UNIVERSITY OF SOUTHERN DENMARK

Patrícia Isabel Teixeira dos Santos

Investigação de um gene *cfr*-like em *Clostridium*

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Patrícia Isabel Teixeira dos Santos

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Keywords

Antibiotic resistance, $PhLOPS_A$, *Clostridium sporogenes*, *cfr*, *cfr*-like, Cfr methyltransferase

Abstract

The aim of this project was, primarily, to clone, express and investigate the function of a *cfr*-like gene from *Clostridium sporogenes* (*clcs*) in *E. coli* and, subsequently, to investigate the function of the *clcs* gene in *C. sporogenes*, its natural host, and ascertain possible variations in function. The *cfr* and *cfr*-like genes were cloned into a plasmid, which allowed their constitutive expression in *E. coli*. The ClCs protein was not able to mediate changes in the antibiotic susceptibility of *E. coli* compared to the $PhLOPS_A$ phenotype conferred by the Cfr methyltransferase. The lack of function of the expressed protein was also investigated by combining parts of the *cfr* and *clcs* genes, but no MIC changes were observed. Attending to the Cfr methyltransferase function, the verification of the presence or absence of the RNA methylation at A2503 in 23S rRNA from *E. coli* was checked by primer extension, showing that the ClCs-containing strain *E. coli* JW2501-1 does not give rise to any stop at A2503, revealing that the Cfr-like protein from *C. sporogenes* does not methylate E. coli 23S RNA, which is consistent with the MIC results. Since it was not possible to conclude that ClCs does not have a Cfr-like function, further investigation was required to determine if ClCs could be able to methylate *C. sporogenes* 23S RNA and act as Cfr.

Two *C. sporogenes* strains reported as *cfr*-like gene carriers were investigated. The attempts to amplify the *cfr*-like genes revealed that the assumption that both these *C. sporogenes* strains contained a *cfr*-like gene seemed to be true for only one of them. MICs showed that the *cfr*-like gene-containing *C. sporogenes* strain has lower susceptibility to all the $PhLOPS_A$ antibiotics tested than the presumably Cfr-lacking *C. sporogenes* strain. The uncertain function of the ClCs protein was then investigated by primer extension to look for an indication of modification at A2503 23S RNA from *C. sporogenes* (*E. coli* numbering). As a similar stop was observed for both strains, mass spectrometry was performed revealing a mono-methylation at A2503, probably caused by the housekeeping RlmN protein and not by Cfr. Another possible modification in the area around A2503 was detected and should be further analyzed.

Palavras-chave

Resistência a antibióticos, $PhLOPS_A$, *Clostridium sporogenes*, *cfr*, *cfr*-like, metiltransferase Cfr

Resumo

O objectivo deste projeto foi, inicialmente, clonar, expressar e investigar a função de um gene *cfr*-like de *Clostridium sporogenes* (*clcs*) em *E. coli* e, posteriormente, investigar a função do gene *clcs* em *C. sporogenes*, o seu hospedeiro original, verificando possíveis variações na sua função. Os genes *cfr* e *cfr*-like foram clonados num plasmídeo, o que permitiu a expressão constitutiva dos genes em *E. coli*. A proteína ClCs não mediou alterações na susceptibilidade de *E. coli* aos antibióticos, em comparação com o fenótipo $PhLOPS_A$ conferido pela metiltransferase Cfr. A ausência de função das proteínas expressas foi também investigada através da combinação de partes dos genes *cfr* e *clcs*, contudo não foram observadas alterações nas MICs. Tendo em conta a função da metiltransferase Cfr, a verificação da presença ou ausência da metilação na posição A2503 do rRNA 23S de *E. coli* foi analisada por *primer extension*, mostrando que a estirpe *E. coli* JW2501-1 que compreende a proteína ClCs não dá origem a qualquer *stop* na posição A2503, demonstrando que a proteína Cfr-like de *C. sporogenes* não metila o RNA 23S de *E. coli*, o que é consistente com os resultados das MICs. Uma vez que não foi possível concluir que a proteína ClCs não possui a função de uma proteína Cfr-like, foi necessária uma investigação mais aprofundada para determinar se a proteína ClCs poderia metilar o RNA 23S de *C. sporogenes* e funcionar como Cfr.

Duas estirpes de *C. sporogenes* apontadas como portadoras do gene *cfr*-like foram investigadas. As tentativas para amplificar os genes *cfr*-like revelaram que a hipótese de ambas as estirpes conterem um gene *cfr*-like parecia ser verdade para apenas uma delas. As MICs mostraram que a estripe *C. sporogenes* que compreende o gene *cfr*-like tem menor susceptibilidade a todos os antibióticos $PhLOPS_A$ testados do que a presumível estirpe de *C. sporogenes* que não possui a proteína Cfr. A função dúbia da proteína ClCs foi então investigada por *primer extension* na tentativa de encontrar alguma modificação na posição A2503 do RNA 23S de *C. sporogenes* (numeração em *E. coli*). Como foi observado um *stop* semelhante em ambas as estirpes, foi realizada espectrometria de massa, revelando uma mono-metilação na posição A2503, provavelmente causada pela proteína *housekeeping* RlmN e não pela Cfr. Uma outra possível modificação em torno da posição A2503 foi detectada e deverá ser posteriormente analisada.

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1. Introduction

1.1. Antibiotic resistance in bacteria

The discovery of antibiotics in the early part of the last century completely altered the treatment of common infections, and it was almost taken for granted by the patients that infections would be diagnosed and immediately treated [1]. However, almost as soon as antibacterial drugs were released, bacteria were able to evolve and become resistant to antibiotics, revealing various forms of resistance, such as mutation and acquisition of new DNA, inactivation of the drugs or reduction of their access to the sites of action, and modifying the drug target [2]. The mechanisms that will predominate depend on the nature of the antibiotic, its target site, the bacterial species and if it is mediated by a resistance plasmid or by a chromosomal mutation [3].

Most antibiotics can be classified according to their main mechanism of action: interference with cell wall synthesis, inhibition of protein synthesis, interference with nucleic acid synthesis, and inhibition of a metabolic pathway [4]. A wide range of antibiotics affect protein synthesis by modification of the specific target [3]. One of the most preferred targets is the ribosome. The bacterial ribosome is disturbed by the antibiotics at crucial components, interfering with the synthesis of new proteins [5]. Ribosomes (sedimentation coefficient of 70S) are large RNA-proteins complexes and comprise of a small (30S) and a large (50S) subunits: the small subunit decodes the genetic information mediating the interaction between transfer RNA (tRNA) and messenger RNA (mRNA), and the large subunit catalyses peptide bond formation. Many antibiotic classes bind to the 16S ribosomal RNA (rRNA) in the 30S ribosomal subunit (aminoglycosides and tetracyclines, for example) or to the 23S rRNA in the 50S ribosomal subunit (oxazolidinones, macrolides, lincosamides, among others) [6-8]. The large subunit is targeted by an extensive range of drugs that interfere with GTP hydrolysis (substrate for the synthesis of RNA), formation of peptide bonds, and channelling the peptide through the exit tunnel, interfering with tRNA transition between the binding sites located within the tunnel that crosses the two subunits. Three tRNA binding sites were identified and characterized: the aminoacyl (A), peptidyl (P), and exit (E) sites. On these subunits, most of the antibiotic binding sites cluster at or near the peptidyl transferase centre (PTC), where peptide-bond formation occurs (Table 1, Figure 1A). PTC comprises of two major components: the A site, which interacts with the CCA end of aminoacylated tRNAs, and the P site, where the CCA ends of peptidyl tRNAs are bound when peptide bonds form. PTC-targeting antibiotics inhibit peptide-bond formation by perturbing or preventing the correct positioning of the aminoacylated ends of tRNAs in the PTC. The binding sites of PTC-targeting antibiotics overlap with the A-site tRNA or with the P-site tRNA, or span both the A- and P-sites (Figure 1B) [5, 9-11].

Table 1: Mechanisms of action and resistance mechanisms of selected 50S subunit-targeting antibiotics [11].

Antibiotic	Inhibition mechanism	Resistance mechanisms*
Blasticidin S	PTC, termination	DM, TM
Chloramphenicol	PTC	DM, E, TA, TM
Clindamycin, lincomycin	PTC	DM, E, TA, TM
Dalfopristin (Streptogramin A)	PTC	E, TA, TM
Quinupristin (Streptogramin B)	PTC	E, TA, TM
Erythromycin, telithromycin	Nascent chain elongation	DM, E, TA, TM
Evernimicin, avilamycin	Initiation	TA, TM
Linezolid	PTC	E, TA, TM
Puromycin	PTC	DM
Sparsomycin	PTC	E, TM
Thiostrepton	Factor binding	TA, TM
Tiamulin	PTC	E, TA, TM

DM: drug modification/degradation; E: efflux/membrane permeability; PTC: peptidyl transferase center; TA: target alteration via modification (or lack thereof); TM: target mutation. *Typical resistance mechanisms (although others might exist).

Resistance may be either inherent by the processes of genetic mutation or be acquired by gene transfer, through several genetic mechanisms, such as transformation, conjugation or transduction. This is termed horizontal evolution, and may occur between strains of the same species or different bacterial species or genera [4]. Although in some bacteria gene exchange occurs mainly by transformation, for many bacteria the most important vector of genetic exchange are plasmids, extra-chromosomal pieces of DNA. Plasmids are directly implicated in the acquisition of resistance to many antibiotics and their transmission is a key factor influencing plasmid-borne antibiotic resistance [12, 13].

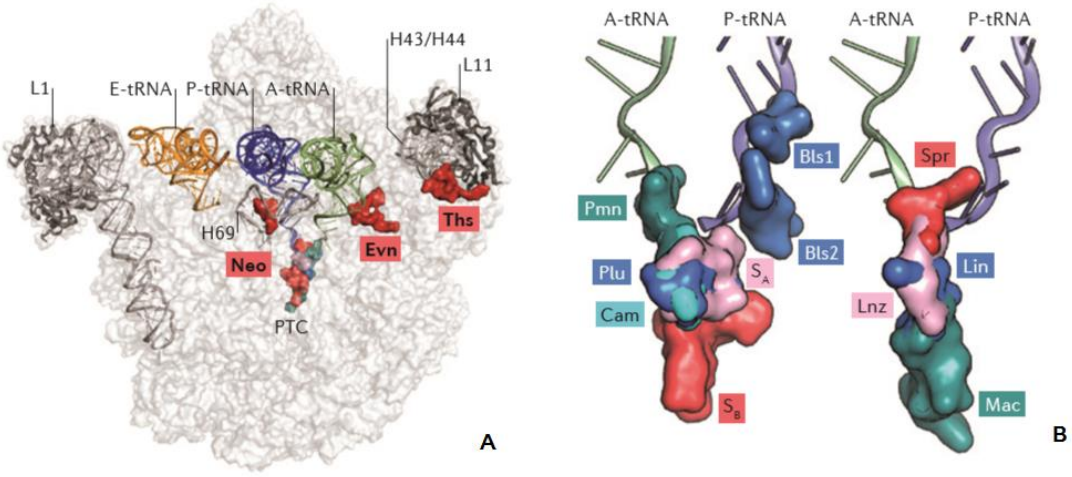


Figure 1: Antibiotic binding sites on the 50S ribosomal subunit. (A) Overview of the binding sites of neomycin (Neo), evernimicin (Evn) and thiostrepton (Ths). The A-site tRNA (green), P-site tRNA (blue), E-site tRNA (orange), H43/H44, H69, peptidyl-transferase centre (PTC) and the L1 and L11 stalks are highlighted for reference. (B) Enlargement of the binding sites of blasticidin S (Bls1 and Bls2), sparsomycin (Spr), lincomycin (Lin), linezolid (Lnz), macrolides (Mac), puromycin (Pmn), pleuromutilins (Plu), chloramphenicol (Cam) and streptogramins A and B (SA and SB) relative to the A-site and P-site tRNAs [11].

1.2. The *cfr* gene and the Cfr methyltransferase

1.2.1. Identification of the plasmid-borne *cfr* gene

The *cfr* gene was originally identified on a multi-resistance plasmid isolated from *Staphyloccocus sciuri* obtained from the nasal swab of a calf. Studies from the 90´s showed that antimicrobial resistance is common among this species, and some plasmids were identified as carriers of one or more resistance genes. This particular plasmid – pSCFS1 – carried the *cfr* gene, and was identified as a chloramphenicol-florfenicol resistance determinant and exhibited an unknown mechanism of resistance regarding both drugs, not only in its host but also in *Escherichia coli* [14]. To clarify how the gene was responsible for this resistance property, the inferred amino acid sequence of the Cfr protein was compared with other proteins known as responsible for resistance to the referred drugs, such as the multidrug efflux pumps family [15]. The results showed no homology to the efflux proteins and also to acetyltransferases, as well as the absence of characteristics of a transmembrane protein and the lack of ATP binding domains, which could help to predict the function of the protein [14, 16].

1.2.2. Cfr methyltransferase methylates at the PTC in the ribosome

The mechanism of resistance towards chloramphenicol and florfenicol (phenicol) remained unknown. Thus, the possibility of Cfr affecting the drug target site was taken into account. Kehrenberg *et al.* investigated the binding of florfenicol, chloramphenicol and clindamycin to the ribosome to find out how Cfr was acting. They observed that cells with Cfr showed a reduced binding of the drugs to the ribosome [17]. Chloramphenicol binds to the 50S ribosomal subunit, more specifically to the 23S ribosomal RNA domain in the peptidyl transferase centre. This drug protects sites in the highly conserved central loop of domain V and inhibits peptidyl transferase activity [18]. The search for a Cfr target confirmed that the mechanism of resistance was due to an altered ribosomal binding site. Kehrenberg *et al.* showed that Cfr was a methyltransferase that adds an extra methylation at position A2503. Besides, Cfr also caused a reduced ribose methylation at C2498 [17]. However, its specific identity and position remained to be clarified.

1.2.3. Cfr methyltransferase belongs to the Radical SAM superfamily and methylates C-8 at A2503 23S ribosomal RNA

Database searches indicated that Cfr had some homology to a protein superfamily called Radical SAM (S-adenosylmethionine). Radical SAM proteins are associated with several ring-forming reactions, pathways with sulphur transfer, anaerobic or oxygen-independent mechanisms, methylation reactions, isomerization and protein radical formation [19]. Proteins belonging to this family are related by the cysteine motif CxxxCxxC, which nucleates the [4Fe-4S] cluster, associated with generation of an oxidizing agent [20]. To corroborate that Cfr was a radical SAM protein, Giessing *et al.* performed some mutagenesis experiments in the cysteine motif CxxxCxxC, where each cysteine was replaced by alanine. *E. coli* strains expressing the mutated Cfr proteins showed that they were inactive, proving the essential role of the cysteine motif for Cfr activity and thus indicating a radical SAM methyltransferase mechanism [21].

The same study, also, showed that the Cfr-mediated methylation at nucleotide A2503 of 23S rRNA was in fact caused by methylation of the C-8. The m^8A2503 modification is then responsible for the antibiotic resistance because of a steric interaction between the antibiotic and the methyl group added, that points into the drug-binding site [21]. In addition, this Cfr-mediated methylation was shown to be independent of the natural m^2A2503 modification already reported in *E. coli* [22], and proved to be mediated by the RlmN methyltransferase in the ribosomes of Gram-positive and Gram-negative bacteria [23]. Actually, the amino acid sequence of this RlmN methyltransferase also showed characteristics associated to the radical SAM superfamily [24].

Therefore, the enzymes RlmN and Cfr methylate C-2 and C-8 of adenosine 2503, respectively, using SAM to methylate electrophilic rather than nucleophilic carbon centres. New mechanisms of methylation of A2503 were proposed for both RlmN and Cfr. It looks like Cfr evolved from RlmN and uses the same strategy to methylate C-8, but still have some capacity to also methylate C-2. The explanation is that Cfr has a less rigid substrate-binding pocket, allowing two different conformations, where a lysil residue acts as a general acid/base, while in RlmN this is a monoprotic residue [25]. Also, bioinformatics analysis of the Cfr/RlmN family suggested that it has a bacterial origin and also that RlmN had evolved in a vertical matter while Cfr evolved from horizontal transfer. RlmN may represent the ancestral form whereas *cfr* gene might have evolved from an *rlmn* gene, probably following duplications and horizontal gene transfer, but the lineage in which it happened was unidentified [26]. Despite their similarities, the methylation mediated by the RlmN methyltransferase is considered to be a housekeeping modification rather than a genuine antibiotic resistance determinant [21]; on the other hand, Cfr was proven to mediate resistance to various antibiotics, as explained below.

1.2.4. Multiple resistance conferred by the Cfr methyltransferase

Cfr methyltransferase was first confirmed as the responsible for three drugs resistance phenotype through methylation at position A2503, meaning resistance to phenicols (chloramphenicol and florfenicol) and lincosamide (clindamycin). If the Cfr affected the binding site of these drugs, probably other drugs that bind to the ribosomal peptidyl transferase centre could be affected too [27]. Pleuromutilins, oxazolidinones and streptogramin A antibiotics, all of clinical or veterinary importance, are known to bind close to A2503, becoming optimal candidates to susceptibility testing of strains carrying the *cfr* gene. Only some representatives of these antibiotic classes were used: tiamulin and valnemulin (pleuromutilin), linezolid (oxazolidinone) and virginiamycin M_1 (streptogramin A). As observed for chloramphenicol, florfenicol and clindamycin, the presence of Cfr conferred resistance to these new drugs. Thus, we could expect that bacterial strains expressing Cfr would present decreased susceptibility to these five families of antibiotics. A new resistance phenotype was then established and named $PhLOPS_A$ for Phenicol, Lincosamide, Oxazolidinone, Pleuromutilin and Streptogramin A resistance, functioning in both Gram-positive and Gram-negative bacteria [27]. A later study showed that Cfr did not only provide resistance to these antimicrobial families but also decreased the susceptibility to 16-member-ring (large) macrolides (josamycin, spiramycin and tylosin), through the modification of A2503 [28].

These classes of antibiotics are known to bind close to A2503 in the 23S rRNA. Thus, it is important to determine ribosome-antibiotic interactions of each drug to understand how the modification caused by the Cfr protein can affect their binding. The structure model of the 50S subunit of *Deinococcus radiodurans* in complex with some antibiotics has been used as a reference to explain some of the ribosome-antibiotic interactions. Chloramphenicol (phenicol) is known to block peptidyl transferase activity by inhibiting the binding of tRNA to the A-site, showing several reactive groups that can form hydrogen bonds with various nucleotides of the peptidyl transferase cavity (Figure 2A). Clindamycin (lincosamide) interacts with the A- and P-sites, mainly through hydrogen bonds with the nucleotides of the 23S rRNA, and sterically blocks the progression of the nascent peptide towards the tunnel (Figure 2B) [29]. Schlünzen *et al.* showed the antimicrobial activity of the pleuromutilins at the molecular level, using tiamulin as a representative. Tiamulin was found to bind strongly in a cavity at the PTC, binding to the 23S rRNA through hydrophobic interactions involving exclusively nucleotides of domain V (Figure 2C) [30]. Streptogramins A act to prevent protein biosynthesis by interfering with substrate binding at the PTC, and thus blocking peptide bond formation. Dalfopristin, a representative of this class, is located in a tight pocket within the PTC, bound by several hydrophobic interactions as shown in Figure 2D [31]. Linezolid (oxazolidinone) binds in the A-site of the peptidyl transferase center of the ribosome and interacts with many 23S rRNA nucleotides in the neighborhood (Figure 2E) [32].

If only one methyltransferase confers resistance to six classes of antimicrobial agents, then it is necessary to evaluate the *cfr* gene dissemination, to try to define how harmful it is and how to fight its spread. Otherwise many of these antibiotics of clinical importance will become useless.

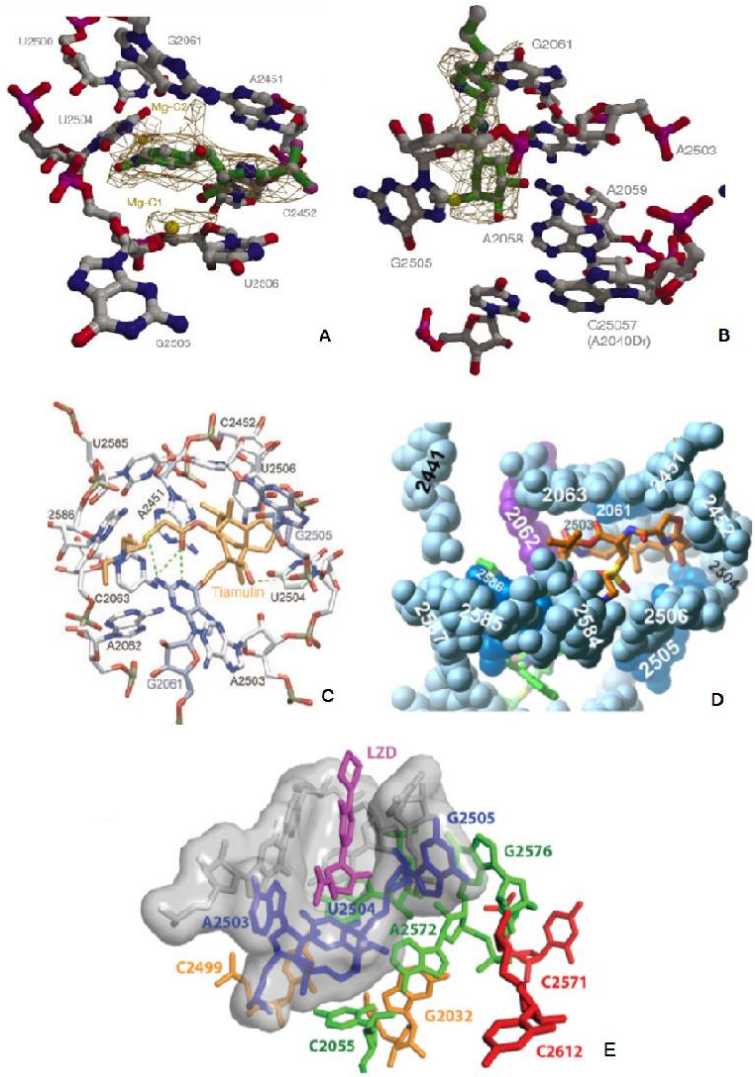


Figure 2: Local environment of the antibiotic and its interaction with the peptidyl transferase cavity. Nucleotides interacting with (A) chloramphenicol (shown in green), (B) clindamycin (shown in green), (C) tiamulin (shown in orange), (D) dalfopristin (shown in orange) and (E) linezolid (shown in purple). Nucleotide numbering is according to the *E. coli* sequence [24-27].

1.2.5. Dissemination of the *cfr* gene

The *cfr* gene only with minor sequence differences has been found on mobile genetic elements in different *Staphylococcus* isolates of animal and human origin, in different locations, such as Germany, Denmark and the United States [33-35]. In fact, the *cfr* gene has been found worldwide and in different organisms. In China, for example, the gene was detected in plasmids in *Bacillus* strains [36] and in the commensal bacteria *Macrococcus caseolyticus* and *Jeotgalicoccus pinnipedialis* [37], in novel plasmid types from five different species of staphylococci [38], in an *Enterococcus faecalis* isolate [39] and in three transferable plasmids obtained from other *Enterococcus* species [40]. Although in the beginning the gene was mainly found in animal sources,

it started to be frequently detected in clinical environments, in places like Italy [41, 42], Spain [43, 44], Ireland [45] and Mexico [46], in many medical centres from the United States [47, 48], in Thailand [49] and in a clinical methicillin-resistant *Staphylococcus aureus* (MRSA) strain from Colombia, showing the capacity of the gene to disseminate among Gram-positive and other pathogenic strains [50]. Recently, another MRSA and a methicillin-resistant *Staphylococcus haemolyticus* strain were identified in a hospital in Spain [51]. Moreover, the *cfr* gene was also identified in naturally occurring Gram-negative bacteria: *Proteus vulgaris* [52] and *Escherichia coli* [53, 54].

These studies indicate how dispersed is the gene, which can be explained by the low fitness cost associated with gene acquisition and expression. LaMarre *et al.* investigated the fitness cost of *cfr* expression and they were able to conclude that acquisition of the *cfr* gene does not significantly reduce the cell growth rate. Thus, genes like *cfr* that come at a low cost can stably persist in the cells [55]. Furthermore, the *cfr* gene is found on plasmids or transposons (mobile genetic elements), allowing horizontal gene transfer. Also, the use of the already referred drugs can promote the maintenance of this gene in the population. All these findings demonstrate how worrying the *cfr* gene can be.

1.2.6. Cfr-like proteins found in various bacteria

The *cfr* gene aroused the attention of many scientists. Given its dissemination worldwide, the next step was clearly to find similar genes encoding Cfr-like proteins and assess if they could confer resistance in a similar way as the Cfr methyltransferase. Through bioinformatics searches, a phylogenetic tree comprising predicted Cfr-like sequences was constructed. Three Cfr-like proteins from the order *Bacillales* (*Bacillus amyloliquefaciens*, *Bacillus clausii* and *Brevibacillus brevis*) were present [56], as well as genes from *Enterococcus*, *Paenibacillus*, and *Clostridium*, which led to the assumption that this group of Cfr-like proteins could contain real Cfr enzymes [57].

The three genes from the order *Bacillales* were considered *cfr*-like because when expressed in *E. coli* they were able to decrease the susceptibility to the five classes of antibiotics in the $PhLOPS_A$ phenotype (florfenicol, clindamycin, linezolid, tiamulin, and streptogramin A/streptogramin B were used as representatives). Likewise, they verified the RNA methylation at A2503 in 23S rRNA by primer extension, to prove that the three Cfr-like proteins were acting through the same mechanism to confer the tested resistance [56]. Similarly, genes from *Paenibacillus* and *Clostridium* were cloned and expressed in *E. coli*. Induced expression was investigated by SDS gel analysis showing high levels of expression and MICs (minimum inhibitory concentrations) were determined

with five antibiotics from the $PhLOPS_A$ phenotype (florfenicol, clindamycin, linezolid, tiamulin and quinupristin-dalfopristin): ClPa (Cfr-like from *Paenibacillus*) conferred some resistance although less effective than the original Cfr methyltransferase (from *S. sciuri*); ClCs (Cfr-like from *Clostridium*) apparently did not mediate any changes in the MICs. The relationship between modification at A2503 in 23S rRNA and phenotype was also checked by primer extension. The results showed a clear stop at the position of interest in the RNA from ClPa (m^8A2503 checked by mass spectrometric analysis), but no stop in the case of ClCs (in accordance with the MICs). The possible conclusion was that ClCs does not methylate 23S RNA from *E. coli*, but may be able to methylate *Clostridium* 23S RNA, due to sequence differences in the ribosome assembly process or in RNA and/or r-proteins [57]. These findings require further studies in order to try to establish if the *cfr*-like gene in *Clostridium* act as the Cfr methyltransferase and provide antibiotic resistance.

1.3. *Clostridium*: the focus of the study

Sequence alignments have shown that some *Clostridium* strains contain *cfr*-like genes, such as several *C. botulinum* and *C. acidurici*, *C. sporogenes*, *C. phytofermentans* and *C. difficile* [57]. The genus *Clostridium* includes diverse bacteria of medical and environmental importance, and mainly consists of Gram-positive, spore-forming, anaerobe rods, comprising toxin-producing species, such as *C. botulinum* and *C. difficile*, and also non-pathogens like *C. sporogenes* [58]. As several *C. botulinum* strains were suggested as *cfr*-like gene carriers, this species could represent an important subject of study regarding *cfr*-like genes. However, due to its pathogenic nature, working with *C. botulinum* requires Biosafety Levels for Laboratories – class 2 or 3 (BSL-2 and BSL-3) [59]. Thus, *C. sporogenes*, a similar species but non-pathogenic (in the sequence alignments mentioned above represented by two different strains), seemed to be the best species to work with.

1.3.1. *Clostridium sporogenes*: the selected species

Phylogenetic analysis suggested that *Clostridium sporogenes* is closely related to *C. botulinum*, and thus it has been used as a non-toxigenic surrogate for proteolytic *C. botulinum* strains (because of the morphological similarities) in the derivation and validation of thermal processes in food [60, 61]. Two *C. sporogenes* strains were suggested as carriers of a *cfr*-like gene and the *cfr*-like gene of one of these strains was cloned and expressed in *E. coli* to investigate resistance to the $PhLOPS_A$ antibiotics [57]. As mentioned above, ClCs (Cfr-like from *Clostridium*) protein expressed in *E. coli* apparently did not mediate resistance to the tested antibiotics, which

calls for further studies. The obvious choice is thus to use *C. sporogenes* PA 3679 and *C. sporogenes* ATCC 15579 [57] to investigate how the Cfr-like protein function in *Clostridium*.

1.4. Aim of the study

Previous experiments of cloning and plasmid expression of the *cfr*-like gene from *Clostridium sporogenes* ATCC 15579 (*clcs* gene) in *E. coli* showed protein expression but no effect on antibiotic resistance and no modification of A2503 in ribosomal 23S RNA. In the first part of this study, the expression of the *clcs* gene in *E. coli* will be investigated by studying the strength of the induction of the gene as well as the effect of a lower constitutive expression of the gene by transfer it into another plasmid. The lack of function of the expressed protein will also be investigated by combining parts of the *cfr* gene with parts of the *clcs* gene to try to identify the differences and thereby determine why the *Clostridium cfr*-like gene does not function in *E. coli*.

Furthermore, the question raised by Atkinson *et al.* [57] about the ability of the *clcs* gene to methylate its own host, the *Clostridium* 23S RNA, remained unanswered. So, the second part of the project will be the investigation of the function of the *clcs* gene in two *Clostridium sporogenes* strains to establish if the *cfr*-like genes in *Clostridia* act as Cfr and provide antibiotic resistance. *Clostridium sporogenes* will be obtained and established, as this is a new organism in the laboratory. Growth conditions for obtaining an exponential growing culture, procedures for RNA and DNA isolation will be developed. After RNA purification, primer extension will be performed to see if there is an indication of modification (a primer extension stop) at A2503 23S RNA. Moreover, a fragment around A2503 has to be isolated and investigated by mass spectrometry to determine whether the strain has m^8A2503 methylation in its 23S ribosomal RNA. The antibiotic resistance pattern of the *Clostridium sporogenes* strains will also be investigated.

2. Results and Discussion

2.1. Part I – Cloning of *cfr* and *cfr*-like genes in *E. coli*

2.1.1. Preliminary analysis of growth after induction of *cfr*-like genes

In 2012, Hansen *et al*. identified three *cfr*-like genes from the order *Bacillales*. These genes were cloned into plasmids under the control of an inducible promoter and transformed into *E. coli* AS19 [62]. The expression of the *cfr*-like genes was induced and SDS gel analysis showed expression of the Cfr-like proteins [56]. However, a previous study performed in the "*BV lab*" indicated poor growth after induction of the *clcs* gene – a *cfr*-like gene from *Clostridium sporogenes* [57]. The apparent growth inhibition of *E. coli* caused by expression of the *clcs* gene was therefore investigated in the beginning of this study. Growth curves with induced plasmids were performed and *E. coli* AS19 cells expressing pClCs (a derivative of the plasmid pLJ102 carrying the *clcs* gene) seemed to be impaired following the induction of the ClCs protein with 1mM IPTG, the amount shown to be needed for the expression of the protein. Simultaneously, the growth of cells that were not induced with IPTG or induced with less IPTG (0.2 mM) was not affected (Figure 3).

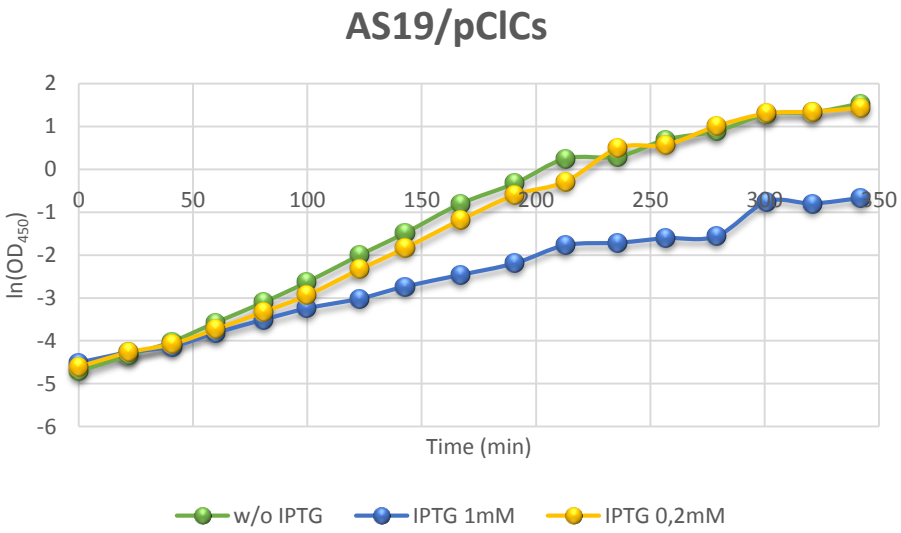


Figure 3: The green line shows the growth of *E. coli* AS19/pClCs cells that have not been induced with IPTG. The blue and yellow lines show the growth of cells that have been induced with 1 mM IPTG and 0.2 mM IPTG, respectively, and thus, express ClCs.

Based on the growth curves made, a clear difference in growth was observed between cells that carry pClCs and have been induced with 1mM IPTG compared to those that have not been

induced with IPTG (and hence, do not express the protein) or with 0.2mM IPTG. These growth curves indicated that the expression of the ClCs protein somehow impairs the growth of these cells.

Since the strength of the *clcs* gene induction was investigated and the inducible plasmid seemed to hamper the study of the protein function, *clcs* gene was transferred into another plasmid. The new plasmid should then allow a constitutive expression of the gene, avoiding the need of induction. Thus, the vector chosen was pBR322 [63]. Three new constructs were made to obtain plasmids expressing constitutively the *clcs, cfr* and *scfr* genes: the plasmid pCfrHisN [21], containing the wild-type *cfr* gene cloned into the NdeI and HindIII sites of plasmid pLJ102 [64] was provided by *"BV group"*; the synthetic *scfr* and *clcs* genes were designed to be codon optimized for *E. coli*. The *scfr* gene was designed to contain AvrII and XhoI restriction sites, and *clcs* gene to contain HindIII and NdeI restriction sites at the ends, but it was already cloned into the plasmid pLJ102 for previous experiment in the *"BV lab"*. The genes were inserted into plasmid pBR322 replacing the *tet* gene. The vector part of pBR322 with the *amp* gene was constructed by PCR to contain AvrII-CCTAGG and XhoI-CTCGAG restriction sites near the ends. The *clcs* and *cfr* genes were also amplified by PCR and were constructed to contain AvrII-CCTAGG and XhoI-CTCGAG restriction sites (the same sites as the vector) near the ends. The *scfr* gene already contained AvrII and XhoI restriction sites at its ends. The new plasmids were named pBRClCs, pBRCfr and pBRsCfr, respectively.

New growth curves were performed using *E. coli* AS19 cells expressing pBRClCs, pBRCfr and pBRsCfr. Figure 4 shows that the growth of the tested strains was not inhibited. As presented in the figure, *E. coli* AS19 cells harbouring the plasmid pBR322 grew at the same rate as *E. coli* AS19 cells without plasmid, meaning that carrying the plasmid has no effect on *E. coli* AS19 growth. Similarly, the cells harbouring the plasmids that carry the genes of interest, pBRsCfr and pBRClCs, are growing equally fast. Considering this result, changing the plasmid to obtain a constitutive expression of the genes was considered the best solution to obtain a growth similar to the wild-type *E. coli* AS19.

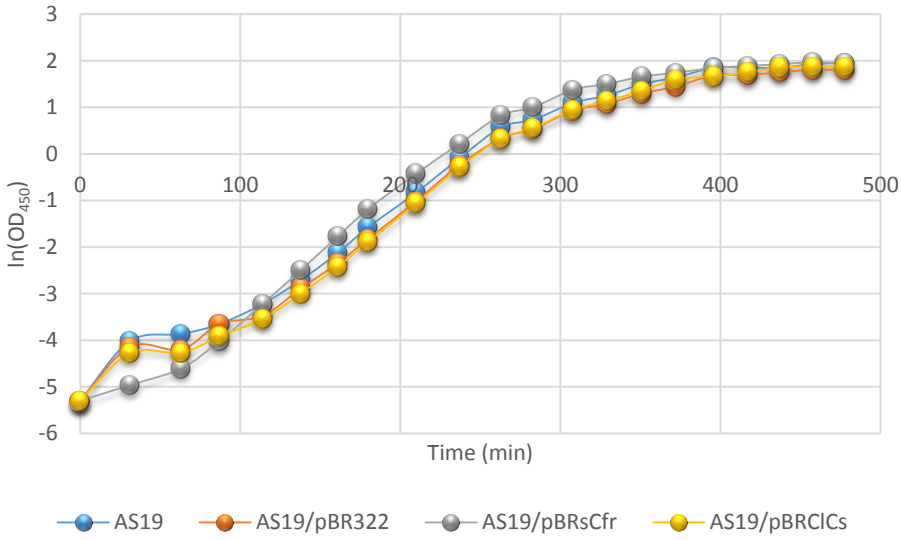


Figure 4: The blue line shows the growth of *E. coli* AS19 cells with no plasmid. The orange, grey and yellow lines follow the growth of *E. coli* AS19 cells harboring the plasmids pBR322, pBRsCfr and pBRClCs, respectively.

2.1.1. Antibiotic susceptibilities of the strains expressing Cfr and Cfr-like proteins

Recent studies demonstrated the antibiotic susceptibilities of strains expressing the wild-type Cfr and Cfr-like proteins, namely the three Cfr-like proteins from the order *Bacillales* and the ClCs from *Clostridium sporogenes* [56, 57]. However, the genes encoding for these proteins were cloned into an inducible plasmid. Since the genes encoding for sCfr and ClCs proteins were cloned into pBR322, it was necessary to perform a Minimum Inhibitory Concentration (MIC) analysis to evaluate whether the inserted *cfr* and *cfr*-like genes confer changes in the antibiotic susceptibility and to establish if the Cfr-like protein ClCs confers a resistance pattern similar to that of the Cfr methyltransferase. Thus, the MICs were conducted including controls with Cfr-positive and Cfr-negative *E. coli* AS19 [62], a hyperpermeable strain used to emphasize the observed effects because it is much more sensitive to antibiotics than other *E. coli* strains that have a relatively low susceptibility to many drugs.

The strains *E. coli* AS19/pBRCfr, AS19/pBRsCfr and AS19/pBRClCs together with the control strains without plasmid, the parent pBR322 plasmid and the pBglII plasmid that constitutively expresses Cfr [17] were exposed to three antibiotics at various concentrations: tiamulin, chloramphenicol and linezolid. These antibiotics represent three out of the five antibiotic classes in the $PhLOPS_A$ phenotype conferred by the Cfr methyltransferase and thus represent a good initial indicator of the phenotype [27]. The minimum inhibitory concentration for each strain was defined as the drug concentration at which the growth of the cultures was completely inhibited after 24 h

of incubation at 37°C. The ODs of the growth in media plus 20% were used as cut-off value. The MIC results are shown in Table 2.

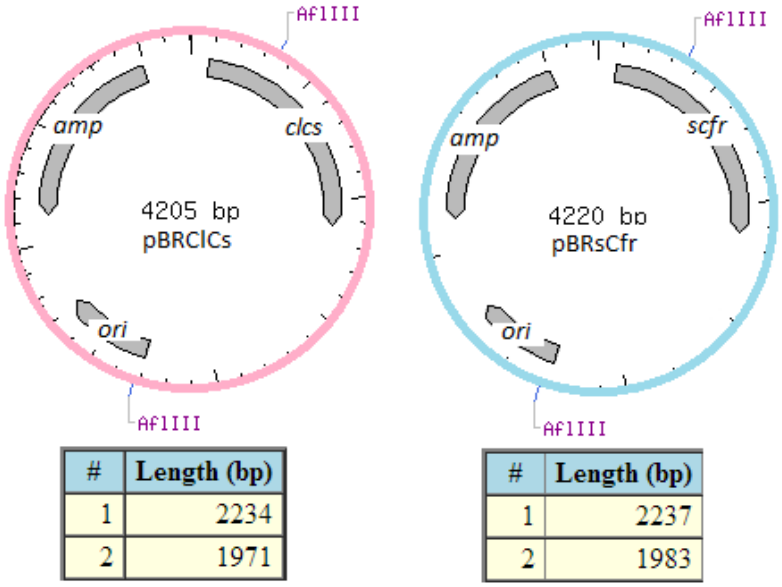
Table 2: Comparison of antimicrobial susceptibilities to 3 antibiotics in the presence or absence of the Cfr or Cfr-like methyltransferases in *E. coli* AS19.

E. coli AS19 strain/plasmid	*cfr*/*cfr*-like gene	MIC (µg/mL)		
		Tiamulin	Chloramphenicol	Linezolid
none	-	1	1	8
pBRCfr	+	4	2	16
pBRsCfr	+	>32	8	>64
pBRClCs	+	1	1	8
pBR322	-	1	1	8
pBglII	+	32	4	32
pBRsCAC	+	2	2	---
pBRCAsC	+	2	1	---

As expected, sCfr confered resistance to the tested antibiotics being as effective as the positive control pBglII and lowering the sensitivity to all three antibiotics. In contrast, Cfr showed lower effectiveness than sCfr, probably because it is not codon optimized for *E. coli*, which resulted in a minor protein expression. As far as ClCs is concerned, it does not mediate MIC changes (MICs comparable to the negative controls). According to the results published by Atkinson *et al.* [57], where the inducible plasmid pLJ102 was used to clone *cfr* and *clcs* genes, the effect of the sCfr and ClCs proteins on tiamulin and linezolid MICs is similar to the effect observed here for the same antibiotics. Hence, the constitutive expression of the proteins did not change its effect in *E. coli* AS19 cells and the results obtained are reliable, showing once again the inability of ClCs to confer resistance.

ClCs is not fully characterized yet and few information about its function and amino acids sequence is available. Therefore, the lack of function of the expressed protein was also investigated by combining parts of the *scfr* and *clcs* genes to try to identify any differences. The genes *scfr* and *clcs* have a common restriction site (A$^{\blacktriangledown}$CRYG$_{\blacktriangle}$T), also present in the vector pBR322. Hence, starting from the plasmids pBRsCfr and pBRClCs cut with the double cutter restriction enzyme AflIII (Figure 5), two other plasmids were obtained: pBRsCfrAflIIIClCs (initial part of *scfr* and final part of *clcs*) and pBRClCsAflIIIsCfr (initial part of *clcs* and final part of *scfr*). They were named pBRsCAC and pBRCAsC,

respectively (Figure 6). *E. coli* AS19 strains harboring these constructs were only tested with tiamulin and chloramphenicol and the MIC results (Table 2) showed no ability of the sCAC and CAsC to lower the susceptibility of the cells, as already observed for the ClCs and negative controls. The combination of parts of *scfr* and *clcs* genes was an attempt to understand the lack of function of the ClCs and to try to define if parts of ClCs could be functioning as the corresponding areas of Cfr or to define which part prevents the function. However, the results did not allow us to reach a conclusion other than the combinations made did not produce a *cfr*-like effect and did not reduce the antibiotic susceptibility. Thus, further studies are needed where more combinations should be tried.



#	Length (bp)
1	2234
2	1971

#	Length (bp)
1	2237
2	1983

Figure 5: Plasmids pBRClCs and pBRsCfr showing the restriction sites where AflIII cuts producing two fragments in each plasmid.

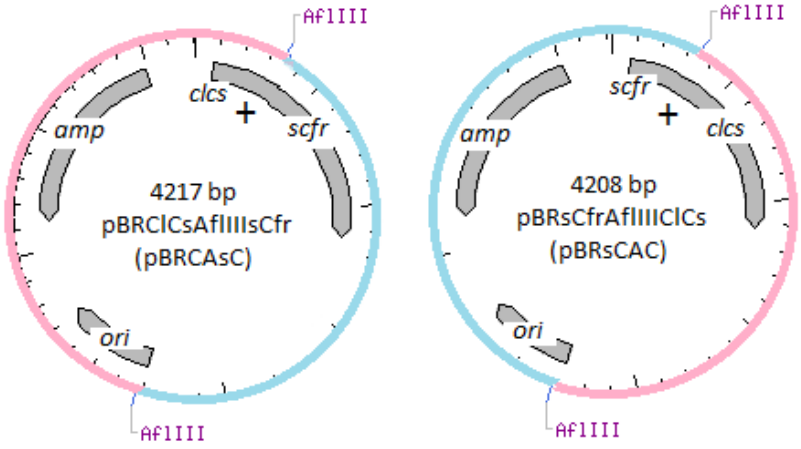


Figure 6: Plasmids constructed from pBRClCs and pBRsCfr. pBRsCfrAflIIIClCs (pBRsCAC) contains the first part of scfr and second part of clcs, and pBRClCsAflIIIsCfr (pBRCAsC) comprises the first part of clcs and second part of scfr.

2.1.2. Verification of *cfr* and *cfr*-like genes expression by SDS gel analysis and protein identification by mass spectrometry

As mentioned above, the *cfr* and *cfr*-like genes were cloned into an inducible plasmid and the expression of these genes was induced by addition of IPTG and then investigated by SDS gel analysis of total protein from the used strains. The MICs revealed how Cfr and ClCs proteins affected the cells expressing them, but the expression level of Cfr and Cfr-like proteins in the expression system used (proteins are constitutively expressed and does not require induction) also had to be investigated.

As shown in Figure 7, no protein bands were detected with the expression of sCfr and ClCs at the expected masses (sCfr, 39.86 kDa and ClCs 39.18 kDa). Based on the MIC results, expression of sCfr and positive control was expected, or at least visible differences in the bands' intensities. The plasmid pBglII expresses the Cfr constitutively and was used both in previous studies and this study, so similar results were expected. Hansen *et al.* [56] could not correlate quantitatively the expression level of each protein and the MIC effects, but a good expression of the Cfr-like proteins was always observed. As far as ClCs is concerned, strong protein band appeared with its expression [57]. The SDS gel analysis did not show the expression of the proteins, which was not expected taking into account the MIC results and previous results with the same positive control, pBglII.

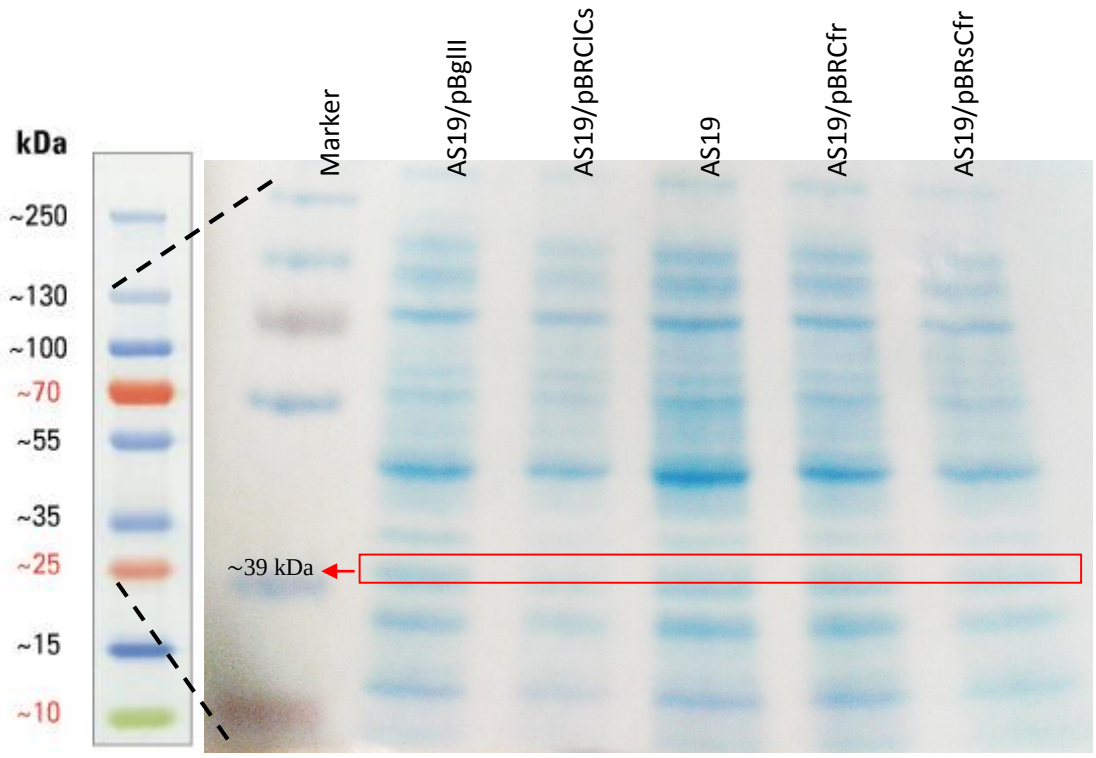


Figure 7: Analysis of cell extracts by SDS-PAGE, to verify expression of Cfr and Cfr-like proteins. The cell extracts are from E. coli AS19 cells alone and harboring the plasmids pBglII, pBRClCs, pBRCfr and pBRsCfr, as indicated above the gel. The marker indicates size markers at 130, 100, 70, 55, 35 and 25 kDa (from the top). The red box indicates the presumable expression of Cfr or Cfr-like proteins.

In order to detect the non-visible proteins, protein identification by mass spectrometry was performed. A thicker and bigger SDS gel was prepared to obtain higher amounts of the proteins and the presumed bands were cut to be investigated by mass spectrometry (Figure 8). The analysis revealed that all samples, including the negative control, had the same protein, which was in fact a Glyceraldehyde-3-phosphate dehydrogenase with a mass of almost 36 kDa (Figure 9). In the "normal" SDS gel, the assumed correct bands were slightly above the 35 kDa, corresponding to the area of the ~39 kDa. Some differences in the intensity were detected but they were not significant enough to reach a conclusion. However, the mentioned region was identified as the region of interest. Comparisons were made between the "normal" and the "adapted" gels, bands and blank spaces were analyzed. The region showed in Figure 8 was considered the corresponding one to the previously identified in the "normal" gel. The bands in this region were cut, digested with proteases and further analysed in the mass spectrometer. Results indicated that the analyzed bands were not the correct ones. Given the results, protein expression should be further analyzed because there is no visible reason or cause for inhibition or no expression. One possible direction would be performing a *Western Blot* analysis of the Cfr and Cfr-like proteins since it allows the detection of a specific protein. Though, this technique requires primary antibodies against the protein of interest, which are not available since the protein was never purified. Some companies can design the intended antibody but the process is not cheap and is time consuming.

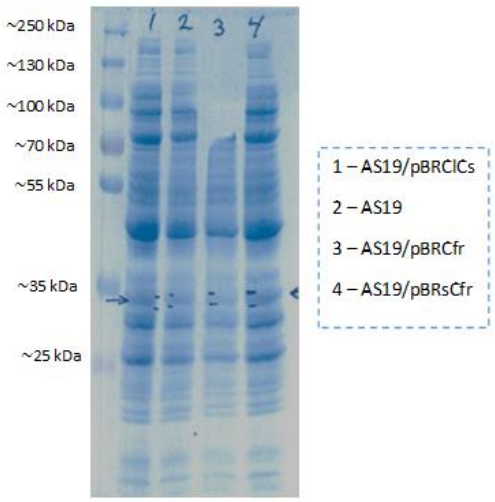


Figure 8: SDS gel to obtain higher amounts of the proteins for further mass spectrometry analysis. The same *E. coli* AS19 cell extracts were used as in the "normal" SDS gel (except pBglII). The marker indicated in the picture as M, indicates size markers at 250, 130, 100, 70, 55, 35 and 25 kDa (from the top). The arrows indicate the bands cut to be analyzed by mass spectrometry.

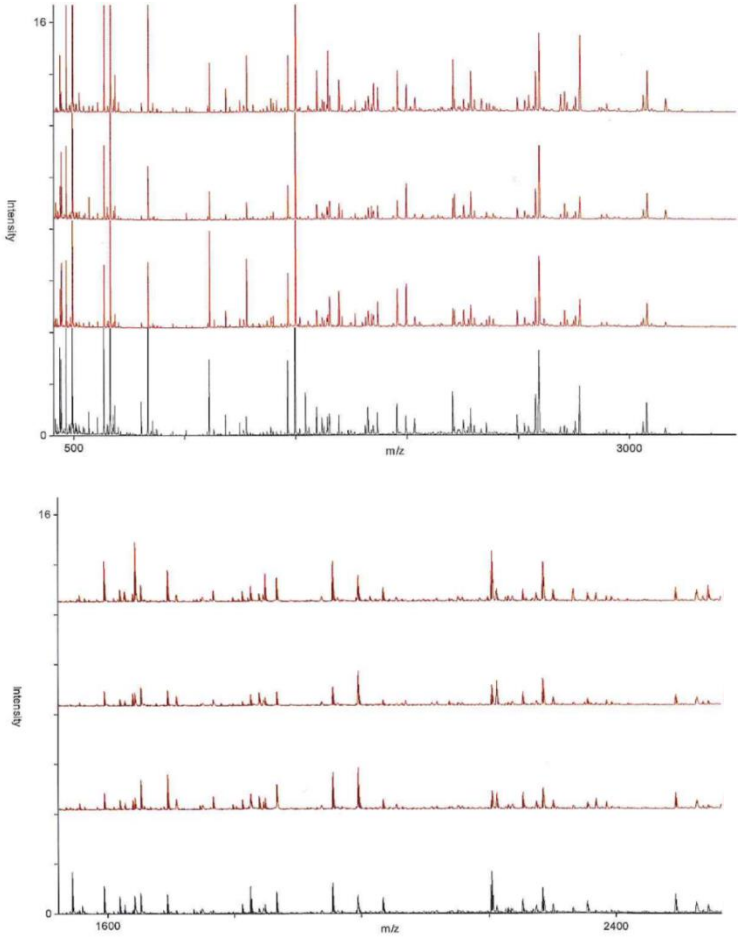


Figure 9: Resulting spectra from protein mass spectrometry of the bands excised from the SDS gel. The spectra show that all bands contain the same proteins.

2.1.3. Primer extension analysis to verify modification at A2503 23S rRNA

Although the lack of resistance to the three antibiotics is an indicator that ClCs and the combined proteins sCAC and CAsC are not acting as the Cfr methyltransferase, the verification of the presence or absence of the RNA methylation at A2503 in 23S rRNA was checked by primer extension. The plasmids pBRCfr, pBRsCfr, pBRClCs, pBglII, pBRsCAC and pBRCAsC were transformed into the ΔRlmN strain *E. coli* JW2501-1 [65] once it facilitates identification of Cfr methylation by avoiding interference from the RlmN m^2A2503 methylation. Total RNA was purified and subjected to primer extension by reverse transcriptase. A fluorescently labeled oligonucleotide (Cy5-labeled, 5'–GAACAGCCATACCCTTG–3') was annealed to the desired region (nucleotides 2540 to 2556 of *E. coli* 23S ribossomal RNA) and extended until stopped by structures in RNA or modifications, or by breakage of RNA caused by enzymatic degradation or simple water hydrolysis. An RNA modification that interferes with base pairing will stop or pause reverse transcriptase one nucleotide before the modified base, which is expected to occur in case of methylation of the position A2503. The

resulting cDNA extension products were separated on 6% polyacrylamide sequencing gel alongside dideoxy sequencing reactions.

The analysis is presented in Figure 10A and shows a clear stop at A2503 of 23S RNA from sCfr (Cfr from pBglII also mediates a strong stop), and a weaker stop in the case of Cfr. These observations are consistent with the resistance observed in the MIC experiment. As expected, the negative control *E. coli* JW2501-1 does not give rise to any stop at the A2503 position. There is also no stop in the ClCs-containing strain, consistent with no observed MIC changes, revealing that Cfr-like from *Clostridium sporogenes* does not methylate *E. coli* 23S RNA. Figure 10A also shows a reverse transcriptase stop at position C2498, caused by the 2'-O-ribose methylation [17]. This stop is decreased in the case of pBRsCfr and pBglII, meaning that sCfr and Cfr from pBglII reduce the amount of Cm2498 in ribosomes as reported [17]. On the other hand, pBRClCs does not affect the stop at this position. ClCs does not modify A2503 and consequently does not affect the nucleotide C2498. Figure 10B shows the primer extension of the plasmids carrying the combined genes, psCAC and pCAsC. Due to the acquisition of a new device (fluorescence scan) used for the visualization of the gels, the picture obtained does not allow the reading of the sequence. However, some of the plasmids showed in Figure 10A are also present in Figure 10B, allowing the comparison. Thus, it is possible to recognize the stops at A2503 mediated by sCfr and Cfr from pBglII, and no stops in the same region are observed in the case of the combined proteins.

It is not possible to conclude that ClCs does not have a Cfr-like function. All in all, this protein may be able to methylate *Clostridium* 23S RNA but not *E. coli* 23S RNA, because of some differences in RNA sequence or r-proteins, or in the ribosome assembly process. Further studies are needed to investigate if ClCs play a similar role in its natural host.

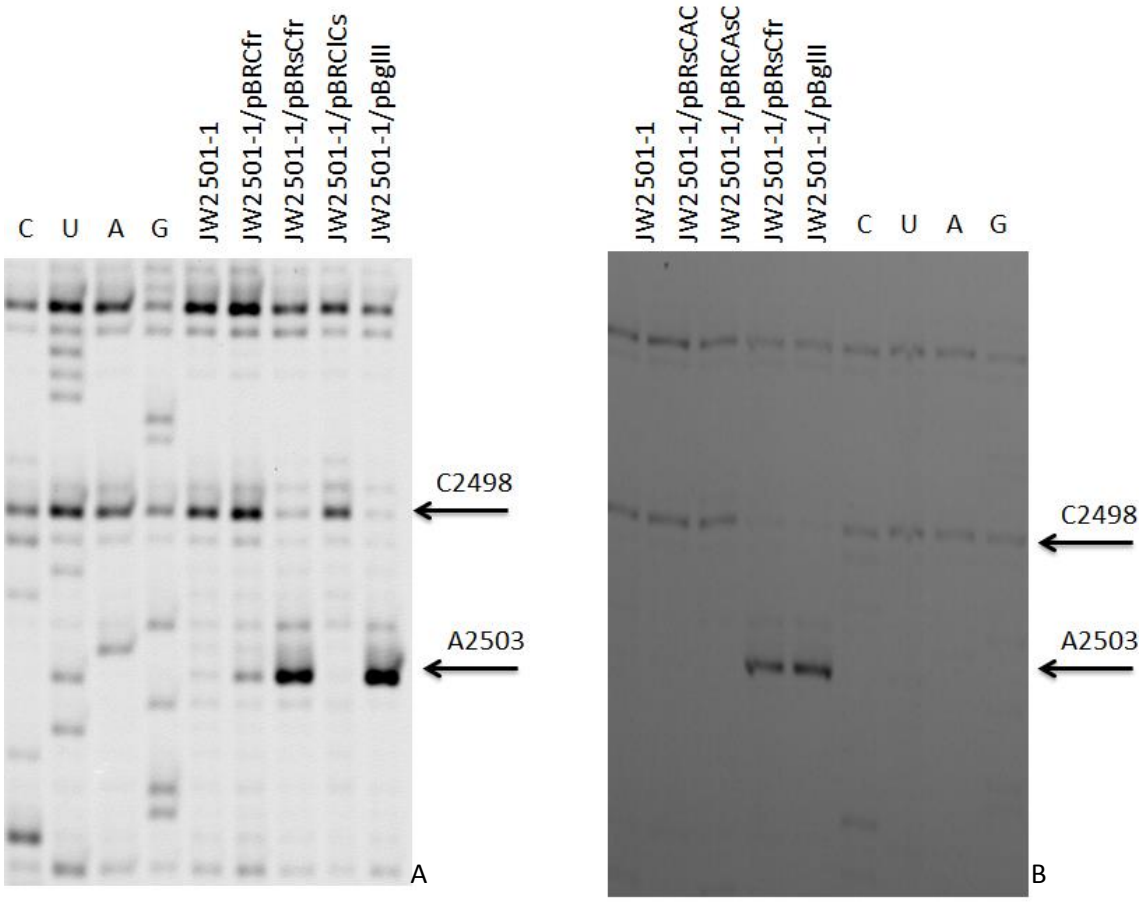


Figure 10: Primer extension analysis made on a 6% acrylamide gel with urea. The picture presented here is limited to the nucleotides flanking A2503 and shows the stops on 23S rRNA from *E. coli* JW2501-1 strain alone and harboring the plasmids (A) pBRCfr, pBRsCfr, pBRClCs and pBgIII and (B) pBRsCAC, pBRCAsC, pBRsCfr and pBgIII. The first four lanes in (A) and last four in (B) (C, U, A and G) correspond to the sequencing reactions. The nucleotides of interest (C2498 and A2503) are pointed by the arrows (one position below the sequencing position, once the extension stops before the modified nucleotide).

2.2. Part II – Investigation of the function of the *Clostridium cfr*-like gene in *C. sporogenes* PA 3679 and *C. sporogenes* ATCC 15579

The cloning and expression of the *cfr*-like gene from *Clostridium sporogenes* (*clcs*) in *E. coli* showed that the ClCs protein does not confer resistance to the $PhLOPS_A$ antibiotics tested and does not methylate the position A2503 in *E. coli* 23S rRNA. Thus, the main purpose of the second part of the study was to establish if the *cfr*-like gene in *Clostridium* could act as Cfr and provide antibiotic resistance and also verify whether the strains *C. sporogenes* PA 3679 and *C. sporogenes* ATCC 15579 had a methylation at A2503 in their 23S ribosomal RNA.

2.2.1. Confirmation of the identity of the strains

Clostridium sporogenes PA 3679 and *C. sporogenes* ATCC 15579 were newly obtained strains in the laboratory and no previous knowledge about their growth conditions was available, which required some prior preparation. The strains were then grown at 37°C ([66], [67] and ATCC recommendation) and due to the anaerobic atmosphere required for the growth of these *Clostridium* strains, Hungate-tubes [68] were used since they allow the removal of the oxygen present in the broth, by the addition of nitrogen, in order to obtain an anaerobic gas mixture of approximately 80% N_2, 10% O_2 and 10% H_2, as recommended by the suppliers of the strains. To incubate the strains after plating, an anaerobic jar with a sachet of Anaerocult A or AnaeroGen was used. These sachets contain components, which chemically bind oxygen quickly and completely, creating an oxygen-free (anaerobic) environment – Figure11.

To verify the identity of the strains, PCR reactions were performed to amplify the 16S ribosomal RNA (Table 3) and then the PCR products were sequenced and analysed using nucleotide blast (http://blast.ncbi.nlm.nih.gov/) in order to determine the genus of the strains. In both situations the identity of the strain was confirmed.

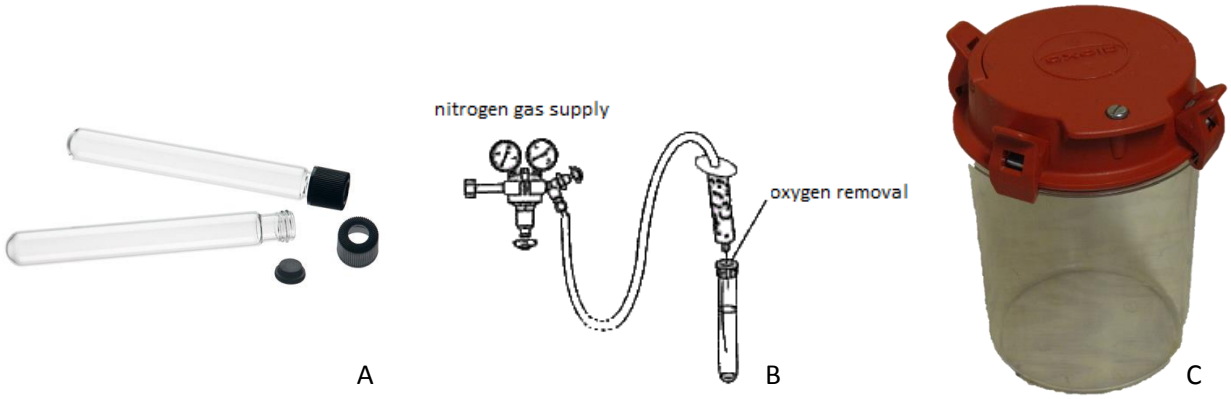


Figure 11: (A) Hungate-tubes used to maintain the anaerobic conditions. (B) Addition of nitrogen gas into the Hungate-tubes causes oxygen removal. (C) Anaerobic jars used to incubate the strains after plating.

2.2.2. Amplification and sequencing of the *cfr*-like gene

Likewise, the *cfr*-like gene had to be amplified, sequenced and analysed as done for the 16S rRNA. We first started with *C. sporogenes* PA 3679, and many trials were made in order to amplify the *cfr*-like gene, as shown in Table 3. Also, gradient PCR with some of the primers was performed in an attempt to find the proper annealing temperature, if that was the cause of the negative results. Still, no gene presence could be detected. Thus, some combinations of the primers were made such as BV472 (forward) and BV471 (reverse), BV470 (forward) and BV473 (reverse), BV477

(forward) and BV471 (reverse), and BV470 (forward) and BV478 (reverse), but again it was not possible to amplify the gene.

Table 3: Primers and PCR conditions used to isolate 16S rRNA and cfr-*like* gene from *Clostridium sporogenes* PA 3679 and *C. sporogenes* ATCC 15579. Note: The annealing temperature for the primers was calculated using NEB Calculator, according to the manufacturer's instructions.

		Primers (5' → 3')	PCR conditions
Both strains	16S rRNA	BV468 (forward) GTGAAATGCGTAGAGATTAGGAA BV469 (reverse) GATTCGCGATTACTAGCAACTC	Initial denaturation (98°C): 1 min; 30 cycles, denaturation (98°C): 10 sec, annealing (64°C): 30 sec, extension (72°C): 20 sec and final extension (72°C): 10 min
***C. sporogenes* PA 3679**	*cfr*-like gene (primers flanking the gene)	BV470 (forward) AGGTTCGAGCAAAGGCTATTTCA BV471 (reverse) GATGTTTTGGGCGACGCTTG	Initial denaturation (98°C): 1 min; 30 cycles, denaturation (98°C): 10 sec, annealing (67°C): 30 sec, extension (72°C): 38 sec and final extension (72°C): 10 min
	cfr-like gene (primers flanking the gene)	BV472 (forward) GTAGCCCATTATGCTCCCTCC BV473 (reverse) CAATAGGCTCCATGCAAAGCC	Initial denaturation (98°C): 1 min; 30 cycles, denaturation (98°C): 10 sec, annealing (69°C): 30 sec, extension (72°C): 36 sec and final extension (72°C): 10 min
	cfr-like gene (primers flanking the gene)	BV477 (forward) TACTCCACGTTGAGCACACA BV478 (reverse) CTTTCCAGCTCGGCAAAACG	Initial denaturation (98°C): 1 min; 30 cycles, denaturation (98°C): 10 sec, annealing (63°C): 30 sec, extension (72°C): 72 sec and final extension (72°C): 10 min
	cfr-like gene (primers within the gene)	BV480 (forward) CCGGATTTGCAAAAGCCTCA BV481 (reverse) AAACAGGGGTGGGAATCGTT	Initial denaturation (98°C): 30 sec; 30 cycles, denaturation (98°C): 10 sec, annealing (67°C): 30 sec, extension (72°C): 10 sec and final extension (72°C): 2 min
	Upstream *cfr*-like gene (gene encoding ATP-binding protein)	BV479 (forward) TCACAAAATGCACTATCATGCTCAA BV478 (reverse) CTTTCCAGCTCGGCAAAACG	Initial denaturation (98°C): 30 sec; 30 cycles, denaturation (98°C): 10 sec, annealing (66°C): 30 sec, extension (72°C): 23 sec and final extension (72°C): 2 min
	Downstream *cfr*-like gene	BV482 (forward) GGACTACGCTATGCTCGGTT BV471 (reverse) GATGTTTTGGGCGACGCTTG	Initial denaturation (98°C): 30 sec; 30 cycles, denaturation (98°C): 10 sec, annealing (68°C): 30 sec, extension (72°C): 84 sec and final extension (72°C): 2 min
***C. sporogenes* ATCC 15579**	*cfr*-like gene (primers flanking the gene)	BV473 (forward) CAATAGGCTCCATGCAAAGCC BV472 (reverse) GTAGCCCATTATGCTCCCTCC	Initial denaturation (98°C): 30 sec; 30 cycles, denaturation (98°C): 10 sec, annealing (68°C): 30 sec, extension (72°C): 52 sec and final extension (72°C): 2 min

At this point, it is required to refer that the *C. sporogenes* PA 3679 sequence is in the databases but divided in more than a hundred contigs, and the *cfr*-like gene belongs to one small contig, together with two other genes (one gene encoding for an ATP-binding protein and other encoding for a hypothetical protein) – Figure 12. The division of the sequence in so many parts may be more likely to contain mistakes, as well as the impossibility to amplify the gene may indicate some problem with the contig containing the *cfr*-like gene, or even that the presence of a *cfr*-like gene may not be as certain as primarily assumed. Thus, two pairs of primers were designed to isolate the genes upstream and downstream the *cfr*-like gene (both genes are in the same contig). Once again, it was not possible to amplify any of the genes. Given the absence of results, DNA degradation was considered to be the problem. A new kit for DNA extraction "DNeasy and Blood Tissue Kit (Quiagen) – Pretreatment for Gram Positive Bacteria" was then obtained. However, despite no degraded DNA, the amplification of the genes was still not possible. As a last try, a new pair of primers complementary to an essential gene (DNA polymerase III) placed in a different contig of the *cfr*-like gene was designed. This PCR amplified the desired gene showing that the chromosomal DNA was in good condition. A possible explanation is the absence of the *cfr*-like gene in the genomic DNA and maybe it could be placed on a plasmid. So, we tried to isolate potential plasmids and amplify the gene, but no PCR products were obtained. The only likely conclusion was the absence of the *cfr*-like gene in *C. sporogenes* PA 3679, unlike what the databases suggest. We decided to obtain another strain, *C. sporogenes* ATCC 15579. A PCR reaction was performed and the *cfr*-like gene was amplified, immediately. Notice that the sequence of this strain is in the databases divided in just three scaffolds and hence, probably less likely to contain mistakes.

The assumption of these two *C. sporogenes* strains to contain a *cfr*-like gene [57], seemed to be true for only one of them. Thus, it was possible to try to establish if the *cfr*-like gene in *C. sporogenes* ATCC 15579 could act as Cfr and provide antibiotic resistance, and also compare the ATCC 15579 with the presumably Cfr-lacking strain *C. sporogenes* PA 3679.

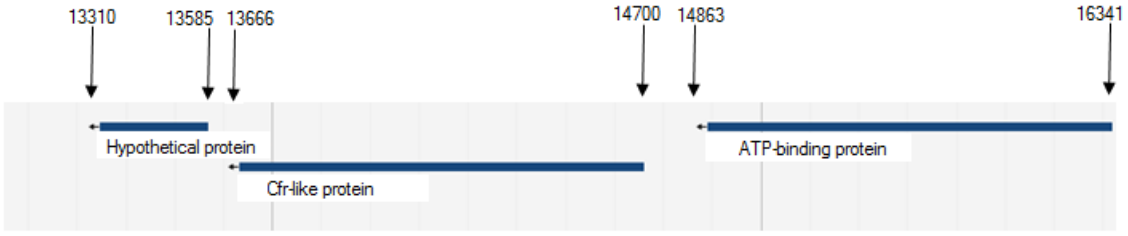


Figure 12: Picture adapted from the information system PATRIC (Bacterial Bioinformatics Resource Center), showing the Cfr-like protein and the two other proteins upstream (ATP-binding protein) and downstream (hypothetical protein) present in the same contig. The arrows and numbers indicate the nucleotides, showing how the proteins are positioned relative to each other.

2.2.3. Antibiotic susceptibilities of the *Clostridium sporogenes* strains

Little information about antibiotic susceptibilities of *C. sporogenes* strains can be found in the literature. However, it is relevant for the study to investigate if the *cfr*-like gene in *C. sporogenes* ATCC 15579 can act as Cfr and provide antibiotic resistance, and compare the results with the presumably Cfr-lacking strain *C. sporogenes* PA 3679. MICs were conducted to assess if the Cfr-like protein found in *C. sporogenes* ATCC 15579 could confer a resistance pattern similar to that of the Cfr methyltransferase. In this experiment, *C. sporogenes* PA 3679 could be used as a negative control, assuming that it does not have the *cfr*-like gene.

Both strains were exposed to nine antibiotics, at various concentrations. Tiamulin and valnemulin (pleuromutilin), chloramphenicol (phenicol), linezolid (oxazolidinone), clindamycin (lincosamide) and synercid (streptogramin) represent the five antibiotic classes in the $PhLOPS_A$ phenotype conferred by the Cfr methyltransferase, which means that those are drugs binding to overlapping sites at the peptidyl transferase center. To verify if any of the strains could provide natural resistance or lower susceptibility to the $PhLOPS_A$ antibiotics, and thus invalidate the results obtained, three other antibiotics were tested: erythromycin, a small macrolide (since Cfr confers resistance to large macrolides) and kanamycin and streptomycin, both aminoglycosides that perturb peptide elongation at the 30S ribosomal subunit. The minimum inhibitory concentration for each strain was defined as the drug concentration at which the growth of the cultures was completely inhibited after 24 h of incubation at 37°C. The MIC results are shown in Table 4.

Table 4: Comparison of antimicrobial susceptibilities of both *C. sporogenes* strains to 9 antibiotics, six representatives of the $PhLOPS_A$ phenotype, one macrolide and two aminoglycosides.

MIC (μg/mL)	*C. sporogenes* PA 3679	*C. sporogenes* ATCC 15579
Tiamulin	8-16	128 - >128
Valnemulin	---	32
Chloramphenicol	0.25	1
Linezolid	0.25	0.5
Clindamycin	<0.125	1-2
Synercid	<0.125	0.5
Erythromycin	0.5	0.5
Kanamycin	8 - >8	4
Streptomycin	8 - >8	8 - >8

Analyzing the MICs and comparing the results obtained from both strains, *C. sporogenes* ATCC 15579 showed lower susceptibility to all the $PhLOPS_A$ antibiotics tested than *C. sporogenes* PA 3679. As far as the aminoglycosides and the small macrolide are concerned, both strains showed similar MICs, which may indicate that none of the strains is naturally more resistant than the other. A study from the 80's [69] showed the antibiotic susceptibilities of six *C. sporogenes* strains isolated from animals. When all the six strains showed resistance to an agent, they were considered naturally resistant to the agent. It was seen for tiamulin (pleuromutilin), clindamycin and lincomycin (lincosamides), and virginiamycin component M (streptogramin A), which are three of the $PhLOPS_A$ antibiotic classes tested in our study. Thus, we can suggest that *C. sporogenes* PA 3679 is not a real *C. sporogenes* strain and therefore does not show similar MICs for tiamulin and clindamycin as *C. sporogenes* ATCC 15579, or that Cfr-like protein may confer some resistance to *C. sporogenes* ATCC 15579, or another mechanism in this strain is responsible for the resistance observed. Since the function of the Cfr-like protein is uncertain and no further information regarding resistance to antibiotics is available in the literature, the most suitable approach was to investigate if the Cfr-like protein was acting as the Cfr methyltransferase and methylates the A2503 in the 23S rRNA from *C. sporogenes* ATCC 15579.

2.2.4. Primer extension analysis to verify modification at A2503 23S rRNA from *C. sporogenes* strains (*E. coli* numbering)

The possible resistance conferred by the Cfr-like protein from *C. sporogenes* ATCC 15579, the uncertainty about its function, and the presumable absence of the *cfr*-like gene in *C. sporogenes* PA 3679, led us to the necessity to verify by primer extension if there was an indication of modification (a primer extension stop) at A2503 23S rRNA from *C. sporogenes* (*E. coli* numbering) as the one caused by Cfr methyltransferase and other Cfr-like proteins in *E. coli*.

Fresh cultures of both strains were growing for around 6.5 hours to allow new ribosomal RNA to be transcribed, modified, and incorporated into ribosomes. Then, the total RNA was purified and a 5'-[32P]-labeled deoxyoligonucleotide (5'– GAACAGCCCAACCCTTG–3') was annealed to the nucleotides 2540 to 2556 of *C. sporogenes* 23S ribosomal RNA (*E. coli* numbering). The reaction was extended until stopped by modifications or secondary structures as explained in part I. The resulting cDNA extension products were separated on 6% polyacrylamide sequencing gel alongside dideoxy sequencing reactions.

The analysis is presented in Figure 13 in two different gels, since the gel that comprises both strains is missing a lane (U). Thus, the gel with only *C. sporogenes* PA 3679 is used to compare the

sequence reading. Figure 13 shows a visible but not too strong stop at A2503 of 23S rRNA from either *C. sporogenes* strains. In the case of *C. sporogenes* PA 3679 (Figure 13A), a strong stop was not expected due to the absence of the *cfr*-like gene. However, *C. sporogenes* ATCC 15579 was expected to mediate a strong stop, if its Cfr-like protein was acting as Cfr in *E. coli*. In both cases (Figure 13B), a slight band is visible, which can be explained due to the presence of an *rlmN*-gene in both strains. To verify the conclusion, mass spectrometry was performed to identify any modification around A2503 23S ribosomal RNA in the strains *C. sporogenes* PA 3679 and *C. sporogenes* ATCC 15579.

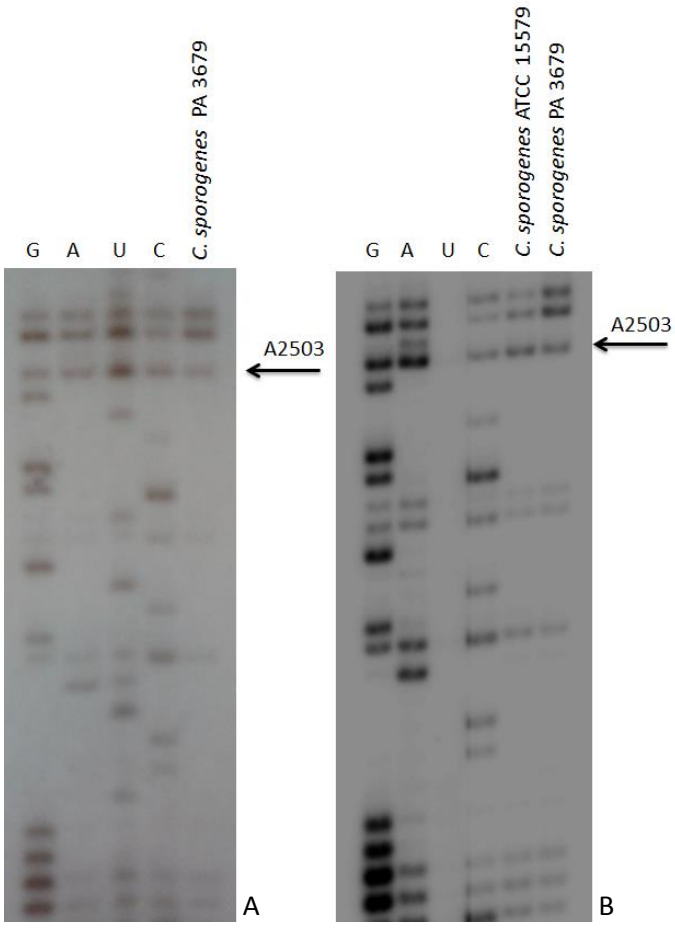


Figure 13: Primer extension analysis made on a 6% acrylamide gel with urea. The picture presented here is limited to the nucleotides flanking A2503 and shows the stops on 23S rRNA from both Clostridium sporogenes ATCC 15579 and PA 3679 (*E. coli* numbering). (A) *C. sporogenes* PA 3679 and (B) *C. sporogenes* ATCC 15579 and *C. sporogenes* PA 3679. The first four lanes (C, U, A and G) correspond to the sequencing reactions. The nucleotide of interest A2503 are pointed by the arrows (reverse transcriptase stops one nucleotide before the modified nucleotide in the sequencing lanes).

2.2.1. MALDI-TOF analysis of a defined rRNA sequence around A2503

To investigate the inconclusive effects observed on the primer extension gels and further define any modification, a matrix-assisted laser desorption ionization – time of flight (MALDI-TOF)

mass spectrometry (MS) analysis was performed. MALDI has been demonstrated as an alternative method to analyse RNA modifications [70]. This technique is generally advantageous in terms of sensitivity and the final result is the generation of singly charged molecular ions, which reduce the complexity of the spectrum [71]. The MALDI technique in this study was used to detect and localize a predicted but unknown modification (A2503) in domain V central loop of 23S rRNA from *C. sporogenes*.

A fragment composed by 48 nucleotides as seen in Figure 14 around A2503 (5'–GCCCCAGGATGCGACGAGCCGACATCGAGGTGCCAAACCTCCCCGTCG–3'), comprising positions 2480-2527 of 23S rRNA from *C. sporogenes* (*E. coli* numbering) was isolated and investigated by mass spectrometry to determine if the modification was m^2A (from RlmN methyltransferase) or m^8A (from Cfr-like enzyme methyltransferase) or maybe both. After fragment purification, the RNA was digested with the G-specific RNase T1, which gives small characteristic fragments suitable for detailed analysis. RNase T1 cleaves after guanosine at the 3' –end through a 2' -, 3' –cyclic phosphate intermediate reaction [70]. The theoretical masses of the generated fragments from the gene sequence of the rRNA fragment isolated were calculated using GPMAW (Table 5).

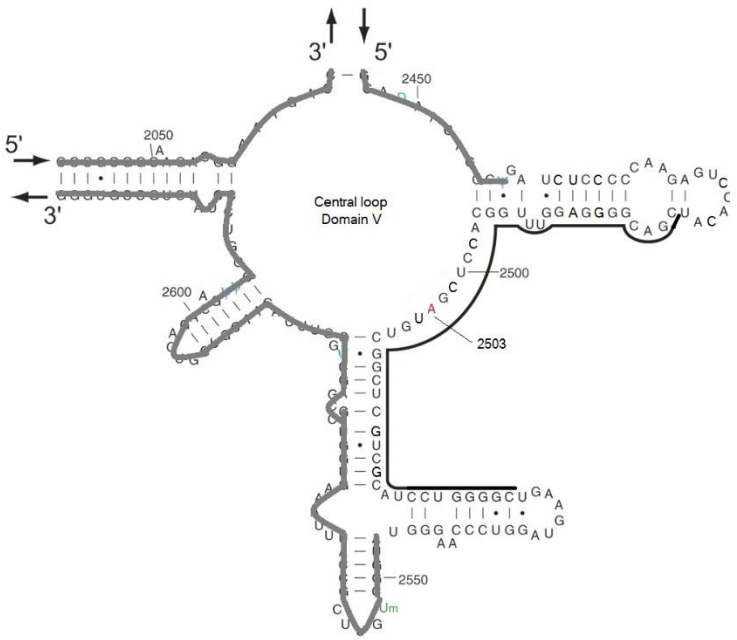
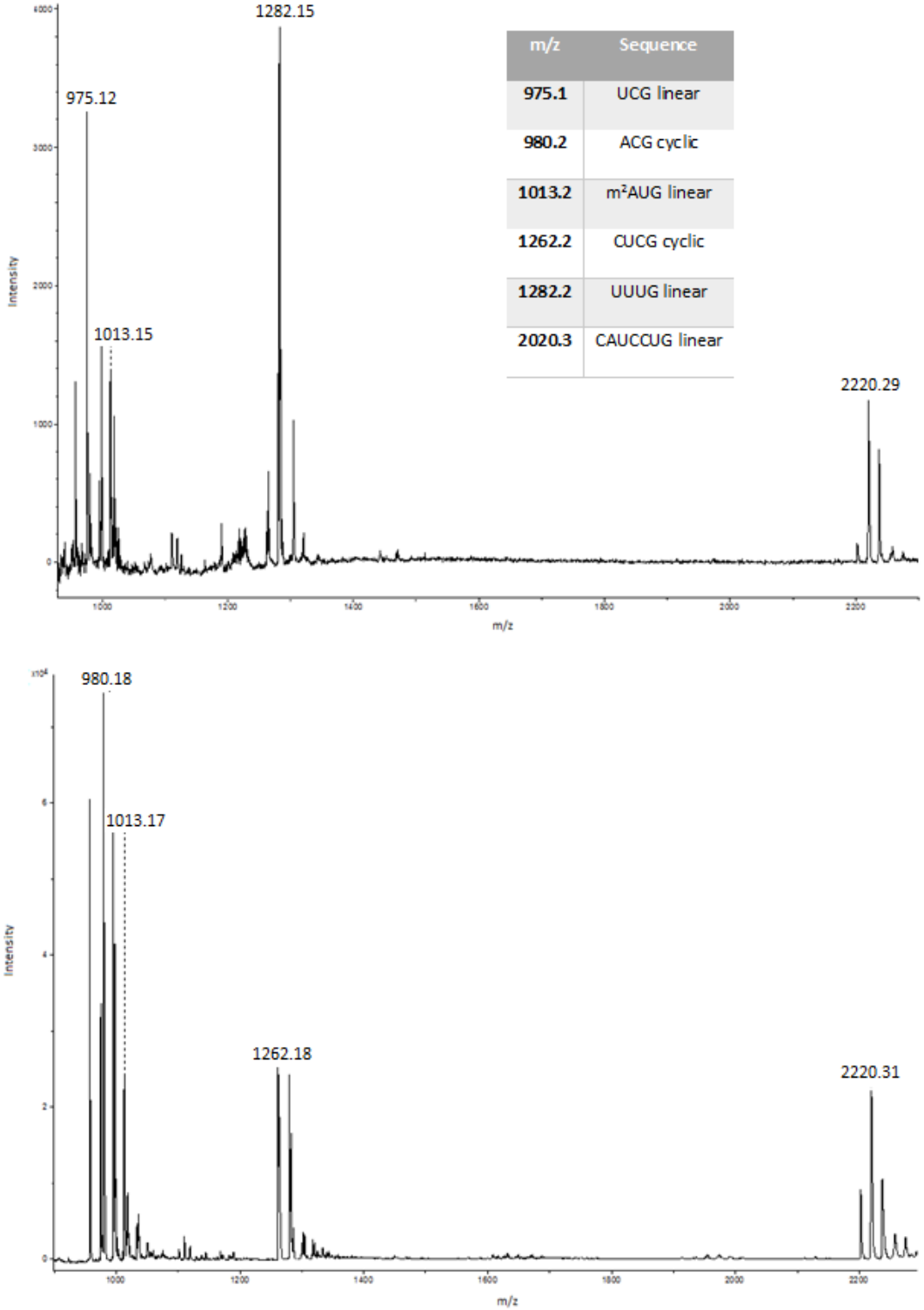


Figure 14: Secondary structure of domain V central loop of 23S rRNA from *C. sporogenes*, adapted from *E. coli* 23S rRNA secondary structure. The region in grey corresponds to *E. coli*, thus shouldn't be taken into account. The black line limits the fragment isolated and investigated by mass spectrometry. The red A corresponds to the adenosine in position 2503 (*E. coli* numbering).

Table 5: Theoretical calculated masses of the 48nt fragment around A2503 from *C. sporogenes* after RNase T1 digestion. *Dinucleotides were not considered.

Mass (linear)	Position	Fragment
669.11	2480 – 2481	CG*
693.12	2488 – 2489	AG*
975.13	2506 – 2508	UCG
975.13	2514 – 2516	UCG
998.16	2482 – 2484	ACG
999.14	2503 – 2505	AUG
1280.17	2510 – 2513	CUCG
1282.14	2491 – 2494	UUUG
2219.31	2496 – 2502	CACCUCG
2220.29	2517 – 2523	CAUCCUG

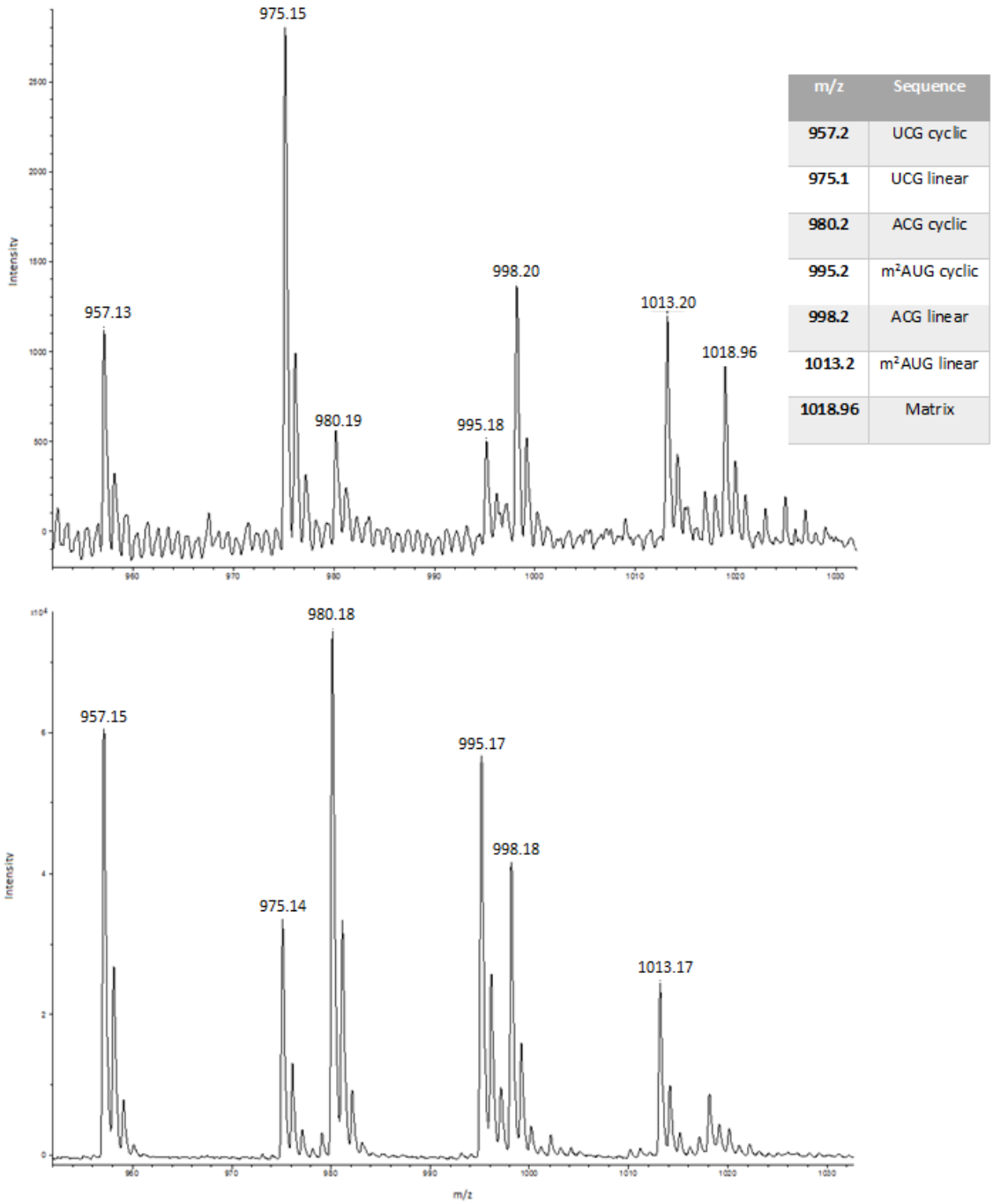
The obtained signals were compared to the theoretical values listed above. Mono- and dinucleotides were not considered because their mass region is dominated by signals from the matrix or buffers in the MALDI-TOF mass spectra. Figure 15 shows the general spectrum and the relevant peaks obtained from the fragment isolated from *C. sporogenes* PA 3679 and *C. sporogenes* ATCC 15579, from m/z 900 to 2300 Da. The m/z value of each signal represents the mass of a protonated RNA fragment in Daltons (Da). All the fragments in the spectra were seen as doublets consisting of the linear fragment and the cyclic phosphate intermediates. The cyclic phosphate intermediates are formed during the RNaseT1 digestion: the cyclic intermediates come from the loss of a water molecule and thus they have a mass of 18 Da lower [70]. The peaks seen in the spectra were compared to the theoretical digestion and, as a first approach, it was possible to recognize most of the peaks expected, although in the *C. sporogenes* ATCC 15579 spectrum the cyclic forms were predominant. Also, a non-theoretically expected peak at m/z 1013.2 Da can be seen in both spectra. This peak corresponds to a mono-methylation in the fragment AUG (999.2+14).



m/z	Sequence
975.1	UCG linear
980.2	ACG cyclic
1013.2	m^2AUG linear
1262.2	CUCG cyclic
1282.2	UUUG linear
2020.3	CAUCCUG linear

Figure 15: MALDI-TOF MS spectra (m/z 900 to 2300 Da) of RNaseT1 digestion of the fragment comprising positions 2480-2527 (*E. coli* numbering) of 23S rRNA from *C. sporogenes* PA 3679 (above) and *C. sporogenes* ATCC 15579 (below), and table of the masses of the observed signals.

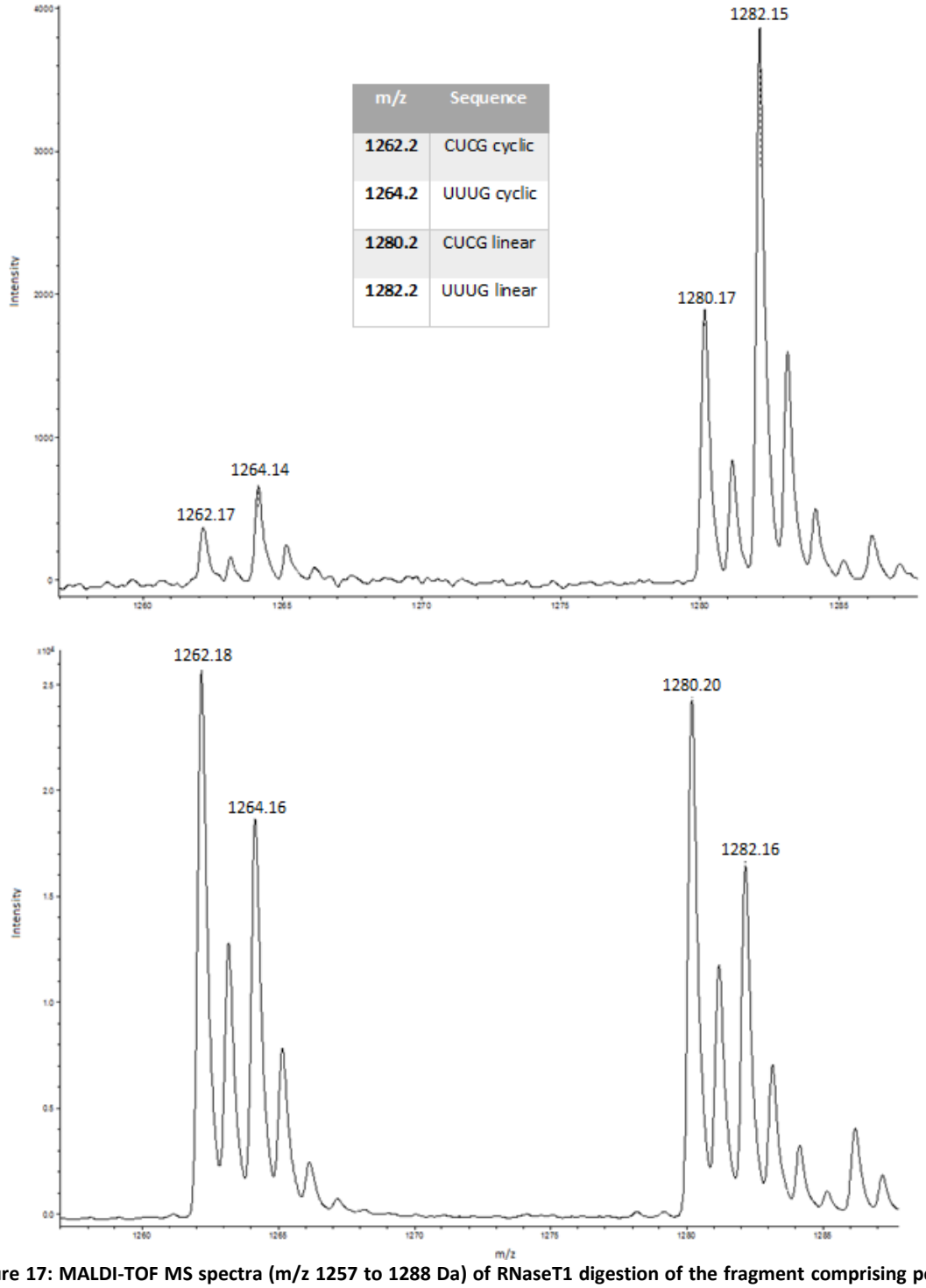
As shown above, not all the signals are seen due to overlapping of the peaks with similar m/z. Hence, each region is further analyzed in more detail. In Figure 16 is shown the initial area correspondent to m/z 952 to 1032 Da, where the methylation at A2503 occurs. All the fragments in this area are represented: linear and cyclic form of UCG, which has really intense signal, because there are two UCG in the RNA fragment isolated; linear and cyclic form of ACG can be seen clearly in both spectra; as far as AUG is concerned, it can be barely seen in the spectra, probably because most of the fragments are methylated, and then we can see the peak corresponding to AUG mono-methylated. If the Cfr-like protein was functioning as the Cfr methyltransferase, it would di-methylate the adenosine at position 2503 and then a peak at m/z 1027 would be seen. However, as shown in Figure 16, no peaks are visible in that area. Hence, the methylation observed is probably caused by RlmN (*rlmn* gene is present in both genomes of *C. sporogenes*) and this methylation occurs at C-2 of A2503. To be sure about the methylation additional experiments have to be performed, such as the tandem mass spectrometry approach described by Giessing *et al.*, where they use an online nanoliquid chromatography electrospray ionization tandem mass spectrometry (nano-LC-ESI-MS^n) in order to structurally characterize the modification [21]. Finally, there is a peak at m/z 1018.96 that cannot be explained by the theoretical fragmentation. This fragment was assumed as a non-RNA fragment. Comparing the m/z of all peaks, the RNA fragments always have low decimals values, typical of the RNA fragments. In contrast, the peak at m/z 1018.96 has a really high decimal value, suggesting that this is in fact a Matrix signal.



m/z	Sequence
957.2	UCG cyclic
975.1	UCG linear
980.2	ACG cyclic
995.2	m²AUG cyclic
998.2	ACG linear
1013.2	m²AUG linear
1018.96	Matrix

Figure 16: MALDI-TOF MS spectra (m/z 952 to 1032 Da) of RNaseT1 digestion of the fragment comprising positions 2480-2527 (*E. coli* numbering) of 23S rRNA from *C. sporogenes* PA 3679 (above) and *C. sporogenes* ATCC 15579 (below), and table of the masses of the observed signals.

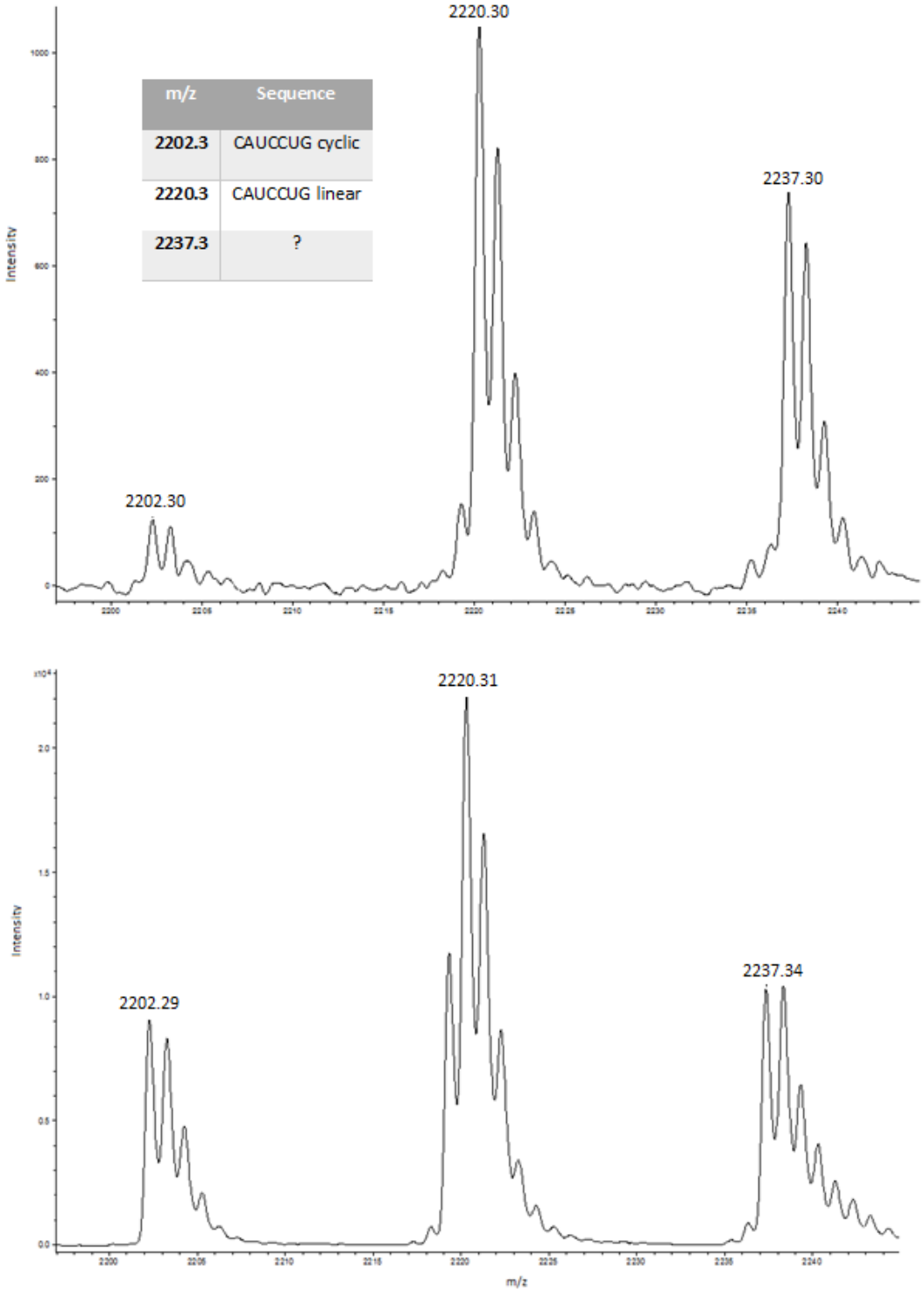
The area comprising m/z 1257 to 1288 (Figure 17) shows undoubtedly the linear and cyclic forms of CUCG and UUUG. Here, the predominance of the cyclic forms in the spectrum of *C. sporogenes* ATCC 15579 is once more denoted.



m/z	Sequence
1262.2	CUCG cyclic
1264.2	UUUG cyclic
1280.2	CUCG linear
1282.2	UUUG linear

Figure 17: MALDI-TOF MS spectra (m/z 1257 to 1288 Da) of RNaseT1 digestion of the fragment comprising positions 2480-2527 (*E. coli* numbering) of 23S rRNA from *C. sporogenes* PA 3679 (above) and *C. sporogenes* ATCC 15579 (below), and table of the masses of the observed signals.

Finally, the last area shown in Figure 18 (m/z 2197 to 2245) contains the cyclic and linear forms of CAUCCUG. The peak corresponding to the fragment CACCUCG should also be seen, according to the theoretical digestion, but in both spectra it is not visible. Also, a non-expected peak appears at m/z 2237.3. The most likely explanation is that the fragment CACCUCG is somehow modified and gives origin to the non-expected peak. In fact, if we look carefully to the spectra, a smaller peak is seen around m/z 2219 that could be associated to the linear form of CACCUCG. However, no cyclic form can be seen around m/z 2201, which is not consistent with what happens in all the other fragments. Thus, the peak around m/z 2219 can be explained as the cyclic form of the linear fragment at m/z 2237.3, telling us how trustable this peak can be. The non-expected peak at m/z 2237.3 corresponds to 2219+18. There isn't an explanation about what modification could give rise to this additional 18 Da. Hence, further studies should be performed, such as tandem mass spectrometry (MS-MS) on the ions corresponding to the fragment CACCUCG. In this technique, the fragment of RNA is completely hydrolysed into its nucleoside constituents (electrospray ionisation – ESI), which are then identified by combined liquid chromatography and MS-MS. To locate the nucleotide modifications in the primary sequence, another aliquot of the RNA in question is digested to completion with a nucleotide-specific RNase, and the digestion mixture is analysed by online high performance liquid chromatography (HPLC)/ESI-MS. By comparing the observed masses with the ones expected from the gene sequence, RNase digestion fragments harbouring posttranscriptional modifications can be identified [72].



m/z	Sequence
2202.3	CAUCCUG cyclic
2220.3	CAUCCUG linear
2237.3	?

Figure 18: MALDI-TOF MS spectra (m/z 2197 to 2245 Da) of RNaseT1 digestion of the fragment comprising positions 2480-2527 (*E. coli* numbering) of 23S rRNA from *C. sporogenes* PA 3679 (above) and *C. sporogenes* ATCC 15579 (below), and table of the masses of the observed signals.

3. Conclusions

In the first part of the project, five new constructs were made to obtain plasmids expressing constitutively the *clcs, cfr, scfr*, *scac* and *casc* genes. The expression level of Cfr and Cfr-like proteins was investigated but no protein bands were detected at the expected masses and it could not be verified by MS either (probably because we excised the wrong area of the gel). Nevertheless, MIC analysis showed that sCfr lowered the sensitivity to the $PhLOPS_A$ antibiotics tested, conferring a resistance pattern similar to the *S. sciuri* Cfr methyltransferase. Cfr showed lower effectiveness than sCfr, probably because it is not codon optimized for *E. coli*. ClCs did not mediate MIC changes, as well as sCAC and CAsC proteins, showing no Cfr methyltransferase function in *E. coli*. The RNA methylation at A2503 in 23S rRNA was checked by primer extension and the analysis showed a clear stop at A2503 of 23S RNA from sCfr and a weaker stop in the case of Cfr. Thus, both sCfr and Cfr confer decreased susceptibility to three classes of antibiotics by modification of position A2503 in 23S rRNA from *E. coli*. There was no stop in the ClCs- and combined proteins-containing strains, which is consistent with no observed MIC changes. Thus, the Cfr-like from *Clostridium sporogenes* does not methylate *E. coli* 23S RNA. However, it was not possible to conclude that ClCs does not have a Cfr-like function and it may be able to methylate *Clostridium* 23S RNA but not *E. coli* 23S RNA, which should be investigated in the second part of the project.

In the second part, the amplification of the *cfr*-like gene from *C. sporogenes* PA 3679 was not possible, leading to the hypothesis that the *cfr*-like gene is absent in this strain. In contrast, the amplification of the *cfr*-like gene from *C. sporogenes* ATCC 15579 was achieved. Both strains were exposed to representatives of the five antibiotic classes in the $PhLOPS_A$ phenotype conferred by the Cfr methyltransferase. MICs obtained from *C. sporogenes* ATCC 15579 showed lower susceptibility to all the $PhLOPS_A$ antibiotics tested than the presumably Cfr-lacking strain *C. sporogenes* PA 3679. Thus, the Cfr-like protein may confer some resistance to *C. sporogenes* ATCC 15579 or another mechanism, responsible for the resistance observed, is present in this strain. The function of the Cfr-like protein from *C. sporogenes* ATCC 15579 was investigated by primer extension, using *C. sporogenes* PA 3679 as a negative control. A visible but not too strong stop at A2503 of 23S RNA from both *C. sporogenes* strains was detected, which required further analysis. MALDI-TOF MS showed two theoretically non-expected peaks in both strains spectra. The first corresponds to a mono-methylation in the fragment $_{2503}AUG_{2505}$. Since *rlmN*-gene is present in both strains, it probably corresponds to a methylation in C-2 at A2503 (m^2AUG). The second peak can be explained by a possible modification in the fragment $_{2496}CACCUCG_{2502}$. The spectra from both strains showed the same peaks, revealing that the presence of the Cfr-like protein in *C. sporogenes* ATCC 15579

does not mediate additional modification in the isolated RNA fragment comparing with the presumably Cfr-lacking strain *C. sporogenes* PA 3679.

4. Future Work

Future work could include the construction of more combinations of parts of *scfr* and *clcs* genes in order to investigate the lack of function of the ClCs when expressed in *E. coli*, and to try to define if parts of ClCs could be functioning as the corresponding areas of Cfr or to define which part prevents the function. Also, the expression of Cfr and Cfr-like proteins in *E. coli* should be analyzed because there is no visible reason or cause for inhibition or no expression. MS should be performed again to analyze the bands near the ones already studied in this project. Another possible direction would be performing a *Western Blot* analysis of the Cfr and Cfr-like proteins since it allows the detection of a specific protein. For the second part of the project, in order to ascertain the source of the primer extension stop, further studies should be made in the presence of antibiotics, to try to clarify if the resistance phenotype is inducible by its presence. In order to ascertain the position of the methylation at A2503, additional tandem MS should be performed to structurally characterize the modification. Last but not least, the supposed modification in fragment $_{2496}$CACCUC$_{2502}$ should be analysed by combined liquid chromatography and MS-MS to identify posttranscriptional modifications.

5. Materials and Methods

5.1. Part I – Cloning of *cfr* and *cfr*-like genes in *E. coli*

5.1.1. Strains and growth conditions

The *E. coli* TOP10 strain was used for transformation of ligated constructs. The hyperpermeable *E. coli* AS19 strain [62] was used for MIC analysis to emphasize the observed effects because it is much more sensitive to antibiotics than other *E. coli* strains that have a relatively low susceptibility to many drugs. The RlmN-lacking strain *E. coli* JW2501-1 [65] was used for methylation analysis to facilitate identification of Cfr methylation by avoiding interference from the RlmN methylation at the same nucleotide. All strains were grown in Luria-Bertani (LB) broth and Luria-Agar (LA) plates at 37°C.

5.1.2. Growth curves – *E. coli* AS19/pClCs

An overnight culture was diluted in LB, to a final OD_{450} of 0.005. The cells were incubated at 37°C, with shaking, and induced with IPTG after 30 minutes. The final concentration of IPTG was 0, 0.2 and 1 mM. The OD450 was measured, approximately, every 20 minutes over a period of 6 hours until stationary phase was reached.

5.1.3. Construction of plasmids encoding *cfr* and *cfr*-like genes

The plasmid pCfrHisN, containing the wild-type *cfr* gene was provided by "BV group". The synthetic *scfr* and *clcs* genes were designed to be codon optimized for *E. coli* and both were purchased from GenScript USA Inc.. The plasmids bearing *cfr* and *clcs* genes were constructed by PCR amplification of the genes from genomic DNA or synthetic genes, followed by cloning into plasmid pBR322 for expression of the proteins. Standard PCR amplification of the genes and vector (vector part with the *amp* gene) was performed with the proper primers (Table 6), each containing AvrII-CCTAGG and XhoI-CTCGAG restriction sites for cloning. The plasmid carrying *scfr* gene was only cut with AvrII and XhoI because it already had the restriction sites. The PCRs were performed with NEW ENGLAND BioLabs® Inc. – Phusion® High Fidelity DNA polymerase, which includes the procedure and program followed.

Table 6: Primers and PCR conditions used to construct the plasmids encoding *cfr*- and *ClCs*- genes. The annealing temperature for the primers was calculated using NEB Calculator, according to the manufacturer's instructions.

Vector or gene	Primers (5' → 3')	PCR conditions
pBR322	BV431 (forward) TATACTCGAGGCCGGCGGCACCTCGCTA BV432 (reverse) TCATCCTAGGTGCCTGACTGCGTTAGCA	Initial denaturation (98°C): 1 min; 5 initial cycles, denaturation (98°C): 8 sec, annealing (65°C): 66 sec, extension (72°C): 20 sec; 25 cycles, denaturation (98°C): 8 sec, annealing (72°C): 66 sec, extension (72°C): 20 sec and final extension (72°C): 8 min
cfr	BV462 (forward) GGTTCCTAGGATGAATTTTAATAATAAAAC BV463 (reverse) TTAACTCGAGCTATTGGCTATTTTGATAAT	Initial denaturation (98°C): 1 min; 5 initial cycles, denaturation (98°C): 8 sec, annealing (45°C): 20 sec, extension (72°C): 20 sec; 25 cycles, denaturation (98°C): 8 sec, annealing (59°C): 20 sec, extension (72°C): 20 sec and final extension (72°C): 8 min
clcs	BV475 (forward) GATACCTAGGATGAAACAAACCAAAACCAA BV476 (reverse) CGCGCTCGAGTTACAGTTCATTTTCATAAC	Initial denaturation (98°C): 1 min; 5 initial cycles, denaturation (98°C): 8 sec, annealing (49°C): 20 sec, extension (72°C): 30 sec; 25 cycles, denaturation (98°C): 8 sec, annealing (72°C): 20 sec, extension (72°C): 30 sec and final extension: 8 min

The PCR products were electrophoresed in an agarose gel (1% for the vector and 1.5% for the fragments) to determine the size of the fragments and were subsequently purified using Gen Elute™ Gel Extraction Kit (Sigma-Aldrich). After cleavage with the AvrII and XhoI enzymes (followed by purification using Gen Elute PCR Clean-up Kit – Sigma-Aldrich), the fragments and vector were ligated according to the ratio 50 fmol: 10 fmol, respectively. To calculate the concentration from ng/µl to fmol/µl the following formula was used: $\frac{x\ ng/\mu L \times 10^{15} fmol/mol}{xx\ bp \times 600 g/mol \times 10^{9} ng/g} = fmol/\mu L$. Ligation was performed according to the following procedure (for 20µL reaction): X µL vector, Y µL fragment, 2 µL 10 X Ligase Buffer (Roche), 1 µL T4 DNA Ligase (Roche), 1 µL of 10 mM ATP and H_2O up to 20µL. It was incubated at 16°C overnight. The new plasmids were named pBRCfr, pBRsCfr and pBRClCs, according to the expressed proteins.

The transformation of the plasmids into *E. coli* Top10 was carried out by the $CaCl_2$-method: 75 µL of an overnight culture of *E. coli* Top10 were added to 25 mL LB and incubated at 37°C with shaking until the culture reached an OD_{450} of 0.5. The cells (2x 10 mL) were then harvested by

centrifugation for 5 min at 3000g, at 4°C, and resuspended with 5 mL ice-cold 50 mM $CaCl_2$. Once again, the cells were harvested by centrifugation for 5 min at 3000g, at 4°C, and resuspended in 800 µl ice-cold 50 mM $CaCl_2$. The cells were left on ice for at least 30 min. Transformation was carried out by mixing 10 ng of the plasmids with 200 µL of the competent cells. After 30 min on ice, heat chock at 42°C for 1.5 min, and ice again for 5 min. Then 800 µL of LB were added and the cells incubated at 37°C for 1 hour. Finally, the cells were centrifuged at 4000g for 1 min and the pellet was resuspended in 100 µL LB. All the volume was applied to LA plates, supplemented with 100 µg/mL ampicillin. Transformants were observed after 17 h at 37°C.

After obtaining single colonies, plasmids were isolated from these clones using the Gen Elute™ Plasmid Miniprep Kit (Sigma-Aldrich) and retransformed into *E. coli* AS19 and *E. coli* JW2501-1 for the further experiments. As negative and positive controls, cells harboring the plasmid pBR322 alone and pBglII were used, respectively. All three plasmid constructs were sequenced at the inserted gene (Eurofins MWG Operon) to verify the identity of the cloned genes. The transformation into *E.coli* AS19 was conducted using a method with minor differences, the $MgCl_2$-method (first resuspension with 5 mL ice-cold 0.1 mM $MgCl_2$ and final resuspension in 1 mL ice-cold 0.1 M $CaCl_2$), and the cells were spread on plates with 50 µg/mL ampicillin instead of the usual 100 µg/mL.

5.1.4. Construction of plasmids encoding combined parts of s*cfr* and *clcs* genes

The plasmids pBRsCfr and pBRClCs were cut with the same restriction enzyme, a double cutter, AflIII. The digestion products were electrophoresed in an agarose gel and purified as described above. Then, the fragments were ligated according with the ratio 30 fmol: 30 fmol, to produce two different plasmids, one containing the first part of *scfr* + the second part of *clcs* (pBR**sC**fr**A**flII**C**lCs), and the second one comprising the first part of *clcs* + the second part of *scfr* (pBR**C**lCs**A**flII**sC**fr). The new plasmids were named pBRsCAC and pBRCAsC. The plasmids were then transformed as explained above.

5.1.5. Growth curves – *E. coli* AS19/pBR322, AS19/pBRsCfr and AS19/pBRClCs

Overnight cultures of all strains were diluted in LB, to a final OD_{450} of 0.005. The cells were incubated at 37°C, with shaking. The OD450 was measured, approximately, every 20 to 30 minutes over a period of 8 hours until stationary phase was reached.

5.1.6. Antibiotic susceptibility testing

LB medium was inoculated with single colonies and incubated overnight and then the cultures were diluted to OD_{450} = 0.01. The strains were tested using three different antibiotics, tiamulin, chloramphenicol and linezolid. The antibiotic concentration ranges were: tiamulin and chloramphenicol 0.5 – 32 µg/mL and linezolid 1 – 64 µg/mL. Drug susceptibility testing was done in a microtiter plate format, where 100µl of diluted culture was mixed with 100µl of antibiotic solutions in a series with 2-fold concentration steps and with triplicates for each strain. The plates were incubated for 24 hours at 37°C and optical density values at 450 nm were measured with a Victor 3 plate reader (Perkin Elmer).

5.1.7. SDS gel analysis

E. coli AS19 cells harbouring the plasmids were grown at 37°C with shaking until reaching an OD450 of 1 and then harvested. The pellet was dissolved in 1xSDS/dithiothreitol (DTT) loading buffer, incubated at 95°C for 5 min, centrifuged at 12000 rpm for other 5 min and then placed on ice. The samples were then loaded onto a 4-12% SDS gel along with the marker (Fermentas #1811 – Page Ruler™ Plus Prestained Protein Ladder). The gel was run at 50 V for around 30 min and then at 180 V for approximately 70 min in Tris-Glycine-*S*DS (TGS) buffer. Before visualization, the gel was stained with Coomassie brilliant blue G and then transferred into water to decolourise.

To isolate protein for mass spectrometry, cells from *E. coli* AS19, AS19/pBRCfr, AS19/pBRsCfr and AS19/pBRClCs were loaded onto a thicker (1 mm) and bigger (15x15 cm) SDS gel along with Fermentas #1811 marker (run at 24 mA for around 9 hours). After running the gel, the bands of interest were cut for mass spectrometry investigation, conducted by a technician from the Protein Research Group, another group from our department.

5.1.8. Primer extension analysis

Diluted overnight cultures of *E. coli* JW2501-1 cells harbouring the plasmids were grown at 37°C, until reaching an OD450 between 0.3 and 0.4, corresponding to the time when the cells are in exponential phase. A volume of cells corresponding to 4 ml culture with an OD450 of 0.375 was harvested. RNA was subsequently extracted using Gene JET RNA Purification Kit (Thermo Scientific). 1 µL of 4,5X Hybridization buffer (250 mM K. Hepes pH 7.0, 500 mM KCl) and 1 µL of 10 pmol/µL primer (Cy5-labeled, 5'–GAACAGCCATACCCTTG–3') were added to 2.5 µL of 1600 ng of RNA. Samples were boiled for 2 minutes at 80°C and slowly cooled down until they reached a temperature of 45°C. To each sequencing reaction, one kind of ddXTP (1 µl of 100 µM, X=G, A, T or C) was added, resulting in four different mixtures. 0.6 µL of extension buffer (1.3 M Tris-HCl pH 8.4,

100 mM MgCl2, 100 mM DTT), 0.3 µL of dNTP (1.1 mM of each), 2.1 µL of water and 1.5U of reverse transcriptase (Roche) were then added to all samples. Samples were mixed and incubated at 42°C for 30 minutes. The cDNA, produced by reverse transcriptase, was precipitated by adding 20 µl 0.25 M NaOAc pH 6.5 and 45 µl 96 % EtOH. The samples were centrifuged for 10 minutes at full speed and the supernatant was removed, leaving only the pellet. The pellet was washed with 70 µl 70 % EtOH and centrifuged at full speed for 5 minutes. The pellet was left to dry and afterwards dissolved in 5 µl UREA loading buffer (8 M UREA, 20 mM Tris-HCl pH 7.8, 1 mM EDTA and bromophenol blue dye). Before loading on the gel, the samples were incubated at 80°C for 2 minutes. The resulting cDNA extension products were separated on 6% polyacrylamide sequencing gel (pre-run at 45 W for 30 minutes) and run in 0.9 x Tris-Borate-EDTA (TBE) buffer, until bromophenol blue reached the bottom. The visualization was achieved by fluorescence scan with Typhoon TRIO Variable Mode Imager (Amersham Bioscience).

5.2. Part II – Investigate the function of the *Clostridium cfr*-like gene in *C. sporogenes* PA 3679 and *C. sporogenes* ATCC 15579

5.2.1. Growth conditions

The strain *Clostridium sporogenes* PA 3679 (bougth under the name NCTC 8594) was obtained from Public Health England and *C. sporogenes* ATCC 15579 from American Type Culture Collection. To grow the strains, ATCC® Medium: 2107 Modified Reinforced Clostridial Agar/Broth (pre-reduced) was used as well as 37°C as the optimal temperature. To maintain the anaerobic atmosphere required for these strains, Hungate-tubes were used for broth cultures (addition of N_2 to remove O_2) and the plates were incubated in an anaerobic jar with a sachet of Anaerocult A or AnaeroGen.

5.2.2. 16S rRNA and *cfr*-like gene amplification

PCR reactions were performed to amplify the 16S ribosomal RNA and the *cfr*-like gene. Proper primers were designed for all PCR reactions and the DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen) – Pretreatment for Gram Positive Bacteria.

Primers and PCR conditions are resumed in Table 3. The PCRs were performed with NEW ENGLAND BioLabs® Inc. – Phusion® High Fidelity DNA polymerase and NEW ENGLAND BioLabs® Inc. – Q5® High-Fidelity DNA Polymerase, which includes the procedure and program followed. All PCR products were measured and sent for sequencing to Eurofins MWG Operon.

5.2.3. Primer extension analysis

100 µL of an overnight culture of each *C. sporogenes* strain were added to 10 mL of fresh broth and incubated at 37°C, for around 6,5h. A volume of cells corresponding to 4 ml culture with an OD_{450} of 0.375 was harvested. RNA was then extracted using the Gene JET™ RNA Purification Kit (Thermo Scientific), 100µl of extract were obtained and the concentration was measured by Nanodrop. The extract was run in an agarose gel (1.5 %) at 75 volts for about 30 minutes, to confirm that an isolation of total RNA was obtained (RNA from *E. coli* used as control). The same procedure described in part I was applied, but a phosphorylated primer (5'– GAACAGCCCAACCCTTG–3') was used and the gel was visualized by a phosphor scan with the same equipment.

5.2.4. Purification of total RNA for MALDI-TOF analysis

1 mL of an overnight culture of each *C. sporogenes* strain were added to 150 mL of fresh broth and incubated at 37°C, for around 6h. After that, the cells were placed on ice for 10 min and then centrifuged at 6000 rpm, for 10 min at 4°C. The pellets were washed and resuspended in 100 mL of cold TMN buffer (100 mM NH_4Cl, 10 mM $MgCl_2$, 50 mM Tris-HCl pH 7.5) and centrifuged again at 6000 rpm, for 10 min at 4°C. Another 2 mL of cold TMN buffer were added and the pellets were resuspended by vortexing. Then, the cells were lysed by sonication, and cell debris was removed by centrifugation (2 X 10 min at 4°C). Afterwards, RNA was extracted using phenol and chloroform (3 times phenol, 1 time phenol/chloroform, 1 time chloroform). The reagent was added to the supernatant (1:1), vortex for 10 seconds, shaking for 3 minutes and centrifugation at full speed for 3 minutes. After all successive extractions, the aqueous phase was precipitated with 1/10 volume of 3M NaOAc pH 6.5 and 3 times volume of 96% ethanol. The mixture was incubated at -20ºC for 1 hour and centrifuged for 40 min at max speed. The pellet was washed with 1/3 volume of 70% cold ethanol and centrifuged again for 5 min at max speed. The pellet was left to dry at room temperature and finally dissolved in 40µL of water.

5.2.5. Isolation of defined rRNA sequence for MALDI-TOF analysis

To 100 pmol of RNA, 4X hybridization buffer (250 mM HEPES pH 7.5, 500 mM NH_4Cl) and 500 pmol of synthetic oligodeoxunucleotide were added. The oligodeoxunucleotide composed by 48 nt (5'–GCCCCAGGATGCGACGAGCCGACATCGAGGTGCCAAACCTCCCCGTCG–3') is complementary to the site of interest and binds to nucleotides 2480 to 2527 of *C. sporogenes* 23S ribosomal RNA (*E. coli* numbering). The mixture for annealing was heated at 80°C for 5 min and then cooled down until reach 35°C. The digestion was performed by adding 20 units of Mung bean nuclease, 1 volume

of Mung bean nuclease buffer (50 mM NaOAc pH 5.0 at 25°C, 30 mM NaCl, 1 mM $ZnCl_2$), and 0.25 µg of RNaseA to the hybridisation mixture and incubating at 35°C for 60 minutes. The reaction was stopped and the enzymes removed by extracting with 1 volume of phenol/chloroform. The RNA:DNA hybrid was precipitated with 1/10 volume of 3M NH_4Ac pH 6.5 and 3 volumes of 96% ethanol. The mixture was placed at 0°C for 45 minutes and centrifuged at max speed for other 45 minutes. The pellet was washed with ½ volume of 70% cold ethanol and centrifuged for 15 minutes. The pellet was left to dry at room temperature and dissolved in 6µL of water. To isolate the RNA fragment, the mixture was separated on 13% polyacrylamide gel (1:30 7 M urea). Before loading the sample, control (hybridization mixture without oligo) and 50 pmol of oligo, 12µL of formamide were added. The gel ran 75 min at 18W in 1 x Tris-Borate-EDTA (TBE) buffer. The gel was transferred to a solution of 1 x TBE+EtBr (0.5 µg/mL) and incubated for 30 minutes shaking. Afterwards, it was visualized by UV light and the band corresponding to the nuclease protected RNA sequence was excised. To the excised fragment cut into pieces, 120uL of 2M NH_4Ac pH 5.3 were added. It was then placed at 4°C, shaking vigorously overnight. To the supernatant, 3 volumes of 96% ethanol were added. The mixture was placed at 0°C for 45 minutes and centrifuged at max speed for other 45 minutes. The pellet was washed with ½ volume of 70% cold ethanol and centrifuged for 15 minutes. The pellet was left to dry at room temperature and dissolved in 4µL of water. Finally, the RNA was digested with RNase T1 to obtain a mass fingerprint of the sequence. To 2 µL of dissolved rRNA, 0.5 µL of 0.5 M 3-HPA (3-Hydroxypicolinic Acid) matrix and 0.5 µL of RNase T1 (10U/µL) were added. The mixture was incubated at 37°C overnight. Half of the digested RNA (1.5 µL), 0.5 µL of 0.5 M 3-HPA matrix and 0.1 µL of suspended ammonium loaded ion exchange beads were mixed on the target plate. The sample was left to dry and the beads removed hereafter. Spectra were recorded in reflector and positive ion mode on a Bruker – The new ultrafleXtreme™ mass spectrometer and smoothed using the software 'm/z' (FlexAnalysis version 3.4).

5.2.6. Antibiotic susceptibility testing

ATCC® Medium: 2107 Modified Reinforced Clostridial broth was inoculated with 100 µL cells of both *C. sporogenes* strains and incubated overnight at 37°C. The cultures were diluted to OD_{450} = 0.01 to perform a Minimum Inhibitory Concentration (MIC) analysis. *C. sporogenes* PA 3679 and *C. sporogenes* ATCC 15579 were tested using nine different antibiotics: tiamulin, chloramphenicol, linezolid, clindamycin, synercid, erythromycin, valnemulin, kanamycin and streptomycin. The antibiotic concentration ranges were: tiamulin 2 – 128 µg/mL, chloramphenicol 0.125 – 8 µg/mL, linezolid 0.125 – 8 µg/mL, clindamycin 0.125 – 8 µg/mL, synercid 0.25 – 16 µg/mL,

erythromycin 0.125 – 8 µg/mL, valnemulin 2 – 128 µg/mL, kanamycin 0.125 – 8 µg/mL and streptomycin 0.125 – 8 µg/mL. Drug susceptibility testing was done as described in part I, but the plates were incubated in an anaerobic jar.

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