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1 ***cfr*(B), *cfr*(C), and a new *cfr*-like gene, *cfr*(E), in *Clostridium difficile* strains recovered across**
2 **Latin America**
3

4 Vanja Stojković[1], María Fernanda Ulate[2], Fanny Hidalgo-Villeda[3], Emmanuel Aguilar[4], Camilo Monge-
5 Cascante[2], Marjorie Pizarro-Guajardo[6,7], Kaitlyn Tsai[1], Edgardo Tzoc[3], Margarita Camorlinga[5], Daniel
6 Paredes-Sabja[6,7], Carlos Quesada-Gómez[2], Danica Galonić Fujimori[1,8], César Rodríguez[2]*
7
8 [1]Department of Cellular and Molecular Pharmacology, University of California San Francisco, USA
9 [2]CIET and Facultad de Microbiología, Universidad de Costa Rica, Costa Rica
10 [3]Escuela de Microbiología, Facultad de Ciencias, Universidad Nacional Autónoma de Honduras,
11 Honduras
12 [4]Medical Bacteriology, Department of Microbiology. Escuela Nacional de Ciencias Biológicas del
13 Instituto Politécnico Nacional (ENCB-IPN), México.
14 [5]Unidad de Investigación en Enfermedades Infecciosas y Parasitarias. Instituto Mexicano del Seguro
15 Social, México
16 [6]Microbiota-Host Interactions and Clostridia Research Group, Facultad Ciencias de la Vida, Universidad
17 Andres Bello, Chile
18 [7]Millennium Nucleus in the Biology of Intestinal Microbiota, Santiago, Chile
19 [8]Department of Pharmaceutical Chemistry, University of California San Francisco, USA
20
21 [*]Correspondence author: Dr. César Rodríguez Sánchez, Centro de Investigación en Enfermedades
22 Tropicales (CIET) and Facultad de Microbiología, Universidad de Costa Rica, Ciudad Universitaria
23 Rodrigo Facio, 11501-2060 San José, Costa Rica; Tel: +506 2511-8615 Fax: +506 2225-4384. Email:
24 cesar.rodriguezsanchez@ucr.ac.cr

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25 **ABSTRACT**

26 Cfr is a radical *S*-adenosyl-L-methionine (SAM) enzyme that confers cross-resistance to antibiotics
27 targeting the 23S rRNA through hypermethylation of nucleotide A2503. Three *cfr*-like genes implicated
28 in antibiotic resistance have been described, of which *cfr*(B) and *cfr*(C) have been sporadically detected
29 in *Clostridium difficile*. However, the methylase activity of Cfr(C) has not been confirmed. We found
30 *cfr*(B), *cfr*(C), and a *cfr*-like gene that shows only 51-58% of protein sequence identity to Cfr and Cfr-like
31 enzymes in clinical *C. difficile* isolates recovered across nearly a decade in Mexico, Honduras, Costa
32 Rica, and Chile. This new determinant was termed *cfr*(E). In agreement with the anticipated function of
33 the *cfr*-like genes detected, high minimum inhibitory concentrations of drugs from four groups of
34 antibiotics targeting the ribosomal peptidyl transferase center were recorded for the isolates. In addition,
35 *in vitro* assays confirmed that purified Cfr(C) and Cfr(E) methylate *Escherichia coli* and, to a lesser
36 extent, *C. difficile* 23S rRNA fragments at the expected positions. The analyzed isolates do not have
37 mutations in 23S rRNAs genes or genes encoding the ribosomal proteins L3 and L4 and lack *poxtA*,
38 *optrA*, and pleuromutilin resistance genes. Moreover, their *cfr*-like genes were found into Tn*6218*-like
39 transposons or ICE-elements that could facilitate their transfer. These results indicate selection of
40 potentially mobile *cfr*-like genes in *C. difficile* from Latin America and provide the first assessment of the
41 methylation activity of Cfr(C) and Cfr(E), which belong to a cluster of Cfr-like proteins that does not
42 include the functionally characterized enzymes Cfr, Cfr(B), and Cfr(D).

43

44 **KEYWORDS**

45 *C. difficile*, Cfr, Cfr(B), Cfr(C), Cfr(E), 23S rRNA methylation, PhLOPS$_A$ phenotype

46

47 **1. Introduction**

48 The bacterial ribosome is one of the most common targets for antibiotics of clinical and veterinary
49 relevance. Resistance to ribosome-targeting antibiotics occurs primarily through modification of drug's
50 binding sites, specifically through mutation or modification of ribosomal RNAs (rRNA) or proteins [1].
51 Several rRNA modifying enzymes implicated in antibiotic resistance have been discovered [2], and

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52 among them, the radical *S*-adenosyl-L-methionine (SAM) enzyme Cfr is noteworthy because it provides
53 cross-resistance to Phenicols (e.g. thiamphenicol), Lincosamides (e.g. clindamycin), Oxazolidinones
54 (e.g. linezolid), Pleuromutilins (e.g. tiamulin), and Streptogramin A (e.g. virginiamycin M1) through C8
55 methylation of the A2503 residue in 23S rRNA (*Escherichia coli* numbering), which is located in the
56 peptidyl transferase center (PTC) of the bacterial ribosome [3]. In addition to this so-called $PhLOPS_A$
57 phenotype [4], Cfr-mediated methylation leads to resistance to 16-member macrolides, the aminocyclitol
58 hygromycin A, and the nucleoside antimicrobial agent A201A [4–6].
59 *cfr* and *cfr*-like genes are typically found on mobile genetic elements (MGEs). Moreover, since *cfr*
60 acquisition exhibits low fitness costs[7], the spread of these genes threatens the utility of PTC-targeting
61 antibiotics in the clinic. The *cfr* gene was first discovered on a *Staphylococcus sciuri* plasmid [8], but it is
62 nowadays found in nearly twenty different genetic contexts in isolates of *Enterococcus*, *Bacillus*,
63 *Proteus vulgaris*, *Escherichia coli*, *Macrococcus caseolyticus*, *Jeotgalicoccus pinnipedialis*, and
64 *Streptoccocus suis* from Europe, Latin America, USA, and Asia [2,3]. Homologues of *cfr* have been
65 identified in non-pathogenic Bacillales [9] and three additional *cfr*-like genes sharing less than 80%
66 protein sequence identity to Cfr have been described in *Clostridium* and *Enterococcus* [2]. These genes
67 are known as *cfr*(B), *cfr*(C), and *cfr*(D).

68 In *C. difficile, cfr*(B) was first detected in strain 11140508 contained within Tn*6218*-like elements [10,11].
69 Afterwards, Candela *et al.* defined *cfr*(C) after analysis of *C. difficile* T10 and found it in three types of
70 integrative and conjugative elements (ICEs) in several strains, including the non-toxigenic strain *C.*
71 *difficile* F548[12]. Subsequently, Hansen and Vester demonstrated by primer extension that a codon-
72 optimized version of *cfr*(B) of *C. difficile* 11140508 modifies A2503 in 23S rRNA when expressed in *E.*
73 *coli* [13]. Equivalent evidence is missing for Cfr(C), though it has been shown to confer $PhLOPS_A$
74 resistance upon introduction into the linezolid-susceptible strain *C. difficile* 630Δ*erm* [12].

75 Despite its confirmed utility to prevent *C. difficile* infections (CDI) in patients with ventilator associated
76 pneumonia [14] and to reduce *C. difficile* toxin gut levels in a mice model [15], linezolid is not used to treat
77 CDI. Nonetheless, the closely related antibiotic cadazolid inhibits moxifloxacin-resistant *C. difficile*

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78 NAP1/027 strains without affecting gut commensals [16] and though it did not pass a Phase III trial [17],
79 novel oxazolidinones to treat CDI may appear in the future.
80 Based on the potential utility of oxazolidinones in CDI therapy and the wide use of linezolid in Latin
81 America for treatment of MRSA and VRE infections, we investigated seven clinical *C. difficile* isolates
82 with predicted rRNA dimethylases to determine whether they carry functional *cfr* or *cfr*-like genes. To
83 this end, we determined minimum inhibitory concentrations (MICs) of PTC-targeting antibiotics from four
84 different groups and evaluated the *in vitro* activity of their Cfr-like enzymes, including a new determinant
85 termed Cfr(E).
86
87 **2. Methods**
88 2.1. Strains
89 This study included ribotype- or PFGE-confirmed NAP1/027 clinical isolates from Mexico (DF11),
90 Honduras (HON06, HON10, HON11) and Chile (PUC51, PUC347), and one NAP_{CR1}/012 isolate from
91 Costa Rica (LIBA5707). These bacteria were recovered between 2009 and 2016 from stool samples of
92 human patients and were selected among ca. 450 sequenced *C. difficile* isolates from Latin America
93 because an automated annotation indicated that their genomes include sequences for putative rRNA
94 dimethylases ([18,19], unpublished data). With a single exception (DF11, recovered from a 3-years old
95 patient with diarrhea), all isolates were obtained from adults with CDI. Moreover, DF, PUC, and LIBA
96 isolates were obtained during confirmed CDI outbreaks. *C. difficile* LIBA5701 was used as a negative
97 control in the determinations of minimum inhibitory concentrations (MICs) because it is a NAP_{CR1}/012
98 strain that naturally lacks MGEs with *cfr*-like genes and therefore does not display a $PhLOPS_A$
99 phenotype (see below) [18].
100 2.2.Detection of *cfr*-like and other resistance genes
101 Whole genome sequences (WGS) were obtained by sequencing-by-synthesis using multiplexed paired-
102 end libraries and HiSeq2000 or Miseq instruments (Illumina). After trimming with sickle
103 (https://github.com/najoshi/sickle), reads were assembled using Spades v.3.12 [20] and annotated with
104 Prokka v.1.13 [21]. The identity of resistance genes identified by automated annotation or with Abricate

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105 was confirmed using resfinder, the CARD database v.3.0.1 [22], and through BLAST, BLASTP, eggNOG3
106 [23], UniProt, and Structure Function Linkage Database (SFLD) searches. Trimmed reads and
107 assemblies for the DF and HON isolates can be downloaded from the MicrobesNG portal
108 (https://microbesng.uk/portal/projects/405FF6AC-A5E0-E04A-AECF-A5C9371B8B60/). Data for
109 isolates PUC51 and PUC347 can be retrieved using the NCBI accession numbers CAADRH000000000
110 and CAADRI000000000, respectively, and raw data for LIBA5707 is available at the European
111 Nucleotide Archive (run ERR467555).

112 2.3 MIC determinations

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

119 2.4. Comparison of RlmN and Cfr protein sequences

120 Though both RlmN and Cfr modify A2503, the former is a housekeeping gene and the latter an acquired
121 antibiotic resistance gene [24]. To examine the phylogenetic relationship between Cfr-like sequences
122 mentioned in this study to Cfr and RlmN sequences, Cfr-like and RlmN-like orthologs from selected
123 Firmicutes species were retrieved from the Integrated Microbial Genomes-Joint Genome institute
124 (IMG/JGI) database by BLAST using the RlmN sequence from *Bacillus subtilis* as a query, as done
125 elsewhere [25] (Supplementary Table 1). Additional RlmN/Cfr paralogous sequences from *Paenibacillus*
126 *durus* were retrieved from the NCBI. These protein sequences were aligned using MUSCLE [26] and the
127 resulting alignment was used to generate a phylogenetic tree using PhyML and the Akaike Information
128 Criterion for model selection [27].

129 2.5. Expression and purification of Cfr(C) and Cfr(E)

130 Codon-optimized sequences of *cfr*(C) (Genescript) and *cfr*(E) (Twist Bioscience) from isolates HON10
131 and DF11 were cloned into the pET21a and pET28b vectors, respectively, and overexpressed in *E. coli*

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132 BL21-CodonPlus (DE3)-RIPL and *E. coli* Rosetta™(DE3)pLysS, in that order. Enzymes were purified
133 by Talon chromatography (Clontech) and underwent iron-sulfur cluster reconstitution using previously
134 published protocols [25,28,29].

135 2.6. Preparation of truncated rRNA substrates

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

141 *2.7. In vitro* methylation assay

142 *In vitro* reactions were performed in 100 µL volumes using 100 mM HEPES pH 8.0 (Cfr(C)) or pH 7.0
143 (Cfr(E)), 100 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 20 µM Flavodoxin, 2 µM Flavodoxin reductase, 4 µM
144 RNA, 0.14 µCi [14C-methyl]-SAM (58 mCi/mmoL) and 5-10 µM purified enzyme. Two final pH
145 conditions were required because Cfr(E) was found to be poorly active at pH 8.0. Reactions were
146 initiated by addition of 1 mM NADPH (final concentration) and proceeded for 1.5 h at 37°C. RNA was
147 recovered from the reaction mixtures using the RNA Clean & Concentrator kit (Zymo Research) and
148 added to vials containing Ultima Gold scintillation fluid (Perkin Elmer). The amount of radioactivity
149 incorporated in the products was measured using a Beckman–Coulter LS6500 multipurpose scintillation
150 counter (Fullerton, CA, USA). Each value represents the average of at least duplicate measurements,
151 with one standard deviation (SD) indicated.

152 2.8. HPLC separation and identification of methylated adenosines

153 Purified, methylated rRNA from *in vitro* reactions was enzymatically digested to mononucleosides using
154 nuclease P_1 (Sigma-Aldrich), snake venom phosphodiesterase (Sigma-Aldrich), and alkaline
155 phosphatase from calf intestine (New England Biolabs) as described before [25,28]. The digested samples
156 were separated by HPLC using a Luna analytical C18 column (10 µm, 4.6 mm × 250 mm)
157 (Phenomenex, Torrance, CA, USA) and a previously published protocol [25,28]. Mononucleosides and
158 synthetic methyladenosine standards were detected by their UV absorption at 256 nm, while the ^{14}C-

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159 labeled methyladenosines were either detected with a Packard radiomatic 515TR flow scintillation
160 analyzer (Perkin Elmer) or with a Beckman–Coulter LS6500 multipurpose scintillation counter
161 (Fullerton, CA, USA).

162 2.9. Comparative genomics

163 To determine the genomic context of the *cfr*-like genes detected among the DF, HON, PUC, and LIBA
164 isolates, contigs with *cfr*-like genes were compared to selected MGEs and sequences deposited in the
165 GenBank non-redundant database using BLASTn and MegaBLAST searches. SNPs and indels in 23S
166 rRNA genes and genes for ribosomal proteins L3 and L4 were searched for through bwa mapping of
167 trimmed reads to WGS from reference strains R20291 (accession number FN545816, linezolid^{s}) or
168 CD630 (accession number AM180355, linezolid^{s}). This was done on account of the recognized role of
169 these mutations in resistance to linezolid [30]. Genomes and genome comparisons were visualized in
170 Artemis or ACT, respectively. Linear comparison figures were prepared with Easyfig.

171 2.10. *cfr*(B) expression in PUC isolates

172 Biomass harvested from *C. difficile* PUC51 and PUC347 cultures in exponential (8h) and stationary
173 growth phase (20h) was used for RNA isolation with the PowerMicrobiome™ RNA Isolation Kit (Mo
174 Bio). RNA yield and quality was assessed using 0.5% chlorine - 1% agarose gels [31]. DNA traces were
175 removed from the RNA preparations using RQ1 RNAsa free-DNAse I (Promega) and cDNA was
176 thereafter synthetized with the ImProm-II™ reverse transcription system and random primers
177 (Promega). *cfr*(B) expression was corroborated by final point PCR amplification of a 150 bp fragment
178 using primers cfr_PUC_FOR (CTGCGTTGTTTGCTTTAAGTC) and cfr_PUC_REV
179 (GCATTAACTCACTTCGCTGTTC).

180 **3. Results**

181 3.1. Detection of *cfr*-like genes

182 Isolates HON06, HON11, PUC51, and PUC347 carry a *cfr*(B) allele that is identical to that of *C. difficile*
183 11140508 (Table 1). On the other hand, isolates HON10 and LIBA5707 have the *cfr*(C) allele previously
184 seen in *C. difficile* T10 (Table 1). Interestingly, the genome of isolate DF11 includes a gene for a radical
185 SAM RNA methylating enzyme whose product only shares 51-58% sequence identity with Cfr, Cfr(B),

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186 Cfr(C), and Cfr(D) and therefore represents a new *cfr*-like gene according to the MLS nomenclature
187 system maintained by Dr. Marilyn Roberts (Table 1). This gene was termed *cfr*(E). BLASTp, EggNOG,
188 UniProt, and SFLD searches confirmed that the predicted protein sequence of Cfr(E) shows homology
189 to C8 RNA methylating enzymes (Supplementary Table 2).
190 Examination of 2134 publicly available *C. difficile* genomes using a 75% coverage and 75% sequence
191 identity threshold revealed that *cfr*(C) (4% detection rate), followed by *cfr*(B) (1.3% detection rate) and
192 *cfr*(E) (0.09% detection rate), are infrequent in this species.
193 The protein sequences of Cfr(B) and Cfr(D) clustered with sequences of Cfr enzymes with
194 demonstrated activity mechanisms. By contrast, predicted Cfr(C) and Cfr(E) sequences were more
195 closely related to sequences of Cfr-like proteins awaiting functional characterization (Figure 1).
196 <u>3.2 MIC</u>
197 We obtained MICs for linezolid, tiamulin, thiamphenicol, and virginiamycin M1 to evaluate whether
198 carriage of *cfr*-like genes was associated with a $PhLOPS_A$ phenotype (Table 2). HON, LIBA, and DF
199 isolates invariably showed higher MICs of linezolid (16 to >256 µg/ml), tiamulin (32 to >256 µg/ml),
200 thiamphenicol (32 to >256 µg/ml), and virginiamycin M1 (80-320 µg/ml) than the negative control and
201 the quality control strains, for which MICs below 2 µg/ml (linezolid, tiamulin, and thiamphenicol) or 20
202 µg/ml (virginiamycin M1) were recorded (Table 2).
203 Despite expressing a *cfr*(B) gene both at 8 and 20h (Supplementary Figure 1), MICs of linezolid (2
204 µg/ml), tiamulin (4-16 µg/ml), thiamphenicol (4-8 µg/ml), and virginiamycin (20-80 µg/ml) determined for
205 PUC51 and PUC347 were lower than those obtained for the other test isolates, but equal or higher than
206 the MICs obtained for the control strains (Table 2).
207 <u>3.3 Functional analysis of Cfr(C) and Cfr(E)</u>
208 To investigate whether Cfr(C) and Cfr(E) are indeed C8-methylating enzymes, we overexpressed
209 codon-optimized versions of the *cfr*(C) sequence of HON10 and the *cfr*(E) sequence of DF11 in *E. coli.*
210 The resulting proteins were purified under anaerobic conditions and their iron-sulfur cluster
211 reconstituted. Thereafter, we performed an *in vitro* methylation assay with *in vitro* transcribed 23S rRNA
212 *E. coli* and [^{14}C-methyl]-*S*-adenosyl methionine ([^{14}C-methyl]-SAM), and the amount of radioactivity

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213 incorporated into the RNA products was determined. These assays revealed that Cfr(C) and Cfr(E) do
214 methylate *E. coli* 23S rRNA *in vitro* (Figure 2A). However, while significantly above the background, the
215 methylation levels detected in the 2447-2625 *E. coli* rRNA fragment for both Cfr(C) and Cfr(E) were
216 lower than that observed in the reaction of the same rRNA fragment with *E. coli* RlmN (Figure 2A). A
217 lower level of activity of Cfr(C) towards *C. difficile* fragments as compared to the *E. coli* fragment was
218 also observed (Figure 2B).
219 To establish the regioselectivity of the modification on the adenosine ring catalyzed by Cfr(C) and
220 Cfr(E), radiolabeled RNA product isolated from the *in vitro* assays with *E. coli* RNA was purified,
221 digested to individual nucleosides, and analyzed by HPLC. Unlike the 2-methyladenosine product of the
222 reaction with *E. coli* RlmN, the products of the reactions with purified Cfr(C) or Cfr(E) co-eluted with the
223 8-methyladenosine standard, demonstrating that these enzymes methylate A2503 at the C8 position
224 (Figure 3).
225 <u>3.4. Comparative genomics</u>
226 The *cfr*-like genes detected were found on four types of putative MGEs with anticipated mobilization or
227 conjugation potential (Table 3). These MGEs are without exception chromosomally encoded.
228 While isolates HON06 and HON11 have *cfr*(B) within a Tn*6218*-like element, isolates PUC51 and
229 PUC347 have *cfr*(B) elsewhere in their genomes in an unreported genetic structure (Table 3). The best
230 hit for this novel MGE was a genomic fragment of *Faecalibacterium prausnitzii* L2/6 (Query cover=74%,
231 E-value=0, Identity=99%); a species that has not been previously reported to carry *cfr*-like genes.
232 The *cfr*(C) genes of isolates HON10 and LIBA5707 were traced back to a MGE that resembles the
233 *cfr*(C)$^+$ ICE of *C. difficile* F548 [12] (Table 3). On the other hand, the putative new *cfr*-like gene of isolate
234 DF11 was found integrated into an undescribed MGE that shows partial hits to genomic sequences of
235 various intestinal Firmicutes (Table 3), including *Lachnoclostridium* sp. YL32 (Query cover=60%, E-
236 value=0, Identity=94%), *Roseburia intestinalis* XB6B4 (Query cover=60%, Evalue=0, Identity=92%),
237 *Faecalibacterium prausnitzii* A2165 (Query cover=60%, Evalue=0, Identity=88%), and *C. difficile* Z31
238 (Query cover=60%, Evalue=0, Identity=87%). In all cases, shared regions did not include *cfr*(E) nor its
239 adjacent genes (Table 3).

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240 None of the WGS studied included mutations or indels in 23S RNA genes or the ribosomal proteins L3
241 and L4. Furthermore, *optrA*, *poxtA*, and pleuromutilin resistance genes were not detected
242 (Supplementary Table 3). Whereas LIBA 5707 and LIBA5701 carry a *catP* gene for phenicol resistance,
243 only the former displays a PhLOPSa phenotype (Supplementary Table 3). All isolates had *ermB*.
244 However, this gene was also found in the linezolid-susceptible strain LIBA5701. *tet*(M) and various
245 aminoglycoside-resistance genes were sporadically detected in the WGS analyzed (Supplementary
246 Table 3).
247
248 **4. Discussion**
249 We report carriage of a rather large diversity of *cfr*-like genes associated with different MGEs by clinical
250 *C. difficile* strains from Latin America and provide for the first-time *in vitro* evidence of the
251 m^8A2503 methyltransferase activity of Cfr(C) and a novel *cfr*-like enzyme, Cfr(E). These two enzymes
252 do not clade with Cfr and therefore implicate a different group of Cfr-like proteins in antibiotic-
253 resistance.
254 The finding of *cfr*-like genes in various types of MGEs with partial hits to genomic sequences reported
255 for other intestinal Firmicutes lends evidence to the plasticity of the *C. difficile* genome [32] and supports
256 the role of this pathogen as a reservoir of resistance genes in the human gut [33]. This situation is
257 worrisome because linezolid is used for the treatment of methicillin-resistant *Staphylococcus aureus* [34]
258 and vancomycin-resistant enterococci [35], which reside in the same Phylum as clostridial organisms.
259 Indeed, versions of Tn*6218* such as those detected in isolates HON06 and HON10 have been found in
260 *Enterococcus faecium* isolates from German hospital patients [36].
261 The widespread detection of *cfr*-like genes among various epidemic NAP1/RT027 strains deserves
262 attention to clarify whether this situation contributes to virulence. This notion is reinforced by the fact
263 that linezolid and moxifloxacin resistance, a marker of highly virulent *C. difficile* strains, are often linked
264 in this ribotype [37]. Furthermore, since antibiotics are crucial both for the induction, progression, and
265 treatment of CDI, multidrug-resistance (MDR) is particularly worrisome when present in epidemic types
266 such as the NAP1/027 strain, which has been linked to severe CDI outcomes [38].

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267 Although the *cfr*(B) allele of isolates HON06, HON11, PUC51, and PUC347 is expressed, the last two
268 isolates did not show an evident $PhLOPS_A$ phenotype. It has been shown that Cfr(B) is functional when
269 encoded by Tn*6218* [10,13], hence we propose that it is not as active in PUC51 and PUC347 due to
270 neighboring-gene effects or different translation requirements in this new genetic background.
271 To further support the role of *cfr*-like enzymes in antibiotic resistance, we have provided the first *in vitro*
272 evidence that both Cfr(C) and Cfr(E) methylate the C8 position of A2503 in *E. coli* 23S rRNA. In this
273 regard, the poor activity of these enzymes towards the assayed rRNA fragments could reflect
274 differences in substrate requirements between clostridial Cfrs and *E. coli* RlmN [25] or result from the lack
275 of unknown modifications in the RNA substrate that may be necessary for efficient methylation by
276 Cfr(C) and Cfr(E).
277 Our results encourage analyses of further resistance phenotypes in strain collections from Latin
278 America. This can be achieved through a combination of classical phenotypic tests, whole genome
279 sequencing, and biochemical validation, as exemplified here. As already noted [39], a prompt phenotypic
280 and genotypic identification of resistance genes, effective antimicrobial stewardship and infection
281 control programs, and alternative therapies are needed to prevent and contain the spread of MDR *C.*
282 *difficile* strains.
283
284 **Declarations**
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291 **Competing Interests**
292 None
293 **Ethical Approval**

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294 None

295 **References**

296 1. Wilson DN. 2014. Ribosome-targeting antibiotics and mechanisms of bacterial resistance. Nature
297 Reviews Microbiology 12:35-48.

298 2. Roberts M. Mechanisms of MLS resistance. Available at:
299 http://faculty.washington.edu/marilynr/ermwebA.pdf. Accessed October 8, 2019.

300 3. Vester B. 2018. The *cfr* and *cfr*-like multiple resistance genes. Research in Microbiology 169:61-66.

301 4. Long KS, Poehlsgaard J, Kehrenberg C, Schwarz S, Vester B. 2006. The Cfr rRNA methyltransferase
302 confers resistance to Phenicols, Lincosamides, Oxazolidinones, Pleuromutilins, and Streptogramin A
303 antibiotics. Antimicrob Agents Chemother 50:2500-5.

304 5. Smith LK, Mankin AS. 2008. Transcriptional and translational control of the mlr operon, which confers
305 resistance to seven classes of protein synthesis inhibitors. Antimicrobial Agents and Chemotherapy
306 52:1703-1712.

307 6. Polikanov YS, Starosta AL, Juette MF, Altman RB, Terry DS, Lu W, Burnett BJ, Dinos G, Reynolds
308 KA, Blanchard SC, Steitz TA, Wilson DN. 2015. Distinct tRNA accommodation intermediates observed
309 on the ribosome with the antibiotics hygromycin A and A201A. Mol Cell 58:832-44.

310 7. LaMarre JM, Locke JB, Shaw KJ, Mankin AS. 2011. Low fitness cost of the multidrug resistance
311 gene *cfr*. Antimicrob Agents Chemother 55:3714-9.

312 8. Schwarz S, Werckenthin C, Kehrenberg C. 2000. Identification of a plasmid-borne chloramphenicol-
313 florfenicol resistance gene in *Staphylococcus sciuri*. Antimicrob Agents Chemother 44:2530-3.

314 9. Hansen LH, Planellas MH, Long KS, Vester B. 2012. The order Bacillales hosts functional homologs
315 of the worrisome *cfr* antibiotic resistance gene. Antimicrob Agents Chemother 56:3563-7.

316 10. Marin M, Martin A, Alcala L, Cercenado E, Iglesias C, Reigadas E, Bouza E. 2015. *Clostridium*
317 *difficile* isolates with high linezolid MICs harbor the multiresistance gene *cfr*. Antimicrob Agents
318 Chemother 59:586-9.

319 11. Schwarz S, Wang Y. 2015. Nomenclature and functionality of the so-called *cfr* gene from *Clostridium*
320 *difficile*. Antimicrob Agents Chemother 59:2476-7.

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321 12. Candela T, Marvaud JC, Nguyen TK, Lambert T. 2017. A *cfr*-like gene *cfr*(C) conferring linezolid resistance
322 is common in *Clostridium difficile*. Int J Antimicrob Agents 50:496-500.
323 13. Hansen LH, Vester B. 2015. A *cfr*-like gene from *Clostridium difficile* confers multiple antibiotic
324 resistance by the same mechanism as the *cfr* gene. Antimicrobial Agents and Chemotherapy 59:5841-
325 5843.
326 14. Valerio M, Pedromingo M, Munoz P, Alcala L, Marin M, Pelaez T, Giannella M, Bouza E. 2012. Potential
327 protective role of linezolid against *Clostridium difficile* infection. International Journal of Antimicrobial Agents
328 40:94-94.
329 15. Baines SD, Noel AR, Huscroft GS, Todhunter SL, O'Connor R, Hobbs JK, Freeman J, Lovering AM, Wilcox
330 MH. 2011. Evaluation of linezolid for the treatment of *Clostridium difficile* infection caused by epidemic strains
331 using an in vitro human gut model. Journal of Antimicrobial Chemotherapy 66:1537-1546.
332 16. Chilton CH, Crowther GS, Baines SD, Todhunter SL, Freeman J, Locher HH, Athanasiou A, Wilcox MH.
333 2014. In vitro activity of cadazolid against clinically relevant *Clostridium difficile* isolates and in an in vitro gut
334 model of C. difficile infection. Journal of Antimicrobial Chemotherapy 69:697-705.
335 17. https://www.pharmaceutical-technology.com/comment/jj-discontinues-development-c-difficile-
336 antibiotic-cadazolid/. Date of last access: October 5, 2019
337 18. Ramirez-Vargas G, Quesada-Gomez C, Acuna-Amador L, Lopez-Urena D, Murillo T, Gamboa-Coronado
338 MD, Chaves-Olarte E, Thomson N, Rodriguez-Cavallini E, Rodriguez C. 2017. A *Clostridium difficile* lineage
339 endemic to Costa Rican hospitals is multidrug resistant by acquisition of chromosomal mutations and novel
340 mobile genetic elements. Antimicrobial Agents and Chemotherapy 61.
341 19. Hidalgo-Villeda F, Tzoc E, Torres L, Bu E, Rodriguez C, Quesada-Gomez C. 2018. Diversity of multidrug-
342 resistant epidemic *Clostridium difficile* NAP1/RT027/ST01 strains in tertiary hospitals from Honduras. Anaerobe
343 52:75-78.
344 20. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S,
345 Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: A new
346 genome assembly algorithm and its applications to single-cell sequencing. Journal of Computational Biology
347 19:455-477.

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348 21. Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068-2069.

349 22. Jia BF, Raphenya AR, Alcock B, Waglechner N, Guo PY, Tsang KK, Lago BA, Dave BM, Pereira S,
350 Sharma AN, Doshi S, Courtot M, Lo R, Williams LE, Frye JG, Elsayegh T, Sardar D, Westman EL, Pawlowski
351 AC, Johnson TA, Brinkman FSL, Wright GD, McArthur AG. 2017. CARD 2017: expansion and model-centric
352 curation of the comprehensive antibiotic resistance database. Nucleic Acids Research 45:D566-D573.

353 23. Huerta-Cepas J, Forslund K, Coelho LP, Szklarczyk D, Jensen LJ, von Mering C, Bork P. 2017. Fast
354 genome-wide functional annotation through orthology assignment by eggNOG-mapper. Molecular Biology and
355 Evolution 34:2115-2122.

356 24. Yan F, LaMarre JM, Rohrich R, Wiesner J, Jomaa H, Mankin AS, Fujimori DG. 2010. RlmN and Cfr are
357 radical SAM enzymes involved in methylation of ribosomal RNA. J Am Chem Soc 132:3953-64.

358 25. Stojkovic V, Noda-Garcia L, Tawfik DS, Fujimori DG. 2016. Antibiotic resistance evolved via inactivation of
359 a ribosomal RNA methylating enzyme. Nucleic Acids Res 44:8897-8907.

360 26. Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic
361 Acids Res 32:1792-7.

362 27. Guindon S, Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by
363 maximum likelihood. Syst Biol 52:696-704.

364 28. Lanz ND, Grove TL, Gogonea CB, Lee K-H, Krebs C, Booker SJ. RlmN and AtsB as Models for the
365 overproduction and characterization of radical SAM proteins. In: Methods in Enzymology, 2012;
366 516:125–52. Available at: http://linkinghub.elsevier.com/retrieve/pii/B9780123942913000307.

367 29. Stojković V, Fujimori DG. Radical SAM-mediated methylation of ribosomal RNA. In: Methods in
368 Enzymology, 2015; 560:355–76.

369 30. Long KS, Vester B. 2012. Resistance to linezolid caused by modifications at its binding site on the
370 ribosome. Antimicrobial Agents and Chemotherapy 56:603-612.

371 31. Aranda PS, LaJoie DM, Jorcyk CL. 2012. Bleach gel: A simple agarose gel for analyzing RNA quality.
372 Electrophoresis 33:366-369.

373 32. Knight DR, Elliott B, Chang BJ, Perkins TT, Riley TV. 2015. Diversity and evolution in the genome of
374 *Clostridium difficile*. Clinical Microbiology Reviews 28:721-741.

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375 33. Johanesen PA, Mackin KE, Hutton ML, Awad MM, Larcombe S, Amy JM, Lyras D. 2015. Disruption of the
376 gut microbiome: *Clostridium difficile* infection and the threat of antibiotic resistance. Genes 6:1347-1360.
377 34. Watkins RR, Lemonovich TL, File TM, Jr. 2012. An evidence-based review of linezolid for the treatment of
378 methicillin-resistant *Staphylococcus aureus* (MRSA): place in therapy. Core Evid 7:131-43.
379 35. Chuang YC, Wang JT, Lin HY, Chang SC. 2014. Daptomycin versus linezolid for treatment of vancomycin-
380 resistant enterococcal bacteremia: systematic review and meta-analysis. BMC Infect Dis 14:687.
381 36. Bender JK, Fleige C, Klare I, Fiedler S, Mischnik A, Mutters NT, Dingle KE, Werner G. 2016. Detection of a
382 *cfr*(B) variant in German *Enterococcus faecium* clinical isolates and the impact on linezolid resistance in
383 *Enterococcus* spp. PLoS One 11:e0167042.
384 37. Freeman J, Vernon J, Vickers R, Wilcox MH. 2016. Susceptibility of *Clostridium difficile* Isolates of Varying
385 Antimicrobial Resistance Phenotypes to SMT19969 and 11 Comparators. Antimicrob Agents Chemother
386 60:689-92.
387 38. See I, Mu Y, Cohen J, Beldavs ZG, Winston LG, Dumyati G, Holzbauer S, Dunn J, Farley MM, Lyons C,
388 Johnston H, Phipps E, Perlmutter R, Anderson L, Gerding DN, Lessa FC. 2014. NAP1 strain type predicts
389 outcomes from *Clostridium difficile* infection. Clin Infect Dis 58:1394-400.
390 39. Spigaglia P. 2016. Recent advances in the understanding of antibiotic resistance in *Clostridium difficile*
391 infection. Ther Adv Infect Dis 3:23-42.
392

393 **Figure legends**

394 **Figure 1.** Evolutionary relationship of RlmN, Cfr, and Cfr-like sequences from selected Firmicutes
395 species. Functionally characterized Cfr enzymes, Cfr-like proteins, Cfr divergent proteins, and known
396 and putative RlmNs sequences are marked. While Cfr-like proteins clade with known Cfrs lacking
397 functional characterization, Cfr-divergent proteins diverged early in evolutionary time and do not clade
398 with either Cfrs or RlmNs. The enzymes of isolates *C. difficile* HON10/LIBA5707 and DF11 appear
399 highlighted in bold. The distance scale underneath the tree indicates the average number of
400 substitutions per site. IMG/JGI database identifiers or accession numbers of protein sequences used in
401 the tree are provided in Supplementary Table 1.

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402 **Figure 2.** Cfr(C)-, Cfr(E)-, and *E. coli* RlmN-mediated methylation of *in vitro* transcribed *E. coli* 2447-
403 2625 23S rRNA fragment (A). Cfr(C)-mediated methylation of *in vitro* transcribed *E. coli* and *C. difficile*
404 23S rRNA fragments (B). Bars represent the mean of at least two replicates ± s.d.
405 **Figure 3.** HPLC analysis of methylation products from Cfr(C) (blue), Cfr(E) (gray), and *E. coli* RlmN
406 reactions (m^2A, green) with *E. coli* 2447-2625 rRNA fragment. A m^8A standard is shown in orange.
407
408 **Supplementary material legends**
409 **Supplementary Table 1**. Sequences used for reconstruction of evolutionary relationships of Cfr, RlmN,
410 and Cfr-like proteins. Letters after scientific names indicate the existence of paralogous genes in the
411 species.
412 **Supplementary Table 2**. Features of the *cfr*-like protein detected in *C. difficile* DF11 that support its
413 classification as CfrE
414 **Supplementary Table 3**. Resistome of Latin American *C. difficile* strains with predicted rRNA
415 dimethylases as determined *in silico* by the Resistance Gene Identifier (RGI CARD) and resfinder tools
416 **Supplementary Figure 1.** *cfr*(B) expression in isolates PUC51 and PUC347 grown for 20h. Agarose
417 gel electrophoresis of PCR products obtained for genomic DNA (2), a reagent control (3), cDNA (4),
418 and RNA (5). Amplification of a 150 bp DNA fragment from retrotranscribed RNA confirms *cfr*(B)
419 expression. A 100 bp ladder is shown in lane 1. Similar results were obtained for cells in logarithmic
420 growth phase (8h).

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

Isolate	Origin/Year of isolation	Type (PFGE/RT)	PFGE pattern[a]	% identity to reference protein sequence(s)[c]	*cfr*-like gene detected	Previously reported in *C. difficile* (Accession number)
HON06	CDI/Honduras/2016	NAP1/027	1057	Cfr(B) KM359438 (99-100%) Cfr(B) KR610408 (99-100%)	*cfr*(B)	Strain 11140508 (KM359438)
HON11	CDI/Honduras/2016		0461			
PUC51	CDI/Chile/2011		ND[b]			
PUC347	CDI/Chile/2011		ND			
HON10	CDI/Honduras/2016	NAP1/027	1056	Cfr(C) CCL89685 (100%) Cfr(C) ENZ41453 (100%)	*cfr*(C)	Strain T10 (CCL89685)
LIBA5707	CDI/Costa Rica/2009	NAP_{CR1}/012	448			
DF11	CDI/Mexico/2015	NAP1/027	1058	Cfr 879565 (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)[d]	No

422
423 [a]NML-Canada database
424 [b]Not determined
425 [c]Reference sequences taken from the MLS nomenclature system (https://faculty.washington.edu/marilynr/ermweb1.pdf)
426 [d]New gene according to the 80% protein sequence identity threshold defined for this group of enzymes

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427 **Table 2.** Minimum inhibitory concentrations of PTC-targeting antibiotics determined for *C. difficile* isolates from Latin America with

428 predicted rRNA dimethylases

429

Isolate	*cfr*-like gene	MIC (µg/ml)			
		Linezolid[b]	Tiamulin[c]	Thiamphenicol[c]	Virginiamycin M1[d]
HON06	*cfr*(B)	24	128	≥256	160
HON11	*cfr*(B)	24	128	≥256	320
PUC51	*cfr*(B)	2	4	4	80
PUC347	*cfr*(B)	2	16	8	20
HON10	*cfr*(C)	24	128	≥256	160
LIBA5707	*cfr*(C)	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

430

431 [a]NAP_{CR1} strain lacking *cfr*-like genes used as negative control

432 [b]As determined by epsilometry

433 [c]As determined by agar macrodilution

434 [d]As determined by broth microdilution

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435 [e]ND: Not determined

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436 **Table 3.** Annotation of the putative mobile genetic elements (MGE) in which *cfr*-like genes were detected

437

Isolate(s)	Element synteny	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 a HTH transcriptional regulator
PUC51 PUC347	Transposase - **Cfr-like protein** - Integrase - RNA methylase - Hypothetical protein - endonuclease - Hypothetical protein - Mobilization protein - Helicase		Undescribed	

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441

442 **Table 3.** Annotation of the putative mobile genetic elements (MGE) in which *cfr*-like genes were detected (Cont.)

443

Isolate(s)	Element synteny	Cfr type	MGE type	Genome insertion site
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*

444

445 [a]Accession number for Tn*6218* in *C. difficile* Ox2167: HG002396.1

446 [b]Accession number for *C. difficile* F548 assembly: GCA_000452325.2

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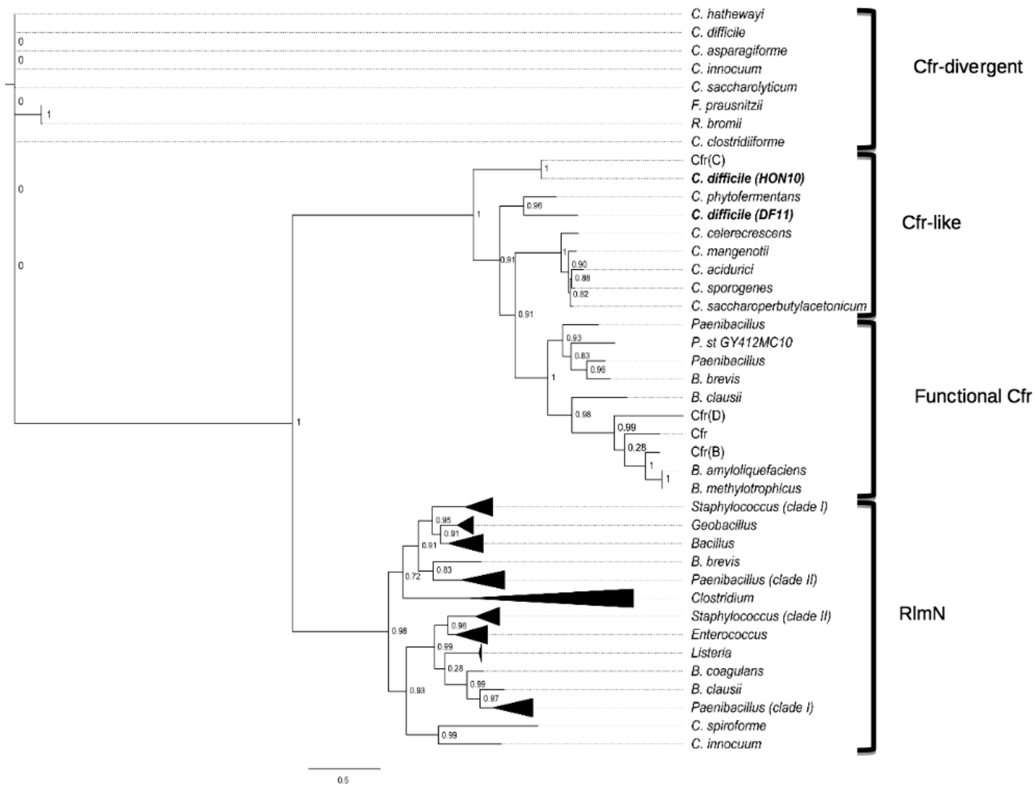

Fig. 1

468 Fig2

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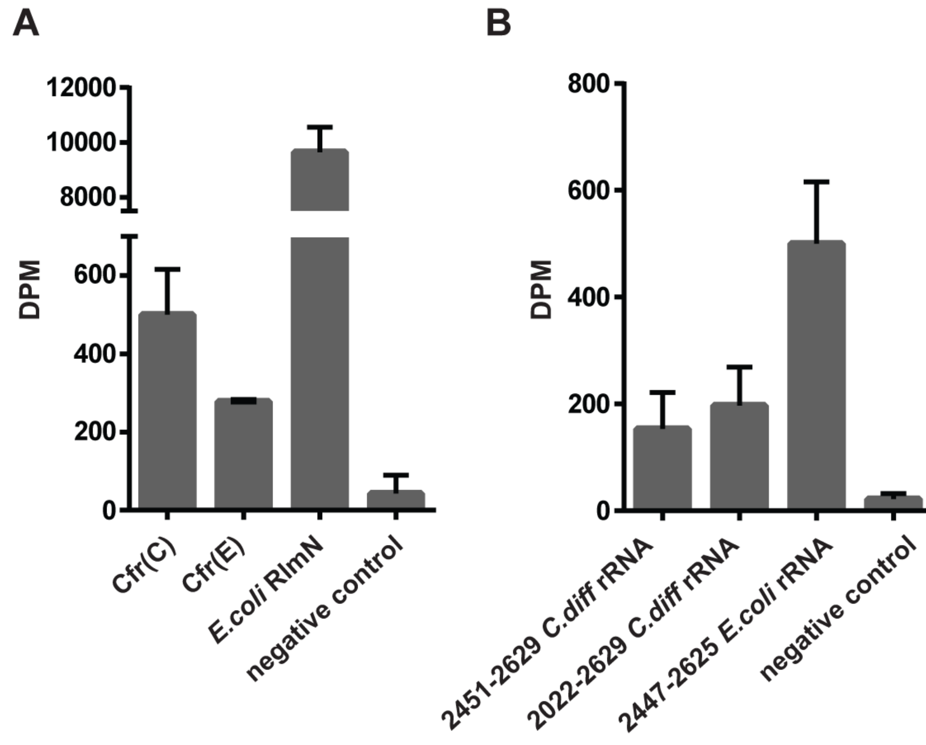

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471 Fig 3.

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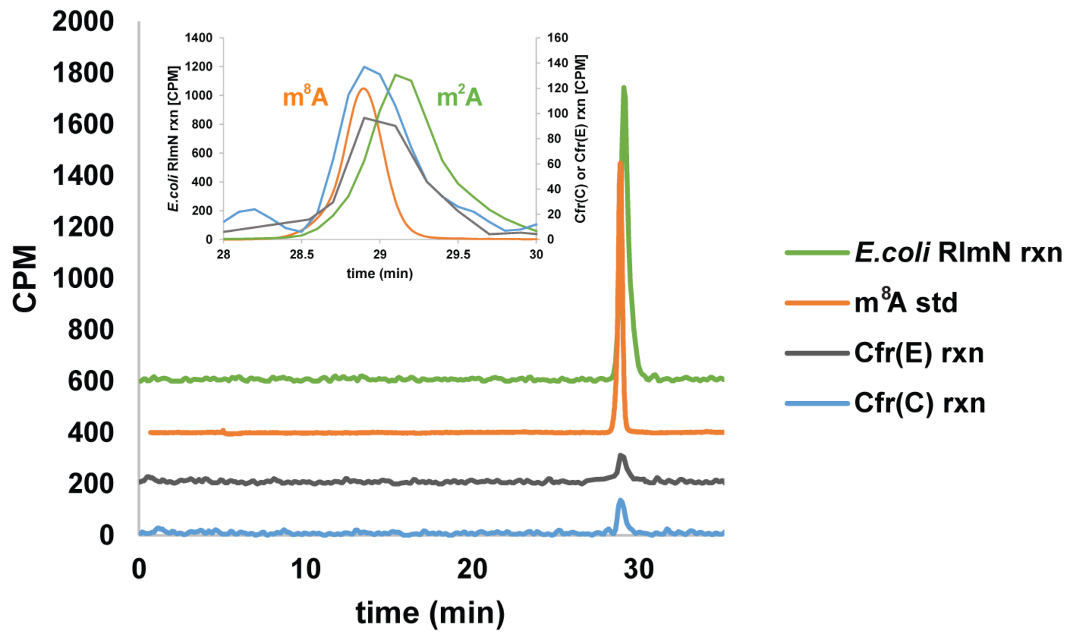

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