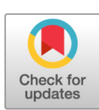
MECHANISMS OF RESISTANCE



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, two 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 to 58% 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 resistance gene was termed *cfr*(E). In agreement with the anticipated function of the *cfr*-like genes detected, all isolates exhibited high MIC values for several ribosome-targeting antibiotics. In addition, *in vitro* assays confirmed that 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 rRNA genes or genes encoding the ribosomal proteins L3 and L4 and lack *poxtA*, *optrA*, and pleuromutilin resistance genes. Moreover, these *cfr*-like genes were found in Tn*6218*-like transposons or integrative and conjugative elements (ICE) 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$_A$ resistance, *cfr*-like genes

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 binding sites, specifically, through mutation or modification of rRNAs or proteins (1). Several rRNA-modifying enzymes implicated in antibiotic resistance have been discovered (2), and among them, the radical *S*-adenosyl-L-methionine (SAM) enzyme Cfr is noteworthy because it provides cross-resistance to phenicols (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

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transferase center (PTC) of the bacterial ribosome (3). In addition to this so-called $PhLOPS_A$ phenotype (4), Cfr-mediated methylation leads to resistance to 16-member macrolides, the aminocyclitol hygromycin A, and the nucleoside antimicrobial agent A201A (4–6).

cfr and *cfr*-like genes are typically found on mobile genetic elements (MGEs). Moreover, since *cfr* acquisition exhibits low fitness costs (7), 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 (8), but it is nowadays found in nearly 20 different genetic contexts in isolates of *Enterococcus* spp., *Bacillus* spp., *Proteus vulgaris*, *Escherichia coli*, *Macrococcus caseolyticus*, *Jeotgalicoccus pinnipedialis*, and *Streptoccocus suis* from Europe, Latin America, the United States, and Asia (2, 3). Homologues of *cfr* have been identified in nonpathogenic *Bacillales* (9), and three additional *cfr*-like genes sharing less than 80% protein sequence identity to Cfr have been described in *Clostridium* and *Enterococcus* spp. (2). 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 Tn*6218*-like elements (10, 11). Then 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 nontoxigenic strain *C. difficile* F548 (12). Subsequently, Hansen and Vester demonstrated by primer extension that a codon-optimized version of *cfr*(B) of *C. difficile* 11140508 modifies A2503 in 23S rRNA when expressed in *E. coli* (13). Equivalent evidence is missing for Cfr(C), though it has been shown to confer $PhLOPS_A$ resistance upon introduction into the linezolid-susceptible strain *C. difficile* 630Δ*erm* (12).

Despite its confirmed utility in preventing *C. difficile* infection (CDI) in patients with ventilator-associated pneumonia (14) and in reducing *C. difficile* toxin gut levels in a mouse model (15), 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 (16), and though it did not pass a phase III clinical trial (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 methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococcus (VRE) infections, we investigated seven clinical *C. difficile* isolates with predicted rRNA dimethylases to determine whether they carry functional *cfr* or *cfr*-like genes. To this end, we determined the MICs of PTC-targeting antibiotics from four different groups and evaluated the *in vitro* activity of their Cfr-like enzymes, including a new determinant termed Cfr(E).

RESULTS

Detection of *cfr*-like genes. *C. difficile* 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, *C. difficile* 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 shares only 51 to 58% sequence identity with Cfr, Cfr(B), Cfr(C), and Cfr(D) and therefore represents a new *cfr*-like gene according to the macrolide-lincosamide-streptogramin B (MLS) nomenclature system maintained by Marilyn Roberts (Table 1). This gene was termed *cfr*(E). BLASTp, eggNOG, UniProt, and Structure Function Linkage Database (SFLD) searches confirmed that the predicted protein sequence of Cfr(E) shows homology to C8 RNA-methylating enzymes (see Table S1 in the supplemental material).

Examination of 2,134 publicly available *C. difficile* genomes using a 75% coverage threshold and a 75% sequence identity threshold revealed that *cfr*(C) (4% detection rate), *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 functionally characterized Cfr enzymes. In contrast, predicted Cfr(C) and Cfr(E) sequences were more closely related to sequences of Cfr-like proteins awaiting functional characterization (Fig. 1).

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TABLE 1 *cfr*-like genes detected among *C. difficile* isolates from Latin America with predicted rRNA dimethylases

Isolate	Origin/yr of isolation	Type (PFGE/RT)[a]	PFGE pattern[b]	Reference protein sequence(s) and GenBank accession no.[d] (identity [%])	*cfr*-like gene detected	Previously reported in *C. difficile* (GenBank accession no.)
HON06	CDI/Honduras/2016	NAP1/027	1057	Cfr(B) KM359438 (99–100%)	*cfr*(B)	Strain 11140508 (KM359438)
HON11	CDI/Honduras/2016		0461	Cfr(B) KR610408 (99–100%)		
PUC51	CDI/Chile/2011		ND[c]			
PUC347	CDI/Chile/2011		ND			
HON10	CDI/Honduras/2016	NAP1/027	1056	Cfr(C) CCL89685 (100%)	*cfr*(C)	Strain T10 (CCL89685)
LIBA5707	CDI/Costa Rica/2009	NAP_{CR1}/012	448	Cfr(C) ENZ41453 (100%)		
DF11	CDI/Mexico/2015	NAP1/027	1058	Cfr AJ879565 (51%) Cfr AM408573 (51%) Cfr(B) KM359438 (53%) Cfr(B) KR610408 (54%) Cfr(C) CCL89685 (58%) Cfr(C) ENZ41453 (58%) Cfr(D) MG707078 (51%)	*cfr*(E)[e]	No

[a]PFGE, pulsed-field gel electrophoresis; RT, ribotype.
[b]From the NML, Canada database.
[c]ND, not determined.
[d]Reference sequences taken from the MLS nomenclature system (https://faculty.washington.edu/marilynr/ermweb1.pdf.)
[e]New gene according to the 80% protein sequence identity threshold defined for this group of enzymes.

MICs. We obtained MICs for linezolid, tiamulin, thiamphenicol, and virginiamycin M1 to evaluate whether the presence of *cfr*-like genes was associated with a $PhLOPS_A$ phenotype (Table 2). The 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 to 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 at both 8 and 20 h (Fig. S1), MICs of linezolid (2 μg/ml), tiamulin (4 to 16 μg/ml), thiamphenicol (4 to 8 μg/ml), and virginiamycin (20 to 80 μg/ml) determined for PUC51 and PUC347 were lower than those obtained for the other test isolates but equal to or higher than the MICs obtained for the negative control and QC strains (Table 2).

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 was reconstituted. Thereafter, we performed an *in vitro* methylation assay with *in vitro* transcribed 23S rRNA of *E. coli* and [(14)C-methyl]-*S*-adenosyl methionine ([(14)C-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* (Fig. 2A). However, while significantly above the background, the methylation levels detected in the 2447 to 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 (Fig. 2A). A lower level of activity of Cfr(C) toward *C. difficile* fragments compared to the *E. coli* fragment was also observed (Fig. 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 using high-pressure liquid chromatography (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) coeluted with the 8-methyladenosine standard, demonstrating that these enzymes methylate A2503 at the C8 position (Fig. 3).

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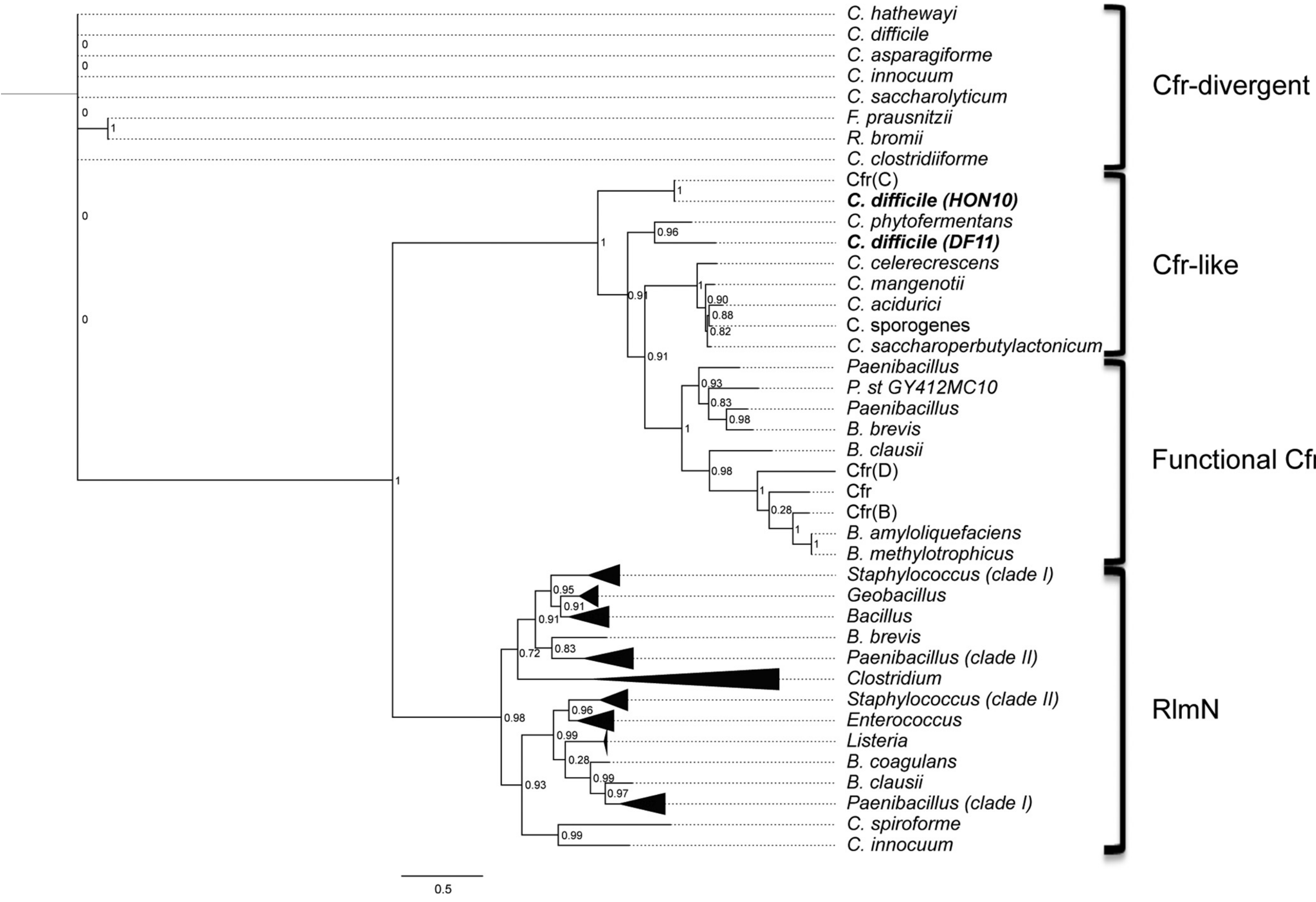

FIG 1 Evolutionary relationship of RlmN, Cfr, and Cfr-like sequences from selected *Firmicutes* species. Functionally characterized Cfr enzymes, Cfr-like proteins, Cfr divergent proteins, and known and putative RlmN sequences are marked. While Cfr-like proteins share clades with known Cfrs lacking functional characterization, Cfr-divergent proteins diverged early in evolutionary time and do not share clades with either Cfrs or RlmNs. The enzymes of *C. difficile* isolates HON10/LIBA5707 and DF11 are 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 Table S3.

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 Tn*6218*-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 an MGE that resembles the *cfr*(C)$^+$ ICE of *C. difficile* F548[12] (Table 3). On the other hand, the 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. strain 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) or its adjacent genes (Table 3).

None of the whole-genome sequences (WGS) studied included mutations or indels in 23S RNA genes or the ribosomal proteins L3 and L4. Furthermore, *optrA*, *poxtA*, and pleuromutilin resistance genes were not detected (Table S2). Whereas LIBA5707 and LIBA5701 carry a *catP* gene for phenicol resistance, only the former displays a $PhLOPS_A$

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TABLE 2 MICs of PTC-targeting antibiotics determined for *C. difficile* isolates from Latin America with predicted rRNA dimethylases

Isolate	*cfr*-like gene	MIC (μg/ml) for:			
		Linezolid[b]	Tiamulin[c]	Thiamphenicol[c]	Virginiamycin M1[d]
HON06	*cfr*(B)	24	128	≥256	160
HON11		24	128	≥256	320
PUC51		2	4	4	80
PUC347		2	16	8	20
HON10	*cfr*(C)	24	128	≥256	160
LIBA5707		16	≥256	≥256	80
DF11	*cfr*(E)	≥256	32	32	ND
LIBA5701[a]	None	1	<0.16	2	20
ATCC 70057	None	1	ND[e]	1.5	1

[a]This is a NAP_{CR1} strain without *cfr*-like genes (negative control).
[b]As determined by epsilometry.
[c]As determined by agar macrodilution.
[d]As determined by broth microdilution.
[e]ND, Not determined.

phenotype (Table S2). All isolates, including the linezolid-susceptible strain LIBA5701, had the *ermB* gene. *tet*(M) and various aminoglycoside resistance genes were sporadically detected in the WGS analyzed (Table S2).

DISCUSSION

We report the presence of a diverse set of *cfr*-like genes associated with different MGEs in clinical *C. difficile* strains from Latin America and provide for the first-time *in vitro* evidence of the m^8A2503 methylsynthase activity of Cfr(C) and a novel Cfr-like enzyme, Cfr(E). These two enzymes are not in the same clade as Cfr and therefore implicate a different group of Cfr-like proteins in antibiotic resistance.

The finding of *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 *C. difficile* genome (18) and supports the role of this pathogen as a reservoir of resistance genes in the human gut (19). This situation is worrisome because linezolid is used for the treatment of MRSA (20) and VRE (21), which reside in the same phylum as clostridial organisms. Indeed, versions of Tn*6218*, such as those detected in isolates HON06 and HON10, have been found in *Enterococcus faecium* isolates from German hospital patients (22).

The widespread detection of *cfr*-like genes among various epidemic NAP1/RT027 isolates deserves attention to clarify whether this situation contributes to virulence. This notion is reinforced by the fact that linezolid and moxifloxacin resistance, markers of highly virulent *C. difficile* strains, are often linked in this ribotype (23). Furthermore, since antibiotics are crucial 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 (24).

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 Tn*6218* (10, 13), and 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 roles of Cfr-like enzymes in antibiotic resistance, we have provided the first *in vitro* evidence that both Cfr(C) and Cfr(E) methylate the C8 position of A2503 in *E. coli* 23S rRNA. In this regard, the poor activity of these enzymes toward the assayed rRNA fragments could reflect differences in substrate requirements between clostridial Cfrs and *E. coli* RlmN (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 classic phenotypic

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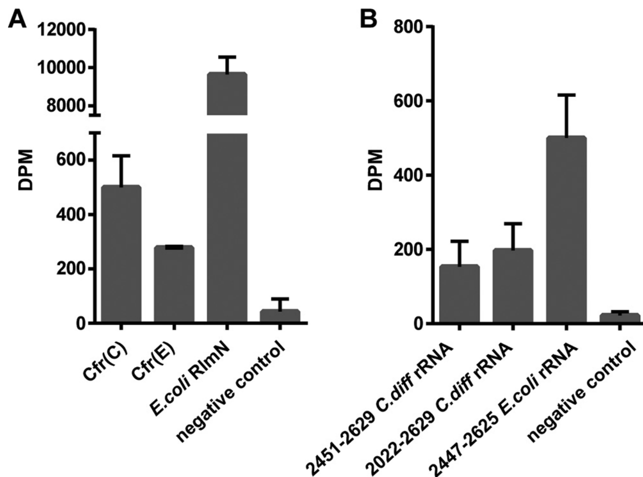

FIG 2 (A) Cfr(C)-, Cfr(E)-, and *E. coli* RlmN-mediated methylation of an *in vitro* transcribed *E. coli* 2447 to 2625 23S rRNA fragment. (B) Cfr(C)-mediated methylation of *in vitro* transcribed *E. coli* and *C. difficile* 23S rRNA fragments. Bars represent the mean of at least two replicates ± the SD.

tests, whole-genome sequencing, and biochemical validation, as exemplified here. As already noted (26), 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 *C. difficile* strains.

MATERIALS AND METHODS

Strains. This study included ribotype- or pulsed-field gel electrophoresis (PFGE)-confirmed NAP1/027 clinical isolates from Mexico (DF11), Honduras (HON06, HON10, HON11), and Chile (PUC51, PUC347) and one NAP_{CR1}/012 isolate from Costa Rica (LIBA5707). These bacteria were recovered from 2009 to 2016 from stool samples from human patients and were selected among ca. 450 sequenced *C. difficile* isolates from Latin America because an automated annotation indicated that their genomes include sequences for putative rRNA dimethylases (27, 28; unpublished data). With a single exception (DF11, recovered from a 3-year-old patient with diarrhea), all isolates were obtained from adults with CDI. Moreover, DF, PUC, and LIBA isolates were obtained during confirmed CDI outbreaks. *C. difficile* LIBA5701 was used as a negative control in the determinations of MICs because it is a NAP_{CR1}/012 strain that naturally lacks MGEs with *cfr*-like genes and therefore does not display a $PhLOPS_A$ phenotype (see below) (27).

Detection of *cfr*-like and other resistance genes. Whole-genome sequences (WGS) were obtained with sequencing-by-synthesis using multiplexed paired-end libraries and HiSeq 2000 or MiSeq instruments (Illumina). After trimming with Sickle (https://github.com/najoshi/sickle), reads were assembled using SPAdes v.3.12 (29) and annotated with Prokka v.1.13 (30). The identity of resistance genes identified by automated annotation or with ABRicate was confirmed using ResFinder and the CARD database v.3.0.1 (31) and through BLAST, BLASTP, eggNOG v.3 (32), UniProt, and Structure Function Linkage Database (SFLD) searches.

MIC determinations. MICs of linezolid were obtained using epsilometry with strips containing 0.016- to 256-μg/ml concentration gradients (Liofilchem). Tiamulin and thiamphenicol MICs were determined using agar macrodilution (1 to 256 μg/ml in brain heart infusion [BHI] plates), and virginiamycin M1 was tested using broth microdilution (1 to 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^s) was tested for quality control purposes.

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 (33). To examine the phylogenetic relationship between Cfr-like sequences mentioned in this study and 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 search using the RlmN sequence from *Bacillus subtilis* as a query, as done elsewhere (25) (Table S3). Additional RlmN/Cfr paralogous sequences from *Paenibacillus durus* were retrieved from the NCBI. These protein sequences were aligned using MUSCLE (34), and the resulting alignment was used to generate a phylogenetic tree using PhyML and the Akaike Information Criterion for model selection (35).

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 using Talon chromatography (Clontech) and underwent iron-sulfur cluster reconstitution using previously published protocols (25, 36, 37).

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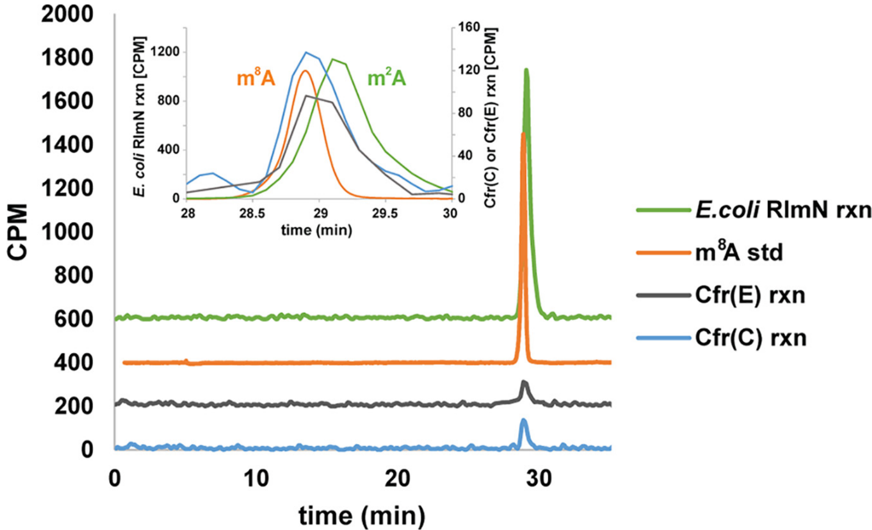

FIG 3 HPLC analysis of methylation products from Cfr(C) (blue), Cfr(E) (gray), and *E. coli* RlmN reactions (m^2A, green) with an *E. coli* 2447 to 2625 rRNA fragment. An m^8A standard is shown in orange.

Preparation of truncated rRNA substrates. The *E. coli* 23S rRNA fragment 2447 to 2625 used in the *in vitro* methylation assay shown in the "*In vitro* Methylation Assay" section was generated by *in vitro* transcription following a previously published protocol (25, 36). *C. difficile* 23S rRNA fragments 2451 to 2629 and 2022 to 2629 were also generated with *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.

***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 $MgCl_2$, 2 mM dithiothreitol (DTT), 20 μM flavodoxin, 2 μM flavodoxin reductase, 4 μM RNA, 0.14 μCi [14C-methyl]-SAM (58 mCi/mmol), and 5 to 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 the 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 shown in Fig. 2 represents the average of at least duplicate measurements, with one standard deviation (SD) indicated.

HPLC separation and identification of methylated adenosines. Purified, methylated rRNA from *in vitro* reactions was enzymatically digested to mononucleosides using nuclease P_1 (Sigma-Aldrich), snake venom phosphodiesterase (Sigma-Aldrich), and alkaline phosphatase from calf intestine (New England Biolabs) as described before (25, 36). The digested samples were separated with HPLC using a Luna analytical C_{18} column (10 μm, 4.6 mm $\times$ 250 mm) (Phenomenex, Torrance, CA, USA) and a previously published protocol (25, 36). Mononucleosides and synthetic methyladenosine standards were detected by their UV absorption at 256 nm, while the (14)C-labeled methyladenosines were either detected with a Packard Radiomatic 515TR flow scintillation analyzer (Perkin Elmer) or with a Beckman-Coulter LS6500 multipurpose scintillation counter.

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 nonredundant database using BLASTn and MegaBLAST searches. Single-nucleotide polymorphisms (SNPs) and indels in 23S rRNA genes and genes for ribosomal proteins L3 and L4 were searched for through Burrows-Wheeler Aligner (BWA) mapping of trimmed reads to WGS from reference strains R20291 (GenBank accession number FN545816, linezolid^s) or CD630 (accession number AM180355, linezolid^s). This was done on account of the recognized role of these mutations in resistance to linezolid (38). Genomes and genome comparisons were visualized in Artemis or ACT (Artemis Comparison Tool), respectively. Linear comparison figures were prepared with Easyfig.

***cfr*(B) expression in PUC isolates.** Biomass harvested from *C. difficile* PUC51 and PUC347 cultures in the exponential (8 h) and stationary growth phases (20 h) was used for RNA isolation with the Power-Microbiome RNA isolation kit (Mo Bio). The RNA yield and quality were assessed using 0.5% chlorine and 1% agarose gels (39). DNA traces were removed from the RNA preparations using RQ1 RNase-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 using final point PCR amplification of a 150-bp fragment using the primers cfr_PUC_FOR (CTGCGTTGTTTGCTTTAAGTC) and cfr_PUC_REV (GCA TTAACTCACTTCGCTGTTC).

Data availability. Reads for isolates PUC51 and PUC347 can be retrieved using the NCBI accession numbers CAADRH000000000 and CAADRI000000000, respectively, and raw data for LIBA5707 is available

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TABLE 3 Annotation of the putative mobile genetic elements (MGE) in which *cfr*-like genes were detected[c]

Isolate(s)	Element synteny[c]	Cfr type	MGE type	Genome insertion site
HON06 HON11	Transposase, excisionase, replication initiation factor, transcriptional regulator, methyltransferase, HTH-type transcriptional regulator, hypothetical protein, Cfr-like protein, MATE efflux protein, RNA polymerase sigma factor, HTH-domain containing protein, hypothetical protein, HTH-type transcriptional regulator, Hypothetical protein	Cfr(B)	Tn*6218*-like transposon[a]	Between genes for a hypothetical protein and an HTH transcriptional regulator
PUC51 PUC347	Transposase, Cfr-like protein, integrase, RNA methylase, hypothetical protein, endonuclease, hypothetical protein, mobilization protein, helicase		Undescribed	
HON10 LIBA5707	Resolvase, resolvase, hypothetical protein, hypothetical protein, RNA polymerase sigma factor, Cfr-like protein, hypothetical protein, hypothetical protein, transcriptional regulator, HTH transcriptional regulator, relaxase	Cfr(C)	F548-like ICE[b]	Gene for ABC transporter permease
DF11	DNA invertase, recombinase, hypothetical protein, *N*-acetyltransferase, ABC transporter ATP binding protein, Cfr-like protein, HTH transcriptional regulator, hypothetical protein	Cfr(E)	Undescribed	Gene for adenine deaminase *adeC*

[a]GenBank accession number for Tn*6218* in *C. difficile* Ox2167, HG002396.
[b]GenBank accession number for *C. difficile* F548 assembly, GCA_000452325.2 (ASM45232v2).
[c]HTH, helix-turn-helix; MATE, multiantimicrobial extrusion.

at the European Nucleotide Archive (run ERR467555). 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/).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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We declare no competing interests.

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