Regulation of antibiotic-resistance in bacteria of the *Burkholderia cepacia* complex: impact of the ncS06 and ncRNA3 small non-coding RNAs

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Abstract

Bcc (*Burkholderia cepacia* complex) bacteria display high-level of resistance to antibiotics, are responsible for severe clinical prognosis in immunocompromised patients, and can chronically persist in infected hosts. The development of alternatives to conventional antimicrobials is crucial and urgent, as the options for current treatment are lacking. sRNAs are key gene regulators that coordinate several bacterial responses whose contribution to antibiotic resistance has become evident. sRNAs identified in *B. cenocepacia* were analyzed and their targets were predicted to select possible molecules involved in Bcc antibiotic susceptibility. Bioinformatics predictions of 167 putative sRNAs targets were performed, and at least a target gene related with antibiotic resistance was found for 78 of these sRNAs. ncS06 and ncRNA3 were chosen for further characterization and the Minimum Inhibitory Concentration was determined for trimethoprim, ciprofloxacin, and tobramycin in clinically relevant Bcc species, overexpressing or repressing these sRNAs. The overexpression of ncS06 increased the susceptibility to ciprofloxacin in *B. multivorans* and the overexpression of ncRNA3 increased trimethoprim resistance in *B. cenocepacia*. When ncRNA3 was overexpressed, its predicted target *dfrA* was upregulated, and the interaction between them was confirmed by EMSA. No direct targets have been demonstrated, although the colony morphology, motility, and the outer membrane profile of *B. multivorans* was altered when ncS06 was overexpressed. These results suggest that ncS06 controls mRNAs involved in multiple physiological processes and can model the composition of the bacterial membrane. These results corroborate the importance of sRNAs in the regulation of antibiotic resistance.

Keywords: Antibiotic Resistance, *Burkholderia cepacia* Complex, Small non-coding RNAs

Introduction

The *Burkholderia cepacia* complex (Bcc) consists of a group of over 20 species from the *Burkholderia* genus that share high similarity[1,2]. Bcc bacteria are Gram-negative, rod shaped, obligate aerobes, with large genomes (6-9 Mb), often containing at least one plasmid and several genomic islands[3–6]. Species from this complex present biotechnological potential, but many of them are also known human and plant pathogens [7,8]. Since the 1980s that species from the Bcc have been noticed to infect immunocompromised patients, namely Cystic Fibrosis (CF) patients. Although Bcc bacteria only infect about 3.5% of CF patients, these bacteria are particularly feared due to the variable and unpredictable infections outcome, which can lead to a fatal combination of necrotizing pneumonia, worsening respiratory failure, and bacteremia, known as Cepacia syndrome [9]. *B. cenocepacia*, *B. multivorans* and *B. dolosa* are three of the species found most often in people with CF[10]. Bacteria from the Bcc are also highly resistant to antimicrobials. Several features of Bcc bacteria are known to confer resistance against some antibiotics, such as their outer membrane structure, the overexpression of efflux pumps, the expression of β-lactamases, and drug target and antibiotic modifications[11,12]. Especially in Bcc infections, the constant use of antimicrobials to attenuate symptoms and avoid the disease progression of CF patients potentiate the rise of Bcc antimicrobial resistance to almost all

antibiotics available, and new strategies to circumvent the present resistance mechanisms are required[13]. Many types of molecules and compounds are being or have been tested for antimicrobial treatment. Small non-coding RNAs (sRNAs) have been studied as possible solutions and can often act as enhancers of the conventional antibiotics [12,14–23].

In bacteria, sRNAs are small molecules (50-500 nucleotides) that act at the posttranscriptional level, regulating the expression of genes, including the ones involved in virulence and antimicrobial resistance[24]. This type of regulation that allows an immediate response can be beneficial for bacterial pathogens when the concentrations of antibiotics increase rapidly and/or for their adaptation to a variable and inhospitable host micro-environment. Usually, sRNAs act by base-pairing with their target mRNAs repressing or, less commonly, activating gene expression. The extensive study of sRNAs in *Escherichia coli* and *Salmonella* strains, but also in *Pseudomonas aeruginosa* and other multidrug resistant pathogens allowed the identification and characterization of some sRNAs that are involved in antibiotic uptake, modification of lipopolysaccharide and cell wall synthesis, drug efflux, and biofilm formation or biofilm-associated antibiotic resistance[1,2]**.**

Compared to other bacteria, riboregulatory sRNAs and the specific pathways of their action are still poorly understood in Bcc bacteria. However, more than a hundred putative sRNAs are encoded in the genome of *B. cenocepacia* J2315, a multidrug-resistant strain isolated from a CF patient. In the present work, bioinformatic analysis will be performed to evaluate if antibiotic resistance genes are predicted targets of some of these *B. cenocepacia* sRNAs. According to that, some *B. cenocepacia* sRNAs will be selected and their possible role in controlling the antimicrobial resistance of Bcc bacteria will be evaluated.

Methods

Bacterial strains and growth conditions

Bacteria were cultured in Miller's LB medium agar (2%) plates, incubated at 37ºC. Growth media would be supplemented with ampicillin (150 µg/mL) or chloramphenicol (25 µg/mL), for *E. coli* DH5α during plasmid construction and maintenance, and with chloramphenicol (200 µg/mL) for Bcc strains (*B. cenocepacia* K56-2, *B. cenocepacia* J2315, *B. multivorans* LMG 1660, *B. dolosa* AUO158).

Construction of plasmids

To silence ncS06, two plasmids expressing ncS06 antisense sequences were constructed. Two single-stranded oligonucleotides with complementary sequences pMBJ1_Fw & pMBJ1_Rv, and the pMBJ2_Fw & pMBJ2_Rv were annealed to form a double strand DNA sequence, subcloned in pUC19 plasmid, and digested with NdeI/XbaI. The resulting fragments were inserted into the pIN29 plasmid yielding the pMBJ1 plasmid and the pMBJ2 plasmid. The ncRNA3 sequence was amplified, digested with NdeI and XbaI restriction enzymes and was inserted into the pIN29 plasmid, yielding the pMBJ3 plasmid. Primers used in this study are represented in **Table 1.**

sRNA Target Search

A list of *B. cenocepacia* sRNAs was compiled by Pita et al., gathering information regarding putative sRNAs detailed in literature beforehand. The targets of 167 sRNAs were predicted using the TargetRNA2 web server[26] from thethe *B. cenocepacia* J2315 genome. For each sRNA, all the putative targets for the three replicons were collected. A list of Bcc genes described in literature as related to antibiotic resistance was constructed. The selection of the sRNAs was performed by filtering the IDs of the predicted targets with the IDs of genes related to antibiotic resistance. For some sRNAs, a second bioinformatic tool, CopraRNA[27], was used to confirm the predicted targets.

Table 1. Primers used in the current study (Fw – Forward; Rv – Reverse). Sequences recognized by restriction enzymes are underlined.

Primer Name	Primer Sequence (5' to 3')	Restriction site	Product Size (bp)
pMBJ1_Fw	CATATGTCTGCTACCCCGTAGAACTTATCTATTCTTTTC ATTTCTAGA	NdeI	-
pMBJ1_Rv	TCTAGAAATGAAAAGAATAGATAAGTTCTACGGGGTAG CAGACATATG	XbaI	-
pMBJ2_Fw	CATATGCAGACGACGGAACGTCGCTTATGTGCAAGTCG GCCTGCATCTAGA	NdeI	-
pMBJ2_Rv	TCTAGATGCAGGCCGACTTGCACATAAGCGACGTTCCG TCGTCTGCATATG	XbaI	-
pMBJ3_RC_Fw	TGTCGTTCGACCGATGTGC	-	1072
pMBJ3_RC_Rv	AGACCAGCGACGGCGAATATG	-	
pMBJ3_Fw	CATATGGGCGGCCGCCGGTGCCAG	NdeI	161
pMBJ3_Rv	CCGTCTAGAGACGCGCGCAAAGCAGC	XbaI	
RT-PCR Primers			
BCAM1421_Mul_Fw	GCTGCCGTCGATCAACATT	-	143
BCAM1421_Mul_Rv	TCGACTCGATGTGCTGGATG	-	
BCAL2915_Fw	TGACGACGTTGACCCTGAT	-	83
BCAL2915_Rv	CTCGGGAAGTTTCCAGGGC	-	
Bmul_4465_Fw	CTCTCGCAATCGATCCTGCTC	-	139
Bmul_4465_Rv	GCCAGTTGTAGCTGTCGGT	-	
ncS06_Fw	AGAATAGATAAGTTCTACGGGGTAGCA	-	169
ncS06_Rv	TCAGACGACGGAACGTCGCTTATG	-	
ncRNA3_Fw	CGCGTCGTTCCGATAAATGCAA	-	80
ncRNA3_Rv	CAAAGCAGCTATGCCCGTAAGT	-	
5SrRNA Fw	ACCATAGCGAGTCGGTCCCA	-	85
5SrRNA Rv	ACACGGGAATCCGCACTATCAT	-	
***In vitro* Transcription Primers**			
BCAL2915_IV_Fw	GTTTTTTTTAATACGACTCACTATAGGAAAATCGGCCCAT TCCGT	-	155
BCAL2915_IV_Rv	AGATCCTCGGGAAGTTTCCA	-	
BCAL2915_RC_Fw	GGAAACGCCGACGTCCCTA	-	471
BCAL2915_RC_Rv	CGTCGAAGTCCGCATCGATCT	-	
ncRNA3_IV_Fw	GTTTTTTTTAATACGACTCACTATAGG GCGGCCGCCGGTGCCAG	-	172
ncRNA3_IV_Rv	GACGCGCGCAAAGCAGC	-	

MIC Determination

The MICs of three antibiotics, ciprofloxacin, tobramycin and trimethoprim were determined using the microbroth dilution method, according to the to the International Standard ISO 20776-1 and EUCAST recommendations. *E. coli* ATCC 25922 strain was used as a control. 96-well polystyrene microtiter plates (Greiner Bio-One) were used, and stock solutions of Tobramycin (5 mg/mL), Ciprofloxacin (5 mg/mL) were prepared with deionized water and the stock solution of Trimethoprim (50 mg/mL) was prepared with DMSO. The plates were incubated at 37ºC for 24h and the wells were examined for turbidity (growth), measuring their optical density in a SPECTROstar Nano microplate reader (BMG Labtech) at 640 nm, after resuspending each well by pipetting. Minimum inhibitory concentration (MIC) values were estimated after data fitting of the OD_{640} mean values using a modified Gompertz equation as described by Lambert and Pearson, using the GraphPad Prism software (version 6.07).

Biofilm and Motility Assays

Biofilm formation on the surface of polystyrene by *B. multivorans* strains was quantified using the dye crystal violet, according with previously described and adapted methodology[28]. Swimming and swarming motility assays were performed for *B. multivorans* using previously described methodologies[29,30]. Agar plates containing 20 mL of swimming or swarming media were spot inoculated with 1 μL of bacterial cultures with an OD_{640} of 1. The plates were incubated for 72 hours, with no agitation at 37 °C. The halos formed in each plate were measured every 24 hours.

Results and Discussion

Searching for Putative sRNAs involved in Bcc Antibiotic Resistance

To select sRNAs putatively involved in Bcc resistance to antibiotics, targets of about a hundred sRNAs identified in *B. cenocepacia* were predicted using bioinformatic tools. For that, the sequences of 167 sRNAs that have been described, observed, or predicted in *B. cenocepacia* strains [25] were collected (Table S1 from Pita et al., 2018), and a list of antibiotic resistance genes described for Bcc bacteria was compiled. TargetRNA2[26] was used for a primary assessment of sRNAs targets, using the three replicons of *B. cenocepacia* J2315. 16 predicted sRNAs were selected for having as predicted targets three or more genes related with antibiotic resistance, or for targeting a gene involved in drug target modification, such as the ncRNA3. As most of the *B. cenocepacia* sRNAs are poorly characterized, the sRNAs ncS03, ncS06 and ncS54, which are validated and are abundant in *B. cenocepacia* J2315 biofilms, were considered for further analysis. Three genes related with antibiotic resistance, two efflux pumps from the RND (Resistance-Nodulation-Cell Division) family and one efflux pump from the MFS (Major Facilitator Superfamily), were predicted to be targeted by the sRNA ncS06, and recent results of our research group indicate that ncS06 influences the virulence of *B. cenocepacia* in a nematode model. Considering this and the importance of efflux pumps for antibiotic resistance this small RNA was the first to be evaluated. While ncS06 has predicted to target several genes associated with antibiotic resistance, the sRNA ncRNA3 was predicted to target the BCAL2915 gene (*dfrA*), which encodes for a dihydrofolate reductase. Dihydrofolate reductase is a key enzyme in the folate metabolism, catalyzing essential reactions for the synthesis of DNA precursors and essential amino acids. Trimethoprim, a heavily used antimicrobial to treat Bcc infections, targets and binds to dihydrofolate reductase, inhibiting its activity[31,32], disrupting the biosynthetic pathways associated with this enzyme, and leading to cell death[33]. Thus, ncRNA3 was also considered an sRNA of interest for further studies due to the specificity of its targets and direct impact in antimicrobial resistance.

How does ncS06 sRNA affect antimicrobial resistance in Bcc bacteria?

ncS06, a sRNA with a predicted sequence of 259 nucleotides, is encoded in chromosome 1 of *B. cenocepacia* J2315. This sRNAs has been reported to be conserved among Bcc species, which is favorable for testing the antimicrobial susceptibility in diverse Bcc bacteria[25]. This sRNA was overexpressed and silenced in three of the most clinically relevant Bcc species (*B. cenocepacia*, *B. multivorans* and *B. dolosa*). To assess whether differences in the expression of ncS06 affect the antibiotic susceptibility of Bcc strains, the MIC values of the antibiotic's tobramycin, ciprofloxacin and trimethoprim were determined for the Bcc strains carrying the different constructs, using the broth microdilution method. A significant decrease in the MIC value of ciprofloxacin was observed for *B. multivorans* LMG 16660 overexpressing the ncS06 (pTAP3). While a MIC value of 16.36 ± 0.87 µg/mL was obtained for the *B. multivorans* strain carrying the empty plasmid pIN29, this value decreased significantly to 11.16 ± 1.64 µg/mL when the plasmid pTAP3 was introduced in *B. multivorans*. It is well known that efflux mechanisms are important determinants for Bcc resistance to antimicrobials[11], and three of the predicted targets for ncS06 were efflux pumps. Chromosomal mutations that alter DNA gyrase or topoisomerase IV, the upregulation of the expression of native efflux pumps and the alteration of the amount or porins types are some of the mechanisms that can be involved in the increased resistance of *B. multivorans* to ciprofloxacin. Considering the lack of specificity of the efflux pumps predicted as ncS06 targets for ciprofloxacin, and the effect of ncS06 overexpression only on the susceptibility of *B. multivorans* to this antibiotic, it is plausible that ncS06 is also regulating the expression of other efflux pumps, porins or other membrane components involved in the resistance of *B. multivorans* LMG 16660 to ciprofloxacin.

As an attempt to identify if the alterations in antimicrobial susceptibility, observed by overexpressing ncS06 in *B. multivorans* LMG 16660, are related to changes in the expression of the ncS06 predicted targets, qRT-PCR assays were performed. For this, in addition to evaluate the ncS06 expression levels in *B. multivorans*, the expression of the predicted targets BCAM1421 and BCAL2915 was determined. The relative expression levels are represented in **Figure 1**. The relative expression levels obtained indicate that in *B. multivorans* LMG 16660 the pTAP3 plasmid is indeed significantly overexpressing the ncS06 sRNA. The silencing of this sRNA was also confirmed in B. multivorans carrying pMBJ2, one of the plasmids containing an antisense sequence for ncS06 (**Figure 1A**).

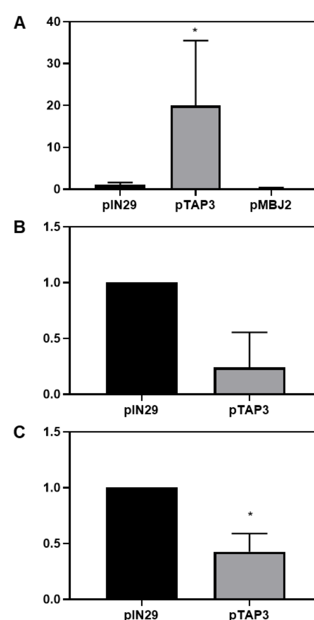

Figure 1. Relative expression levels of ncS06 and predicted gene targets. Relative expression values for ncS06 (**A**); BCAM1421 (**B**) and BCAL2915 (**C**) on *B. multivorans* LMG 16660 carrying the pIN29 (empty vector), pTAP3 (ncS06 overexpression) and pMBJ2 (ncS06 antisense) plasmids. Quantitative real-time PCR was performed, and the resulting Ct values were normalized accordingly between samples. Error bars stand for standard deviation of the mean values for the normalized Ct values. The p-value was determined with one-way ANOVA and represented with * when the p-value<0.05.

As for the expression of the targets, despite a slight decrease in BCAM1421 expression levels, no significant differences were observed in the expression of this gene when ncS06 was overexpressed in *B. multivorans* LMG 16660 (**Figure 1B**). BCAM1421 encodes for an efflux pump from the RND family and the wide abundance of efflux pumps in Bcc bacteria poses an obstacle

for a complete understanding of the underlying mechanisms and direct interactions that lead to antimicrobial resistance[12]. On the other hand, the overexpression of ncS06 in *B. multivorans* LMG 16660 led to a significant reduction in the expression of the BCAL2915 gene (**Figure 1C**).Since the gene BCAL2915 codes for the enzyme dihydrofolate reductase, that binds to trimethoprim inhibiting the folic acid synthesis pathway[31], it would be expected that a slight increase in the expression levels of this gene could be the reason why the overexpression of ncS06 increased trimethoprim MIC values in both *B. multivorans* LMG 16660 and *B. cenocepacia* K56-2 strains. However, in the tested conditions, this was not the case, suggesting that other targets should be regulated by ncS06. In addition to the targets selected as being directly involved in antibiotic resistance, several membrane proteins and genes described as virulence factors were also predicted to be targeted by this sRNA. Unlike the other analyzed species, the colonies morphology of *B. multivorans* LMG 16660 overexpressing ncS06 was altered, presenting changes in the colonies overall shape and margin (**Figure 2**). Taking into account all the previous information, this seems to suggest that the overexpression of ncS06 has an effect in multiple targets that can affect various cellular processes of Bcc bacteria.

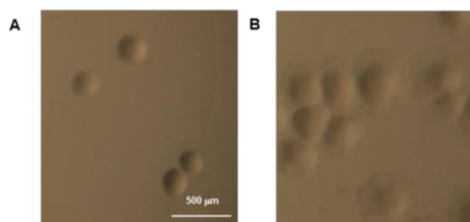

Figure 2. *B. multivorans* LMG 16660 colonies carrying the pIN29 (A) and pTAP3 (B) plasmids. The images of bacterial colonies were captured using an AxioCam 503 color device coupled to the Zeiss Stemi 2000-C Stereo Microscope.

Overexpression of ncS06 influences several phenotypes of *B. multivorans* LMG 16660

Aside from the genes related with antibiotic resistance that were predicted as targets for ncS06, other predicted targets for this sRNA were associated with the synthesis of LPS, motility (flagellum), secretion systems and other membrane proteins. To better understand how *B. multivorans* LMG 16660 carrying the pTAP3 plasmid overexpressing the sRNA ncS06 exhibited different resistance to antibiotics, phenotypic assays such as swimming and swarming assays were performed, as well as crystal violet biofilm assays. The results for these assays are presented in **Figure 3**.

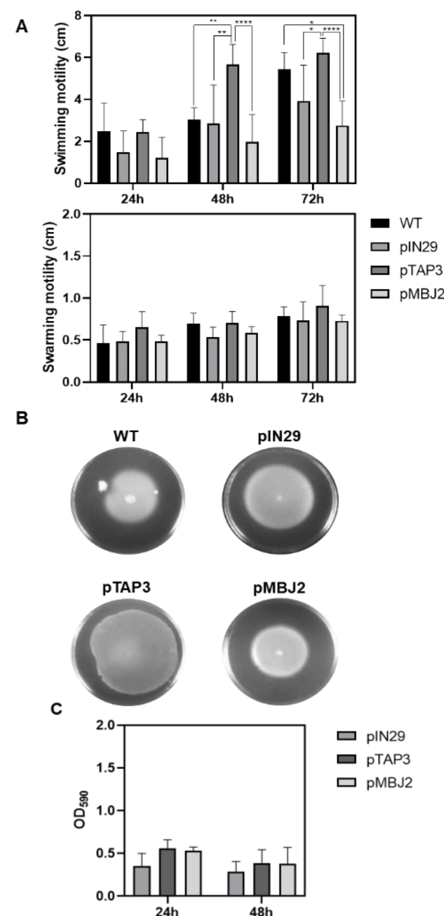

Figure 3. *B. multivorans* LMG 16660 motility and biofilm formation assays. Swimming and Swarming motility measurements for *B. multivorans* LMG 16660 strains (WT, pIN29, pTAP3 and pMBJ2) at 24h,48h and 72h (**A**). Swimming plates after 72h of incubation. Pictures were taken with the gray scale digital camera model CFW-1312M (**B**). Biofilm formation assay of *B. multivorans* LMG 16660 strains (**C**). Error bars stand for standard deviation of the mean values for at least 3 independent assays. The p-value was determined with one-way ANOVA and represented with * when the p-value<0.05, with ** when the p-value<0.01 and **** when the p-value<0.0001.

The motility of bacterial cells, especially for Bcc bacteria, is a crucial factor for bacterial virulence, antimicrobial resistance, and establishment of infections[34].Regarding the motility assays, no significant changes were verified in swarming

motility when ncS06 was overexpressed in *B. multivorans* LMG 16660. *B. multivorans* overexpressing ncS06 displayed greater swimming motility at 48 and 72 hours, when compared with *B. multivorans* without any plasmid (WT), carrying the empty vector pIN29 or the ncS06 silencing plasmid pMBJ2 (***Figure* 3B**). The morphology of the swimming ring exhibited by *B. multivorans* carrying pTAP3 was also notoriously different from those formed by *B. multivorans* carrying the other plasmids (**Figure 3A**). Flagellar motility is considered one of the many virulence factors that Bcc bacteria possess and are reported to be involved with the motility, adhesion, invasion, and biofilm formation of bacterial cells[35,36]. During the target prediction for ncS06, the *fliH* (BCAL0523), *fliP* (BCAL3503) and *flgH* (BCAL0570) genes were predicted targets of this sRNA. Although these targets are not reported to be directly involved with antibiotic resistance, it would be very likely that their dysregulation could influence the motility of *B. multivorans* LMG 16660. Thus, the possible influence of the ncS06 overexpression on the expression of these targets, could justify the changes in swimming motility verified for *B. multivorans* carrying the pTAP3 plasmid. Under the tested conditions, *B. multivorans* LMG 16660 did not form biofilms, which was inferred based on the too low absorbance values obtained for the 24 and 48h experiments. Although it was not possible to establish a direct relationship between the ncS06 overexpression and the differences in the antibiotic resistance of *B. multivorans*, the results obtained in the phenotypical assays suggest that ncS06 can regulate several bacterial processes. This broader regulation can induce changes in bacterial cells that indirectly influence their resistance to antibiotics.

Role of the sRNA ncRNA3 for the Bcc resistance to antibiotics

ncRNA3 is a small RNA identified through RNA-seq by Yoder-Himes *et* al. in 2009[37] as containing 129 nucleotides. ncRNA3 is encoded in chromosome 1 and is extremely conserved in *B. cenocepacia* strains but not conserved in Bcc species. Only one target related with antimicrobial resistance was predicted for ncRNA3, the trimethoprim resistance gene BCAL2915, so this antibiotic was chosen to perform the MIC assays. For this, *B. cenocepacia* K56-2 and *B. multivorans* LMG 16660 strains carrying the empty plasmid pIN29, or the pMBJ3 plasmid overexpressing the sRNA ncRNA3 were used. A significant increase in the MIC value was only observed for *B. cenocepacia* K56-2. While a trimethoprim MIC value of 7.99 ± 1.90 µg/mL was determined for *B. cenocepacia* K56-2 carrying the pIN29 empty vector, a value of 20.42 ± 1.10 µg/mL was obtained for this strain carrying the ncRNA3 expressing plasmid pMBJ3. The BCAL2915 mRNA was also predicted as a ncS06 target when the search was performed against the genome of *B. multivorans*. For both ncRNA3 and ncS06 sRNAs, BCAL2915 was the only target predicted to be involved in antibiotic resistance through a mechanism of drug target alteration (trimethoprim). To verify if the difference in the MIC value obtained corresponds to an effect directly related with BCAL2915, the expression level of this gene was assessed, similarly to what was done previously for ncS06. (**Figure 4**).

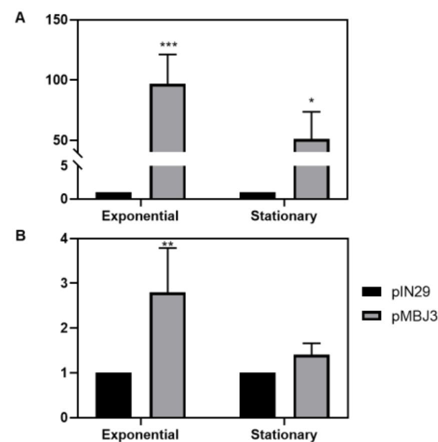

Figure 4. Relative expression levels of ncRNA3 and BCAL2915 in *B. cenocepacia* K56-2. The relative expression levels of ncRNA3 (**A**) and BCAL2915 (**B**) were determined for *B. cenocepacia* K56-2 carrying the pIN29 (empty vector) and pMBJ3 (ncRNA3 overexpression) plasmids in exponential and stationary growth phases. Error bars stand for standard deviation of the mean values for the normalized Ct values. The p-value was determined with one-way ANOVA, represented with * for p-value<0.05, ** when p-value<0.01 and *** when p-value<0.001.

The results clearly show that the pMBJ3 plasmid is significantly inducing the overexpression of ncRNA3 in *B. cenocepacia* K56-2, confirming the desired effect of this plasmid. This overexpression is especially accentuated during the exponential growth phase (**Figure 4A**). When ncRNA3 was overexpressed in *B. cenocepacia* K56-2, the BCAL2915 expression levels also increased.

As previously mentioned, the overexpression of BCAL2915, the gene that encodes for the DfrA enzyme to which trimethoprim binds to, was expected to lead to an increase in the resistance to this antibiotic. That was precisely the effect verified for the expression of BCAL2915 when the ncRNA3 was overexpressed. These results suggest a direct effect of ncRNA3 on its predicted target related with antimicrobial resistance, which actually influences the *B. cenocepacia* K56-2 resistance to trimethoprim. To better understand if ncRNA3 is sorting a direct effect over BCAL2915, the interaction between these two molecules was tested.

<u>Direct RNA-RNA interaction between ncRNA3 and its target BCAL2915</u>

To explore if the sRNA ncRNA3 interacts directly with its predicted target BCAL2915, an Electrophoretic Mobility Shift Assay (EMSA) was performed. It was predicted that the 3' end of the sRNA ncRNA3 interacts with the 5' untranslated region (5' UTR) of the BCAL2915 gene and the energy predicted for this interaction was -12.87 kcal/mol, favoring the interaction. The predicted interaction region of the sRNA is extremely conserved in various species of *B. cenocepacia*. To prove the interaction between these two molecules, the 129 nucleotides of the sRNA ncRNA3, and a 128 nucleotides RNA molecule containing the predicted interaction region of the BCAL2915 mRNA (nucleotides -39 to 89), were *in vitro* transcribed. Electrophoretic mobility shift assays were performed, incubating the RNA molecule labelled with Biotin with increasing concentrations of the unlabelled RNA molecule, and running these samples in a native polyacrylamide gel. Two gels were run, one in which the sRNA ncRNA3 was labelled, and another one where BCAL2915 was labelled, and the results are represented in **Figure 5**.

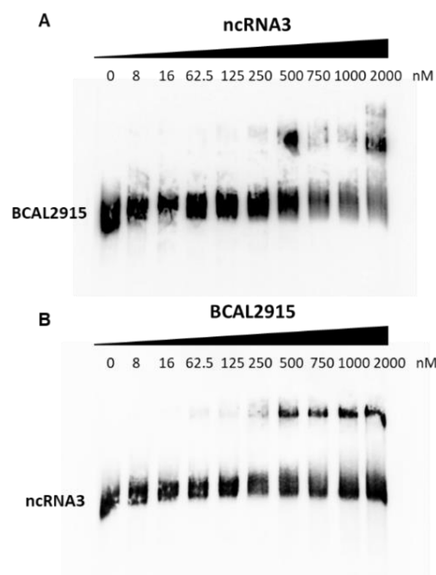

Figure 5. The sRNA ncRNA3 interacts with the BCAL2915 RNA. EMSA interaction results for BCAL2915 (**A**) and for ncRNA3 (**B**) labelled with Biotin. For each assay, increasing concentrations of unlabelled ncRNA3 (**A**) or BCAL2915 (**B**) were used against a fixed concentration of the other molecule.

The results clearly show that ncRNA3 interacts with its predicted target BCAL2915by the gel shift. In both gels, the interaction becomes notoriously visible when the labelled RNA is mixed with 250 nM of the unlabelled RNA. These results suggest a direct regulatory effect of the sRNA ncRNA3 in its predicted target BCAL2915, which had previously been identified as being involved in Bcc resistance to antibiotics. Although other pathways could be affected by the overexpression of this sRNA, the direct regulation of BCAL2915 expression by ncRNA3 should be one of the main reasons for the increased resistance to trimethoprim when the ncRNA3 is overexpressed in *B. cenocepacia* K56-2.

Conclusion

In the present work, 78 sRNAs from *B. cenocepacia* were predicted to target at least one gene related with antimicrobial resistance in Bcc.

Two of these sRNAs were selected, ncS06 and ncRNA3, and the impact of their overexpression in Bcc antimicrobial resistance was demonstrated. The overexpression of ncS06, a sRNA for which 5 target genes related with antimicrobial resistance were predicted, led to an increased susceptibility of *B. mul*tivorans LMG 16660 to ciprofloxacin. Although, the direct effect of ncS06 on a specific target has not been shown, the changes of the overexpression of these sRNA in *B. multivorans* colony morphology, motility, and membrane protein profile suggest that this sRNA could be influencing several genes in regulatory networks that can modulate the membrane composition. On the other hand, the overexpression of ncRNA3 led to an increased resistance of *B. cenocepacia* K56-2 to trimethoprim, which seems to be directly related to the regulation and interaction with the drug target modification gene *dfrA* (BCAL2915). While ncS06 sRNA seems to contribute to multiple antibiotics susceptibility through direct regulatory interactions with mRNAs involved in drug import, efflux, cell-wall synthesis, or even promoting antibiotic-tolerant lifestyles; ncRNA3 confers resistance to a specific antibiotic by interacting with a specific target modification mRNA. The effects sorted by ncS06 still remain uncertain, but the analysis of *B. multivorans* transcriptome would give insights into the messengers that are regulated by this sRNA.

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