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Review

The *cfr* and cfr-like multiple resistance genes

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ABSTRACT

The Cfr methyl transferase causes an RNA methylation of the bacterial ribosomes impeding reduced or abolished binding of many antibiotics acting at the peptidyl transferase center. It provides multi-resistance to eight classes of antibiotics, most of which are in clinical and veterinary use. The *cfr* gene is found in various bacteria in many geographical locations and placed on plasmids or associated with transposons. Cfr-related genes providing similar resistance have been identified in Bacillales, and now also in the pathogens *Clostridium difficile* and *Enterococcus faecium*. In addition, the presence of the *cfr* gene has been detected in harbours and food markets.

1. Introduction

There is an increasing concern about pathogenic bacteria obtaining resistance to the antibiotics used for treatment of humans and animals. To address the problems with antibiotic resistance we need knowledge about the various resistance mechanisms, how they can be disseminated, which bacteria contain them and where they come from. Antibiotic resistance is not a new phenomenon, see e.g. review by [1]. There are many ways bacteria can obtain and exert antibiotic resistance and new resistance determinants are continuously discovered. The strategies bacteria use to evade the effects of antibiotics, can be grouped into three general mechanisms: increased antibiotic efflux out of the cell or reduced antibiotic influx into the cell, enzymatic inactivation of antibiotics through drug modification or cleavage, and the alteration of the antibiotic binding site. A bacterial cell contains many different target sites for antibiotics, but in general, an antibiotic interfere with or inhibits an essential cellular pathway or process. The most common antibiotic targets include bacterial cell wall synthesis; the bacterial membrane; the DNA replication machinery; RNA polymerase; the folate biosynthesis pathway; and the protein synthesis machinery.

The ribosome is a major site of antibiotic action in the bacterial cell and is targeted by a large and chemically diverse group of antibiotics. A number of these antibiotics have important applications in human and veterinary medicine in the treatment of bacterial infections. The antibiotic binding sites are clustered at functional centers of the ribosome, such as the decoding center on the 30S subunit, the peptidyl transferase center (PTC), the GTPase center, and the peptide exit tunnel on the 50S subunit and the subunit interface in the 70S ribosome. The resistance mechanisms to antibiotics targeting the ribosomes include all three mechanism mentioned above. The alterations of the ribosomal antibiotic binding sites providing antibiotic resistance can be either mutation or methylation. While mutations appear spontaneously the gaining of methylations normally need a gene transfer of a methyl transferase gene. Since the 1950s, about 17 methyltransferases providing antibiotic resistance has been discovered, reviewed by [2,3]. One of the most recently discovered and most exceptional regarding nature of modification and mechanism of methylation is the Cfr methyltransferase [4–7] and Cfr and Cfr-like proteins have a potential to become a serious threat for antibiotic treatment. The sections below will present an overview of our current knowledge about Cfr and Cfr-like methyltransferases and especially cover aspects that have not previously been reviewed.

2. The Cfr methyltransferase and its target

The *cfr* gene codes for Cfr, a 349 amino acid long RNA methyltransferase, characterized as a radical SAM methyltransferase [8]. Cfr makes one methylation at the bacterial ribosome, causing reduced or abolished binding of many antibiotics that bind to the peptidyl transferase center (PTC) of the bacterial ribosomes [5] (Fig. 1A). Many different antibiotics act by binding to the ribosomes where their binding inhibits peptide synthesis and thereby bacterial growth [9]. Chemically different antibiotics can bind at the same specific sites in the ribosomes, although not totally

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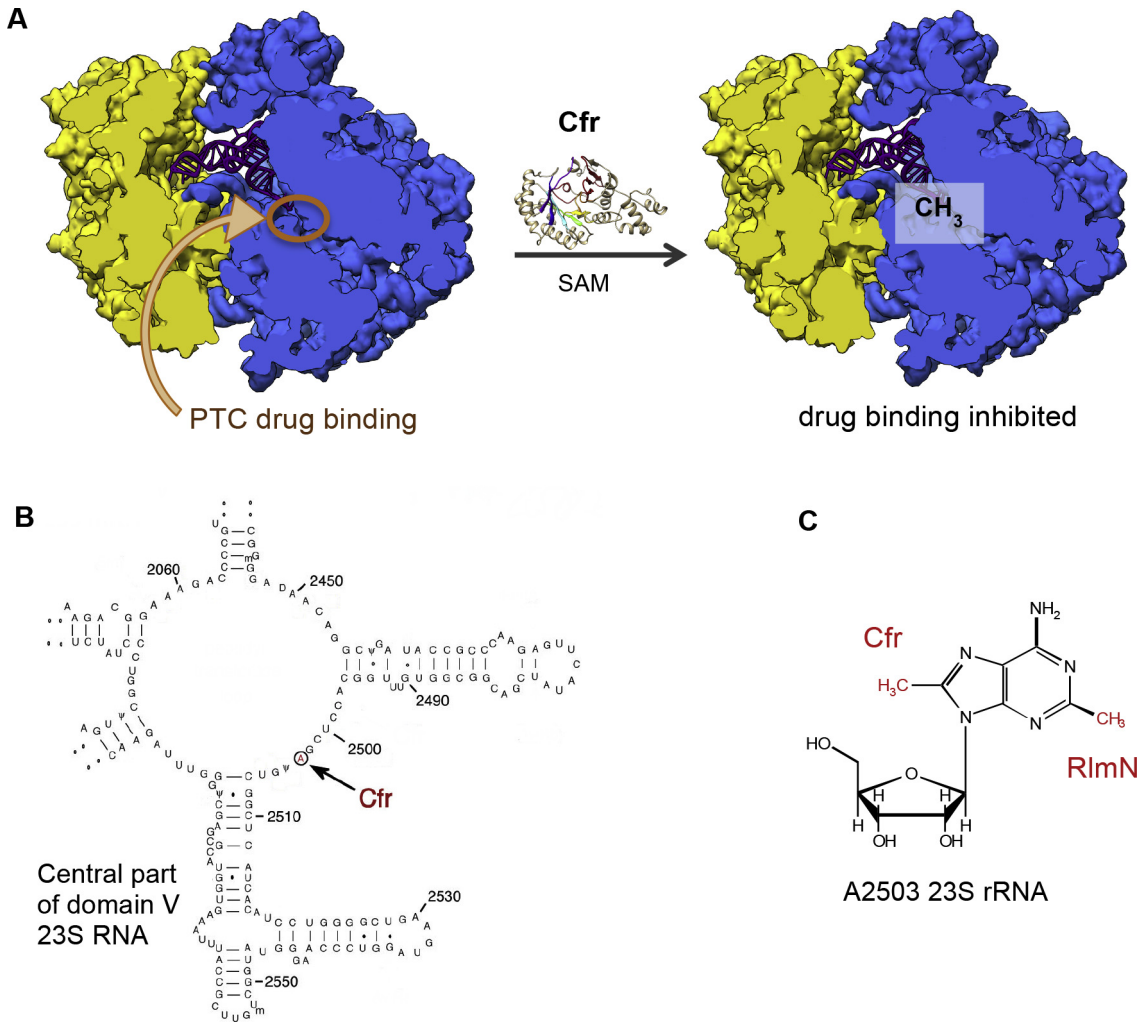

Fig. 1. The Cfr methylation site in the ribosome. A: A cut view of the *E. coli* ribosome with tRNA in P-site (purple) (PDB 4V9D) with a circle indicating the PTC where Cfr methylates A2503 23S RNA and thereby inhibits binding of the antibiotics at this region. B: central part of domain V 23S RNA constituting part of the PTC. The arrow points at position A2503 where Cfr adds a methyl group. C: the chemical structure of the double methylated A2503 with the enzymes shown in red. Cfr acts at the C-8 position and RlmN at the C-2 position. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

overlapping. The PTC is such a site and the Cfr enzyme methylates A2503 in 23S ribosomal RNA in the PTC (Fig. 1B and C). This is so far the only adenine nucleotide found to be methylated at the C-8 position [6] and this provides multi-resistance to eight different classes of antibiotics, most of which are in clinical and veterinary use. At its discovery on a plasmid in *Staphylococcus sciuri* [10] the gene was reported to provide resistance to chloramphenicol and florfenicol. A later study [11] showed that Cfr provided resistance to: Phenicols (including Chloramphenicol and Florfenicol), Lincosamides (including Clindamycin), Oxazolidinones (including Linezolid), Pleuromutilins (including Tiamulin and Valnemulin), and Streptogramin A's (including Pristinamycin II, Viginiamycin M, and Dalfopristin), all relevant for use in animals or humans. Since then, Cfr has also been shown to provide resistance to large macrolides [12], Hygromycin A and A201A [13].

3. Which bacteria contain the *cfr* resistance gene and where are they found

Until now, the cfr gene has mainly been identified in strains belonging to *Staphylococcus* but it has also been found in *Enterococcus, Bacillus, Proteus vulgaris, Escherichia coli, Macrococcus caseolyticus, Jeotgalicoccus pinnipedialis,* and *Streptococcus suis* (reviewed in [14–17]). The *cfr* genes only contain small variations of a few amino acids as clearly seen by a BLAST search that shows 99% identity between Cfr's until the identity jumps to 77% and the following sequences must be characterised as Cfr-like proteins (see below). The *cfr* gene is often found on plasmids and if chromosomal it seems always associated with insertion elements. A detailed investigation detected a novel variant of the phenicol resistance transposon Tn558 in *Staphylococcus* isolates that harboured an additional resistance gene region, including the *cfr* gene, integrated into the tnpC reading frame [18]. They detected transpositionally active forms of the IS21-558 element, known as minicircles, and suggest a pathway for mobility of the *cfr* gene. A more recent study analysed the genetic environment of the *cfr* gene in *Staphylococcus* isolates by sequencing of the up- and downstream regions on various plasmid types [19] and found insertion sequences (IS21-558, IS256, IS257, or IS1216E) as well as other resistance genes. In chromosomes they found the *cfr* gene to be bracketed by insertion sequences, such as IS256 or ISEnfa5 and stability tests confirmed that these cfr-containing regions could be looped out via IS-mediated recombination. Other examples can be found in [17,20–22] and references therein. A recent summary of the

structural variability in the regions surrounding the *cfr* gene on both plasmids or in the chromosomal DNA of various Gram-positive and Gram-negative bacteria is provided in [22]. It reveals twenty different contexts of the *cfr* gene and a considerable diversity and only three of these have no sign of insertion elements or transposition genes. Because of its presence on plasmids and with insertion elements the *cfr* gene is apparently easily disseminated. Spreading between genera is one aspect while geographical spreading is a different matter and the *cfr* gene is also found in many geographical locations. So far, the *cfr* gene is reported found in pathogenic bacteria in Belgium, Brazil, China, Columbia, Denmark, France, Germany, Ireland, Italy, Mexico, Spain, Thailand, and USA. In some cases its presence have been linked to treatment with linezolid e.g. [23]. This apparently global distribution probably picture where studies have looked for it, rather than its actual location. The presence of *cfr* on mobile genetic elements, and in all probability helped by heavily use of antibiotics in farming and disease treatment, suggests how it has been spread among bacteria. The ancestral origin of *cfr* genes has not been determined as is also the case for many other types of antibiotic resistance genes.

4. *cfr* genes in the environment

From 2000 and up till now the *cfr* gene was looked for and found in bacterial strains from humans and animals, but in recent years there had been a few studies of the presence of *cfr* elsewhere and this is of interest for the dissemination of Cfr-mediated antibiotic resistance. Is it already everywhere or just in some specific environments, and caused by extensive antibiotic use? The first identification of the *cfr* gene was in Germany [10] and a recent study from Germany shed some light over the frequency and propagation of the *cfr* gene in staphylococci from nasal swabs [15]. They found *cfr* in 12 of 52 calves at three farms with a history of florfenicol use, no *cfr* in 10 humans living on these farms, no *cfr* in 142 calves at 16 farms in the same area not using florfenicol, *cfr* in 11 of 67 pigs from three of eight farms, *cfr* in 1 of 12 humans living on these farms, *cfr* in 4 of 169 veterinarians from all over Germany, and *cfr* in 3 of 263 persons in contact with veterinarians [15]. Whether or not these numbers are alarming might be a matter of personal opinion but it might suggest that we keep an eye on the development and consider whether florfenicol should be discouraged in areas where *cfr* has been found.

Many findings of *cfr* genes come from China, and mostly from animals. Even without having solid data but based on estimates it can be concluded that the consumption of antibiotics in China for animal therapy and growth promotion antibiotics is huge [24,25]. Some of this ends up in the environment and might further select for presence of drug resistant bacteria. Li et al. 2015 investigated waste water and soil from seven swine feedlots and found approx. 7×10^4–$1{,}3\times10^6$ copies of *cfr* genes/mL in the water and 1.1×10^5–$5\text{x}10^5$/g in the soil [26]. Zhao et al. 2016 investigated soil samples from six Chinese swine farms with a record of florfenicol usage to detect florfenicol resistance genes (FRGs). Quantitative PCR and metagenomic sequencing revealed a significantly higher relative abundance of FRGs in the soils adjacent to the three swine farms where florfenicol was heavily used compared with the other sites. They conclude that it appears that the amount of florfenicol used on swine farms promotes the prevalence and abundance of FRGs, including the linezolid resistance genes *cfr* and *optrA*, in adjacent soils [24]. In another study Zeng et al. 2014 [27] investigated meat samples from Chinese markets for presence of *cfr*. 118 pork and chicken samples from Guangzhou markets were screened by PCR and 22 Staphylococcus isolates obtained from 12 pork and 10 chicken samples harboured *cfr*. The 22 cfr-positive staphylococci isolates, included eight *Staphylococcus equorum*, seven *Staphylococcus simulans*, four *Staphylococcus cohnii*, and three *S. sciuri*, and in 14 isolates, the *cfr* gene was found located on plasmids [27]. In a fourth study Wang et al. 2015 [28] studied the prevalence and genetics of *cfr* in Staphylococcus isolates recovered from pigs, workers, and meat-handling facilities (a slaughterhouse and a hog market). Twenty (4.5%) *cfr*-positive *Staphylococcus* isolates (18 *S. simulans*, 1 *S. cohnii*, and 1 *S. aureus*) were derived from: pigs (16/312), the environment (2/52), and workers (2/80). The authors conclude that both clonal spread and horizontal transmission via mobile elements contributed to the *cfr* dissemination among staphylococcal isolates obtained from different sources [28]. Although it is impossible to make safe conclusions for all China based on the above studies plus the findings of *cfr* in bacteria directly from humans and animals referred to previously, it points to a disturbing development.

In addition to the studies from Germany and China there is also a study of *cfr* in ballast and harbour waters in Singapore [29]. This study screened for 13 antibiotic resistant genes in six samples and found that *cfr* was present in 2.00–5.21 copies/mL. It also found a strong positive correlation between *cfr* and *Pseudomonas aeruginosa* gene copies, but so far *cfr* has not been found in *P. aeruginosa*. There is too little information to even speculate what these findings mean in terms of spreading of Cfr resistance, but hopefully the future will provide us with more information.

5. Cfr-like methyltransferases

Usually genes and the proteins they code for share some homology with others and this can often give a hint to the evolution of the genes, from where they originate and their function. Cfr is a bit special in this aspect, at least based on the available sequence information. The m^8A made by Cfr is so far only found at the A2503 position in 23S ribosomal RNA (*E. coli* numbering) but not in other RNAs or at other positions. The same nucleotide is modified by RlmN that makes an m^2A methylation [30], see Fig. 1C. RlmN is a housekeeping enzyme apparently present in all bacteria and it is not a resistance determinant. Cfr and RlmN show some similarity (34% identity based on RlmN from *E. coli*) and both are so-called radical SAM methyltransferases but not closely related [31,32], although they use the same unique molecular mechanism for methylation [8]. Doing BLAST searches with the Cfr sequence results in various sequences, with some that are very obviously Cfr with small variations plus a huge number of sequences with less similarity to Cfr. Some of these are probably true Cfr-likes (in the sense that they make m^8A as Cfr do); some are RlmN or RlmN-like and some probably something else. Unfortunately, the automatic database annotation for these enzymes is not trustworthy and will not truly distinguish between Cfr-likes and RlmNs. This can be overcome by performing an alignment followed by a detailed analysis of a number of 13 differential conserved amino acids at specific positions [33] that are shown in Fig. 2B.

We have previously been searching for Cfr-likes, by which we mean enzymes that are significantly different from Cfr (>20% identity difference) but perform the same m^8A2503 modification as Cfr. It is thus not sufficient to use gene sequence identity as the only criterion, it has to provide the modification and show the same antibiotic resistance profile as Cfr. In 2011 there were not that many sequences similar to Cfr in the databases but we examined some cfr-like genes from non-pathogenic Bacillales (*Bacillus amyloliquefaciens, Bacillus clausii, Brevibacillus brevis*), which provided the same multi-drug resistance as Cfr when transferred to *E. coli* [34]. Later we also showed the same for a cfr-like gene from *Paenibacillus* sp. Y412MC10 [33]. Their similarity to Cfr is shown in Fig. 2 and in Table 1. They can thus provide antibiotic resistance to *E. coli* but they have not been found in pathogenic strains and whether these

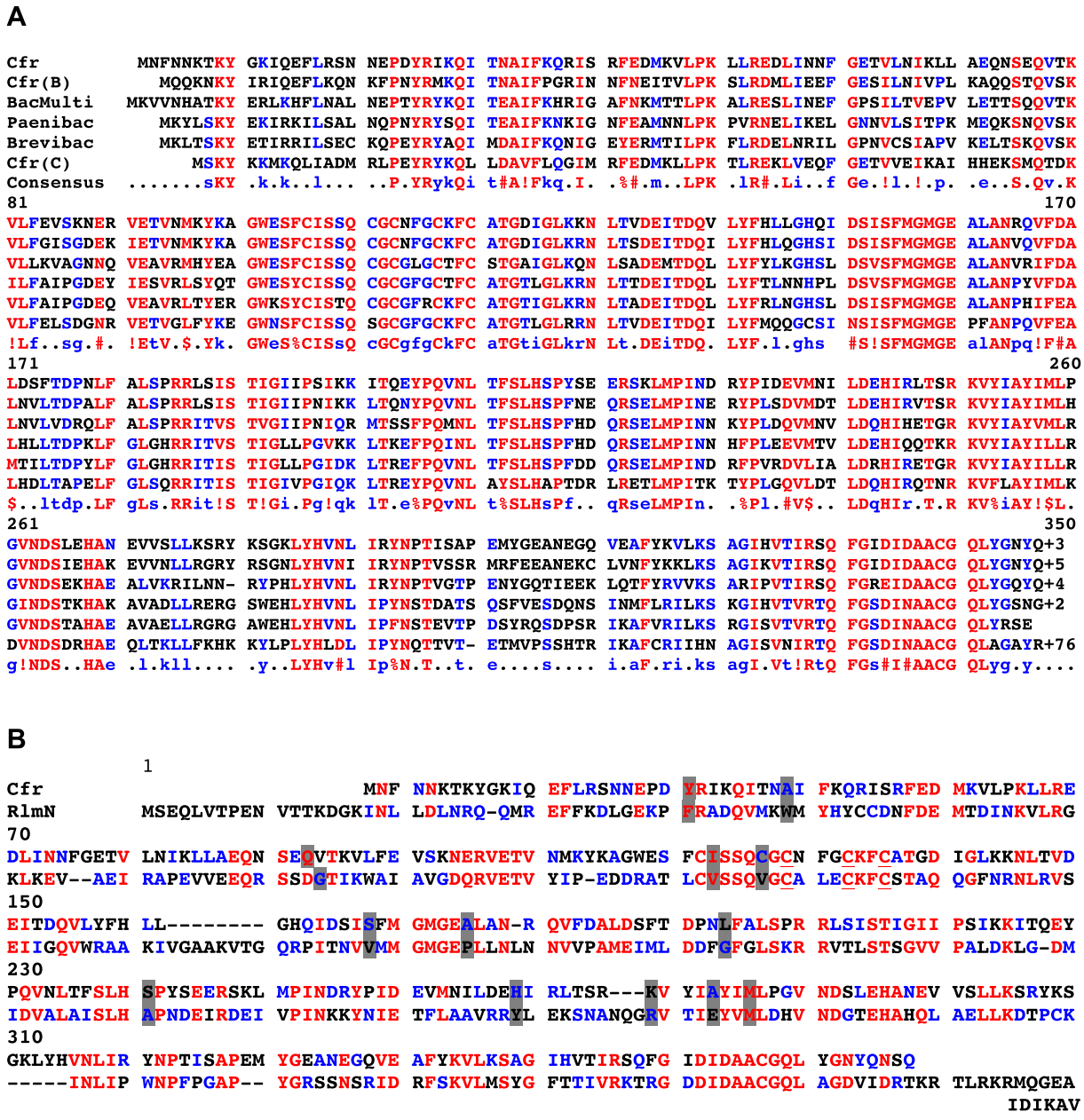

Fig. 2. A. Sequence alignment of Cfr and Cfr-like enzymes to highlight similarities and differences. Red shows amino acids that are >90% conserved in the aligned sequences, blue >50% conserved and black less conserved. The alignment is made using http://multalin.toulouse.inra.fr/multalin/ and the following sequences: Cfr - WP 001010505.1, Cfr(B) - WP0023499811 that includes Cfr-likes from *Clostridium difficile* and *Enterococcus faecium*, BacMulti - WP 011245929 that includes Cfr-like ClBc from *Bacillus clausii*, Paenibac - WP 015735625 that includes Cfr-like ClPa from *Paenibacillus* sp. Y412MC10, Brevibac - WP 015892743 that includes Cfr-like ClBb from *Brevibacillus brevis*, and Cfr(C) -WP002578771 1 that includes Cfr-likes from *Campylobacter* and *Clostridium difficile*. Cfr-like ClBa from *Bacillus amyloliquefaciens* are <20% different from Cfr(B) and can thus be characterised as a Cfr(B) like. B. Sequence alignment of Cfr and RlmN (WP 000003317.1) enzymes showing the 13 positions (grey marking) that can be used to discriminate between these to similar enzymes making m^8A or m^2A respectively. The amino acids marked with grey are selectively conserved in a designated Cfr and RlmN alignment with multiple sequences [33], meaning that 70% have a specific amino acid in all Cfrs and another specific amino acid in RlmNs. The one aa displacement at 94 in RlmN and the identical M at 286 are because this representation are based on only two sequences. Underlining indicate the CxxxCxxC motif cysteines involved in binding of a Fe-S cluster and a characteristic of radical SAM enzymes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

should be called antibiotic resistance genes is a matter of definition as discussed in [35]. These cfr-like genes are found on the chromosomes but no studies of their genetic context have been reported. Recently, a Cfr-like ortologue was also found in *Paenibacillus* sp. LC231 from an isolated ecosystem and with 95% identity to the *Paenibacillus* sp. Y412MC10 Cfr-like enzyme [36]. This is in accordance with the general view that genes providing protection to antibiotics have always been around and will always be present in the bacterial population, see e.g. [1,37].

Recently, Cfr-like methyltransferases have also been verified in pathogenic strains and a new naming for these genes has started (http://faculty.washington.edu/marilynr/). To avoid genes with multiple names and to be able to identify the presence of very similar genes they get single letter alphabetic extensions if >20% identity difference. At present we have Cfr(B) in *Clostridium difficile*/*Peptoclostridium difficile* [38,39] and in *Enterococcus faecium* [40,41] and Cfr(C) in *Campylobacter* [42] and in *C. difficile* and *Clostridium bolteae* [43]. Cfr(B) and Cfr(C) are also included in the alignment

Table 1
% Similarity, as obtained from BLAST searches at NCBI https://blast.ncbi.nlm.nih.gov/Blast.cgi, between all sequences aligned in Fig. 2 and supplemented by comparisons with RlmN. All sequences are >20% different from each other as well as >20% different from Cfr.

% Identity	cfr	cfr(B)	BacMulti	Paenibac	Brevibac	Cfr(C)
Cfr(B)	75	–	–	–	–	–
BacMulti	62	63	–	–	–	–
Paenibac	57	60	59	–	–	–
Brevibac	57	59	59	72	–	–
Cfr(C)	56	52	48	52	56	–
RlmN	34	32	32	31	34	34

Fig. 2 and in Table 1 together with their % similarity. It is expected that more examples in pathogenic strains will appear in the future reinforcing the current concern about Cfr and Cfr-like proteins. Also, there are indications that these cfr-like genes may have been inserted into the chromosomes via transposons [40] [41] or other insertion mechanisms and thus also have the possibility to easily spread further.

6. Future perspectives

The threat of antibiotic resistance to our ability to treat bacterial diseases is truly worrisome but the impacts of the various resistance determinants are difficult to predict. We cannot eradicate antibiotic resistance but only try to minimize selection for resistance and spreading of resistant strains. Cfr and Cfr-like enzymes cause multiple antibiotic resistances to drugs binding at PTC - meaning that selection for the presence of this gene can probably be due to any of these drugs. Thus, one problem with Cfr and Cfr-like resistance is that many different antibiotics can select for its uptake or persistence and that the resistance mechanism functions in many bacterial strains. Also, it is well tolerated and cause very little fitness cost [44]. Nonetheless, our knowledge in the field is still too limited and important questions emerge: How widespread are *cfr* and the cfr-like genes? Are the Cfr-like enzymes as effective as the original Cfr in providing antibiotic resistance? Is m^8A2503 methylation their primary function, or do they also do something else not related to antibiotic resistance?

Conflict of interest

No conflict of interest.

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