Intrinsic Class D β-Lactamases of Clostridium difficile

Marta Toth, Nichole K. Stewart, Clyde Smith, Sergei B. Vakulenko

ABSTRACT  Clostridium difficile is the causative agent of the deadly C. difficile infection. Resistance of the pathogen to β-lactam antibiotics plays a major role in the development of the disease, but the mechanism of resistance is currently unknown. We discovered that C. difficile encodes class D β-lactamases, i.e., CDDs, which are intrinsic to this species. We studied two CDD enzymes, CDD-1 and CDD-2, and showed that they display broad-spectrum, high catalytic efficiency against various β-lactam antibiotics, including penicillins and expanded-spectrum cephalosporins. We demonstrated that the cdd genes are poorly expressed under the control of their own promoters and contribute only partially to the observed resistance to β-lactams. However, when the cdd1 gene was expressed under the control of efficient promoters in the antibiotic-sensitive Clostridium cochlearium strain, it produced high-level resistance to β-lactams. Taken together, the results determined in this work demonstrate the existence in C. difficile of intrinsic class D β-lactamases which constitute a reservoir of highly potent enzymes capable of conferring broad-spectrum, clinically relevant levels of resistance to β-lactam antibiotics. This discovery is a significant contribution to elucidation of the mechanism(s) of resistance of the clinically important pathogen C. difficile to β-lactam antibiotics.

IMPORTANCE  C. difficile is a spore-forming anaerobic bacterium which causes infection of the large intestine with high mortality rates. The C. difficile infection is difficult to prevent and treat, as the pathogen is resistant to many antimicrobial agents. Prolonged use of β-lactam antibiotics for treatment of various infectious diseases triggers the infection, as these drugs suppress the abundance of protective gut bacteria, allowing the resistant C. difficile bacteria to multiply. While resistance of C. difficile to β-lactam antibiotics plays the major role in the development of the disease, the mechanism of resistance is unknown. The significance of our research is in the discovery in C. difficile of β-lactamases, enzymes that destroy β-lactam antibiotics. These findings ultimately can help to combat deadly C. difficile infections.

KEYWORDS  Clostridium difficile, antibiotic resistance, beta-lactamases

Clostridium difficile infection is a life-threatening disease that imposes a significant burden on health care. In 2011, the estimated number of C. difficile infections in the United States exceeded 450,000, resulting in 29,300 deaths (1) and in associated excess health care costs of $4.8 billion for acute care facilities alone (2–4). The number of incidents of C. difficile infections in both hospitals and the outpatient setting is steadily rising, with an average annual rate of 3.3% in the United States (5–8). As a result, the sum of charges for C. difficile infection-associated hospitalizations increased from $20.1 billion in 2005 to $31.4 billion in 2014 (8). Antimicrobial therapy plays a crucial role in the development of C. difficile infection (9–11) due to the ability of many antibiotics to suppress the abundance of the gut microbiota that protects the host from invasion by pathogenic bacteria (12–14). Resistance of C. difficile to antibiotics is an important contributor to the emergence of the disease. C. difficile is notorious for its resistance to antimicrobial agents and is listed by the Centers for Disease Control and Prevention as...
the pathogen that causes the highest antibiotic resistance threat in the United States (15). While several classes of antimicrobial agents have been implicated in outbreaks of *C. difficile* infection, β-lactam antibiotics are recognized as major causative agents of the disease (16–18). β-Lactams differ significantly in their levels of activity against *C. difficile* (19, 20), and those levels correlate with their ability to trigger the infection. β-Lactams with low potency against *C. difficile* and high activity against the protective gut microbiota represent the major risk factor (21–24). Although resistance of *C. difficile* to β-lactams is recognized as a leading contributor to the development of *C. difficile* infection, the underlying mechanisms of resistance are currently unknown (19, 20).

### RESULTS

*C. difficile* ATCC 43255 and *C. difficile* 630Δerm are resistant to β-lactam antibiotics. While published data cumulatively indicate that *C. difficile* is generally resistant to many β-lactams (19, 20), there have been relatively few studies on the susceptibility of the pathogen to these antimicrobial agents. To initiate studies of the mechanism(s) of resistance of *C. difficile* to β-lactam antibiotics, we evaluated MIC values of various β-lactam antibiotics against two clinically important clostridial isolates, *C. difficile* ATCC 43255 and *C. difficile* 630Δerm (25–27), and showed that the two isolates exhibit very similar broad antibiotic resistance profiles (Table 1). They were highly resistant to the expanded-spectrum cephalosporins cefotaxime, ceftriaxone, ceftazidime, and cefepime (MICs, 64 to 256 μg/ml), the cephamycin cephalosporin cefoxitin (MIC, 128 μg/ml), and the monobactam aztreonam (MIC, 2,048 μg/ml). The MICs of the penicillins ampicillin, penicillin G, and oxacillin, of the carbapenems imipenem and meropenem, and of the cephalosporin cephalothin were significantly lower. The MICs of oxacillin, cephalothin, and meropenem were 2-to-8-fold higher for *C. difficile* 630Δerm.

Genomes of *C. difficile* ATCC 43255 and *C. difficile* 630Δerm encode class D β-lactamases. Gram-positive bacteria manifest resistance to β-lactam antibiotics via two major mechanisms: production of antibiotic-degrading enzymes, namely, β-lactamases, and/or production of antibiotic-resistant targets, namely, penicillin-binding proteins (PBPs). Four molecular classes of β-lactamases (classes A through D) are widely disseminated in Gram-negative bacteria, while for a long time, only class A and B enzymes were known in Gram-positive microorganisms. Recently, we demonstrated that class D enzymes are also widely spread in the *Clostridiaceae*, *Bacillaceae*, and *Eubacteriaceae* families of phylum *Firmicutes* of Gram-positive bacteria (28).

In our effort to get insights into the mechanism of resistance of *C. difficile* to β-lactam antibiotics, we analyzed genomes of two strains, *C. difficile* ATCC 43255 and

### TABLE 1 MICs of β-lactam antibiotics against clostridial isolates

| Antibiotic | ATCC 43255 | C. difficile 630Δerm | C. cochlearium ATCC 17787 |
|------------|-------------|----------------------|-----------------|
| Parental strain | *cdd1* | *cdd2* | Δcdd2 | *cdd1* | *cdd2* | Δcdd2 |
| Ampicillin | 4 | 4 | 1 | 1 | 0.03 | 0.25 | 0.25 | 32 | 16 |
| Penicillin G | 4 | 4 | 1 | 1 | 0.015 | 0.25 | 0.25 | 32 | 16 |
| Oxacillin | 8 | 64 | 64 | 64 | 0.25 | 2 | 1 | 64 | 16 |
| Cephalothin | 16 | 32 | 32 | 32 | 0.12 | 0.5 | 0.25 | 32 | 16 |
| Cefotaxime | 128 | 128 | 128 | 128 | 1 | 2 | 2 | 128 | 64 |
| Ceftriaxone | 64 | 64 | 32 | 32 | 1 | 2 | 2 | 256 | 128 |
| Cefoxitin | 128 | 128 | 128 | 128 | 0.25 | 0.25 | 0.25 | 0.5 | 0.5 |
| Ceftazidime | 256 | 256 | 64 | 64 | 32 | 32 | 32 | 1,024 | 512 |
| Cefepime | 64 | 64 | 32 | 32 | 16 | 16 | 16 | 256 | 128 |
| Aztreonam | 2,048 | 2,048 | 2,048 | 2,048 | 512 | 512 | 512 | 512 | 256 |
| Imipenem | 4 | 4 | 4 | 4 | 0.03 | 0.06 | 0.06 | 0.12 | 0.12 |
| Meropenem | 2 | 4 | 4 | 4 | 0.007 | 0.015 | 0.015 | 0.03 | 0.03 |
C. difficile 630Δerm, and found that each contains only one open-reading frame annotated as a putative β-lactamase gene. The enzyme from C. difficile ATCC 43255 is 312 amino acid residues long, while that from C. difficile 630Δerm is slightly shorter (Fig. 1). Both harbor conserved amino acid sequence motifs (SXXK, YGN, KTG, and WXXG) characteristic of class D β-lactamases. We named the enzyme from C. difficile ATCC 43255 CDD-1 (an abbreviation from Clostridium difficile class D β-lactamase) and named the enzyme from C. difficile 630Δerm CDD-2.

CDD β-lactamases are intrinsic to C. difficile. To evaluate how widely CDD-type β-lactamases are spread among C. difficile, we analyzed around 800 completed genomic sequences of C. difficile and found that the vast majority of them harbor a single chromosomal gene for the CDD-type enzyme, showing that these enzymes are intrinsic to the pathogen. To appreciate the diversity of CDD-type β-lactamases on the amino acid sequence level, we utilized BLAST software from the NCBI for the search of homologous proteins using the sequence of the mature CDD-1 enzyme as the template. Results of this study showed that class D β-lactamases from C. difficile strains isolated from different parts of the world are very closely related, with >97% of them sharing at least 95% amino acid sequence identity.

To identify the most common substitutions in CDD β-lactamases, we analyzed the reported amino acid sequences that were identical in at least five different C. difficile strains. We found 477 such strains and subdivided their enzymes into two subgroups, the CDD-1-type and CDD-2-type subgroups (Table 2). We identified the CDD-1-type
enzymes in 47 strains. Of those, 41 strains encoded the CDD-1/H9252-lactamase, while the remaining strains encoded the CDD-1 mutant (CDD-1-M1) with a single C22Y amino acid substitution. This substitution is also present in enzymes of all other 430 strains encoding the CDD-2-type/H9252-lactamases. Among the strains harboring genes for the CDD-2-type enzymes, the vast majority (312) encoded the CDD-2/H9252-lactamase. All remaining strains encoded seven CDD-2-like enzymes that have from one to three amino acid substitutions compared to the amino acid sequence of the CDD-2/H9252-lactamase.

Our analysis also demonstrated that beyond the C. difficile species, the amino acid sequence similarity of CDD-1 to currently known class D enzymes is below 55%. These data indicated that CDD/H9252-lactamases have failed to spread to other bacteria and that they represent a unique family of closely related enzymes that are native to C. difficile.

C. difficile ATCC 43255 and C. difficile 630Δerm produce active CDD β-lactamases. To assess whether C. difficile ATCC 43255 and C. difficile 630Δerm produce active β-lactamases, we utilized a nitrocefin test. We observed a slight change in nitrocefin color in wells with both strains grown in the presence of antibiotics but not in their absence (Fig. 2A, second row). We then investigated whether the observed β-lactamase activity was due to production of the class D CDD-1 and CDD-2 enzymes or if β-lactamases of other classes are involved. Unlike serine β-lactamases of classes A and C, class D enzymes require posttranslational carboxylation of their active-site lysine.

![Image](https://example.com/image.png)

**FIG 2** Evaluation of β-lactamase activity. (A) Induction of β-lactamase activity in C. difficile (C. dif) ATCC 43255 and C. difficile 630Δerm by three representative antibiotics. Their activity increases in the presence of NaHCO₃ and is not inhibited by EDTA. (B) Distribution of β-lactamase between the bacterial cells and growth medium. (C) Expression of CDD-1 and CDD-2 enzymes from their own promoters in C. cochlearium ATCC 17787. The right panel shows 5-fold-concentrated cell pellets. (D) Expression of CDD-1 from two functional promoters in C. cochlearium ATCC 17787. (E) Loss of CDD-2 β-lactamase activity in C. difficile 630Δerm knockouts. Activity was measured in 5-fold-concentrated cell pellets. Abbreviations: BC, bacterial culture; NIT, nitrocefin; NA, no antibiotic; AMP, ampicillin; CTR, ceftriaxone; CAZ, ceftazidime; P, pellet; S, supernatant; CP, control pellet of the parental strain; IK, insertional knockout; DK, deletion knockout.
TABLE 3  Steady-state kinetic parameters of the CDD-1 β-lactamase

| Antibiotic      | \( k_{\text{cat}} \) (s⁻¹) | \( K_m \) (µM) | \( k_{\text{cat}}/K_m \) (M⁻¹ s⁻¹) |
|----------------|-----------------------------|----------------|-----------------------------------|
| Ampicillin     | 3.2 ± 0.1                   | 2.7 ± 0.7      | (1.2 ± 0.3) × 10⁶                 |
| Penicillin G   | 1.8 ± 0.1                   | <1             | >(1.8 ± 0.1) × 10⁶                |
| Oxacillin      | 2.9 ± 0.1                   | 1.6 ± 0.3      | (1.8 ± 0.3) × 10⁶                 |
| Cephalothin    | 0.88 ± 0.02                 | 0.8 ± 0.2      | (1.2 ± 0.2) × 10⁶                 |
| Cefoxitin      | 0.009 ± 0.001               | 40 ± 3         | (2.3 ± 0.3) × 10²                 |
| Ceftazidine    | 21 ± 1                      | 110 ± 10       | (1.9 ± 0.2) × 10³                 |
| Cefepime       | 10.5 ± 0.2                  | 23 ± 1         | (4.5 ± 0.2) × 10⁵                 |
| Ceftriaxone    | 12.0 ± 0.4                  | 7.6 ± 0.9      | (1.6 ± 0.2) × 10⁶                 |
| Cefotaxime     | 12.0 ± 0.2                  | 7.3 ± 0.7      | (1.6 ± 0.2) × 10⁶                 |
| Aztreonam      | 20 ± 1                      | 40 ± 3         | (5.0 ± 0.5) × 10³                 |
| Imipenem       | 0.044 ± 0.001               | 4.7 ± 0.4      | (9.4 ± 0.8) × 10³                 |
| Meropenem      | 0.005 ± 0.001               | <4             | >(1.3 ± 0.3) × 10³                |
| Nitrocefin     | 40 ± 1                      | 5.8 ± 0.8      | (6.9 ± 1) × 10⁶                   |

for catalysis (29). This lysine may undergo decarboxylation under experimental conditions, resulting in a precipitous decrease in the catalytic activity of the enzyme. The activity can be restored by addition of a CO₂ source such as sodium bicarbonate. We monitored hydrolysis of nitrocefin in the presence of 50 mM sodium bicarbonate. We observed a significant change in color when bacteria were grown in the presence of each of the β-lactams that we tested (except for cefoxitin), while the color of antibiotic-free medium changed only slightly (Fig. 2A, third row). These data indicated that both C. difficile ATCC 43255 and C. difficile 630Δerm produce an inducible class D β-lactamase.

We also evaluated whether metallo β-lactamases contribute to the observed change in the nitrocefin color. The presence of EDTA, a chelating agent that strongly inhibits the activity of class B metalloenzymes, did not prevent or delay the nitrocefin reaction, which excludes the possibility of a contribution of metallo β-lactamases to nitrocefin hydrolysis (Fig. 2A, fourth row).

In Gram-positive bacteria, β-lactamases can be anchored to the bacterial cell wall and/or excreted to the milieu. We investigated whether the CDD-1 and CDD-2 enzymes are secreted or attached to the cell wall. We pelleted bacteria by centrifugation, saved the growth medium, and resuspended the pellets in a phosphate buffer. The nitrocefin color change in C. difficile ATCC 43255 was mostly associated with bacterial cell pellets, while the C. difficile 630Δerm color changes were similar in the cell pellets and supernatant (Fig. 2B). These data showed that more CDD β-lactamase is cell wall associated in C. difficile ATCC 43255 than in C. difficile 630Δerm. Cell wall association also has been demonstrated for other classes of β-lactamases from Gram-positive and Gram-negative bacteria, where the enzymes are often anchored to the membrane (30–33).

The CDD-1 β-lactamase displays broad-spectrum activity. To evaluate the substrate profile of CDD enzymes, we purified the CDD-1 β-lactamase to homogeneity and undertook kinetic studies. CDD-1 exhibited high catalytic efficiency for the three penicillins (\( k_{\text{cat}}/K_m \) 1.2 × 10⁶ to >1.8 × 10⁶ M⁻¹ s⁻¹; Table 3) and for five of the six cephalosporins (\( k_{\text{cat}}/K_m \) 1.9 × 10⁵ to 1.6 × 10⁶ M⁻¹ s⁻¹), with the exception of cefoxitin. The catalytic efficiency determined for the monobactam aztreonam (\( k_{\text{cat}}/K_m \) 5.0 × 10⁵ M⁻¹ s⁻¹) was similar to that determined for the penicillins and cephalosporins, while that determined for the carbapenems imipenem and meropenem was 2 orders of magnitude lower. CDD-1 had high apparent affinity levels (\( K_m \) <1 to 2.7 µM) and relatively low turnover numbers (\( k_{\text{cat}} \) 1.8 to 3.2 s⁻¹) for all penicillins tested. For the cephalosporins ceftriaxone, cefotaxime, and cefepime, turnover numbers were 4-to-8-fold higher and the apparent affinity decreased by up to 23-fold compared to penicillins. The steady-state kinetic parameters for aztreonam were similar to those for expanded-spectrum cephalosporins, while very low turnover rates corresponding to \( k_{\text{cat}} \) values of 0.044 and 0.005 s⁻¹ were observed for imipenem and meropenem, respectively. Our kinetic studies demonstrated that CDD-1 is an efficient broad-
We have shown is highly sensitive to most enzymes in a heterologous clostridial host, intrinsically resistant to expanded-spectrum cephalosporins, we expressed the CDD helicase gene in \textit{Clostridium cochlearium} ATCC 17787. The resulting low MIC values for the majority of \(-\text{lactam antibiotics (ampicillin, oxacillin, cefotaxime, and }
\textit{ceftriaxone)} and found they were almost identical to those of CDD-1 (data not shown).

\textbf{The genes for CDD-1 and CDD-2 \(-\text{lactamases express poorly under the control of their own promoters.} \) To test the expression levels of the \textit{cdd1} and \textit{cdd2} genes under the control of their own promoters, we analyzed the genetic composition of their upstream regions. The \textit{cdd1} gene is flanked upstream by the gene for an ATP-dependent DNA helicase \textit{(hlc)} and downstream, \textit{hlc} gene (an excinuclease ABC subunit A paralog of unknown function \textit{[ecn]} and a putative membrane protein \textit{[pmp]}). As a result, the intergenic region between the \textit{cdd} gene and the upstream gene changed from 540 bp for \textit{cdd1} to 27 bp for \textit{cdd2}.

We then introduced the pMTL83151::\textit{cdd1} and pMTL83151::\textit{cdd2} constructs in \textit{C. cochlearium} ATCC 17787, where they were stably maintained. Expression of the \textit{cdd1} and \textit{cdd2} enzymes in the new host was not inducible by \(-\text{lactam antibiotics (ampicillin, oxacillin, cefotaxime, and }
\textit{ceftriaxone)} and were almost identical to those of CDD-1 (data not shown).

\textbf{CDD-1 \(-\text{lactamase produces high-level resistance to \(-\text{lactam antibiotics when expressed from efficient promoters.} \) Next, we investigated whether CDD \(-\text{lactamases are capable of producing high-level resistance to \(-\text{lactam antibiotics when expressed from known functional clostridial promoters.} \) To accomplish this goal, we placed the gene for the CDD-1 \(-\text{lactamase encoding the full-length enzyme under the control of the \textit{Pthl} and \textit{Pfdx} promoters, which are often utilized for expression of clostridial genes in various clostridial species (34, 35).} \) The resulting constructs were cloned into shuttle vectors to produce the pMTL83122::\textit{cdd1-Pthl} and pMTL83123::\textit{cdd1-Pfdx} vectors, which were introduced into \textit{C. cochlearium} ATCC 17787. The nitrocefin color change seen with these constructs was significantly more intense than what we observed with CDD-1 expressed under the control of its own promoter (Fig. 2D and C). As in the case of \textit{C. difficile} ATCC 43255, hydrolysis of nitrocefin was more efficient.
in cell pellets of \( C. \) cochlearium than in the supernatants, an indication that the two bacterial species process the CDD-1 \( \beta \)-lactamase in the same way (Fig. 2D).

Expression of CDD-1 from the \( \text{Pthl} \) and \( \text{Pfdx} \) promoters (\( \text{Pthl cdd1} \) and \( \text{Pfdx cdd1} \)) rendered \( C. \) cochlearium highly resistant to the majority of \( \beta \)-lactam antibiotics tested (Table 1). Overall, the \( \text{Pthl} \) promoter provided 2-fold-higher levels of resistance. \( \text{Pthl cdd1} \) increased the MICs of ampicillin, penicillin G, and oxacillin 256-fold to 2,048-fold (to 32 to 64 \( \mu \text{g/ml} \)). It also significantly increased the MICs of the majority of cephalosporins tested. The parental \( C. \) cochlearium ATCC 17787 strain is highly sensitive to cephalosporins cephalothin, cefotaxime, ceftiraxone, and cefoxitin. \( \text{Pthl cdd1} \) increased the MIC values of the first three of these drugs 128-fold to 256-fold. Noticeably, the MICs of cefoxitin remained practically unchanged, an indication that this antibiotic is not a substrate for the \( \beta \)-lactamase. The two remaining cephalosporins, ceftazidime and cefepime, had much higher MICs against the parental \( C. \) cochlearium ATCC 17787 strain. Expression of the CDD-1 \( \beta \)-lactamase from the \( \text{Pthl} \) promoter further increased the MICs of these antibiotics to 1,024 and 256 \( \mu \text{g/ml} \), respectively. Expression of CDD-1 failed to further increase the high MICs of aztreonam. The MICs of meropenem and imipenem increased 4-fold but remained well below clinically significant levels.

**Inactivation of CDD-2 \( \beta \)-lactamase decreases resistance of \( C. \) difficile to \( \beta \)-lactam antibiotics.** To further evaluate the contribution of clostridial \( \beta \)-lactamases to the observed resistance to \( \beta \)-lactam antibiotics, we attempted to inactivate the genes for the CDD-1 and CDD-2 \( \beta \)-lactamases. Creating gene knockouts in \( C. \) difficile is challenging, as some \( C. \) difficile strains are minimally amenable to genetic manipulations, while the majority are not (36, 37). To inactivate the \( \text{cdd1} \) and \( \text{cdd2} \) genes, we utilized two recently developed methodologies. One of the two, represented by the CloStron system, utilizes a group II intron to create insertions into the gene of interest to cause its inactivation (38). The other employs a two-step allele-exchange methodology (36) to generate targeted deletions of genetic material. Our numerous attempts to knock out the \( \text{cdd1} \) gene failed due to the extreme instability, in \( C. \) difficile ATCC 43255, of the vectors used for generation of knockouts. We succeeded, however, in creating the \( \text{cdd2} \) gene knockout in \( C. \) difficile 630\( \Delta \text{erm} \) using both approaches. Inactivation of the gene abolished the \( \beta \)-lactamase activity as confirmed by the nitrocefin test (Fig. 2E). The MICs of \( \beta \)-lactams against \( C. \) difficile 630\( \Delta \text{erm} \) strain harboring either of the two generated \( \text{cdd2} \) gene knockouts (\( \text{cdd2} \) intron and \( \Delta \text{cdd2} \)) were identical (Table 1). We observed a 4-fold decrease in the MIC values for ampicillin, penicillin G, and ceftazidime and a 2-fold decrease for ceftiraxone and cefepime, while the MICs of other antibiotics remained unchanged. This correlates with poor expression of the gene under the control of its own promoter, which we also observed in \( C. \) cochlearium. These data demonstrated that while \( C. \) difficile 630\( \Delta \text{erm} \) encodes a class D \( \beta \)-lactamase that has high catalytic activity against various \( \beta \)-lactam substrates, the enzyme is poorly expressed in this strain and responsible only partially for its resistance to these antibiotics. Results of our studies also indicate that an additional, as-yet-unidentified mechanism in \( C. \) difficile 630\( \Delta \text{erm} \) contributes significantly to the observed high-level resistance to \( \beta \)-lactams.

We then aimed to reintroduce the \( \text{cdd2} \) gene into the \( C. \) difficile 630\( \Delta \text{erm} \) strain harboring the \( \Delta \text{cdd2} \) gene knockout and to evaluate the effect on the produced resistance. For this purpose, we used the pMTL83151:::*\( \text{cdd2} \) construct, where the gene is expressed under the control of its own promoter, and also constructed the pMTL83122::\( \text{cdd2-Pthl} \) plasmid, where the \( \text{cdd2} \) gene is expressed under the control of the efficient \( \text{Pthl} \) promoter. We then measured the MICs of the two \( \beta \)-lactams, ampicillin and ceftriaxone, against the resulting transconjugants. For the transconjugant harboring the pMTL83151:::*\( \text{cdd2} \) plasmid, we observed only a 2-fold increase in the MICs for one of antibiotics, ampicillin, while for the transconjugant carrying the pMTL83122::\( \text{cdd2-Pthl} \) construct, the MIC of ampicillin increased 16-fold and that of ceftriaxone 2-fold (data not shown). These data clearly show that the \( \text{cdd2} \) gene is expressed more efficiently under the control of the \( \text{Pthl} \) promoter than under that of its own promoter, as was also the case with the \( \text{cdd1} \) gene in \( C. \) cochlearium. The lower magnitude of the
increase in antibiotic resistance in *C. difficile* could have resulted from both its significant level of residual β-lactam resistance and the high degree of instability of the constructs that we observed in this host.

**DISCUSSION**

In our efforts to elucidate the mechanism of resistance of *C. difficile* to β-lactam antibiotics, we discovered that this pathogen encodes intrinsic class D β-lactamases which share a high level of identity of their amino acid sequences. Antibiotic susceptibility testing of two clostridial isolates, *C. difficile* ATCC 43255 and *C. difficile* 630Δerm, showed that they are resistant to expanded-spectrum cephalosporins and the cephamycin cefoxitin and are highly resistant to the monobactam aztreonam but are sensitive to both penicillins and carbapenems. Our kinetic studies of CDD β-lactamases demonstrated that both CDD-1 and CDD-2 possess high catalytic activity against a wide spectrum of β-lactam antibiotics. Comparison of the antibiotic resistance and catalytic profiles of CDD-1 revealed some noticeable discrepancies between them (Tables 1 and 3). While the pathogen was highly resistant to cefoxitin, CDD-1 had very low catalytic efficiency against this substrate, which indicates that the high MIC of cefoxitin likely resulted from low affinity of the drug to its target PBP. The opposite trend was observed for ampicillin, penicillin G, oxacillin, and cephalothin. Although CDD-1 has high catalytic efficiency against these substrates, *C. difficile* ATCC 43255 is relatively sensitive to these antibiotics. Such sensitivity could result from poor expression of the CDD-1 β-lactamase.

To validate this assumption, we knocked out the *cdd2* gene and observed only moderate decreases in the MICs of β-lactams. Subsequent reintroduction of the *cdd2* gene under the control of its own and the strong *Pthl* promoters confirmed that the β-lactamase is indeed poorly expressed under the control of its own promoter. In addition, we expressed the genes for the CDD-1 and CDD-2 β-lactamases in the highly β-lactam-sensitive *C. cochlearium* ATCC 17787 strain and found that both enzymes were also expressed poorly from their own promoters in this bacterium.

Our study also demonstrated that the catalytic efficiency or substrate profile or both of the CDD enzymes are unique among class D β-lactamases. Although CDD-1 has a substrate profile similar to that of the only other characterized class D β-lactamase from Gram-positive bacteria, BPU-1, it has superior (up to 200-fold-higher) catalytic efficiency against expanded-spectrum cephalosporins and aztreonam (28). While the substrate profile of CDD-1 is much broader than that of mutants of OXA-2 and OXA-10 β-lactamases from *Pseudomonas aeruginosa* (39, 40), it is more similar to those of some mutant class D β-lactamases from *Acinetobacter baumannii* (41–44). However, as with BPU-1, the catalytic activity of CDD-1 is much higher. Our analysis demonstrates that, among characterized class D β-lactamases, CDD-1 overall displays the broadest spectrum and the highest level of activity against expanded-spectrum cephalosporins and aztreonam. These results strongly indicate that active site of CDD-1 has a unique architecture that evolved to accommodate and efficiently hydrolyze a wide spectrum of β-lactam substrates.

Combined, our experiments showed that *C. difficile* strains produce intrinsic broad-spectrum CDD β-lactamases. In the two studied strains, the enzymes are poorly expressed and responsible only partially for the observed β-lactam resistance. This implies that these strains also employ an additional, non-CDD β-lactamase-mediated mechanism(s) of resistance. Importantly, our studies demonstrated that the CDD β-lactamases are very potent enzymes, and when one of them, CDD-1, was supplied with functional promoters, it produced high-level, broad-spectrum resistance to β-lactams. Further experiments are warranted to determine whether CDD β-lactamases with efficient promoters are circulating in any of the multiple clinical *C. difficile* isolates. In fact, acquisition of strong promoters has already been well documented with class D β-lactamase genes of *Acinetobacter baumannii*. Originally, these genes were poorly expressed and were of no clinical concern. However, over time, they acquired stronger promoters supplied by insertion sequences, which rendered *A. baumannii* highly...
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resistant to carbapenem antibiotics. As C. difficile also harbors multiple mobile genetic elements, which constitute up to 11% of its genome (45), the acquisition of strong promoters by the cdd genes is highly likely. More-efficient promoters would provide even higher levels and broader spectra of resistance to β-lactam antibiotics. Indeed, clinical C. difficile isolates with such high levels of resistance to β-lactams have already been reported (20, 46, 47). Regardless, these intrinsic enzymes pose a serious potential threat, as they are fully capable of producing a high level of resistance to clinically important antibiotics, which would further complicate the already difficult tasks of preventing and treating C. difficile infection.

MATERIALS AND METHODS

**Bacterial strains.** E. coli DH10B (New England Biolabs) was used in cloning experiments. E. coli CA434 (CHAIN Biotech, United Kingdom) was used as the donor strain for mobilization of shuttle vectors into clostridial strains. C. difficile 630Δerm (CHAIN Biotech, United Kingdom) and C. difficile ATCC 43255 are known toxigenic isolates, widely used to study various aspects of C. difficile infection. C. cochlearium ATCC 17787 was used for expression of cdd genes. All clostridial isolates were grown under anaerobic conditions at 37°C.

**Antibiotic susceptibility testing.** MICs of β-lactam antibiotics against clostridial strains were determined according to Clinical and Laboratory Standards Institute guidelines (M11-A8) (48). Briefly, the broth microdilution method was used with 96-well plates. Serial dilutions of antibiotics were performed in supplemented Brucella medium, which subsequently was inoculated with the final bacterial inoculum of 10^5 CFU/mL. Plates were incubated for 44 to 48 h at 37°C in an anaerobic box prior to recording results. All MIC experiments were performed at least in triplicate.

**Identification of β-lactamases in C. difficile.** To identify β-lactamases in C. difficile ATCC 43255 and C. difficile 630Δerm, we analyzed their genomic sequences (available at https://www.ncbi.nlm.nih.gov). Visual inspection of the amino acid sequences of these enzymes was used to identify conserved motifs of the class D β-lactamases. Around 800 completed genomic sequences of C. difficile (available at www.paticbrc.org) were analyzed to appreciate the spread and diversity of CDD enzymes.

**Nitrocefin test.** To assess whether clostridia produced active β-lactamases, we utilized the chromogenic cephalosporin nitrocefin, which changes color from yellow to dark red upon hydrolysis. Overnight bacterial cultures were used for the analysis of bacterial cultures, pellets resuspended in phosphate buffer, and supernatants. Reactions performed with nitrocefin (200 μM) were monitored for up to 60 min. Sodium bicarbonate and EDTA were used at 50 mM and 10 μM, respectively, where indicated.

**Cloning procedures.** For expressing the CDD-1 β-lactamase in C. cochlearium ATCC 17787, we placed the full-length cdd1 gene under the control of either the Pthl promoter or Pfdx promoter (34, 35) by cloning it between the NdeI and HindIII restriction sites of the pMTL83122 and pMTL83123 shuttle vectors (49) to generate pMTL83122::cdd1 and pMTL83123::cdd1, respectively.

To evaluate whether there are functional promoters upstream of the cdd1 and cdd2 genes, we PCR amplified DNA fragments containing the entire genes and their 182-bp and 177-bp 5′ flanking regions, respectively. We subsequently cloned the amplified fragments between the BamHI and HindIII restriction sites of the pMTL83151 vector to result in pMTL83151::cdd1 and pMTL83151::cdd2, respectively.

To construct the cdd1 and cdd2 gene knockouts, we utilized two alternative methodologies. The Clostron system achieves gene inactivation by the insertion of a group II intron (38), while a two-step allele exchange technique generates targeted deletions of genetic material (36). For the insertional mutagenesis, the introns were retargeted using an online tool (www.clostron.com), custom synthesized, and inserted into the pMTL007C-E5 vector (ATUM, CA), resulting in pMTL007C-E5::Cdi-cdd1-284a and pMTL007C-E5::Cdi-cdd2-272a. For the deletional inactivation of the cdd1 and cdd2 genes, we amplified regions upstream (left arm) and downstream (right arm) of the gene fragment to be deleted. The left arm included the first 30 bp of the genes and 759 bp of their upstream region. The right arm consisted of the 511 bp of the 3′ region of the cdd genes plus 560 bp downstream. Use of this construct is expected to create deletions of 398 bp and 386 bp in the cdd1 and cdd2 genes, respectively. The arms were ligated, and the product was cloned into the Pmel restriction site of the pMTL-SC7315 vector to result in pMTL-SC7315::Δcdd1 and pMTL-SC7315::Δcdd2.

To produce a large amount of the CDD-1 β-lactamase, the gene for the mature enzyme was custom synthesized (GenScript) and cloned into the NdeI and HindIII sites of the pET-24a(+) expression vector (Novagen). The mature enzyme lacks the first 58 N-terminal amino acids, whose deletion is necessary for the optimal expression of the enzyme, as we have shown previously for the BPU-1 class D β-lactamase from another Gram-positive bacterium, Bacillus pumilus (28).

**Conjugation experiments.** Various shuttle vectors were delivered to clostridial cells using an established protocol (49). Briefly, 1 ml of overnight culture of donor E. coli CA434 culture harboring a shuttle vector was pelleted and resuspended in 200 μl of the appropriate C. difficile or C. cochlearium recipient strains. The bacterial suspension was spotted onto the surface of brain heart infusion-supplemented (BHIS) agar and incubated anaerobically overnight at 37°C. Next, grown cells were resuspended in 500 μl BHIS medium and spread on selective plates to recover transconjugants. For C. cochlearium, selection was modified by using 12 μg/ml aztreonam instead of cefoxitin. After 3 days of incubation and subsequent restreaking, individual colonies were grown in selective BHIS medium overnight. DNA was isolated using a DNeasy blood & tissue kit (Qiagen) and analyzed by PCR to identify transconjugants.
Inactivation of cdd1 and cdd2 genes using ClosTron mutagenesis. The mutagenesis procedure was performed as described earlier (37). Briefly, following introduction of pMTL007C-E5::Cdi-cdd1-284a and pMTL007C-E5::Cdi-cdd2-272a into C. difficile ATCC 43255 and C. difficile 630Δerm, respectively, the transconjugants were selected on BHI agar supplemented with 20 μg/ml lincomycin and 250 μg/ml d-cycloserine overnight at 37°C. Next, 120 individual colonies were restreaked onto the same medium, and the resulting individual colonies were grown in BHI agar supplemented with 20 μg/ml lincomycin for DNA isolation. Insertion of the intron into the cdd genes was verified by PCR and sequencing.

Inactivation of cdd1 and cdd2 genes by using the two-step allele exchange procedure. The protocol was used as previously described (36). pMTL-SC7315::Δcdd1 and pMTL-SC7315::Δcdd2 were introduced into C. difficile ATCC 43255 and C. difficile 630Δerm, respectively, by conjugation. The transconjugants were restreaked onto TCC plates (BHI agar supplemented with 15 μg/ml thiamphenicol, 10 μg/ml cefoxitin, and 250 μg/ml d-cycloserine), and the DNA from larger colonies was isolated and checked by PCR for integration of the plasmid into the chromosome. An integrant clone was then restreaked onto nonselective BHI agar plates, and the cells were harvested in 0.5 ml phosphate-buffered saline (PBS) after 3 to 4 days of incubation and spread onto C. difficile minimal medium (CDMM) plates supplemented with 75 μg/ml 5-fluorocytosine after serial 102 to 105 dilutions. Plates with individual colonies were replica plated onto BHI agar with and without 15 μg/ml thiamphenicol to identify clones that lost the plasmid. Deletion of the cdd genes was confirmed by PCR and sequencing.

Protein expression and purification. For expression of the CDD-1 enzyme, BL21(DE3) cells harboring the plasmid with the cdd1 gene were grown at 37°C with shaking to an optical density at 600 nm (OD600) of 0.6. Expression was induced with 0.5 mM isopropyl β-D-thiogalactoside (IPTG), and the cells were grown overnight at 22°C. The cells were harvested by centrifugation, resuspended in 25 mM HEPES (pH 7.0), 1 mM EDTA, and 0.2 mM dithiothreitol (DTT), and disrupted by sonication. Subsequently, the lysate was centrifuged at 32,000 rpm at 4°C and the supernatant was loaded onto a DEAE anion exchange column (Bio-Rad) pre-equilibrated with the same buffer. The protein was eluted with a 0 to 250 mM NaCl gradient. The enzyme was concentrated using a Centricon Plus 70 concentrator (Millipore), and dialyzed against 25 mM HEPES (pH 7.0) using a 10-kDa-molecular-weight-cutoff (MWCO) membrane (Spectrum).

Enzyme kinetics. All data were collected on a Cary 60 spectrophotometer (Agilent). Reaction conditions were identical to those previously reported for class D β-lactamases (50). Observed rates were calculated from the linear phase of each reaction and fitted to the Michaelis-Menten equation using the same software.

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