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The Order *Bacillales* Hosts Functional Homologs of the Worrisome *cfr* Antibiotic Resistance Gene

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The *cfr* gene encodes the Cfr methyltransferase that methylates a single adenine in the peptidyl transferase region of bacterial ribosomes. The methylation provides resistance to several classes of antibiotics that include drugs of clinical and veterinary importance. This paper describes a first step toward elucidating natural residences of the worrisome *cfr* gene and functionally similar genes. Three *cfr*-like genes from the order *Bacillales* were identified from BLAST searches and cloned into plasmids under the control of an inducible promoter. Expression of the genes was induced in *Escherichia coli*, and MICs for selected antibiotics indicate that the *cfr*-like genes confer resistance to PhLOPSa (phenicol, lincosamide, oxazolidinone, pleuromutilin, and streptogramin A) antibiotics in the same way as the *cfr* gene. In addition, modification at A2503 on 23S rRNA was confirmed by primer extension. Finally, expression of the Cfr-like proteins was verified by SDS gel electrophoresis of whole-cell extracts. The work shows that *cfr*-like genes exist in the environment and that *Bacillales* are natural residences of *cfr*-like genes.

The *cfr* gene was first found in 2000 on a plasmid in a *Staphylococcus sciuri* strain in a veterinary bovine sample from Germany (25). Cfr methylates nucleotide A2503 of 23S rRNA at the ribosomal peptidyl transferase center (16). It provides resistance to antibiotics binding to the ribosomal peptidyl transferase center on the ribosome, defining a PhLOPSa phenotype reflecting resistance to phenicol, lincosamide, oxazolidinone, pleuromutilin, and streptogramin A antibiotic classes (19), and it also confers resistance to some macrolide antibiotics (28). The *cfr* gene is thus a serious threat when it spreads in pathogenic bacteria because many clinically important antibiotics will become useless.

In 2007, the *cfr* gene was found in a methicillin-resistant *Staphylococcus aureus* (MRSA) isolate from a patient from Colombia (30). The *cfr* gene has now been found worldwide in *Staphylococcus* spp. isolated from animals in Germany, Denmark, and China (15, 17, 25, 35) as well as in patients from the United States, Spain, Mexico, Italy, and Ireland (3, 4, 8, 13, 20–24, 27). It has also been found in other *Firmicutes*, namely, in an *Enterococcus faecalis* isolate from a patient in Thailand (7) and one of animal origin (18) and in *Bacillus* sp. isolates from swine feces (6, 33, 36). Furthermore, the *cfr* gene has recently been detected in animal isolates of the Gram-negative bacteria *Proteus vulgaris* (34) and *Escherichia coli* (32). All findings concern the same gene with only very minor sequence changes. It is also evident that the *cfr* gene has been transmitted to its hosts, as it is always found either on a plasmid or together with insertion sequences.

In 2008, the identity of the Cfr-mediated methylation was determined to be 8-methyladenosine (m⁸A), a new natural RNA modification (9). It was also established by mutagenesis that Cfr is a radical S-adenosylmethionine (SAM) enzyme. This family includes a wide range of enzymes from a diverse set of bacteria involved in protein radical formation, isomerization, sulfur insertion, anaerobic oxidation, and unusual methylations, as originally described by Sofia et al. (29).

Phylogenetic comparisons have shown that Cfr is similar to the RlmN methyltransferases that add a methyl group at the C-2

position of 23S rRNA nucleotide A2503 (31), the same nucleotide that is methylated by Cfr. It may be that the *cfr* gene evolved from the *rlmN* gene via gene duplication, but no clear path has emerged yet. A new mechanism involving protein methylation and transitory cross-linking has recently been proposed to explain the detailed mechanism of Cfr and RlmN methylation (10, 11), and an X-ray structure of RlmN has been published (2).

The gene and genome databanks contain a wealth of information that can be used to find genes similar to *cfr*. We have selected three Cfr-like proteins from the order *Bacillales*, cloned the genes, and investigated if they indeed confer resistance in a manner similar to that of the Cfr methyltransferase. In addition, the methylation was assayed by primer extension on 23S rRNA and protein expression was assayed by SDS gel electrophoresis. The results confirm that *cfr*-like genes exist in the environment and that the *cfr* gene is not functionally unique.

MATERIALS AND METHODS

Construction of plasmids bearing *cfr*-like genes. Plasmids encoding Cfr-like proteins were constructed by PCR amplification of the genes from genomic DNA (the sources and other information are listed in Table 1), followed by cloning into plasmid pLJ102 for expression of the Cfr-like proteins. *Bacillus amyloliquefaciens* and *Bacillus clausii* were grown in LB. *Brevibacillus brevis* was grown in a medium containing 10 g polypeptone, 2 g yeast extract, and 1 g MgSO₄ · 7H₂O per liter. All strains were grown at 37°C. Genomic DNA was isolated with the High Pure PCR template preparation kit (Roche) or the Aqua Pure genomic DNA kit (Bio-Rad). Standard PCR amplification of the relevant genes was performed with the following primers, each containing NdeI or HindIII cleavage sites for

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TABLE 1 The *cfr*-like genes and plasmids used in this study

Gene or plasmid	Organism (genome source for <i>cfr</i> -like genes) or features	Strain designation	Source or reference ^a	Gene ID ^b
Genes				
<i>clbA</i>	<i>Bacillus amyloliquefaciens</i>	FZB42	BGSC	YP 001420189
<i>clbC</i>	<i>Bacillus clausii</i>	"domuvar"	BGSC	YP 174574.1
<i>clbB</i>	<i>Brevibacillus brevis</i>	100599	NBRC	YP 002773985.1
Plasmids				
pBa	Inducible ClbA expression		This study	
pBc	Inducible ClbC expression		This study	
pBb	Inducible ClbB expression		This study	
pCfrhis	Inducible Cfr expression		9, 14	
pBgIII	Constitutive Cfr expression		16	
pLJ102	Derivative of pQE60		12	
pBluescript	Cloning vector		Stratagene	

^a BGSC, Bacillus Genetic Stock Center; NBRC, NITE Biological Resource Center.

^b Gene identification numbers indicate database accession numbers.

cloning: 5'CTGCATACATATGCAACAAAAACAAGTATAT3' and 5' CAGAATAAGCTTTTATTGGTTCTTATTTTTTGGATA3' for the *Bacillus amyloliquefaciens* gene (*clbA*), 5'CTGCATACATATGAAAGTTGTCA ATCATGCG3' and 5'CAGAATAAGCTTTCACCTTTTTTTCGCCTGATA3' for the *Bacillus clausii* gene (*clbC*), and 5'CTGCATACATATGAACTAACCTCGAAATATGAA3' and 5'CAGAATAAGCTTATTCAGAGCGGTATAACTGGCC3' for the *Brevibacillus brevis* gene (*clbB*). The PCR fragments were cut with the appropriate restriction enzymes and ligated into plasmid pLJ102, cut with the same enzymes (whereby another gene was removed), such that the genes are positioned after an IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible promoter. The ligations were transformed into the *E. coli* TOP10 strain (Invitrogen), and plasmid-containing clones were selected on agar plates with 100 μ g/ml ampicillin. Plasmids were isolated from these clones and retransformed into *E. coli* strains AS19 (26) and JW2501-1 (1). All three plasmid constructs were sequenced at the inserted gene to verify the identity of the cloned genes.

Verification of *cfr*-like gene expression by SDS gel analysis. *E. coli* AS19 cells harboring the plasmids with the *cfr* look-alike genes were grown at 37°C to an optical density at 450 nm (OD₄₅₀) of 0.2 to 0.3, followed by addition of IPTG (to 1 mM) for induction of the genes. Cells were harvested after 3 to 3.5 h of growth and stored at -80°C. For gel analysis, samples were dissolved in 1× SDS/dithiothreitol (DTT) loading buffer, boiled for 5 min, and loaded onto standard SDS gels along with standard markers. Gels were run at 180 V and then stained with brilliant blue G.

Antibiotic susceptibility testing of strains expressing Cfr-like proteins. Drug susceptibility testing was done in a microtiter plate format by measuring optical density values at 450 nm with a Victor 3 spectrophotometer (Perkin Elmer). LB medium was inoculated with single colonies of *E. coli* AS19 strains harboring plasmids with *cfr* or the *cfr*-like genes and incubated overnight. The cultures were diluted to an OD₄₅₀ value of 0.01, followed by mixing of 100 μ l diluted culture with 100 μ l of antibiotic solution in a series with 2-fold concentration steps. Expression of *cfr* and the *cfr*-like genes was induced by addition of 1 mM IPTG. The tested concentration ranges were as follows: florfenicol, 0.5 to 32 μ g/ml; clindamycin and linezolid, 2 to 128 μ g/ml; tiamulin, 0.25 to 128 μ g/ml; and a mixture of streptogramin A and streptogramin B antibiotics (Synercid), 1 to 64 μ g/ml. The MIC was defined as the drug concentration at which the growth of the cultures was completely inhibited after 24 h of incubation at 37°C.

Primer extension analysis. RNA was extracted from *E. coli* JW2501-1 strains harboring the plasmids following induction with 1 mM IPTG and 3 to 4 h of growth using the GeneJET RNA purification kit (Fermentas). Methylation at A2503 was examined by primer extension analysis with avian myeloblastosis virus (AMV) reverse transcriptase (Finnzymes). The Cy5-labeled deoxyoligonucleotide primer (5'-

GAACAGCCATACCTTG-3'), complementary to nucleotides 2540 to 2556 of *E. coli* 23S rRNA, was used. The cDNA extension products were separated on 6% polyacrylamide sequencing gels. The positions of the stops were visualized by fluorescence scan and identified by referencing to dideoxynucleotide sequencing reactions on 23S rRNA that were electrophoresed in parallel.

RESULTS AND DISCUSSION

BLAST search and selection of *cfr*-like genes. BLAST searches were performed against the nonredundant protein sequences database with the *S. sciuri* Cfr protein sequence as the query sequence. As expected from the literature, the top hits corresponded to the Cfr proteins found in *Bacillus* sp., *S. aureus*, and *E. faecalis*, with 99 to 100% identity to the query sequence. Other hits with over 50% identity to Cfr are proteins found in *Firmicutes*, including organisms in the genera *Bacillus*, *Brevibacillus*, *Paenibacillus*, *Clostridium*, and *Enterococcus*. Of these, the proteins with highest identity to Cfr (60 to 80%) are, with one exception, found in *Bacillus* and *Brevibacillus*. Three proteins with high degrees of identity to Cfr that were found in organisms from these two genera, namely, *Bacillus amyloliquefaciens*, *Bacillus clausii*, and *Brevibacillus brevis*, were selected for further analysis. An alignment showing the homology is presented in Fig. 1. As we are interested in discovering Cfr and not RlmN homologs, RlmN is included in the alignment to show that the selected sequences are more similar to Cfr than to RlmN (Fig. 1).

Cloning, sequencing, and expression of the selected Cfr-like proteins. After extraction of genomic DNA from the *Bacillus amyloliquefaciens*, *Bacillus clausii*, and *Brevibacillus brevis* strains, their *cfr*-like genes were amplified by PCR for cloning (see Materials and Methods). The genes were introduced into plasmid pLJ102 (12) in the same way that plasmid pCfrhis was constructed with the *S. sciuri* *cfr* gene (9, 14) except that no histidine tag was added. The insertions were verified by sequencing of the cloned genes, and the plasmids named pBa, pBc, and pBb were transformed into *E. coli* AS19. Expression of the genes was induced by addition of IPTG and investigated by SDS gel analysis of total protein from the strains. Each strain was assayed with and without IPTG induction. Samples of *E. coli* AS19 without plasmid and with plasmid pBgIII (16), which constitutively expresses Cfr, were included as controls. The gels presented in Fig. 2 show expression of Cfr and the Cfr-like proteins (here referred to as ClbA, ClbC, and ClbB), for which the pBb and pBc samples show very strong expression. As the plasmids are identical except the cloned gene sequences, the differences in expression may be due to differences in translation, rare codons, or the stability of the mRNAs or the enzymes themselves.

Antibiotic susceptibilities of strains expressing Cfr-like proteins for PhLOPSa antibiotics. To establish if the Cfr-like proteins ClbA, ClbC, and ClbB confer a resistance pattern similar to that of the Cfr methyltransferase, MICs were determined for *E. coli* AS19 harboring the respective plasmids with the five antibiotics florfenicol, clindamycin, linezolid, tiamulin, and streptogramin A/streptogramin B. These antibiotics represent the five antibiotic classes in the PhLOPSa phenotype defined from the Cfr methyltransferase (19). The *E. coli* AS19 strain is used as a host for these experiments because it is much more sensitive to many antibiotics than other *E. coli* strains that have a naturally low sensitivity level. Expression of the plasmid-borne genes was induced by adding IPTG when the strains were transferred to LB medium containing

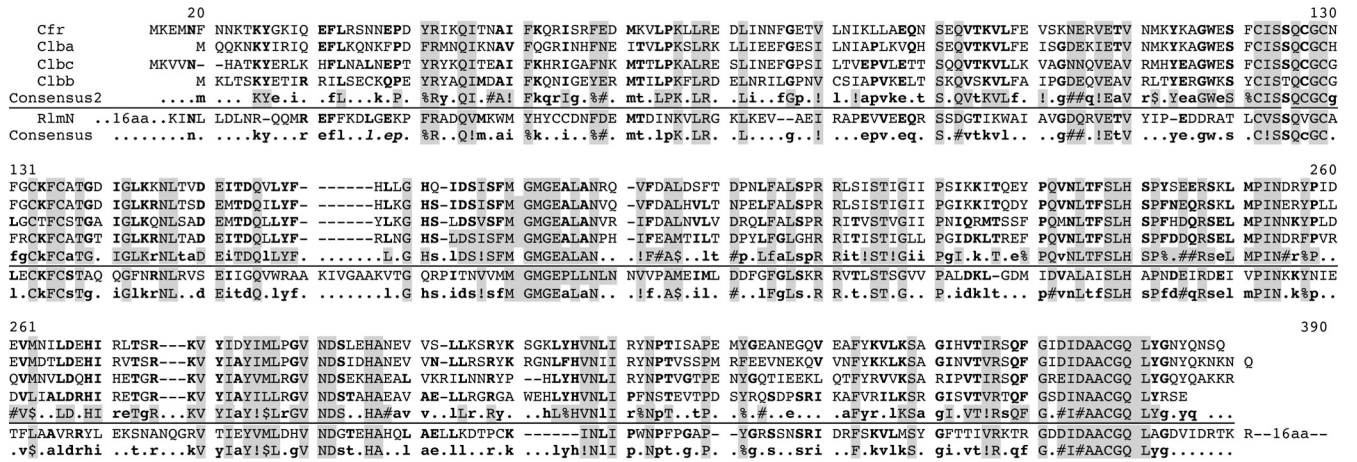


FIG 1 Protein alignment and sequence conservation of Cfr, ClbA, ClbC, ClbB, and RlmN. Alignment of the four investigated proteins plus RlmN, which is placed below the lines. Shaded text indicate >90% consensus. Bolded text indicate >50% consensus. Consensus symbols: !, one of I or V; \$, one of L or M; %, one of F or Y; #, one of N, D, Q, E, B, or Z. The underlined consensus2 sequence is from only Cfr, ClbA, ClbC, and ClbB. The alignment was made using MultAlin (5) (<http://multalin.toulouse.inra.fr/multalin/multalin.html>).

the antibiotics. The MICs are summarized in Table 2 together with those of the control strains without plasmid, the parent pLJ102 plasmid, the pBglII plasmid that constitutively expresses Cfr, and its parent plasmid pBluescript with no *cf*r gene. All in all, expression of the ClbA, ClbC, and ClbB proteins lowers the sensitivity to all five tested antibiotics. Thus, all three proteins are Cfr-like. The general tendency is that Cfr provides more resistance than ClbA, ClbC, and ClbB. The expression level of each protein and the MIC effects do not necessarily correlate quantitatively. This may be because the MIC values reflect a longer induction effect than do the SDS gels and the enzymes are not acting on their natural targets. Although the peptidyl transferase region of the ribosome is well conserved, there are species-specific differences. Despite this, the ClbA, ClbC, ClbB, and Cfr proteins all seem to act relatively efficiently in *E. coli*.

Verification of RNA methylation at A2503 in 23S rRNA by primer extension. Although the combined resistance to five different antibiotic classes is a strong argument for ClbA, ClbC, and ClbB acting via the same mechanism as the Cfr methyltransferase,

a verification of the modified site proves the relationship. The plasmids pBa, pBc, and pBb were transformed into JW2501-1, an *E. coli* RlmN-negative strain (31). The 2-methyladenosine (m^2A) methylation mediated by RlmN causes a minor primer extension stop at A2503 that interferes with detection of the m^8A methylation from Cfr-like enzymes. This is avoided by using rRNA from the JW2501-1 strain as *in vivo* substrates. After induction of Cfr and the Cfr-like proteins, the bacteria were grown for 3 to 4 h to allow new rRNA to be transcribed, modified, and incorporated into ribosomes. Then the total RNA was purified and subjected to primer extension by reverse transcriptase. A fluorescently labeled oligonucleotide was annealed to a region of 23S rRNA 3' to A2503 and extended until stopped by modifications or secondary structures. The products were run alongside dideoxy sequencing reactions, as illustrated in Fig. 3. The induction of Cfr from pCfrhis and the constitutively expressed Cfr from pBglII mediate strong stops at A2503. The ClbA, ClbC, and ClbB proteins cause clear but somewhat less-intense bands. As expected, the control with pLJ102 does not give rise to any stop at the A2503 position. The stops thus confirm that ClbA, ClbC, and ClbB modify A2503 like Cfr does. The intensity of the stops correlates with the MICs in Table 2 in that stronger stops are observed with Cfr, which also yields higher MIC values than the Cfr-like proteins. The data are from different hosts and time intervals after induction, so the correlation is not expected to be absolute. Both m^2A and m^8A methylations give rise to primer extension stops, but m^2A does not cause significant antibiotic resistance (31). Therefore, the conclusion is that ClbA, ClbC, and ClbB are true Cfr-like proteins, providing the same effect on 23S rRNA as that by Cfr. Further studies are needed to investigate if these enzymes play additional roles in their natural hosts. This requires either gene knockouts or a close relative without the gene that can be supplemented with the gene or other genetic manipulations, and even then, it might not be a trivial task.

Concluding remarks. It is still uncertain how widespread the *cf*r gene is and how it evolved, but its presence in pathogenic bacteria and the resulting antibiotic resistance are certainly matters of concern. It is not known if it originally evolved for protection

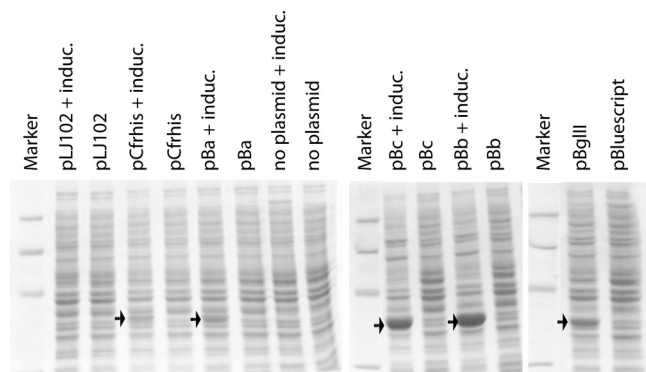


FIG 2 Analysis of whole-cell extracts by SDS-PAGE to verify expression of Cfr and Cfr-like proteins. The extracts are from *E. coli* AS19 harboring the plasmids indicated on top of the gels. Lanes marked with “+ induc.” denote samples with induced expression. The marker protein sizes in kilodaltons are, from the top, 97.4, 66.2, 45, and 31. The arrows point to bands appearing as a result of Cfr or Cfr-like protein expression.

TABLE 2 MICs of *E. coli* AS19 strains in the presence or absence of plasmids expressing *cfr* or *cfr*-like genes

Plasmid	Presence of <i>cfr/cfr</i> -like gene	MIC ($\mu\text{g/ml}$) ^a				Streptogramin A/ streptogramin B
		Florfenicol	Clindamycin	Linezolid	Tiamulin	
No plasmid	–	1	32	8–16	0.5	4
pLJ102	–	1	16	8	0.5	4
pBa	+	4	>128	32	4–8	32
pBc	+	8	>128	16–32	4–8	32
pBb	+	16	>128	16	8–16	32
pCfrhis	+	16	>128	128	32	64
pBluescript	–	1	16–32	8	0.5	4
pBgIII	+	16	>128	64	16	64

^a The tabulated MICs are the averages from at least three independent experiments. An interval is given when no clear distinction between the numbers was obtained. Only greater-than-2-fold differences are considered significant.

against antibiotics or if it also has some other function. The similar *rlmN* genes that do not confer resistance are abundant, but there is also a third group of sequences that code for proteins that are a bit different from RlmN and Cfr (14). This study is a very first step to shed light on some of these questions. Our data clearly confirm that the three investigated genes from *Bacillus amyloliquefaciens*, *Bacillus clausii*, and *Brevibacillus brevis* are *cfr*-like. They confer decreased susceptibility to five classes of antibiotics when expressed in *E. coli* by modification of position A2503 in 23S rRNA. The verification of functional *cfr*-like genes in the environment is a disturbing finding regarding antibiotic resistance that warrants further investigation.

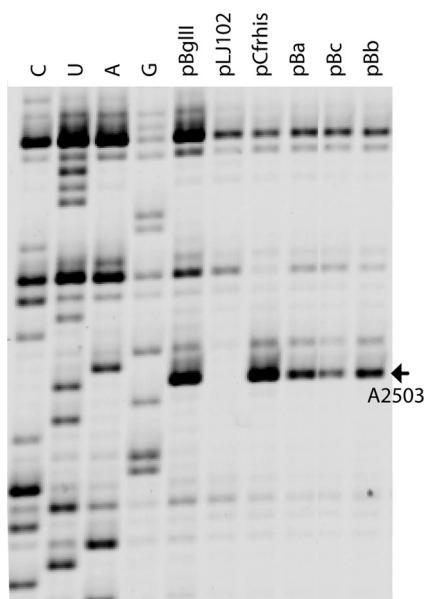


FIG 3 Primer extension analysis of reverse transcriptase stops on 23S rRNA from *E. coli* JW2501-1 strains harboring various plasmids expressing Cfr-like proteins. The region shown is limited to the nucleotides flanking A2503, which is methylated by Cfr. Lanes 1 to 4, marked C, U, A, and G, refer to dideoxy sequencing reactions. Lanes 5 to 10 show primer extension reactions on total RNA from cells harboring the indicated plasmids. Reverse transcriptase stops one nucleotide before the corresponding nucleotide in the sequencing lanes. The arrow points to the stop mediated by methylation from Cfr or the Cfr-like proteins.

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