630Δ*erm* (ribotype 012), R20291 (ribotype 027), M120 (ribotype 078), and VPI 10463 (ribotype 003), were grown in the

presence or absence of ampicillin, a penicillin, pelleted, resuspended in ~30 μL of spent media, and incubated either in an anaerobic chamber at 37°C or in an aerobic incubator at 37°C for 15 minutes. After incubation, cells were applied to a membrane disk impregnated with nitrocefin, a chromogenic cephalosporin, and incubated for another 20 minutes either anaerobically or aerobically at 37°C. As shown in **Figure 1A**, under anaerobic conditions, all four strains of *C. difficile* grown in the presence of ampicillin caused a color change from yellow to red, indicating cleavage of nitrocefin. In the absence of ampicillin, none of the strains demonstrated observable nitrocefin cleavage. These results suggested that *C. difficile* produces a β-lactamase that is inducible by β-lactams and is present in diverse strains. During optimization of these assays, we observed markedly higher β-lactamase activity under anaerobic conditions, suggesting that this activity was impaired by oxygen. Indeed, as indicated by **Figure 1A**, when the nitrocefin assay was performed in the presence of oxygen, the disks did not change color, even under induction by ampicillin, indicating a loss of β-lactamase activity. Quantification of the β-lactamase activities is shown in **Figure 1B**. All four *C. difficile* strains exhibited significantly increased β-lactamase activity in the absence of oxygen. These results demonstrate that *C. difficile* strains produce an inducible β-lactamase, and that the activity of this enzyme is quenched by oxygen.

blaD (CD0458)* is the only β-lactam-induced β-lactamase gene in *C. difficile

Based on the observed induction of β-lactamase activity, we hypothesized that the expression of one or more putative β-lactamases would be induced upon exposure to β-lactams. To test this, *C. difficile* strain 630Δ*erm* was grown in the presence of three classes of β-lactams: cefoperazone (a cephalosporin), ampicillin (a penicillin), and

imipenem (a carbapenem). Using qRT-PCR, we measured the gene expression for 17 putative β-lactamases identified in the *C. difficile* genome (8, 54, 55). **Figure S2** demonstrates that the expression of one of these genes, *CD0458*, was robustly induced upon exposure to each of the three types of β-lactams. None of the other putative β-lactamase genes were induced by any of the β-lactam classes tested (**Figure S2**). Expression of the homologous gene in *C. difficile* strain R20291 was also greatly induced by these three β-lactams (CDR20291_0399, 99% identity; **Figure S2**).

CD0458 is analogous to the loci described recently by Toth *et al.* as CDD-2 (630 genome) and CDD-1 (ATCC 43255 genome) (39). However, other genes are already annotated as *cdd, cdd2, cdd3,* and *cdd4* in *C. difficile* (56, 57). In addition, these β-lactamases share high sequence similarity (≥94% identity), with the greatest variability in a putative N-terminal signal sequence, suggesting that they are gene alleles, rather than distinct genes. To avoid confusion with the previously established *cdd* loci and to adhere to the guidelines on genotypic designation of operons, the locus was renamed *bla* and the β-lactamase *blaD* by the NCBI, in accordance with its function as a class D β-lactamase (58).

CD0457 encodes a putative membrane protein, BlaX, which is co-transcribed with *blaD*

Analysis of the region surrounding *blaD* revealed the presence of another gene, *CD0457*, which appeared to be part of an operon with *blaD*. **Figure 2A** illustrates the putative *bla* operon, in which *CD0457* is located 27 nucleotides upstream of the start codon of *CD0458***.** To determine if expression of *CD0457* is similarly induced upon β-lactam exposure, we measured transcription of *CD0457* in *C. difficile* strain 630Δ*erm*

upon exposure to cefoperazone, ampicillin, and imipenem. **Figure 2B** demonstrates that expression of *CD0457* is comparably induced upon exposure to all three β-lactams. This co-regulation by β-lactams strongly suggested that *CD0457* is co-transcribed with *CD0458* and that the CD0457 predicted membrane protein product could play a role in the β-lactam resistance. The expression of the homologous gene in *C. difficile* strain R20291 was also comparably induced upon exposure to these β-lactams, indicating a similar organization in divergent strains (**Figure S3**).

To determine if the *CD0457* and *blaD* genes are part of a single cistronic unit, we assessed the linkage of these transcripts by amplifying the region between *CD0457* and *blaD* from cDNA generated after exposure of *C. difficile* strains 630Δ*erm* and R20291 to ampicillin (**Figure S4A**). **Figure S4B** illustrates the results of the PCR from cDNA that generated a product of 1 kb, which matches the genomic DNA product from the same strain. These data demonstrate that the transcription of *CD0457* and *blaD* are linked, indicating that they comprise a monocistronic unit. Since *CD0457* and *blaD* form an operon and the function of CD0457 is unknown, we named the *CD0457* gene *blaX*.

To further define the transcriptional organization of the *bla* operon, we examined promoter activity within the *bla* locus. Potential promoter activity was measured for putative promoter regions within the locus using *phoZ* reporter fusions, which produce alkaline phosphatase (50). As illustrated in **Figure 3**, regions of 300 nucleotides directly upstream of the start codons of *blaX* or *blaD* were fused to *phoZ* and expressed in *C. difficile*. The results of these reporter assays indicate that the region 300 nucleotides upstream of *blaX*, but not the region 300 nucleotides upstream of *blaD*, is able to promote transcription, resulting in measurable activity. To confirm the absence

of a cryptic *blaD* promoter located within the *blaX* coding region, the entire region from the translational start of *blaX* to the start codon of *blaD* was also examined for possible promoter activity. However, no transcriptional activity was observed from this region (**Figure 3**). The only segment that produced significant and inducible activity contained the region upstream of the *blaX* coding sequence, strongly suggesting that solely this region drives *blaX* and *blaD* expression.

BlaX and BlaD contribute to ampicillin resistance in *C. difficile*

Notably, 36% of complete *C. difficile* genomes contain a homolog of *blaX*. Other sequenced genomes simply contain the same promoter and *blaD* region without the membrane protein. The membrane protein only shares approximately 23-40% amino acid identity to uncharacterized proteins found in a handful of other bacterial species. Thus, the function of this membrane protein cannot be inferred from other systems. To define the roles of BlaX and BlaD in β-lactam resistance and in β-lactamase activity, we created mutants of the 630Δ*erm* strain with an insertional mutation in the *blaX* gene (MC905) or complete deletion of the *blaX-blaD* locus (MC1327). Compared to the parent strain, *blaX*::*erm* displayed decreased, but still inducible *blaD* expression (**Figure S5**). Although *blaX* transcription is measurable in the *blaX*::*erm* mutant, the product is presumably non-functional because of the insertional mutation. We confirmed that neither the *blaX* nor the *blaD* transcript was expressed in the Δ*blaXD* mutant (**Figure S5**).

Based on the induction of β-lactamase activity and the induction of the *bla* operon by β-lactams, we hypothesized that both genes contribute to *C. difficile* resistance to β-lactams. As shown in **Figure 4**, we performed growth curves with the

Δ*blaXD* and *blaX::erm* strains in cefoperazone, ampicillin, and imipenem to measure the contribution of the *bla* operon to β-lactam resistance in *C. difficile*. While the deletion of *blaX* and *blaD* did not significantly affect growth in cefoperazone, Δ*blaXD* and *blaX*::*erm* growth was impaired in ampicillin compared to the parent strain. These data suggest that the *bla* operon contributes to ampicillin resistance in *C. difficile*. Interestingly, the deletion of *blaX* and *blaD* improved growth in imipenem, supporting the finding by Toth *et al*. that BlaD binds to, but does not hydrolyze imipenem (39).

To further define the contribution of *blaX* and *blaD* to β-lactam resistance in *C. difficile*, we measured the MIC of β-lactams in 630Δ*erm*, Δ*blaXD*, and *blaX*::*erm*. Although the parent strain grew better in ampicillin, the MICs for both cefoperazone and ampicillin were similar in all three strains (**Table S1**), and higher for 630Δ*erm* in imipenem, indicating a modest difference in resistance values.

The *bla* operon exhibits high level, dose-dependent expression in β-lactams

The induction of both *blaX* and *blaD* by β-lactams suggested that these genes are important for β-lactam resistance in *C. difficile*. To determine whether these genes could be induced by other cell wall targeting antimicrobials or if the induction is specific to β-lactam exposure, we measured the levels of gene expression for *C. difficile* strain 630Δ*erm* in various cell wall targeting antibiotics (vancomycin, polymyxin B, and lysozyme) and cationic antimicrobial peptides (nisin and LL-37), as well as a ribosome-targeting antibiotic (kanamycin). The concentrations of each of these antimicrobials was at sub-MIC value and sufficient for robust induction of resistance gene expression based on previous work (59-62). **Figure 5** shows that expression of *blaX* and *blaD* were induced in the presence of vancomycin and polymyxin B. However, these levels of

expression are not statistically significant and were less than 3% of the levels seen for expression after β-lactam exposure, suggesting that the high levels of induction of *blaX* and *blaD* are specific to β-lactams.

Although the levels of *blaX* and *blaD* induction were high in all three β-lactams, expression varied greatly between each β-lactam. These results suggested that the level of induction of the *bla* operon is dependent upon the type of β-lactam *C. difficile* is exposed to and could be dose-dependent. To determine if the *bla* operon exhibits dose-dependent expression in β-lactams, we measured the relative expression of *blaX* and *blaD* in the 630Δ*erm* strain in varying concentrations of cefoperazone, ampicillin, and imipenem. **Figure S6** shows that the *bla* operon did indeed exhibit dose-dependent induction by β-lactams and that the response was different for the various classes of β-lactams. In increased concentrations of cefoperazone, induction of the *bla* operon trended downward, whereas expression trended upward in increased concentrations of ampicillin. Expression of the *bla* operon was high in all concentrations of imipenem, exhibiting only a modest increase in expression as the concentration of imipenem was increased. Furthermore, the level of induction of the *bla* operon was high even at concentrations of β-lactams far below the MIC (0.03125x MIC of cefoperazone, 0.125x of ampicillin, and 0.0625x MIC of imipenem). These results suggest that *bla* expression is controlled in a dose-dependent manner specific to the class of β-lactam administered.

BlaX is not required for β-lactamase activity

Of the 1747 amino acid sequence variants retrieved from a 630Δ*erm* BlaD BLASTp search of *C. difficile* (>90% coverage and >80% identity), 736 isolates (42%) also encode the upstream putative membrane protein (>97% coverage and >86%

identity), suggesting that the membrane protein BlaX may be important for β-lactamase activity in some strains, but not in others. The BlaD enzyme from strains M120 and VPI 10463, which lack BlaX, and strains 630Δ*erm* and R20291 are highly similar, but the 4% variability clearly lies within the N-termini of these proteins (**Figure S7**). As shown in **Figure 1A**, all four of these strains exhibit β-lactamase activity. The variability in the amino acid sequence of these enzymes may be due to differences in signal sequence recognition, but a potential interaction with another protein cannot be ruled out.

As the function of BlaX was not immediately apparent, we examined whether BlaX is necessary to observe the β-lactamase activity of BlaD in strain 630Δ*erm*. To test this, we complemented the Δ*blaXD* strain with *blaX* and/or *blaD* in trans. As expected, no apparent β-lactamase activity was observed for the Δ*blaXD* strain (**Figure 6A**). In comparison, the *blaX*::*erm* strain exhibits a slight change in color to a light pink, indicating that this mutant does not fully abolish production and activity of the β-lactamase, which is in agreement with the decrease in *blaD* gene expression observed for this strain (**Figure S5**). The nitrocefin disk assays in **Figure 6B** demonstrate that expression of *blaD* alone can restore β-lactamase activity in the Δ*blaXD* mutant, indicating that BlaD can act independently of BlaX, despite the co-transcription of these two genes. This result is further supported by the observation that the *blaX*::*erm* strain exhibits some β-lactamase activity (**Figure 6A**).

BlaD contains a predicted signal sequence and is associated with the cell membrane

A common characteristic of β-lactamases is an N-terminal signal sequence that directs the protein out of the cytoplasm. We hypothesized that the N-terminus of BlaD encodes

a signal sequence based on the signal sequence prediction within the first 18 amino acid residues (63, 64). We generated a truncated version of BlaD missing these first 18 residues (BlaDΔ18; p*blaD*Δ18). As shown in **Figure 6B**, the expression of BlaDΔ18 is unable to complement the absence of β-lactamase activity in the Δ*blaXD* mutant in a whole cell assay. qRT-PCR results shown in **Figure S8** confirm that *blaX* and/or *blaD* are expressed in the complemented strains, indicating that the absence of gene expression is not the cause of the lack of observable β-lactamase activity. This suggested that BlaDΔ18 is either not translated, is an unstable or inactive protein, or is active but trapped in the cytosol and unable to hydrolyze nitrocefin.

All of the characterized β-lactamases in Gram-positive bacteria are membrane-bound enzymes, although many of these proteins are cleaved, resulting in a smaller, soluble form that can be found in culture supernatants (29, 31, 34, 36). These findings are consistent with the lack of a periplasmic space for β-lactamases accumulation in Gram-positive bacteria. To determine if a soluble form of BlaD is secreted into the culture medium, we performed a nitrocefin hydrolysis assay using culture supernatants. As shown in **Figure 7A and 7C**, neither the supernatants of Δ*blaXD* cells harboring p*blaD* or p*blaX-blaD*, nor the wild-type strains 630Δ*erm* or M120, react with nitrocefin, indicating that BlaD is not secreted into the medium. To confirm that BlaD is a membrane-associated enzyme, we lysed the cells and performed a nitrocefin hydrolysis assay using lysates containing cell debris (denoted as 'lysates') or the cleared cell lysates (denoted as 'lysate filtrate'). **Figures 7B and 7D** show that when comparing the level of activity in the lysate to the lysate filtrate in strains containing a full-length *blaD*, 74-80% of the total β-lactamase activity is found in the cell debris, indicating that BlaD is associated with the cell surface. Furthermore, BlaDΔ18 activity is not associated with

the cell surface, as demonstrated by the similar levels of activity in the lysate and the lysate filtrate (**Figure 7B**). This result indicates that BlaDΔ18 is an active, soluble form of BlaD that is trapped in the cytosol, and strongly suggests that the first 18 residues at the N-terminus of BlaD encode a signal sequence. Together, these results support the presence of a signal sequence that helps bring the protein to the cell surface.

BlaX aids in BlaD activity

Although BlaX is not necessary for BlaD activity (**Figure 6A, B**), *blaX* is conserved in many *C. difficile* strains. Thus, we examined whether BlaX enhances BlaD activity. The results shown in **Figure 7A and 7B** demonstrate that the presence of BlaX increases β-lactamase activity of the 630Δ*erm* BlaD two to three-fold, suggesting that BlaX plays a role in the function of BlaD. To investigate the activity of a BlaD from a *C. difficile* genome that lacks BlaX, we also complemented the Δ*blaXD* strain with *blaD* cloned from the M120 genome, under the M120 native promoter. **Figure 7A** shows that in cell suspensions of Δ*blaXD* complemented strains, the M120 BlaD (pM120*blaD*) exhibits two-fold higher activity than the 630Δ*erm* BlaD (p*blaD*). This result suggests that the M120 BlaD is superior to the 630Δ*erm* BlaD at translocating to the cell surface when BlaX is not present. However, M120 BlaD is only two-thirds as active as the 630Δ*erm* BlaXD complement (p*blaXD*). In lysed cells, the M120 BlaD β-lactamase activity levels are slightly higher than the 630Δ*erm* BlaD (**Figure 7B**). Interestingly, the wild-type strains 630Δ*erm* and M120 exhibit similar β-lactamase activity levels in both cell suspension and lysate samples, indicating that their overall efficacy is comparable (**Figure 7C and D**). Together, these results demonstrate that in 630Δ*erm*, BlaX enhances BlaD activity, while in M120, β-lactamase activity is not dependent on BlaX.

Finally, because the M120 BlaD does not fully complement the Δ*blaXD* strain, the N-terminal sequence variability of the BlaD proteins likely plays a role in strain-dependent translocation of BlaD to the cell surface.

The *bla* operon is regulated by BlaIR

Transcription of most β-lactamase genes in Gram-positive bacteria is regulated by the two-component BlaRI system (65-67). The *C. difficile* genome encodes several orthologs of the two genes that make up this system, *blaI* and *blaR*. In other bacteria, BlaR is a sensor that is activated upon β-lactam binding (68). Activated BlaR cleaves the BlaI repressor, which is bound as a dimer to the *bla* operon promoter in the absence of β-lactams (69-71). Once cleaved, BlaI can no longer bind to the *bla* promoter, thus allowing for active transcription. Two candidate orthologs *CD0471* (*blaI*) and CD0470 (*blaR*) are located 11 kb downstream of the *blaXD* operon. To determine if these *blaIR* orthologs regulate the *blaXD* operon in *C. difficile*, we created an insertional disruption in *blaI*. **Figure S9** shows that transcription of *blaR* is decreased in the *blaI*::*erm* mutant, confirming that *blaI* and *blaR* are organized in an operon, as is consistent with other bacteria. As seen in **Figure S9B**, transcription of *blaI* is high, even in the absence of β-lactams, which demonstrates that BlaI regulates itself, as the primers used were upstream of the disruptional insertion. As shown in **Figure 8**, in the absence of β-lactams, *blaX* and *blaD* are transcribed at high levels in the *blaI*::*erm* mutant, as compared to the wild-type 630Δ*erm* strain. These results confirm that BlaI acts as a repressor of the *bla* operon. Further, the induction of *blaXD* in β-lactams in the wild-type strain, but not in the mutant, strongly suggests that BlaI repression is relieved by the presence of β-lactams in wild-type strain. To verify that relief of BlaI repression

results in β-lactamase production, we performed a nitrocefin hydrolysis assay on the *blaI*::*erm* mutant. **Figure 6C** confirms that the absence of BlaI results in active β-lactamase, independent of β-lactam presence. Together, these results show that *C. difficile* encodes a BlaRI system that represses *bla* transcription in the absence of β-lactams. Efforts to complement *blaIR* resulted in poor growth of *E. coli* mating strains, as well as *C. difficile*, and were not successful, however two independent strains of *blaI*::erm were successfully created, as shown in **Figure 6C**.

To further confirm that the BlaRI system regulates the *bla* operon and to define its contribution to ampicillin resistance, we examined the growth of the *blaI*::*erm* mutant in multiple β-lactams. **Figure 9A** illustrates that growth of the *blaI* mutant is not significantly different than the wild-type 630Δ*erm* strain in the presence of cefoperazone. However, growth of the *blaI* mutant is significantly improved in the presence of ampicillin, as compared to 630Δ*erm* (**Figure 9B**). FInally, the *blaI*::*erm* mutant shows slightly impaired growth in imipenem, as compared to 630Δ*erm* (**Figure 9C**). These results show that BlaIR contributes to ampicillin and impenem resistance in *C. difficile* through regulation of the *bla* operon.

DISCUSSION

This study provides evidence for robust β-lactam-dependent expression of the β-lactamase, BlaD. The *blaD* gene is located in an operon with *blaX*, which encodes a putative membrane protein (**Figure S4**). Our data indicate that the promoter for the *blaXD* operon is located within a 300 nucleotide region located directly upstream of the *blaX* start codon (**Figure 3**). The high level of *blaD* and *blaX* expression in response to β-lactams far below MICs (**Figure S6**), indicate that the promoter of the *bla* operon is

quite strong, in contrast to a previous report in which part of the *blaD* locus was expressed in a heterologous host (39).

Our work has demonstrated that BlaD is a β-lactamase that is only active under anaerobic (reducing) conditions (**Figure 1**). Analysis of the protein via DiANNA (**Figure S7**) revealed that all of the cysteines encoded in the four BlaD proteins analyzed have a predicted oxidation state probability of 1 (72). The high probability of these cysteines oxidizing under aerobic conditions renders this enzyme sensitive to changes in the redox state of the environment. To our knowledge, no other anaerobic-restricted β-lactamases have been reported, which is not surprising given that β-lactamase assays are generally performed in the presence of oxygen (73, 74). This, however, may be one reason that so few β-lactamases have been identified in anaerobic, Gram-positive bacteria (75-78). Indeed, the addition of 0.2 mM DTT to the nitrocefin hydrolysis assays, or steady-state enzyme kinetics assays (39), allows for observation of BlaD activity (**Figure 7**) by maintaining reducing conditions. Assaying β-lactamases from other anaerobic, Gram-positive bacteria under reducing conditions may lead to the identification of additional anaerobic β-lactamases in other species.

Our data indicate that BlaD acts at the cell surface, which is facilitated by the signal sequence at the N-terminus, which allows for translocation of BlaD to the membrane. BlaD is not secreted into the environment, but remains associated with the cell surface (**Figure 7**). While the exact function of BlaX is unknown, the data demonstrate that BlaD activity is enhanced by the presence of BlaX (**Figure 7B**). BlaX has five predicted transmembrane domains, with an approximate 125 residue-long extracellular loop (79). Because the activity of BlaD is membrane-associated across all samples except BlaDΔ18, and BlaD activity in cell lysates lacking BlaX is 60% less than

when BlaX is present, it is possible that BlaX interacts with BlaD in a way that makes BlaD more accessible to substrates on the cell surface. Alternatively, BlaX may interact with β-lactams to facilitate their interaction with BlaD. Nitrocefin hydrolysis assays showed that in cell lysates, the activity of full length BlaD (p*blaD*) is 45% less than BlaDΔ18 (**Figure 7B**). This could result from BlaD cleavage at the N-terminus after translocation to the cell membrane, or BlaX helping to relieve a steric hindrance caused by insertion into the cell membrane. The absence of β-lactamase activity in cell supernatants does not support cleavage of BlaD, unless BlaD remains anchored to the cell membrane after cleavage. Although BlaD does not contain a canonical lipobox immediately downstream of the signal peptide, BlaD has a putative transmembrane domain at the N-terminus, which may allow for membrane anchoring via a non-canonical mechanism (79, 80). Further experiments are needed to determine how BlaD is processed by *C. difficile*.

To date, only one other published β-lactamase is reported to be co-transcribed with a membrane protein (81). This membrane-bound β-lactamase, PenA, found in the Gram-negative *Burkholderia psuedomallei*, is encoded in an operon with *nlpD1*, a gene annotated as an outer membrane lipoprotein and thought to be involved in cell wall hydrolytic amidase activation (82). However, *C. difficile* does not contain an outer membrane, and *nlpD1* does not exhibit homology with *blaX*. Analysis of the *blaD* locus in the *C. difficile* strain M120, which does not contain a full *blaX* coding sequence, revealed regions of partial homology to the 5' and 3' ends of *blaX*, located between the promoter and the *blaD* start codon. This suggests that over the course of evolution of *C. difficile*, the majority of this gene was deleted. A search of the rest of the M120 genome revealed no other proteins similar to BlaX, further supporting the model that in many *C.*

difficile strains, BlaX is not necessary for sufficient BlaD activity. However, the superior activity levels of M120 BlaD (**Figures 7A and 7B**), the 74% of cell surface-associated activity of M120 BlaD (**Figure 7B**), as well as the equal levels of β-lactamase activity of the 630Δ*erm* strain compared to M120 (**Figure 7D**), suggest that M120 may have a different mechanism of translocation.

We have shown that the *bla* operon confers resistance to ampicillin and is regulated by the BlaRI system in *C. difficile* (**Figures 6, 9**). Disruption of *blaI* resulted in constitutive expression of *blaX* and *blaD* (**Figure 8**), which resulted in improved growth in ampicillin (**Figure 9**), supporting the model that BlaI is a direct repressor of the *bla* operon. We identified a 52-nucleotide region of dyad symmetry in the promoter of the *bla* operon, which contains a canonical BlaI binding site, supporting the model of BlaI-P*blaX* binding, but does not rule out other binding partners. Our results align with previously reported data that BlaD confers resistance to penicillins (39). The discrepancy of the MIC values versus the growth curves can be attributed to the greater sensitivity of growth curves in assessing the impact of antimicrobials on cell growth. Further investigation is needed to fully define the mechanisms of β-lactam resistance in *C. difficile*. Identification and characterization of any additional β-lactam resistance mechanisms may aid in preventing *C. difficile* infections and recurrence in the future.

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this manuscript is solely the responsibility of the authors and does not necessarily reflect the official views of the National Institutes of Health.

TABLES

Table 1. Bacterial Strains and plasmids

Plasmid or Strain	Relevant genotype or features	Source, construction or reference
Strains		
A. *E. coli*		
HB101	F- *mcrB mrr hsdS20*(r_B- m_B-) *recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20*	B. Dupuy
DH5α		
B. *C. difficile*		
630	Clinical isolate	(83)
630Δ*erm*	Erm^S derivative of strain 630	(84)
M120	Clinical isolate	(85)
MC324	630Δ*erm* pMC123	(52)
MC448	630Δ*erm* pMC358	(50)
MC905	*blaX*::*erm*	This study
MC985	*blaI*::*erm* 1.0	This study
MC1316	630Δ*erm* pMC822	This study
MC1317	630Δ*erm* pMC826	This study
MC1318	630Δ*erm* pMC827	This study
MC1327	630Δ*erm* Δ*blaXD*	This study
MC1369	630Δ*erm* pMC842	This study
MC1338	Δ*blaXD* pMC811	This study
MC1399	Δ*blaXD* pMC867	This study
MC1400	Δ*blaXD* pMC123	This study
MC1438	*blaI*::*erm* pMC123	This study
MC1466	Δ*blaXD* pMC897	This study
MC1494	Δ*blaXD* pMC896	This study
MC1538	*blaI*::*erm* 2.0	This study
Plasmids		
pRK24	Tra^+, Mob^+; *bla, tet*	(86)
pCR2.1	*bla, kan*	Invitrogen
pCE240	*C. difficile* TargeTron construct based on pJIR750ai (group II intron, *ermB*::RAM, *ltrA*), *catP*	(43)
pMTL-SC7215	Pseudo-suicide plasmid used for allelic exchange in *C. difficile*	(44)
pMC123	*E. coli-C. difficile* shuttle vector; *bla, catP*	(53)
pMC358	pMC123::*phoZ*	(50)
pMC585	pCR2.1 + group II intron targeted to *blaX*	This study
pMC586	pCE240 + group II intron targeted to *blaX*	This study
pMC593	pCR2.1 + group II intron targeted to *blaI*	This study
	pCE240 + group II intron targeted to *blaI*	This study

pMC622	pMC123 + group II intron targeted to *blaX*, *ermB*::RAM, *ltrA*, *catP*	This study
pMC664	pMC123 + group II intron targeted to *blaI*, *ermB*::RAM, *ltrA*, *catP*	This study
pMC810	pMC123 + P_{blaXD} + *blaX*	This study
pMC811	pMC123 + P_{blaXD} + *blaD*Δ18	This study
pMC822	pMTL-SC7215 + 500bp 5' + 500bp 3' of *blaXD*	This study
pMC826	pMC358 + P_{blaXD} (300 bp 5' UTR of *blaX*)	This study
pMC827	pMC358 + 300 bp 5' UTR of *blaD*	This study
pMC842	pMC358 + *blaX*	This study
pMC867	pMC123 + P_{blaXD} + *blaXD*	This study
pMC896	pMC123 + $P_{M120blaD}$ + M120*blaD*	This study
pMC897	pMC123 + P_{blaXD} + *blaD* (*cdd-2* in (39))	This study

Table 2. Oligonucleotides

Underlined nucleotides denote the restriction sites used for vector construction.

Primer	Sequence (5'→3')	Use/locus tag/reference
oMC44	CTAGCTGCTCCTATGTCTCACATC	qPCR/*rpoC* (87)
oMC45	CCAGTCTCTCCTGGATCAACTA	qPCR/*rpoC* (87)
oMC1184	AACAAGAAGGTCACCATGTTCTAC	qPCR/*blaD*
oMC1185	TACTCTGTACCATCATATCCCATAACT	qPCR/*blaD*
oMC1212	GCTTTGTCTTGATTGATACTGGATATG	qPCR/*CD0527*
oMC1213	CATGAGCATGAGTTAGAAATATGTATCG	qPCR/*CD0527*
oMC1214	GCTCTCACAACTGGAACCTTTAATA	qPCR/*CD3196*
oMC1215	TTGCAATACCTATTAAGGCTGATATAATAC	qPCR/*CD3196*
oMC1216	GTATTGAGTATGTTATTTACTGCTGCTC	qPCR/*CD1469*
oMC1217	TATTGAGCACTTACAGCACCAT	qPCR/*CD1469*
oMC1218	CAATAGTAGGTGTATACGTAGATGGTAAAG	qPCR/*CD1802*
oMC1219	GGTCTGCATTTGAATGAGTGTTTATT	qPCR/*CD1802*
oMC1220	TATAGACCCAGGTGGAAGTTTAGTA	qPCR/*CD2742*
oMC1221	TGCAACTACTTTAGCACCAGTT	qPCR/*CD2742*
oMC1235	AGATAGTACTCGTGGTTCAAATTGTT	qPCR/*CD0464*
oMC1222	GTTACCACATATTTCAGAAGCAGAATATC	qPCR/blaI
oMC1223	TTTAGGACTCCATGTACTTGTTTCTAC	qPCR/blaI
oMC1225	TTCACGGTCTATACGCATTTCTTTA	sequencing of blaI
		Targetron
oMC1236	GCTTAATATCTGTAAGTTTAATGCCAAGT	qPCR/*CD0464*
oMC1237	TTGAAGATAACACAGCACTTATGATAGA	qPCR/*CD0344*
oMC1238	ATTGATTACAAGCTCCATAGTGGTC	qPCR/*CD0344*
oMC1262	GCTGATAGACACACCTGAAGATATTAC	qPCR/*CD0692*
oMC1263	CTCCTGTGATAAAGTCACATCCTATTT	qPCR/*CD0692*
oMC1264	TGATGTTGGACAAGGTGATAGTATTT	qPCR/*CD2478*
oMC1265	GTCTGAATCTGGATGAGTTGCTATTAT	qPCR/*CD2478*
oMC1266	TGGTTGTACTACATCAGATAATGGAAATA	qPCR/*CD1930*
oMC1267	TAATCTACCATTAATCCCTCATCATCATT	qPCR/*CD1930*
oMC1268	TCATCAAATGTATTCGGTGAAGATAAAG	qPCR/*CD0655*
oMC1269	TTAACCTATCAAAGCTCGTGTTACT	qPCR/*CD0655*
oMC1270	TGGTATCCAGAGGAGCACAA	qPCR/*CD0895*
oMC1271	TCAATCATTATGAATTTATCACCTATCTCG	qPCR/*CD0895*
oMC1272	ATTGATAGATACTTTGTTGGAGAACCA	qPCR/*CD0829*
oMC1273	ATATGAATACATCTGAATATCCCGAATCA	qPCR/*CD0829*
oMC1343	GGAGGAGTAATGCTACTATTTATAGGTT	qPCR/*blaX*
oMC1344	GTAAAGCTTAATCATATGTACACAAATCCA	qPCR/*blaX*
oMC1349	AAAAGCTTTTGCAACCCACGTCGATCGTGAA-CGAATCCTCTGC-GTGCGCCCAGATAGGGT	IBS Targetron/*blaI*
oMC1350	CAGATTGTACAAATGTGGTGATAACAGATAAGTC-CTCTGCTA-TAACTTACCTTTCTTTG	EBS1 Targetron/*blaI*
oMC1351	CGCAAGTTTCTAATTTCGGTT-ATTCG-TCGATAGAGGAAAGTGTCT	EBS2 Targetron/*blaI*

oMC1360	AAAAGCTTTTGCAACCCACGTCGATCGTGAA-ACATATGATTAA-GTGCGCCCAGATAGGGT	IBS Targetron/*blaX*
oMC1361	CAGATTGTACAAATGTGGTGATAACAGATAAGTC-GATTAAGC-TAACTTACCTTTCTTTGT	EBS1 Targetron/*blaX*
oMC1362	CGCAAGTTTCTAATTTCGGTT-TATGT-TCGATAGAGGAAAGTGTCT	EBS2 Targetron/*blaX*
oMC1461	GTAATATACTCCAGTCTAGGAGC	sequencing of *blaX* Targetron
oMC1945	GTACTAAAGGAGTTTTGCTCTATATAGACTCCTCCTTTCAGTTTGTGAGGTAATTATTTATTC	*blaXD* allelic replacement cloning
oMC1946	GAATAAATAATTACCTCACAAACTGAAAGGAGGAGTCTATATAGAGCAAAACTCCTTTAGTAC	*blaXD* allelic replacement cloning
oMC1970	TACGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGACATGAATGTTAAATCCTTTCTGAGTAC	*blaX* Gibson assembly
oMC1971	ACGGCCAGTGAATTCGAGCTCGGTACCCGGGGATCCCATCTCCTCTACATAAGTTTATAGTTCACC	*blaX* Gibson assembly
oMC1974	ACGGCCAGTGAATTCGAGCTCGGTACCCGGGGATCCGTACTAAAGGAGTTTTGCTCTATATAGACTC	*blaD* Gibson assembly
oMC1999	GTAGAAATACGGTGTTTTTTGTTACCCTAAGTTTAAACGGAGTTTGGTCTACGATTACAGAAG	5’ flank for *blaXD* Gibson assembly
oMC2000	GGATTTTGGTCATGAGATTATCAAAAAGGAGTTTAAACCTGCAAGAGCTTCTTCCTTTAAAC	3’ flank for *blaXD* Gibson assembly
oMC2019	CAGTCACGACGTTGTAAAACGACGGCCAGTGAATTCGTAAAGCAATTATATTATGTAACCATATTA	P_{blaX} cloning via Gibson assembly
oMC2020	AAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCTATGTCCTCCTTTCAGTTTG	P_{blaX} cloning via Gibson assembly
oMC2021	CAGTCACGACGTTGTAAAACGACGGCCAGTGAATTCGAAAAAACTAAACAGAAATTTAGATGTAG	5’ *blaD* cloning via Gibson assembly
oMC2022	AAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCAGCTACAACAACTAGAAGAATAAC	5’ *blaD* cloning via Gibson assembly
oMC2062	CAGTCACGACGTTGTAAAACGACGGCCAGTGAATTCCCTCACAAACTGAAAGGAGGA	*blaX* cloning via Gibson assembly
oMC2110	GCGCGGATCCGGCTACCAAATATAACACCATC	*blaIR* cloning
oMC2111	GCGCGAATTCGAGGGAGAGTTGCCACTATTTG	*blaIR* cloning
oMC2338	TATCCAAATAAAATTATTTTTTCTTTTCATTATGTCCTCCTTTCAGTTTGTGAGGTAATT	P_{blaXD} SOE PCR to *blaD*
oMC2339	AATTACCTCACAAACTGAAAGGAGGACATAATGAAAAGAAAAAATAATTTTATTTGGATA	P_{blaXD} SOE PCR to *blaD*
oMC2340	ATGCTTTCTTCCTACATAATATACTCCCATTATGTCCTCCTTTCAGTTTGTGAGGTAATT	P_{blaXD} SOE PCR to *blaD*Δ18
oMC2341	AATTACCTCACAAACTGAAAGGAGGACATAATGGGAGTATATTATGTAGGAAGAAAGCAT	P_{blaXD} SOE PCR to *blaD*Δ18
oMC2342	TACGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGACTCAAACTAACTTGACTTTTAAAACTTACTATTG	M120 *blaD* cloning (CDM120_RS02980)
oMC2343	ACGGCCAGTGAATTCGAGCTCGGTACCCGGGGATCCGGAGTTTTGCTCTATGTAAACTCAATTTAG	M120 *blaD* cloning (CDM120_RS02980)

FIGURES

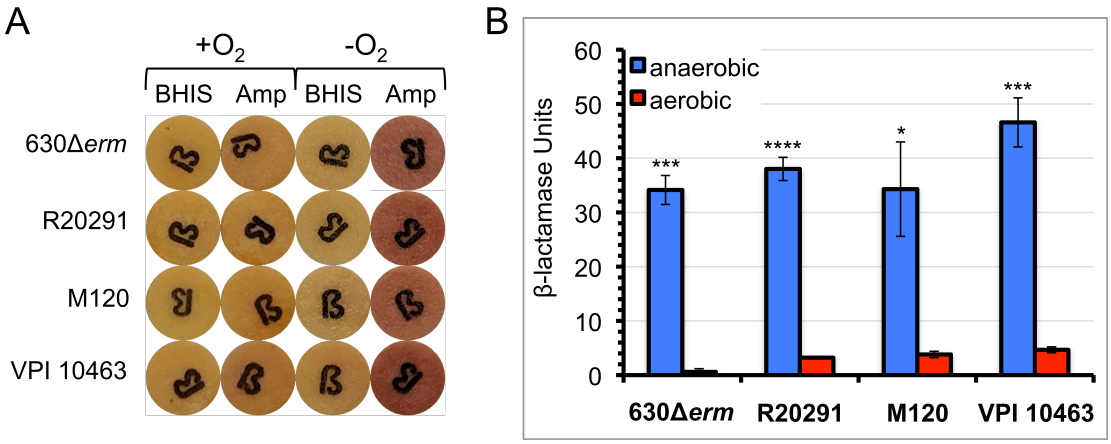

Figure 1. *C. difficile* strains exhibit inducible, anaerobic β-lactamase activity. Hydrolysis of the chromogenic cephalosporin nitrocefin was assessed for strains 630Δ*erm*, R20291, M120, and VPI 10463. Strains were grown anaerobically to mid-log in BHIS medium +/- 2 μg/mL ampicillin and pelleted. **A)** Cell pellets were resuspended in non-reduced ($+O_2$) or reduced ($-O_2$) BHIS and incubated anaerobically or aerobically for 15 min. Cell pellets in ~30 μL remaining media were spotted onto nitrocefin disks for 20 min. Color change from yellow to red indicates cleavage of nitrocefin. **B**) Frozen cell pellets were resuspended in reduced or non-reduced BHIS and assayed for nitrocefin cleavage either anaerobically or aerobically, respectively. Columns represent the means +/- SEM from three independent replicates. Data were analyzed by a two-tailed unpaired Student's *t*-test, compared to aerobic conditions for each strain. Adjusted P values indicated by *≤0.05, ****<0.0001.

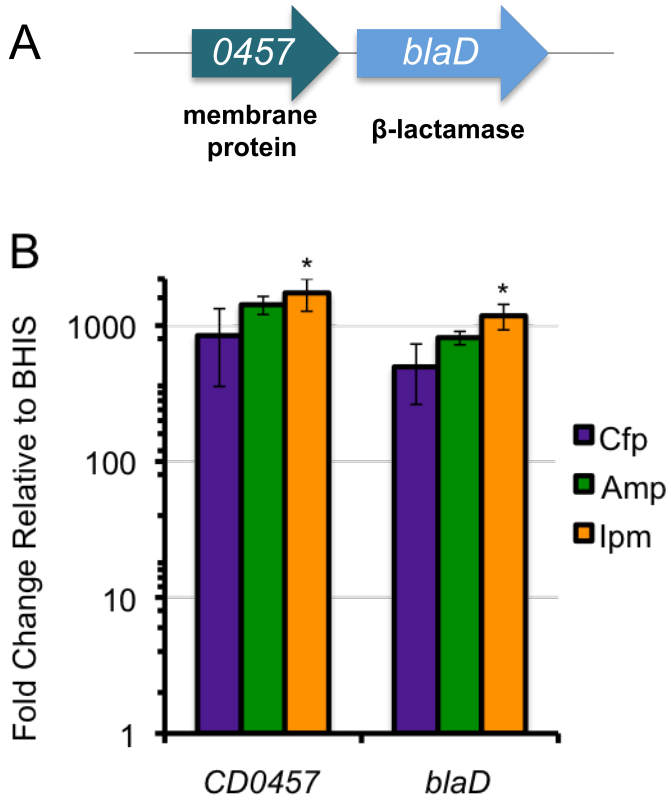

Figure 2. The putative β-lactamase, *CD0458*, and the upstream gene, *CD0457* are induced by β-lactams. **A**) The putative β-lactamase gene *CD0458* is 27 bp downstream of the predicted membrane protein, *CD0457*. **B**) Relative expression of each gene was measured via qRT-PCR. *C. difficile* strain 630Δ*erm* was grown to mid-log in BHIS medium supplemented with sub-inhibitory concentrations of β-lactams (Cfp: cefoperazone 50 μg/mL; Amp: ampicillin 2 μg/mL; Ipm: imipenem 1.5 μg/mL). mRNA levels are normalized to expression levels in BHIS alone. Columns represent the means +/- SEM from three independent replicates. Data were analyzed by one-way ANOVA with Dunnett's multiple comparisons test, compared to no antibiotic. Adjusted *P* values indicated by *≤0.05.

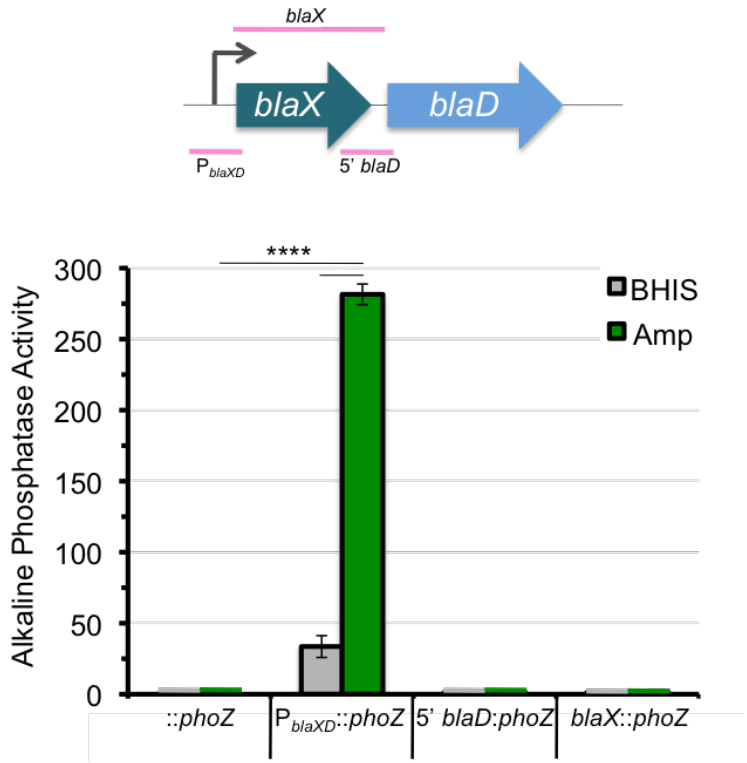

Figure 3. Alkaline phosphatase activity from P*blaXD*::*phoZ* is induced in the presence of ampicillin. *C. difficile* 630Δ*erm* cultures were grown to an OD_{600} of ~0.5 in BHIS with 2 µg/mL thiamphenicol for plasmid maintenance in the presence or absence of 2 µg/mL ampicillin . Strains: MC448 (::*phoZ* - empty vector); MC1317 (P*blaXD*::*phoZ)*; MC1318 (5' *blaD*::*phoZ)*; MC1369 (*blaX*::*phoZ)*. The means and standard errors of the means of three biological replicates are shown. Data were analyzed by one-way ANOVA with Dunnett's multiple comparison test. Adjusted *P* value indicated by ****<0.0001.

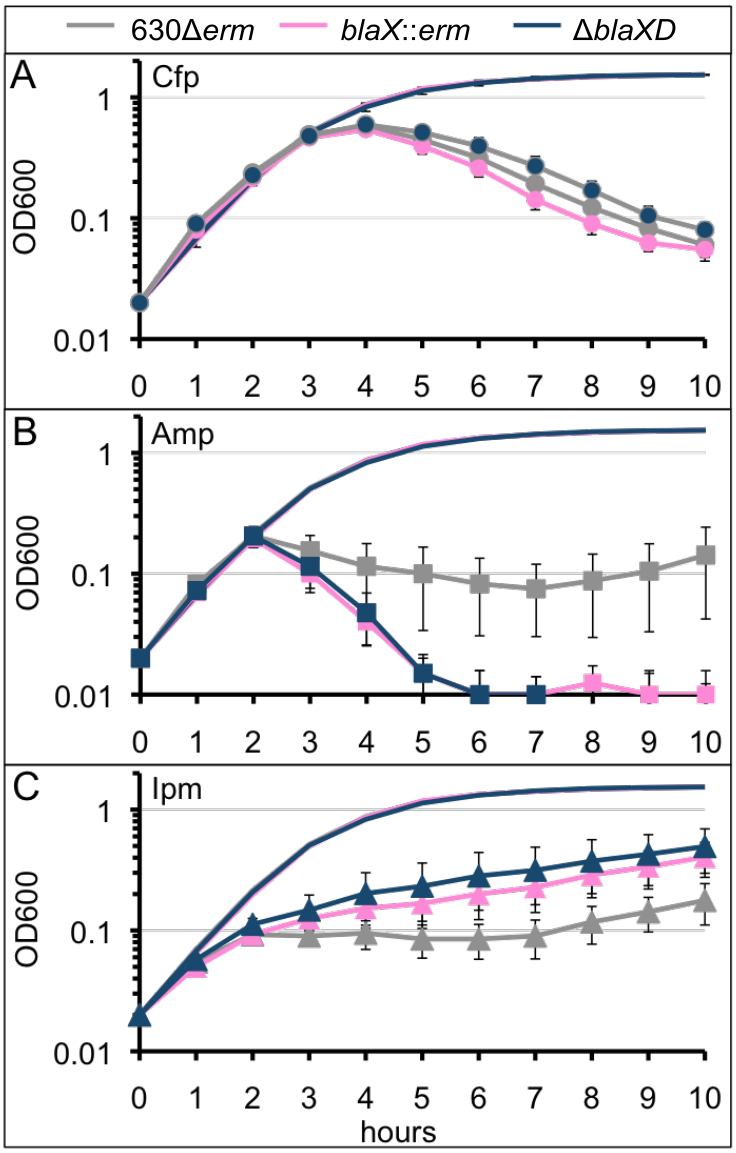

Figure 4. *blaX* and *blaD* contribute to β-lactam resistance in *C. difficile*. *C. difficile* strains 630Δ*erm* (grey), *blaX*::*erm* (MC905; pink), and Δ*blaXD* (MC1327; blue) were grown to mid-log, backdiluted to OD 0.05, and grown in BHIS supplemented with **A**) Cfp: cefoperazone 60 μg/mL, **B**) Amp: ampicillin 4 μg/mL, or **C**) Ipm: imipenem 2 μg/mL. Lines represent the means +/- SEM from four independent replicates. Data were analyzed by one-tailed paired Student's *t*-test, compared to 630Δ*erm*. No statistically significant differences found.

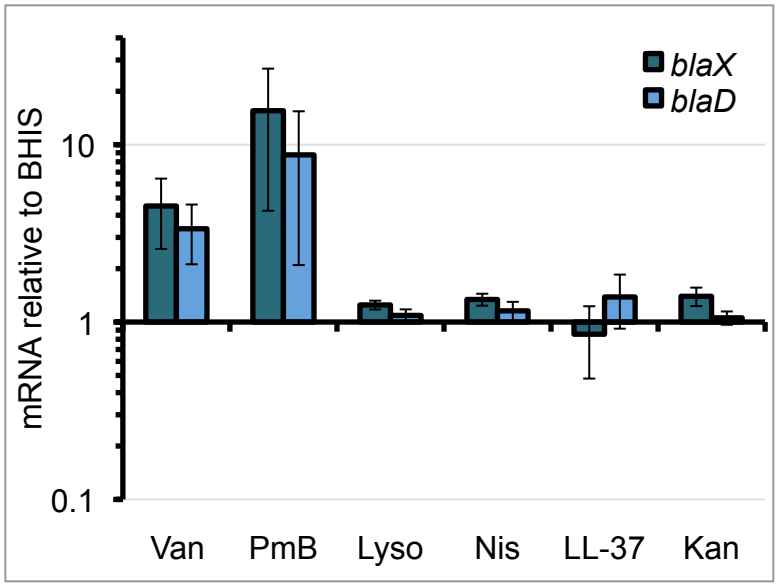

Figure 5. *blaXD* transcription is modestly induced by vancomycin and polymyxin B. Relative expression of each gene was measured via qRT-PCR. *C. difficile* strain 630Δ*erm* was grown to mid-log in BHIS medium supplemented with sub-inhibitory concentrations of cell wall targeting antimicrobials (Van: vancomycin 0.75 µg/mL, PmB: polymyxin B 75 µg/mL, Lys: lysozyme 1 mg/mL, Nis: nisin 7.5 µg/mL, LL-37 2 µg/mL, and Kan: kanamycin 250 µg/mL). mRNA levels are normalized to expression levels in BHIS alone. Columns represent the means +/- SEM from four independent replicates. Data were analyzed by one-way ANOVA with Dunnett's multiple comparisons test, compared to expression in 630Δ*erm* without antibiotic. No statistically significant values found.

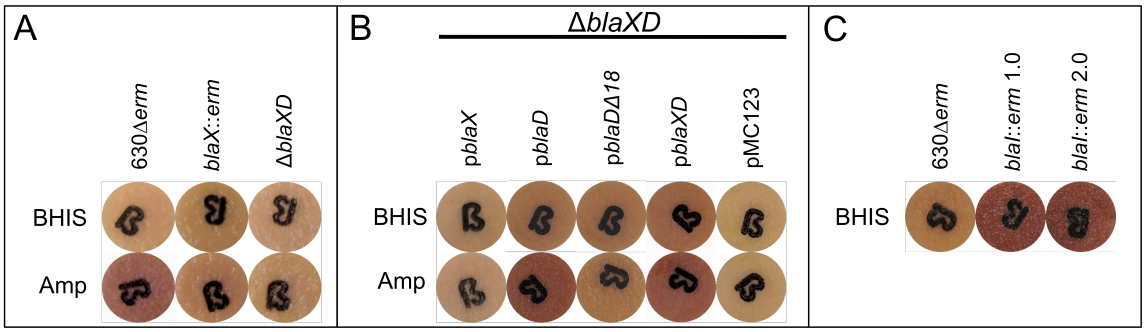

Figure 6. The N-terminus of BlaD is necessary for β-lactamase secretion, independent of BlaX. Hydrolysis of the chromogenic cephalosporin nitrocefin was assessed for **A**) strains 630Δ*erm*, *blaX*::*erm* (MC905), and Δ*blaXD* (MC1327), **B**) strain Δ*blaXD* complemented with *blaX* and/or *blaD*, expressed from their native promoter, and **C**) strains 630Δ*erm*, *blaX*::*erm* 1.0, *blaX*::*erm* 2.0. Strains were grown anaerobically to mid-log in BHIS medium (with 2 μg/mL thiamphenicol for plasmid maintenance in **B**) +/- 2 μg/mL ampicillin and pelleted. Cell pellets in ~30 μL of remaining media were incubated anaerobically on nitrocefin disks for 2 h. Color change from yellow to red indicates cleavage of nitrocefin. Results shown are representative of three independent assays.

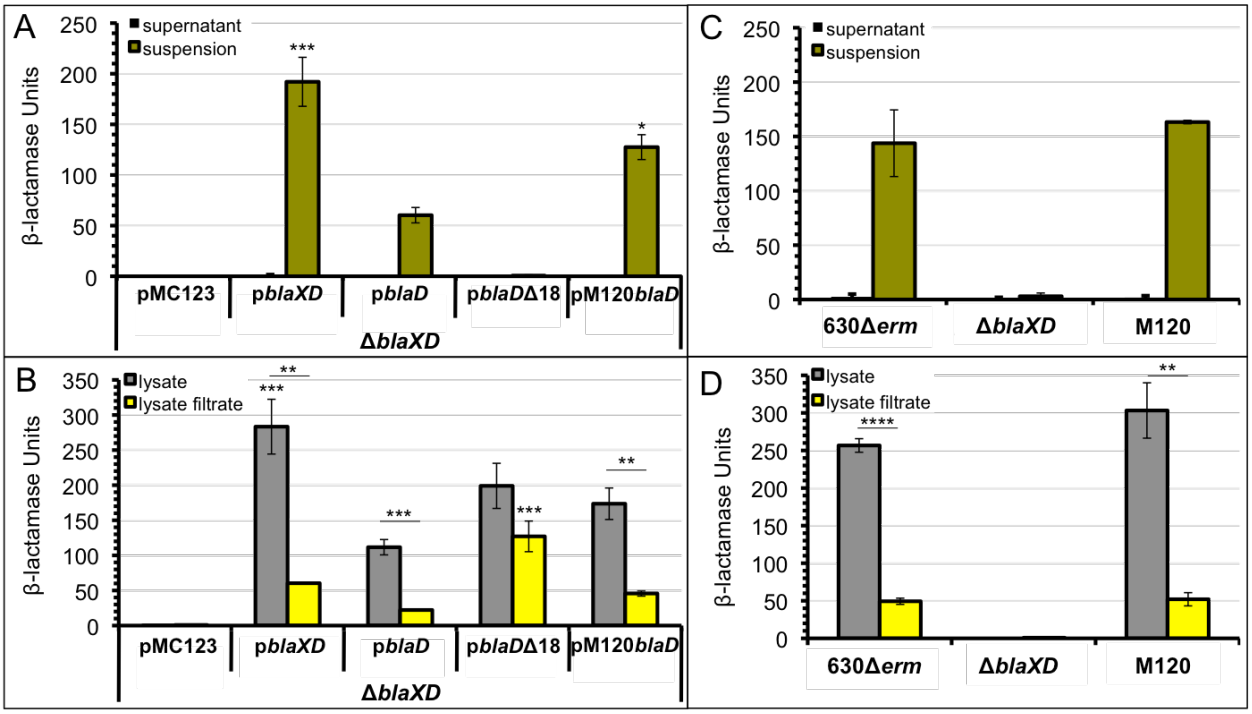

Figure 7. BlaD utilizes a signal sequence to act at the cell membrane. Δ*blaXD* (**A, B**) or 630Δ*erm*, Δ*blaXD*, and M120 (**C, D**) *C. difficile* were grown to mid-log phase in 2 μg/mL thiamphenicol and 2 μg/mL ampicillin and assayed for β-lactamase activity via a nitrocefin assay in **A, C**) supernatant or cell suspension and **B, D**) cell lysate or cell lysate filtrate. Δ*blaXD* pMC123 (MC 1400); Δ*blaXD* p*blaXD* (MC1399); Δ*blaXD* p*blaD* (MC1466); Δ*blaXD* p*blaD*Δ18 (MC1338); Δ*blaXD* pM120*blaD* (MC1494). Columns represent the means +/- SEM from at least three independent replicates. Data were analyzed by one-way ANOVA with Dunnett's multiple comparisons test, compared to p*blaD* in A) and B) or 630Δ*erm* in C) and D), or by a two-tailed unpaired student's *t*-test, where indicated by bars. Absence of asterisk indicates no statistically significant difference found. Adjusted *P* values indicated by *≤0.05, ****<0.0001.

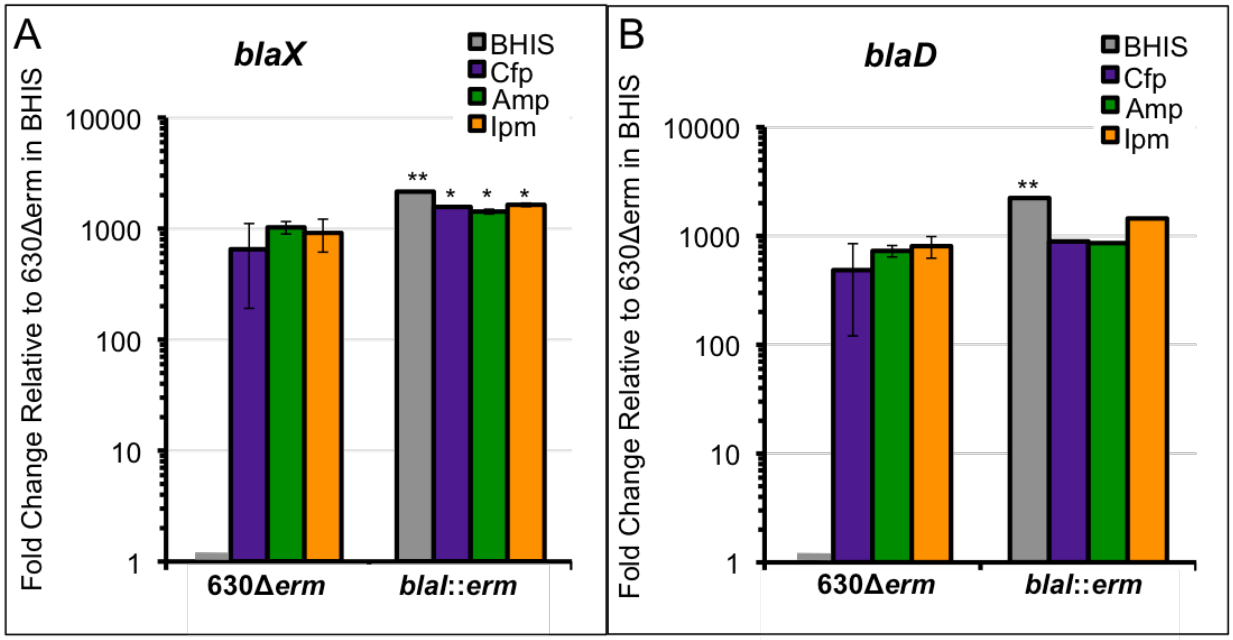

Figure 8. *blaXD* is derepressed in the *blaI*::*erm* strain. qRT-PCR was performed to measure expression of **A**) *blaX* and **B**) *blaD* in *C. difficile* 630Δ*erm* and *blaI*::*erm* strains grown to mid-log in BHIS media with or without β-lactam (Cfp: cefoperazone 60 μg/mL; Amp: ampicillin 2 μg/mL; Ipm: imipenem 1.5 μg/mL). mRNA levels are normalized to expression levels in 630Δ*erm* in BHIS alone. Columns represent the means +/- SEM from three independent replicates. Data were analyzed by one-way ANOVA with Dunnett's multiple comparisons test, compared to expression in 630Δ*erm* without antibiotic. Adjusted *P* values indicated by *≤0.05, **≤0.005.

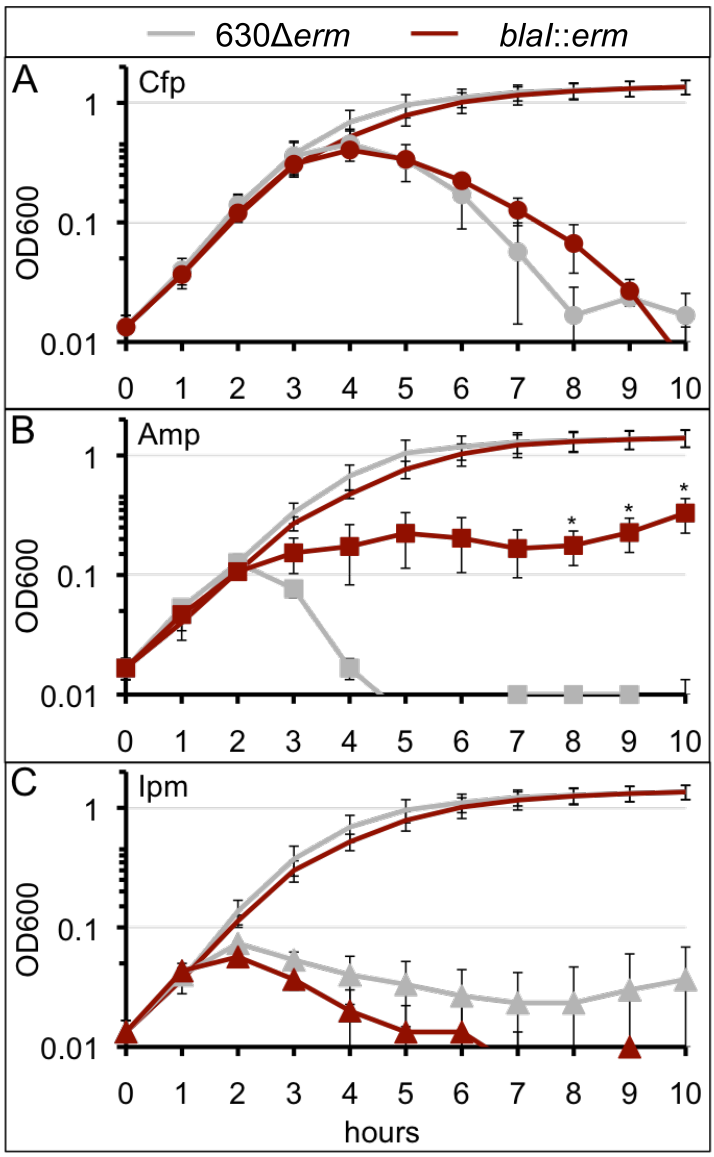

Figure 9. *blaI* regulates resistance to ampicillin. *C. difficile* strains 630Δ*erm* (gray) and *blaI*::*erm* (MC985; red) were grown to mid-log, backdiluted to OD 0.05, and grown in BHIS (no marker) or BHIS supplemented (filled marker) with **A**) 60 μg/mL cefoperazone (Cfp), **B**) 4 μg/mL ampicillin (Amp), or **C**) 2 μg/mL imipenem (Ipm). Lines represent the means +/- SEM from three independent replicates. Data were analyzed by one-tailed paired Student's *t*-test, compared to 630Δ*erm*. Adjusted *P* values indicated by *≤0.05.

SUPPLEMENTAL TABLE

	β-lactam (μg/mL)		
	Cfp	**Amp**	**Ipm**
630Δ*erm*	**100**	**2**	**2**
blaX*::*erm	**100**	**2**	**1**
Δ*blaXD*	**100**	**2**	**1**

Table S1. MIC values for 630Δ*erm*, *blaX*::*erm*, and Δ*blaXD* strains. MIC values were determined for strains 630Δ*erm*, *blaX::erm* (MC905), and Δ*blaXD* (MC1327) in Cfp (cefoperazone), Amp (ampicilin), and Ipm (imipenem) using liquid broth dilution. Values represent the highest MIC value of three biological replicates.

SUPPLEMENTAL FIGURES

Figure S1. DNA cloning and vector details.

pMC585: The group II intron of pCE240 was retargeted to *blaX* (*CD0457*) by splicing by overlap extension PCR using primers oMC1360/1361/1362 and EBSu as outlined in the Targetron users manual (Sigma-Aldrich). The primers used for intron retargeting were obtained by using the algorithm provided by J.P. van Pijkeren and Rob Britton of Michigan State University. This region was TA-cloned into pCR2.1.

pMC586: The *blaX*-targeting intron was digested from pMC585 and ligated into pCE240 with BsrGI and HindIII.

pMC593: The group II intron of pCE240 was retargeted to *blaI* at nucleotide 187 by splicing by overlap extension PCR using primers oMC1349/1350/1351 and EBSu as outlined in the Targetron users manual (Sigma-Aldrich). The primers used for intron retargeting were obtained by using the algorithm provided at ClosTron.com (82). This region was TA-cloned into pCR2.1.

The *blaI*-targeting intron was digested from pMC593 and ligated into pCE240 with BsrGI and HindIII.

pMC622: The *blaX*-targeting intron was digested from pMC586 with SphI and SfoI and ligated into the SphI and SnaBI sites of pMC123.

pMC664: The *blaI*-targeting intron was digested from the plasmid derivative of pMC593 with SphI and SfoI and ligated into the SphI and SnaBI sites of pMC123.

pMC810: A 1.07 kb *blaX* PCR product was amplified with primers oMC1970/1971 from *C. difficile* 630Δ*erm* genomic DNA and cloned into pMC123 cut with XbaI and BamHI using Gibson Assembly.

pMC811: A 1.34 kb *blaD*Δ54 (*CD0458*) PCR product was generated by splicing by overlap extension PCR using primers oMC1970/2340/2341/1974 from *C. difficile* 630Δ*erm* genomic DNA and cloned into pMC123 using XbaI and BamHI using Gibson Assembly.

pMC822: A 1 kb PCR product of the 5' and 3' regions of the *bla* operon was generated by splicing by overlap extension PCR using primers oMC1999/1945/2000/1946 from *C. difficile* 630Δ*erm* genomic DNA and cloned into pMTL-SC7215 at the PmeI site using Gibson Assembly.

pMC826: A 373 bp PCR product of the 5' UTR of *blaX* was amplified with primers oMC2019/2020 from *C. difficile* 630Δ*erm* genomic DNA and cloned into pMC358 at the EcoRI and BamHI sites.

pMC827: A 373 bp PCR product of the 5' UTR of *blaD* was amplified with primers oMC2021/2022 from *C. difficile* 630Δ*erm* genomic DNA and cloned into pMC358 at the EcoRI and BamHI sites.

pMC842: An 895 bp *blaX* PCR product was amplified with primers oMC2062/2022 from *C. difficile* 630Δ*erm* genomic DNA and cloned into pMC358 at the EcoRI and BamHI sites.

pMC867: A 2.1 kb *blaXD* PCR product was amplified using primers oMC1970/2340/2341/1974 from *C. difficile* 630Δ*erm* genomic DNA and cloned into pMC123 using XbaI and BamHI.

pMC896: A 1.24 kb *blaD* PCR product was amplified using primers oMC2342/2343 from *C. difficile* M120 genomic DNA and cloned into pMC123 using XbaI and BamHI using Gibson Assembly.

pMC897: A 1.4 kb *blaD* PCR product was generated by splicing by overlap extension PCR using primers oMC1970/2338/2339/1974 from *C. difficile* 630Δ*erm* genomic DNA and cloned into pMC123 using XbaI and BamHI using Gibson Assembly.

pMC920: An 875 bp *blaI* PCR product was amplified with primers oMC2110/2432 from *C. difficile* 630Δ*erm* genomic DNA and cloned into pMC123 at the EcoRI and BamHI sites.

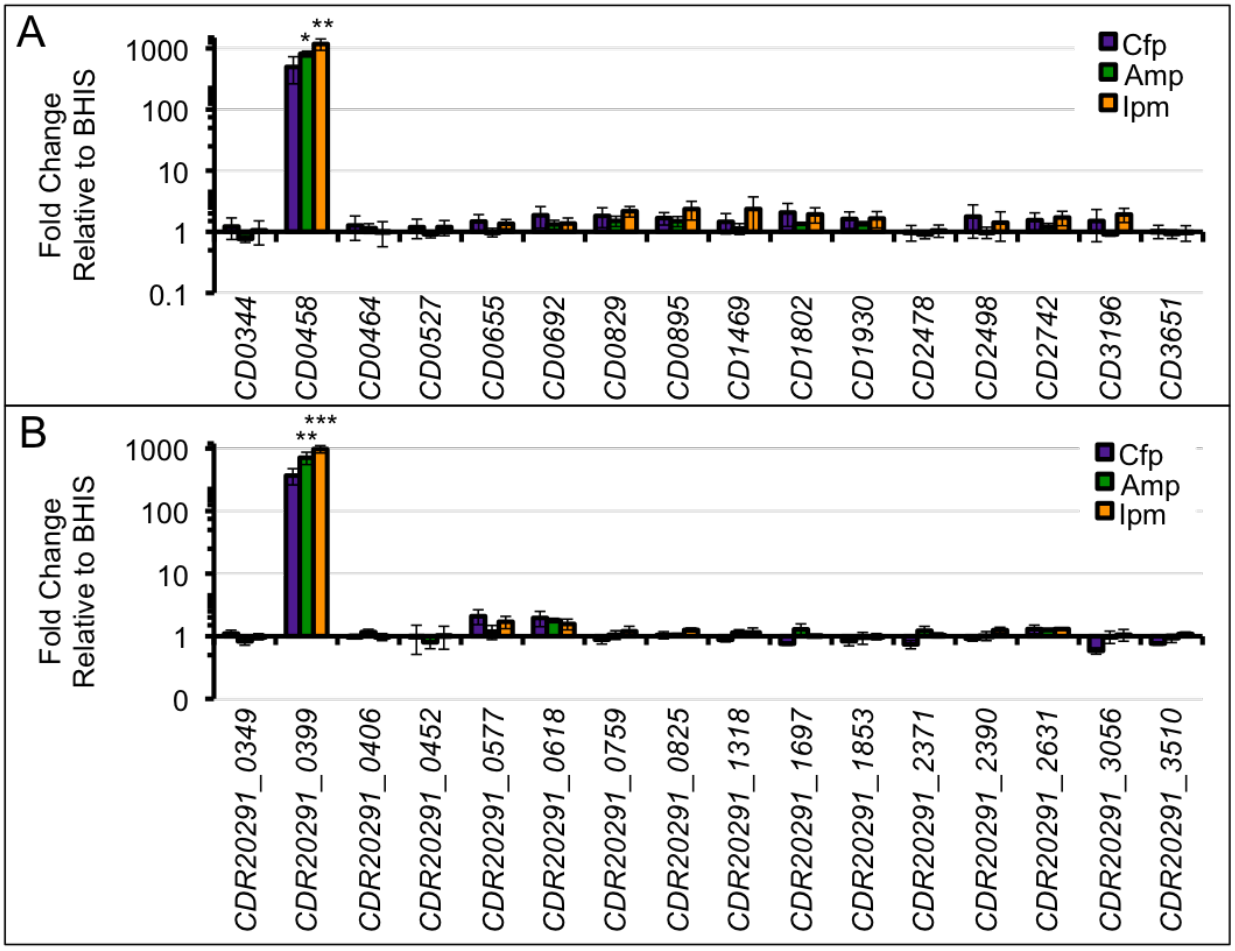

Figure S2. The putative β-lactamase gene, *CD0458 (CDR20291_0399)*, is induced by β-lactams. Putative β-lactamase genes in strains **A**) 630Δ*erm* and **B**) R20291 were measured for relative expression to the housekeeping gene, *rpoC*, in β-lactams via qRT-PCR (Cfp: cefoperazone 50 μg/mL; Amp: ampicillin 2 μg/mL; Ipm: imipenem 1.5 μg/mL). mRNA levels are normalized to expression levels in BHIS alone. Columns represent the means +/- SEM from three independent replicates. Data were analyzed by one-way ANOVA with Dunnett's multiple comparisons test, compared to no antibiotic. Adjusted *P* values indicated by *≤0.05, ***≤0.001.

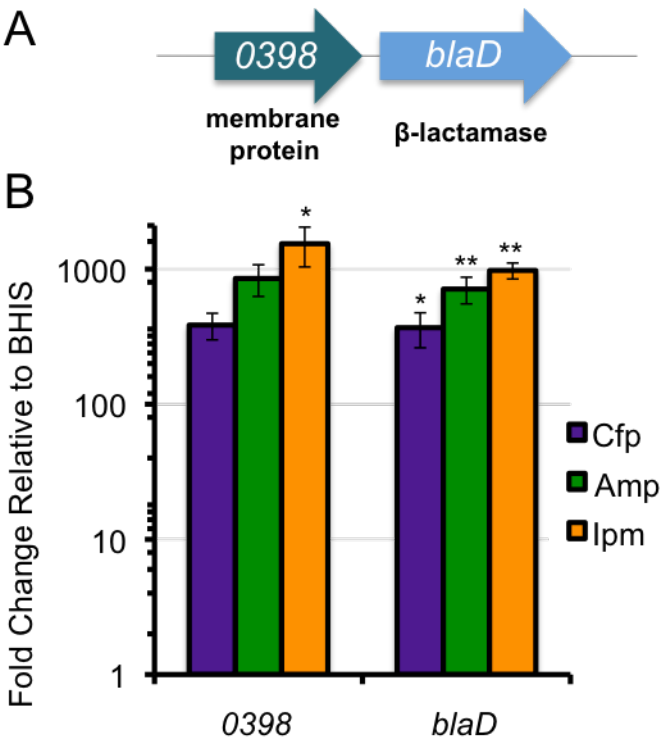

Figure S3. The putative β-lactamase, *CDR20291_0399*, and its upstream gene are induced by β-lactams. A) The putative β-lactamase gene *CDR20291_0399* is 27 bp downstream of the putative membrane protein, *CDR20291_0398*. **B**) Relative expression of each gene was measured via qRT-PCR. *C. difficile* strain 630Δ*erm* was grown to mid-log in BHIS medium supplemented with sub-inhibitory concentrations of β-lactams (Cfp: cefoperazone 50 μg/mL; Amp: ampicillin 2 μg/mL; Ipm: imipenem 1.5 μg/mL). mRNA levels are normalized to expression levels in BHIS alone. Columns represent the means +/- SEM from three independent replicates. Data were analyzed by one-way ANOVA with Dunnett's multiple comparisons test, compared to no antibiotic. Adjusted *P* values indicated by *≤0.05, **≤0.01.

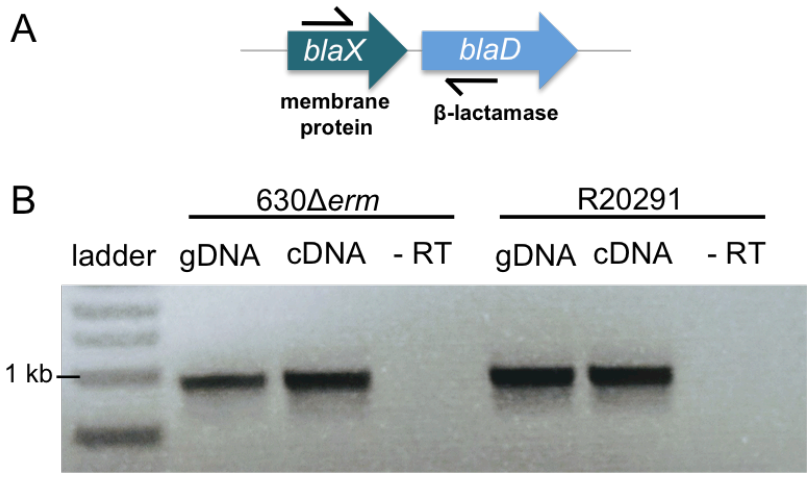

Figure S4. *blaX* and *blaD* form the *bla* operon. **A**) PCR was performed using a forward primer (oMC1184) within *blaX* and the reverse primer (oMC1185) within *blaD* (*CD0457* and *CD0458* in 630Δ*erm* and *CDR20291_0398* and *CDR20291_0399* in R20291). **B**) cDNA was created from *C. difficile* strains 630Δ*erm* and R20291 treated with 2 μg/mL ampicillin. gDNA: genomic DNA from each strain served as a positive control; -RT: RNA from a reverse transcription reaction lacking enzyme served as a negative control.

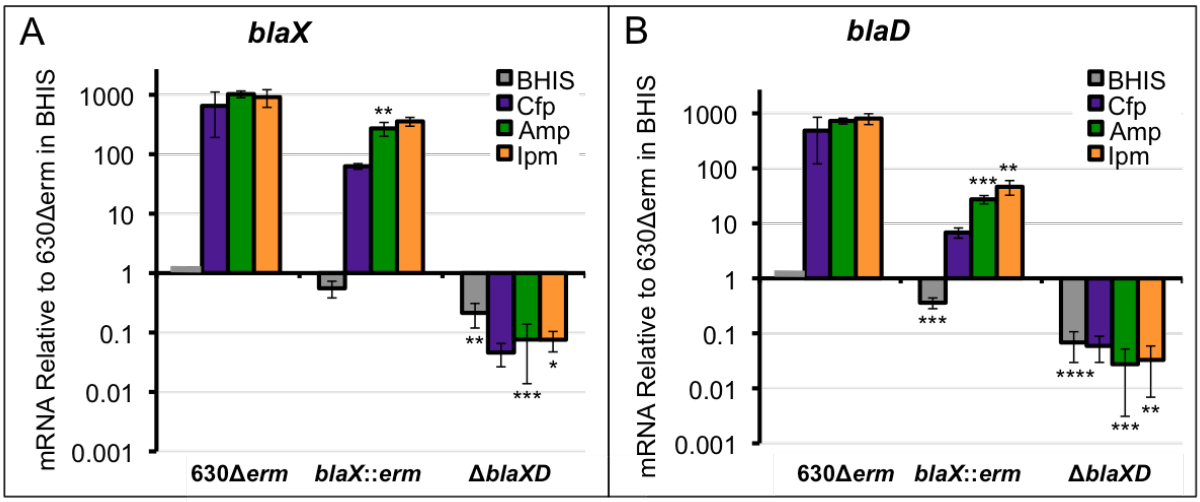

Figure S5. Analysis of gene expression of mutants *blaX*::*erm* and Δ*blaXD*. Relative expression of **A**) *blaX* and **B**) *blaD* in 630Δ*erm* compared to *blaX*::*erm* (MC905), and Δ*blaXD* (MC1327) was measured via qRT-PCR. *C. difficile* was grown to mid-log in BHIS media supplemented with sub-inhibitory concentrations of β-lactams (Cfp: cefoperazone 60 μg/mL, Amp: ampicillin 2 μg/mL, and Ipm: imipenem 1.5 μg/mL). mRNA levels are normalized to expression levels in 630Δ*erm* in BHIS alone. Columns represent the means +/- SEM from three independent replicates. Data were analyzed by one-way ANOVA with Dunnett's multiple comparisons test, compared to expression in 630Δ*erm* without antibiotic. Adjusted *P* values indicated by *≤0.05, ****<0.0001.

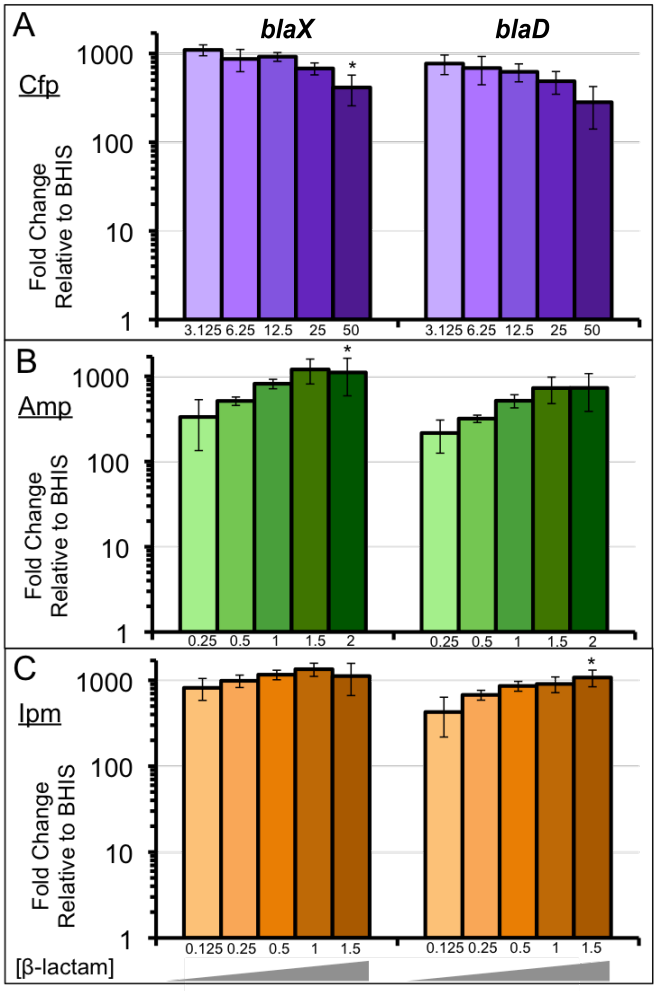

Figure S6. The *blaXD* operon exhibits dose dependent expression for different classes of β-lactams. Relative expression of *blaX* and *blaD* in 630Δ*erm* was measured using qRT-PCR. *C. difficile* was grown to mid-log in BHIS medium supplemented with increasing sub-inhibitory concentrations of **A**) cefoperazone (μg/mL: 3.125, 6.25, 12.5, 25, 50), **B**) ampicillin (μg/mL: 0.25, 0.5, 1, 1.5, 2), or **C**) imipenem (μg/mL: 0.125, 0.25, 0.5, 1, 1.5). mRNA levels are normalized to expression levels in 630Δ*erm* in BHIS alone. Columns represent the means +/- SEM from three independent replicates. Data were analyzed by one-way ANOVA with Dunnett's multiple comparisons test, comparing to expression with lowest concentration antibiotic. Adjusted *P* values indicated by *≤0.05, ****<0.0001.

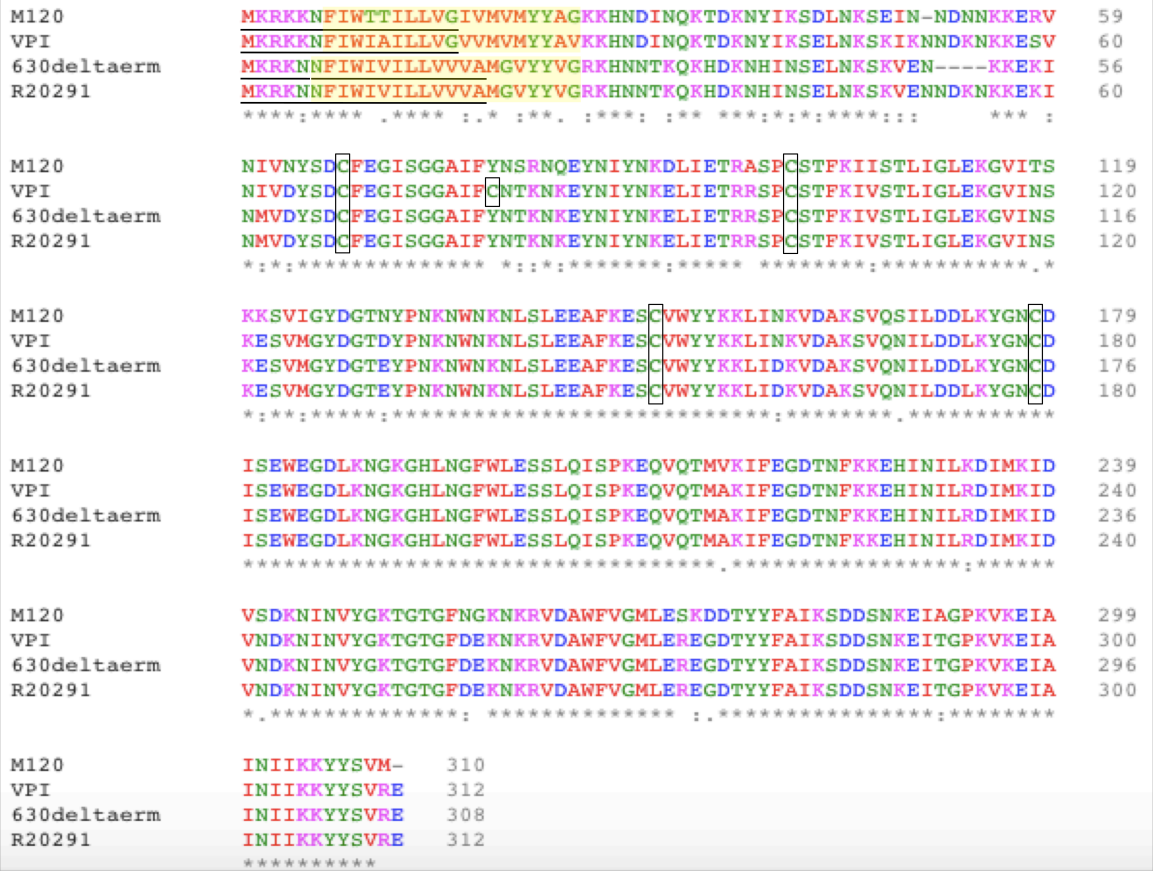

Figure S7. BlaD displays variance at the N-terminus in diverse *C. difficile* strains. Alignment of the BlaD protein from strains M120, VPI 10463, 630Δ*erm*, and R20291 via Clustal omega (88). (*) indicates fully conserved residues, (:) indicates conservation of strongly similar residues, and (.) indicates conservation of weakly similar residues. The black lines indicate the signal peptides predicted by Signal-3L 2.0 (63). The yellow highlighting indicates transmembrane domains predicted by Phobius (79). Boxes indicate cysteines with an oxidation state probability of 1, predicted by DiANNA analysis (72).

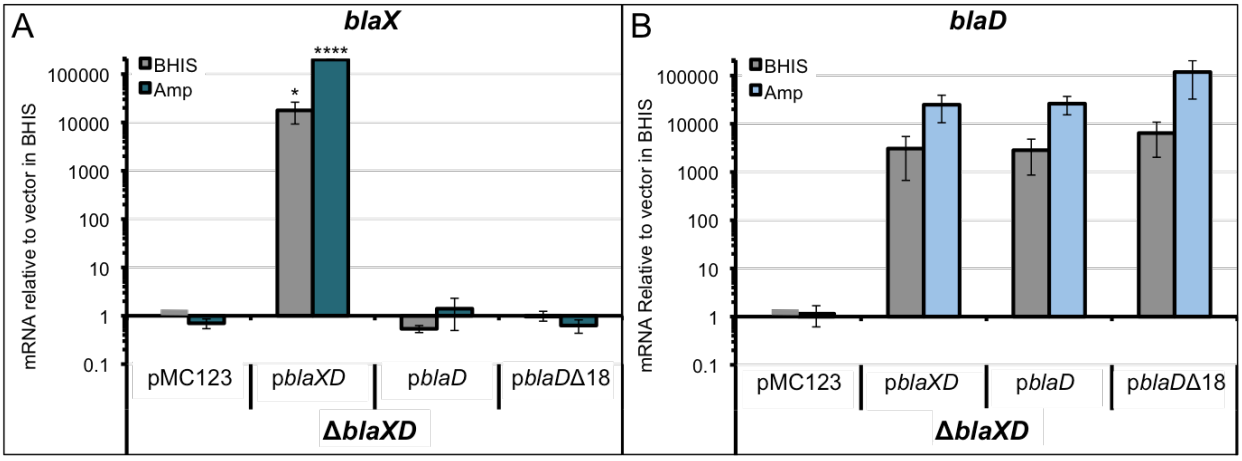

Figure S8. Expression of *blaX* or *blaD* from Δ*blaXD* complemented strains. qRT-PCR was performed to examine expression of **A**) *blaX* or **B**) *blaD* from a plasmid maintained in Δ*blaXD (*MC1327) grown to mid-log in BHIS media supplemented with 2 ug/mL ampicillin. mRNA levels are normalized to expression levels in Δ*blaXD (*MC1327) expressing an empty vector (pMC123) in BHIS alone. p*blaXD*: pMC867; p*blaD*: pMC897; p*blaD*Δ18: pMC811. Columns represent the means +/- SEM from three independent replicates. Data were analyzed by one-way ANOVA with Dunnett's multiple comparisons test, comparing to expression without antibiotic. Absence of asterisk indicates no statistically significant difference found. Adjusted *P* values indicated by *≤0.05, **<0.0001.

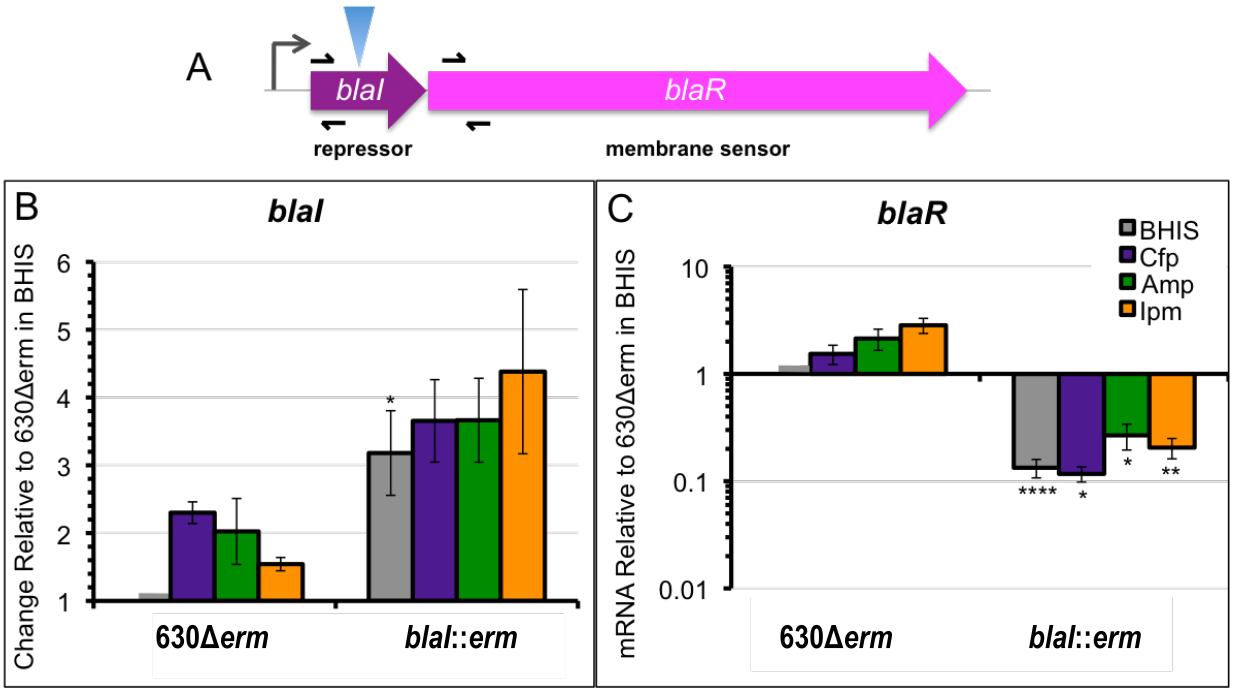

Figure S9. *blaIR* is derepressed but disrupted in the *blaI*::*erm* strain. A) *blaI* was disrupted by an insertion. qRT-PCR was performed to measure expression of **B**) *blaI* and **C**) *blaR* in *C. difficile* 630Δ*erm* and *blaI*::*erm* strains grown to mid-log in BHIS media with or without β-lactam (Cfp: cefoperazone 60 μg/mL; Amp: ampicillin 2 μg/mL; Ipm: imipenem 1.5 μg/mL). mRNA levels are normalized to expression levels in 630Δ*erm* in BHIS alone. Columns represent the means +/- SEM from three independent replicates. Data were analyzed by one-way ANOVA with Dunnett's multiple comparisons test, compared to expression in 630Δ*erm* without antibiotic. Adjusted *P* values indicated by *≤0.05, ****<0.0001.

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Chapter 3: Discussion

C. difficile is a worldwide health concern and costs the United States billions of dollars in healthcare expenses every year (1). With a broad antibiotic resistance profile and the ability to form spores, *C. difficile* is difficult to eliminate, especially for recurrent infections (2). *C. difficile* is particularly dangerous as this bacterium exhibits resistance to the most commonly prescribed class of antibiotics, the β-lactams, and thrives in an intestinal environment in which most commensal bacterial species have been eliminated (3, 4). Understanding the nature of β-lactam resistance in *C. difficile* will bring us closer to preventing *C. difficile* colonization of the intestinal tract.

We have investigated one mechanism of β-lactam resistance in *C. difficile*, a β-lactamase. This gene was originally identified in late 2018, and the enzyme was biochemically assayed to show that it hydrolyzes the penicillin, cephalosporin, and monobactam classes of β-lactams (5). Interestingly, this enzyme can bind to, but does not cleave, the carbapenem class of β-lactams (5). To add to our current knowledge of this β-lactamase, which we named *blaD*, we characterized the induction, regulation, and transcriptional organization of *blaD* and the gene found immediately upstream, *blaX*. In addition, we characterized the anaerobic nature of BlaD function, further investigated the contributions of this β-lactamase to β-lactam resistance in *C. difficile*, identified a signal sequence required for translocation of the protein out of the cytosol, and established the localization of the enzyme within the cell.

I. An anaerobic β-lactamase

Before we identified *blaD*, we observed that *C. difficile* produces a β-lactamase that is inactivated by oxygen (**Ch. 2 Fig. 1**). To our knowledge, no published β-

lactamases have been reported to function exclusively under strictly anaerobic conditions. This finding suggest that prior identification of β-lactamases in anaerobes may have incorrectly characterized enzymes as having poor hydrolysis rates due to the presence of oxygen in the assay (6).

Understanding that β-lactamases can be inactivated by oxygen increases the potential to discover many more β-lactamases from anaerobes. Furthermore, eliminating ambient air or adding reducing agents to antibiotic susceptibility tests of clinical isolates could improve the accuracy of these diagnostic tests and reduce false negative results.

II. *blaD* transcription and regulation

We demonstrated that *blaD* encodes the only functional β-lactamase of *C. difficile* (**Ch. 2, Fig. 5A**), and that expression of *blaD* is inducible up to 1000-fold by at least three classes of β-lactams, including penicillins and cephalosporins, the two classes that make up 30% and 18%, respectively, of all prescribed antibiotics in the U.S. (**Ch. 2 Figs. 2, S2, S3**) (7). Interestingly, although *blaD* is induced by many β-lactams, our growth curve and MIC data showed that this enzyme likely only provides *C. difficile* with resistance to penicillins (**Ch.2 Fig. 4, Table S1**). Because of this, we know that *C. difficile* possesses additional resistance mechanisms to survive β-lactam effects. An intriguing result of growth curves in imipenem was an advantage in growth of the *blaXD* mutant strain versus the parent strain, 630Δ*erm* (**Ch 2. Fig. 4C**). This result is consistent with the kinetic data that showed that BlaD binds to, but does not hydrolyze, carbapenems (5). Our data show that BlaD can sequester free imipenem, providing modest protection against PBP-cleavage (**Ch. 2 Fig. 4C**). This result further supports

the theory that β-lactamases evolved from PBPs, as they can both bind β-lactams, but β-lactamases evolved to hydrolyze β-lactams particularly well. In the case of BlaD in *C. difficile*, this enzyme did not evolve to hydrolyze carbapenems, similar to most class D β-lactamases (8).

The difference in broad specificity for induction of *blaD* versus the ability of BlaD to hydrolyze particular β-lactams lies in the regulation of *blaD* transcription. As is the case in other Gram-positive bacteria, *C. difficile* encodes BlaR and BlaI, two proteins that regulate expression of β-lactamases. BlaR is a sensor that localizes to the cell membrane and binds β-lactams. Upon β-lactam binding, BlaR cleaves dimerized BlaI, releasing BlaI from the promoter of β-lactamase- (and PBP) encoding genes (9-13). Because BlaR binding to β-lactam antibiotics is independent of β-lactamase function, BlaR has the potential for broader substrate recognition than the BlaD enzyme (14). Our data show that expression of *blaR* and *blaI* are induced by penicillins, cephalosporins, and carbapenems. The induction of *blaIR* transcription by various β-lactams strongly suggests that BlaR binds to the β-lactam ring (**Ch. 2 Fig. S10**). In contrast to the *blaD* mutant, the *blaIR* mutant demonstrates enhanced growth in β-lactams, further corroborating the repression of *blaD* transcription by BlaIR (**Ch. 2 Figs. 4, 8**). Additional experiments, such as transcriptional analyses (RNA-seq) of the *blaI* mutant, could be performed to identify the complete BlaI regulon, and potentially lead to the discovery of additional BlaIR regulated β-lactam resistance mechanisms.

III. BlaX and β-lactamase localization

A particularly intriguing feature of *blaD* is the localization of the gene within an operon with the putative membrane protein, *blaX* (**Ch. 2 Fig. S4**). This genomic

arrangement of a β-lactamase gene has only been observed in one other bacterial genome—the Gram-negative pathogen *Burkholderia pseudomallei.* In *B. pseudomallei,* a membrane-bound class A β-lactamase gene, *penA*, is co-transcribed downstream of *nlpD1*, which encodes an activator of periplasmic amidase that is involved in cell division (15, 16). BlaX does not have any apparant similarity to NlpD1. Further, a BLASTp search for BlaX orthologs outside of *Clostridia* did not reveal proteins with greater than 40% similarity to BlaX (17). A Phyre2 analysis of BlaX was inconclusive, due to the lack of similarity to known protein structures (12% coverage, with 37.4% confidence) (18). Our data show that in the 630Δ*erm* strain, BlaX is not required for β-lactamase activity, but BlaX increases the β-lactamase activity of BlaD by three-fold (**Ch. 2 Figs. 5B, 6A, B**). However, a comparison of β-lactamase activity between 630Δ*erm* and the *blaX* negative strain M120 revealed no significant differences (**Ch. 2 Fig. 6C, D**). Analysis of the promoter regions of *blaD* in these two strains revealed that the promoter regions are conserved, though M120 contains an extra approximately 200 nucleotides of the 5' and the 3' regions of *blaX*, joined together, between the promoter and *blaD* start codon. The ribosome-binding site for *blaXD* is also conserved in the M120 genome, but the 60 nucleotides that follow the rbs are not predicted to be translated, as the region is riddled with stop codons. This strongly suggests that the ancestral genome of M120, and other *C. difficile* strains that lack *blaX*, contained the entire operon with both *blaX* and *blaD*, but that in some isolates the majority of *blaX* was deleted, leaving an intact promoter driving a non functional protein, followed by *blaD*. A BLASTp search for BlaD in *C. difficile* returns 1747 isolates (>90% coverage and >80% identity), and 736 of those encode a BlaX protein (>97% coverage and >86%

identity) (17). This conservation of BlaX in 42% of *C. difficile* isolates implies an evolutionary conservation of the *blaX* gene, for yet to be uncovered reasons.

We observed that BlaD is mainly associated with the cell membrane (**Ch. 2 Fig. 6B, D**) and that a signal peptide is required for translocation of the protein to the cell surface (**Ch. 2 Fig 5B**). This is expected, given the localization of β-lactamase activity at the cell wall. Since β-lactams inhibit peptidoglycan synthesis, an effective hydrolase could be tethered to the cell membrane or secreted outside of the cell. Keeping an enzyme membrane-bound prevents diffusion of the enzyme into the surrounding environment and facilitates contact with the β-lactam substrate. Given that BlaD is membrane-associated, and that BlaX is a putative membrane protein, it is tempting to speculate that BlaD is tethered to the cell membrane via BlaX. A Phobius analysis of BlaX predicted four to five transmembrane domains, with a ~170 residue extra-cytoplasmic region between the fourth and fifth transmembrane regions (19). This region could interact with BlaD as a way to anchor BlaD to the membrane, and at the same time, maintain accessibility to the β-lactam substrates.

An alternative hypothesis is that BlaX acts as a substrate-binding partner to BlaD. An experiment that could be performed to determine whether BlaD and BlaX interact would be a split-luciferase assay (20). In this assay, each gene is fused to a part of the luciferase enzyme on a single plasmid, the plasmid is transformed into the *blaXD* mutant, cells are lysed, and luciferase activity is detected if these proteins interact with each other. If these two proteins do interact, purification and nitrocefin hydrolysis assays of BlaD, with and without the addition of BlaX, would test the hypothesis that BlaX acts as a substrate-binding partner.

Interestingly, alignments of protein sequences between strains that encode BlaX and those that do not, revealed that the N-terminus of BlaD demonstrates the greatest variability of this protein. The N-terminal variability could be due to simple divergence of this non-enzymatic region or changes due to signal sequence processing differences for strains lacking BlaX. One way to determine if the N-terminus is important for translocation or function of BlaD in different strains would be to swap the N-terminal domains of BlaD in BlaX+ and BlaX- strains, and measure β-lactamase activity in these backgrounds. The N-terminus of BlaD from strain M120 could replace the N-terminus of BlaD from strain 630Δ*erm*, and vice versa. Nitrocefin assays could then be performed to determine if the protein is able to reach the cell surface (intact cells), and if the overall level of activity is different between these chimeric enzymes (lysed cells).

IV. Final Summary

A *blaD* mutant of *C. difficile* does not exhibit decreased resistance to cephalosporins or carbapenems, strongly suggesting that other β-lactam resistance mechanisms, as yet undiscovered, contribute to the β-lactam resistance profile of *C. difficile*. It is known that 3-3 cross-links, created by L,D-transpeptidases, or LDTs, make up ~70% of *C. difficile* peptidoglycan, while 4-3 cross-links, created by D,D-transpeptidases, make up the remaining ~30% (21). D,D-transpeptidases, or PBPs, are the proteins traditionally targeted by β-lactams. Two of the three LDTs in *C. difficile*, however, are acylated by carbapenems (22). Whether the four known PBPs of *C. difficile* are targets of β-lactams is unknown. Further understanding of β-lactam targets in *C. difficile* could allow for the identification of the remaining β-lactam resistance mechanisms in *C. difficile*.

Overall, we have characterized the transcription and regulation of the *blaXD* operon, as well as characterized the localization, contribution to β-lactam resistance, and anaerobic function of the BlaD enzyme. Our data suggest that BlaD is associated with the cell membrane, but we have not shown whether the enzyme is anchored to the cell membrane or to the cell wall by non-canonical mechanisms. Furthermore, our data suggest that BlaD is not completely responsible for the β-lactam resistance observed in *C. difficile*, and likely only contributes to resistance to penicillins. Other resistance mechanisms likely include LDTs and/or PBPs that do not bind β-lactams and thus evade inhibition by these antibiotics. A better understanding of β-lactam resistance mechanisms may illuminate potential therapeutic targets to inhibit cell wall synthesis and reduce *C. diffiicile* infection or recurrence.

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