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The order Bacillales hosts functional homologs of the worrisome *cfr* antibiotic resistance gene

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Abstract

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 towards 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 control of an inducible promoter. Expression of the genes was induced in *E. coli* and MICs for selected antibiotics indicate that the *cfr*-like genes confer resistance to PhLOPSa antibiotics like 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 a natural residence *cfr*-like genes.

Introduction

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 ribosomal RNA (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 *S. 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 USA, 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 *E. 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, a new natural RNA modification (9). It was also

established by mutagenesis that Cfr is a radical 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 crosslinking 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 like 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 encoding 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 10g polypeptone, 2g yeast extract, and 1g $MgSO_4 \cdot 7H_2O$ per l. All strains were grown at 37°C. Genomic DNA was isolated with the High Pure PCR Template Preparation Kit (Roche) or Aqua Pure Genomic DNA Kit (Biorad). Standard PCR amplification of the relevant genes were performed with the following primers each containing *Nde*I or *Hind*III cleavage sites for cloning: 5'CTGCATACATATGCAACAAAAAAACAAGTATAT3' and 5'CAGAATAAGCTTTTATTGGTTCTTATTTTTTTGATA3' for the *Bacillus amyloliquefaciens* gene (*clba*); 5'CTGCATACATATGAAAGTTGTCAATCATGCG3' and 5'CAGAATAAGCTTTCACCTTTTTTTCGCCTGATA3' for the *Bacillus clausii* gene (*clbc*) and 5'CTGCATACATATGAAACTAACCTCGAAATATGAA3' 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 inducible promoter. The ligations were transformed into the *E. coli* TOP10 strain (Invitrogen) and plasmid containing clones selected on agar plates with 100 µg/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 harbouring the plasmids with the *cfr* look-alike genes were grown at 37°C to an OD_{450} of 0.2-0.3, followed by addition of IPTG (to 1mM) for induction of the genes. Cells were harvested after 3-3.5 h of growth and stored at -80°C. For gel analysis, samples were dissolved in 1 x SDS/DTT-loading buffer, boiled 5 min. and loaded on standard SDS gels along with standard markers. Gels were run at 180V 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 the *cfr* or *cfr*-like genes and incubated overnight. The cultures were diluted to an OD_{450} value of 0.01, followed by mixing of 100 µL diluted culture with 100 µL of antibiotic solution in a series with two-fold concentration steps. Expression of the *cfr* and *cfr*-like genes was induced by addition of 1 mM IPTG. The tested concentration ranges were: florfenicol, 0.5-32 µg/ml; clindamycin and linezolid, 2-128 µg/ml; tiamulin, 0.25-128 µg/ml; and Synercid (a mixture of streptogramin A and streptogramin B antibiotics.), 1-64 µg/ml. The MIC was defined as the drug concentration at which the growth of the cultures was completely inhibited after 24 h 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 3-4 h of growth using GeneJET RNA

Purification Kit (Fermentas). Methylation at A2503 was examined by primer extension analysis with AMV reverse transcriptase (Finnzymes). The Cy5-labeled deoxyoligonucleotide primer (5´-GAACAGCCATACCCTTG-3´), complementary to nucleotides 2540-2556 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 non-redundant protein sequences database with *S. sciuri* Cfr protein sequence as the query sequence. As expected from the literature, the top hits correspond to the Cfr proteins themselves found in *Bacillus* sp., *S. aureus*, and *E. faecalis* with 99-100% 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 (60-80%) are with one exception found in *Bacillus* and *Brevibacillus*. Three proteins with high degrees of identity to Cfr 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 Figure 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 (Figure 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 as 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) that constitutively expresses Cfr were included as controls. The gels presented in Figure 2 show expression of Cfr and the Cfr-like proteins (hereafter referred to as Clba, Clbc and Clbb), where the pBb and pBc samples show a 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 stability of the mRNAs or the enzymes themselves.

Antibiotic susceptibilities of strains expressing Cfr-like proteins for PhLOPS$_A$ antibiotics

To establish if the Cfr-like proteins Clba, Clbc, and Clbb confer a resistance pattern similar to the Cfr methyltransferase, minimal inhibitory concentrations

(MICs) were determined for *E. coli* AS19 harboring the respective plasmids with the five antibiotics florfenicol, clindamycin, linezolid, tiamulin and Synercid. These antibiotics represent the five antibiotics classes in the PhLOPSa phenotype defined from the Cfr methyltransferase (19). The *E. coli* AS19 strain is used as host for these experiments because it is much more sensitive to many antibiotics than other *E. coli* strains that have a natural low sensitivity level. Expression of the plasmid-encoded genes was induced by adding IPTG when the strains were transferred to LB media containing the antibiotics. The MICs are summarized in Table 2 together with controls of strains without plasmid, with the parent pLJ102 plasmid, with the pBgIII plasmid that constitutively expresses Cfr and its parent plasmid pBluescript with no *cfr* 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 compared to 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, and 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 minus strain (31). The m2A methylation mediated by RlmN causes a minor primer extension stop at A2503 that interferes with detection of the m8A 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 are grown for 3-4 hours to allow new rRNA to be transcribed, modified and incorporated into ribosomes. Then total RNA is purified and subjected to primer extension by reverse transcriptase. A fluorescently labeled oligonucleotide is annealed to a region of 23S rRNA 3´ relative to A2503 and extended until stopped by modifications or secondary structures. The products are run alongside dideoxysequencing reactions as illustrated in Figure 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 that also yields higher MIC values relative to 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 m2A and m8A methylations will give rise to primer extension stops but m2A 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 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 *cfr* gene is and how it evolved but its presence in pathogenic bacteria and the resulting antibiotic resistance is certainly a matter of concern. It is not known if it originally evolved for protection 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.

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Figure legends

Figure 1. Protein alignment and sequence conservation of Cfr, Clba, Clbc, Clbb, and RlmN.

Alignment of the four investigated proteins plus RlmN that is placed below the underlining. Shaded is >90% consensus, bold is >50%. Consensus symbols: ! denotes one of IV, $ one of LM, % one of FY, # one of NDQEBZ. The underlined consensus2 sequence is only from Cfr, Clba, Clbc

and Clbb. The alignment is made using MultAlin (5): http://multalin.toulouse.inra.fr/multalin/multalin.html.

Figure 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.

Figure 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 that is methylated by Cfr. Lanes 1-4 marked C, U, A, and G refer to dideoxysequencing reactions. Lanes 5-10 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 Cfr–like proteins.

422 Table 1. The *cfr*-like genes and plasmids used in this study.

Gene label	**Organism** (genome source for *cfr*-like genes)	**Strain designation**	**Source**	**Gene ID**
clba	*Bacillus amyloliquefaciens*	FZB42	BGSC	5463020
clbc	*Bacillus clausii*	"domuvar"	BGSC	7717399
clbb	*Brevibacillus brevis*	100599	NBRC	3201806
Plasmid	**Features**	**References**		
pBa	Inducible Clba expression	This study		
pBc	Inducible Clbc expression	This study		
pBb	Inducible Clbb expression	This study		
pCfrhis	Inducible Cfr expression	(9) (14)		
pBglII	Constitutive Cfr expression	(16)		
pLJ102	Derivative of pQE60	(12)		
pBluescript	Cloning vector	Stratagene		

423 BGSC: Bacillus Genetic Stock Center, NBRC: NITE Biological Resource

424 Center

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

Plasmids	*cfr*/*cfr*-like gene	florfe-nicol	clinda-mycin	linezolid	tiamulin	Synercid
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
pBglII	+	16	>128	64	16	64

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432 The tabulated MIC values are given in units of □icrograms/mL and are the
433 average from at least three independent experiments. An interval is given when
434 no clear distinction between the numbers was obtained. Only greater than two-
435 fold differences are considered significant.