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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I declare that this document is an original work of my own authorship and that it fulfils all the requirements of the Code of Conduct and Good Practices of the *Universidade de Lisboa*.

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Abstract

Bacteria of the *Burkholderia cepacia* complex (Bcc) display high-level of resistance to antibiotics, are responsible for severe clinical prognosis in immunocompromised patients, and can chronically persist in the infected host. The development of alternative therapies to conventional antimicrobials is urgent, as the options for current treatment are lacking. sRNAs are key gene expression regulators that coordinate several bacterial responses, contributing to antibiotic resistance. The targets of 167 sRNAs identified in *B. cenocepacia* were predicted bioinformatically to select possible molecules involved in Bcc antibiotic resistance. At least a target gene related with antibiotic resistance was found for 78 of these sRNAs. Two sRNAs, ncS06 and ncRNA3, were selected and the effect of their overexpression or silencing in the susceptibility of three clinically relevant Bcc strains to different antibiotics was tested. The overexpression of ncS06 increased the susceptibility to ciprofloxacin in *B. multivorans* and the overexpression of ncRNA3 increased trimethoprim resistance in *B. cenocepacia*. The *dfrA* gene, a ncRNA3 target involved in *B. cenocepacia* resistance to trimethoprim, was upregulated when ncRNA3 was overexpressed, and the interaction between them was confirmed *in vitro* by Electrophoretic mobility shift assay. Although no direct targets have been demonstrated, the colony morphology, motility, and the outer membrane profile of *B. multivorans* were altered when ncS06 was overexpressed, suggesting that ncS06 controls mRNAs involved in multiple physiological processes. These results corroborate the importance of sRNAs in the regulation of antibiotic resistance, and the understanding of these regulatory mechanisms is an asset for the development of new antimicrobial therapies.

Keywords

Antibiotic Resistance

Burkholderia cepacia Complex

Small non-coding RNAs

Resumo

Bactérias do complexo *Burkholderia cepacia* (Bcc) apresentam elevados níveis de resistência a antibióticos, são responsáveis por prognósticos clínicos graves em indivíduos imunocomprometidos e podem persistir cronicamente em hospedeiros. O desenvolvimento de alternativas às terapias convencionais com antimicrobianos é crucial e urgente. Os pequenos RNAs não codificantes (sRNAs), importantes reguladores da expressão genética, coordenam variadas respostas bacterianas incluindo a resistência a antimicrobianos. Os alvos de 167 sRNAs identificados em *B. cenocepacia* foram previstos, selecionando os que pudessem estar envolvidos na resistência a antibióticos. Pelo menos um gene alvo descrito como estando envolvido na resistência a antibióticos foi previsto para 78 sRNAs. Os sRNAs ncS06 e ncRNA3 foram selecionados, testando os seus efeitos de sobrexpressão e silenciamento na sensibilidade a antibióticos em três espécies do Bcc. A sobrexpressão de ncS06 em *B. multivorans* levou a um aumento da suscetibilidade destas bactérias à ciprofloxacina. A sobrexpressão de ncRNA3 em *B. cenocepacia* levou a um aumento da resistência destas bactérias ao trimetoprim. O gene *dfrA*, um alvo previsto para o ncRNA3 envolvido na resistência de *B. cenocepacia* ao trimetropim, está mais expresso quando este sRNA é sobrexpresso, sendo a sua interação confirmada *in vitro*. Embora não se demonstre efeitos do ncS06 nos alvos previstos, a morfologia das colónias, motilidade e perfil da membrana externa de *B. multivorans* estavam alterados quando o ncS06 foi sobrexpresso, sugerindo que o ncS06 regula mRNAs envolvidos em múltiplos processos fisiológicos. Estes resultados corroboram a importância dos sRNAs bacterianos na regulação da expressão de genes envolvidos na resistência a antimicrobianos.

Palavras-chave

Resistência a Antibióticos

Complexo *Burkholderia cepacia*

Pequenos RNAs não codificantes

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List of Abbreviations

Bcc - Burkholderia cepacia Complex

sRNAs - Small non-coding RNAs

RNA - Ribonucleic acid

MIC - Minimum Inhibitory Concentration

LPS - Lipopolysaccharide

EPS - Exopolysaccharide

MDR - Multidrug Resistant

IFN - Interferon

RT-PCR - Quantitative Real-Time PCR

EMSA - Electrophoretic Mobility Shift Assay

Ct - Cycle Threshold

rRNA - Ribosomal RNA

DNA - Deoxyribonucleic acid

MLST - Multilocus Sequence Typing

CF - Cystic Fibrosis

CFTR - Cystic fibrosis Transmembrane Conductance Regulator

PHDC - Philadelphia District of Columbia

CGD - Chronic Granulomatous Disease

ROS - Reactive Oxygen Species

Ara4N - Amino-4-deoxyarabinose

AHL - Acyl Homoserine Lactones

ABC - ATP-binding Cassette

MFS - Major Facilitator Superfamily

RND - Resistance Nodulation Division

PUFAs - Polyunsaturated Fatty Acids

PAAG - Poly (acetyl, arginyl) glucosamine

CAMPs - Cationic Antimicrobial Peptides

ppGpp - Guanosine 5′-diphosphate 3′-diphosphate

pppGpp - Guanosine 5′-triphosphate 3′-diphosphate

QSI - Quorum Sensing Inhibitors

BH - Baicalin hydrate

CA - Cinnamaldehyde

RBS - Ribosome Binding Site

mRNA - Messenger RNA

LB - Lysogeny Broth

EDTA - Ethylenediamine tetraacetic acid

PCR - Polymerase Chain Reaction

OD - Optical Density

EUCAST - European Committee on Antimicrobial Susceptibility Testing

MHB - Mueller Hinton Broth

CFU - Colony Forming Unit

SDS-PAGE - Sodium dodecyl sulfate–polyacrylamide Gel Electrophoresis

APS - Adenosine 5′-phosphosulfate

TEMED - Tetramethylethylenediamine

TBE - Tris/Borate/EDTA

kDa - kilodalton

UTR - Untranslated Region

1. Introduction

1.1 The *Burkholderia cepacia* complex

The *Burkholderia cepacia* complex (Bcc) comprises a group of over 20 closely related species that often act as opportunistic pathogens in diverse hosts ranging from plants, animals, and humans, and are widespread in diverse environments [1,2]. Ubiquitous in soil, frequently found in the rhizosphere as well as in man-made environments and even water, organisms from this complex often present as much biotechnological potential as they pose serious health threats [3].

The bacterial species to which this complex is named was originally described in 1950 by Burkholder from samples of rotten onion bulbs, being formally named *Pseudomonas cepacia*. Later on, the introduction of the *Burkholderia* genus in 1992 by Yabuuchi *et* al. assigned the now *Burkholderia cepacia* as its type-species [4].

The Bcc grouping was created due to the high heterogenicity in many different *B. cepacia* isolates from distinct niches [1]. These isolates were initially divided into genomovars, according to their phylogeny and phenotypes, and naming the genomovar groups collectively as the *Burkholderia cepacia* complex [5]. As typing and sub-typing techniques developed and evolved, more species of the *Burkholderia* genus were added into the complex and the species included were better characterized and described as separate species with formal terminology [6].

Members of the Bcc are characterized as gram-negative, rod-shaped, obligate aerobes, belonging in the β-proteobacteria class [7, 8]. They share higher 16S rRNA similarity amongst themselves rather than with other species that belong in the *Burkholderia* genus. That is also verified for DNA-DNA hybridization, which is higher in species within the Bcc and relatively lower comparing with *Burkholderia* sp. species that do not belong to the mentioned complex. [9]

Bcc species have shown to be extremely versatile and have high adaptability to surrounding environments, presenting large genomes that range from 6 to over 9 Mb [10], usually organized in 3 chromosomes and one to five plasmids, and with an approximate GC content of 67% [3]. Part of the adaptability of Bcc bacteria derives from the large genomic content, enabling the acquisition or loss of genetic elements. In fact, it has been shown that over 10% of Bcc genomes were acquired through horizontal gene transfer, and over time bacteria from this complex accumulated genomic islands that contribute to the virulence of epidemic strains [3].

B. cenocepacia J2315, was the first strain from the Bcc to have its genome sequenced [11]. Currently, 217 genomes from Bcc strains have been fully sequenced and annotated, and many others remain incomplete [12].

Bacteria from the *Burkholderia* genus are widely diverse, especially regarding their phenotypes [3]. While the correct identification of these bacteria with standardized methods is challenging, distinguishing between Bcc and non-Bcc bacteria becomes impossible with methods such as biochemical testing [9]. Especially in clinical cases, it is crucial to determine efficiently the Bcc species and strains that are causing the infections, since it will influence the treatment decisions. If the conventional phenotypical methods fail to identify Bcc species, the use of molecular typing methods becomes crucial. Several techniques have been implemented successfully but, currently, the MLST

(Multilocus Sequence Typing), a DNA sequence-based typing method based on the comparison of the genomic sequences of seven house-keeping genes common to Bcc species, is used to characterize the isolates based on their differential genomic profiles [13]. MLST currently stands as the golden standard of Bcc typing techniques, especially for isolates of infected patients [3]. This technique offers comparable and reproducible results and is relatively accessible, despite its expenses and time-consuming nature [14].

Species belonging to the Bcc have been described as a great source of natural products, since they often establish symbiotic relationships with other organisms in the rhizosphere, producing a vast range of antimicrobial and antifungal metabolites, promoting plant growth, and fixating nitrogen [15]. The potential of bacteria in this complex is also shown through their ability to degrade several compounds including soil and water pollutants, herbicides, and even xenobiotics [3]. Although these features of species in the Bcc sparked interest for industrial and agricultural use, many species of this complex are known human pathogens and therefore not recommended for industrial use [16]. The commercial use of Bcc species may lead to environmental contamination and consequently increase human exposure, resulting in possible outbreaks.

1.2 Bcc Bacteria as Pathogens

Since the 1980s members of the Bcc have been identified in opportunistic human infections, most commonly in patients with Cystic Fibrosis (CF). This autosomal recessive disease, the most common in Europe and Caucasians [14], is characterized by the accumulation of thick mucus in the epithelium of patients' airways, due to a mutation in the chloride channel CFTR (Cystic fibrosis transmembrane conductance regulator). Besides the natural progression and degradation of organs and tissues that leaves these patients severely vulnerable and immunocompromised, the overflow of secretions in respiratory tissues generates an optimal niche for bacterial infections to spread and form biofilms [14]. In fact, CF patients are often subject to chronic bacterial infections that are extremely difficult to control and treat, representing ultimately over 80% of the mortality of these patients. Various species of bacteria can inhabit these damaged tissues, but the major bacterial threats to CF patients' health are *P. aeruginosa* and bacteria from the Bcc [17]. Bacteria from the Bcc only infect around 3.5% of patients with Cystic Fibrosis, but their infections are particularly aggressive and highly transmissible between patients, with very limited options for treatment due to their intrinsic resistance to most antibiotics [18].

Several Bcc species are capable of causing infections, but the most prevalent species, namely from CF patients, are *B. cenocepacia* and *B. multivorans*. In fact, these species account for over 80% of Bcc infections in CF patients worldwide [13]. The prevalence of Bcc strains is heavily discriminated by geographical location, as some areas have *B. multivorans* strains as the most prevalent in infections. *B. cepacia*, *B. dolosa* and *B. contaminans*, although taking part in a much smaller percentage of Bcc infections, present some patterns and relevant occurrence in certain countries and CF centers. This may have occurred due to the preventive measures imposed to mitigate the spread of *B. cenocepacia* and *B. multivorans*, allowing these less-occurring species to become more prevalent [3,6].

One of the best-known groups of epidemiological strains is the Edinburgh-Toronto lineage, also known as electrophoretic type 12 (ET-12) [11]. *B. cenocepacia* highly transmissible strains are frequently associated with "cepacia syndrome". This syndrome is characterized by the rapid health decline in patients infected with Bcc strains, including high fevers, severe decline in lung function, necrosis, organ failure, and sepsis, being associated with higher rates of mortality [19]. ET12 strains are of extreme relevance due to their violent symptom progression and transmissibility, which can occur via air droplets or patient's direct contact. In the late 1980's and 1990s, an intercontinental spread of this lineage occurred amongst CF patients in the UK and Canada [7,20]. Other highly epidemic lineages of the *B. cenocepacia* species include the PHDC (Philadelphia-District of Columbia) and the MidWest strains, that although being originated from certain regions, are currently prevalent and widespread through different geographical regions [10], [21].

Being transmissible, able to cause severe symptoms, extremely resistant to antimicrobials, highly tolerant and persistent in diverse environments, and having a flexible genome, Bcc bacteria are not only threatening to CF patients but also to immunocompromised patients in hospital settings. Hospital outbreaks with Bcc bacteria have been associated with material and equipment contaminations, including catheters, air conditioning, and disinfectants[10,19]. Bcc bacteria are also commonly a threat for patients that suffer from CGD (Chronic Granulomatous Disease), a hereditary disease, caused by mutations in genes involved with the production of reactive oxygen species (ROS)[22]. The inability to properly produce these very important radicals makes these individuals much more susceptible to bacterial infections [23].

1.3 Virulence Factors

Species belonging to the Bcc can cause quite severe and difficult to treat infections, with multiple factors that improve and intensify their virulence and pathogenicity and allow their adaptation to new conditions and environmental changes. These highly mutable specimens present various structures, mechanisms, and metabolites that are critical to establish infection and allow their survival at the infection site.

Among the structures that play relevant roles in the early stages of Bcc-oriented infections, the flagella and pili are essential for the adhesion to surfaces and for early steps of invasion and interaction with the host cells. Many bacteria from this complex, especially most of the *B. cenocepacia* strains, are also capable of intracellular replication in epithelial cells and macrophages, evading then first-line responders from the host immune system [11].

Several factors are involved in Bcc virulence, including exopolysaccharides (EPS) and lipopolysaccharides (LPS) synthesis, quorum sensing systems, alternative sigma factors, biofilm formation, protein secretion systems, and siderophores synthesis[11].

1.3.1 LPS and EPS

Lipopolysaccharides (LPS) are one of the most important components of the outer membrane of Gram-negative bacteria, providing structure, stability, protection, and integrity to their membrane.

These molecules are composed of a core oligosaccharide, Lipid A and O-antigen. This last component has a very variable structure, and it is strain specific [24]. In addition to the variable O-antigen, the composition of LPS in Bcc bacteria is by itself unique, even at its core oligosaccharide (substitution of a Ko for a Kdo residue) and Lipid A composition. The presence of residues of amino-4-deoxyarabinose (Ara4N) attached to phosphate residues from Lipid A and the lack of negatively charged residues modifies the membranes' overall charge, restricting the binding of many antimicrobial substances [11]. Many *Burkholderia* species have also the ability to produce EPS, connected to the outermost layer of these bacteria. EPS have been described as involved with processes of cell recognition, environment-related stresses, absorption of water and ions, and biofilm formation. These long and variable polysaccharides, having Cepacian as the most common EPS produced in species belonging to the Bcc, have been described by their capability to interfere with phagocytosis by human neutrophils and to scavenge reactive oxygen species, essential for the viability of the cells in diverse environments [11,25].

1.3.2 Quorum Sensing Systems

Quorum sensing systems act as a form of communication between communities of bacteria, stimulating specific responses by emitting signals according to the current cell density [11]. When a certain threshold of cell density is achieved, many signaling molecules (given the name autoinducers) are produced by these bacteria, and gene responses are regulated accordingly, whether it consists of gene expression or repression [21]. These systems are often associated with stress responses, environment changes, biofilm formation, and virulence. Quorum sensing in Gram-negative bacteria usually implies the production of acyl homoserine lactones (AHL) as signaling molecules, produced by a LuxI homolog that binds to a transcriptional regulator (similar to LuxR in *Vibrio fischeri*) [26].

An AHL-directed system, CepIR, is conserved among Bcc species, but other systems are also used by these bacteria to form these intricate networks. The CciIR quorum system is found in *B. cenocepacia*, and the BviIR system is found in *B. vietnamensis*, both driven by the production of AHL molecules with LuxR and LuxI homologs [11]. In *B. cenocepacia* strains, a conserved quorum system non-dependent of AHLs, the CepR2, was also identified. In Bcc bacteria, there is also a quorum sensing system that is dependent on the production of BDSF, a diffusible signaling molecule that has been shown to regulate diGMP levels with the help of the receptor protein RpfR [21]. The *B. cenocepacia* J2315 and K56-2 strains contain all the QS systems described above, being then widely used in studies related to Bcc virulence.

Quorum sensing systems in the Bcc regulate several cell processes as motility, iron acquisition, biofilm formation, protease production, secretion systems, and overall enhancement of virulence, heavily contributing to the pathogenicity of some strains in this complex [11, 27].

1.3.3 Alternative Sigma Factors

Alternative sigma factors allow the initiation of the RNA transcription process, binding with prokaryotic RNA polymerases in a specific way in order to express genes involved with environment responses and virulence [28]. The RpoE factor is commonly associated with extracytoplasmic stress

response in bacteria. This factor in *B. cenocepacia* has been implicated in aiding bacterial intracellular survival within macrophages [11]. The RpoN factor has been reported to be involved in the nitrogen metabolism, metabolic adaptation to stress and environment responses, as well as virulence of some *B. cenocepacia* strains [11]. In some conditions, the synthesis of the exopolysaccharide Cepacian is also dependent of this sigma factor, since it leads to the expression of genes that are essential to the process [2129].

1.3.4 Biofilm Formation

Many bacterial species rely on biofilm formation for their establishment of virulence. The regulation of biofilm formation is highly dependent on metabolites and regulatory networks, and as mentioned above the production of exopolysaccharides, the alternative sigma factors, quorum sensing systems, and iron availability can affect the formation and composition of biofilms. The formation of these dynamic structures is intricate and complex, requiring the allocation of many cellular resources and restructuring of the bacterial communities, which involves fine control and regulation, but it also allows the exhibition of features that are most favorable for the survival of bacteria. While some bacterial functions such as motility are lost, when Bcc bacteria form biofilms, they are notoriously more tolerant to antibiotics, even at high concentrations, have enhanced defense against neutrophils, and produce fewer ROS (reactive oxygen species) [11]. Biofilms have been associated with persistence of infection, being quite common structures displayed by Bcc species and thereby making Bcc chronic infections so difficult to treat.

1.3.5 Protein Secretion Systems

Protein secretion systems are prevalent in all bacterial species, being used to, as the name refers, secrete proteins and other molecules to the extracellular space, whether it is into their surrounding environment or into other cells [11]. Bacteria from the Bcc present several types of secretion systems, most of them presenting some correlation with the virulence and pathogenesis of these bacteria. Many of these systems were studied and mostly characterized in *B. cenocepacia*. The type I and type II secretion systems are widespread in Bcc, namely in *B*. *cenocepacia* and *B. vietnamensis,* and they have been associated with hemolytic protein secretion [30]. Type III secretion systems of *B. cepacia* have shown to influence virulence in a murine model of infection[31]. In *B. cenocepacia*, two type IV secretion systems, related with virulence and intracellular survival; four type V secretion systems, involved in bacterial adhesion; and a type VI secretion system, that alters the cytoskeleton structure of invaded macrophages, were described [11].

1.3.6 Iron Acquisition

Iron is a crucial resource in all living beings for regular biologic function. Bacteria often present difficulties in acquiring iron when invading a host, especially mammals due to their differential iron acquisition and retaining abilities [21]. Bcc bacteria make use of siderophores to capture and transport iron through their cells, presenting specific receptors in their membranes for this purpose. Four different siderophores were described in Bcc bacteria: ornibactin, pyochelin, cepabactin, and cepaciachelin [11].

Although these iron uptake mechanisms are not directly associated with virulence, they are often necessary for host interaction and, ultimately, for bacteria to be able to activate their other virulence factors correctly [21].

1.3.7 Two-Component Systems

Two-component systems are yet another mechanism that cells use to respond to environmental changes. These systems are formed by a histidine kinase, that in response to a specific stimulus autophosphorylates and transfers this phosphoryl group into a response regulator. The phosphorylation of the response regulator, will mediate the expression of specific genes, allowing a response to the changes in the cell's surroundings. 28 putative two-component systems have been predicted in bacteria from the Bcc, yet many remain uncharacterized. Systems like the BceSR system, the AtsR/AtsT system, and the FixLJ system have been linked with motility, biofilm formation, intracellular invasion, virulence, quorum sensing, and protease production [21], [29].

1.4 Antibiotic Resistance

In addition to virulence factors and many other molecules that enhance their pathogenicity, Bcc bacteria are incredibly persistent when invading hosts and possess innate resistance to several classes of antibiotics.

1.4.1 Induced vs. Acquired Resistance

Antibiotic resistance can be intrinsic if certain bacterial traits are universally expressed among the species evaluated, and not dependent on exposure to the antibiotic. One of the most common examples, which applies to Bcc bacteria, is the intrinsic resistance of Gram-negative bacteria to antibiotics due to the structure of their outer membrane [32], [33].

Induced resistance is derived from naturally present resistance-related genes in bacteria. Usually, these genes are expressed in response to antibiotic exposure, as seen by the activity of efflux pumps, for example. Acquired resistance, on the other hand, is dependent on selective pressures and horizontal gene transfers, in which the mechanisms, molecules, and genes expressed when the bacteria are presented to antibiotics were obtained from foreign origins or by mutation of a pre-existing genetic element in the organism [32]. The essence of acquired resistance is that no natural trait of the bacterium is able to provide the resistance observed, which can be acquired by a mutation on its genome, the transfer of foreign genes via plasmids, transformations, or even transduction processes with phages [33].

Bcc bacteria are heavily packed with various traits that confer them intrinsic resistance, preventing the use of a myriad of antimicrobials just from the natural characteristics of these organisms. Due to the widespread nature of Bcc-related infections, and their elevated ability to adapt, bacteria of this complex have acquired a fair share of foreign resistance genes, and many species are indeed multidrug resistant. Thus, the treatment of Bcc bacterial infections with antibiotics can be quite challenging.

1.4.2 Outer Membrane Structure

A key part of Bcc antibiotic resistance relies on the structure of its membrane and the composition of the elements that form it. Bcc bacteria, as Gram-negative bacteria, present an inner membrane, periplasmic space, and an outer membrane composed by LPS with an unusual structure. As described before, Bcc bacteria produce LPS molecules with a specific composition, that decreases the overall negative charge of Bcc cell surface. These modifications often occur upon exposure to antimicrobials or in other stress situations [14].

The lack of binding sites of the LPS molecules on this outer membrane and decreased permeability have contributed to the resistance to polymyxins, β-lactams, aminoglycosides, and other cationic peptides [14], [32]. Although the outer membrane itself is a major contributor for the resistance to the antimicrobials mentioned, other mechanisms have been also described to be involved in these phenomena.

Resistance to polymyxins has also been attributed to a two-component system that modifies LPS molecules; to the alternative sigma factor RpoE, that coordinates the expression of many extra-cytoplasmic stress-related genes and, finally, the action of the metalloproteases ZmpA and ZmpB, that contribute to overall cationic peptide resistance by antimicrobial degradation [14].

1.4.3 β-Lactamases

Many Bcc strains are resistant to β-lactams, derived from the production of class A β-lactamases encoded by the *penA* gene [32]. These proteins repress antimicrobial binding to the precursors of peptidoglycan, in which the terminal D-Ala-D-Ala of this molecule usually poses as a target. The mechanisms of action and the spectrum in which these enzymes provide resistance are variable and strain dependent. However, overall, due to the presence of these β-lactamases, Bcc bacteria generally present reduced susceptibility to β-lactams such as penicillins, cephalosporins, clavams, carbapenems, and monobactams. The resistance to this class of antimicrobials is highlighted by the capability of Bcc bacteria to use penicillin G as a carbon source, showing the high resistance and the ability of these bacteria to utilize the antimicrobials themselves as energy sources for their benefit [7]. Efflux pumps are also a major contributor to β-lactam resistance.

1.4.4 Efflux Pumps

Efflux pumps are a major contributor to overall drug resistance in bacteria from the Bcc. Transport systems from different families such as ABC (ATP-binding cassette), MFS (major facilitator superfamily), and RND (resistance nodulation division) family are involved in the extrusion of many antimicrobials from Bcc bacteria. Currently, at least 16 RND efflux pumps were identified as being encoded by *B. cenocepacia*, 6 of them related with antibiotic resistance [32,34]. Amongst the compounds that are excluded by these systems, ciprofloxacin, tobramycin, chloramphenicol, fluoroquinolones, trimethoprim-sulfamethoxazole, macrolides, and tetracyclines stand out [14]. Oftentimes, many strains tend to overexpress the genes that express these pumps, enhancing Bcc drug resistance [11].

1.4.5 Antibiotic and Drug Target Modification

Besides the reduced permeability of the cell envelope and the overexpression of efflux pumps, bacteria that belong to the Bcc can acquire antimicrobial resistance through other methods such as drug target modification and antibiotic modification. In Bcc bacteria, drug target modification has been reported mostly associated with fluoroquinolone resistance [32]. Drug target modification has been observed in *B. cenocepacia* strains that are resistant to levofloxacin and ciprofloxacin, presenting mutated species with a significant increase in the MIC (minimal inhibitory concentration) value. Drug target alteration has been reported for trimethoprim, in which the target enzyme seems to be modified to deflect the action of this antimicrobial [14].

Antibiotic resistance that results from antibiotic modification is commonly found in Bcc bacteria to acquire resistance to β-lactams and aminoglycosides. In many antimicrobials from the β-lactams class of antibiotics, this is achieved by the degradation of the β-lactam ring, degrading then part of the antimicrobial itself [14].

1.4.6 Biofilm Formation

Biofilm formation can also further develop antibiotic resistance. The formation of a thick matrix of cellular components decreases the diffusion of several molecules, including antimicrobials [14]. To reach bacteria in the intricated polysaccharides, protein, and DNA matrix the antimicrobials must be administered in higher concentrations. The complex network system, upon formation, also undergoes several metabolic changes to adapt themselves to the new phenotype, generating cells that are not only more resistant to antibiotics but are also extremely persistent in infections. These persistent cells are very tolerant to environmental factors and seem to be indifferent to the action of antimicrobials, remaining stagnant until the end of the drug treatment and then displaying virulent traits that intensify the infection [14]. Many of these cells are also considered sessile, slowing cell division and metabolism, which make cell division-related antimicrobials less efficient [33].

1.5 Bcc as Multidrug resistant (MDR) Bacteria

Bcc bacteria are an emergent group of multidrug resistant bacteria, that are not only intrinsically resistant to several drugs but can also quickly acquire new traits and mechanisms of resistance to novel antimicrobials used in therapy.

MDR bacteria are a serious health threat since the adaptable nature of these organisms limits the number of antibiotics available for the treatment of infections. To increase the success of antibiotic treatments, combinations of two or three drugs or antibiotics with greater toxicity are often used. So far, the advances to design new synthetic antimicrobials are not fast enough to respond the constant mechanisms developed by bacteria to acquire resistance, which many times occurs shortly after the new drugs are approved [33]. It is thereby being crucial to resort to new alternatives that can help overcome these infections [35].

CF patients are susceptible to repeated and intensive antibiotic therapy due to the chronic

nature of their lung infections. This, naturally, promotes acquired resistance, especially in Bcc bacteria. The constant use of antimicrobials ends up not completely eradicating the bacterial populations, but they are used to alleviate symptoms, and to control the growth and spread of these persistent bacteria [36].

1.6 Antibiotic Treatment

Bcc-driven infections are then difficult to treat. There is no ideal standard drug combination for therapy, looking into each patient case-by-case, ideally considering the patient's history with antimicrobials and antibiotic susceptibility data [8, 14].

Overall, trimethoprim-sulfamethoxazole, ceftazidime, meropenem, and doripenem are considered the most efficient drugs for Bcc therapy, with trimethoprim-sulfamethoxazole being the most recommended [8, 14]. When these cannot be employed, combinations of ceftazidime, meropenem, and doripenem can be used when paired with tobramycin, amikacin, or other β-lactams, depending on antimicrobial susceptibilities. In fact, for multidrug-resistant Bcc bacteria, the triple combination of meropenem, high doses of tobramycin, and a third antibiotic such as ciprofloxacin are most efficient for CF patients, due to their synergistic effect. Other third agents can also include piperacillin-tazobactam, ceftazidime, trimethoprim-sulfamethoxazole, or amikacin. High dosages of tobramycin or other aminoglycosides (gentamicin and amikacin) are often administered in a nebulized form to avoid side effects. β-lactams paired with β-lactamase inhibitors such as ceftazidime-avibactam and meropenem-vaborbactam aid the action of β-lactamases but not the action of efflux pumps [8].

Other drugs such as doxycycline and minocycline are considered oral alternatives for treatment, when the strains involved are resistant to trimethoprim-sulfamethoxazole, although presenting aggravated side effects[37]. Temocillin has been considered salvage therapy for *B. cepacia* acute multidrug-resistant infections [38].

1.7 Alternative Antimicrobial Therapy

Bcc bacteria are, as stated, highly resistant to several antibiotics making the drugs treatment choice difficult. By the time new drugs are approved, resistance mechanisms are quick to develop. To counteract this, alternative therapies with other types of molecules are considered an attractive path to fight infections, caused by MDR bacteria. These alternative therapies can be administrated alone or in combination with antibiotics.

Various molecules have been tested either *in vivo* or *in vitro*, like immunosuppressors and corticosteroids, IFN- γ, Cysteamine, Imidazoles, phages, quorum sensing inhibitors, small RNAs, and other molecules derived from natural products such as plant nanoparticles, fish oils, and glycopolymers [14] (**Figure 1**).

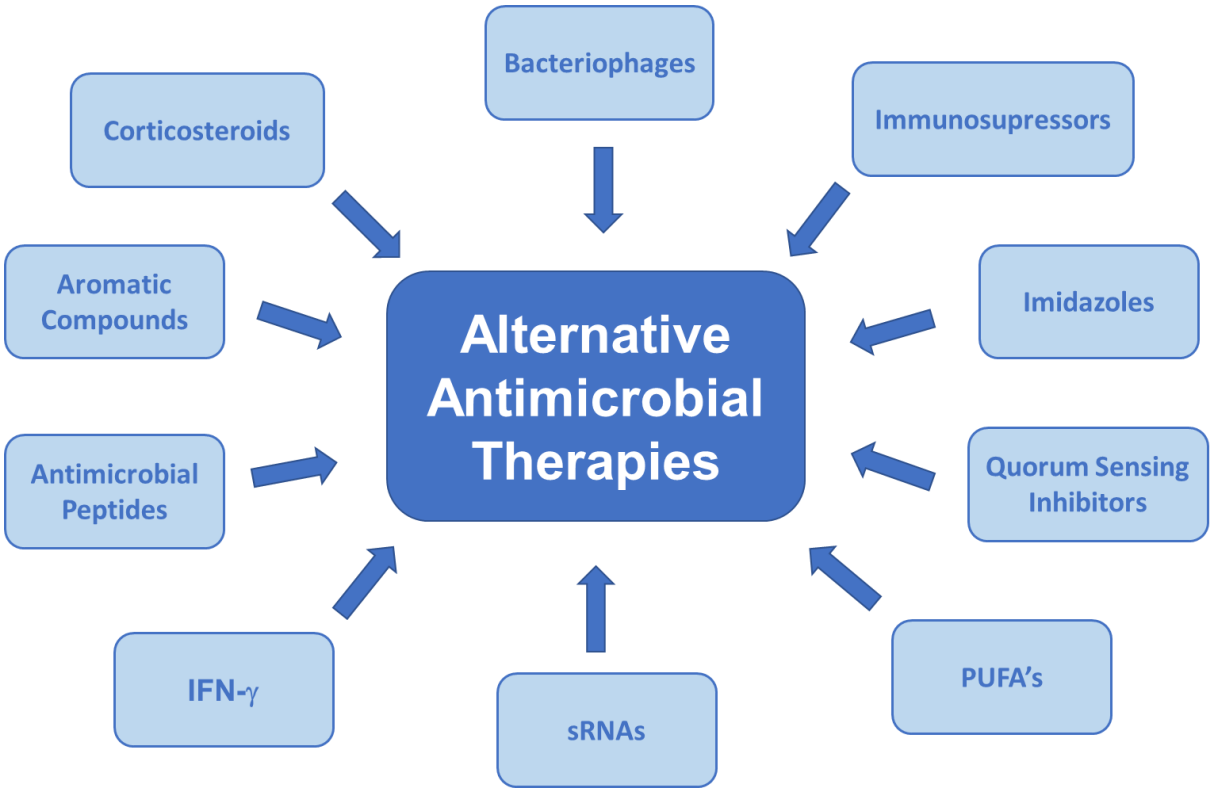


Figure 1. Examples of molecules used for alternative antimicrobial therapy.

1.7.1 Immunosuppressors and Corticosteroids

In patients with severe prognosis, such as cepacia syndrome cases, the use of immunosuppressors, such as mycophenolate mofetil, cyclosporine, and tacrolimus, or corticosteroids, such as prednisolone alongside antimicrobials, seem to contribute to the efficiency of the drug responses, possibly attenuating the exacerbated response from the cepacia syndrome progression [39].

1.7.2 IFN-γ

One of the first-line agents of defense against infections are macrophages. Now, Bcc bacteria can not only survive inside macrophages but also affect their biological functions, including autophagy, whose defective functioning allows the pathogens to thrive. Stimulation and attempting to restore autophagy in macrophages allow these agents to tackle Bcc bacteria efficiently. The interferon gamma (IFN-γ), an important macrophage activator, has been used *in vitro* to reestablish proper autophagosome formation, which can help in fighting bacterial infections [40].

1.7.3 Cysteamine

Cysteamine is a decarboxylated derivative of cysteine that was initially used to treat cystinosis, and nowadays it is approved for other diseases [41]. This compound shows potential for Bcc infections by being able to disrupt disulfide bonds, that can help antimicrobials break through biofilms and act against bacteria. In fact, this molecule has been shown to enhance the antimicrobial activity of tobramycin, ciprofloxacin, and trimethoprim-sulfamethoxazole. The interest of cysteamine goes even beyond

antimicrobial therapy, as it may be of help to restore proper CFTR function when combined with other elements [42].

1.7.4 Imidazoles

Compounds that can help to penetrate through biofilms and eradicate them, especially when going against Bcc chronic infections, are always of interest. Imidazoles with antifungal properties such as econazole, miconazole, oxiconazole, and ketoconazole showed promise in potentiating the effects of tobramycin. Tobramycin is usually used in high concentrations which also pertains heavy side effects. In combination with imidazoles, lower concentration of the mentioned antibiotic can be used to eradicate Bcc biofilms [43].

1.7.5 Aromatic Compounds

In addition to organic compounds and immunotherapy, and chemotherapy-related molecules, tracing natural products revealed potential compounds with antimicrobial activity that can enhance a patient's response to antibiotics.

Essential oils, extracted from aromatic plants, have been shown to contain antimicrobial activity. A few essential oil extracts may help to slow down or even inhibit the growth of Bcc bacteria, by altering their membrane permeability and disrupting important biological processes. The usage of polymers derived from these extracts may be useful for treatment since essential oils by themselves present a few stepbacks regarding stability [14].

1.7.6 Polyunsaturated Fatty Acids

Fish oils, rich in polyunsaturated fatty acids (PUFAs) such as omega-3 and omega-6, present not only many metabolic benefits but also potential antimicrobial activity. Purified eicosapentaenoic acid, docosahexaenoic acid, linoleic acid, and arachidonic acid were reported to be able to act against bacterial infections, including *P. aeruginosa* and *B. cenocepacia*. PUFAs' mechanism of action relies quite possibly on membrane disruption and its underlying gradients, besides altering other enzymatic activities of pathogenic bacteria. CF patients lack fatty acids metabolism, which leaves them with a lipid imbalance. The intake of PUFAs can be beneficial for the overall health and nutrition of the host [44].

1.7.7 Glycopolymers

Poly (acetyl, arginyl) glucosamine (PAAG), a glycopolymer with antimicrobial activity has been reported to disrupt cell membranes of Gram-negative bacteria, increasing its permeability. The polycationic nature of this compound competes with the naturally present divalent cations on the outer membrane of Bcc bacteria, depolarizing it. The administration of glycopolymers in combination with antibiotics, such as tobramycin and meropenem, could then enhance antimicrobial activities and facilitate the diffusion of such molecules [45].

1.7.8 Phage Therapy

Phage therapy traces back before the commercial use of antibiotics, with its application being commercially available around the 1930s [14]. Early reports of this kind of therapy remote to the late 19th century, and until the takeover of antibiotics, this kind of therapy was prevalent in medicine. In the 20th century antibiotics became the go-to treatment for bacterial infections. Now that multidrug resistant bacteria are an overwhelming health issue, the employment of bacteriophages has been experiencing a renaissance in the 21st century [23].

Bacteriophages (bacterial viruses) infect bacterial cells intracellularly, taking over their cellular machinery to replicate themselves, and produce infectious copies that can infect other hosts. Phages are dependent on their hosts to thrive, in a parasitic relationship, and when their replication is complete, they dispose themselves of the host, lysing it. The use of bacteriophages in clinic presents many advantages and benefits such as high specificity to target bacteria, efficiency, abundance, and reduced toxicity. Phages rely on specific binding to receptors in the outer membrane to infect a host, which makes them highly specific. However, this specificity can also be burdensome if the host strain mutates loses such receptors. Phages have an incredible ability to produce millions of copies of themselves, but the chances of infecting a specific strain from a bacterial species decrease due to its narrow spectrum. Contrasting with conventional antibiotics, this target specificity is naturally more beneficial for the surrounding bacterial communities of the host, not interfering with the natural microbiomes and the host's health. Antibiotics are also known for their significant toxicity and side effects, which are very low on bacteriophages. These viruses are very abundant and mutable according to needs, and their biological nature makes them more efficient in a cellular environment [23].

To increase the clearance of the strains involved in infection, phages can also be used in combination with antibiotics, helping them, to break through biofilms and act upon non-specific targets that phages have. Phage therapy for Bcc infections is promising and seems to be extremely beneficial as an alternative treatment [23].

1.7.9 Antimicrobial Peptides

The synthesis of cationic peptides, derived from cationic antimicrobial peptides (CAMPs) found in diverse environments and organisms has shown to be efficient for disruption of Bcc biofilms. These molecules prevent cell adhesion by promoting electrostatic bonds with the bacterial outer membrane [14]. When bacteria are stimulated by the environment to form biofilms, among many other factors, they release two signaling molecules, guanosine 5′-diphosphate 3′-diphosphate (ppGpp) and guanosine 5′-triphosphate 3′-diphosphate (pppGpp), which are collectively called (p)ppGpp. An antimicrobial peptide, Peptide 1018, was found to inhibit biofilm formation by targeting (p)ppGpp and degrading it. The degradation of these signaling molecules not only permitted the inhibition of biofilm formation but also prevented the dispersion of mature biofilms and promoted cell death from within the biofilms [46].

1.7.10 Quorum Sensing Inhibitors

Bcc strains are rich in virulence factors, whose expression can be controlled by quorum sensing

systems. Looking into molecules that can inhibit the activation of these systems may be beneficial to control the exacerbated and virulent response that bacteria from the Bcc exert in their infected hosts. Molecules that resemble AHLs have been developed, modifying the lactone portion or the amide portion of these compounds [14]. Quorum sensing inhibitors (QSI) such as baicalin hydrate (BH) or cinnamaldehyde (CA), increased the susceptibility of the cellular structures of Bcc bacterial species to tobramycin. Diketopiperazines, another class of QSI, were found to inhibit the activity of *B. cenocepacia* AHL synthase CepI, preventing not only the expression of virulence factors but also biofilm formation, [26,47].

Alternative therapies, other than the use of conventional antibiotics, have shown potential to help eradicate Bcc infections independently of the resistance mechanisms that the strains *in situ* may offer. The *in vivo* efficacy of many of the molecules described above is still under study. Some alternatives are still dependent or are, perhaps, more effective in combination with antibiotics, but the perspective of having a range of molecular alternatives that can further improve the patient's condition and reduce the toxicity of the current treatments is promising to overcome the antibiotic resistance problem.

Many of the molecules explored for alternative therapies are related to bacterial virulence, antibiotic resistance mechanisms, and biofilm formation. Targeting the molecules that are directly or indirectly related with these processes seems to be promising to attenuate or inhibit the virulence expressed by Bcc bacteria, and to help the penetration of conventional antimicrobials in bacterial cells. It has been shown that antibiotic-induced regulatory small non-coding RNAs (sRNAs) control mRNAs involved in essential processes of the bacterial cell envelope and cell-wide physiological changes that promote tolerant bacterial lifestyles[48]. These sRNA-dependent responses have been shown to significantly contribute to antibiotic susceptibility, but the potential of these molecules as possible alternative therapies for Bcc bacteria needs to be explored.

1.8 sRNAs as Key Gene Regulators

Small non-coding RNAs (sRNAs) are a heterogeneous group of RNA molecules in terms of biogenesis and biological functions, whose size ranges from 50 to 500 nucleotides. sRNAs are widespread among bacterial species and are of extreme importance for the post-transcriptional regulation of the gene expression, with fast and efficient responses. These regulators most commonly act on RNA transcripts or small peptides [17].

sRNAs can regulate a myriad of different cell processes, such as DNA assembly, transcription, translation, synthesis of various metabolites, virulence, and plasmid replication, since they can target a wide range of molecules. sRNAs also play a significant role in several metabolic processes, such as quorum sensing systems, biofilm formation, stress responses, overall homeostasis, and pathogenesis [17, 35, 49].

sRNAs that interact with mRNAs act by the partial or full pairing with a target mRNA. Depending on the complementarity with the target, sRNAs can be classified as *cis* or *trans* encoding. *Cis*-encoded sRNAs are fully complementary to their specific target, while *trans*-encoding sRNAs only share partial

complementarity with their targets [49]. Since the binding of trans-encoded sRNAs is not as specific as observed for *cis*-encoded sRNAs, this class of small RNAs may present more than one possible mRNA targets, often included in intricate regulatory networks [17].

The regulation mediated by sRNAs can result in the repression of the expression of a gene or subset of genes, or it can lead to the activation of the mRNA function. While the inhibition of RNA transcription by sRNA is more common, the activation of targets has been verified in a few cases [17].sRNAs can induce these regulatory effects through various mechanisms. The inhibition of target mRNAs is often done by the blockage of the ribosome binding site (RBS), or other close elements downstream of the target molecule by the sRNA. This way, the translation of the transcript is affected, and the gene is not expressed. Some sRNAs can also induce the degradation of the mRNA itself. Regarding the activation mechanisms, the binding of the sRNA to the target mRNA causes conformational changes, exposing a previously inaccessible RBS. sRNAs can also bind to other sRNAs, impairing the action of the other over a certain target [17], [50].

Most sRNAs are dependent on the Hfq-like proteins to successfully bind to mRNAs. The RNA binding proteins that belong to the Hfq family are highly conserved amongst bacteria and are involved in the regulation of several biological functions. Structurally, these proteins arrange as hexamers that resemble a donut in shape, leaving a central pore in its center. These proteins promote the binding of the sRNAs to their respective targets through different mechanisms: exposing the binding regions of the RNA structures, modulating them; increasing the possibility of encounter between the RNA molecules, maximizing the chances of sRNAs form a stable duplex with the mRNA transcript and sort the desired effect in post-transcriptional gene regulation [50]. Hfq proteins often possess more than one RNA binding site, and are capable of binding to several molecules simultaneously, contributing to the efficient response mediated by sRNAs [17]. Two Hfq-like proteins are expressed in Bcc bacteria, encoded by the *hfq* and *hfq2* genes [21]. Such expression suggests not only the crucial role of Hfq proteins as global gene regulators, but also the importance of sRNA regulation in Bcc bacteria [51].

The interest of sRNAs as modulators of virulence and antimicrobial resistance has been increasing, especially since MDR bacteria became an overwhelming health threat, and few antimicrobials are available to act upon many of these highly resistant pathogenic strains. Modulating the expression of sRNAs has been previously shown to contribute to increased antimicrobial sensitivity in various bacterial species [52,53]. Studies have been made in many species, namely *Escherichia coli* and *Pseudomonas aeruginosa*, in which the overexpression or depletion of sRNAs influenced antibiotic sensitivity (**Figure 2**).

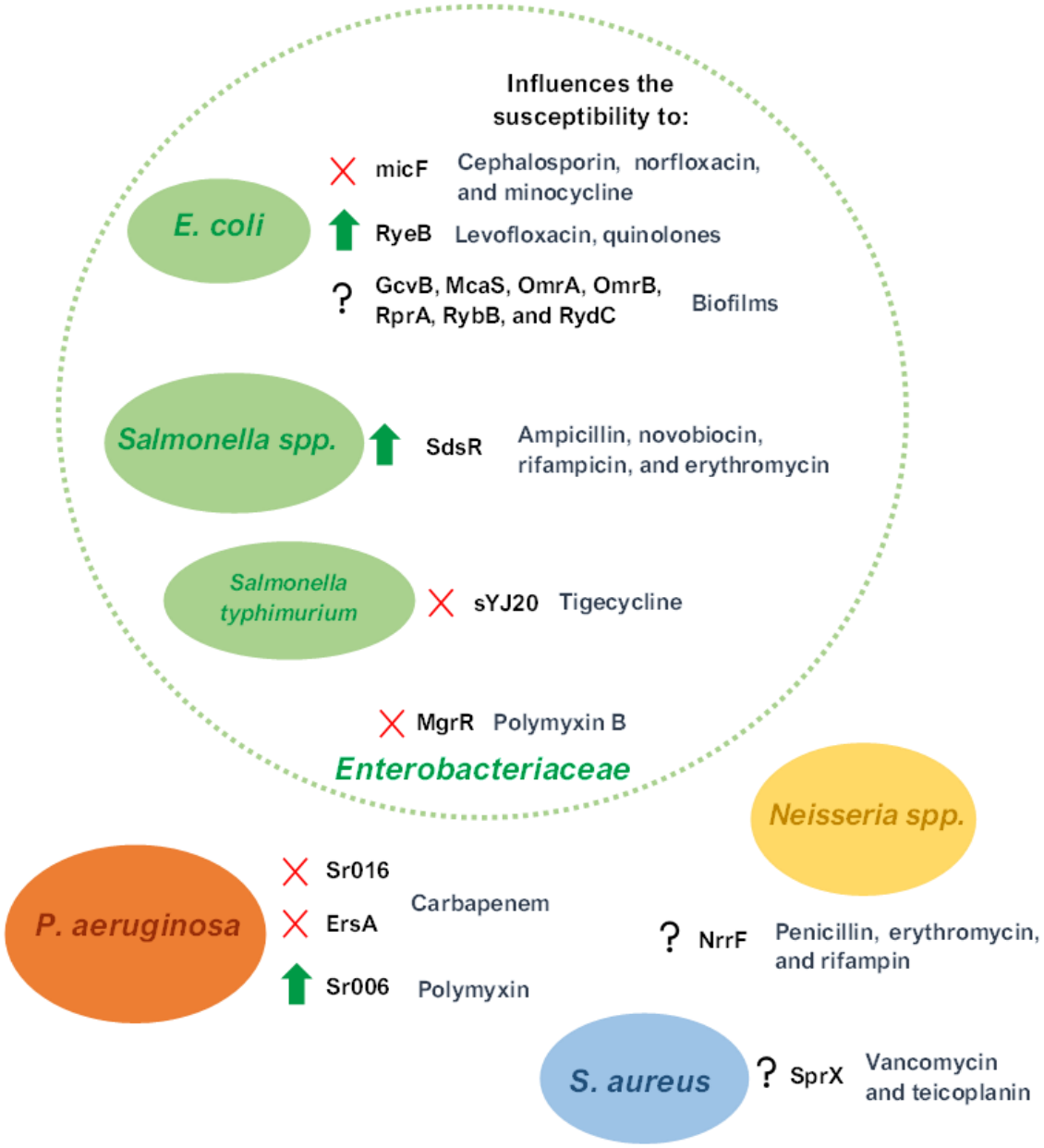


Figure 2. Examples of bacterial sRNAs described as being involved in antibiotic resistance and the antimicrobials affected. Green arrows represent sRNA overexpression, the red X represents sRNA inhibition and question marks represent unspecified effects.

The inhibition of the *micF* sRNA in *E. coli*, involved in the bacterial expression of porins, lead to increased susceptibility of bacteria to antimicrobials such as cephalosporin, norfloxacin, and minocycline [54]. The overexpression of the RyeB sRNA, on the other hand, improved the action of levofloxacin against *E. coli* MDR strains and increased antimicrobial susceptibility to quinolones [54]. The overexpression of this sRNA, also known as SdsR, in *Salmonella* spp. was also shown to reduce the expression of the TolC protein, an important component of *E. coli* major efflux pumps systems. This sRNA is relatively conserved in Enterobacteriaceae, and the repression of TolC protein by SdsR was observed in *Salmonella* strains, leading to increased sensitivity to antimicrobials such as ampicillin, novobiocin, rifampicin, and erythromycin [55]. The sRNA MgrR, expressed in *E. coli* and other Enterobacteriaceae species, is associated with LPS metabolism and modifications in the bacterial outer membrane. This

molecule downregulates genes involved with LPS modifications and allows increased polymyxin B resistance [56].

In *Salmonella enterica* serovar Typhimurium, the inhibition of sYJ20, a regulator of antibiotic tolerance, reduced the survival rates of these bacteria when exposed to tigecycline [57].

In *P. aeruginosa*, the Sr0161 and ErsA sRNAs are involved in carbapenem resistance, interacting with porins that allow the intake of this class of antimicrobials. Strains of this species lacking these sRNAs were, therefore, more susceptible to the action of carbapenems [58]. The Sr006 sRNA, when overexpressed, allows increased susceptibility of *P. aeruginosa* cells to polymyxins through LPS modifications [58].

In *Neisseria gonorrhoeae* and *Neisseria meningitidis*, the sRNA NrrF was shown to attenuate the expression of MtrF, a membrane protein associated with hydrophobic antimicrobial resistance to antibiotics such as penicillin, erythromycin, and rifampin. The mRNA that encodes for the efflux pump-mediated system becomes compromised with the action of NrrF and the antibiotic resistance is attenuated [59].

Staphylococcus aureus methicillin resistant strains are becoming highly prevalent, many being resistant to glycopeptides. The sRNA SprX modulates the resistance to the mentioned antimicrobial, showing its altered expression an increased susceptibility to vancomycin and teicoplanin [60].

It was also described that at least seven sRNAs in *E. coli* affect the expression of transcription factors that regulate the biofilm formation process. The sRNAS GcvB, McaS, OmrA, OmrB, RprA, RybB, and RydC downregulate the *E. coli* transcription factor CsgD, and thereby reduce the formation of biofilms, increasing the antimicrobial susceptibility of this species [61].

The mentioned studies highlight the vast potential of the sRNAs as alternatives to antimicrobial therapy, increasing the efficiency of antimicrobials or compromising the performance of bacteria during the infection process. Although the rising interest in using sRNA as alternative antimicrobial therapy, the Bcc sRNAs are still poorly characterized and their influence in antibiotic resistance needs to be explored.

While the presence of many putative sRNAs in the Bcc have been indicated, the expression of few of them was confirmed *in vitro* or *in vivo*, and very few have been fully characterized. Pita et al. compiled 167 putative sRNAs in *B. cenocepacia* strains, most identified by Sass et al. in *B. cenocepacia* J2315 growing under biofilm conditions[51]. These sRNAs are conserved at the species level and relatively conserved within the Bcc. Most of them are located in chromosome 1. Many sRNAs, as specific gene regulators, are only expressed in certain conditions. [17,51].

The importance of sRNAs in bacterial virulence, biofilm formation and antibiotic resistance is of great interest when considering the Bcc infections, especially if these small molecules can regulate these processes [48]. The functional characterization of these regulatory molecules can be crucial for a better elucidation of Bcc infections and to find strategies to fight them.

1.9 Thesis Focus

Infections by bacteria belonging to the *Burkholderia cepacia* complex can be, as highlighted before, extremely hard to erradicate, especially in already vulnerable hosts. Although conventional antimicrobial drugs are consistently administered, it is often not enough to attenuate the violent prognosis that species from this complex can induce. The intrinsic flexibility of Bcc bacteria allows them to remain adaptable and develop antimicrobial resistant mechanisms to most available drugs. With the rise of multidrug resistance strains as a serious health threat, novel alternative therapies need to be explored.

With the unveiling of Bcc mechanisms for virulence expression, small RNAs are presented as key gene regulators. These small molecules often coordinate responses that induce pathogenicity of Bcc strains, being then considered attractive targets for alternative therapies. Very little is still known about these small but promising molecules in the Bcc, therefore efforts to further understand the function and effects of these sRNAs in Bcc bacteria are extremely important.

The main objectives of the work developed for this Masters dissertation were to identify *B. cenocepacia* sRNAs that can target genes related with antibiotic resistance in Bcc, and evaluate the effect of their overexpression and/or silencing on the Bcc susceptibility to commonly used antibiotics. For that, the targets of the putative 167 sRNAs compiled by Pita et al. were predicted with bioinformatics tools, and two of the most promising sRNAs to be involved in antimicrobial resistance were chosen. For different antibiotics, the MIC values of clinical isolates from the most feared Bcc species overexpressing or silencing sRNAs were assessed to evaluate the impact of these molecules in antimicrobial resistance mechanisms. Further characterization and functional evaluation were made to test the potential synergistic efficacy of the sRNA in combination with traditional antibiotics to treat Bcc infections.

2. Material and Methods

2.1 Bacterial strains and growth conditions

The strains and plasmids that were used for this work are represented in **Table 1**. Bacteria cultures were preserved in 40% (v/v) glycerol at -80ºC. Bacteria were cultured in Miller's LB (Lysogeny Broth) medium (NZyTech) agar (2%) plates, incubated at 37ºC for 16-24h, and later kept under 4ºC for posterior uses. Growth media was supplemented with Amp (ampicillin ;150 µg/mL, NZYTech) or chloramphenicol (Cm) (25 µg/mL, NZYTech), for *E. coli* DH5α during plasmid construction and maintenance, and with chloramphenicol (200 µg/mL, NZYTech) for Bcc strains (*B. cenocepacia* K56-2, *B. cenocepacia* J2315, *B. multivorans* LMG 1660, *B. dolosa* AUO158). Overnight cultures were inoculated in 3 mL of LB media supplemented with antibiotics when required and incubated 16 hours with orbital agitation (250 rpm) at 37ºC.

Table 1. Strains and plasmids employed in the current study.

Strains	Genotype or Description	Reference
E. coli DH5α	Maintenance of replicative plasmids. F− Φ80*lac*Z∆M15 ∆(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_k−, m_k+) *pho*A *sup*E44 *thi*-1*gyr*A96 *rel*A1 λ−	Invitrogen
B. cenocepacia J2315 (LMG 16656)	Cystic fibrosis clinical isolate (Edinburgh, UK); ET12 lineage reference strain	62
B. cenocepacia K56-2 (LMG 18863)	Cystic fibrosis clinical isolate (Toronto, Canada); ET12 lineage	63
B. dolosa AU0158	Cystic fibrosis clinical isolate (outbreak at Children's Hospital Boston, USA).	64
B. multivorans LMG 16660	Cystic fibrosis clinical isolate (Glasgow, UK)	65,66
E. coli ATCC 25922	MIC control strain. Clinical isolate, FDA strain Seattle 1946	67
Plasmids		
pUC19	Cloning vector Lac promoter; LacZ; Amp^r; Multiple cloning site	68
pIN29	ori_{pBBR} Δ*mob*, Cm^r, DSRed	69
pTAP3	pIN29 overexpressing the sRNA ncS06 from *B. cenocepacia* J2315	Kindly provided by Tiago Pita
pMBJ1	pIN29 overexpressing the 5' antisense sequence of the sRNA ncS06	This study
pMBJ2	pIN29 overexpressing the 3' antisense sequence of the sRNA ncS06	This study
pMBJ3	pIN29 overexpressing the sRNA ncRNA3 from *B. cenocepacia*	This study

Table 2. 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	CATATGTCTGCTACCCCGTAGAACTTATC TATTCTTTTCATTTCTAGA	NdeI	-
pMBJ1_Rv	TCTAGAAATGAAAAGAATAGATAAGTTCT ACGGGGTAGCAGACATATG	XbaI	-
pMBJ2_Fw	CATATGCAGACGACGGAACGTCGCTTAT GTGCAAGTCGGCCTGCATCTAGA	NdeI	-
pMBJ2_Rv	TCTAGATGCAGGCCGACTTGCACATAAG CGACGTTCCGTCGTCTGCATATG	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	GTTTTTTTTAATACGACTCACTATAGGAAA ATCGGCCCATTCCGT	-	155
BCAL2915_IV_Rv	AGATCCTCGGGAAGTTTCCA	-	
BCAL2915_RC_Fw	GGAAACGCCGACGTCCCTA	-	471
BCAL2915_RC_Rv	CGTCGAAGTCCGCATCGATCT	-	
ncRNA3_IV_Fw	GTTTTTTTTAATACGACTCACTATAGG GCGGCCGCCGGTGCCAG	-	172
ncRNA3_IV_Rv	GACGCGCGCAAAGCAGC	-	

2.2 Construction of plasmids

The two single-stranded oligonucleotides with complementary sequences pMBJ1_Fw & pMBJ1_Rv, and the pMBJ2_Fw & pMBJ_Rv were annealed to form a double strand DNA sequence. For this, each oligonucleotide was diluted to a final concentration of 100 µM using the Annealing Buffer (10 mM Tris, pH 8.0; 50 mM NaCl; 1 mM EDTA). Equal volumes of the equimolar oligonucleotides were mixed and incubated at 95 °C for 5 min to break all the hydrogen bonds. The reaction mixture was allowed to cool slowly to room temperature (<60 min). The double strand sequences were inserted into the pUC19 cloning vector previously linearized with the HincII restriction enzyme (Thermo Scientific), yielding the pUC19- ncS06sil5' and pUC19-ncS06sil3' plasmids. After sequencing, each plasmid was digested with NdeI/XbaI, and the resulting fragments were inserted into the pIN29 plasmid yielding the pMBJ1 plasmid and the pMBJ2 plasmid (**Table 1**).

A Nested PCR was performed to amplify a 1072 bp fragment containing the sequence of the sRNA ncRNA3 using the pMBJ3_RC_Fw and pMBJ3_RC_Rv pair of primers, and then to amplify a 161 bp fragment using the pMBJ3_Fw and pMBJ3_Rv primers. The small fragment was inserted into the pUC19 cloning vector previously linearized with the HincII restriction enzyme (Thermo Scientific), yielding the ncRNA3 plasmid. After sequencing, the fragment from the plasmid was amplified with the M13 primers (**Table 2**) and the resulting PCR product was inserted into the pIN29 plasmid, yielding the pMBJ3 plasmid (**Table 1**). Plasmid maps for pTAP3, pMBJ1, pMBJ2 and pMBJ3 are represented in **Figure S1**.

2.3 sRNA Target Search

A list of *B. cenocepacia* sRNAs was compiled by Pita et al., gathering information regarding putative sRNAs detailed in literature beforehand. 167 sRNAs were used for target search with the TargetRNA2 web server[70]. As input, the sequences of the sRNAs previously identified were entered in FASTA format, and each replicon of the *B. cenocepacia* J2315 genome was selected. 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 (**Table S1**). The selection of the sRNAs was performed by filtering the IDs of the predicted targets with the IDs of genes related to antibiotic resistance and finding matches. For some sRNAs, a second bioinformatic tool, CopraRNA[71], was used to confirm

the predicted targets.

2.4 Molecular Biology Techniques

The isolation of plasmid DNA was performed using the GeneJET Plasmid Miniprep Kit (Thermo Scientific), following the manufacture instructions. Agarose gel electrophoresis were performed according to standard procedures [72]. *E. coli* competent cells were transformed with plasmid DNA using the heat shock method [72]. Transformed cells were incubated in LB medium at 37ºC for 1 hour and plated in LB agar plates supplemented with appropriate antibiotics. For Bcc strains, the insertion of the pIN29, pTAP3, pMBJ1, pMBJ2 and pMBJ3 was done by electroporation. Electrocompetent cells stored at -80ºC in 10% glycerol were incubated with 3 µL plasmid DNA. Electroporation was performed using a resistance of 200 ohms, 2.5 volts and a capacitance of 25 µFD and the *Burkholderia* cells were incubated in LB medium for 2 hours at 37º C. The cells were plated in LB agar plates supplemented with Cm 200 µg/mL. PCR amplification of *B. cenocepacia* K56-2 ncRNA3 and BCAL2915 gene was made with the oligonucleotides presented in **Table 2** (*in vitro* transcription section). The primers were designed with the AmplifX[73] and were synthesized by Stabvida.

2.5 *B. cenocepacia* Growth Curve

Overnight cultures of *B. cenocepacia* K56-2 pIN29 and pTAP3 cells grown in 25 mL of LB medium supplemented with 100 µg/mL were prepared to perform a growth curve. Adjusting the initial OD_{640}(Optical Density) to 0.05, 50 mL of LB medium were inoculated, and incubated at 37ºC with orbital agitation (250 rpm). Growth curve points were taken at 0h, 2h, 4h,5h, 6h, 8h, 10h, 12h and 24h. At each point, the OD of the culture was measured at λ= 640 nm, diluting 100 µL of each culture in 900 µL of NaCl 0.9%. Serial dilutions up to 10^{-8} were also performed using sterile NaCl 0.9%. The dilutions were plated as spots into 2% LB agar plates and incubated at 37º C for 24h, for colony count.

2.6 MIC determination

The MICs of three antibiotics, ciprofloxacin and tobramycin and trimethoprim (Sigma-Aldrich) were determined using the microbroth dilution method, according to the to the International Standard ISO 20776-1 and EUCAST (European Committee on Antimicrobial Susceptibility Testing)[74,75] recommendations.

Mueller Hinton Broth (MHB; Sigma-Aldrich) was used to perform the MIC assays, preparing it according to the suppliers' instructions and kept at 4ºC. Overnight cultures of the Bcc strains were inoculated in 3 mL MHB supplemented with Cm 200 µg/mL and incubated at 37ºC with orbital agitation. The cells were diluted in fresh medium without antibiotics with an initial OD_{640} of 0.05 and incubated at 37 ºC until the middle of exponential phase (about 4h). *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. The stock solution of Trimethoprim (50 mg/mL) was prepared with DMSO. These solutions were then further diluted to 1137.8 µg/mL (Tobramycin and Trimethoprim) and 568.9 µg/mL (Ciprofloxacin) with MHB to obtain the final antibiotic concentrations desired in the wells.To each well of the first column of the 96-well plate, 180

µL of the antibiotic stock solutions were added, and serial dilutions (1:2) were performed in MHB in order to obtain final concentrations ranging from 1 to 512 µg/mL for Tobramycin and Trimethoprim, and 0.5 to 256 µg/mL for Ciprofloxacin assays. Then, 10 µL of adequately diluted bacterial suspensions were added to each well and mixed to obtain a final optical density of ~0.01 (5.0×10^5 CFU/mL), measured at 640 nm. In each experiment, a positive (without antibiotic) and a negative control (no bacterial inoculum) were also included. 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). In each experiment, the initial number of bacteria (CFUs/mL) was determined, performing serial dilutions of the initial suspension up to 10^{-4} and plating in 2% LB agar plates. These plates were incubated for 24h at 37ºC and then counted with the help of a magnifier.

2.7 Biofilm 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[76]. 96-well polystyrene microtiter plates (Greiner Bio-One) containing LB liquid medium were inoculated with the strains' cultures at an initial OD_{640} of 0.05 and were left to incubate during 24 or 48 hours at 37 °C, with no agitation. The staining of adherent cells, previously washed with deionized water (three times), was made with a crystal violet solution (1% wt/v) for 15 minutes, that was followed by another set of three washes with deionized water. Biofilm quantification was made after dissolving the bound crystal in 190 µl of 95% EtOH and reading its absorbance at 590 nm with a microplate reader (SpectroStar Nano, BMG Labtech).

2.8 Motility Assays

Swimming and swarming motility assays were performed for *B. multivorans* using previously described methodologies[77,78]. Agar plates containing 20 mL of swimming media [1% (wt/v) Tryptone, 0.5% (wt/v) NaCl, 0.3% (wt/v) Agar] or swarming media (composition presented below) 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. At least three plates were used for each strain per experiment and two independent assays were made.

Swarming solid medium: 10% Component A, 89% Component B, 1% Component C, 0.1% Casamino acids, 0.4% Agar

Component A: $(NH_4)_2SO_4$ 20 g/L, Na_2HPO_4 60 g/L, KH_2PO_4 30 g/L, NaCl 30 g/L

Component B: 2 mM $MgCl_2.6H_2O$, 100 µM $CaCl_2.2H_2O$, 3 µM $FeCl_2.6H_2O$

Component C: 1M Sodium Citrate

2.9 Fractionation of Cell proteins

Bacterial cells in exponential and stationary growth phases from *B. multivorans* strains were collected and centrifuged at 7000x g for 30 minutes, at 4 °C. The pellet was washed twice with 10 mM Tris buffer (pH 7.5) and resuspended in 7 mL of this buffer. The samples were kept at -80 °C until further use. The bacterial cells from the samples were lysed by ultrasonic vibration with the Branson sonifier 250 (Branson), and large cell debris were deposited with centrifugation at 6700x g, for 10 min, 4 °C. Cytoplasmic proteins were separated with ultracentrifugation (6 mL of the samples) using the Beckman XL-90 Ultracentrifuge (108,726x g, 10 min at 4 °C), after which the supernatant contained the cytoplasmic proteins and the pellet contained the total membrane proteins. An aliquot of 50 µL of the cytoplasmic membranes was set apart with 12.5 µL of SDS sample buffer [100 mM Tris (pH 6.8); 4% (w/v) SDS; 0.2% (w/v) bromophenol blue; 20% (v/v) glycerol; 200 mM DTT (dithiothreitol)]. The remaining pellet was washed twice in 6 mL of 10 mM Tris buffer (pH 7.5) and resuspended in 500 µL of the same buffer for exponential growth samples and 1 mL for stationary growth samples. An aliquot of the total membrane proteins for SDS-PAGE was taken (40 µL of sample, 25 µL SDS sample buffer) and, to further separate the inner and outer membrane proteins, the samples were resuspended in 10 mM Tris buffer supplemented with 2% (v/v) Triton X-100, incubated for 30 min at room temperature and centrifuged (108,726x g, 10 min at 4 °C). The pellet, containing the outer membrane proteins, was resuspended in 250 µL of 10 mM Tris buffer (pH 7.5) for exponential growth samples and 500 µL for stationary growth samples. An aliquot of 50 µL was taken for SDS-PAGE, adding 25 µL of SDS sample buffer.

2.10 Polyacrylamide Gel Electrophoresis

B. multivorans cytoplasmatic proteins, total membrane proteins and outer membrane proteins were separated in a SDS-PAGE. 12.5% polyacrylamide gels were used employing the discontinuous gel system[79] .The APS and TEMED solutions were added immediately before pouring the gel to avoid premature polymerization. 8 µl of previously boiled samples (5 min, 100 °C) were loaded into the gels that ran at 160V until the dye nearly reached the end of the gel system. Gel runs were made with Running Buffer 1x (25 mM Tris base; 192.4 mM glycine; 3.5 mM SDS). Gel staining was done with Coomassie Blue R-250 (Sigma).

2.11 RNA Isolation

For total RNA extraction, bacterial cultures were grown to an OD_{640} of 0.6 or 2.0, were mixed with 0.2 volumes of STOP solution (5% water-saturated phenol, 95% ethanol), and snap-frozen in liquid nitrogen. After thawing on ice, bacteria were pelleted by centrifugation (5 min, 15 000 g, 4°C), and RNA was extracted using the hot-phenol method and treated with DNAse I as previously described[80]. Total RNA quality was analyzed in gel and the concentration was quantified in a NanoDrop ND-1000 Spectrophotometer.

2.12 qRT-PCR

cDNA was synthesized from 2 µg RNA with Thermo Scientific RevertAid Reverse Transcriptase (RT) (Thermo Scientific), its provided Buffer (5X Buffer) and Random Hexamers (1 µL per reaction), following the instructions provided by the manufacturers. qPCR was performed in an Applied Biosystems QuantStudio 5 Real-Time PCR System, using the NZYSpeedy qPCR Green Master Mix (2x), ROX (NZYTech). Each reaction contained 100 ng of cDNA, 400 nM of each specific primer (**Table 2**) and the NZYSpeedy qPCR Green Master Mix. Real time PCR was performed at 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 seconds and 60°C for 30 seconds. A calibration curve was performed for each pair of primers and every sample was run in two technical replicates and three independent assays. Relative expression was calculated with the $2^{-\Delta\Delta CT}$ method[81] using the 5S rRNA for normalization (2 ng/µl cDNA per reaction). Changes in expression were analysed by One-Way ANOVA with a Tukey's multiple comparisons test using GraphPad Prism 6.

2.13 *In vitro* transcription and RNA labelling

The DNA fragments used as templates for *in vitro* transcription of the ncRNA3 sRNA and BCAL2915 RNA (nucleotides -39 to 89) were amplified from *B. cenocepacia* K56-2 genome using forward primers containing the T7 promoter at the 5' end (**Table 2**). RNA sequences were transcribed *in vitro* using the MEGAscript T7 kit (Ambion) with native UTP, or native UTP and Biotin-16-UTP (3:1), followed by DNase I digestion (1 unit) for 30 min at 37°C. The RNA synthesized was purified following standard procedures[82].

2.14 Electrophoretic mobility shift assay (EMSA)

The interactions between the sRNA ncRNA3 and BCAL2915 RNA were assessed using mobility shift assays with increasing amounts of the unlabelled RNA and a fixed concentration of the labelled RNA (0.04 pmol). Labelled RNAs, previously denatured for 1 min at 95 °C and cooled for 5 min on ice, were mixed in a total volume of 10 µL with 1 µg yeast tRNA (Ambion) and increasing amounts of unlabeled RNA in structure buffer (10 mM Tris-HCl pH 7.5, 50 mM KCl, 1 mM DTT, pH 7.5). The reaction mixtures were incubated at 37ºC for 30 min and mixed with 3 µL of native loading dye [50% (v/v) glycerol, 0.5x TBE, 0.2% (w/v) bromophenol blue] immediately before loading on 6% polyacrylamide, 0.5x TBE gels. After electrophoresis on 6% polyacrylamide (250V 4ºC), samples containing biotin labelled RNA were electroblotted to BrightStar®-Plus positively charged Nylon Membranes (Ambion) using a trans-Blot® SD (BIORAD) device at 15V and 120 mA for 50 min. Labelled RNA was detected by chemoluminescence using a Pierce Chemoluminescent Nucleic Acid Detection Module kit (Thermo Scientific) according to the manufacturer's instructions.

3. Results and Discussion

3.1 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 already identified in *B. cenocepacia* were predicted using two bioinformatic tools. For that, the sequences of 167 small RNAs that have been described, observed, or predicted in *B. cenocepacia* strains [17] were collected (Table S1 from Pita et al., 2018), and a list of antibiotic resistance genes described for Bcc bacteria was compiled (**Table S1**). The genes selected include genes involved in efflux pump mediated multidrug resistance (e.g., efflux pumps of the RND family), permeability of the outer membrane barrier (genes involved in modification of lipid A from the LPS molecule, alternative sigma factor RpoE, porins and other membrane proteins), and alteration of drug targets (e.g., GyrA, dihydrofolate reductase).

TargetRNA2[70] was used for a primary assessment of sRNAs targets. This tool provided the location of the predicted targets for each sRNA in the three replicons of *B. cenocepacia* J2315, the genes that are encoded by these regions, and the interaction site within the sRNA. Depending on the sRNA, the targets lists obtained ranged from a few to over a hundred targets in the three *B. cenocepacia* J2315 replicons. At least a target involved in antibiotic resistance was predicted for 78 of the sRNAs analyzed (data not shown). 16 of these 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, which was predicted to target a gene directly involved in trimethoprim resistance (**Table S2**). Except for ncRNA3, for all selected sRNAs at least one predicted target gene seems to be related with antibiotic resistance by efflux pumps. Antibiotic resistance genes related with the permeability of cell envelope were predicted to be targeted by six sRNAs (ncRNA11, nc5U1, ncRI9, nc5U23, nc5U59 and nc5U60), and a putative beta-lactamase (penB) seems to be targeted by four sRNAs (ncRNA11, nc5U23, nc5U19, ncS54).

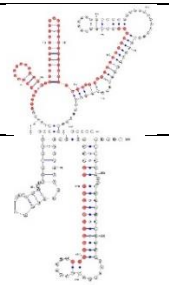
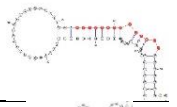
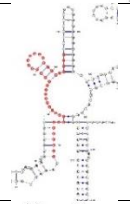
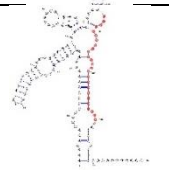
As most of the *B. cenocepacia* sRNAs are poorly characterized, the sRNAs ncS03, ncS06 and ncS54, which have a rho-independent terminator, are validated and are abundant in *B. cenocepacia* J2315 biofilms, were considered for further analysis[51]. Three genes related with antibiotic resistance, two efflux 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. In addition, recent results from 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, especially in Bcc bacteria that encode in their genomes several of these transporters, allowing the excretion of various antimicrobials[32], 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 (**Table 3**). 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[83,84], disrupting the biosynthetic pathways associated with this enzyme, and leading to cell death[85]. Thus, ncRNA3 was also

considered an sRNA of interest for further studies due to the specificity of its targets and the expected direct impact in antimicrobial resistance.

Considering the limitations of the bioinformatics tools available for target prediction, the tool CopraRNA[71] was also employed to confirm and evaluate the information collected regarding ncS06 and ncRNA3 mRNA targets. Target predictions performed using this tool are not as simple and fast as those with TargetRNA2[70], since CopraRNA[71] requires at least three homologous sRNA sequences from three distinct organisms as input. Using CopraRNA[71] three mRNA targets were predicted for ncS06. Out of the three targets, two were also predicted by TargetRNA2[70] (BCAM1421 and BCAS0583), being related with the RND efflux pumps. Interestingly, the MFS-related efflux pump previously targeted by TargetRNA2[70] was not detected by CopraRNA[71], but an additional target, the *arnT* gene that encodes for a 4-amino-4-deoxy-L-arabinose transferase, was predicted as an mRNA target of ncS06. This enzyme is crucial to the formation of Ara4N (Amino-4-deoxyarabinose), a key component of the LPS molecules of Bcc bacteria[24]. These data provide a good indication that the sRNA ncS06 could be involved in antibiotic resistance, presenting putative targets that not only affect efflux pumps but also the bacterial cell membrane. Interestingly, using CopraRNA[71] additional targets involved in antibiotic resistance were also identified within the genome of *B. multivorans*, the second most prevalent species of Bcc infections in CF patients. Curiously, the BCAL2915 gene, which was predicted to be a target of ncRNA3 in TargetRNA2[70], and the Bmul_4465 gene, which encodes for an MFS transporter not annotated in *B. cenocepacia* J2315 genome, were two of these predicted targets. These additional targets predicted for *B. multivorans* may potentiate possible effects of ncS06 on antimicrobial resistance, especially in this species.

The list of predicted targets for ncS06 and ncRNA3 collected by both bioinformatic tools is summarized below (**Table 3**).

Table 3. ncS06 targets predicted by TargetRNA2[70] and/or CopraRNA[71] using the genome of *Burkholderia cenocepacia* J2315, respective features and predicted sRNA structure, obtained with VARNA[86].The predicted interaction sites of ncS06 or ncRNA3 with the predicted targets are highlighted in red. ncS06* represents a target predicted for ncS06 using the genome of *B. multivorans* for target search.

sRNA	NCBI Locus Tag	NCBI Old Locus Tag	Coordinates	Gene Length	Product Description	Antibiotic resistance	CopraRNA[71]	TargetRNA2[70]	sRNA Structure
ncS06	QU43_RS46190	BCAL1929	2128443..2130119(+)	1677	4-amino-4-deoxy-L-arabinose transferase ArnT	Permeability of the cell envelope-LPS	-151|-117	-	
	QU43_RS55415	BCAM0199	232941..234377(+)	1437	outer membrane efflux protein	Efflux pump-MFS	-	-39|-25	
	QU43_RS61465	BCAM1421	1579322..1582489(-)	3168	RND family efflux system transporter protein	RND efflux pump-Acriflavine	-37|-8	-33|-14	
	QU43_RS71570	BCAS0583	636385..637803(+)	1419	efflux system transport protein	RND efflux pump-Acriflavine	1301|1337	1391|1405	
ncS06*	QU43_RS51220	BCAL2915	3196499..3196999 (+)	501	dihydrofolate reductase	Drug target modification-Trimethoprim	44|80	-	
ncRNA3							-14|10	-19|-7	

3.2 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, between the BCAL0549 and BCAL0550 genes, that encode for a TraB family protein and a LamB/YcsF family protein, respectively. This sRNAs has been reported to be conserved among Bcc species, which is favorable for testing the antimicrobial susceptibility in diverse Bcc bacteria[17].Structure predictions made with RNAfold WebServer[87] reveal that this molecule has the conformation represented in **Figure 3**, and a Minimum Free Energy of -98.20 kcal/mol.

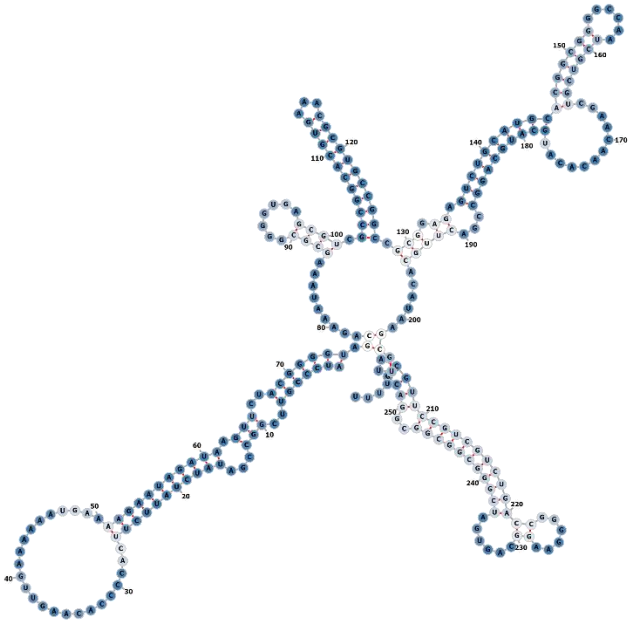


Figure 3. ncS06 predicted sRNA structure. Predicted structure was obtained with RNAfold WebServer[87] and visualized with Forna[88]. Different shades of blue represent the base pair probability of the sRNA nucleotides; The darker the blue, the higher the base pair probability is.

3.2.1 Influence of ncS06 in Antibiotic Resistance to Ciprofloxacin, Tobramycin and Trimethoprim

In order to evaluate the impact of ncS06 in Bcc resistance to antibiotics, this sRNA was overexpressed and silenced in three of the most clinically relevant Bcc species (*B. cenocepacia*, *B. multivorans* and *B. dolosa*) using appropriate plasmids. Three different plasmids were used: pTAP3, overexpressing ncS06, pMBJ1 and pMBJ2, encoding, respectively, an antisense sequence complementary with the initial or final region of this sRNA. All these plasmids derived from the pIN29 plasmid, a stable plasmid in Bcc strains [89]. Regarding the silencing of ncS06 based on antisense pairing, two silencing constructs were made due to the size of the sRNA and different locations of the sRNA are involved in the interaction with the predicted targets. As the antisense based silencing is more effective using small antisense molecules, oligonucleotides were designed (~50 bp) to encode an antisense RNA that pairs with the nucleotides 44 to 80 of ncS06, and another one that pairs with the nucleotides 180 to

221. The double strand short sequence was cloned in pIN29 plasmid, under control of a strong promoter (*tac* promoter), and the resulting constructions were confirmed by sequencing.

To evaluate the impact of the overexpression and silencing of ncS06 using the mentioned plasmids, three Bcc pathogenic strains were selected based on their prevalence in infections of CF patients: *B. cenocepacia* K56-2, *B. multivorans* LMG 16660 and *B. dolosa* AU0158. These isolates were collected from outbreaks in hospital settings, and have been studied throughout the years, being readily available for testing. Each isolate was transformed with the pIN29, pTAP3, pMBJ1 or pMBJ2 plasmids. To verify if the growth of these strains was influenced by the insertion of the transformed plasmids, growth curves for *B. cenocepacia* K56-2 carrying the pIN29 and pTAP3 plasmids were performed, measuring the OD_{640} of the bacterial culture and counting the number of colony forming units (CFU's). No major differences were found between the strain carrying pTAP3 and the strain with the empty plasmid in terms of growth curve (**Figure 4**) and number of bacteria (data not shown). The results obtained were extrapolated for the other strains in study, since no differences were found in the optical density measurement of bacterial cultures.

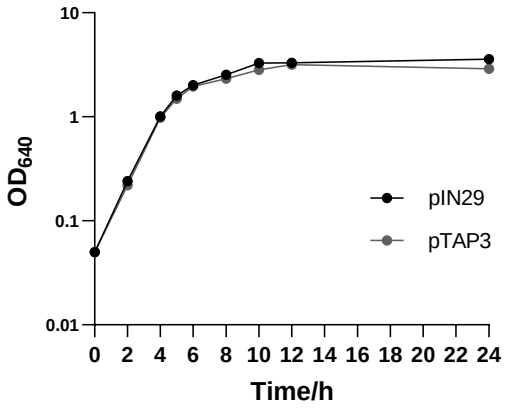


Figure 4. *B. cenocepacia* K56-2 growth curve. Growth curve was made with *B. cenocepacia* K56-2 carrying pIN29 (empty vector) and pTAP3 (ncS06 overexpression).

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[1]. The three antibiotics selected are extensively used for the treatment of Bcc-caused infections, especially in CF patients. Tobramycin and ciprofloxacin are often employed in combination with a third antibiotic, such as meropenem, for administration to MDR Bcc bacteria. Nebulized tobramycin is also administered

[1] There are currently no standardized methods for MIC determination in Bcc bacteria. According to the European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org/), the MIC breakpoints for Bcc bacteria cannot be established. This happens because Bcc bacteria are frequently part of mixed infections, the wide MIC distribution of Bcc for relevant antimicrobials, and finally, the lack of correlation between the MIC values determined to define the wild-type population as either susceptible or resistant. EUCAST indicates that MIC determination via broth microdilution with Mueller-Hinton broth could provide reproducible results. This was thereby the method chosen for antimicrobial susceptibility testing in Bcc strains. A control strain was included, the well-studied clinical isolate *E. coli* ATCC 25922, for which the MIC values are well defined.

to avoid side-effect on patients[8,14]. Trimethoprim, often used alongside with sulfamethoxazole, is a common and recommended antibiotic for Bcc infections[14]. The extensive use in clinic and the availability were the reasons for choosing these antibiotics for the antimicrobial susceptibility testing.

The MIC values of the different strains transformed with the plasmids overexpressing or silencing the sRNA ncS06 were compared with the parental strain carrying the empty plasmid pIN29. The results obtained are represented in **Table 4** (values obtained for all replicates are indicated in **Table S3, Table S4** and **Table S5**). Although slight differences can be observed between the MIC values obtained for the different constructs, a significant decrease in the MIC value of ciprofloxacin was observed for *B. multivorans* LMG 16660 overexpressing the ncS06. 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*. Although not statistically significant, an increase in the trimethoprim MIC value was also observed when the sRNA ncS06 was overexpressed in *B. cenocepacia* K56-2, MIC value variation from 7.99 ± 1.90 µg/mL (pIN29) to 15.14 ± 6.35 µg/mL (pTAP3), and *B. multivorans* LMG 16660, MIC value variation from 7.03 ± 1.20 µg/mL (pIN29) to 12.39 ± 2.58 µg/mL (pTAP3).

Table 4. MIC values of Bcc strains tested with Ciprofloxacin, Tobramycin and Trimethoprim for ncS06 (µg/mL). Significant values are represented with **** when the p-value<0.0001 (one-way ANOVA test).

	Strain	pIN29 (empty vector)		pTAP3 (ncS06 overexpression)		pMBJ2 (ncS06 Antisense)	
		MIC	S.D.	MIC	S.D.	MIC	S.D.
Ciprofloxacin	*B. multivorans* LMG 16660	16.36	0.87	11.16****	1.64	16.28	2.40
	B. dolosa AU0158	3.80	0.69	3.75	0.18	3.64	0.55
	B. cenocepacia K56-2	3.38	0.30	4.17	0.32	4.53	-
Tobramycin	*B. multivorans* LMG 16660	406.74	354.27	628.20	65.81	660.90	-
	B. dolosa AU0158	249.27	57.46	187.30	23.13	181.10	17.82
	B. cenocepacia K56-2	514.45	24.38	559.50	17.59	930.15	349.10
Trimethoprim	*B. multivorans* LMG 16660	7.03	1.20	12.39	2.58	9.34	0.34
	B. cenocepacia K56-2	7.99	1.90	15.14	6.35	14.19	-

The significant change in the susceptibility to ciprofloxacin suggests that the overexpression of ncS06 is more effective in *B. multivorans* LMG 16660, which is intrinsically more resistant to ciprofloxacin than *B. cenocepacia* K56-2. However, it remains unclear whether this effect is related to a

direct action of ncS06 in the expression of the antibiotic resistance predicted targets, or if it results from an indirect effect of the overexpression of this sRNA.

It is well known that efflux mechanisms are important determinants for Bcc resistance to antimicrobials[32], and three of the predicted targets for ncS06 were efflux pumps (**Table 3**). The efflux pumps play a major role in Bcc resistance, being able to secrete various antimicrobials such as ciprofloxacin, tobramycin, chloramphenicol, fluoroquinolones, trimethoprim-sulfamethoxazole, macrolides, and tetracyclines[14]. The RND pumps predicted to be targeted by ncS06 were described as involved in the extrusion of acriflavine, an old antibacterial drug with potential for antitumoral activity[90], but that is not currently used as a treatment for Bcc infections. 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.

3.2.2 How does the overexpression of ncS06 affect the expression of the predicted targets?

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 (listed previously in **Table 3**), qRT-PCR assays were performed. For this, in addition to evaluate the ncS06 expression levels in *B. multivorans*, only the expression of the predicted targets BCAM1421 and BCAL2915 could be determined. Although BCAM0199 and BCAS0583 genes were predicted to be targeted by ncS06 in *B. cenocepacia*, no homology was found between the predicted binding sequence of these targets and the available genomes of *B. multivorans* (it is noteworthy that the genome sequence of *B. multivorans* LMG 16660 is not available). Considering the location of the predicted interaction, the expression of the BCAL1929 gene was not selected to be analyzed, since it was unclear whether the predicted ncS06 binding site could interfere with the expression of the BCAL1928 or BCAL1929 genes. Finally, several attempts were also made to assess the expression of the Bmul_4465 predicted target, however, a linear calibration curve was never obtained for the designed primers when using the *B. multivorans* LMG 16660 genome. Due to all these limitations, only the expression of BCAL2915 and BCAM1421 was assessed by qRT-PCR in *B. multivorans* LMG 16660. For this, primers to amplify the sRNA ncS06 were designed, as well as primers to amplify part of the BCAM1421 and BCAL2915 transcripts. The expression of the 5S rRNA was used as the reference gene. RNA from *B. multivorans* LMG 16660 was extracted from the strains containing the pIN29, pTAP3 and pMBJ2 plasmids and was converted into cDNA to perform the qRT-PCR assays. RNA samples were tested to verify the purity of the extraction products and possible genomic DNA contaminations. The relative expression levels for ncS06, BCAM1421 and BCAL2915 were calculated for *B. multivorans* carrying different plasmids with the $2^{-\Delta\Delta ct}$ method[81], normalizing the Ct values obtained with the ones obtained for *B. multivorans* LMG 16660 carrying the empty plasmid. The relative

expression levels are represented in **Figure 5**.

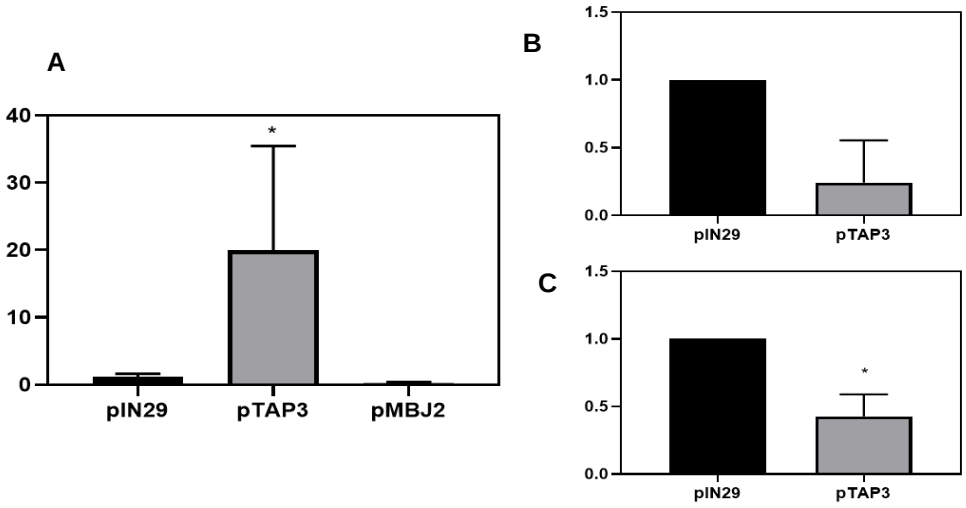


Figure 5. Relative expression levels of ncS06 and predicted gene targets. Relative expression values for ncS06 (**A**); BCAM1421 (**B**) and BCAL2915 (**C**) on *B. multivorans* LMG 16660 carrying the pIN29 (empty vector), pTAP3 (ncS06 overexpression) and pMBJ2 (ncS06 antisense) plasmids. Quantitative 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.

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 5A**). Both plasmids have the intended effects on ncS06 expression levels, strengthening the results obtained when testing the bacterial strains carrying these plasmids.

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 5B**). BCAM1421 encodes for an efflux pump from the RND family and as mentioned previously, the absence of specificity of this efflux pump for ciprofloxacin 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 [14]. Then, testing the relative expression levels of a single efflux pump target can be quite a limitation to justify differences in antibiotic resistance, since a subset of the pumps usually exhibits a considerable degree of substrate promiscuity, and the effects of the dysregulation of some efflux pumps can be compensated by others.

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 5C**). Since the gene BCAL2915 codes for the enzyme dihydrofolate reductase, that binds to trimethoprim inhibiting the folic acid synthesis pathway[83], 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, the opposite was verified, suggesting that targets involved in other mechanisms of resistance 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. Thus, considering the high number of predicted targets, a more global analysis, such as RNA-seq, would allow us to understand which genes would be differently expressed and facilitate the identification of genes regulated by ncs06 that may influence the Bcc resistance to antibiotics.

Curiously, 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 6**). 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. These effects are especially relevant in *B. multivorans* LMG 16660 since an influence on antimicrobial resistance was also detected.

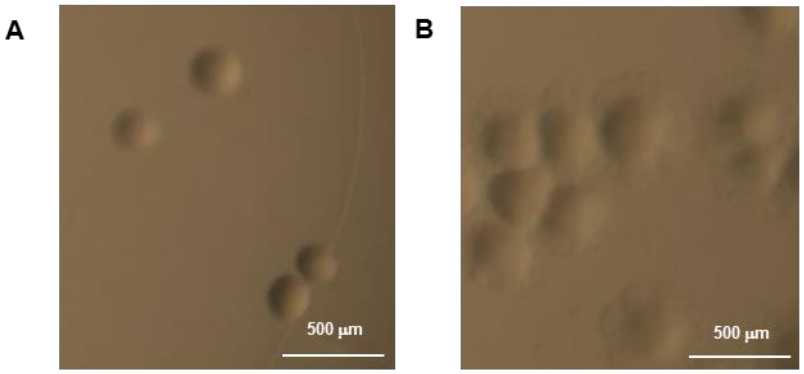


Figure 6. *B. multivorans* LMG 16660 colonies carrying the pIN29 (A) and pTAP3 (B) plasmids. The images of bacterial colonies were captured using an AxioCam 503 color device coupled to the Zeiss Stemi 2000-C Stereo Microscope.

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

To better understand how *B. multivorans* LMG 16660 carrying the pTAP3 plasmid overexpressing the sRNA ncS06 exhibited different resistance to antibiotics, several phenotypic assays were performed to find out if other global cellular processes would be altered in these bacteria.

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 test if the ncS06 overexpression could interfere with *B. multivorans* LMG 16660 motility and biofilm formation, swimming and swarming assays were performed, as well as crystal violet biofilm assays. The results for these assays are presented **Figure 7**.

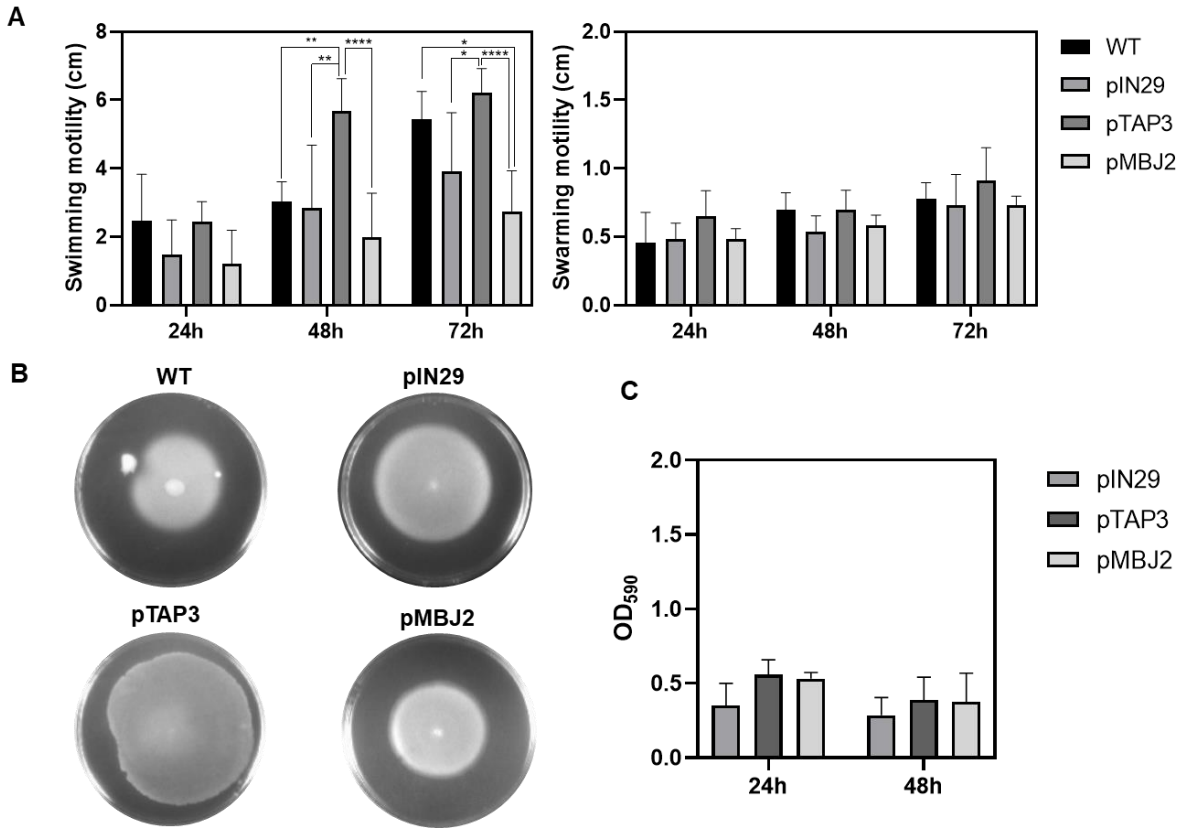


Figure 7. *B. multivorans* LMG 16660 motility and biofilm formation assays. Swimming and Swarming motility measurements for *B. multivorans* LMG 16660 strains (WT, pIN29, pTAP3 and pMBJ2) at 24h,48h and 72h (**A**). Swimming plates after 72h of incubation. Pictures were taken with the gray scale digital 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[91]. In fact, to colonize and persist in the lungs of CF patients, Bcc bacteria often experience loss of bacterial motility, which is accompanied by the formation of biofilms, thick matrixes of extracellular components and proteins that greatly limit the entrance of many antimicrobials[11]. Regarding the motility assays, no significant changes were verified in swarming motility when ncS06 was overexpressed in *B. multivorans* LMG 16660. However, *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 7B**). Beside the measurements made, 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 7A**). While the WT, pIN29 and antisense plasmid-carrying strains present their swimming patterns with circular or oval shapes with well-defined borders and halos, the strain carrying pTAP3 presented irregular borders and overall shape. Flagellar motility is considered one of the many virulence factors that Bcc bacteria possess. Flagella are reported to be involved with the motility, adhesion, invasion, and biofilm formation of bacterial cells[11,92]. During the target prediction for ncS06, a few targets related with the formation of the flagellum were identified:

fliH (BCAL0523), *fliP* (BCAL3503) and *flgH* (BCAL0570). 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.

The formation of biofilms is considered an important virulence factor in Bcc bacteria, which is involved with the establishment and persistence of Bcc infections[93]. To assess biofilm formation in *B. multivorans* LMG 16660, bacterial cells were incubated in LB medium, and the extent of biofilm formation was measured using the dye crystal violet. 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.

As previously mentioned, several genes related with secretion systems (BCAM2050, BCAL0337, BCAL3522), transporters (BCAL1110-BCAL1117), two-component regulatory systems (BCAL2011-BCAL2012), and several membrane proteins (BCAL0894, BCAL3114, BCAL3503) were predicted to be ncS06 targets. Considering that the protein composition of the outer membrane has a strong impact on the sensitivity of bacteria to many types of antibiotics, it was hypothesized whether the overexpression of the ncS06 sRNA could interfere with the expression of some components of the *B. multivorans* membrane. In an attempt to verify this hypothesis, a gram-negative fractionation method was applied and the cytoplasmic, total membrane and outer membrane proteins profiles of *B. multivorans* carrying the empty plasmid pIN29, overexpressing ncS06, or silencing this sRNA were analysed. The bacterial cell extracts were collected and fractionated from bacterial cultures in exponential and stationary phases. The protein profiles for these strains are shown in **Figure 8**.

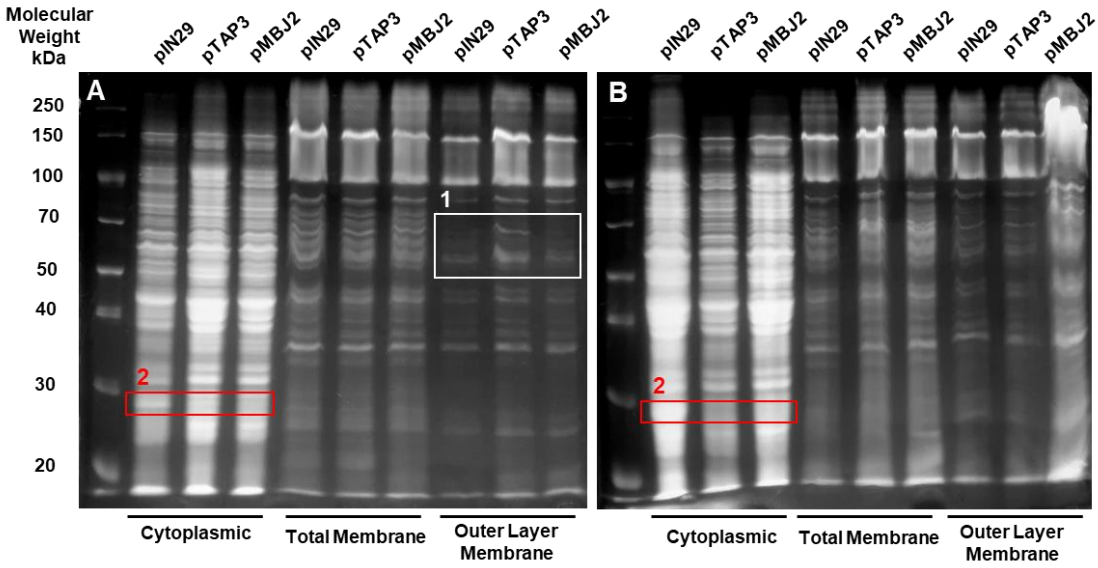


Figure 8. Protein profiles of *B. multivorans* LMG 16660. Protein extracts of *B. multivorans* during exponential (**A**) and stationary (**B**) growth phases. Cytoplasmic, total membrane and outer layer proteins from *B. multivorans* LMG 16660 strains carrying the pIN29 (empty vector), pTAP3 (ncsS06 overexpression) and pMBJ2 (ncS06 antisense) were separated on 12.5% acrylamide gels. White and red boxes (1- white; 2- red) signal bands that are different amongst the given profiles. First lane of each gel image represents the protein ladder, and the respective molecular weights are pointed next to it.

Despite the low resolution obtained for some samples, some profiles seem to have less protein content than others. As the same initial number of cells was used, probably these differences resulted from inefficiency of the cell lysis during the extractions or loss of material throughout the separations. These differences were evident in the gels since the protein concentration of each fraction was not quantified, and samples with different amounts of proteins were loaded onto the gel. Despite this limitation, results show that most of the protein profiles are relatively similar between the various samples for each of the collected fractions. In cytoplasmic phase protein fractions from exponential and stationary *B. multivorans* carrying pIN29, a band (<30 kDa) that does not appear in the other cytoplasmic samples is notorious. As the gene that encodes for DsRed (26.8 kDa) in pIN29 under control of *tac* promoter was replaced by smaller fragments in pTAP3 and pMBJ2 plasmids, it is quite likely that this band corresponds to the mentioned fluorescent protein. Regarding the exponential growth phase profiles, two protein bands from the outer membrane fraction (that are signaled in **Figure 8A, box 1**) seem to be more intense in *B. multivorans* carrying the plasmid that overexpress the sRNA ncS06. These bands have a molecular weight between 50-70 kDa and can contribute to a significant alteration of the outer membrane, so their identification should be done. The mentioned differences in the band profile do not seem to be maintained in the stationary phase, suggesting that these alterations are growth phase-dependent.

The molecular weights of the proteins that are translated from the target genes predicted for ncS06 were analyzed, and three genes encoding for proteins whose molecular weight ranges between 50 and 70 kDa were identified. The gene BCAL1929, encoding for the ArnT protein (~63 kDa), essential for the formation of the key component of the Bcc LPS Ara4N and directly related with antibiotic resistance; the gene BCAL2012, encoding a histidine kinase (~51kDa) that regulates the OmpR channel; and the BCAL0621, encoding a putative cyclic-diGMP signaling protein (~66 kDa). The modulation of the cyclic-diGMP has been reported to affect swimming motility in *B. cenocepacia* [94]. Although interesting targets, these proteins are all classified as cytoplasmatic proteins, being unlikely targets to justify the differences found in the outer membrane fractions.

Other genes related with LPS assembly and formation were also predicted to be targeted by ncS06: a BCAL3103 (encode for a UreD-family accessory protein), BCAL3116 (glycotransferase WbxB), BCAL3136 (diadenosine tetraphosphatase ApaH), BCAL3114 (the putative O-antigen exporter Wzx) and BCAL0894 (an LPS assembly protein). To avoid host antimicrobial factors, Gram negative bacteria can change the structure of their LPS, which allow them to evade host immunity and promote further virulence. The results obtained highlight the difficulty of finding a specific and direct mechanism of action that explains the role of the ncS06 overexpression in the *B. multivorans* phenotypes and resistance to antibiotics. Alterations in the cell membrane may explain the differences caused by the overexpression of ncS06 in *B. multivorans* colony morphology, motility and in the profile of the outer membrane proteins.

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.

3.3 Role of the sRNA ncRNA3 in Bcc resistance to antibiotic

ncRNA3 is a small RNA identified through RNA-seq by Yoder-Himes *et* al. in 2009[95] as containing 129 nucleotides. ncRNA3 is encoded in chromosome 1, in the intergenic region between the BCAL1338 gene, which encodes for a hypothetic protein, and the *bcsB* gene (BCAL1389), encoding for a cellulose synthase regulator protein. This sRNA is extremely conserved in *B. cenocepacia* strains but not conserved in Bcc species. Structure predictions (**Figure 9**) were made using the RNAfold WebServer[87], and the Minimum Free Energy calculated for the predicted structure was -40.80 kcal/mol. Only one target related to antimicrobial resistance was predicted for this sRNA, the BCAL2915 that encodes for a dihydrofolate reductase, with high affinity to the antibiotic trimethoprim.

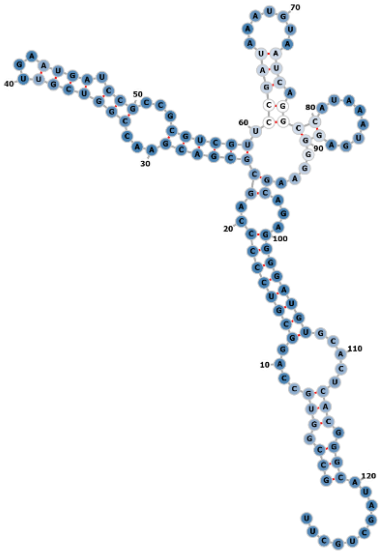


Figure 9. ncRNA3 predicted structure. The predicted structure was obtained with RNAfold WebServer[87] and visualized with Forna[88]. Different shades of blue represent the base pair probability of the sRNA nucleotides; The darker the blue, the higher the base pair probability is.

3.3.1 ncRNA3 affects the resistance of Bcc bacteria to Trimethoprim

Similar to ncS06, to evaluate the impact of the sRNA ncRNA3 in antimicrobial resistance, this sRNA was cloned in the pIN29 vector under control of the strong tac promoter. The resulting plasmid, pMBJ3, was transferred into two of the most relevant Bcc species in CF infections, *B. multivorans* and *B. cenocepacia*, and the Minimum Inhibitory Concentration was tested with the broth microdilution method. Since only one target related with antimicrobial resistance was predicted for ncRNA3, the trimethoprim resistance gene BCAL2915, this antibiotic was chosen to perform the MIC assays. For this purpose, *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. The results obtained for these strains are presented in **Table 5** (values obtained for all replicates are indicated in **Table S5**).

Table 5. Trimethoprim MIC values obtained for two Bcc clinical strains carrying the pIN29 empty vector or the pMBJ3 plasmid overexpressing the ncRNA3 (μg/mL). Significant values are represented with ** when the p-value<0.01 (one-way ANOVA test).

Trimethoprim	Strain	pIN29 (empty vector)		pMBJ3 (ncRNA3 overexpression)	
		MIC	S.D.	MIC	S.D.
	B. multivorans LMG 16660	7.03	1.20	10.10	0.52
	B. cenocepacia K56-2	7.99	1.90	20.42**	1.10

When the bacterial cells were transformed with the ncRNA3 expressing vector, 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 *B. cenocepacia* K56-2 carrying the ncRNA3 expressing plasmid pMBJ3. Therefore, the overexpression of ncRNA3 seems to increase the *B. cenocepacia* K56-2 resistance to trimethoprim. This increased resistance was not verified when the sRNA ncRNA3 was expressed in *B. multivorans*, possible due to lack of ncRNA3 conservation among Bcc species, being this sRNA only conserved amongst *B. cenocepacia* strains.

Although not significant, an increased resistance to trimethoprim was also verified when ncS06 was overexpressed in *B. cenocepacia.* 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). When the sRNA ncRNA3 was overexpressed in *B. cenocepacia*, the trimethoprim MIC value was considerably higher than the value obtained when overexpressing ncS06. 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.

3.3.2 The effect of the ncRNA3 overexpression in BCAL2915 expression levels

To assess if the overexpression of ncRNA3 was affecting the BCAL2915 expressing levels, qRT-PCR assays were performed. For this, total RNA from *B. cenocepacia* K56-2 strains carrying the pIN29 plasmid and the pMBJ3 plasmid was extracted from both exponential and stationary growth phases, and the extracted RNA was converted into cDNA. Specific primers for BCAL2915 and ncRNA3 were designed, and the 5S rRNA was used as the reference gene. The relative expression levels of ncRNA3 and BCAL2915 for the mentioned strains and growth conditions are represented in **Figure 10**.

The results clearly show that in both exponential and stationary growth phases 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 10A**).

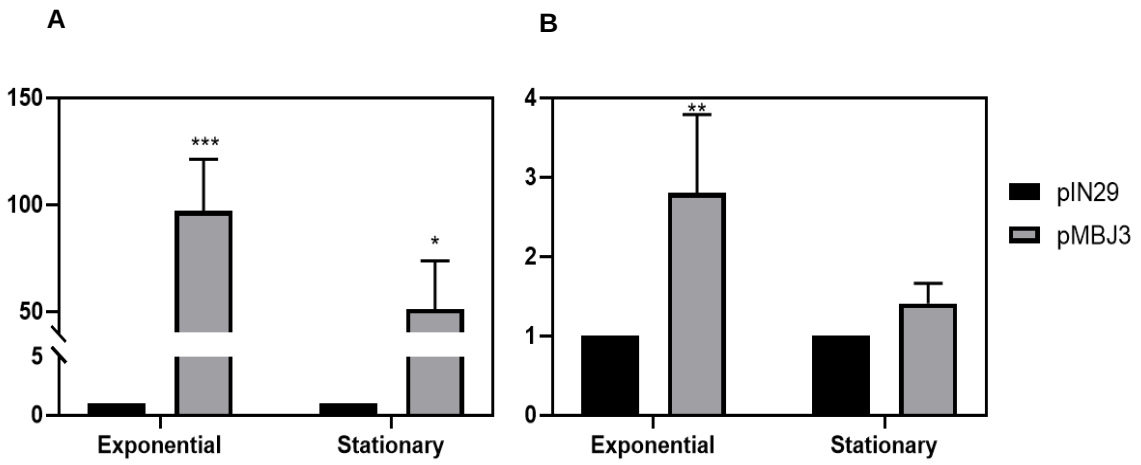


Figure 10. Relative expression levels of ncRNA3 and BCAL2915 in *B. cenocepacia* K56-2. The relative expression levels of ncRNA3 (**A**) and BCAL2915 (**B**) were determined for *B. cenocepacia* K56-2 carrying the pIN29 (empty vector) and pMBJ3 (ncRNA3 overexpression) plasmids in exponential and stationary 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.

When the sRNA ncRNA3 was overexpressed in *B. cenocepacia* K56-2, the BCAL2915 expression levels also increased. An increase in the expression of BCAL2915 was verified in both exponential and stationary growth phases of *B. cenocepacia* overexpressing the ncRNA3, however this effect was only statistically significant during the exponential growth phase. As previously mentioned, the overexpression of BCAL2915, the gene that encodes for the dihydrofolate reductase (DfrA) enzyme to which trimethoprim binds to, was expected to lead to an increase in the resistance to this antibiotic. This is due to one of the bacterial resistance mechanisms for this antibiotic, which includes the overexpression of the DfrA enzyme, leading to an increase in its bioavailability in the cell and requiring higher concentrations of trimethoprim to exert its antimicrobial effect[84]. 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 exerts a direct effect over BCAL2915, the interaction between these two molecules should be tested.

3.3.3 Direct RNA-RNA interaction between ncRNA3 and its target BCAL2915

To explore if the sRNA ncRNA3 interacts directly with its predicted target BCAL2915, an Electrophoretic Mobility Shift Assay (EMSA) was performed. To better understand the interaction between these two molecules, the IntaRNA program was used[71], where both sequences were inserted to provide a prediction of the interaction site, and its respective features.

It was predicted that the 3' end of the sRNA ncRNA3 interacts with the 5' untranslated region (5' UTR) of the BCAL2915 gene. The predicted interaction region of the sRNA, that range from the 86th nucleotide to 110th nucleotide of its sequence, is extremely conserved in various species of *B. cenocepacia* (**Figure S2**). The schematic view of the interaction predicted for ncRNA3 and BCAL2915 is represented in **Figure 11**.

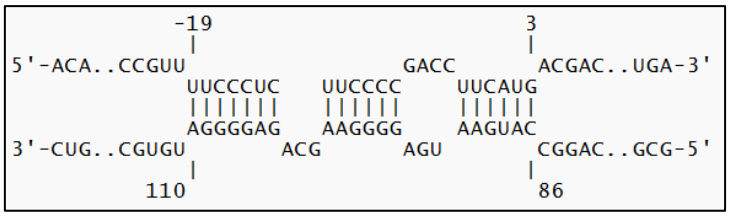


Figure 11. ncRNA3 predicted interaction site with BCAL2915. Top query represents the BCAL2915 sequence and bottom query represents the ncRNA3 sequence. The Energy calculated for this interaction is -12.87 kcal/mol and Hybridization Energy is -26.12 kcal/mol. Interaction scheme was calculated and produced with the IntaRNA[71] tool.

Although there is no perfect complementarity throughout the interaction site for ncRNA3 and BCAL2915, the energy predicted for this interaction was -12.87 kcal/mol, favoring the interaction.

To experimentally 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. If an interaction between these two molecules occurs, the sRNA-target RNA complex will migrate slower relatively to unbound labelled RNA molecule during electrophoresis in a nondenaturing 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 12**.

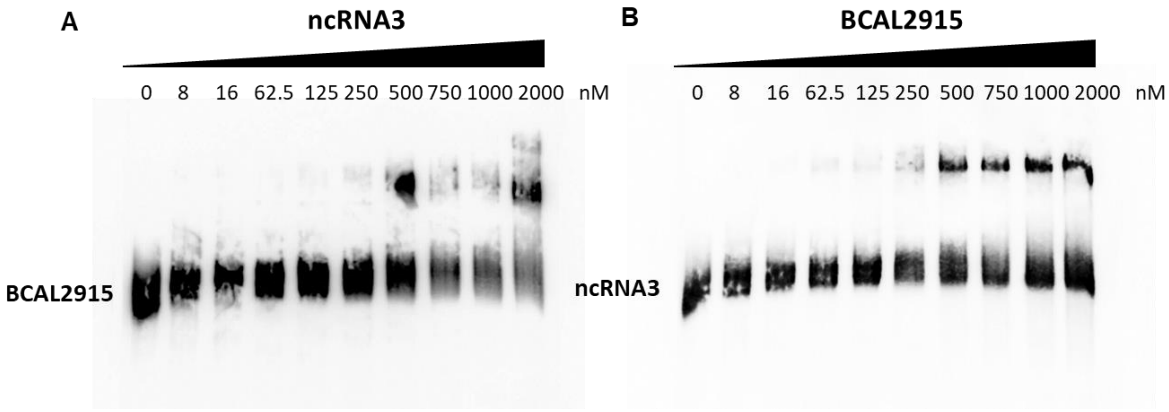


Figure 12. The sRNA ncRNA3 interacts with the BCAL2915 RNA. EMSA interaction results for BCAL2915 (**A**) and for ncRNA3 (**B**) labelled with Biotin. For each assay, increasing concentrations of unlabelled ncRNA3 (**A**) or BCAL2915 (**B**) were used against a fixed concentration of the other molecule.

From the results obtain through EMSA, it is clear that ncRNA3 interacts with its predicted target BCAL2915. In both gels, the two molecules are shown to interact with each other by the gel shift. In both cases, the interaction becomes notoriously visible when the labelled RNA is mixed with ~250 nM of the unlabeled RNA.

The chemoluminescence signal of each bound and unbound band was quantified, and a graph plotting the fraction of the labelled ncRNA3 bound in each reaction versus the concentration of unlabelled BCAL2915 (nM) was constructed. The data were fit with a binding equation, the specific binding equation with Hill slope, to determine the Kd (equilibrium dissociation constant). Although more replicates are required to calculate the binding affinity, the calculated Kd value was around 190 nM

(**Figure S3**).

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. As the expression of the target increases when the sRNA is overexpressed, it is possible that the ncRNA3 somehow promotes the stabilization of mRNA BCAL2915 *in vivo*.

In *B. multivorans* LMG 16660, the overexpression of the ncRNA3 had no effects on the resistance of this strain to trimethoprim, and it was assumed that this could be due to the low conservation of this sRNA in Bcc bacteria. It was interesting to note that the region of the BCAL2915 gene that was predicted to interact with ncRNA3 is not very conserved either in Bcc bacteria (**Figure S2**). This could be a reason for the ncRNA3 overexpression fail to influence antibiotic resistance in other Bcc species, namely in *B. multivorans* that was previously tested and showed no changes in resistance to trimethoprim (**Table 5**). Although this precludes a wider use, the specificity of ncRNA3 for *B. cenocepacia* targets could be useful in combating infections caused by one of the worst Bcc species.

Unlike ncS06, ncRNA3 results strongly suggest that this sRNA interacts directly with a specific *B. cenocepacia* target, influencing the antimicrobial resistance of these bacteria.

It is well stablished that bacterial sRNAs influence the expression of mRNA targets, and can even regulate the expression of multiple targets, making them key regulators in bacteria[17]. The sRNAs characterized in this study differ in size, and also in the number and type of predicted targets that they can regulate. While ncS06 seems to influence several targets, which can potentiate and expand the scope of the effects seen in antimicrobial resistance to different antimicrobials, ncRNA3 was mostly associated with one specific target, related with the resistance to the antimicrobial trimethoprim. RNAs with these two profiles have also been described for other bacteria.

The AS1974 sRNA, identified in *P. aeruginosa*, was reported to influence multiple pathways simultaneously and its overexpression/repression was associated with resistance to various antimicrobials such as aminoglycosides, chloramphenicol, and most β-lactams[35]. This sRNA seems to modulate the expression of genes involved in efflux pumps, cell wall turnover, biofilm formation, motility and iron acquisition. ncS06, similarly to the *P. aeruginosa* AS1974 sRNA, also presents various predicted gene targets that are related with these functions, which broadens the spectrum of effects seen with antimicrobials.

The CsiR sRNA (ciprofloxacin stress-induced ncRNA) identified in *P. vulgaris* was shown to interact with the *erm*B gene, which heavily influenced the resistance of this species to ciprofloxacin[96]. Similarly to ncRNA3, the action of this sRNA was associated to a specific target, which influenced the bacterial resistance to a specific antimicrobial.

Studying RNAs that have their function associated with a reduced number of targets can be beneficial for linking possible phenotypical effects to their molecular source. However, this can also be a limitation in achieving significant and lasting changes in antimicrobial susceptibility, especially to more

than one antibiotic. This should be considered in future therapies due to the vast resistance that Bcc bacteria have evolved to almost all antimicrobials clinically available.

4. Final Remarks and Future Prospects

The rapid emergence of bacteria resistant to multiple antibiotics is occurring worldwide, and the threat of untreatable antimicrobial resistant infections is now a reality for many individuals. Members of the *Burkholderia cepacia* complex have high inherent resistance to conventional antibiotics and it is urgent to find alternatives to antibiotic therapy to treat the infections caused by these bacteria. Small RNAs have notable roles in modulating the composition of the bacterial envelope, and through these functions control intrinsic antimicrobial resistance in many human pathogens. Despite being studied as alternatives to antimicrobial therapies, the effect of most sRNAs, especially in Bcc bacteria, is still poorly understood[51,52]. In the present work, 78 sRNAs from *B. cenocepacia* were predicted to target at least one gene related to 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, and slightly increased the resistance of these bacteria to trimethoprim. 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.

The world of sRNAs is still beginning to be unravelled in Bcc bacteria: most sRNAs are still poorly characterized, and their regulatory pathways and influence in antibiotic resistance are now beginning to be more studied. However, in the course of this work it became clear that Bcc sRNAs produce impactful effects in bacterial cells, and their use as possible molecular alternatives to antimicrobial therapy is quite promising.

5. Supplementary Materials

5.1 Supplementary Figures

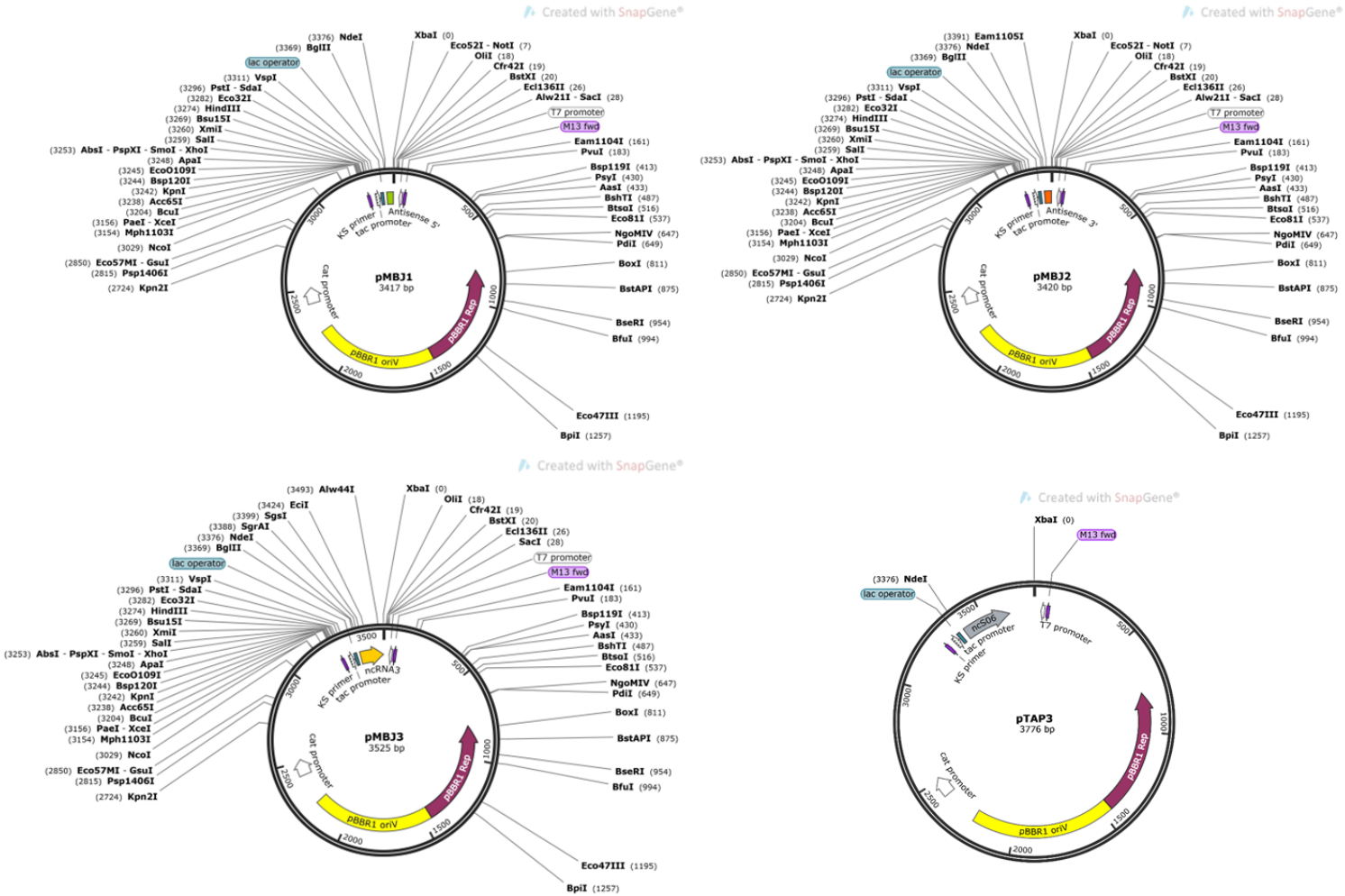


Figure S1. Plasmid maps for pMBJ1 (ncS06 antisense), pMBJ2 (ncS06 antisense), pMBJ3 (ncRNA3 overexpression) and pTAP3 (ncS06 overexpression).

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A

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BcepD          CCGATAAATGCAATCAGGCCATGAATGAGGGGAAGCAGAGGGGATGTGCACTTACGGGCA
BcenoCR        CCGATAAATGCAATCAGGCCATAAATGAGGGGAAGCAGAGGGGATGTGCACTCACGGGCA
BcenoJ         CCGATAAATGTAATCAGGCCATAAATGAGGGGAAGCAGAGGGGATGTGCACTCACGGGCA
BcenoFD        CCGATAAATGTAATCAGGCCATAAATGAGGGGAAGCAGAGGGGATGTGCACTCACGGGCA
BcenoH         CCGATAAATGTAATCAGGCCATAAATGAGGGGAAGCAGAGGGGATGTGCACTCACGGGCA
BcenoV         CCGATAAATGCAATCAGGCCATAAATGAGGGGAAGCAGAGGGGATGTGCACTCACGGGCA
BcenoFL        CCGATAAATGCAATCAGGCCATAAATGAGGGGAAGCAGAGGGGATGTGCACTCACGGGCA
BcenoCM        CCGATAAATGCAATCAGGCCATAAATGAGGGGAAGCAGAGGGGATGTGCACTCACGGGCA
BcenoM         CCGATAAATGCAATCAGGCCATAAATGAGGGGAAGCAGAGGGGATGTGCACTCACGGGCA
               ********** *********** ***************************** *******
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B

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Bdol           TAGAATCGCCGCCATCCCGGTTTTCCTTTTTCTACCCTCTCATGACGACGTTGACCCTGA
BmulA          TAGAATCGGCGCCATTCCGGTTCTTCCTCTCTCCGACCTTCATGACGACGTTGACCCTGA
BmulF19        TAGAATCGGCGCCATTCCGGTTCTTCCTCTCTCCGACCTTCATGACGACGTTGACCCTGA
BcenoJ         TAAAATCGGC-CCATTCCGTTTTCCCTCTTCCCCGACCTTCATGACGACGTTGACCCTGA
BcenoK         TAAAATCGGC-CCATTCCGTTTTCCCTCTTCCCCGACCTTCATGACGACGTTGACCCTGA
Bamf           TAGAATCGGCGCCATTCCGGCTCTCCTCTTCCCCGACCTTCATGACGACGTTGACCCTGA
Bcep           TAGAATCGGCGCCATTCCGGTTTTCCTC-TCCCCGACCTTCATGACGACGTTGACCCTGA
               ** ***** * **** ***  *   *   *      *  ********************
```

Figure S2. Sequence alignments for ncRNA3 (A) and BCAL2915 (B), in their predicted regions for interaction. **A** - ncRNA3 sequence alignment from the 61st nc to 120th nc using homologs of this sRNA from Bcc bacteria. Highlighted part (black) represents the region of interaction with BCAL2915 (86-110 nc). Sequences from *B. cepacia* DDS 7H-2; *B. cenocepacia* CR318, J2315, FDAARGOS_720, HII2424, VC7848, FL-5-3-30-s1-D7, CMCC (B) 23006 and MC0-3 were used in multiple sequence alignment. **B** – sequence alignment from the -39th nc to 20th nc of BCAL2915 sequence using homologs from Bcc bacteria (*B. dolosa* AU0158, *B. multivorans* ATCC 17616, *B. multivorans* FDAARGOS_719, *B. cenocepacia* J2315, *B. ambifaria* MC40-6 and *B. cepacia* ATCC 25416). Highlighted zone (black) represents the region for predicted interaction with ncRNA3 (-19 to 10 nc). Asterisks show conservation in the ranging nucleotide sequences. Multiple sequence alignment was performed using the MUSCLE software[97].

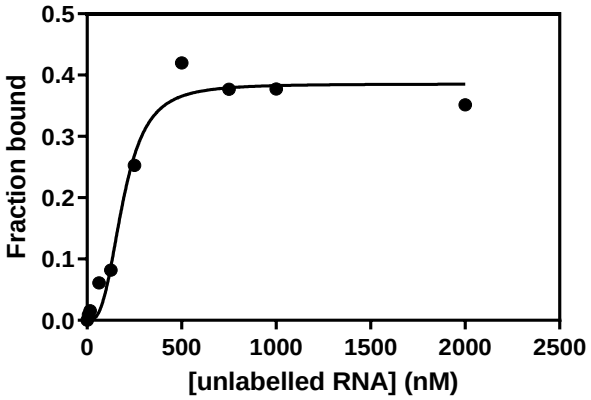

Figure S3. Quantification of the fraction bound during the EMSA. Labelled RNA (ncRNA3) chemoluminescent signal was quantified and fitted into a binding equation versus the concentration of unmarked RNA (BCALI2915). The slope of the binding equation was used to calculate the Kd.

5.2 Supplementary Tables

Table S1. Genes reported so far as being involved in *B. cenocepacia* J2315 antimicrobial resistance.

Locus ID	Description	Type of resistance	Family	Antibiotic Substrate
BCAS0591	RND-1	RND efflux pump	HAE-RND	Essential oils
BCAS0592	RND-1	RND efflux pump	HAE-RND	Essential oils
BCAS0593	RND-1	RND efflux pump	HAE-RND	Essential oils
BCAS0764	RND-2	RND efflux pump	HAE-RND	Fluoroquinolones, Tetracycline, rifampicin, novobicin, essential oils
BCAS0765	RND-2	RND efflux pump	HAE-RND	Fluoroquinolones, Tetracycline, rifampicin, novobicin, essential oils
BCAS0766	RND-2	RND efflux pump	HAE-RND	Fluoroquinolones, Tetracycline, rifampicin, novobicin, essential oils
BCAS0767	RND-2: LysR Family transcriptional regulator	RND efflux pump		Fluoroquinolones, Tetracycline, rifampicin, novobicin, essential oils
BCAS0768	RND-2: AraC family transcriptional regulator	RND efflux pump		Fluoroquinolones, Tetracycline, rifampicin, novobicin, essential oils
BCAL1674	RND-3	RND efflux pump	HAE-RND	Nalidixic acid, ciprofloxacin, tobramycin, meropenem, chlorhexidine
BCAL1675	RND-3	RND efflux pump	HAE-RND	Nalidixic acid, ciprofloxacin, tobramycin, meropenem, chlorhexidine
BCAL1676	RND-3	RND efflux pump	HAE-RND	Nalidixic acid, ciprofloxacin, tobramycin, meropenem, chlorhexidine
BCAL1672	RND-3: TetR type regulator	RND efflux pump		Nalidixic acid, ciprofloxacin, tobramycin, meropenem, chlorhexidine
BCAL2820	RND-4	RND efflux pump	HAE-RND	Aztreonam, chloramphenicol, fluoroquinolones, tobramycin, tetracycline, rifampicin, novobiocin,

				essential oils, ethidium bromide, 2-thiocyanatopyridine derivative (11026103)
BCAL2821	RND-4	RND efflux pump	HAE-RND	Aztreonam, chloramphenicol, fluoroquinolones, tobramycin, tetracycline, rifampicin, novobiocin, essential oils, ethidium bromide, 2-thiocyanatopyridine derivative (11026103)
BCAL2822	RND-4	RND efflux pump	HAE-RND	Aztreonam, chloramphenicol, fluoroquinolones, tobramycin, tetracycline, rifampicin, novobiocin, essential oils, ethidium bromide, 2-thiocyanatopyridine derivative (11026103)
BCAL2823	RND-4: TetR type regulator	RND efflux pump		Aztreonam, chloramphenicol, fluoroquinolones, tobramycin, tetracycline, rifampicin, novobiocin, essential oils, ethidium bromide, 2-thiocyanatopyridine derivative (11026103)
BCAL1079	RND-6-7	RND efflux pump	HAE-RND	Essential oils
BCAL1080	RND-6-7	RND efflux pump	HAE-RND	Essential oils
BCAL1081	RND-6-7	RND efflux pump	HAE-RND	Essential oils
BCAM0925	RND-8	RND efflux pump	HAE-RND	Tobramycin
BCAM0926	RND-8	RND efflux pump	HAE-RND	Tobramycin
BCAM0927	RND-8	RND efflux pump	HAE-RND	Tobramycin
BCAM1945	RND-9	RND efflux pump	HAE-RND	Tobramycin, chlorhexidine, Essential Oils, 2-thiocyanatopyridine derivative (11026103), 2,1,3-benzothiadiazol-5-yl family compound (10126109)
BCAM1946	RND-9	RND efflux pump	HAE-RND	Tobramycin, chlorhexidine, Essential Oils, 2-thiocyanatopyridine derivative (11026103), 2,1,3-benzothiadiazol-5-yl family compound (10126109)
BCAM1947	RND-9	RND efflux pump	HAE-RND	Tobramycin, chlorhexidine, Essential Oils, 2-thiocyanatopyridine

				derivative (11026103), 2,1,3-benzothiadiazol-5-yl family compound (10126109)
BCAM1948	RND-9: Mer-R type regulator	RND efflux pump	HAE-RND	Tobramycin, chlorhexidine, Essential Oils, 2-thiocyanatopyridine derivative (11026103), 2,1,3-benzothiadiazol-5-yl family compound (10126109)
BCAM2548	RND-10	RND efflux pump	HAE-RND	Chloramphenicol, fluoroquinolones, Trimethoprim, Essential Oils
BCAM2549	RND-10	RND efflux pump	HAE-RND	Chloramphenicol, fluoroquinolones, Trimethoprim, Essential Oils
BCAM2550	RND-10	RND efflux pump	HAE-RND	Chloramphenicol, fluoroquinolones, Trimethoprim, Essential Oils
BCAM2551	RND-10	RND efflux pump	HAE-RND	Chloramphenicol, fluoroquinolones, Trimethoprim, Essential Oils
BCAM0711	RND-11	RND efflux pump	Heavy-metal efflux-RND	Divalent cations (Zn2+, Co2+, Cd2+ and Ni2+)
BCAM0712	RND-11	RND efflux pump	Heavy-metal efflux-RND	Divalent cations (Zn2+, Co2+, Cd2+ and Ni2+)
BCAM0713	RND-11	RND efflux pump	Heavy-metal efflux-RND	Divalent cations (Zn2+, Co2+, Cd2+ and Ni2+)
BCAM0433	RND-12	RND efflux pump	Heavy-metal efflux-RND	Monovalent cations (Cu+ and Ag+), Essential Oils
BCAM0434	RND-12	RND efflux pump	Heavy-metal efflux-RND	Monovalent cations (Cu+ and Ag+), Essential Oils
BCAM0435	RND-12	RND efflux pump	Heavy-metal efflux-RND	Monovalent cations (Cu+ and Ag+), Essential Oils
BCAL2134	RND-16	RND efflux pump	Uncertain function -RND	Minocycline, meropenem ciprofloxacin
BCAL2135	RND-16	RND efflux pump	Uncertain function -RND	Minocycline, meropenem ciprofloxacin
BCAL2136	RND-16	RND efflux pump	Uncertain function -RND	Minocycline, meropenem ciprofloxacin
BCAM1417	RND:two-component regulatory system	RND efflux pump	HAE-RND	Acriflavine

	sensor kinase			
BCAM1418	RND: two-component regulatory system response regulator protein	RND efflux pump	HAE-RND	Acriflavine
BCAM1419	RND	RND efflux pump	HAE-RND	Acriflavine
BCAM1420	RND	RND efflux pump	HAE-RND	Acriflavine
BCAM1421	RND	RND efflux pump	HAE-RND	Acriflavine
BCAS0582	RND	RND efflux pump	HAE-RND	Acriflavine
BCAS0583	RND	RND efflux pump	HAE-RND	Acriflavine
BCAS0584	RND	RND efflux pump	HAE-RND	Acriflavine
BCAS0585	RND:two-component regulatory system sensor kinase	RND efflux pump	HAE-RND	Acriflavine
BCAS0586	RND: two-component regulatory system response regulator protein	RND efflux pump	HAE-RND	Acriflavine
BCAM0199	outer membrane efflux protein	Efflux pump	MFS	
BCAM0200	efflux system transport protein	Efflux pump	MFS	
BCAM0201	major facilitator superfamily protein	Efflux pump	MFS	
BCAM2186	putative macrolide-specific efflux system transport protein	Efflux pump	ABC	
BCAM2187	putative macrolide-specific ABC-type efflux carrier protein	Efflux pump	ABC	

BCAM2188	outer membrane efflux protein	Efflux pump	ABC	
BCAL2957	DNA gyrase subunit A (gyrA)	Drug target modification		
BCAL2454	DNA topoisomerase IV subunit A (parC)	Drug target modification		quinolone
BCAL2915	dihydrofolate reductase (dfrA)	Drug target modification		Trimethoprim
BCAL1927	Ara4N	Permeability of the cell envelope	LPS	
BCAL1928	Ara4N	Permeability of the cell envelope	LPS	
BCAL1929	Ara4N	Permeability of the cell envelope	LPS	
BCAL1930	Ara4N	Permeability of the cell envelope	LPS	
BCAL1931	Ara4N	Permeability of the cell envelope	LPS	
BCAL1932	Ara4N	Permeability of the cell envelope	LPS	
BCAL1933	Ara4N	Permeability of the cell envelope	LPS	
BCAL1934	Ara4N	Permeability of the cell envelope	LPS	
BCAL1935	Ara4N	Permeability of the cell envelope	LPS	
BCAL1936	Ara4N	Permeability of the cell envelope	LPS	
BCAL1937	Ara4N	Permeability of the cell envelope	LPS	
BCAL2946	putative UDP-glucose dehydrogenase	Permeability of the cell envelope	LPS	
BCAM0855	UDP-Glucose	Permeability of the cell	LPS	

	Dehydrogenase BceC	envelope		
BCAL2872	RNA polymerase sigma factor RpoE (rpoE1)	Permeability of the cell envelope		
BCAL0998	RNA polymerase sigma factor RpoE (rpoE2)	Permeability of the cell envelope		
BCAL2831	two-component regulatory system, response regulator protein	Permeability of the cell envelope		
BCAL2907	putative multidrug resistance protein (norM)	Permeability of the cell envelope		
BCAL2869	serine protease MucD 1 (mucD)	Permeability of the cell envelope		
BCAL1001	serine protease MucD 2 (mucD)	Permeability of the cell envelope		
BCAL2710	4-hydroxy-3-methylbut-2-enyl diphosphate reductase (ispH)	Permeability of the cell envelope		
BCAM2738	4-hydroxy-3-methylbut-2-enyl diphosphate reductase (ispH)	Permeability of the cell envelope		
BCAS0409	zinc metalloprotease ZmpA	Permeability of the cell envelope		
BCAM2307	zinc metalloprotease ZmpB	Permeability of the cell envelope		
BCAM2166	LysR family regulatory protein (penR)	Antibiotic modification		
BCAM2165	putative beta-lactamase (penB)	Antibiotic modification		

BCAL3430	N-acetyl-anhydromuranmyl-L-alanine amidase (ampD?)	Antibiotic modification		

Table S2. sRNA *B. cenocepacia* J2315 targets predicted with TargetRNA2[70] and brief description of its influence in antibiotic resistance.

RNA	Gene	Locus ID	Description	Type of resistance	Antibiotic Substrate	sRNA start	sRNA stop	mRNA start	mRNA stop
ncRNA2	czcB	BCAM0712	RND-11	RND efflux pump	Divalent cations (Zn2+, Co2+, Cd2+ and Ni2+)	40	52	-27	-13
	ceoA	BCAM2551	RND-10	RND efflux pump	Chloramphenicol, fluoroquinolones, Trimethoprim, Essential Oils	44	61	-8	12
	bpeA	BCAS0766	RND-2	RND efflux pump	Fluoroquinolones, Tetracycline, rifampicin, novobicin, essential oils	68	82	-43	-29
ncRNA7	mdtB	BCAL1080	RND-6-7	RND efflux pump	Essential oils	52	62	-69	-59
	-	BCAM2186	putative macrolide-specific efflux system transport protein	Efflux pump		291	306	-56	-41
	-	BCAS0591	RND-1	RND efflux pump	Essential oils	266	279	-7	8
ncRNA11	norM	BCAL2907	putative multidrug resistance protein (norM)	Permeability of the cell envelope		163	179	-10	7
	mucD 1	BCAL2869	serine protease MucD 1 (mucD)	Permeability of the cell envelope		39	52	-6	7
	mucD 2	BCAL1001	serine protease MucD 2 (mucD)	Permeability of the cell envelope		39	52	-6	7
	czcC	BCAM0711	RND-11	RND efflux pump	Heavy-metal efflux-RND	158	173	-73	-58
	penA	BCAM2165	putative beta-lactamase (penB)	Antibiotic modification		164	179	-26	-11
nc5U1	-	BCAM1421	RND	RND efflux pump	Acriflavine	146	160	-21	-7
	bceC	BCAM0855	UDP-Glucose Dehydrogenase BceC	Permeability of the cell envelope		154	165	-16	-5
	zmpA	BCAS0409	zinc metalloprotease ZmpA	Permeability of the cell envelope		72	80	11	19
ncS03	-	BCAM0433	RND-12	RND efflux pump	Monovalent cations (Cu+ and Ag+), Essential Oils	44	52	-76	-68
	-	BCAS0583	RND	RND efflux pump	Acriflavine	44	52	-46	-38
	-	BCAS0584	RND	RND efflux pump	Acriflavine	44	52	-48	-40

ncS06	-	BCAM1421	RND	RND efflux pump	Acriflavine	195	214	-33	-14
	-	BCAM0199	outer membrane efflux protein	Efflux pump		208	222	-39	-25
	-	BCAS0584	RND	RND efflux pump	Acriflavine	54	67	-59	-45
	-	BCAS0583	RND	RND efflux pump	Acriflavine	271	287	-31	-15
ncS54	penA	BCAM2165	putative beta-lactamase (penB)	Antibiotic modification		78	87	-26	-17
	-	BCAM0199	outer membrane efflux protein	Efflux pump		71	87	-47	-31
	-	BCAS0593	RND-1	RND efflux pump	Essential oils	67	85	-19	1
ncRI9	-	BCAL1927	Ara4N	Permeability of the cell envelope		179	194	-11	4
	-	BCAS0584	RND	RND efflux pump	Acriflavine	47	63	-21	-6
	-	BCAS0768	RND-2: AraC family transcriptional regulator	RND efflux pump	Fluoroquinolones, Tetracycline, rifampicin, novobicin, essential oils	102	116	8	20
nc5U19	penA	BCAM2165	putative beta-lactamase (penB)	Antibiotic modification		185	193	-17	-9
	-	BCAS0584	RND	RND efflux pump	Acriflavine	185	193	-17	-9
	-	BCAS0593	RND-1	RND efflux pump	Essential oils	183	194	-17	-6
nc5U23	-	BCAL1928	Ara4N	Permeability of the cell envelope		48	67	1	20
	penA	BCAM2165	putative beta-lactamase (penB)	Antibiotic modification		46	60	-38	-24
	ispH	BCAM2738	4-hydroxy-3-methylbut-2-enyl diphosphate reductase (ispH)	Permeability of the cell envelope		3	20	-25	-8
	-	BCAS0591	RND-1	RND efflux pump	Essential oils	29	41	-6	8
nc5U25	-	BCAL2136	RND-16	RND efflux pump	Minocycline, meropenem ciprofloxacin	50	62	-29	-17
	-	BCAS0591	RND-1	RND efflux pump	Essential oils	201	214	-14	-1
	-	BCAS0584	RND	RND efflux pump	Acriflavine	201	214	-27	-14
nc5U48	oqxA	BCAM1947	RND-9	RND efflux pump	Tobramycin, chlorhexidine, Essential Oils, 2-thiocyanatopyridine derivative (11026103), 2,1,3-benzothiadiazol-5-yl family compound (10126109)	38	53	2	18

	macB	BCAM2187	putative macrolide-specific ABC-type efflux carrier protein	Efflux pump		33	43	9	20
	czcC	BCAM0711	RND-11	RND efflux pump	Divalent cations (Zn2+, Co2+, Cd2+ and Ni2+)	2	14	-5	9
nc5U59	bpeR	BCAL2823	RND-4: TetR type regulator	RND efflux pump	Aztreonam, chloramphenicol, fluoroquinolones, tobramycin, tetracycline, rifampicin, novobiocin, essential oils, ethidium bromide, 2-thiocyanatopyridine derivative (11026103)	21	36	-29	-14
	-	BCAL1929	Ara4N	Permeability of the cell envelope		15	32	1	19
	zmpA	BCAS0409	zinc metalloprotease ZmpA	Permeability of the cell envelope		38	47	10	19
nc5U60	arnB	BCAL1931	Ara4N	Permeability of the cell envelope		105	115	10	20
	-	BCAM2188	outer membrane efflux protein	Efflux pump		18	36	1	17
	-	BCAS0591	RND-1	RND efflux pump	Essential oils	144	154	-17	-8
nc5U66	-	BCAL2135	RND-16	RND efflux pump	Minocycline, meropenem ciprofloxacin	344	358	-30	-16
	-	BCAM1945	RND-9	RND efflux pump	Tobramycin, chlorhexidine, Essential Oils, 2-thiocyanatopyridine derivative (11026103), 2,1,3-benzothiadiazol-5-yl family compound (10126109)	484	503	-31	-12
	-	BCAS0583	RND	RND efflux pump	Acriflavine	193	205	-28	-16
ncRNA3	dfrA	BCAL2915	dihydrofolate reductase (dfrA)	Drug target modification	Trimethoprim	88	102	-19	-7

Table S3. MIC values of all replicas of Bcc strains tested with Ciprofloxacin.

	Strain	Replica	pIN29			pTAP3			pMBJ1			pMBJ2		
			MIC	Average	S.D.	MIC	Average	S.D.	MIC	Average	S.D.	MIC	Average	S.D.
Ciprofloxacin	*B. multivorans* LMG 16660	R1	16.810	16.360	0.875		11.161	1.641	16.210	15.863	0.492	14.990	16.277	2.404
		R2	16.790			9.314			15.300			14.790		
		R3	17.050			11.720								
		R4	14.880			12.450								
		R5	16.270						16.080			19.050		
	B. dolosa AU0158	R1		3.797	0.694		3.753	0.179	4.776	4.175	0.553	4.239	3.640	0.549
		R2				3.603			4.062			3.521		
		R3	3.553			3.698								
		R4	3.727			4.030								
		R5	3.138			3.610								
		R6	4.770			3.823			3.687			3.160		
	B. cenocepacia K56-2	R1	3.326	3.385	0.304	4.059	4.173	0.322		3.967	-		4.534	-
		R2	3.115			3.924								
		R3	3.715			4.537			3.967			4.534		

Table S4. MIC values of all replicas of Bcc strains tested with Tobramycin.

	Strain	Replica	pIN29			pTAP3			pMBJ1			pMBJ2		
			MIC	Average	S.D	MIC	Average	S.D	MIC	Average	S.D	MIC	Average	S.D
Tobramycin	*B. multivorans* LMG 16660	R1	0.322	406.741	354.274	690	628.2	65.813		760.6	-		660.9	-
		R2	569.6			559								
		R3	650.3			635.6			760.6			660.9		
	B. dolosa AU0158	R1	325.4	249.275	57.456		187.3	23.132	239.2	229.05	14.354	193.7	181.1	17.819
		R2	232.7			164.9								
		R3	251.6			211.1								
		R4	187.4			185.9			218.9			168.5		
	B. cenocepacia K56-2	R1	548.9	514.45	24.382		559.5	17.589	953.5	764.3	267.569	1177	930.15	349.099
		R2	513.9			575.9								
		R3	494.2			556.4								
		R4	500.8			569.6								
		R5	486.8			536.1			575.1			683.3		

Table S5. MIC values of all replicas of Bcc strains tested with Trimethoprim.

	Strain	Replica	pIN29			pTAP3			pMBJ2			pMBJ3		
			MIC	Average	S.D.	MIC	Average	S.D.	MIC	Average	S.D.	MIC	Average	S.D.
Trimethoprim	*B. multivorans* LMG 16660	R1	6.790	7.028	1.200	10.78	12.390	2.583	9.588	9.344	0.345	10.47	9.800	0.630
		R2	5.891			11.02			9.10			9.734		
		R3	8.498			15.37			17.66			15.72		
		R4	6.201			8.341						9.210		
		R5	8.564											
		R6	6.224											
	B. dolosa AU0158	R1	>512			>512						>512		
		R2												
		R3												
	B. cenocepacia K56-2	R1	10.66	7.989	1.900	23.91	12.216	3.033	7.309	14.190	-	31.41	20.425	1.096
		R2	5.653			9.348			7.358			17.77		
		R3	7.097			15.39			14.19			19.65		
		R4	7.684			11.91						21.20		
		R5	9.866											
		R6	6.976											

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