cfr(B), cfr(C), and a new cfr-like gene, cfr(E), in Clostridium difficile strains recovered across Latin America

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ABSTRACT

Cfr is a radical S-adenosyl-L-methionine (SAM) enzyme that confers cross-resistance to antibiotics targeting the 23S rRNA through hypermethylation of nucleotide A2503. Three cfr-like genes implicated in antibiotic resistance have been described, of which cfr(B) and cfr(C) have been sporadically detected in Clostridium difficile. However, the methylase activity of Cfr(C) has not been confirmed. We found cfr(B), cfr(C), and a cfr-like gene that shows only 51-58% of protein sequence identity to Cfr and Cfr-like enzymes in clinical C. difficile isolates recovered across nearly a decade in Mexico, Honduras, Costa Rica, and Chile. This new determinant was termed cfr(E). In agreement with the anticipated function of the cfr-like genes detected, high minimum inhibitory concentrations of drugs from four groups of antibiotics targeting the ribosomal peptidyl transferase center were recorded for the isolates. In addition, in vitro assays confirmed that purified Cfr(C) and Cfr(E) methylate Escherichia coli and, to a lesser extent, C. difficile 23S rRNA fragments at the expected positions. The analyzed isolates do not have mutations in 23S rRNAs genes or genes encoding the ribosomal proteins L3 and L4 and lack poxA, optrA, and pleuromutilin resistance genes. Moreover, their cfr-like genes were found into Tn6218-like transposons or ICE-elements that could facilitate their transfer. These results indicate selection of potentially mobile cfr-like genes in C. difficile from Latin America and provide the first assessment of the methylation activity of Cfr(C) and Cfr(E), which belong to a cluster of Cfr-like proteins that does not include the functionally characterized enzymes Cfr, Cfr(B), and Cfr(D).

KEYWORDS

C. difficile, Cfr, Cfr(B), Cfr(C), Cfr(E), 23S rRNA methylation, PhLOPS\textsubscript{A} phenotype

1. Introduction

The bacterial ribosome is one of the most common targets for antibiotics of clinical and veterinary relevance. Resistance to ribosome-targeting antibiotics occurs primarily through modification of drug’s binding sites, specifically through mutation or modification of ribosomal RNAs (rRNA) or proteins\textsuperscript{1}. Several rRNA modifying enzymes implicated in antibiotic resistance have been discovered\textsuperscript{2}, and
among them, the radical S-adenosyl-L-methionine (SAM) enzyme Cfr is noteworthy because it provides 
cross-resistance to Phenics (e.g. thiamphenicol), Lincosamides (e.g. clindamycin), Oxazolidinones 
(e.g. linezolid), Pleuromutilins (e.g. tiamulin), and Streptogramin A (e.g. virginiamycin M1) through C8 
methylation of the A2503 residue in 23S rRNA (Escherichia coli numbering), which is located in the 
peptidyl transferase center (PTC) of the bacterial ribosome. In addition to this so-called PhLOPSA 
phenotype, Cfr-mediated methylation leads to resistance to 16-member macrolides, the aminocyclitol 
hygromycin A, and the nucleoside antimicrobial agent A201A.

cfr and cfr-like genes are typically found on mobile genetic elements (MGEs). Moreover, since cfr 
acquisition exhibits low fitness costs, the spread of these genes threatens the utility of PTC-targeting 
antibiotics in the clinic. The cfr gene was first discovered on a Staphylococcus sciuri plasmid, but it is 
nowadays found in nearly twenty different genetic contexts in isolates of Enterococcus, Bacillus, 
Proteus vulgaris, Escherichia coli, Macrococcus caseolyticus, Jeotgalicoccus pinnipedialis, and 
Streptococcus suis from Europe, Latin America, USA, and Asia. Homologues of cfr have been 
identified in non-pathogenic Bacillales and three additional cfr-like genes sharing less than 80% 
protein sequence identity to Cfr have been described in Clostridium and Enterococcus. These genes 
are known as cfr(B), cfr(C), and cfr(D).

In C. difficile, cfr(B) was first detected in strain 11140508 contained within Tn6218-like elements. Afterwards, Candela et al. defined cfr(C) after analysis of C. difficile T10 and found it in three types of 
integrative and conjugative elements (ICEs) in several strains, including the non-toxigenic strain C. 
difficile F548. Subsequently, Hansen and Vester demonstrated by primer extension that a codon-
onimized version of cfr(B) of C. difficile 11140508 modifies A2503 in 23S rRNA when expressed in E. 
coli. Equivalent evidence is missing for Cfr(C), though it has been shown to confer PhLOPSA 
resistance upon introduction into the linezolid-susceptible strain C. difficile 630Δerm.

Despite its confirmed utility to prevent C. difficile infections (CDI) in patients with ventilator associated 
pneumonia and to reduce C. difficile toxin gut levels in a mice model, linezolid is not used to treat 
CDI. Nonetheless, the closely related antibiotic cadazolid inhibits moxifloxacin-resistant C. difficile
NAP1/027 strains without affecting gut commensals\textsuperscript{16} and though it did not pass a Phase III trial\textsuperscript{17}, novel oxazolidinones to treat CDI may appear in the future. Based on the potential utility of oxazolidinones in CDI therapy and the wide use of linezolid in Latin America for treatment of MRSA and VRE infections, we investigated seven clinical \textit{C. difficile} isolates with predicted rRNA dimethylases to determine whether they carry functional \textit{cfr} or \textit{cfr}-like genes. To this end, we determined minimum inhibitory concentrations (MICs) of PTC-targeting antibiotics from four different groups and evaluated the \textit{in vitro} activity of their Cfr-like enzymes, including a new determinant termed Cfr(E).

\section*{2. Methods}

\subsection*{2.1. Strains}
This study included ribotype- or PFGE-confirmed NAP1/027 clinical isolates from Mexico (DF11), Honduras (HON06, HON10, HON11) and Chile (PUC51, PUC347), and one NAP\textsubscript{CR1}/012 isolate from Costa Rica (LIBA5707). These bacteria were recovered between 2009 and 2016 from stool samples of human patients and were selected among ca. 450 sequenced \textit{C. difficile} isolates from Latin America because an automated annotation indicated that their genomes include sequences for putative rRNA dimethylases (\textsuperscript{18,19}, unpublished data). With a single exception (DF11, recovered from a 3-years old patient with diarrhea), all isolates were obtained from adults with CDI. Moreover, DF, PUC, and LIBA isolates were obtained during confirmed CDI outbreaks. \textit{C. difficile} LIBA5701 was used as a negative control in the determinations of minimum inhibitory concentrations (MICs) because it is a NAP\textsubscript{CR1}/012 strain that naturally lacks MGEs with \textit{cfr}-like genes and therefore does not display a PhLOPS\textsubscript{A} phenotype (see below)\textsuperscript{18}.

\subsection*{2.2. Detection of \textit{cfr}-like and other resistance genes}
Whole genome sequences (WGS) were obtained by sequencing-by-synthesis using multiplexed paired-end libraries and HiSeq2000 or Miseq instruments (Illumina). After trimming with sickle (https://github.com/najoshi/sickle), reads were assembled using Spades v.3.12\textsuperscript{20} and annotated with Prokka v.1.13\textsuperscript{21}. The identity of resistance genes identified by automated annotation or with Abricate
was confirmed using resfinder, the CARD database v.3.0.1, and through BLAST, BLASTP, eggNOG3, UniProt, and Structure Function Linkage Database (SFLD) searches. Trimmed reads and assemblies for the DF and HON isolates can be downloaded from the MicrobesNG portal (https://microbesng.uk/portal/projects/405FF6AC-A5E0-E04A-AECF-A5C9371B8B60/). Data for isolates PUC51 and PUC347 can be retrieved using the NCBI accession numbers CAADRH000000000 and CAADRI000000000, respectively, and raw data for LIBA5707 is available at the European Nucleotide Archive (run ERR467555).

2.3 MIC determinations

MICs of linezolid were obtained by epiillumetry with strips containing 0.016 to 256 µg/ml concentration gradients (Liofilchem). Tiamulin and thiamphenicol MICs were determined by agar macrodilution (1-256 µg/ml in BHI plates) and virginiamycin M1 was tested by broth microdilution (1-320 µg/ml in Brucella broth). These antibiotics are not recommended for *C. difficile* treatment; hence no breakpoints for susceptibility categorization are available. *C. difficile* ATCC 70057 (linezolid*) was tested for quality control purposes.

2.4. Comparison of RlmN and Cfr protein sequences

Though both RlmN and Cfr modify A2503, the former is a housekeeping gene and the latter an acquired antibiotic resistance gene. To examine the phylogenetic relationship between Cfr-like sequences mentioned in this study to Cfr and RlmN sequences, Cfr-like and RlmN-like orthologs from selected Firmicutes species were retrieved from the Integrated Microbial Genomes-Joint Genome institute (IMG/JGI) database by BLAST using the RlmN sequence from *Bacillus subtilis* as a query, as done elsewhere (Supplementary Table 1). Additional RlmN/Cfr paralogous sequences from *Paenibacillus durus* were retrieved from the NCBI. These protein sequences were aligned using MUSCLE and the resulting alignment was used to generate a phylogenetic tree using PhyML and the Akaike Information Criterion for model selection.

2.5. Expression and purification of Cfr(C) and Cfr(E)

Codon-optimized sequences of *cfr*(C) (Genescript) and *cfr*(E) (Twist Bioscience) from isolates HON10 and DF11 were cloned into the pET21a and pET28b vectors, respectively, and overexpressed in *E. coli*.
BL21-CodonPlus (DE3)-RIPL and E. coli Rosetta™(DE3)pLysS, in that order. Enzymes were purified by Talon chromatography (Clontech) and underwent iron-sulfur cluster reconstitution using previously published protocols 25,28,29.

2.6. Preparation of truncated rRNA substrates

The E. coli 23S rRNA fragment 2447-2625 used in the in vitro methylation assay shown in Section 2.7 was generated by in vitro transcription following a previously published protocol 25,28. C. difficile 23S rRNA fragments 2451-2629 and 2022-2629 were also generated by in vitro transcription, using forward PCR primers that contain the T7 RNA polymerase promoter sequence TAATACGACTCACTATAGG and several nucleotides of the C. difficile 23S rRNA fragments of interest.

2.7. In vitro methylation assay

In vitro reactions were performed in 100 µL volumes using 100 mM HEPES pH 8.0 (Cfr(C)) or pH 7.0 (Cfr(E)), 100 mM KCl, 10 mM MgCl₂, 2 mM DTT, 20 μM Flavodoxin, 2 μM Flavodoxin reductase, 4 μM RNA, 0.14 μCi [14C-methyl]-SAM (58 mCi/mmol) and 5-10 μM purified enzyme. Two final pH conditions were required because Cfr(E) was found to be poorly active at pH 8.0. Reactions were initiated by addition of 1 mM NADPH (final concentration) and proceeded for 1.5 h at 37°C. RNA was recovered from the reaction mixtures using the RNA Clean & Concentrator kit (Zymo Research) and added to vials containing Ultima Gold scintillation fluid (Perkin Elmer). The amount of radioactivity incorporated in the products was measured using a Beckman–Coulter LS6500 multipurpose scintillation counter (Fullerton, CA, USA). Each value represents the average of at least duplicate measurements, with one standard deviation (SD) indicated.

2.8. HPLC separation and identification of methylated adenosines

Purified, methylated rRNA from in vitro reactions was enzymatically digested to mononucleosides using nuclease P₁ (Sigma-Aldrich), snake venom phosphodiesterase (Sigma-Aldrich), and alkaline phosphatase from calf intestine (New England Biolabs) as described before 25,28. The digested samples were separated by HPLC using a Luna analytical C18 column (10 μm, 4.6 mm × 250 mm) (Phenomenex, Torrance, CA, USA) and a previously published protocol 25,28. Mononucleosides and synthetic methyladenosine standards were detected by their UV absorption at 256 nm, while the ¹⁴C-
labeled methyladenosines were either detected with a Packard radiomatic 515TR flow scintillation analyzer (Perkin Elmer) or with a Beckman–Coulter LS6500 multipurpose scintillation counter (Fullerton, CA, USA).

2.9. Comparative genomics

To determine the genomic context of the cfr-like genes detected among the DF, HON, PUC, and LIBA isolates, contigs with cfr-like genes were compared to selected MGEs and sequences deposited in the GenBank non-redundant database using BLASTn and MegaBLAST searches. SNPs and indels in 23S rRNA genes and genes for ribosomal proteins L3 and L4 were searched for through bwa mapping of trimmed reads to WGS from reference strains R20291 (accession number FN545816, linezolid) or CD630 (accession number AM180355, linezolid). This was done on account of the recognized role of these mutations in resistance to linezolid. Genomes and genome comparisons were visualized in Artemis or ACT, respectively. Linear comparison figures were prepared with Easyfig.

2.10. cfr(B) expression in PUC isolates

Biomass harvested from C. difficile PUC51 and PUC347 cultures in exponential (8h) and stationary growth phase (20h) was used for RNA isolation with the PowerMicrobiome RNA Isolation Kit (Mo Bio). RNA yield and quality was assessed using 0.5% chlorine - 1% agarose gels. DNA traces were removed from the RNA preparations using RQ1 RNAsa free-DNase I (Promega) and cDNA was thereafter synthetized with the ImProm-II reverse transcription system and random primers (Promega). cfr(B) expression was corroborated by final point PCR amplification of a 150 bp fragment using primers cfr_PUC_FOR (CTGCGTTGTTTGCTTAAAGT) and cfr_PUC_REV (GCATTAACACTTCGTCTGTT).

3. Results

3.1. Detection of cfr-like genes

Isolates HON06, HON11, PUC51, and PUC347 carry a cfr(B) allele that is identical to that of C. difficile 11140508 (Table 1). On the other hand, isolates HON10 and LIBA5707 have the cfr(C) allele previously seen in C. difficile T10 (Table 1). Interestingly, the genome of isolate DF11 includes a gene for a radical SAM RNA methylating enzyme whose product only shares 51-58% sequence identity with Cfr, Cfr(B),
Cfr(C), and Cfr(D) and therefore represents a new cfr-like gene according to the MLS nomenclature system maintained by Dr. Marilyn Roberts (Table 1). This gene was termed cfr(E). BLASTp, EggNOG, UniProt, and SFLD searches confirmed that the predicted protein sequence of Cfr(E) shows homology to C8 RNA methylating enzymes (Supplementary Table 2).

Examination of 2134 publicly available C. difficile genomes using a 75% coverage and 75% sequence identity threshold revealed that cfr(C) (4% detection rate), followed by cfr(B) (1.3% detection rate) and cfr(E) (0.09% detection rate), are infrequent in this species. The protein sequences of Cfr(B) and Cfr(D) clustered with sequences of Cfr enzymes with demonstrated activity mechanisms. By contrast, predicted Cfr(C) and Cfr(E) sequences were more closely related to sequences of Cfr-like proteins awaiting functional characterization (Figure 1).

3.2 MIC

We obtained MICs for linezolid, tiamulin, thiamphenicol, and virginiamycin M1 to evaluate whether carriage of cfr-like genes was associated with a PhLOPSA phenotype (Table 2). HON, LIBA, and DF isolates invariably showed higher MICs of linezolid (16 to >256 µg/ml), tiamulin (32 to >256 µg/ml), thiamphenicol (32 to >256 µg/ml), and virginiamycin M1 (80-320 µg/ml) than the negative control and the quality control strains, for which MICs below 2 µg/ml (linezolid, tiamulin, and thiamphenicol) or 20 µg/ml (virginiamycin M1) were recorded (Table 2).

Despite expressing a cfr(B) gene both at 8 and 20h (Supplementary Figure 1), MICs of linezolid (2 µg/ml), tiamulin (4-16 µg/ml), thiamphenicol (4-8 µg/ml), and virginiamycin (20-80 µg/ml) determined for PUC51 and PUC347 were lower than those obtained for the other test isolates, but equal or higher than the MICs obtained for the control strains (Table 2).

3.3 Functional analysis of Cfr(C) and Cfr(E)

To investigate whether Cfr(C) and Cfr(E) are indeed C8-methylating enzymes, we overexpressed codon-optimized versions of the cfr(C) sequence of HON10 and the cfr(E) sequence of DF11 in E. coli. The resulting proteins were purified under anaerobic conditions and their iron-sulfur cluster reconstituted. Thereafter, we performed an in vitro methylation assay with in vitro transcribed 23S rRNA_E. coli and [14C-methyl]-S-adenosyl methionine ([14C-methyl]-SAM), and the amount of radioactivity...
incorporated into the RNA products was determined. These assays revealed that Cfr(C) and Cfr(E) do methylate *E. coli* 23S rRNA *in vitro* (Figure 2A). However, while significantly above the background, the methylation levels detected in the 2447-2625 *E. coli* rRNA fragment for both Cfr(C) and Cfr(E) were lower than that observed in the reaction of the same rRNA fragment with *E. coli* RlmN (Figure 2A). A lower level of activity of Cfr(C) towards *C. difficile* fragments as compared to the *E. coli* fragment was also observed (Figure 2B).

To establish the regioselectivity of the modification on the adenosine ring catalyzed by Cfr(C) and Cfr(E), radiolabeled RNA product isolated from the *in vitro* assays with *E. coli* RNA was purified, digested to individual nucleosides, and analyzed by HPLC. Unlike the 2-methyladenosine product of the reaction with *E. coli* RlmN, the products of the reactions with purified Cfr(C) or Cfr(E) co-eluted with the 8-methyladenosine standard, demonstrating that these enzymes methylate A2503 at the C8 position (Figure 3).

### 3.4. Comparative genomics

The *cfr*-like genes detected were found on four types of putative MGEs with anticipated mobilization or conjugation potential (Table 3). These MGEs are without exception chromosomally encoded.

While isolates HON06 and HON11 have *cfr*(B) within a Tn6218-like element, isolates PUC51 and PUC347 have *cfr*(B) elsewhere in their genomes in an unreported genetic structure (Table 3). The best hit for this novel MGE was a genomic fragment of *Faecalibacterium prausnitzii* L2/6 (Query cover=74%, E-value=0, Identity=99%); a species that has not been previously reported to carry *cfr*-like genes.

The *cfr*(C) genes of isolates HON10 and LIBA5707 were traced back to a MGE that resembles the *cfr*(C)+ ICE of *C. difficile* F548 12 (Table 3). On the other hand, the putative new *cfr*-like gene of isolate DF11 was found integrated into an undescribed MGE that shows partial hits to genomic sequences of various intestinal Firmicutes (Table 3), including *Lachnoclostridium* sp. YL32 (Query cover=60%, E-value=0, Identity=94%), *Roseburia intestinalis* XB6B4 (Query cover=60%, E-value=0, Identity=92%), *Faecalibacterium prausnitzii* A2165 (Query cover=60%, E-value=0, Identity=88%), and *C. difficile* Z31 (Query cover=60%, E-value=0, Identity=87%). In all cases, shared regions did not include *cfr*(E) nor its adjacent genes (Table 3).
None of the WGS studied included mutations or indels in 23S RNA genes or the ribosomal proteins L3
and L4. Furthermore, \textit{optR}, \textit{poxA}, and pleuromutilin resistance genes were not detected
(Supplementary Table 3). Whereas LIBA 5707 and LIBA5701 carry a \textit{catP} gene for phenicol resistance,
only the former displays a PhLOPS\textsubscript{a} phenotype (Supplementary Table 3). All isolates had \textit{ermB}.
However, this gene was also found in the linezolid-susceptible strain LIBA5701. \textit{tet}(M) and various
aminoglycoside-resistance genes were sporadically detected in the WGS analyzed (Supplementary
Table 3).

4. Discussion

We report carriage of a rather large diversity of \textit{cfr}-like genes associated with different MGEs by clinical
\textit{C. difficile} strains from Latin America and provide for the first-time \textit{in vitro} evidence of the
\textit{m}^3\text{A}2503 methyltransferase activity of \textit{Cfr}(C) and a novel \textit{cfr}-like enzyme, \textit{Cfr}(E). These two enzymes
do not clade with \textit{Cfr} and therefore implicate a different group of \textit{Cfr}-like proteins in antibiotic-
resistance.
The finding of \textit{cfr}-like genes in various types of MGEs with partial hits to genomic sequences reported
for other intestinal Firmicutes lends evidence to the plasticity of the \textit{C. difficile} genome \textsuperscript{32} and supports
the role of this pathogen as a reservoir of resistance genes in the human gut \textsuperscript{33}. This situation is
worrisome because linezolid is used for the treatment of methicillin-resistant \textit{Staphylococcus aureus} \textsuperscript{34}
and vancomycin-resistant enterococci \textsuperscript{35}, which reside in the same Phylum as clostridial organisms.
Indeed, versions of Tn6218 such as those detected in isolates HON06 and HON10 have been found in
\textit{Enterococcus faecium} isolates from German hospital patients \textsuperscript{36}.
The widespread detection of \textit{cfr}-like genes among various epidemic NAP1/RT027 strains deserves
attention to clarify whether this situation contributes to virulence. This notion is reinforced by the fact
that linezolid and moxifloxacin resistance, a marker of highly virulent \textit{C. difficile} strains, are often linked
in this ribotype \textsuperscript{37}. Furthermore, since antibiotics are crucial both for the induction, progression, and
treatment of CDI, multidrug-resistance (MDR) is particularly worrisome when present in epidemic types
such as the NAP1/027 strain, which has been linked to severe CDI outcomes \textsuperscript{38}. 
Although the cfr(B) allele of isolates HON06, HON11, PUC51, and PUC347 is expressed, the last two isolates did not show an evident PhLOPS_{A} phenotype. It has been shown that Cfr(B) is functional when encoded by Tn6218 \textsuperscript{10,13}, hence we propose that it is not as active in PUC51 and PUC347 due to neighboring-gene effects or different translation requirements in this new genetic background.

To further support the role of cfr-like enzymes in antibiotic resistance, we have provided the first \textit{in vitro} evidence that both Cfr(C) and Cfr(E) methylate the C8 position of A2503 in \textit{E. coli} 23S rRNA. In this regard, the poor activity of these enzymes towards the assayed rRNA fragments could reflect differences in substrate requirements between clostridial Cfrs and \textit{E. coli} RlmN \textsuperscript{25} or result from the lack of unknown modifications in the RNA substrate that may be necessary for efficient methylation by Cfr(C) and Cfr(E).

Our results encourage analyses of further resistance phenotypes in strain collections from Latin America. This can be achieved through a combination of classical phenotypic tests, whole genome sequencing, and biochemical validation, as exemplified here. As already noted \textsuperscript{39}, a prompt phenotypic and genotypic identification of resistance genes, effective antimicrobial stewardship and infection control programs, and alternative therapies are needed to prevent and contain the spread of MDR \textit{C. difficile} strains.

\textbf{Declarations}

\textbf{Funding}

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\textbf{Competing Interests}

None

\textbf{Ethical Approval}
References


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**Figure legends**

**Figure 1.** Evolutionary relationship of *RlmN*, *Cfr*, and *Cfr*-like sequences from selected Firmicutes species. Functionally characterized *Cfr* enzymes, *Cfr*-like proteins, *Cfr* divergent proteins, and known and putative *RlmN* sequences are marked. While *Cfr*-like proteins clade with known *Cfr*s lacking functional characterization, *Cfr*-divergent proteins diverged early in evolutionary time and do not clade with either *Cfr*s or *RlmN*s. The enzymes of isolates *C. difficile* HON10/LIBA5707 and DF11 appear highlighted in bold. The distance scale underneath the tree indicates the average number of substitutions per site. IMG/JGI database identifiers or accession numbers of protein sequences used in the tree are provided in Supplementary Table 1.
**Figure 2.** Cfr(C)-, Cfr(E)-, and *E. coli* RlmN-mediated methylation of *in vitro* transcribed *E. coli* 2447-2625 23S rRNA fragment (A). Cfr(C)-mediated methylation of *in vitro* transcribed *E. coli* and *C. difficile* 23S rRNA fragments (B). Bars represent the mean of at least two replicates ± s.d.

**Figure 3.** HPLC analysis of methylation products from Cfr(C) (blue), Cfr(E) (gray), and *E. coli* RlmN reactions (m2A, green) with *E. coli* 2447-2625 rRNA fragment. A m8A standard is shown in orange.

**Supplementary material legends**

**Supplementary Table 1.** Sequences used for reconstruction of evolutionary relationships of Cfr, RlmN, and Cfr-like proteins. Letters after scientific names indicate the existence of paralogous genes in the species.

**Supplementary Table 2.** Features of the *cfr*-like protein detected in *C. difficile* DF11 that support its classification as CfrE

**Supplementary Table 3.** Resistome of Latin American *C. difficile* strains with predicted rRNA dimethylases as determined *in silico* by the Resistance Gene Identifier (RGI CARD) and resfinder tools

**Supplementary Figure 1.** *cfr*(B) expression in isolates PUC51 and PUC347 grown for 20h. Agarose gel electrophoresis of PCR products obtained for genomic DNA (2), a reagent control (3), cDNA (4), and RNA (5). Amplification of a 150 bp DNA fragment from retrotranscribed RNA confirms *cfr*(B) expression. A 100 bp ladder is shown in lane 1. Similar results were obtained for cells in logarithmic growth phase (8h).
Table 1. cfr-like genes detected among *C. difficile* isolates from Latin America with predicted rRNA dimethylases

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Origin/Year of isolation</th>
<th>Type (PFGE/RT)</th>
<th>PFGE pattern</th>
<th>% identity to reference protein sequence(s)</th>
<th>cfr-like gene detected</th>
<th>Previouly reported in <em>C. difficile</em> (Accession number)</th>
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<tr>
<td>HON06</td>
<td>CDI/Honduras/2016</td>
<td>NAP1/027</td>
<td>1057</td>
<td>Cfr(B) KM359438 (99-100%) Cfr(B) KR610408 (99-100%)</td>
<td>cfr(B)</td>
<td>Strain 11140508 (KM359438)</td>
</tr>
<tr>
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<td>CDI/Honduras/2016</td>
<td>ND</td>
<td>0461</td>
<td>ND</td>
<td>cfr(C)</td>
<td>Strain T10 (CCL89685)</td>
</tr>
<tr>
<td>PUC51</td>
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<td>NAP_Cfr/012</td>
<td>1056</td>
<td>Cfr(C) CCL89685 (100%) Cfr(C) ENZ41453 (100%)</td>
<td>cfr(C)</td>
<td></td>
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<tr>
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<td>ND</td>
<td>448</td>
<td>Cfr(B) KM359438 (53%) Cfr(B) KM359438 (53%)</td>
<td>cfr(E)</td>
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<td>CDI/Mexico/2015</td>
<td>NAP1/027</td>
<td>1058</td>
<td>Cfr 879565 (51%)</td>
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</tr>
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</table>

*NML-Canada database

1Not determined

Reference sequences taken from the MLS nomenclature system (https://faculty.washington.edu/marilynr/ermweb1.pdf)

New gene according to the 80% protein sequence identity threshold defined for this group of enzymes
**Table 2.** Minimum inhibitory concentrations of PTC-targeting antibiotics determined for *C. difficile* isolates from Latin America with predicted rRNA dimethylases

<table>
<thead>
<tr>
<th>Isolate</th>
<th>cfr-like gene</th>
<th>MIC (μg/ml)</th>
<th>Linezolid&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Tiamulin&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Thiampenicol&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Virginiamycin M1&lt;sup&gt;d&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>HON06</td>
<td>cfr(B)</td>
<td>24</td>
<td>128</td>
<td>≥256</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td>HON11</td>
<td></td>
<td>24</td>
<td>128</td>
<td>≥256</td>
<td>320</td>
<td></td>
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<tr>
<td>PUC51</td>
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<td>2</td>
<td>4</td>
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<tr>
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<td>16</td>
<td>8</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>HON10</td>
<td>cfr(C)</td>
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<td>128</td>
<td>≥256</td>
<td>160</td>
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<tr>
<td>LIBA5707</td>
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<td>≥256</td>
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<td>DF11</td>
<td>cfr(E)</td>
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<td>32</td>
<td>ND</td>
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<tr>
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<td>&lt;0.16</td>
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<td>ND&lt;sup&gt;*&lt;/sup&gt;</td>
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</table>

<sup>a</sup>NAP<sub>CR</sub> strain lacking cfr-like genes used as negative control  
<sup>b</sup>As determined by epsilometry  
<sup>c</sup>As determined by agar macrodilution  
<sup>d</sup>As determined by broth microdilution
ND: Not determined
Table 3. Annotation of the putative mobile genetic elements (MGE) in which cfr-like genes were detected

<table>
<thead>
<tr>
<th>Isolate(s)</th>
<th>Element synteny</th>
<th>Cfr type</th>
<th>MGE type</th>
<th>Genome insertion site</th>
</tr>
</thead>
<tbody>
<tr>
<td>HON11</td>
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<tr>
<td>PUC347</td>
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</table>
Table 3. Annotation of the putative mobile genetic elements (MGE) in which cfr-like genes were detected (Cont.)

<table>
<thead>
<tr>
<th>Isolate(s)</th>
<th>Element synteny</th>
<th>Cfr type</th>
<th>MGE type</th>
<th>Genome insertion site</th>
</tr>
</thead>
</table>
Fig 3.