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Analysis of RNA methylation by phylogenetically diverse Cfr radical SAM enzymes reveals an iron-binding accessory domain in a clostridial enzyme

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Abstract

Cfr is a radical *S*-adenosylmethionine (SAM) RNA methylase linked to multi-drug antibiotic resistance in bacterial pathogens. It catalyzes a chemically challenging C-C-bond forming reaction to methylate C8 of A2503 [*Escherichia coli* (*Ec*) numbering] of 23S ribosomal RNA during ribosome assembly. The *cfr* gene has been identified as a mobile genetic element in diverse bacteria and in the genome of select Bacillales and Clostridiales species. Despite the importance of Cfr, few representatives have been purified and characterized *in vitro*. Here we show that Cfr homologs from *Bacillus amyloliquefaciens*, *Enterococcus faecalis*, *Paenibacillus lautus*, and *Clostridioides difficile* act as C8 adenine RNA methylases in biochemical assays. *C. difficile* Cfr contains an additional Cys-rich C-terminal domain that binds a mononuclear Fe²⁺ ion in a rubredoxin-type Cys₄ motif. The C-terminal domain can be truncated with minimal impact on *C. difficile* Cfr activity, but turnover is diminished upon disruption of the Fe²⁺ binding site by Zn²⁺ substitution or ligand mutation. These findings indicate an important purpose for the observed C-terminal iron in the native fusion protein. Bioinformatic analysis of the *C. difficile* Cfr Cys-rich domain shows that it is widespread (~1000 homologs) as a stand-alone gene in pathogenic or commensal bacilli and clostridia, with >10% encoded adjacent to a predicted radical-SAM RNA methylase. Although the domain is not essential for *in vitro* *C. difficile* Cfr activity, genomic co-

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ACCESSION CODES

WP_002578771 - *Clostridioides difficile* str. QCD63q42

WP_015735625 - *Paenibacillus lautus* sp. Y412MC10

WP_013351126 - *Bacillus amyloliquefaciens* str. DSM-7 (ATCC 23350)

WP_002405682 - *Enterococcus faecalis* str. TX0635

AIT41446 - *Staphylococcus aureus* (plasmid)

WP_003483120 - *Clostridium sporogenes* str. ATCC 15579

Supporting information includes supplementary tables S1–S4, and supplementary figures S1–9.

occurrence and high abundance in the human microbiome suggests a possible functional role for a specialized rubredoxin in certain radical-SAM RNA methylases with relevance to human health.

Graphical Abstract

INTRODUCTION

Radical *S*-adenosylmethionine (SAM) enzymes compose one of the largest enzyme superfamilies defined to date, with more than 400,000 members.¹ Radical SAM enzymes are essential in primary anaerobic metabolism and in the synthesis of diverse secondary metabolites.^{2,3} Nucleic acid modification is a third central function of radical SAM proteins, and this activity has been demonstrated in all three domains of life.^{4,5} A pair of adenine RNA methylases, RlmN and Cfr, are among the most widespread and well-characterized radical SAM nucleic acid modification enzymes in bacteria.^{6–8} Although originally discovered to methylate C2 of A2503 in ribosomal RNA (rRNA), a base located in the peptide exit tunnel of the peptidyltransferase center of the bacterial ribosome, RlmN also modifies six additional tRNA substrates at A37.^{9,10} In *Escherichia coli*, RlmN is non-essential, but its activity improves translational fidelity and organism fitness.¹⁰ More than 20,000 RlmN homologs have been identified in the sequenced genomes of prokaryotes of diverse origin.¹ Related Cfr enzymes are smaller in number, with approximately 200 homologs annotated in Interpro and other protein sequence databases. Cfrs were initially found encoded as mobile genetic elements in staphylococcal pathogens,⁹ and *Staphylococcus aureus* (*Sa*) Cfr has been characterized extensively *in vitro*. The enzyme methylates C8 of A2503 in 23S rRNA, and this modification confers resistance to multiple classes of antibiotics.¹¹ Although quite relevant to human health, few of the other Cfrs have been characterized *in vitro*, and the structure of the enzyme is not known.

The common methyl donor SAM is the source of the newly appended methyl carbon in RlmN and Cfr.⁶ In their function as C-methylases, RlmN and Cfr perform a chemically challenging C-C bond forming reaction involving functionalization of inert *sp*²-hybridized C-H bonds.¹² This activity cannot be accomplished by the standard polar mechanism characteristic of SAM-dependent methyl transferases. Instead, radical chemistry is required and enabled here by a second SAM-dependent activity in RlmN and Cfr – transient

formation of a potentially oxidizing 5'-deoxyadenosyl radical (5'-dA•).^{6, 7, 15} Nearly all enzymes in the radical SAM superfamily share this intermediate,^{13, 16} generated by reduction of a SAM-coordinated [4Fe4S]²⁺ cluster that, in turn, triggers reductive cleavage of the coordinated cosubstrate. In RlmN and Cfr, the 5'-dA• abstracts a hydrogen atom from a Cys-appended -CH₃ group (mC338, *Sa* Cfr numbering) to yield a methylene radical (Figure 1).⁷ Radical addition to the adenine nucleobase at either C2 or C8 generates a transient protein-RNA crosslink containing an unpaired electron on the adenine nucleobase.¹⁷ Proton transfer from C2/C8 to an active site base, a second conserved Cys residue (C105, *Sa* Cfr numbering), resolves the crosslink.^{14, 18, 19} This step is proposed to yield a thiyl radical at the C-terminal C338, which must be reduced prior to subsequent turnover.¹⁴ Interestingly, several other redox-neutral radical SAM C-C bond forming enzymes also oxidize an active site Cys side chain¹² to balance the initial two-electron reductive cleavage of SAM.^{12, 20} In these systems, auxiliary iron-sulfur cluster or cobalamin-binding domains are often present and proposed to provide necessary reducing equivalents.¹² The RlmN and Cfr homologs characterized to date are distinct in that they do not harbor an obvious universal dedicated thiyl radical reductant.

The proposed mechanism of the RNA methylases has been validated through spectroscopic and biochemical analysis of *E. coli* RlmN and *S. aureus* Cfr.^{7, 14, 17, 21} The two key catalytic Cys residues (C338 and C105) are strictly conserved in these homologs and all others identified to date. X-ray crystal structures of *E. coli* RlmN have been solved, both alone²² and in complex with tRNA,¹⁸ the latter obtained by trapping the covalent protein-RNA intermediate via substitution of C118 with a non-functional alanine (C105A equivalent).^{18, 22} To date, no structures have been reported of either enzyme in complex with a ribosomal RNA substrate. Additionally, Cfr homologs have not been structurally characterized in any form, even though such information would be useful in the design of inhibitors that could rescue the activities of existing antibiotics to which Cfr currently confers bacterial resistance.

To identify Cfr candidates suitable for ongoing structural studies, we performed phylogenetic analyses of *Sa* Cfr homologs identified in database searches. As reported previously, at least four distinct groups of enzymes emerge, including a clostridial clade classified as Cfr-like.²³ Cfr-like homologs from *Clostridium sporogenes*²³ and *Clostridium phytofermantas*²⁴ have been proposed to harbor a different, still undefined, activity because they fail to confer antibiotic resistance and lack C8 methylation activity in primer extension assays when overexpressed in *E. coli*.^{23, 24} Here we show that heterologously expressed and purified Cfr homologs from *Bacillus amyloliquefaciens* (*Ba*), *Enterococcus faecalis* (*Ef*), *Paenibacillus lautus* (*Pl*), and *Clostridioides difficile* (*Cd*) are capable of rRNA methylation *in vitro*. All four enzymes contain a full occupancy [4Fe-4S] cluster in their wild-type (wt) forms, as established by Mössbauer spectroscopy and other analytical techniques. All four homologs modify A2503 at C8 but with different rates. The enzymes also vary in their capacity for subsequent C2 methylation of the same base. *C. difficile* Cfr is active as a C8 methylase with limiting RNA substrate but exhibits distinctly slower methylation when challenged with excess RNA. Interestingly, this Cfr homolog contains a unique ~50-amino-acid Cys-rich C-terminal extension related by sequence to a much larger family of clostridial and streptococcal proteins of unknown function. In *Cd* Cfr, this domain binds a mononuclear

Fe²⁺ ion in a 4-Cys binding site with a midpoint potential [Fe²⁺/Fe³⁺ couple] of +105 mV vs. NHE, similar to those of rubredoxin electron transfer proteins. Substitution of the C-terminal metal binding site with Zn²⁺, or Cys → Ala mutation of one of the predicted Fe²⁺ ligands, significantly impedes turnover. However, complete removal of the C-terminal domain has no effect on *in vitro* activity. While only a few select Cfr homologs contain a Cys-rich C-terminal fusion, genome neighborhood analysis of the larger family of stand-alone domains reveals that >10% are encoded next to clostridial radical SAM enzymes. The findings suggest that these iron-binding rubredoxin-like proteins are widespread in Clostridiales and, to a lesser extent, Bacillales organisms, including pathogens and human commensals. While the metal-binding domain is dispensable for *in vitro* Cfr activity in *C. difficile*, an essential role for this specialized rubredoxin (or another protein partner) *in vivo* could explain the reported lack of RNA methylation activity in other clostridial Cfr enzymes.

MATERIALS AND METHODS

Materials.

Kanamycin was obtained from Teknova (Hollister, CA). Ampicillin was purchased from Dot Scientific (Burton, MI). *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), DNaseI (RNase-free), Bradford reagent, and His-Pur Co²⁺-TALON resin were obtained from Thermo Fisher Scientific (Waltham, MA). Sodium sulfide, 2-mercaptoethanol, sodium dithionite, glucose, *S*-adenosylmethionine, and p1 nuclease from *Penicillium citrinum* were purchased from Sigma Aldrich (St. Louis, MS). Iron (III) chloride hexahydrate was acquired from BDH Analytical Chemicals, VWR (Radnor, PA). DL-dithiothreitol (DTT) was obtained from Alfa Aesar (Tewksbury, MA). ⁵⁷Fe metal (98%) was purchased from Isoflex USA (San Francisco, CA). Bovine serum albumin (BSA), *Xho*I, *Nde*I, antarctic phosphatase, Q5 DNA polymerase, and T4 DNA ligase were obtained from New England Biolabs (Ipswich, MA). PD-10 and HiPrep Sephacryl 16/60 S200 columns were purchased from GE Healthcare (Little Chalfont, United Kingdom).

Phylogenetic analysis of Cfrs.

Homologs were identified by BLASTp analysis using *Sa* Cfr as the query against the non-redundant protein sequence database (NCBI, accessed March 2018) with default search parameters. The top 500 matches were aligned in MUSCLE after consolidation with ExPASy (<98% minimum identity filter in the decrease redundancy tool) to remove duplicate sequences. The final pool contained 361 unique sequences.^{25, 26} The program MEGA7²⁷ was used to compute a phylogenetic tree using the minimum evolution method with the JTT model of substitution, allowance of pairwise deletions, and closest neighbor set to level = 2. Bootstrap values were computed from 1000 replicates.

Cloning and overexpression of full-length Cfr proteins.

DNA sequences encoding genes for the Cfrs selected for study (Table S1) (with the exception of *Cd* Cfr) were codon-optimized for over-expression in *E. coli*, synthesized, and inserted into a pMK-T vector (GeneArt). PCR primers (Table S1) were designed with Primer3 and appended at the 5'-end with *Nde*I and *Xho*I restriction sites. The coding sequences were amplified by PCR using Q5 DNA polymerase. The PCR products were

digested using *NdeI* and *XhoI* restriction enzymes, purified by gel electrophoresis, re-isolated using an Omega Biotek gel extraction kit, and ligated into pET26b. The *C. difficile cfr* overexpression plasmid was constructed using the Gibson assembly method.²⁸ The gene was codon optimized for overexpression in *E. coli* and synthesized in two pieces, each containing 20 base-pair regions that overlap with the pET26b vector and with each other. The gene and linearized vector were combined with a 5'-exonuclease, DNA polymerase, and DNA ligase to allow for pET26b-*cfr* assembly. All four pET26b-*cfr* sequences were verified by Sanger sequencing at the Pennsylvania State University Nucleic Acid Facility, and the coding regions can be found in Table S1 of the Supporting Information. All plasmids additionally encode a C-terminal His₆-tag with a short two amino acid (-Leu-Glu-) linker.

The C363A *CdCfr* variant was generated by inverse PCR using a new forward primer containing the relevant substitution in the annealing region (Table S1). Amplification was performed using the pET26b/Cfr (*Cd*) plasmid (generated as described above) as the DNA template. *DpnI* digestion was used to remove the original template DNA and the vector was circularized by ligation with T4 DNA ligase.

Overexpression of all constructs was accomplished in *E. coli* BL21(DE3) cells co-transformed with pET26b-*cfr* and pDB1282,²⁹ the latter a vector containing inducible iron-sulfur cluster assembly machinery. Successful transformants were selected on kanamycin (50 µg/mL) and ampicillin (100 µg/mL) supplemented LB plates. 1.5 L cultures of the overexpression strains were grown shaking (180 rpm) at 37 °C in M9 minimal medium supplemented with kanamycin (50 µg/mL), ampicillin (100 µg/mL), iron (III) chloride (30 µM), and L-cysteine (300 µM) to maximize iron-sulfur cluster incorporation.³⁰ Growths for samples suitable for Mössbauer analysis utilized ⁵⁷Fe dissolved in 1 M H₂SO₄. At an OD₆₀₀ = 0.3, L-arabinose was added [final concentration 0.08 % (w/v)] to induce overexpression of the genes harbored on the pDB1282 plasmid. At an OD₆₀₀ = 0.6, IPTG was added to a final concentration of 100 µM to induce overexpression of the pET26b-*cfr*. After incubation overnight at 18 °C at 180 rpm, cells were pelleted by centrifugation at 5000g for 10 min at 4 °C. Approximately 3.2 g cell paste was obtained per liter of culture. Cell paste was flash-frozen in liquid nitrogen and stored at -80 °C. Flavodoxin and flavodoxin reductase were purified as described previously.³⁰

Purification of full-length Cfrs.

All purification steps were performed anaerobically in an anoxic chamber with a mixed H₂/N₂ atmosphere (Coy Laboratory Products). Cells were resuspended in lysis buffer (50 mM HEPES pH 7.5, 300 mM KCl, 4 mM imidazole, 10 mM MgCl₂, 10% (v/v) glycerol, 10 mM β-mercaptoethanol) in a nickel-coated beaker containing 1 mg/mL lysozyme, 100 µg/mL DNase, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The suspension was stirred at room temperature for 30 min and lysed by sonication (QSonica Q500, 10 s pulse, 30 s rest, 60% amplitude) on ice. The resulting cell debris was pelleted by centrifugation at 11,300g at 4 °C for 1.25 h in a sealed centrifuge bottle. The supernatant was applied to Co²⁺-TALON resin. Protein fractions were eluted in 50 mM HEPES, pH 7.5, 300 mM KCl, 300 mM imidazole, 10 mM MgCl₂, 30% (v/v) glycerol, 10 mM β-mercaptoethanol. Reconstitution of [4Fe4S]²⁺ clusters was performed on ice for all samples except *Cs* Cfr.

Protein samples (200 μ M) were exchanged into gel-filtration chromatography buffer [10 mM HEPES pH 7.5, 500 mM KCl, 5 mM DTT, 10 mM MgCl_2 , 25% (v/v) glycerol] and incubated for 30 min at room temperature. Iron (III) chloride (800 μ M) was added to the solution, which was subsequently incubated for 1 h at room temperature. Sodium sulfide was added to the solution (to a final concentration of 800 μ M) in six equal volumes over the course of 2 h. The solution was allowed to incubate on ice overnight. A PD-10 column was used to remove excess iron and sulfide. Size-exclusion chromatography was performed using a Sephacryl S200 column. Protein concentrations were determined by Bradford analysis with correction factors (Molecular Structural Facility at UC Davis) of 0.891 (*Cd*), 0.627 (*Ba*), 0.828 (*Ef*), and 0.687 (*Pl*). Iron-sulfur cluster incorporation (Table 2) was assessed by measuring the number of iron equiv per protein with the ferrozine assay.³¹

Overexpression and purification of truncated and single-site *Cd Cfr* variant proteins.

Truncated *Cd Cfr* variants consisting of either the radical SAM domain (*Cd-N*), residues 1–345, or the rubredoxin/KTR domain (*Cd-C*), residues 346–416, were generated using the approach described above (see Table S1 for primer sequences). A C363A variant (*M*) of full-length *Cd Cfr* was generated as described. Overexpression and purification of all variant proteins was performed as described for the wt enzymes. Zinc replacement of the C-terminal metal binding site in *Cd-C* was accomplished by addition of ZnCl_2 (7 mM) to minimal medium cultures both upon initial inoculation and at $\text{OD}_{600} \sim 0.6$ in place of iron supplementation. Both iron- and zinc-bound versions of *Cd-C* were overexpressed without pDB1282 present, and the iron-sulfur cluster reconstitution step was omitted. Iron and zinc occupancies were determined by inductively coupled plasma optical emission spectroscopy (ICP-OES) at the Pennsylvania State University Laboratory for Isotopes and Metals in the Environment (LIME). *Cd-C* contains 0.45 equiv iron, *Cd-Z* contains 0.36 equiv zinc, and full-length *Cd C363A* contains 2.53 equiv iron.

Synthesis and purification of RNA.

To generate a 155 nucleotide (nt) fragment of *Ec 23S* rRNA corresponding to A2454-G2608 (Figure S1), previously shown to be efficiently methylated by *Sa Cfr*,⁵ a DNA template was amplified using primers 2454 and 2608 (Table S1), and transcribed in a reaction with T7 RNA polymerase (500 μ g/mL). The transcription reaction was performed in 30 mM Tris, pH 8.4, 20 mM DTT, 36 mM MgCl_2 , 0.01% (w/v) Triton X-100, 2 mM spermidine, 2 mM NTPs (4 mM GTP). The template DNA PCR product was diluted to a final concentration of 5 ng/ μ L and incubated for 3 h at 37 °C with the components above. DNaseI was added to the solution (to a final concentration of 0.005 U/ μ L) and incubated at 37 °C for an additional hour. EDTA (pH 8.5) was added to a final concentration of 50 mM, and the solution was frozen at –20 °C for at least 2 h. Full-length 155mer RNA transcripts were purified under anaerobic conditions (Coy Laboratory Products) by PD-10 column equilibrated in filtered nanopure water. RNA concentrations were determined by UV-visible spectrophotometry ($\epsilon_{260} = 1.87 \mu\text{m}^{-1} \text{cm}^{-1}$).¹⁷ The A2503G variant RNA was prepared similarly, except the first PCR step was performed with a synthetic template (Integrated DNA Technologies, see Supporting information for sequence) containing the desired base change.

RNA methylation activity assays with limiting substrate.

In experiments using a protein-based low-potential reducing system [flavodoxin (Flv), flavodoxin reductase (Flx), and NADPH], RNA methylation was assayed in a total volume of 150 μ L at room temperature in an anoxic glovebox (Coy Laboratory Products) over a time course of 2.75 h. Samples contained 48 μ M Cfr and 50 μ M 155mer RNA in 100 mM sodium EPPS, pH 8.0, 10 mM MgCl_2 , 12.5% (v/v) glycerol, 250 mM KCl, 200 μ M Flv, 20 μ M Flx, and 2 mM NADPH. The solution was incubated at room temperature for 5 min before initiating the reaction by addition of SAM to a final concentration of 1 mM. At designated time points, a 10 μ L aliquot of the reaction solution was removed and added to 10 μ L of quench solution containing 250 μ M tryptophan and 95 mM H_2SO_4 . Quenched reactions were diluted 1:1 in P1 nuclease buffer (250 mM sodium acetate, pH 6.0, 45 mM NaCl, 4 mM ZnCl_2). Antarctic phosphatase (10 U) and P1 nuclease (1 U) were added to digest the RNA into individual nucleosides. The digestion reactions were incubated at 37 $^\circ\text{C}$ overnight and subsequently analyzed by liquid chromatography (Agilent 1960/1990) coupled to mass spectrometry (LC-MS) (Agilent 6460 QQQ) (LC-MS). Assay mixtures were separated on an Agilent Extend-C18 column (4.6 $\times$ 50 mm) equilibrated in a 40 mM ammonium acetate, 5% (v/v) methanol (solvent A) / 100% methanol (solvent B) system using the conditions listed in Table 3 below.

MS detection of products (Figure S2) and assignment of peaks were accomplished as described previously.²¹ Results were analyzed with the MassHunter software package, and quantitative analyses were facilitated by comparison to a standard curve calculated from data acquired from the tryptophan internal standard. Unproductive cleavage of SAM was determined by dividing the number of SAM cleavage events, obtained from the concentration of 5'-dA in LC-MS samples, by the estimated number of methylation events ($[\text{m}8\text{A}] + 2 \cdot [\text{m}2,8\text{A}]$). Six full-length and four variant Cfr proteins were tested for methylase activity. Assays using *Cd*Cfr in which the C-terminal domain of *Cd*Cfr (Cd-C) was removed (giving Cd-N) are indicated as such in activity measurement plots (48 μ M protein). Cd-C was also added back separately at a 1:1 ratio. These samples are renamed Cd Fe res (iron-bound Cd-C) or Cd Zn res (zinc-bound Cd-C) in plots. All assays were performed at least three times on different days. Figures show the average product concentration for three selected experiments, with an error bar showing the standard deviation in these values. The assay metrics in Table S2 are the average $\pm$ standard deviation over the selected trials.

Time courses for experiments using limiting substrate for *Ba*, *Ef*, and *Pl*Cfrs were initially simulated in KinTek Explorer⁵² with the following model.

(1)

In this model, enzyme (E) and substrate (RNA) bind and react to form the first product, m8a (EM). This monomethylated compound can be further converted to m2,8a (EP), as shown above. Both steps are considered to be irreversible. In initial simulations, we found that the best fit for k_1 (k_{obs} m8a) falls below a lower limit set by the steady-state turnover rate ($v/[E]$) determined in subsequent experiments with excess RNA. This discrepancy indicated that the simple kinetic model in (1) is not sufficient. However, in the absence of additional experimental data to constrain the rates of other steps (substrate binding, product release, etc.), we instead fixed k_1 to the lower limit stated above, obtained from fits of m8a production with excess RNA to a single exponential function (described below). We then used KinTek Explorer to simulate the reaction to obtain the apparent rate constant (k_2 or k_{obs} m2,8a) for the second methylation step. Note that in our model, the rate constant for the second dimethylation reaction includes, in addition to the catalysis step, m8a product release and substrate rebinding steps. The fits we obtained are reasonable but, given these complexities, this k_2 value should be viewed as a lower limit for the rate of the second methylation reaction. For *Ba/Efl* Cfr, the active enzyme fraction was allowed to vary and fell below 48 μM for ideal performance in the simulation. Because the limiting-substrate time course of *Cd* Cfr and its variants yields little dimethylated product, product vs. time traces were fit to a single exponential function to extract the apparent reaction rate (k_{obs} m8a) with equation (2), in which a is the product concentration at completion and k is equivalent to k_{obs} for m8a formation. We assumed a fully active enzyme pool in this analysis.

$$[\text{m8a}] = a \cdot (1 - e^{-kt})$$

(2)

RNA methylation activity assays with excess substrate.

Activity assays with excess substrate were performed as described above but with 100 μM 155mer RNA substrate and 3 μM Cfr enzyme. The reaction solutions were prepared in 150 μL total volume with 100 mM sodium EPPS, pH 8, 10 mM MgCl_2 , 12.5% (v/v) glycerol, 250 mM KCl, 200 μM Flv, 20 μM Flx, and 2 mM NADPH. Reactions were initiated by addition of 1 mM SAM. Time courses were analyzed via linear fit to the initial portion of the progression curve for m8a formation to obtain the initial velocities of the reactions. This approach allowed us to obtain a reaction rate, $v/[E]$, for each Cfr enzyme and variant (Table S3). A single exponential fit, using equation (2), to the full m8a time course allowed us to obtain the amplitude of each enzymatic reaction. This value reflects the total amount of methylated product generated by each enzyme for the entire experiment. The information was used to determine a total turnover number (TON, Table S3), after division by the initial enzyme concentration (3 μM). The goal of the latter analysis was to ascertain whether each enzyme is capable of performing more than one full substrate conversion.

Spectroscopic analysis of Cfr proteins.

Reconstituted ^{57}Fe protein samples were transferred to EPR tubes or Mössbauer cups and flash-frozen in liquid nitrogen in an anoxic chamber (Coy Laboratory Products). Mössbauer spectra were recorded on constant acceleration Mössbauer spectrometers (SEE Co., Edina, MN) equipped either with a Janis SVT-400 variable-temperature cryostat (weak-field) or a Janis 8TMOSS-OM-12SVT variable-temperature cryostat (strong-field) (Janis, Woburn, MA). All isomer shifts are quoted relative to the centroid of the spectrum of α -iron metal at room temperature. Simulations of Mössbauer spectra were carried out using WMOSS (SEE Co., Edina, MN). Some of the simulations are based on the spin Hamiltonian formalism (see equation below), in which the first term describes the electron Zeeman effect, the second and third terms describe the axial and rhombic zero-field splitting of the total electron spin ground state, the fourth term represents the interaction between the electric field gradient and the nuclear quadrupole moment, the fifth term describes the magnetic hyperfine interactions of the ^{57}Fe nucleus with respect to the total electron spin ground state, and the last term represents the nuclear Zeeman interactions of ^{57}Fe . All symbols are conventional. ³² Spectra were calculated in the slow relaxation limit.

$$H = \beta S \cdot g \cdot B + D \left(S_z^2 - \frac{S(S+1)}{3} \right) + E \left(S_x^2 - S_y^2 \right) + \sum_i \frac{eQV_{zz,i}}{4} \left[I_{z,i}^2 - \frac{I_i(I_i+1)}{3} + \frac{\eta}{3} (I_{x,i}^2 - I_{y,i}^2) \right] + \sum_i S \cdot A_i \cdot I_i - \sum_i g_n \beta_n B \cdot I_i$$

EPR samples were analyzed on a Bruker ESP 300 spectrometer (Billerica, MA) equipped with an ER 041 MR microwave bridge and an ER 4116DM resonator.

UV-visible spectrophotometry.

Oxidation of the CdCfr C-terminal domain was analyzed spectrophotometrically using an Agilent 8453 G1103A spectrometer. In an anoxic glovebox, a 50 μL sample of 9.4 mM Cd-C [10 mM HEPES pH 7.5, 500 mM KCl, 5 mM DTT, 10 mM MgCl_2 , 25% (v/v) glycerol] was diluted to 1 mL in water and sealed in a screw cap quartz cuvette (Starna Cells, Inc). The anoxic protein solution was scanned from 190 nm to 1100 nm with a baseline correction at 800 nm. The sample was oxidized by opening the cuvette and pipetting the sample intermittently to introduce oxygen into the solution. The cuvette remained open for the duration of the experiment. The solution was scanned at the following times: 1 min, 2.5 min, 5 min, 7.5 min, 10 min, 20 min, and 30 min. A difference spectrum was calculated by subtracting the initial anoxic spectrum from the final spectrum.

Protein film voltammetry.

Square-wave (SW) voltammetry experiments were performed on a VersaSTAT 3 (Princeton Applied Research) attached to a three-electrode electrochemical cell housed in an anaerobic chamber (Coy Laboratory Products). Protein films were generated via incubation of 9.4 mM Cd-C [10 mM HEPES pH 7.5, 500 mM KCl, 5 mM DTT, 10 mM MgCl_2 , 25% (v/v) glycerol] on a pyrolytic graphite edge (PGE) working electrode for 10 min at room temperature. The working electrode surface was prepared by polishing the electrode surface

with 0.3 μm alumina slurry (Allied High Tech) followed by washing in nanopure H_2O in a sonication bath for 6 min. The electrode was dried and brought into the glovebox. All subsequent steps were performed under anoxic conditions. The background current was measured with the bare PGE working electrode, Ag/AgCl reference electrode (Pine Research), and Pt wire auxiliary electrode in 50 mM HEPES, pH 7.5, 300 mM KCl. SW voltammograms were collected using a step potential of 10 mV, a pulse amplitude of 25 mV, and a SW frequency of 10 Hz. A current range gate of 20 μA was applied and a 1 kHz filter was used as a background filter. A significant decrease in current amplitude was observed between the first and second scans with smaller decreases evident upon successive scans.

Genome neighborhood network (GNN) and isoelectric point analysis of cysteine-rich KTR proteins.

The complete FASTA file for the cysteine-rich KTR family was downloaded from Interpro (IPR025957) and used to generate sequence similarity networks (SSNs) with the EFIEST server³³ via default parameters. An SSN calculated with an alignment score of 31 was used to make a genome neighborhood network with the EFI-GNT server³⁴ (± 10 ORFs, 5% co-occurrence). Results were visualized and analyzed in Cytoscape.

To calculate the predicted isoelectric points of cysteine-rich KTRs, the full FASTA file for IPR025957 was pruned with the ExPASy decrease redundancy tool (<98% minimum identity), and the resulting FASTA file was used as input to the protein isoelectric point calculator web server (<http://isoelectric.org/>).³⁵ The results for each individual KTR were averaged to obtain the overall predicted isoelectric point for the entire family.

Calculating enzyme abundances from metagenomic analysis.

The EFI-CGFP/shortBRED webtool was used to determine the abundance of KTRs in metagenomic samples obtained as part of the Human Microbiome Project (HMP).^{33, 36–40} The KTR SSN described above (alignment score = 31) was used as the input file for marker identification (UniRef90 database, CD-hit identity = 85, DIAMOND pairwise comparison algorithm). Marker quantification with USEARCH in 380 healthy microbiome datasets yielded a genome-normalized median protein abundance heatmap. The dataset compares the relative quantity of KTR coding sequences (separated by cluster) per microbial genome at six different body sites. To highlight the relative abundance of radical SAM-adjacent KTR proteins in gut microbiome samples, boxplots for clusters 1–3 were generated in Kaleidagraph using the genome-normalized median protein abundance. The shaded area represents the median quartile (Q2), and the error bars extend to the first (Q1) and third (Q3) quartiles. Data points outside of these quartile limits are plotted as open circles.

RESULTS

Selection of phylogenetically diverse Cfr candidates for biochemical and biophysical characterization.

To identify Cfrs of diverse origin for biochemical and biophysical studies, we performed BLAST searches of the NCBI non-redundant protein sequence database using *Sa* Cfr as a query. The resulting set of 361 representative sequences was used to construct a minimum

evolution phylogenetic tree (Figure 2A) with an unrelated radical SAM class B methylase, TsrM,¹³ as the outgroup. The initial search included two groups of annotated RlmN enzymes that share 37% sequence identity with *Sa* Cfr but belong to distinct sequence clusters. The more distantly branched RlmN group is composed of sequences from Firmicutes and Chloroflexi organisms. The second group includes putative RlmN sequences from Candidatus phyla and obligate intracellular parasites. Annotated Cfrs cluster into a eukaryotic group and two different bacterial clades, as described previously.^{24, 41} Plasmid-encoded Cfrs with validated C8 methylation activity from staphylococcal clinical isolates⁹ belong to a group that also includes genomically-encoded Bacillales sequences⁴² and plasmid-encoded Cfr proteins from *Enterococcus faecalis* (*Ef*) and other enterococcal pathogens. From the latter group, we selected a *Ef* plasmid-borne Cfr and a genomic sequence from a mesophilic strain of *B. amyloliquefaciens* (*Ba*) for study here. The similarity (~75%, Table 1) of these sequences to *Sa* Cfr suggests that the *Ef* and *Ba* proteins are likely to have the same activity, methylation of rRNA at A2503 C8. In support of this hypothesis, several antibiotic-resistant enterococcal clinical isolates have recently been demonstrated to harbor a transposable element with a Cfr that is 99% sequence identical to *Ef* Cfr from strain TX0635 analyzed here.^{43–46} Cfr homologs from an alternative *Ba* strain (92% identity to the Cfr from DSM-7 ATCC 23350 targeted in this work), *B. clausii*, and *B. brevis* all confer multidrug antibiotic resistance when expressed heterologously in *E. coli* AS19, a strain with high antibiotic susceptibility.⁴² In the latter systems, the mechanism of drug resistance has been directly linked to methylation of A2503, confirmed in a primer extension analysis of total rRNA from an *rlmN* knockout of *E. coli* following induction of Bacillales Cfr expression.⁴² In all of these studies, conversion of A2503 to the singly- and di- methylated forms was not confirmed *in vitro* with purified proteins or analyzed quantitatively.

The second bacterial clade is dominated by genomic sequences from Firmicutes and partitioned into three distinct groups. One of these clusters is composed largely of sequences from the *Paenibacillus* genus.²³ It includes a representative [*Paenibacillus lautus* sp. Y412MC10 (*Ph*)] originally isolated from the Obsidian hot spring at Yellowstone National Park.⁴⁷ This *PIC* Cfr sequence was selected for characterization, owing to the utility of thermotolerant proteins in structural and biophysical studies. Many of the remaining sequences are found in Clostridial organisms. Some of these proteins have been annotated as Cfr-like and are postulated to be functionally divergent from *Sa* Cfr.²⁴ Our analysis shows that this subset breaks down into two smaller groups that consist of genomically-encoded Cfrs from a set of pathogenic *Clostridium difficile* (*Cd*) strains, now reclassified to the *Clostridioides* genus, and a larger group of Cfrs from more diverse clostridial organisms. The second group includes Cfr sequences from *C. sporogenes* and *C. phytofermentas* recently demonstrated to lack rRNA methylation activity.^{23, 24} We purified a Cfr from *C. sporogenes* and validated this finding but further characterization of the enzyme was not feasible owing to low soluble expression in *E. coli*.

Intriguingly, five members of the *Cd* Cfr cluster have an extended C-terminal motif (Fig. 1B). A BLAST search of the corresponding amino acid sequence shows similarity to an InterPro family (IPR025957)¹ designated “cysteine-rich KTR” with more than 1400 members identified as of May 2019. The group was initially named for a common internal

Arg-Thr-Lys sequence, although among the current set of IPR025957 sequences, this motif is not universally conserved. Members are most often found as stand-alone proteins approximately 60 amino acids in length, with two additional strictly conserved CXXC motifs. To date, none of these proteins has been functionally characterized in purified form. Cys-rich KTRs are found almost exclusively in Firmicutes, most in the order Clostridiales (968 annotated genes). A Cfr-KTR fusion sequence from *Clostridioides difficile* str. QCD-63q42⁴⁸ was selected for *in vitro* characterization in this study. In evaluating the activity and properties of this fusion enzyme, we sought to understand whether it lacks known Cfr activity upon heterologous production in *E. coli*, as shown for the *C. sporogenes* and *C. phytofermentans* homologs. In work by other laboratories, a *C. difficile* Cfr homolog has been shown to confer antibiotic resistance via A2503 modification, but the activity was promoted by a *cfr* on a mobile genetic element that shares only 52% sequence identity with our chromosomally-encoded *Cd* homolog.^{49, 50} A *Clostridium boltae* Cfr-KTR fusion has also been shown to confer moderate drug resistance in cell-based assays.⁵¹ Neither of these proteins has been purified and characterized *in vitro*, however. In our work, we also aim to elucidate the function and properties of the distinctive C-terminal Cys-rich domain in our selected *Cd* enzyme.

Diverse Cfr homologs exhibit RNA methylation activity.

To test for activity in the new Cfr homologs and probe possible functions of the fused Cys-rich domain, we viewed two types of activity measurements as likely to be most informative. We assessed (i) sequential mono- and di-methylation of the relevant adenine in a previously reported 155mer RNA substrate derived from 23S rRNA under conditions of high enzyme concentration and limiting substrate and (ii) catalytic monomethylation of the same base/substrate under conditions of low enzyme concentration and excess RNA. The RNA substrate is a transcribed 155mer of 23S rRNA substrate (nt 2454–2608) from an *Ec* rRNA template. *Ec* rRNA has been used to characterize the activity of heterologous Cfr enzymes, both in assays with purified protein/RNA components⁷ and in cells.¹¹ We elected to use the same substrate to facilitate comparison to *Sa* Cfr enzymes. The sequence identities of these rRNA fragments to the corresponding region of native host organism 23S rRNA range from 87 to 90% (Figure S1). Reactions were initiated by addition of excess SAM to reaction mixtures containing NADPH and a protein-based *Ec* flavodoxin/flavodoxin reductase (Flv/Flx) system for reduction of the [4Fe-4S]²⁺ cluster of Cfr.^{52, 53} In LC-MS analyses of the digested RNA products in both assays, new peak(s) can be assigned to C8-methyladenosine (m8a) or C2,C8-dimethyl-adenosine (m2,8a) based on retention times, *m/z* values, and MS/MS fragmentation (Figure 3A and Figure S2).^{6, 7} Assignment of the methylated adenine products to A2503 was confirmed by comparison to an A2503G RNA substrate in *Ba* and *Cd* Cfr assays. The AàG variant yields negligible turnover (Figure S3). Methylation of A2503 by *PICfr* was demonstrated in a previous study.²³

HPLC-MS analysis of the limiting substrate reactions (i) revealed detectable levels of mono-methylated and dimethylated products for three of the four enzymes (Figure 3B–D). The quantities of the two products were consistent with sequential reactions. Build-up of the m2,8a product initially lagged while the m8a product grew in, and the m2,8a product then accumulated significantly as the m8a product was depleted (Figure 3B–D). In general, these

enzymes resemble *Sa* Cfr in their ability to catalyze sequential mono- and dimethylation reactions, albeit with varying efficiencies. The *Cd* homolog is an outlier in that it produced very little of the dimethylated product (Figure 3E).

In the limiting substrate time course, *Ba* Cfr accumulates the greatest amount of singly and dimethylated product and exhibits the fastest initial rate of catalysis for both reactions (Figure 3B). The kinetic profiles of the *Ba*, *Ef*, and *Pl* enzymes were analyzed⁵⁴ to extract k_{obs} for the first methylation step (Figure S4 and Table S2). The rate (0.23 min^{-1}) and extent of *Ba* Cfr C8 methylation (~80 % substrate consumption within 20 min of initiation) is comparable to previously reported values for *Sa* Cfr analyzed under similar (limiting RNA) conditions ($0.7 \pm 0.2 \text{ min}^{-1}$ with >80% of the substrate consumed within the first 5 min of the reaction).⁵⁵ By contrast, the *Pl* and *Ef* enzymes exhibit rates of C8 methylation that are 5- to 10-fold lower ($0.07\text{--}0.14 \text{ min}^{-1}$), while the *Cd* Cfr reaction proceeds even more slowly (0.02 min^{-1}) (Figure 3E, Table S2). To understand whether uncoupled SAM consumption could account for these differences, we quantitatively analyzed the 5'-dA and SAH byproducts (Table S2). All enzymes exhibited a near 1:1 ratio of SAH:methylated product. Abortive SAM cleavage was assessed by comparing the amount of methylated RNA produced to the 5'-dA coproduct (Figure S5). All of the homologs tested exhibited slightly elevated levels of 5'-dA (1.3–1.7), suggesting modest uncoupling under these conditions. However, the similarities between the enzymes indicate that abortive SAM cleavage cannot account for the differences in initial methylation rate. Nevertheless, the *Ba* enzyme is the most efficiently coupled with a 1.3:1 ratio of 5'-dA:methylated product. Under limiting substrate conditions, with the exception of *Ba* Cfr, none of the enzymes consumed the rRNA substrate completely (Table S2). This outcome could be due to a number of different factors. It may arise from use of a non-native RNA substrate in our assays. It could also reflect a propensity for enzyme inactivation or product inhibition.

In the second type of activity assay (ii), we incubated low concentrations of enzyme ($3 \mu\text{M}$) with excess RNA substrate ($100 \mu\text{M}$) at room temperature (Figure 4A–D). In these reactions, the m2,8a dimethylated product was barely detectable (Figure S6), implying that none of the Cfr homologs methylate the RNA processively and that the first C8 methylation site is vastly preferred over the second, C2. The excess substrate assays show that all enzymes exhibit the capacity to undergo more than one m8 methylation event, with 2–14 conversions observed within the ~3 h assay window (Table S3). All are appreciably slower than *Sa* Cfr ($0.24 \pm 0.06 \text{ min}^{-1}$ when assayed under similar multi-turnover conditions)¹⁹ with the exception of *Ba* Cfr ($v/[E] = 0.23 \text{ min}^{-1}$) (Figure S6, Table S3). The *Cd* enzyme is the slowest catalyst in this assay, with an m8 methylation rate ($v/[E]$) that is 5–30% that of the other homologs and the lowest level (~5%) of total RNA substrate consumption. In *Cd* Cfr, we also considered that the low rate of *in vitro* turnover with excess substrate could be linked to the presence of the extra C-terminal domain. To address this question, we characterized the function and properties of this domain and analyzed the activity of truncated variants.

Spectroscopic analysis of Cfrs reveals an additional iron-binding site in *Cd* Cfr.

In order to verify the [4Fe4S] cluster content and interrogate the capacity of the *Cd* Cfr C-terminal domain to bind iron, we examined Cfr homologs by Mössbauer spectroscopy

(Figure 5). Spectra from *Ba*, *Ef*, and *Pl*Cfr are dominated by a single quadrupole doublet with parameters consistent with $[4\text{Fe}_4\text{S}]^{2+}$ clusters ($\delta = 0.44\text{--}0.45$ mm/s and $E_Q = 1.11$ mm/s in each case).⁵⁶ From the relative area of the subspectrum associated with $[4\text{Fe}_4\text{S}]^{2+}$ clusters and the amount of Fe/protein after reconstitution, the number of clusters per protein can be estimated.³⁰ After reconstitution, *Ba*, *Ef*, and *Pl*Cfr contain 4.6, 4.2, and 3.9 Fe per protein, corresponding to 1.1, 1.0, and 0.9 $[4\text{Fe}_4\text{S}]^{2+}$ clusters per protein, respectively (Table 2). In addition to the features of a $[4\text{Fe}_4\text{S}]$ cluster ($\delta = 0.45$ mm/s and $E_Q = 1.14$ mm/s), the Mössbauer spectrum of *Cd*Cfr contains a second quadrupole doublet feature with parameters ($\delta = 0.70$ mm/s and $E_Q = 3.08$ mm/s), similar to those of a Fe^{2+} -Cys₄ rubredoxin center.⁵⁶ We propose that this Fe^{2+} center is coordinated by the four conserved cysteine residues in the C-terminal KTR region of the protein (*vide supra*). Analysis of all four Cfrs by electron paramagnetic resonance spectroscopy did not result in any detectable signals for the as-isolated reconstituted proteins, thus ruling out assembly of any iron-sulfur clusters with an $S = 1/2$ ground state in these samples.

To test the hypothesis that the $[4\text{Fe}_4\text{S}]^{2+}$ cluster and rubredoxin-like center in *Cd*Cfr reside in the N-terminal and C-terminal domains, respectively, N-terminal (residues 1–345, Cd-N) and C-terminal (residues 346–416, Cd-C) fragments of *Cd*Cfr were produced. Mössbauer spectra of these truncated proteins (Figure 5) unambiguously demonstrate that the $[4\text{Fe}_4\text{S}]$ cluster resides in the N-terminal radical SAM domain, whereas the ferrous rubredoxin-like center resides in the C-terminal cysteine-rich KTR domain. In order to gain additional insight into the electronic structure of the rubredoxin-like species in *Cd*Cfr, we examined Cd-C using Mössbauer spectroscopy at 4.2 K in variable externally applied magnetic fields (Figure 5). The spectra can be simulated with the following parameters and assuming a $S = 2$ electron spin ground state: $D = 5.7$ cm⁻¹, $E/D = 0.2$, $\delta = 0.70$ mm/s, $E_Q = 3.08$ mm/s, $\eta = 0.9$, and $\mathbf{A}/g_N\beta_N = [-14.4, -8.3, -23.3]$ T, similar to those previously reported for ferrous rubredoxin centers coordinated by four cysteines.^{56, 57}

The *Cd* Cfr C-terminal domain is not essential for RNA methylation activity but functions less efficiently in the absence of Fe^{2+} .

The truncated version of *Cd*Cfr, *Cd*-N, lacking the C-terminal Cys-rich domain, is as efficient in m8a production as full-length *Cd*Cfr in experiments using the Flv/Flx/NADPH reducing system with limiting RNA (Figure 6A–B, Figure S7A). Attempts to modulate activity by addition of the isolated Fe^{2+} -loaded C-terminal domain *in trans* (*Cd*Fe res) did not yield any significant difference in product formation. To investigate the functional role of the Fe^{2+} center in the *Cd*Cfr C-terminal domain, a variant of one of the putative metal-binding residues in the CXXC motif, a Cys363 → Ala substitution (C363A, renamed Cd M), was generated in the full-length enzyme. Unexpectedly, this substitution alters iron-sulfur cluster stability, giving a sample with < 3 equiv Fe/protein after reconstitution, as determined by ICP-OES analysis. Similar cluster instability is preceded in $[4\text{Fe}_4\text{S}]^{2+}$ DNA repair enzymes upon mutation of an auxiliary metal binding site Cys ligand.^{58, 59} As a consequence, the C363A *Cd*Cfr enzyme exhibits negligible activity. A Zn^{2+} -loaded version of the C-terminal domain was also tested (*Cd*Zn res) for rescue of RNA methylation activity. Inclusion of the mismetallated Cd-C domain gives a slight decrease in activity in limiting substrate assays (Figure 6B, Figure S7A). We also evaluated the activity of variant

forms of *CdCfr* with excess RNA (3 μ M enzyme, 100 μ M 155mer rRNA) (Figure 6C). The trends observed in the limiting substrate assays are largely maintained, with little effect on endpoint product formation upon truncation of *CdCfr*. However, at early time points, a detectable lag in product formation is evident in the rescue experiment (Figure S8). Interestingly, the impact of Zn²⁺-substitution in the C-terminal domain is magnified with excess substrate. We observe only a small amount of product after 2.75 h (Figure 6C, Figure S7B), underscoring the initial findings from single turnover assays. Disruption of the C-terminal domain iron-binding site via ligand mutation or metal ion substitution impedes turnover.

The isolated *CdCfr* C-terminal domain exhibits rubredoxin-like redox properties.

Although our activity assays do not indicate an essential role in *in vitro* catalysis for the metal-bound C-terminal domain in *CdCfr*, particularly under limiting substrate conditions, we speculated that the domain could mediate electron transfer (ET) steps in the rRNA methylation reaction. This function may be dispensable with a non-native reductant *in vitro* but more critical *in vivo* (e.g., with a clostridial protein-based redox donor). Our Mössbauer experiments revealed a Rdx-type Cys₄-coordinated Fe²⁺ center in the version of this protein isolated from an *E. coli* heterologous host and expressed under iron-replete conditions in minimal medium. Given that Rdx proteins typically serve as high-potential electron-transfer shuttles that operate by cycling between Fe²⁺ and Fe³⁺ oxidation states,⁶⁰ we sought to characterize the redox properties of as-isolated Cd-C. Anaerobic isolation of Cd-C yields a clear/bluish protein solution that is exclusively Fe²⁺ by Mössbauer analysis. Exposure of the domain to O₂ results in a visible color change to a red solution that, when monitored spectrophotometrically, exhibits absorption bands at 370, 485, and 582 nm (Figure 7A). These spectral features resemble those of oxidized stand-alone Rdx proteins.⁶¹

Previously characterized Rdx proteins also exhibit reduction potentials in the -50 to +50 mV range (vs. NHE).⁶⁰ To measure the Fe^{2+/3+} midpoint potential of the isolated Cd-C domain, protein films were generated on pyrolytic edge graphite (PGE) electrodes under anaerobic conditions by incubation of protein [9.4 mM in 10 mM HEPES pH 7.5, 500 mM KCl, 5 mM DTT, 10 mM MgCl₂, 25% (v/v) glycerol] on the electrode surface for 10 min. Modified electrodes were transferred to an electrochemical cell (50 mM HEPES, pH 7.5, and 300 mM KCl buffer), and evaluated by square-wave (SW) voltammetry. Voltammograms obtained at a SW frequency of 10 Hz showed a quasi-reversible waveform with a midpoint potential of +106 mV vs. NHE (Figure 7B). The relatively high midpoint potential compared to prototypical Rdx proteins can be rationalized by differences in the predicted charge of Cd-C at pH 7.5. Cd-C has a calculated pI of 9.2, compared to ~4.0 for a canonical Rdx. At physiological pH, Rdx would be largely negative while Cd-C would have a net positive charge. This scenario would destabilize the Fe³⁺ state in Cd-C, rendering the protein more difficult to oxidize and resulting in a higher midpoint potential. Interestingly, subsequent electrochemical scans of the isolated domain show loss of current amplitude over time, suggesting that the domain may not be stable in the oxidized form. In the presence of O₂, Cd-C visibly precipitated over the 30 min time period in which the protein was monitored electrochemically. These observations could preclude a role for the Fe²⁺ center as an explicit electron donor in *Cfr* catalysis. However, the stability/reduction potential may be further

modulated via interaction with the radical SAM domain of *CdCfr* and/or the RNA substrate. The protein could also require binding to the protein reducing system prior to ET to ensure rapid re-reduction to the Fe^{2+} oxidation state.

Homologs of the *Cd* C-terminal domain are widespread as stand-alone proteins in Clostridiales.

Spectroscopic and electrochemical analyses of the isolated *CdCfr* C-terminal domain suggest properties similar to Rdx ET proteins. Although traditional Rdx proteins are similar in length (~60 aa) and contain identical CXXC metal binding motifs separated by ~20–30 amino acids, the overall sequence identity between the *Cd*-C domain and a model Rdx from *C. pasteurianum* is only 30% (Figure 7D). *Cp* Rdx (and other Rdx proteins with validated function/properties) additionally contain a conserved Gly immediately adjacent to the C-terminal Cys in each metal binding motif. This sequence pattern enables key H-bonds to form between peptide backbone N-H groups and the metal-coordinated thiolates of Cys residues. These interactions can influence Rdx reduction potential.⁶² Interestingly, *Cd*-C deviates from this sequence pattern significantly because it contains a Lys residue immediately adjacent to each CXXC motif. In general, *Cd*-C has a high proportion of positively charged residues, accounting for its aforementioned $\text{pI} > 9$, while *Cp* Rdx has a net negative charge at neutral pH. These observations leave open the possibility that while *Cd*-C functionally resembles Rdx proteins, it may adopt a distinct structure.

Our initial identification of the *Cd*-C Fe^{2+} -binding domain via sequence inspection revealed homology to a large group of bacterial stand-alone proteins annotated as Cys-rich KTR proteins. The latter naming scheme comes from an Arg-Thr-Lys sequence that resides in between the two strictly conserved CXXC motifs. Additionally, analysis of the Cys-rich KTR sequence group shows that the stand-alone proteins are also dominated by positively charged residues, resulting in an average isoelectric point of 8.6. Our discovery of an essential Fe^{2+} binding site in the *Cfr*-fused version of this domain raises the possibility that the larger family of stand-alone Cys-rich KTR proteins might have the same property, with widespread functional significance for the metal-bound form. To gain insight into this possibility, we analyzed patterns in genome neighbors (± 10 open reading frames) for the entire InterPro family.³³ The resulting sequence similarity network revealed that 13% of the Cys-rich KTR family can be found in two large clusters (133 sequences, clusters 1 and 3 in Fig. 8A). For the 85 of the 133 sequences from organisms with fully sequenced genomes, 84 are encoded directly adjacent to a putative RNA methylase. As in the *CdCfr* fusion enzyme studied here, nearly all of these Cys-rich KTRs are from organisms belonging to the clostridiales order (Table S4). This subset of Cys-rich KTR proteins may function similarly to *Cd*-C, sharing its requirement for Fe^{2+} and exhibiting Rdx-type redox properties. Interestingly, the organisms with paired *Cfr*-KTR gene clusters identified in this analysis are commonly found as commensal human gut microbiome constituents (Table S4). We further validated this observation by analyzing the healthy human microbiome metagenome abundance of Cys-rich KTR genes via the Enzyme Function Initiative chemically-guided functional profiling (CGFP) web tool (Figure 9).^{35–37} The KTR genes associated with clusters 1 and 3 are significantly enriched in human stool microbiome metagenomes. These

patterns could indicate a reservoir of Cys-rich KTR-Cfr genes in the beneficial gut flora to protect against antibiotic exposure with the possibility of transfer to pathogens.

DISCUSSION

In this study, we establish that four different Cfr enzymes from distinct phylogenetic groups assemble a necessary $[4Fe4S]^{2+}$ cofactor and exhibit adenine C8 RNA methylation activity *in vitro*, similar to the well-characterized Cfr homolog from *S. aureus*. Under the assay conditions tested, three of the four enzymes analyzed exhibit both mono- and di-methylation activity. We also show that a genomically encoded Cfr from a Bacillales organism consumes substrate at a comparable rate to *Sa* Cfr with both limiting and excess RNA. These results establish that diverse Cfr homologs are viable targets for structural and biophysical study.

In vitro characterization of a clostridial Cfr homolog fused to a Cys-rich C-terminal domain also reveals an active mono-methylase with limiting substrate. However, with a >30-fold excess of RNA substrate, the clostridial fusion enzyme is markedly less active. Combined with the unexpected detection of a rubredoxin-like Fe^{2+} binding site in the C-terminal domain of *Cd*Cfr, these findings prompted a more detailed investigation of the properties of this module. Complete removal of the domain fails to either increase or decrease the rate of RNA methylation in reactions initiated by a protein-based reducing system, but disruption of the Fe^{2+} binding site by ligand mutagenesis or Zn^{2+} substitution decreases turnover. While the effect of Zn^{2+} is modest with limiting substrate, the mismetallated enzyme is nearly inactive when challenged with excess RNA. Our spectroscopic and electrochemical analysis of the stand-alone domain suggests a similarity to rubredoxin-type electron transfer cofactors, often described as a particularly minimalist member of the iron-sulfur cluster cofactor family.

Many radical SAM enzymes harbor additional iron-sulfur clusters with diverse functions, including electron transfer.^{63, 64} The redox-neutral carbon methylation catalyzed by RlmN/Cfr enzymes shares in common with other radical SAM-mediated C-C bond forming systems a requirement for reductive quenching of a substrate radical at the end of the reaction.¹² In other superfamily members, auxiliary cofactors or redox-active amino acids in the active site can mediate controlled provision of redox equivalents.^{65, 66} In Cfr and RlmN, a conserved C-terminal Cys residue is oxidized to a thiyl radical to facilitate product release (Figure 1).^{14, 18} The midpoint potential of the Fe^{2+} cofactor in the *Cd*Cfr C-terminal rubredoxin-like domain could enable its use as an electron donor to regenerate the Cys 355 thiolate for subsequent methylation.¹² We note, however, that the domain is not universally conserved as a Cfr neighbor. Therefore, a KTR-rubredoxin cannot be the sole mechanism of C355 reduction and other means to quench the radical must exist in other Cfr/RlmNs. Diversity in reductant usage has precedent in other oxidoreductase enzymes. For example, class III ribonucleotide reductases have adapted to use either protein-based (thioredoxin or ferredoxin) or small molecule (formate) reducing systems to resolve an oxidized active site Cys disulfide or a thiosulfuranyl radical.^{67–69} Also, we cannot rule out other roles for the KTR-rubredoxin such as RNA-binding or structural stabilization. However, our work shows that the *Cd*-N truncated protein is stable and active in the absence of the C-terminal domain *in vitro*.

The *CdCfr* rubredoxin-like domain may also serve as an intermediary or coordinator of Cfr reaction initiation via interaction with protein reducing partners. The reduction potential of the Cd-C domain is likely too high (+100 mV vs NHE) to donate an electron to the [4Fe4S]²⁺ radical SAM cluster directly, even though a similar role was recently proposed for a stand-alone CSL-family Zn finger protein, Dph3, encoded adjacent to a non-canonical radical SAM enzyme, diphthamide biosynthesis protein Dph2.^{70, 71} Dph3 is the only other example of a biochemically characterized mononuclear metal binding protein found as a genome neighbor to a radical SAM enzyme. The Fe²⁺-bound form of Dph3 was reported to function in iron-sulfur cluster reduction in Dph2, initiating the reaction in the absence of reductant. However, the redox potential of Dph3 has not been experimentally determined, and no known rubredoxin-type electron transfer protein has a sufficiently low reduction potential (< -400 mV) for activation of a radical SAM [4Fe4S] cluster.⁷²

Our findings also raise the possibility that reported inactivity in RNA methylation in heterologously expressed Cfrs from clostridial clades could be due to lack of a necessary accessory factor or redox partner. The Cys-rich KTR domain is a candidate for such a factor, although this protein is not essential for activity in *CdCfr* and it is not found universally adjacent to the inactive clostridial enzymes. Genome analysis of the remainder of the clostridial Cfr subgroup reveals a second conserved neighbor, an Lsa ABC-F ribosomal protection protein. Our query shows 100% co-occurrence of clostridial *cfr* genes with either a KTR or an Lsa neighbor. Lsa has not been structurally characterized in complex with rRNA, but another ABC-F protein, VmlR, binds to the fully-assembled 70S ribosome to prompt a structural change that drives drug release from the peptidyl transferase center.⁷³ While *Sa*-type Cfrs methylate A2503 solely in a protein-free 23S rRNA context,⁵ clostridial Cfrs could instead modify fully assembled 70S ribosomal rRNA following Lsa remodeling. Such an approach would be particularly efficient, eliminating the fitness cost of pre-emptively methylating nascent ribosomes.

The genome context of other Cys-rich KTR orthologs suggests the protein may additionally play a more generic role in nucleic acid binding or antibiotic resistance. Apart from the clostridial Cys-rich KTR group found adjacent to Cfr radical SAM enzymes, the second most frequent gene neighbor is TetM, a ribosomal protection protein that confers antibiotic resistance by triggering a conformational change in the tetracycline binding site to release the drug.⁷⁴ Interestingly, this group of KTR homologs is almost exclusively associated with known bacterial pathogens originating from Clostridiales and Bacillales. The remaining KTR proteins are often found near drug efflux systems, with the majority annotated as ABC family transporters or MATE/MFS effluxers. All Cys-rich KTR genes are proximal to transposable elements including helix-turn-helix proteins (HTH3/HTH16), RNA polymerase σ ⁷⁰ components, and TetR-like repressors.⁷⁵ These patterns suggest KTRs are transferable elements, consistent with a general role in antibiotic resistance. In the other GNN clusters, a functional role for Fe²⁺ and Rdx-like activity is not immediately evident, although it is interesting to note that the protein families listed above all exhibit some overlap with the biological function of Cfr in drug resistance. The prevalence of Cys-rich KTR genes in pathogens and in members of the human microbiome warrants further investigation of their biological function and metal ion requirements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Proposed mechanism of C8 adenine methylation by Cfr.^{7, 13, 14}
 Schematic diagram of the catalytic Cys residues located in the Cfr RNA binding pocket (gray box) and the A2503 rRNA target nucleobase.

Figure 2. Selection of phylogenetically diverse Cfr enzymes for biochemical characterization. (A) Minimum evolution phylogenetic tree containing 361 representative sequences obtained by NCBI BLAST. The sequence of *Sa* Cfr was the initial query. Bootstrap values (1000 replicates) were calculated for branch support and red circles denote values > 85% in selected branches. Dominant genera and the genomic context in each cluster are indicated in colored text/schematics. (B) Cartoon diagram of the domain architecture in Cfrs selected for study. Locations of conserved Cys residues (*Sa* numbering) in the radical SAM domain are shown.

Figure 3. Methylation of a 155mer rRNA substrate by Cfr enzymes under limiting substrate conditions initiated with a protein-based reductant.

Reactions used the *Ec* Flv/Flx/NADPH reducing system in mixtures containing 100 mM EPPS, pH 8, 10 mM MgCl₂, 12.5 % glycerol, 250 mM KCl, 200 μM Flv, 20 μM Flx, 2 mM NADPH, 1 mM SAM, 50 μM 155mer RNA, and 48 μM protein. (A) LC-MS analysis of methylated RNA products via single-ion monitoring at $m/z = 282.1$ (bottom trace, m8A) and $m/z = 296.1$ (top trace, m2,8A). Time course experiment monitoring formation and decay of singly-methylated m8A (solid bars) and doubly methylated m2,8a (patterned bars) with (B) *Ba* Cfr, (C) *Ef* Cfr, (D) *PI* Cfr, and (E) *Cd* Cfr. Error bars correspond to standard deviation of three averaged trials.

Figure 4. Methylation of a 155mer rRNA by Cfr enzymes with excess substrate in reactions initiated with a protein-based reductant.

Reactions used the *Ec* Flv/Flx/NADPH reducing system in mixtures containing 100 mM EPPS, pH 8, 10 mM MgCl₂, 12.5 % glycerol, 250mM KCl, 200 μM Flv, 20 μM Flx, 2 mM NADPH, 1 mM SAM, 100 μM 155mer RNA, and 3 μM protein. Time course experiment monitoring formation and decay of singly-methylated m8A with (A) *Ba Cfr*, (B) *EfCfr*, (C) *PlCfr*, and (D) *CdCfr*. Error bars correspond to standard deviation of three averaged trials. The inset in panel D shows that *CdCfr* can promote multiple turnovers (red line).

Figure 5. Mössbauer spectra of Cfr enzymes.

Left panel: comparison of reconstituted samples of Cfr from *Bacillus amyloliquefaciens* (*Ba*), *Enterococcus faecalis* (*Ef*), *Paenibacillus lautus* (*Pl*), and *Clostridioides difficile* (*Cd*). Spectra of N-terminal and C-terminal truncations of *Cd* Cfr are also shown; features of the $[4\text{Fe}4\text{S}]^{2+}$ cluster and ferrous rubredoxin-like site are denoted by red and blue dashed lines, respectively. Right panel: Mössbauer spectra of the C-terminal domain of *Cd* Cfr in varying applied magnetic fields. Experimental data are displayed as black vertical bars, with simulations overlaid (blue solid lines).

Figure 6. Methylation of a 155mer rRNA substrate by variant *Cd* Cfr enzymes under limiting substrate and excess substrate conditions and initiated by a protein-based reductant.

Reactions were performed with the *Ec* Flv/Flx/NADPH reducing system in mixtures containing 100 mM EPPS, pH 8, 10 mM MgCl₂, 12.5 % glycerol, 250 mM KCl, 200 μM Flv, 20 μM Flx, and 2mM NADPH. (A) Limiting substrate LC-MS analysis of methylated RNA products via single-ion monitoring at $m/z = 282.1$ (bottom trace, m8A) and $m/z = 296.1$ (top trace, m2,8A). Cd-N = radical SAM domain only, Cd-C = C-terminal KTR domain with Fe²⁺ or Zn²⁺, Cd-M = C363A variant (full-length), rescue (res) experiments contain Cd-C added to Cd-N *in trans*. Endpoint analysis (165 min) of the formation of singly-methylated m8A under (B) limiting substrate and (C) excess substrate conditions. Error bars correspond to standard deviation of three averaged trials.

Figure 7. Electrochemical and UV-visible characterization of Cd C-terminal domain.

(A) UV-visible difference spectrum of the oxidation of the C-terminal domain of Cd Cfr.

Absorbance maxima are visible at 370 nm, 485 nm, and 582 nm. (B) Square-wave voltammogram of KTR domain of CdCfr. The midpoint potential is +106 mV. (C) Size exclusion chromatogram illustrating the interaction of Cd-N and Cd-C through co-elution.

(D) Comparison of the C-terminal domain of Cd Cfr (Cd-C), the KTR from Cb, and the rubredoxin from Cp. Metal coordinating cysteine pairs are highlighted in yellow, the structurally important residues directly following are highlighted in green, the positively charged residues are colored blue, and the negatively charged residues are colored red. The number of positively and negatively charged residues (bottom, numbers in parentheses) differentiates cys-rich KTRs from typical rubredoxins.

Figure 8. Sequence similarity and genome neighborhood network analyses of stand-alone Cys-rich KTR proteins.

(A) SSN of IPR025957 constructed with an alignment score of 31. Nodes are arranged in an organic layout and each represents a single sequence. Nodes are shaded by host organism and colored by co-occurring gene type. Clusters without numbering represent groups that do not fall into one of the three major gene type categories. Additional clusters numbering two or less are not shown. (B) Representative gene clusters from each major category as determined by GNN analysis (± 10 ORFs, 5% co-occurrence). Host organism and cluster number are displayed (left). KTR proteins are marked by a “*” symbol. (C) Breakdown by organism type in each of the first three SSN clusters. Clostridia = blue, Bacilli = purple, and other = green. Pathogenic organisms are highlighted with dark shading and non-pathogens are shown in light shading.

Figure 9. Metagenome abundance of KTR proteins in healthy human microbiome samples. (A) Heatmap for clusters 1–10 in the KTR SSN (see Fig. 8) showing marker abundance in 380 HMP datasets collected from six different areas of the body. Cluster 4 is omitted due to low abundance in the metagenomic sample set. (B–D) Box plots of the per-site abundance for the top three clusters. This analysis shows that the KTR proteins encoded adjacent to radical SAM enzymes are abundant in the gut microbiome.

Table 1.

Sequence identity for Cfr homologs tested for RNA methylation.

Homolog	<i>Bacillus amyloliquefaciens (Ba)</i>	<i>Enterococcus faecalis (Ef)</i>	<i>Paenibacillus lautus (Pl)</i>	<i>Clostridioides difficile (Cd)</i>	<i>Clostridium sporogenes (Cs)</i>
% ID (<i>Sa</i> Cfr)	75.8	74.1	51.5	55.7	57.2

Table 2.

Iron incorporation for Cfr homologs analyzed by Mössbauer spectroscopy.

Homolog	Fe/protein	Cluster	Area of subspectrum (%)	Cofactor/protein
<i>Clostridioides difficile</i>	4.4	[4Fe-4S] ²⁺ Fe ²⁺ Cys ₄	77 22	0.8 1.0
<i>Enterococcus faecalis</i>	4.2	[4Fe-4S] ²⁺	97	1.0
<i>Paenibacillus lautus</i>	3.9	[4Fe-4S] ²⁺	94	0.9
<i>Bacillus amyloliquefaciens</i>	4.6	[4Fe-4S] ²⁺	100	1.2

Table 3.

Liquid chromatography elution gradient.

Time (min)	A	B
0.0 – 5.0	95%	5%
5.0 – 6.5	87%	13%
6.5 – 7.0	73%	27%
7.0 – 8.0	47%	53%
8.0 – 12.0	95%	5%