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STUDIES ON THE INVOLVEMENT OF QUORUM SENSING IN THE REGULATION OF EXOPOLYSACCHARIDE BIOSYNTHESIS BY *BURKHOLDERIA CEPACIA* COMPLEX ISOLATES

Vítor Hugo Jorge de Oliveira

MESTRADO EM MICROBIOLOGIA APLICADA

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Dissertação orientada pela Profª. Doutora Leonilde Moreira (IBB-IST)

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MASTER THESIS

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Prof. Dr. Mário Santos was the internal designated supervisor in the scope of the Master in Applied Microbiology of the Faculty of Sciences of the University of Lisbon.

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Abstract

Quorum sensing (QS) is a cell-to-cell communication mechanism used by bacteria that produce and recognize signaling molecules named autoinducers. It is known to be involved in the regulation of different virulence factors including exopolysaccharide (EPS) biosynthesis in several pathogens. Bacteria belonging to *Burkholderia cepacia* complex (*Bc*c) are a group of related species that had emerged as important opportunistic pathogens mainly in cystic fibrosis (CF) patients. EPS produced by these bacteria plays an important role in the development of *Bc*c infections. Like in other bacteria, *Bc*c possess several QS-regulated phenotypes but little is known about the effect of this regulation mechanism in the *Bc*c EPS biosynthesis. Therefore, to evaluate the role of QS in such regulation, we tested the effects of acyl-homoserine lactones (AHLs) degradation by AiiA lactonase and the presence of a quorum sensing inhibitor (QSI) 4-nitro-pyridine-1-oxide (4NPO) in EPS biosynthesis inhibition. The results indicate that indeed QS regulates EPS biosynthesis in *Bc*c. However the signaling molecules involved are not dependent on the CepI/R QS system since an insertion mutant in the *cepI* gene did not inhibit the EPS production. Thus, bioinformatics analyses led to the identification of protein CbsI as another putative AHL synthase of *Burkholderia*.

*Bc*c bacteria are resistant to most of the conventional antibiotic treatments making its eradication a challenge. Thus, it has become essential to identify and develop alternative therapies to deal with these infections. The use of QSI seems a promising field to be explored. Since 4NPO was able to inhibit EPS production in *Burkholderia*, it was decided test its effect in several other *Bc*c QS-dependent phenotypes, such as motility, production of extracellular proteases and siderophores, and biofilm formation. Furthermore, its potential use as co-adjuvant of antibiotics in different *Bc*c species was also evaluated. The results confirmed that 4NPO is indeed affecting the tested phenotypes and increases the susceptibility to the antibiotics tested in both *Bc*c planktonic and sessile cells. The results also showed that 4NPO potentiates the activity of detergents such as Triton X100 and Tween 20. Since the clinical application of this compound is limited, its usage to clean and disinfect abiotic surfaces could be a possibility.

Keywords: *Burkholderia complex* cepacia (*Bc*c), virulence factors, exopolysaccharide (EPS), quorum sensing (QS), quorum sensing inhibitors (QSI), 4-nitro-pyridine-1-oxide (4NPO)

Resumo

As bactérias pertences ao complexo *Burkholderia cepacia* (Bcc) estão divididas em 17 espécies e são consideradas patogénicas oportunistas infectando sobretudo doentes com fibrose quística (FQ). Apesar de representarem apenas uma pequena percentagem da totalidade das infecções em doentes com FQ, os danos causadas por estas bactérias apresentam uma grande heterogeneidade, uma vez que as infecções que causam podem ser infecções crónicas ou podem estar associadas a quadros clínicos mais graves, levando muitas vezes à falência das funções pulmonares, desenvolvimento de pneumonia e de septicémia, designada síndrome da cepacia. A severidade destas infecções é agravada pela possibilidade de transmissão cruzada entre pacientes e pela partilha de equipamentos de terapêutica inalatória. Consequentemente, a maioria dos centros de FQ em todo o mundo teve que implementar regras especiais de higiene hospitalar e medidas severas de isolamento de indivíduos colonizados e indivíduos não colonizados com *Bcc*. Adicionalmente, estas bactérias são intrinsecamente resistentes à maioria dos antibióticos clinicamente usados e à maioria dos desinfectantes. Os compostos antibacterianos, com acção bacteriostática ou bacteriocida, têm-se assim revelado pouco eficazes no tratamento destas infecções, sendo necessário recorrer a elevadas concentrações de antibióticos e ao uso combinado desses mesmos antibióticos. Torna-se, portanto, fundamental o desenvolvimento de novas estratégias que permitam um tratamento mais rápido e eficaz desta infecções.

Umas das estratégias que tem sido fortemente estudada e que se tem revelado promissora é a interferência dos mecanismos de quorum sensing (QS). Este mecanismo é dependente de uma densidade populacional elevada, permitindo a comunicação entre as bactérias de uma população através da libertação de sinais químicos, sendo os mais comuns em bactérias Gram-negativa as acil-homoserina lactonas (AHLs). Quando esses sinais se acumulam no meio e atingem um determinado nível, ocorre a activação de proteínas reguladoras que vão activar ou reprimir a expressão de genes regulados por QS, levando a comportamentos sincronizados dentro dessa população. Tem sido demonstrado que o QS está envolvido na regulação da expressão de diversos genes relacionados com a virulência. No caso das bactérias do complexo *Bcc*, o sistema mais conservado é constituído pela sintase cepI, que produz C6-HSL e C8-HSL, e pelo regulador de transcrição cepR, que está envolvido na expressão de genes codificantes para proteases e sideróforos, motilidade e formação de biofilmes, entre outros. Ao contrário do que ocorre em outras espécies, pouco se sabe sobre os efeitos do QS, e em

concreto do sistema CepI/R ao nível da regulação da síntese de exopolissacárido, um importante factor de virulência em *Bc*c. Trabalhos feitos anteriormente no nosso laboratório sugerem que a biossíntese deste polímero deverá ser regulada por QS. Assim, um dos objectivos deste trabalho foi compreender qual o papel do mecanismo de QS na regulação do exopolissacárido em diferentes espécies de *Burkholderia*. Para tal, estudaram-se os efeitos da degradação de AHLs pela lactonase AiiA e do inibidor de QS, 1-óxido- 4-nitro-piridina (4NPO) na síntese de exopolissacárido. Os resultados obtidos, sugerem que o QS está efectivamente a regular a produção de exopolissacárido em *Bc*c mas as moléculas sinalizadoras não são as dependentes do sistema CepI/R. Isto porque a construção de um mutante de inserção no gene *cepI* não aboliu a síntese do exopolissacárido. Assim sendo, utilizaram-se ferramentas bioinformáticas para identifcar outro possível sistema de QS em *Burkholderia*.

O segundo objectivo deste trabalho foi determinar os efeitos de um inibidor de QS descrito em *Pseudomas aeruginosa*, o 4NPO, em diferentes espécies do complexo *Bc*c, ao nível de diversos fenótipos regulados por QS, nomeadamente a mobilidade, produção de proteases extracelulares e de sideróforos e a formação de biofilmes. Uma vez que se confirmou que efectivamente o 4NPO inibe o QS em *Burkholderia*, decidiu-se estudar a potencial utilização deste composto como co-adjuvante de antibióticos. Para tal, testaram-se diferentes antibióticos e os resultados mostraram que o 4NPO aumenta a capacidade antibacteriana desses antimicrobioanos, tanto ao nível da inibição do crescimento das células planctónicas como ao nível da inibição da formação de biofilmes. Embora o uso clínico deste produto esteja limitado devido às suas propriedades mutagénicas, o uso de 4NPO em superfícies abióticas poderá ser uma potencial aplicação deste composto, uma vez que, também se verificou potenciar a actividade de detergentes como o Triton X100 e o Tween 20, ao nível do crescimento planctónico e do desenvolvimento de biofilmes. De acordo com estes resultados, os inibidores de QS, e o 4NPO em particular, poderão de facto ter aplicações a nível da desinfecção de superfícies e de material clínico, prevenindo o aparecimento de casos de contaminações hospitalares, que já levaram a graves surtos de *Bc*c em doentes com FQ.

Index

1 Introduction

1.1 The *Burkholderia* genus

Bacteria belonging to the *Burkholderia* genus are motile, metabolically diverse Gram-negative β-proteobacteria that occupy a wide range of ecological niches including soil, industrial waste, water, clinical sources and many others [1, 2]. *Burkholderia* species have unusual large genomes, which contribute to the high plasticity, adaptability and capacity to colonize different environments and to survive under several stress conditions. These bacteria are able to use many unusual carbon and energy sources, being highly versatile from the metabolic point of view [3]. Some beneficial *Burkholderia* species establish symbiotic rhizospheric interactions with fungi, bring benefits to crops due to their ability to produce several antimicrobial compounds; promote plant growth by fixing atmospheric nitrogen in symbiosis with several plants; and can degrade natural and man-made pollutants (reviewed in [4]). On the other hand, some *Burkholderia* can be pathogens of plants, animals and humans [5, 6]. Among the pathogenic *Burkholderia* species are included *B. mallei*, which causes glanders in horses; *B. pseudomallei* that causes melioidosis; and the species belonging to *Burkholderia cepacia* complex (*Bc*c) which have emerged as important opportunistic human pathogens, especially in patients with chronic granulomatous disease (CGD), of immunocompromised individuals, and most importantly in cystic fibrosis (CF) patients [7]. *Burkholderia cepacia* complex comprises a group of related species that share a high level of similarity at 16S rRNA gene sequence (>97.5%) and of *rec*A gene sequence (94% to 95%), and moderate levels of DNA-DNA hybridization (30% to 60%) [8-11].

1.1.1 *Burkholderia cepacia* complex: a group of opportunistic CF pathogens

Cystic fibrosis is a genetic disease caused by mutations in a gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) that leads to the accumulation of a thick mucus in different organs including lungs, favoring bacterial colonization of several pathogens like *Burkholderia* [12, 13]. Even though *Burkholderia* infections affect less than 10% of the CF patients [14], it has become an extremely important opportunistic pathogen, which is associated with a worst clinical outcome and lower life expectancy. Furthermore, lung transplants are usually denied to these patients, due to the possibility of the development of cepacia syndrome characterized by necrotizing pneumonia and septicemia [15]. *Bc*c are highly transmissible from patient-to-patient and by contact with contaminated clinical devices, such as respiratory

therapy equipment, reusable temperature probes and catheters, being these bacteria resistant to the most commonly used disinfectants [16-19]. *Burkholderia* is also intrinsically resistant to antibiotics. Although poorly understood, the antibiotic resistance mechanisms in *Bc*c strains can be divided into three categories: enzyme modification, alteration of drug targets and limited permeability [20]. Permeability alterations at the membrane level play a key role in the *Bc*c defense mechanisms against antimicrobial agents, due to the existence of modified LPS, porins and efflux pumps, as well as the ability to produce EPS and/or form biofilms, which are thought to limit drugs access to the cell [20]. *Bc*c are able to cause enzyme drug modifications and target changes. These organisms are intrinsically resistant to aminoglycoside antibiotics and have high levels of β-lactam resistance due to the production of inducible chromosomal β-lactamases and altered penicillin-binding proteins [21]. Moreover, the capacity of antibiotic resistance in the *Bc*c can be highlighted by its capacity to use penicillin G as a sole carbon source [22]. Accordingly, the majority of *Bc*c strains are multidrug resistant and conventional antimicrobial therapies which includes the combination of two or sometimes three different antibiotics are often ineffective [19].

1.1.2 Virulence factores in *Burkholderia*

Multiple factors contribute to the pathogenicity of *Bc*c bacteria giving them the capacity to overwhelm the host defenses, to establish chronic infections that are rarely eradicated, to invade the epithelial cells, causing their necrosis, or to cross the epithelium paracelularly, enabling their dissemination into the blood stream [23]. These proprieties are due to the production of numerous virulence factors, the intrinsic resistance to antibiotics and the ability to form biofilms.

Among the virulence factors produced by *Bc*c bacteria there is the synthesis of lipopolysaccharide (LPS), which contains particular structural properties that neutralize the anionic charge of cell surface, being involved in the resistance to polymyxin, cationic antimicrobial peptides like protegin-1 [24] and in the prevention of bacterial phagocytosis by macrophages [25-28]. Other virulence factors are the expression of a cable pili and 22 kDa adhesin, which allow the binding to epithelial cells, induce cytotoxicity and initiate cellular apoptosis [29]; flagella, which are involved in bacteria dissemination, epithelial cells invasion, biofilm formation and induction of host responses [30]; and the biosynthesis and secretion of haemolysins, lipases, siderophores and extracellular proteases (eg. ZmpA and ZmpB), which were shown to be controlled by quorum sensing (QS) [31-33]. In particular, ZmpA and ZmpB are

metalloproteases involved in the disruption of host tissues and the enhancement of the host immune system, contributing to an increase of inflammation [32, 34].

The ability of *Bcc* bacteria to form biofilms has been shown in both environmental and clinical *Bcc* isolates grown in abiotic and biotic surfaces [35]. Biofilms are defined as a sessile community of bacterial cells in the stationary phase irreversibly attached to a surface, being embedded by a matrix of extracellular polymeric substances. The matrix of mature biofilms is composed of diverse substrates that include polysaccharides, proteins, nucleic acids and lipids. Biofilm communities exhibit gradients of nutrients and oxygen through the different layers of the biofilm, having the cells of the bottom lower availability of nutrients and therefore less metabolic activity [36]. These characteristics protect bacteria-forming biofilm from many environmental factors, including antibiotics, disinfectant chemicals and the host immune system. The reason it could be the limited drug diffusion and inactivation of compounds by the biofilm matrix [37, 38]. Biofilm formation involves several temporal phases as resumed in the Figure 1. The role of extracellular polymeric substances on biofilm matrix cohesiveness and biofilm disruption have been an interesting target to find chemical compounds that acting at that level, could promote biofilm detachment [39].

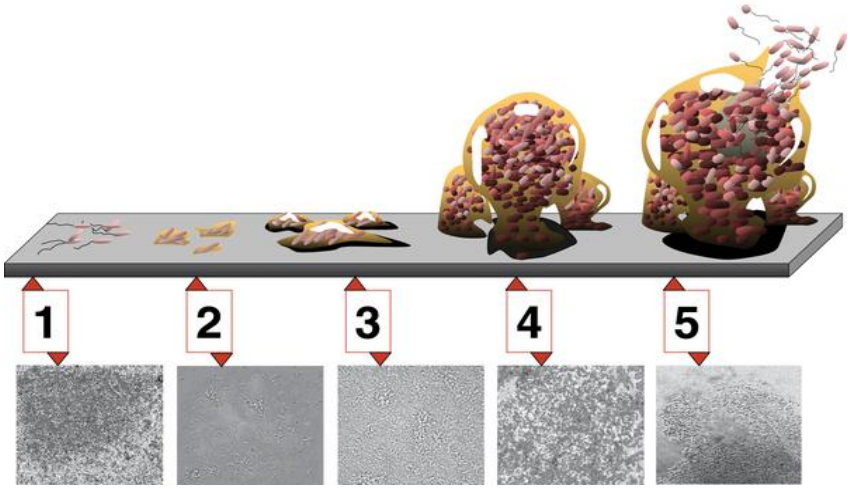


Figure 1 - Steps required for biofilm formation. [1] initial attachment, **[2]** irreversible attachment, **[3-4]** maturation, **[5]** dispersion. Each stage of development in the diagram is paired with a photomicrograph of a developing *P. aeruginosa* biofilm. All photomicrographs are shown to same scale. The planktonic bacteria start to swim towards the substratum using flagella, to form loose attachments **[1]** on the surface forming microcolonies **[2]**. The microcolonies multiply and differentiate into mature biofilms where cells are embedded into a thick extracellular polymeric matrix. The biofilm can acquire mushroom- or tower-like structures **[3-4]**. Subsequently, it is observed a detachment of bacterial cells that can be spread to other places giving origin to new biofilms **[5]** (adapted from [40])

Besides being involved in biofilm formation, extracellular polysaccharides or exopolysaccharides (EPS) are considered to be important virulence features for several pathogens. In the case of *Burkholderia* the role of EPS in infection is not well

established, as some authors point out that it might have a virulence role and others consider EPS a persistence factor. Still the importance of EPS in *Burkholderia* adaptation to different environments, stress conditions, and to overcome the immune system has been demonstrated experimentally (reviewed in [41]), as summarized in Figure 2. Several *Burkholderia* EPSs have been identified and structurally characterized, but the EPS cepacian is the most common one, being produced by both *Bcc* and non-*Bcc* *Burkholderia* strains of clinical and environmental origin [42, 43]. Cepacian biosynthesis involves many genes, most of them encoded by *bce*-I and *bce*-II gene clusters [43-46]. Still, little is known about the mechanisms involved in the regulation of cepacian biosynthesis, with the exception of the BY-kinase BceF and the phosphotyrosine phosphatase BceD by exerting regulation at the post-translational level [47].

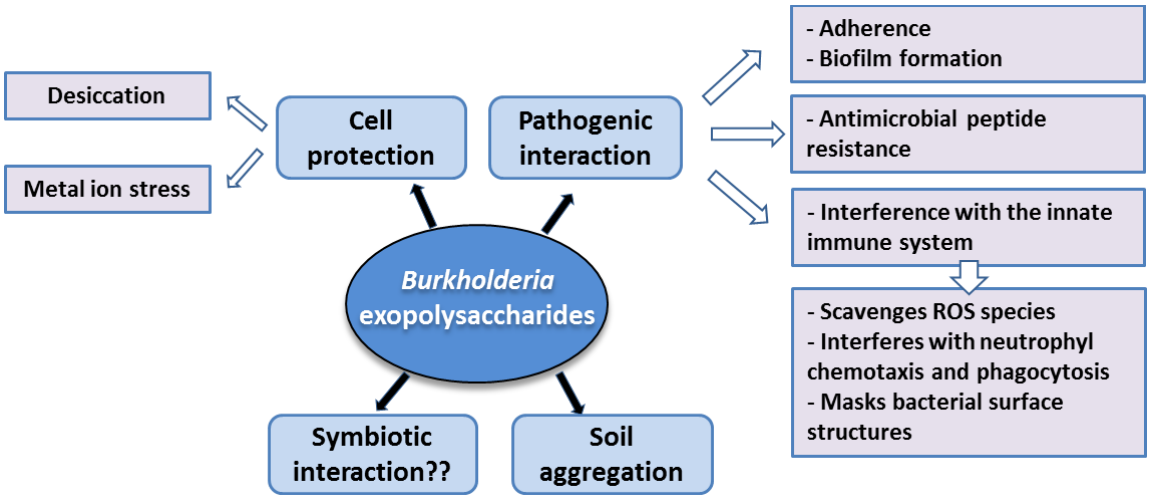


Figure 2 – Summary of the role of *Burkholderia* EPS in the adaptation to different niches (adapted from [41]). Cepacian was shown to interact with antimicrobial peptides [48]; to scavenge reactive oxygen species (ROS), to interfere with neutrophil chemotaxis [49], to be required for the formation of mature biofilms structures [50] and to be involved in the resistance to desiccation and metal ion stress, contributing to Bcc capacity to thrive in adverse environments [43].

Quorum sensing (QS) mechanisms are known to control EPS production in other bacterial species, including nonpathogenic *Sinorhizobium meliloti* and the plant pathogens *Erwinia stewartii* and *Pseudomonas syringae* [51-53]. Recently, Soarez-Moreno and collaborators had shown that QS mechanisms also control EPS biosynthesis in non-*Bcc* plant-associated *Burkholderia* [54]. Accordingly, we had hypothesized that QS may also regulate cepacian biosynthesis in *Bcc* by acting at the transcriptional level, and that will be one of the aims of this work.

1.2 Insights into quorum sensing mechanism and functions

Cell-to-cell communication can be mediated by different chemical signals that are secreted, diffused and detected throughout the community. In response to such factors, signal transduction cascades are activated and induce alterations in gene expression which enable bacteria to survive in different stress conditions and consequently adjust to the surrounding environment [55]. These mechanisms are mediated by a cell-density-dependent regulatory mechanism designated by quorum sensing.

QS signaling is based on the intracellular production of low molecular weight molecules, known as autoinducers, that are either passively or actively released to external milieu. When these signal molecules reach a specific threshold, which is usually dependent on bacterial population density they bind to specific receptors (regulatory proteins) that trigger signal transduction cascades, resulting in the alteration of gene expression [56]. QS plays a central role in both symbiotic and pathogenic interactions. For instance, it regulates several virulence phenotypes and allows a coordinated "attack" to the host that is triggered only at high population density, increasing the bacterial ability to overwhelm host defenses and to establish an infection [57]. In symbiotic associations, QS has also crucial roles in root nodulation by legumes/*Rhizobiaceae* associations and the production of bioluminescence in squid/*Vibrio fisheri* symbiosis [58, 59].

The autoinducers chemical structure is highly diverse, being the most common structures presented by Gram-negative bacteria based on N-acylated-L-homoserine lactones (AHLs), while in Gram-positive bacteria QS is based on small peptides synthesis and detection [60].

The first QS system was described in the marine bacterium *Vibrio fischeri*, which establishes symbiotic associations with the squid *Euprymna scolopes* and is involved in the activation of the luciferase operon that enables bacterial bioluminescence [59, 61]. This system is composed by two proteins: LuxI, a N-acylhomoserine lactone synthase, and LuxR, a cytoplasmatic transcriptional regulator which recognizes the signal molecules constituting the LuxI/R QS-system [62]. This system represents the paradigm of QS systems that are usually composed by a synthase and a regulator protein. Several types of QS systems have been described in Gram-negative bacteria, each one presenting distinct class of signal molecules, such as N-acyl homoserine lactones, quinolones (AQs), long-chain fatty acids and fatty acid methyl esters as well as autoinducer-2 (AI-2), a group of furanone derivatives [63].

Due to substrate specificity of the LuxI-homologues and conformation restrictions of the LuxR-homologues, only specific AHLs can bind to the regulator

protein [64, 65]. Therefore, AHL autoinducers tend to be species specific, as only particular acyl-chains are recognized by the species producing them being associated to intraspecies communication [64, 65]. Interspecies communication is usually associated with another autoinducer class, known as autoinducer-2 (AI-2), which is present in both Gram-negative and Gram-positive bacteria being proposed as an "universal signal" for interspecies communication (reviewed in [66]).

In the case of *Bcc* bacteria, the most well conserved QS system identified so far is based on CepI, the synthase LuxI-homologue, and CepR, the LuxR-like protein [67, 68]. The CepI protein synthesizes two AHL molecules: N-octanoyl-L-homoserine lactone (C8-HSL) and, in lower amounts, N-hexanoyl-L-homoserine lactone (C6-HSL) [69] (Figure 3). This system was shown to play a crucial role in *B. cenocepacia* virulence being essential for full pathogenicity in several infection models [70, 71]. CepI/R system positively regulates expression of extracellular proteases, chitinases and a polygalactunorase, as well as the swarming motility and biofilm formation; and negatively regulates the biosynthesis of the siderophore ornibactin [72-74]. Besides CepI/R homologue systems, an orphan LuxR homologue was also described in *B. cenocepacia* [75]. Orphan LuxR homologues are QS regulators that do not have an associated AHL synthase but respond to endogenous or exogenous synthesized AHLs [76] (Figure 3).

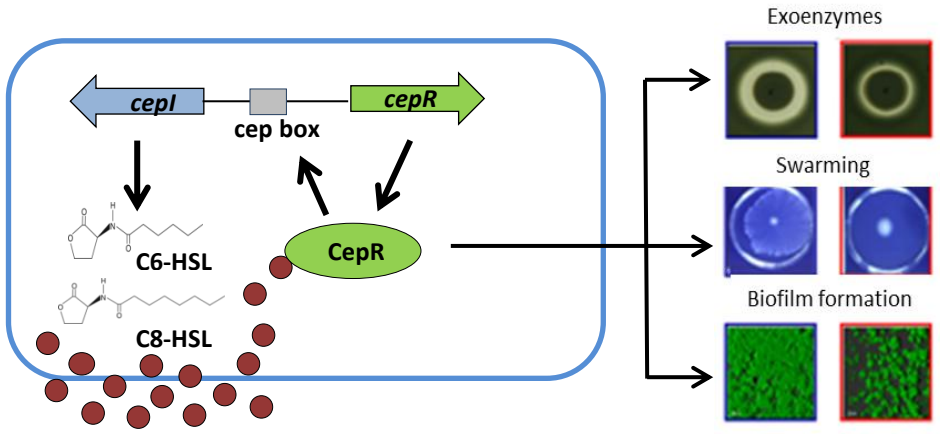


Figure 3 - Schematic representation of CepI/R *Burkholderia* QS system and some QS-regulated phenotypes (adapted from http://botserv1.uzh.ch/microbio/site/research/introduction/BurkholderiaCepacia.php).

Other AHL- based QS systems have been identified in several *Burkholderia* species. For instance, the *B. cenocepacia* CciI/R system produces and is activated by C6-HSL and C8-HSL [77]. *B. vietnamiensis* BviI/R system produces and responds to C10-HSL [78] and the BraI/R system from *B. kururiensis* synthesizes and responds to 3-oxo-C12-HSL [54]. System BraI/R was shown to control EPS biosynthesis in the

plant-associated *Burkholderia* species [79]. Even though no BraI/R homologues can be found in the sequenced *Bcc* species and the *Bcc* EPS is slightly different from the one produced by the plant-associated *Burkholderia*, the results obtained by Suarez-Moreno and co-authors give a further indication that QS may control EPS production in *Bcc* species. Besides AHLs, other molecules have been described to be involved in QS within the *Burkholderia* genus such as 4-hydroxy-2-alkylquinolines (HAQ) derivative. These compounds are thought to function as iron chelators, immune modulators and antimicrobial compounds, able to inhibit bacterial growth [80]. *B. cenocepacia* is able to produce a diverse set of 2-alkyl-4(1H)-quinolones shown be involved in colony morphology and elastase production, and cis-2-dodecenoic acid (BDSF), a molecule structurally related to the diffusible signal factor found in *Xanthomonas campestris* contributing to interspecies and intraspecies communication [81-83].

1.3 Quorum sensing inhibitors as antimicrobial compounds

Considering the rapid growth of bacterial resistance against many antimicrobial compounds and the link between QS and virulence, the discovery of QS antagonists may provide a possible means to achieve new antimicrobial strategies and it has thus attracted significant attention in recent years. Many QS inhibitors (QSIs) have been described and there are a growing number of studies showing their ability to prevent and/or disrupt biofilm formation, diminish the production of virulence factors, impair the responses to oxidative stress and increase neutrophils activity against bacteria [84-88]. Accordingly, these studies point out that the use of QSIs might be the next generation of drugs against bacterial infections, as QS is directly related with the production of virulence factors that are not essential for bacterial survival. Therefore, with the selective disruption of QS, pathogens can no longer adapt to the host environment being eliminated by innate host defenses without the selective pressure associated with the conventional antibiotic treatments, which have biocide activity [86, 89].

QSI can act at several levels. For instance, the modulation of QS can occur by interfering with the synthase, with the regulator protein or with the signal itself (Figure 4) [86, 90]. Regarding QSI that act by preventing the production of autoinducers, there are studies that use small-molecule agents such as 59-methylthioadenosine (MTA) and S-adenosylmethionine (SAM) analogs to target LuxI-type synthase proteins [91]; others that use substitutes of SAM, one of the AHL's precursors [86, 92]; and others that use autoinducer synthase blocking compounds, such as thiol derivatives and homoserine lactone derivatives [93]. Another approach used is to block the signal at the receptor level, which can be achieved by the use of antagonist compounds, capable of

competing or interfering with the cognate AHL signal for binding to LuxR-type receptors and both designed synthetic and natural naturally occurring substances, such as extracts from plants and food, have been tested to modulate QS at LuxR-homologues levels [94, 95]. Finally, QS inhibition can occur by manipulation of the QS signal itself. Some plants and bacteria present defense mechanisms that destroy the invading or competing bacteria autoinducers to protect themselves. For instance, some plants increase the pH at infection sites, causing hydrolysis of the lactone ring; others secrete of oxidized halogenated compounds that are capable of reacting with the 3-oxo-AHLs; some bacterial species are capable of using AHLs as a carbon and nitrogen source, others are able to breakdown AHL by secreting lactonases and acylases enzymes, others produce secondary metabolites, such as brominated furanones, that can block the action of AHLs [86, 96, 97].

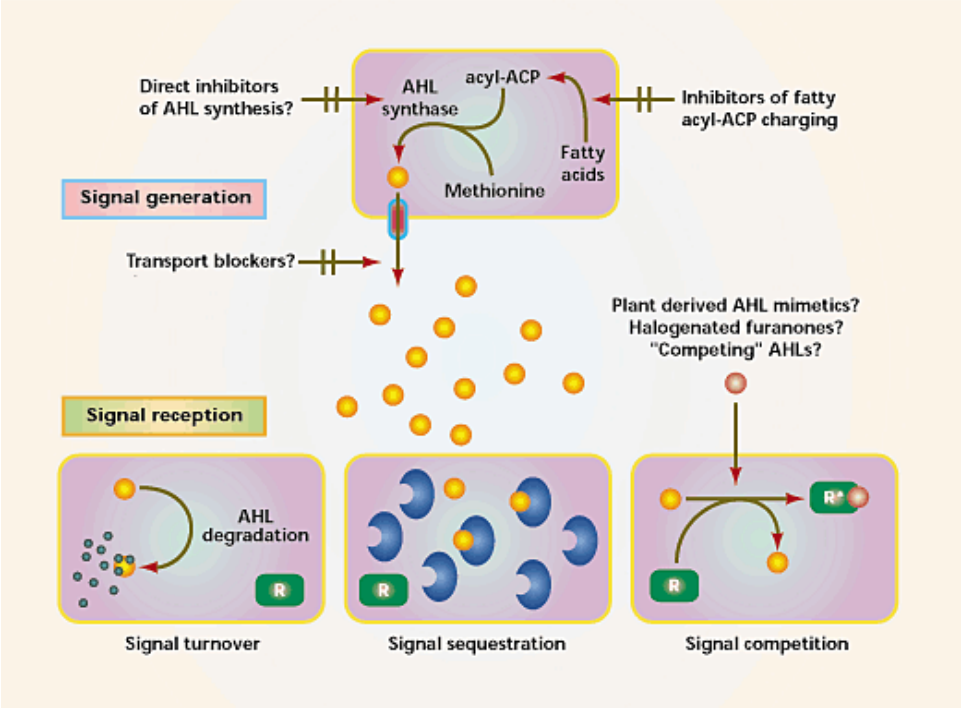


Figure 4 – Different levels of quorum-sensing (QS) disrupting strategies in bacteria using quorum sensing inhibitors (QSI) (adapted from [98]).

The use of QSI as co-adjuvant of antibiotic action has been widely studied, particularly against *P. aeruginosa* causing CF lung infections where halogenated furanone compounds were able to inhibit biofilm formation [99] and garlic extract in combination with tobramycin enhanced the clearance of infecting bacteria in the mice pulmonary infection model [100]. Still, little is known about the potential use of QSIs against *Bcc*, probably due to the fact that these bacteria are able to degrade a wide variety of compounds, as some of the QSIs known to be efficient in *P. aeruginosa* did

not seem to have any effect on *Bcc* strains [101]. Nevertheless, Brackman and co-authors (2009, 2011) showed that same QSIs can interfere with biofilm formation and maturation in *B. multivorans* and *B. cenocepacia* [84, 102].

1.4 Objectives

Quorum sensing is known to regulate the expression of several genes that are involved in the production of virulence factors. Among the phenotypes controlled by QS, the biosynthesis of exopolysaccharides was shown to be regulated at the transcriptional level in several bacterial systems, including the plant pathogens *Erwinia stewartii* and *Pseudomonas syringae* [51, 52] and nonpathogenic species of *Sinorhizobium meliloti* [53]. Regarding *Burkholderia* species, the work of Suarez-Moreno and co-authors (2008, 2010) developed in plant-associated *Burkholderia*, which are non-*Bcc* and non-virulent species, showed that the QS system BraI/R, directing the production of *oxo*-C12-HSL and *oxo*-C14-HSL, is involved in the control of EPS biosynthesis in these species [43, 44].

Previous results from our laboratory also indicate that cepacian biosynthesis is most likely regulated by QS. In the presence of the lactonase Aiia from *Bacillus subtilis* which is able to hydrolyze the lactone ring of AHLs and sub-lethal concentrations of a QSI, 4-nitro-pyridine-1-oxide (4NPO), cepacian production by *B. cepacia* IST408 was inhibited. Therefore, one of the goals of this work was to determine if QS is indeed controlling cepacian biosynthesis in *Bcc* bacteria and if CepI/R system, the most well conserved QS system among *Bcc* species, was involved in such control or there might be another conserved system controlling this feature. To accomplish this goal we tested lactonase expression in different *Bcc* and non-*Bcc* species and evaluate whether the QSI 4NPO is also able to inhibit EPS biosynthesis. In parallel we constructed unmarked *cepI* and *cepR* genes deletion mutants to evaluate whether the AHLs produced by CepI/R QS system are the one controlling EPS expression. If these mutants still produce EPS, it is our aim to identify other QS system in a strain with the genome sequenced such as *B. multivorans* ATCC 17616.

The second major goal of this work is to determine if the QSI 4NPO can be used to control biofilm formation by *Bcc* strains, if applied as a co-adjuvant of antibiotics or detergent/disinfectants. Our previous results had shown that EPS biosynthesis by *B. cepacia* IST408 was inhibited by the *P. aeruginosa* QSI 4NPO [85] when used in sub-lethal concentrations which do not affect bacteria growth. Since 4NPO seemed to be an efficient QSI against this strain, further work was done to determine its potential as an antibiotic adjuvant. The results indicate that indeed *B. cepacia* IST408 susceptibility to trimethoprim, kanamycin, amikacin and piperacillin

was enhanced in vitro as well as in *Galleria mellonella* virulence model of infection, where significant survival differences were observed between the presence and absence of 4NPO supplementation with trimethoprim and kanamycin (Ferreira *et. al.* unpublished results). Even though the results obtained were quite promising, the application of 4NPO to humans is compromised, as previous studies in *E. coli* and fibroblasts showed that this compound has mutagenic activity [103, 104]. Still, 4NPO could have other biotechnological applications such as being used as disinfectant of surfaces or anti-biofouling agent, being used together with detergents or other antimicrobial compounds. The work of Vanoyan *et al.* (2010) showed that 4NPO has interesting physico-chemical properties that can reduce the extent of bacterial adhesion to surfaces [105]. Currently, a CF patient infected with *Bc*c needs to be isolated from other CF patients, requiring special treatment rooms, equipment and nursing teams, leading to an increase of costs [106]. The potential use of 4NPO or others QSI as co-adjuvant of detergents or disinfectants could prevent cross-contaminations in CF centers and hospitals. Thus, in this work, different classes of antibiotic and detergents were used to test the efficiency of 4NPO as a QSI. The chosen antibiotics are the two aminoglycosides kanamycin and amikacin (inhibitors of protein synthesis); the sulfonamide trimethoprim (folate pathway inhibitor) and the β-lactam piperacillin and ceftazidime (inhibitors of cell wall synthesis). As disinfectant agent it were used bleach solution and as detergents the anionic sodium dodeyl sulphate (SDS) and non-anionic Triton X100 (TX100) and Tween 20.

2 Material and Methods

2.1 Bacterial strains, plasmids and oligonucleotides

Bacterial strains and plasmid used in this work are listed on Table 1.

Table 1 – List of strains and plasmids used in this work.

Strain	Relevant Characteristics	Reference
***Burkholderia* strains**		
B. cepacia IST408	Cystic fibrosis clinical isolate	[107]
B. cenocepacia K56-2	Cystic fibrosis clinical isolate	[108]
B. multivorans ATCC 17616	Soil isolate	[9]
B. multivorans D2095	Mucoid cystic fibrosis clinical isolate	[109]
B. dolosa AUO158	Cystic fibrosis clinical isolate	[110]
B. ambifaria AMMD	Root-colonizing bacterium	[111]
B. lata 383	Soil isolate	[112]
B. xenovorans LB400	Soil isolate	[113]
B. phymatum STM815	Soil isolate; nitrogen fixation	[114]
B. phytofirmans PsJN	Soil isolate; plant growth-promoting bacterium	[115]
B. multivorans ATCC 17616 *cepI*::pVO1105-1	pVO1105-1 integrated into *cepI* gene region	This work
B. multivorans ATCC 17616 *cepR*::pVO1106-1	pVO1106-1 integrated into *cepR* gene region	This work
B. multivorans ATCC 17616 *cepI*::pVO1105-1+pDAI-SceI	*B. multivorans* ATCC 17616 derivative containing pVO1105-1 integrated into *cepI* gene region and the replicative plasmid pDAI-SceI	This work
B. multivorans ATCC 17616 *cepR*::pVO1106-1+pDAI-SceI	*B. multivorans* ATCC 17616 derivative containing pVO1106-1 integrated into *cepR* gene region and the replicative plasmid pDAI-SceI	This work
B. cepacia IST 408 *cepI*::pIS410-1	pIS410-1 integrated into *cepI* gene region	Ferreira *et al.* unpublished results
***E.coli* strains**		
E. coli αDH5	*sup*E44 (ϕ80 *lac*ZΔM15) *hsd*R17(*rK*$^{-}$ *mK*$^{+}$) *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1 *deo*R Δ(*lac*ZYA-*arg*F)U169	Invitrogen
E. coli HB101	*thi*-1 *hsd*S20(rB -, mB-) *sup*E44 *rec*A13 *ara*-14 *leu*B6 *pro*A2 *lac*Y1 *gal*K 2*rps*L20 (Str^{R}) *xyl*-5 *mti*-1	Promega

Plasmids		
pMLBAD	pBBR1 ori, *araC*-PBAD, Tp^R *mob*$^+$	[116]
pMLBAD-*aiiA*	Broad-host-range vector carrying *araC*-PBAD-*aiiA* for expression of AiiA; Tp^R	[117]
pGPI-SceI	ori_{R6K} TP^R, mob$^+$, carries I-SceI cut site	[118]
pDAI-SceI	pDA17 carrying the I-*SceI* gene	[118]
pVO1105-1	pGPI-SceI derivative containing Δ*cepI* gene	This work
pVO1106-1	pGPI-SceI derivative containing Δ*cepR* gene	This work
pDrive	Cloning vector, Amp^R Km^R	Qiagen
pK18mob	Cloning vector, Km^R	[119]
pUC18	Cloning vector, Amp^r	[120]
pBBRIMCS	Cloning vector, containing *cat* gene, Cm^r	[121]
pVO412-1	pK18mob derivative containing *cbsI* gene (upstream region)	This work
pVO412-2	pDrive derivative containing *cbsI* gene (downstream region)	This work
pVO412-3	pK18mob derivative containing *cbsI* gene regions	This work

Abbreviations: Tp^R trimethoprim resistance; Km^R: kanamycin resistance; Amp^R: ampicillin resistance; Cm^R, chloramphenicol resistance.

The list of oligonucleotides used in PCR and a RT-PCR amplifications is shown in Table 2. Oligonucleotides primers were designed using AmplifX 1.5.4, available at http://ifrjr.nord.univ-mrs.fr/AmplifX and synthesized by MWG Biotech AG (Germany).

Table 2 - Oligonucleotides used for PCR amplification. Abbreviations: A, Adenine; C, Cytosine; G, Guanine; T, Thymine.

Primer name	**Sequence (5`- 3´)**	**Primer name**	**Sequence (5`- 3´)**
1997A-fw	AAGATTCAGTCTGAGATGAAGGCACGAGT	RTbceH-rev	CGATGTCGTCGCCTTTCC
1997A-rev	AAGGTACCTCGGCAGTTCTCGCATTAG	RTbceI-fw	AAGTTTCGAGCGTGACCAGTTC
1997B-fw	GTCTCTAGACTTCCAGACCTTCATGGCGTA	RTbceI-rev	AACAGCGACTTCAGCAGATACG
1997B-rev	TAGGTACCTGTTCCGACTGTCCGACATC	RTbceP-fw	GGACAAAGGCATACTCAAGAACGT
catKpn-up	ATGGTACCTATCACGAGGCCCTTTCGTCTTC	RTbceP-rev	CGAAGGTCGGCAGGATCA
catKpn-low	CTGGTACCTGTCGTGCCAGCTGCATTA	RTbceQ-fw	TTCGGCGAGGACGACTATG
RTbceB-fw	TTCGTGAACATCCGCTTCATT	RTbceQ-rev	TGGAACCCGAGGAAATGC
RTbceB-rev	CCGAGCACCTCGACCACTT	proC-fw	GTCGGCGAGATCGTATGGTT
RTbceE-fw	CCGAGACCTATCCGGTTCATT	proC-rev	CTGCAGCGCTTCGATGAAA

RTbceE-rev	CTTTCTGCAGCTGGTCCATCA	cepRa-fw	TCGAATTCTGTTCCTCGGCGTGACGATTCC
RTbceF-fw	AAACACTCCTACGCGGATCTGT	cepRa-rev	ATGGATCCATCGAAGCACCCTGACGCAA
RTbceF-rev	CAGCCAGATGTCGTCCATGA	cepRb-fw	ATGGATCCTATACCGAATGGCATCGCA
RtbceH-fw	ACGAAAGTCCACGTCCATCTG	cepRb-rev	CATCTAGAGTGCCACAGCAATTCGTCA
cepIa-fw	TGAATTCTTGCGTCAGGGTGCTTCGAT	cepIb-fw	ATGGATCCTCGATCCGCAAACGTTTGCT
cepIa-rev	TAGGATCCTCGTGAACGAAGGTCTGCAT	cepIb-rev	GCGTCTAGAGTAGGGAACTGACGAATGGGTA

2.2 Bacterial growth conditions

Burkholderia fresh cultures were obtained by inoculating a portion of the frozen material at -80^{o}C into plates of *Pseudomonas* isolation agar media (PIA, Difco), followed by incubation at 37^{o}C overnight. The cultures were then maintained at 4^{o}C until further use. Pre-inocula required for all assays were done as followed: overnight liquid cultures were prepared by transferring one isolated bacterial colony of *Burkholderia*, previously grown on solid media, into LB liquid medium followed by incubating overnight at 30^{o}C or 37^{o}C with orbital agitation (250 rpm). Then, these cultures would be used to inoculate working cultures in the proper conditions. *Burkholderia* strains were cultured in S medium or MM medium (see below), at 30ºC with orbital agitation (250 rpm). *E. coli* strains were always grown in LB medium (Difco) and incubated at 37ºC with orbital agitation (250 rpm).

S liquid medium – 12.5 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 1 g/l K_2SO_4, 1 g/l NaCl, 0.2 g/l $MgSO_4.7H_2O$, 0.001 g/l $FeSO_4.7H_2O$, 0.01 g/l $CaCl_2 \cdot 2H_2O$, 20 g/l glucose, 1 g/l yeast extract, 1 g/l casamino acids

MM liquid medium – 2 g/l of yeast extract (Difco), 20 g/l of mannitol (Merck)

2.3 DNA manipulation techniques

2.3.1 DNA extraction

Plasmid DNA was extracted from overnight cultures of *E. coli* host strains growing in LB medium supplemented with appropriate antibiotics, by a QIAprep Spin Miniprep kit (QIAGEN), following the manufacturer's instructions. Total genomic DNA from *Burkholderia* strains was extracted according standard protocol [122]. Concentration of genomic and plasmid DNA solutions was determined on a Spectrophotometer ND-1000 (NanoDrop).

2.3.2 DNA amplification by PCR

B. multivorans ATCC 17616 genomic DNA was used as template for PCR amplification of different DNA fragments required in this work. General conditions used were: 100 ng template DNA, 0.4 µM of each primer, 200 µM of deoxynucleotides and 1 U Platinum Taq DNA polymerase (Invitrogen); or 1 U TaqMed DNA polymerase in the case of *cat* gene amplification. Enhancer (Invitrogen) and $MgSO_4$ concentrations were optimized for each case according to the Table 3. PCR amplification was performed on GeneAmp® PCR System 2700 (Applied Biosystems). The annealing temperature (T_a) and extension time (T_e) were also optimized for each set of primers and DNA fragments as described in the Table 3. The general PCR conditions were: samples were subjected to an initial denaturation at 95ºC for 2 minutes, followed by 34 cycles of three steps: denaturation at 95ºC for 45 seconds, annealing at the optimized T_a for 30 seconds and elongation at 68ºC for text, calculated as 1 minute per kb of product expected. After the cycles, samples were submitted to a final elongation at 68ºC for 8 minutes and stored at 4ºC.

Table 3 - PCR amplification conditions and restriction endonucleases used.

Gene amplified	Primers name	Enhancer	[$MgSO_4$]	Annealing Temperature	Product size	Restriction Endonucleases
cepR (flanking region A)	cepRa-fw cepRa-rev	1×	1 mM	65 ºC	1472 bp	EcoRI BamHI
cepR (flanking region B)	cepRb-fw cepRb-rev	1×	2 mM	60 ºC	1479 bp	BamHI XbaI
cepI (flanking region A)	cepIa-fw cepIa-rev	1x	5 mM	59 ºC	1488 bp	EcoRI BamHI
cepI (flanking region B)	cepIb-fw cepIb-rev	1×	2 mM	61 ºC	1415 bp	BamHI XbaI
*Bmul*1997 (flanking region A)	1997A-fw 1997A-rev	1x	5 mM	59 ºC	1328 bp	EcoRI KpnI
*Bmul*1997 (flanking region B)	1997B-fw 1997B-rev	1x	4 mM	61ºC	1323 bp	KpnI XbaI
cat	catKpn-up catKpn-low	1x	6 mM	59º C	974 bp	KpnI

2.4 Construction of unmarked deletion mutants

Burkholderia deletion mutations were designed according to Flannagan *et al*. [118]. Fragments of around 700 bp of each flanking regions of *cepI* and *cepR* genes were obtained by PCR amplification, digested with the appropriate restriction endonucleases (Table 3) and inserted into pK18mob and pUC18 cloning vectors to increase their number of copies, and selected according to white/blue selection in the respective selection marker. The digested PCR fragments were subsequently ligated into pGPI-SceI (a suicide plasmid that cannot replicate in *Burkholderia* and contains the I-SceI recognition site) digested with EcoRI and XbaI in a triple ligation mixture, giving rise to the plasmids pVO1105-1 (deletion of *cepI*) and pVO1106-1 (deletion of *cepR*). Selection of vectors containing the inserted regions was made on *E. coli* αDH5 by electrotransformation and candidates were selected based on pGPI-SceI selection marker (trimethoprim). Then, the vectors obtained were introduced into *B. multivorans* ATCC 17616 by triparental mating and candidates were selected based on trimethoprim resistance phenotype.

Next, pDAI-SceI, constitutively expressing the I-SceI nuclease, was introduced by triparental mating into the strains carrying the integrated mutagenic plasmid. I-SceI will causes a double strand break into the inserted plasmid sequence, stimulating the intramolecular recombination between the mutant and parental alleles. From this recombination it can be observed either the restoration of the parental allele or gene deletion. Thus, exconjungants were selected for tetracycline resistance (to select for pDAI-ISceI) and trimethoprim sensitivity (indicating the loss of the integrated pGPI-SceI plasmid). Then, the colonies were picked to LB agar in the absence to tetracycline until the loss of pDAI-SceI occurs and the mutants confirmed by PCR and Southern blot hybridization.

2.5 Construction of a marked deletion mutant on *cbsI* gene

Fragments of around 1400 bp of each flanking regions of *cbsI* gene were obtained by PCR amplification, digested with the proper restriction endonucleases (Table 3) and inserted into pK18mob and pUC18 cloning vectors to increase its number of copies and selected according to white/blue selection in the respective selection marker. Two constructions were obtained, pVO412-1 and pVO412-2. Subsequently, pVO412-2 was digested with EcoRI and KpnI, and the fragment of interest was then inserted in the digested pVO412-1, originating the pVO412-3 plasmid, carrying the flanking areas of *cbsI*. To insert the Cm^r cassette between *cbsI* flanking regions, pBBRIMCS plasmid was used as template to amplify by PCR the *cat* gene encoding chloramphenicol acetyltransferase that was subsequently digested with KpnI. The PCR

fragment with around 980 bp was obtained and it was then inserted in the digested pVO412-3 plasmid. Selection of vectors containing the inserted regions and the Cm^r cassette were made on *E. coli* αDH5 by electrotransformation and candidates were selected based on plasmid selection markers. The obtained construction was subsequently, inserted into *B. multivorans* ATCC 17616 by triparental mating and candidates selected by the Cm^r phenotype.

2.6 *Burkholderia* triparental mating

Triparental conjugation was used to transfer plasmids of interest from *E. coli* to *Burkholderia* strains. The *E. coli* donor strain and the helper strain *E. coli* HB101 (pRK2013) were grown overnight at 37ºC in 30 ml of LB supplemented with appropriate antibiotics. The recipient *Bcc* strain was grown overnight at 30ºC in 30 ml of LB. Cells from 0.6 ml of *Burkholderia* culture, of donor and helper strains were harvested by centrifugation, washed with sterile 0.9% (wt/v) NaCl twice and suspended in saline solution. The three bacterial cultures were mixed together and harvested by low speed centrifugation for 5 minutes. The pellet was suspended in 80 µl of 0.9% (wt/v) NaCl and spot-inoculated on the surface of a filter Supor®-200 (13 mm diameter; 0.2 µm pore size, Pall corporation), placed onto the surface of an LB agar plate. After overnight incubation at 30ºC, the bacterial layer on the surface of the filter was suspended in 1 ml of saline solution and appropriate serial dilutions were plated on PIA supplemented with the proper antibiotic.

2.7 RNA manipulation techniques

2.7.1 RNA extraction and purification

The time point chosen to extract RNA was the 24^{th} hour of growth that is the time where EPS production is detected in *B. multivorans* ATCC 17616. Therefore, strains under study were inoculated with an initial OD_{640} nm 0.1 in MM medium supplemented with 1% (wt/v) of arabinose to promote the expression of Aiia lactonase, and incubated at 30ºC with orbital agitation. Similar approach was assayed with 4NPO at sub-lethal concentration. Strains carrying pMLBAD-*aiiA* that allows the expression of the lactonase and the addition of 4NPO to the pre-inocula were performed to evaluate their effects by comparison with the wild type strain (without lactonase/4NPO). RNA was extracted with RNeasy Mini Kit (Qiagen) following an optimized procedure. Briefly, samples of 400 µl of culture were taken and two volumes of RNA protect reagent were added, promoting mRNA stabilization. After 5 minutes of incubation at room temperature, the mixture was centrifuged and the pellet was suspended in 200 µl of TE buffer (30 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 15 mg/ml lysozyme and 15 µl

of proteinase K (Qiagen) were added to the suspension. After 10 min of enzymatic lysis 700 µl of RLT buffer were added, the mixture was vortexed for 10 s and 500 µl of 100% ethanol were added, mixed and transferred to a RNeasy Mini spin column. The mixture, in the columns, was then centrifuged and washed with RW1. To avoid contamination with genomic DNA, a step of DNA digestion using RNase-free DNA digestion kit (Qiagen) was introduced after wash with RW1 buffer. RNA elution was made after a second wash with RW1 buffer and a two-step RPE wash.

All steps described above were executed using RNase-free material. RNA concentration was estimated using a UV spectrophotometer (ND-1000 UV-Vis, NanoDrop Technologies, USA). RNA samples were stored at -80ºC, immediately after the extraction.

2.7.2 Quantitative Real-Time PCR

qRT-PCR was performed using a relative quantification method based on a two steps protocol. In the first step RNA was converted into cDNA and in the second step the cDNA formed was quantified. TaqManR Reverse Transcription Reagents (Applied Biosystems) were used to convert 1000 ng of total RNA to cDNA, according to the manufacturer's instructions. The cDNA samples obtained were diluted to a proper concentration and mixtures containing 400 ng of template cDNA, 2x SYBR Green PCR Master Mix (Applied Biosystems) and 0.4 mM of reverse and forward primer for each gene under study, in a total amount of 25 µl, were prepared. Reactions were run on 7500 Instrument from Applied Biosystem. The expression ratio of the target gene was determined relative to a reference gene, *proC*, encoding L-proline oxidase, which did not show variation in the transcription under the conditions tested. Results were obtained from the average of three technical and three biological replicates.

2.8 Determination of 4NPO sub-lethal concentration

To determine the highest concentration of 4NPO that can be added to each *Burkholderia* species under study without affecting their growth, a 96-well plate microtiter dish was inoculated with the bacteria and different 4NPO concentrations and grown for 15 hours. Briefly, a stock solution of 50 mM 4NPO was prepared and 150 µl of S medium were added to each well. Two-fold serial dilutions of 4NPO were made from 500 µM to 0 µM. The wells were then inoculated with 50 µl of each bacterial species to a final $OD_{640\ nm}$ of 0.2. Growth was followed on a Spectro star nano (BMG Labtech) at 640 nm using an appropriate kinetic program. The microtiter plate was thermostatized at 30ºC during the growth. Rows containing only S medium were used

as blank and negative controls to show that wells were not contaminated during the growth period.

2.9 Determination of Minimal Inhibitory Concentration (MIC)

MICs of antibiotics and disinfectant agents, in presence or absence of 4NPO, used in this study were determined in triplicate using a microdilution assay in 96-well microtiter plates. The proper amount of the disinfectant compounds, stock solutions of each antibiotic and 4NPO sub-lethal concentrations were added to the respective wells containing 300 µl of S medium and subsequently, two-fold serial dilutions of each compound were carried to test the concentrations ranging from 3300 mg/l to 6.4 mg/l, in the case of antibiotics tested, and 50% (v/v) to 0.1 % (v/v) for the disinfectants. Two rows of dilutions were made for each condition (each compound alone and in combination with 4NPO sub-lethal concentrations) being the final volume of each sample 150 µl. Wells were then inoculated with more 150 µl of the respective *Burkholderia* cultures to a final $OD_{640\ nm}$ of 0.05. Rows containing only S medium or *Burkholderia* culture were used as blank and positive controls, respectively. The plates were incubated for 24 h at 30^{o}C and absorbance at 590 nm was measuring in a Spectro star nano (BMG Labtech). The results were standardized by subtraction of the negative controls absorbance from those of the corresponding inoculated wells. To each condition, the lowest concentration for which no growth was observed it was recorded as the MIC.

2.10 Phenotypic tests

Several phenotypic properties of *B. multivorans* ATCC 17616, *B. cepacia* IST408 and *B. cenocepacia* K56-2, in 4NPO presence and absence, were tested, as will be described.

2.10.1 EPS precipitation and quantification

To test the EPS production, strains under study were grown in S medium for 3 days, at 30ºC with orbital agitation. Samples of 2 ml were taken overtime for EPS quantification.

EPS quantification was based on the dry weight of ethanol-precipitated polysaccharide [50]. Samples were taken from *Burkholderia* cultures and centrifuged at 8000 rpm for 15 min to separate bacterial cells. Supernatant was then added to 2.5 volumes of cold ethanol. The EPS precipitates obtained was dried and weighted.

2.10.2 Motility assays: Swimming and Swarming

Several optimizations had to be performed to infer how 4NPO could affect *Burkholderia* motility. These included testing different media, which would allow following Bcc growth over 2 days; incubation at 30°C or 37°C; and 4NPO supplementation over the solid media surface or by incorporating the QSI into the media under test. All assays were done using 3 µl of overnight *Bcc* cultures standardized to an $OD_{640\ nm}$ of 0.3 and were inoculated in swimming and swarming media with or without 4NPO supplementation. Plates were then incubated for 2 days and the halos were measured at 24 h and 48 h of incubation. The best results were obtained using the following media:

Swarming medium – 20 g/l of LB medium (Difco) supplemented with agar 0.5% (wt/v) and 5 g/l glucose

Swimming medium – 20 g/l of LB medium (Difco) supplemented with agar 0.3% (wt/v) and 5 g/l glucose

2.10.3 Proteases and Siderophores production

To test the 4NPO effect in the production of proteases and siderophores, different media were prepared. Thus, to proteases production, AL medium (12 g/l skin milk (Difco), 10 g/l peptone (Difco) and 25 g/l agar) and to siderophores production, Chrome azurol S (CAS) agar diffusion, were made. In the latter case, the modified CAS agar diffusion assay was carried out. A CAS agar diffusion assay made according to the method described by Shin *et al.* [123] was modified adding 20 g/l of mannitol, 2 g/l yeast extract and 15 g/l of agar (Noble, Difco) and pH adjusted to 7.0. Then, 5 µl of *B. cepacia* IST408 strains overnight liquid cultures standardized to an $OD_{640\ nm}$ of 1, were inoculated into plates of AL medium or MM medium supplemented with CAS. From those plates, only half of them were supplemented with 4NPO sub-lethal concentration. During incubation 30°C for 3 days, halos were measured every 24h.

2.10.4 Antibiotic and disinfectant susceptibility in the presence of 4NPO

To evaluate whether 4NPO could be used to enhance *Burkholderia* susceptibility to antibiotics and disinfectant agents, growth differences with and without 4NPO were measured. The antibiotics and disinfectant/detergents tested were inoculated in 96-well plates with *B. mulitovorans* D2095 *B. cepacia* IST408 and *B. cenocepacia* K6-2 to a final $OD_{640\ nm}$ of 0.2 using LB medium. Half of the inoculum was supplemented with 4NPO sub-lethal concentrations. Sequential two-fold dilutions of each compound were carried to test the concentrations ranging from 1000 mg/l to 8 mg/l, for each antibiotic tested and 10 % (v/v) to 0 % (v/v) in the case of the disinfectants. Rows containing only bacteria with and without 4NPO were used as

controls to guarantee that 4NPO did not affect bacteria growth within each assay; as well as rows containing only medium to account for possible contaminations. Growth was followed on a Spectro star nano (BMG Labtech) read at 640 nm using an appropriate kinetic program.

2.10.5 Biofilm formation assays

Biofilm assays were conducted to determine 4NPO possible role as anti-biofouling agent. Differences in biofilm formation upon antibiotic and disinfectant addition and in presence or absence of 4NPO sub-lethal concentrations were analyzed. The antibiotic and disinfectant concentrations tested correspond to those where different growth behaviors were observed due to 4NPO presence.

Biofilm assays were performed at least in triplicate and based on the methodology described by O'Toole and Kolter [124]. LB overnight liquid cultures of *Bcc* species were performed and grown at 30 or 37^{o}C with orbital agitation until the mid-exponential phase was reached. The cultures were then diluted to a standardized culture OD640 nm of 0.1, and 20 µl of this cell suspension were used to inoculate the wells of a 96-well microtiter plate containing 180 µl of liquid medium. The compounds under study were supplemented in the medium at appropriate concentrations. Plates were incubated at 30 (disinfectants) or 37°C (antibiotics) for 48 hours without agitation. Wells containing sterile medium were used as negative controls.

For biofilm quantification, the culture medium and unattached bacterial cells were removed by washing the wells with 200 µl of distilled water, three times. Adherent bacteria were stained with 200 µl of a 1% (wt/v) crystal violet solution for 20 minutes at room temperature. After three gentle rinses with 200 µl of distilled water, the dye associated with the attached cells was solubilized in 200 µl of 95% ethanol and the biofilm was quantified by measuring the absorbance of the ethanol solution at 590 nm in a Spectro star nano (BMG Labtech) reader.

Crystal violet solution 1% was prepared as described by dissolving 0.5 g crystal violet in 10 ml of 95% (v/v) ethanol and 40 ml of water containing 0.4 g of ammonium oxalate.

2.11 Bioinformatic analyses

BLAST [125] algorithm was used to compare sequences of the deduced amino acids to database sequences available at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Expasy (http://www.expasy.org/). Alignments were performed using the program CLUSTALW [126]. Swiss model (http://swissmodel.expasy.org/) was used to predict protein 3D-structure Protein function was predicted using Tm-align [127]. SWISSDOCK

prediction (http://swissdock.vital-it.ch/docking) was performed do study molecular interactions between a protein target and its ligands.

3 Results and Discussion

3.1 Role of quorum-sensing in cepacian biosynthesis regulation

The first step to test the hypothesis that QS may regulate EPS biosynthesis in *Bcc* strains was to introduce the plasmid pMLBAD-*aiiA* into several isolates of *Bcc* (*B. cepacia*, *B. multivorans*, *B. dolosa*, *B. ambifaria* and *B. lata*) and non-*Bcc* species (*B. xerovorans, B. phytofirmans*, *B. phymatum*) and verify if polymer production was inhibited. This plasmid contains the *aiiA* gene encoding a lactonase enzyme under the control of an arabinose inducible promoter and this enzyme is responsible for AHLs degradation. The pLMBAD vector alone was introduced into the same strains as a negative control. The results shown in Figures 5 and 6 indicate that lactonase expression inhibited EPS production in all tested strains, which indeed confirms that QS mediated AHL molecules are involved in such regulation.

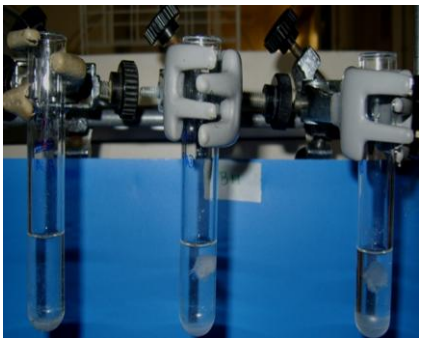


Figure 5 – Ethanol- precipitated exopolysaccharide production by *B. multivorans* ATCC 17616 in presence of pMLBAD-*aiiA* (left); vector pMLBAD only (centre) and wild-type strain (right). This result is representative of the behavior of the other strains studied.

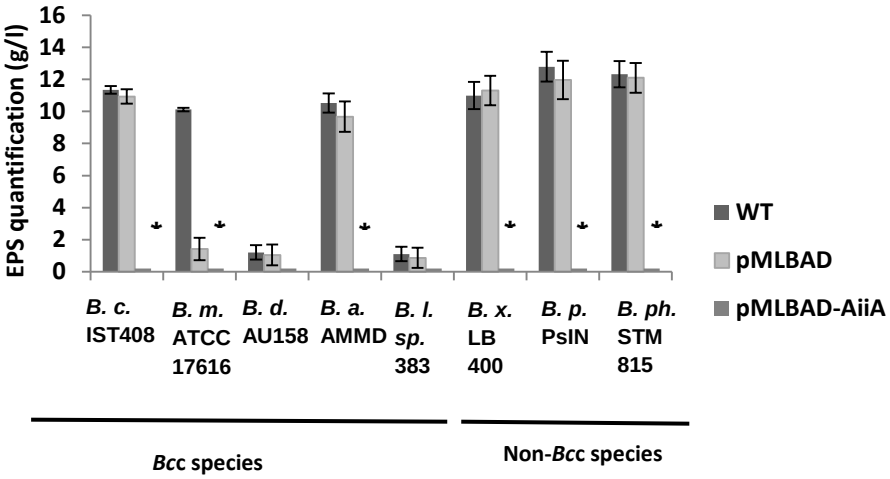


Figure 6 – EPS production by the Bcc and non-Bcc species in presence or absence of the AiiA lactonase. Error bars represent the standard error of the mean. ANOVA analysis was performed. A *P* value of <0.05 was considered significant compared with the condition of pMLBAD only (*). Abbreviations: *B. c.* IST408: *B. cepacia* IST408; *B. m.* ATCC 17616: *B. multivorans* ATCC 17616; *B. d.* AU158: *B. dolosa* AU158; *B. a.* AMMD: *B. ambifaria* AMMD; *B. lata sp* 383; *B. x.* LB400: *B. xenovorans* LB400; *B. p.* PsIN: *B. phytofimans* PsIN; *B. ph*. STM815: *B. phymatum* STM815.

In order to confirm these results at the transcriptional level, quantitative real-time RT-PCR assays were performed. Expression of some *bce* genes involved in EPS biosynthesis was studied by growing *B. multivorans* ATCC 17616 harbouring pMLBAD

or pMLBAD-*aiiA* in MM medium supplemented with 1% (wt/v) of arabinose. Cultures were grown at 30ºC for 24 hours and total RNA was extracted. The relative expression of genes belonging to the *bce*-I and *bce*-II gene clusters under the two tested conditions indicate that lactonase production caused a repression in the expression of all genes studied, with exception for the *bceE* gene (Figure 7). Consequently, these results support the hypothesis of EPS biosynthesis in *Burkholderia* being regulated by QS at a transcriptional level.

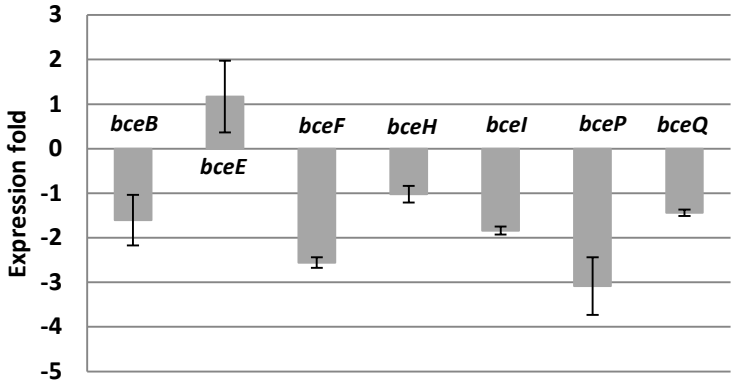


Figure 7 - **Quantitative real-time RT-PCR analysis** of the relative transcript abundance of *B. multivorans* ATCC 17616 containing pMLBAD-*aiiA* plasmid relatively to the parental strain harbouring pMLBAD growing in MM medium supplemented with 0.1% (wt/v) arabinose. Data was standardized to the internal control gene *proC*. The results were obtained from three independent experiments. Error bars represent the standard error of the mean.

3.1.1 Role of a quorum sensing inhibitor in preventing EPS biosynthesis

As mentioned before, *Bcc* species are intrinsically resistant to many toxic compounds, being able to degrade them and sometimes to use them as alternative carbon sources. Due to this feature, little is known about QSI that can efficiently interfere with *Burkholderia* QS. Previous work from our laboratory led to the identification of a QSI that was able to inhibit EPS production by *B. cepacia* IST408. Such QSI, 4-nitro-pyridine-N-oxide (4NPO), was firstly identified by random screening of pure compound libraries using QSI selector systems, based on killing/survival of reporter bacteria upon AHL presence/absence, respectively [85]. Expression studies using microarrays showed that sub-lethal concentrations of 4NPO down regulated 37% of the genes known to be dependent on QS in *Pseudomonas aeruginosa* [85].

Even though 4NPO was able to inhibit EPS production in *B. cepacia* IST408, nothing was known for other EPS-producer *Bcc* species, namely for *B. multivorans* ATCC 17616 whose genome sequence is freely available. To test that, we had to find the highest 4NPO concentration that does not affect the growth of *B. multivorans* and this value was 50 μM (Figure 8A). Therefore, the supplementation of the EPS-

producing medium with this sub-lethal concentration of 4NPO confirmed that no EPS was present in the culture supernatant of *B. multivorans* ATCC 17616 (Figure 8 B).

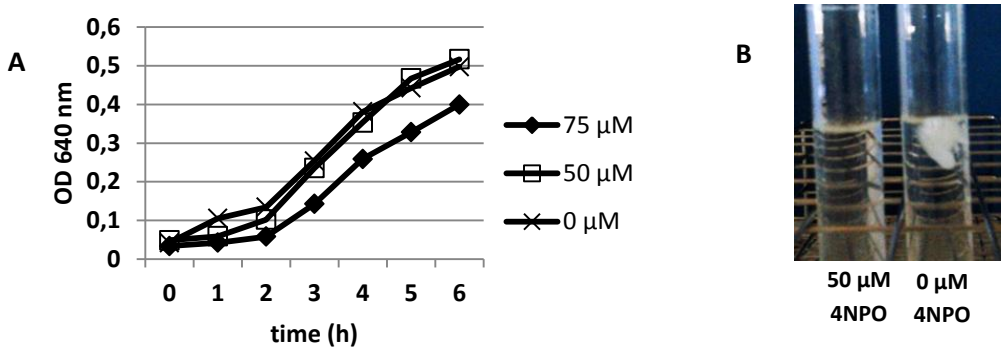


Figure 8 - Determination of 4NPO sub-lethal concentration of 4NPO (A) and EPS precipitation by addition of cold ethanol by *B. multivorans* ATCC 17616 growing in 4NPO absence or presence (B).

The next step was to determine if CepI/R, the most well conserved QS system in *Bcc* species was involved in EPS biosynthesis regulation. To accomplish this we followed the strategy described by Flannagan *et al.* to construct unmarked deletion mutants in *cepI* and *cepR* genes [118]. This strategy is based on the use of the endonuclease I-SceI to promote recombination events after double strand DNA breakage. The sequences flanking *B. multivorans* ATCC 17616 chromosomal regions of *cepI* and *cepR* genes were cloned into the suicide plasmid (pGPI-SceI) that is unable to replicate in *Burkholderia*. The plasmids obtained, pVO1105-1 and pVO1106-1 (see Annex A), were introduced into *B. multivorans* ATCC 17616 by triparental mating and recombinants with the plasmid inserted into the genome selected. These single recombinant strains were named *B. multivorans* ATCC 17616 *cepI*::pVO1105-1 and *B. multivorans* ATCC 17616 *cepR*::pVO1106-1. The next step was the introduction of plasmid pDAI-SceI, that constitutively expresses the I-SceI nuclease into the single recombinant strains. The nuclease recognizes a 15bp sequence present in vector pVO1105-1 or pVO1106-1, causing a double strand break in the bacterial replicon, stimulating the recombination events, mediated by the DNA repair system, between the mutant and parental alleles. This recombination can originate either the gene deletion or restore the parental allele depending on the site of the cross-over [118]. Although we successfully introduced pGPI-SceI into each strain, we were unable to obtain the unmarked deletion mutants. In spite of that, the few candidates obtained had the parental allele. Accordingly, it was hypothesized that perhaps the nuclease was not be expressed in *B. multivorans*. To test this, RNA was extracted from *B. multivorans* ATCC 17616 *cepI*::pVO1105-1 and *B. multivorans* ATCC 17616 *cepR*::pVO1106-1 with or without pGPI-SceI and *I-SceI* gene expression was evaluated by qRT-PCR

techniques. The results indicate that *I-SceI* is being expressed (data not shown) and therefore the for the failure in obtaining the deletion mutants, should be another one..

As an alternative to the previous strategy, it was used an available insertion mutant for the *cepI* gene encoding the AHL synthase in *B. cepacia* IST 408 (Ferreira *et al*, unpublished results). After growing this *cepI* mutant strain in EPS-producing medium, we were still able to recover EPS (data not shown), suggesting that C6- and C8-HSL are not the signals regulating cepacian biosynthesis in *B. cepacia* IST 408 and another synthase encoding gene must exist in this strain.

3.1.2 Search of other AHL synthases that might control EPS biosynthesis

Due to the previous results, it was decided to use bioinformatic approaches to find new candidates of AHL synthase proteins that might be responsible for cepacian biosynthesis regulation. For that, the sequences of several experimentally characterized QS synthases (see Annex B) were collected and used in a blast search against the genome *B. multivorans* ATCC 17616, available on line. As expected, the best score was obtained for *B. multivorans* CepI protein and no other obvious one was identified. Then, we looked for proteins with lower sequence identity and the results included diverse proteins, such as a metelloprotease (E:$4e^{-1}$); a type VI secretion protein (E:$5e^{-1}$); a flavin-containing monooxygenase (E:$7e^{-1}$) and a hemolysin-like protein (E:$6e^{-3}$). Since these proteins are unrelated and their putative function is different from the one we were looking for, we searched the literature for a common motif of the AHLs synthases. In fact, according to Fuqua *et al.* (1996), the homology between LuxI homologues has low scores that are often not higher than 28-35% [128]. However, the AHL synthase proteins have conserved regions composed by ten conserved residues [129]. Among the genes identified through our BLAST search, such local conservation was only identified in a putative protein annotated as a hemolysin-like protein from *B. multivorans* ATCC 17616, encoded by the gene that we named *cbsI* (Figure 9). This protein has six conserved amino acids among the conserved ten of these proteins.

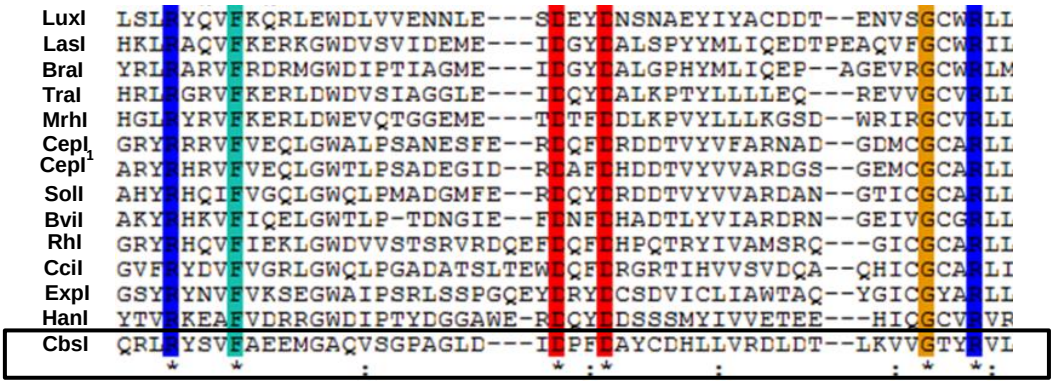


Figure 9 - Conserved domains of autoinducer synthases. Alignment of *cbsI* protein from B. multivorans ATCC 17616 with other N-acylhomoserine lactone proteins from *Vibrio fischeri* ES114 (AAQ90197), *Pseudomonas aeruginosa* PAO*1* (NP_250123 and ACI26688), *Burkholderia kururiensis* (CAP91066), *Bradyrhizobium sp*. BTAi1 (ABQ39897), *Mesorhizobium huakuii* (ABY91284), *Burkholderia cenocepacia* J2315 (YP_002232872 and CAR55728), *B. multivorans* ATCC 17616 (YP_001948920)[1], *Ralstonia solanacearum* PSI07 (YP_003750860), *Burkholderia vietnamiensis* (ABK32015), *Erwinia amylovora* CFBP1430 (YP_003530770) and *Halomonas anticariensis* (ADN33402) was performed using CLUSTALW [130]. Asterisks and coloured bars indicate amino acid residues that are identical in the analysed proteins; two dots indicate conserved substitutions.

Further *in silico* analyses were made to characterize the protein encoded by *cbsI* gene and access its possible role as an AHL synthase. Like *P. aeruginosa* LasI, the CbsI protein is predicted to have cytoplasmic localization, using to the PSORTb tool prediction. Through SWISS-Model tool was possible to obtain the predicted 3D-structure of CbsI (Figure 10) revealing overlap between CbsI and LasI from *P. aeruginosa* PAO1 with an Evalue: $8.8e^{-26}$ that corresponds to a 20% of homology. In order to confirm these results, the TM-align algorithm was also used to predict protein structure and function [131]. The output from TM-align is the result of an optimal alignment between two proteins based in the TM-score, which calculates the similarity of topologies of two protein structures. TM-score >0.5 indicates that the proteins share the same fold. From the results obtained using this model, two experimentally characterized AHL synthases were identified as being structural analogues of CbsI. These enzymes were the *P. aeruginosa* LasI (TM-score: 0.615) and *Burkholderia glumae* TofI (TM-score: 0.567).

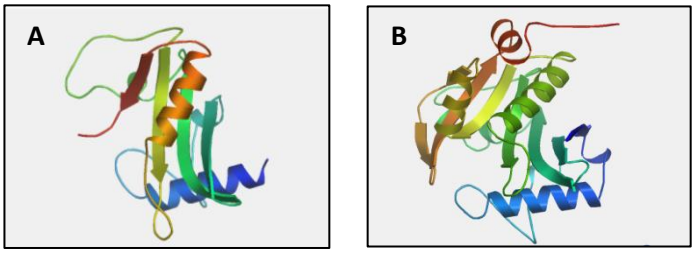


Figure 10 - Predicted 3D-structure of CbsI performed by SWISS-Model (A). The crystal structure of CbsI presents 20% homology with *Pseudomonas aeruginosa* PAO1 LasI synthase (B). Resolution: 2.30Ä and Evalue: $8.8e^{-26}$.

Additionally, SWISSDOCK program was used to predict molecular interactions between the candidate protein and possible subtracts. If CbsI is indeed an AHL synthase it will bind to SAM, which is an AHL precursor to form the QS molecules. The results obtained in SWISSDOCK indicate that indeed the protein encoded by *cbsI* has high affinity to the SAM molecule (data not shown).

Taken all together, even though no significant homology was observed between the CbsI protein and the other AHL synthases at the amino acid level, the *in silico* results, and particularly the ones obtained using 3D predictions approaches, indicate

that this candidate protein could be indeed a N-acyl homoserine lactone synthase. Furthermore, a search of *cbsI* gene homologues within the available *Burkholderia* genomes, indicate that the gene and its genetic neighborhood is highly conserved, which makes the protein a good candidate for being involved in EPS biosynthesis.

To confirm if *cbsI* gene encodes an AHL synthase involved in cepacian biosynthesis, it was decided to construct a deletion mutant on this gene. Briefly, the vector pVO412-3 was obtained by cloning the flanking regions of *cbsI* into pk18mob vector. Next, an interposon cassette harbouring the *cat* gene (for chloramphenicol resistance) was amplified using catKpn_up and catKpn_low primers, digested with KpnI restriction enzyme, to be introduced into the middle of *cbsI* flanking regions. This construction should be then introduced into *a* mucoid *Bc*c strains to evaluate AHL production and EPS biosynthesis. Due to time limitation of this study, it was only possible to obtain plasmid pVO412-3, harbouring *cbsI* flanking regions.

3.2 4NPO as a QSI in *Bc*c species

As early mentioned, *Bc*c bacteria are intrinsically resistant to several compounds, including antibiotics and disinfectants. Consequently, there is a strong need to develop alternative approaches to target those pathogenic bacteria. One of the most promising approaches to cope with multi-drug resistance in bacteria is the use of antimicrobial compounds that attenuate pathogens virulence features, making them more susceptible to the host immune system, to antibiotics and to toxic compounds. The main drawback to the use of antibiotic therapies is the selective pressure imposed, affecting cellular growth. Since QS is known to control the expression of several virulence factors but not affecting bacterial growth, it became a good target to develop antimicrobial drugs that could be administrated together with other drugs to prevent or to eliminate infections. Several compounds were shown to affect QS, being generally designated as QS inhibitors (QSI). A well known QSI in *P. aeruginosa* is 4NPO which was identified by screening systems in natural and synthetic compound libraries [85]. This compound has unique physico-chemical characteristics (Figure 11) and it was shown to reduce the extent of bacterial adhesion in abiotic surfaces [105]. 4NPO also seems to interfere with QS in *B. cepacia* IST408 as its supplementation in sub-lethal concentration inhibited the expression of both *cepI* and *cepR* genes [132]. Accordingly to this, it was decided to evaluate the potential use of 4NPO in *Bc*c

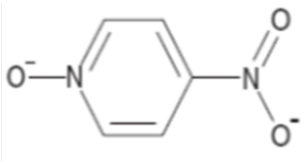


Figure 11 - Molecular structure of the quorum sensing inhibitor 4-nitro-pyridine-N-oxide (4NPO). This compound is hydrophilic, do not serve as hydrogen bond donor and is electrically neutral. These properties make this QSI a good candidate to be used against bacterial infections.

bacteria by analyzing its effect against several QS-dependent phenotypes. All assays described were done using sub-lethal concentrations of 4NPO, so that it does not affect the bacterial growth, lowering the selective pressure on the bacteria.

3.2.1 Evaluation of the effect of 4NPO in QS-dependent phenotypes

To determine the effect of 4NPO in QS-dependent phenotypes there was the need to estimate the 4NPO sub-lethal concentration for each of the *Bcc* strains under study. This concentration is defined as the highest concentration of 4NPO that did not affect cell growth. To do that, each *Burkholderia* species was grown in S medium supplemented with increasing 4NPO concentrations (a range between 0 and 1600 µM) at 30ºC. The results indicate that *B. cenocepacia* K56-2 4NPO sub-lethal concentration was 25 µM and for *B. cepacia* IST408, *B. multivorans* ATCC 17616 and *B. multivorans* D2095 was 50 µM (data not shown). After determination of 4NPO sub-lethal concentration for the different species several QS-regulated phenotypes associated with *Bcc* virulence, such as motility, extracellular proteases secretion and siderophore production were evaluated in the presence of this compound.

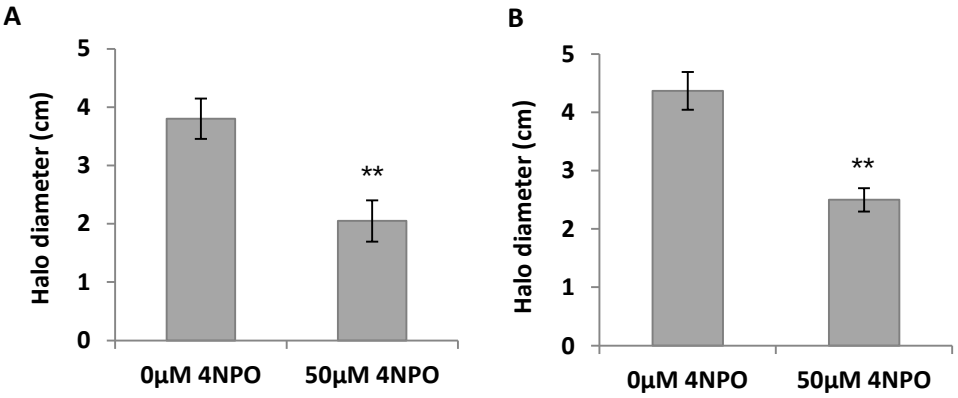


Figure 12 - *B. cepacia* IST408 swimming (A) and swarming (B) motilities in the absence or in the presence of 4NPO sub-lethal concentration, respectively. Error bars represent the standard error of the mean. *P* value < 0.01 was considered significant using ANOVA analysis compared with the condition of no 4NPO supplementation (**).

Motility allows bacteria to move toward a surface, to colonize broader spaces and move to locations where higher substrate concentrations are available. In this work we tested changes in swimming and swarming motility of several *Burkholderia* strains caused by 4NPO supplementation to the tested media. The technique required several optimization steps, such as, best medium; best temperature for growth; 4NPO incorporation into the hot medium before platting; spreading onto the medium surface after solidification; and incorporation into the pre-inocula. The best results were obtained using LB medium supplement with glucose and incorporation of 4NPO in the

pre-inoculum and into the agar. Motility was tested at 37^{o}C, with exception of *B. cepacia* IST408 that was only motile at 30^{o}C. Figure 12 shows the 48 h motility results obtained for *B. cepacia* IST408. Both swimming (A) and swarming (B) motilities decreased after adding 4NPO to the medium, suggesting that the compound affects *Bc*c motility. Similar results were obtained for *B. cenocepacia* K56 and *B. multivorans* D2095 (data not shown).

Secretion of proteases such as ZmpA and ZmpB and siderophores by *Bc*c bacteria are known to be important in host/pathogen interaction and for CF lung infections [32, 133]. Siderophores are involved in iron uptake from the environment and therefore have an important role in adaptation, contributing to the severity of *Bc*c lung infections [134]. Both proteases and siderophores production is known to be controlled by QS at the transcriptional level. To confirm the efficiency of 4NPO in the disruption of QS communication in *Bc*c, proteases and siderophores secretion were tested in the presence of the QSI compound. To test this, *B. cepacia* IST408 and *B. cenocepacia* K56-2 were inoculated into appropriate medium in the presence or absence of 4NPO. AL medium was used to follow proteases activity over time. Proteases secretion was evaluated by measuring the formation of a transparent halo around the bacterial colony spot. Chrome azurol S modified medium was used to observe siderophore production, which can be observed by the formation of an orange halo formed due to iron chelation. The results showed that both proteases and siderophores production by *B. cepacia* IST408 (Figure 13) and *B. cenocepacia* K56-2 (data not shown) were lower upon 4NPO supplementation. The proteolytic activity showed the highest differences in the diameter of the halos with 120h (Figure 13 A), while the differences in siderophore production were visible after 24h of incubation (Figure 13 B). The decrease of siderophore biosynthesis when QS is inhibited seems to be in disagreement with previous results found in the literature where synthesis of the siderophore ornibactin was negatively regulated by QS [73]. One possible explanation for this is that *Burkholderia* species produce several siderophores, besides ornibactin, such as pyochelin, salicylic acid, cepaciachelin, among other [135] and their biosynthesis might become up-regulated by the indirect effect of 4NPO.

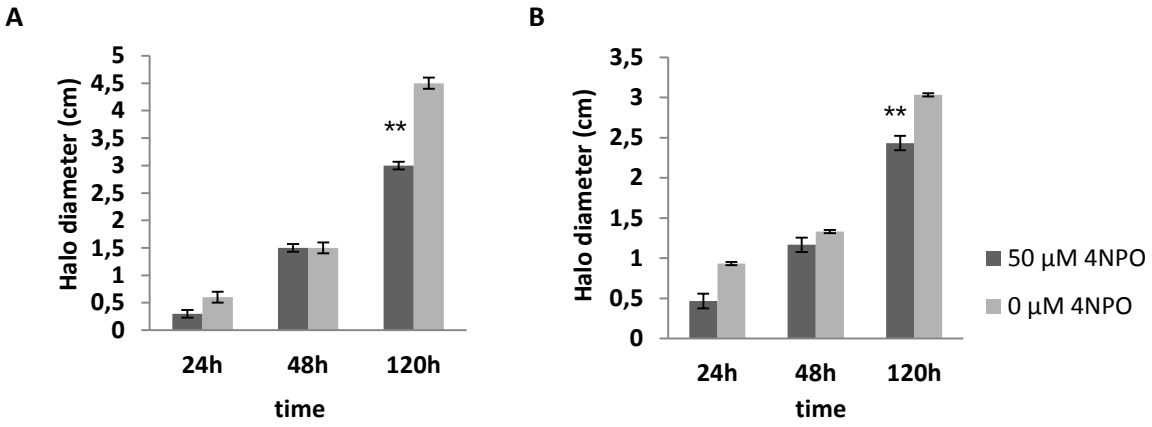


Figure 13 – Extracellular proteases (A) and siderophores (B) secretion by *B. cepacia* IST408 upon 4NPO supplementation (dark grey bars) and without (light grey bars). Error bars represent the standard error of the mean. A *P* value < 0.01 was considered significant compared with the condition of no 4NPO supplementation (**).

Taken together, the results obtained for swimming and swarming motilities and for proteases secretion, phenotypes that are positively controlled by QS indicate that indeed 4NPO interferes efficiently with *Bc*c QS mechanisms. Still, how 4NPO inhibits QS in these bacteria remains to be elucidated.

3.2.2 Effect of 4NPO as enhancer of *Bc*c susceptibility against antibiotics

*Bc*c species are among the most resistant bacteria to antibiotics being intrinsically resistant due to the presence of β-lactamases, permeability changes of the membrane due to modified LPS, reduction of the porin number and size and the existence of efflux pumps [19]. This obligates the application of high doses of antibiotics but even so, it is almost impossible to eradicate these bacteria.

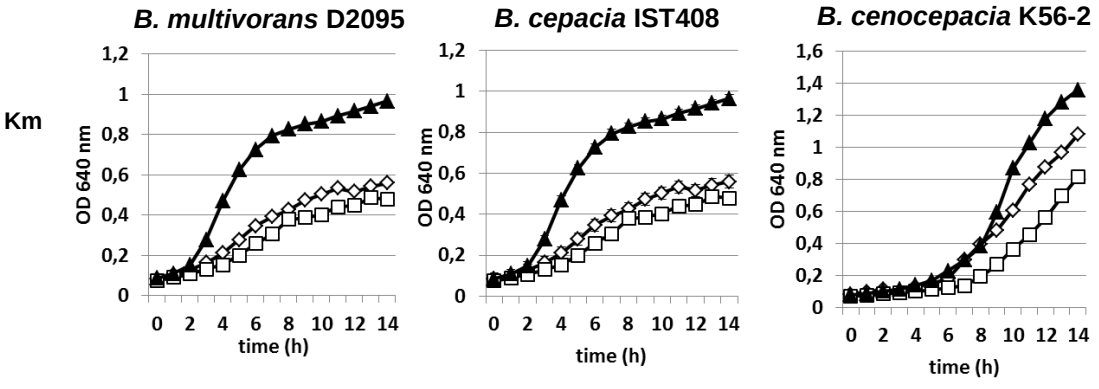
Considering the observed anti-QS properties of 4NPO, the next stage of this work was evaluate whether the use of 4NPO in sub-lethal concentrations induced changes in *Burkholderia* antibiotic resistance profile. The first step to accomplish this objective was to determine if the addition of 4NPO changed *Bc*c strains minimal inhibitory concentrations (MICs) of several antibiotics. The antibiotics chosen belong to different classes: kanamycin and amikacin are aminoglycosides, trimethoprim is a sulfonamide and piperacillin and ceftazidime are β-lactam antibiotics. Three *Bc*c species were tested, namely: *B. cenocepacia* K56-2, *B. multivorans* D2095 and *B. cepacia* IST408. *B. cenocepacia* and *B. multivorans* are the most common species infecting CF patients in the world, Portugal being an exception, where *B. cepacia* is the most common species [136]. The MIC values were determined in 96-well plates using S medium with two-fold dilutions of each antibiotic ranging from 3300 mg/l to 6.4 mg/l, in the presence or absence of 4NPO added in the sub-lethal concentration. The MIC concentration corresponded to the lowest concentration of antibiotic that inhibited the bacterial growth. The results obtained are summarized in the table 4, showing that no

MICs differences were observed due to 4NPO supplementation. These results are similar to the ones obtained by Brackman and co-authors while analyzing the effect of different QSIs in several *B. multivorans* and *B. cenocepacia* strains [102]. These authors evaluated the effects of baicalin hydrate and cinnamaldehyde in combination with some antibiotics against *Burkholderia* planktonic and biofilm cells and similarly to our data they did not observed differences in the MIC values due to supplementation with the QSI´s used.

Table 4 – Minimal inhibitory concentration (MIC) values in absence or presence of 4NPO for *B. multivorans* D2095, *B. cepacia* IST408 and *B. cenocepacia* K6-2. Strains not inhibited by 3300 mg/l are depicted as an MIC of >3300 mg/l

	Antibiotic	4NPO	
		0 µM 4NPO	50 µM 4NPO
***B. multivorans* D2095**	Kanamycin	1650 mg/l	1650 mg/l
	Amikacin	412 mg/l	412 mg/l
	Trimethoprim	25,8 mg/l	25,8 mg/l
	Piperacillin	>3300 mg/l	>3300 mg/l
	Ceftazidime	25,8 mg/l	25,8 mg/l
***B. cepacia* IST408**	Kanamycin	1650 mg/l	1650 mg/l
	Amikacin	412 mg/l	412 mg/l
	Trimethoprim	25,8 mg/l	25,8 mg/l
	Piperacillin	>3300 mg/l	>3300 mg/
	Ceftazidime	25,8 mg/l	25,8 mg/l
***B. cenocepacia* K56-2**	Kanamycin	1650 mg/l	1650 mg/l
	Amikacin	412 mg/l	412 mg/l
	Trimethoprim	103,2 mg/l	103,2 mg/l
	Piperacillin	>3300 mg/l	>3300 mg/l
	Ceftazidime	103,1 mg/l	103,1 mg/l

Even though there were no differences in MIC concentrations, there could be differences during bacterial growth. Accordingly, several antibiotic concentrations lower than the MIC were tested to determine whether the 4NPO supplementation in sub-lethal concentrations did interfere with the bacterial growth behavior. All antibiotics from Table 4 were tested with *B. multivorans* D2095, *B. cepacia* IST408 and *B. cenocepacia* K56-2 (Figure 14).



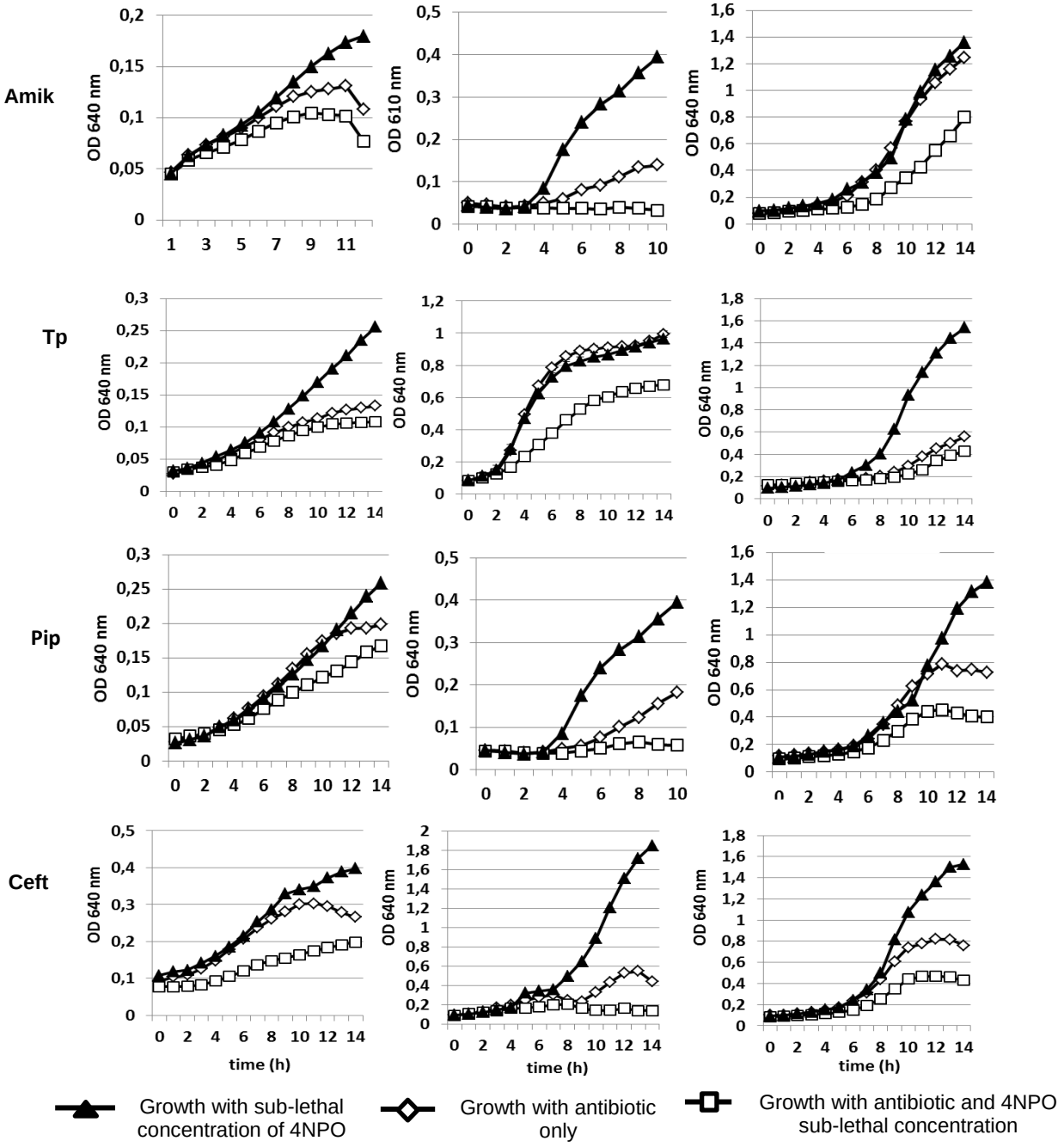


Figure 14 – Antibiotic susceptibility in presence of 4NPO of different *Bcc* strains. The antibiotic concentration used were for *B. multivorans* D2095: kanamycin (400 mg/l), amikacin (400 mg/l), trimethoprim (7.8 mg/l), piperacillin (31.25 mg/l) and ceftazidime (31.25 mg/l); *B. cepacia* IST408: kanamycin (250 mg/l), amikacin (50 mg/l) trimethoprim (15.6 mg/l), piperacilin (12.5mg/l) and ceftazidime (15.6 mg/l); and *B. cenocepacia* K56-2: kanamycin (500 mg/l), amikacin (250 mg/l), trimethoprim (31.25 mg/l), piperacillin (31.25 mg/l) and ceftazidime (1.9 mg/l). Growth was in presence of 4NPO sub-lethal concentrations. Abbreviations: **Km**- kanamycin; **Amik**- amikacin; **Tp**- trimethoprim, **Pip**- piperacillin; **Ceft** – ceftazidime

In the case of *B. multivorans* D2095, the strongest growth differences due to the supplementation of 4NPO and an antibiotic simultaneously were observed for ceftazidime. In the case of *B. cepacia* IST408, 4NPO supplementation seemed enhance the activity of trimethoprim, amikacin, piperacillin but no significant differences were found using kanamycin. Finally for *B. cenocepacia* K56-2, the growth inhibition was higher in the assays done with amikacin, piperacillin and ceftazidime and no differences were observed when using trimethoprim. Overall, the results from Figure 14

indicate a general increased susceptibility to antibiotics in the presence of 4NPO by all three *Burkholderia* species tested. These five antimicrobial compounds, belonging to different families of antibiotics, when used in combination with the QSI enhanced bacterial growth inhibition when compared to their individual use. Similarly to *P. aeruginosa*, *Bc*c are intrinsically resistant to aminoglycosides. However, while in *P. aeruginosa* the aminoglycosides affect the outer membrane, promoting its partial disruption and leaving the membrane partially permeabilized, in *Bc*c these compounds seem to have no effect on the membrane probably due to differences in its composition, structure and/or function [137]. Furthermore, cationic drugs self-promote the entry of these antibiotics by disrupting the outer membrane structure as described for *E. coli* and *P. aeruginosa*. However, the susceptibility of *Bc*c to aminoglycosides is not enhanced by cationic drugs [138]. This resistance to aminoglycosides was confirmed in these experiments. Even upon 4NPO supplementation, high doses of kanamycin and amikacin were still necessary to observe a substantial growth inhibition in the three *Bc*c species tested. Nevertheless, among these species, *B. cepacia* IST408 was the most susceptible to amikacin in the presence of 4NPO, as the use of relatively low concentrations (50 mg/l) inhibited almost completely bacterial growth. In the case of *Bc*c, studies on clinical isolates indicate that very low membrane permeability is the main mechanism of resistance to amikacin [139]. *Bc*c bacteria are also intrinsically resistant to β-lactams due to the presence of β-lactamase proteins that degrade these antibiotics. This can explain why we were unable to determine piperacillin MIC values. Interestingly, the presence of 4NPO seemed to reduce this resistance in all the three studied *Bc*c species as can be observed in Figure 14 showing that 4NPO supplementation in conjunction with the piperacillin had more effect in bacterial growth of the three species than the administration of piperacillin alone.

Some studies showed a significant susceptibility of *Bc*c bacteria to trimethoprim and ceftazidime, being extensively used in CF patients therapies [18, 140]. Furthermore, antibiotic combinations are used many times to efficiently treat CF patients with *Bc*c infections, such as, the combination of trimethoprim-sulfamethoxazole [140]. Our experiments, confirmed that *B. multivorans* D2095 and *B. cepacia* IST408 have a higher susceptibility to trimethoprim and ceftazidime then *B. cenocepacia* K56-2, which according to the MIC values determined is more tolerant to these antibiotics (Table 4). The resistance to trimethoprim might be due to alterations in outer membrane porin level and/or specific LPS characteristics which are responsible for neutralization of cell anionic charge [141]. Regarding to ceftazidime, previous work in *P. aeruginosa* showed that in low concentrations this antibiotic has QSI activity [142]. Skindersoe and colleagues have hypothesized that ceftazidime and other antibiotics

might exert its action on QS by interfering with PprB, a multifunctional protein that controls the influx of AHLs in *P. aeruginosa*, thereby globally influencing the expression of QS-regulated genes, and is also involved in the regulation of sensitivity to antibiotics, probably due to a decrease in membrane permeability. Thus, even though we cannot exclude the possibility that 4NPO may directly modify membrane permeability, leaving the cells more susceptible to antibiotics, it might also be that this QSI could be acting at the transcriptional level to down-regulate the expression of membrane proteins or transporters that would reduce membrane permeability against the toxic effects of the antibiotics. This is a possible explanation for the increased susceptibility to ceftazidime and other antibiotics observed in our experiments in the presence of 4NPO (Figure 14).

Taken together, these data suggest that 4NPO can increase the antimicrobial potential of some antibiotics against planktonic cells. Still, further studies are needed to understand the role of this QSI in *Burkholderia* physiology. However, one of the major factors leading to antibiotic resistance is the bacterial capacity to form biofilms. Thus, our next aim was to evaluate if 4NPO had a role in biofilm formation.

3.2.2.1 Effect of the QSI 4NPO in biofilm formation

Bacterial biofilms are an important source of persistent bacterial infection, contributing to a continuous inflammation and tissue damage [143]. After entering the respiratory airways, *Bc*c is known to attach epithelial cells, forming microcolonies that become extremely difficult to eradicate. Furthermore, *Bc*c species can also colonize abiotic surfaces, forming biofilm structures that difficult the material cleaning and disinfection and might represent a vehicle for infection propagation. Consequently, conventional therapies, such as antibiotic treatments and disinfectants were shown to be useless against biofilm populations due to their special physiology and physical characteristics. Furthermore, biofilms are a concern since billions of dollars are spent every year worldwide to deal with equipment damage, contamination of products and infections in human beings as a result of microbial growth as biofilms [144].

Biofilm formation has been shown to be dependent on QS in several pathogens including *Burkholderia* [74]. Thus, the application of QSI seems to be an interesting field to inhibit biofilm formation. As a first approach, the effect of 4NPO itself in *Bc*c biofilm formation was evaluated by testing how its supplementation in sub-lethal concentrations would affect the biofilm biomass of the three species under study. The results obtained showed that 4NPO has no significant effects on biofilm formation (Figure 15). Therefore, the next step would be to study the additive effect of 4NPO

supplementation and antibiotics, especially the ones that were shown to have some inhibitory effect in planktonic bacterial growth.

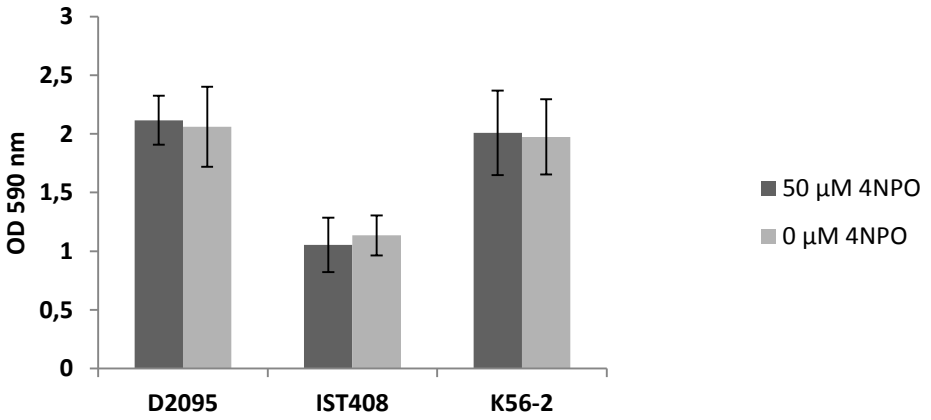


Figure 15 – *Burkholderia* biofilm formation in presence of 4NPO sub-lethal concentration. Biofilm formation was evaluated in static 96-well plates after 48 h of growth in S medium, at 30º C. The 4NPO sub-lethal concentrations used were 50 µM to *B. multivorans* D2095 and *B. cepacia* IST408 and 25 µM to *B. cenocepacia* K6-2. Error bars represent the standard error of the mean Abbreviations: **D2095**: *B. multivorans* D2095; **IST408**: *B. cepacia* IST408; **K56-2:** *B. cenocepacia* K6-2.

B. cepacia IST408 was used as a test strain and the results showed that the size of the biofilm formed in the presence of antibiotics and 4NPO is significantly reduced when compared to the biofilm formed with antibiotic supplementation only (Figure 16). However, these differences in biofilm formation were only observed when higher doses of antibiotic other than the ones used to obtain bacterial growth inhibition against planktonic cells were used. These observations are in agreement with the current knowledge that biofilm formation by *Burkholderia* plays a key role in the persistence of *Bc*c infections, since this form of growth is known to protect cells against several stress conditions, including antibiotics. The only exception was observed for ceftazidime, where a lower dose of antibiotic was required to reduce the size of the biofilm formed, compared to the concentration required to observe inhibition of planktonic growth. A possible explanation could be the fact that ceftazidime may affect the cell envelope structure/function, interfering with the flux of AHLs and therefore, affecting biofilm formation as shown in *P. aeruginosa* [142].

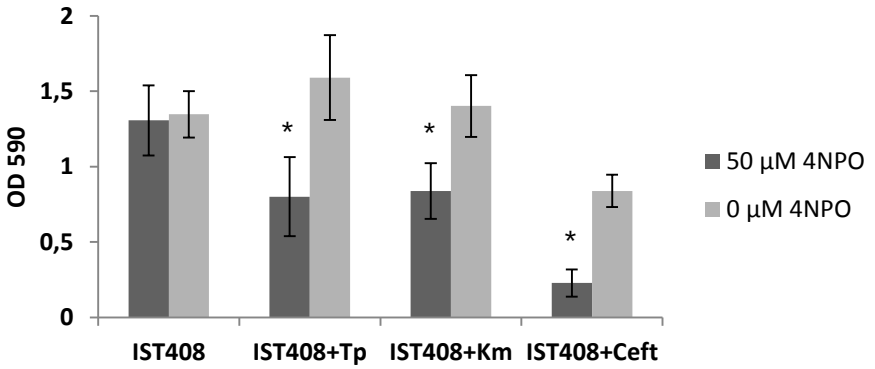


Figure 16 - *B. cepacia* IST408 biofilm formation in static 96-well plates after 48 h of growth in S medium, at 30ºC. Supplementation of 4NPO at sub-lethal concentration reduces the biofilm formation when used in combination with trimethoprim (25 mg/l), kanamycim (400 mg/l) and ceftazidime (8 mg/l). Error bars represent the standard error of the mean. Abbreviations: **IST408**, *B. cepacia* IST408; **Tp,** Trimethoprim; **Km,** Kanamycin; **Ceft,** Ceftazidime. *P* value < 0.05 was considered significant compared with the condition of no 4NPO supplementation (*).

3.2.3 Effect of 4NPO as co-adjuvant of detergents and disinfectants

Even considering that clinical application of 4NPO might be restricted due to its possible mutagenic activity this QSI might still present important characteristics that would allow its industrial use as co-adjuvant of detergents and other disinfectant agents. Thus, it was decided to analyze the effects of 4NPO together with three detergents and the commercial disinfectant agent bleach. For that, we started by determining if the MIC values for each compound varied due to QSI presence at sub-lethal concentrations for the three bacterial species under study. Two-fold dilutions to test concentrations ranging from 50 % (v/v) to 0% (v/v) for each compound were assayed. The results obtained are summarized in the Table 5 and, contrastingly to the observed for the antibiotics, there were some MICs differences. *B. cepacia* IST408 was more susceptible to sodium dodecyl sulfate (SDS) and *B. cenocepacia* K56-2 was more susceptible triton X100 in presence of 4NPO, suggesting a synergy between these compounds and 4NPO. Nevertheless, it was not possible to determine the MIC values of tween 20 for the three *Bcc* species neither of bleach for *B. cepacia* IST408 revealing the ineffectiveness of these commercial compounds in *Bcc* clearance.

Table 5 – Minimal inhibitory concentration (MIC) values in the absence or presence of 4NPO for *B.multivorans* D2095, *B. cepacia* IST408 and *B. cenocepacia* K6-2. Strains not inhibited by 50% (v/v) are depicted as an MIC of >50% (v/v).

	Stress	4NPO	
		0 µM	50 µM
***B. multivorans* D2095**	Bleach	1,56%	1,56%
	SDS	0.47%	0.47%
	Triton X100	25%	25%
	Tween 20	>50%	>50%
***B. cepacia* IST408**	Bleach	>50%	>50%
	SDS	1.88%	0.47%
	Triton X100	25%	25%
	Tween 20	>50%	>50%
***B. cenocepacia* K56**	Bleach	50%	50%
	SDS	7.5%	7.5%
	Triton X100	50%	25%
	Tween 20	>50%	>50%

To perceive the combined effects of 4NPO and the surfactant agents, different sub-MIC concentrations of these compounds were used to determine differences in biofilm formation of the three species duo to 4NPO activity. Cultures were grown in S medium statically at 30^{o}C, and biofilm quantification was assessed after 48 h of growth. The results indicate that the effect of 4NPO combined with the compounds studied is species-dependent. However, the same tendency was observed for the three *Bc*c species, where 4NPO enhanced the antimicrobial activity of all the compounds tested. Figure 17 describes the results obtained for *B. multivorans* D2095, being representative of the results obtained for the other species under study.

The usage of commercial bleach solution at sub-MIC concentration revealed to be completely inefficient against *Bc*c biofilms (Figure 17 A). Even, in presence of 4NPO there were no significant differences in biofilm formation for the three tested species using bleach. This resistance might be due to transport limitations of the disinfectant through the biofilm due to reaction-diffusion interactions [145]. Although the basis for biofilm resistance to NaOCl remains unknown, there are evidences that chlorine penetration (a substance released from the reaction of hypochlorous acid and the organic material) into biofilms can be retarded due to quicker consumption of the active chlorine in the biofilm surface layers compared to its rate of diffusion into the biofilm [146]. Moreover, a study in *B. cenocepacia* J2315 revealed that the presence of NaOCl resulted in a significant up-regulation of genes involved in the oxidative stress response as well as in the general stress response [147]. Accordingly, bleach, one of the most common disinfectants used, is inefficient against *Bc*c.

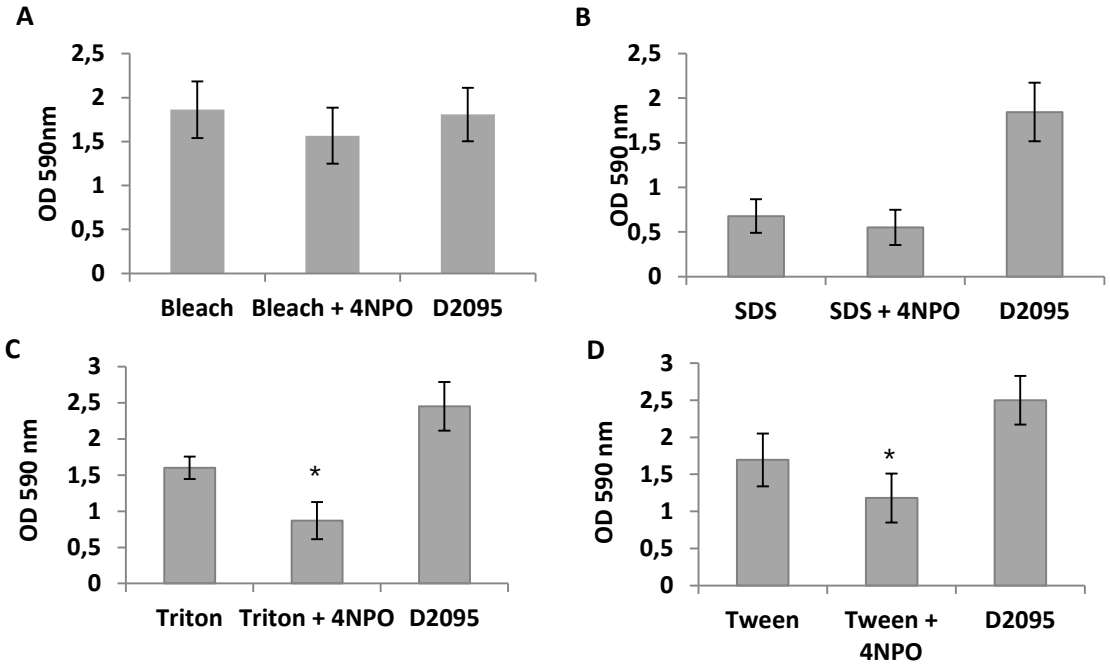


Figure 17 – ***B. multivorans*** **D2095 biofilm formation in presence of 4NPO sub-lethal concentration.** Bleach, SDS, TritonX100 and Tween 20 at concentration 0.17% (v/v) were evaluated in combination with 4NPO, to test the efficacy of this QSI in biofilm formation. A *P* value < 0.05 was considered significant compared with condition of no 4NPO supplementation but with the detergent/disinfectant (*).

Also, the use of 4NPO sub-lethal concentrations in combination with SDS does not seem to increase the efficiency of this detergent against *B. multivorans* D2095 biofilm (Figure 17 B). SDS is an anionic detergent commonly used to disrupt bacterial membrane. Contrastingly, 4NPO decreased biofilm formation when used in combination with the non-anionic detergents triton X100 and tween 20 (Figures 17 C and D), suggesting that their combined application might bring benefits in the disinfection process.

The high impact of biofilm formation in a broad range of surfaces is undoubted. Although it was desirable a total biofilm formation inhibition, this is rarely accomplished. Thus, using the detergents that exhibited a greater antibacterial activity upon 4NPO supplementation we assayed for disruption of pre-established biofilm. After 24 h of biofilm formation in 96-well plates at 30ºC, *Bcc* biofilms were rinsed and consequently treated with a detergent alone; sub-lethal of 4NPO alone (control) or a combination of the QSI and the detergent. However, no significant biofilm formation was observed either using triton X100 or tween 20 only or in combination with 4NPO in the three *Bcc* species under study (data not shown) being only observed biofilm formation in the control conditions. Nevertheless, these are preliminary results that need further confirmation and optimization.

4 Final remarks

One of the goals of this work was to study the influence of QS in *Burkholderia* EPS biosynthesis regulation. Here we present evidence that EPS is regulated by AHL-based QS systems since the presence of the lactonase enzyme AiiA from *Bacillus sp.* inhibits EPS production in several *Bc*c and non-*Bc*c species. Moreover, the presence of a known QSI also inhibited cepacian production in the tested *Bc*c strains. Even though CepI/R system does not seem to be involved in EPS biosynthesis in *Bc*c, the identification of a new putative autoinducer gene in *B. multivorans* ATCC 17616 will allow us to get additional insights into the underlying mechanism of cepacian biosynthesis regulation.

The second goal of this research work was the evaluation of the QSI 4NPO as a possible agent to decrease *Bc*c bacteria virulence. It was first demonstrated that 4NPO interferes with *Bc*c QS since several phenotypes known to be positively regulated by QS (motility, biofilm formation, EPS production, extracellular proteases production showed decreased levels in the presence of this inhibitor. These results are promising since we are looking for compounds that do not interfere directly with bacterial growth, but instead affect virulence factor expression and expose unprotected bacteria to killing by for example the host immune system.

Another great problem of *Bc*c bacteria is their intrinsic resistance to most of the clinically used antibiotics, making eradication of infections (namely, lung infections of CF patients) very difficult. Here we tested whether 4NPO could be co-adjuvant of antibiotics to decrease *Bc*c resistance to these last antimicrobials. Indeed, we showed that 4NPO potentiate the antimicrobial action of the tested antibiotics against both planktonic and sessile (biofilm) *Bc*c cells. Although the use of 4NPO for therapeutic purposes is limited due to the mutagenic properties of this compound, its use to prevent biofilm formation in abiotic surfaces of clinical or industrial origin it is perhaps a possibility to explore. Similarly, we had shown that 4NPO combined with some detergents such as Triton X100 or Tween 20 also affects biofilm development. Since the use of antibiotics to prevent *Bc*c biofilm formation is not feasible/ desirable, these detergents could be a good alternative that should be considered in future studies.

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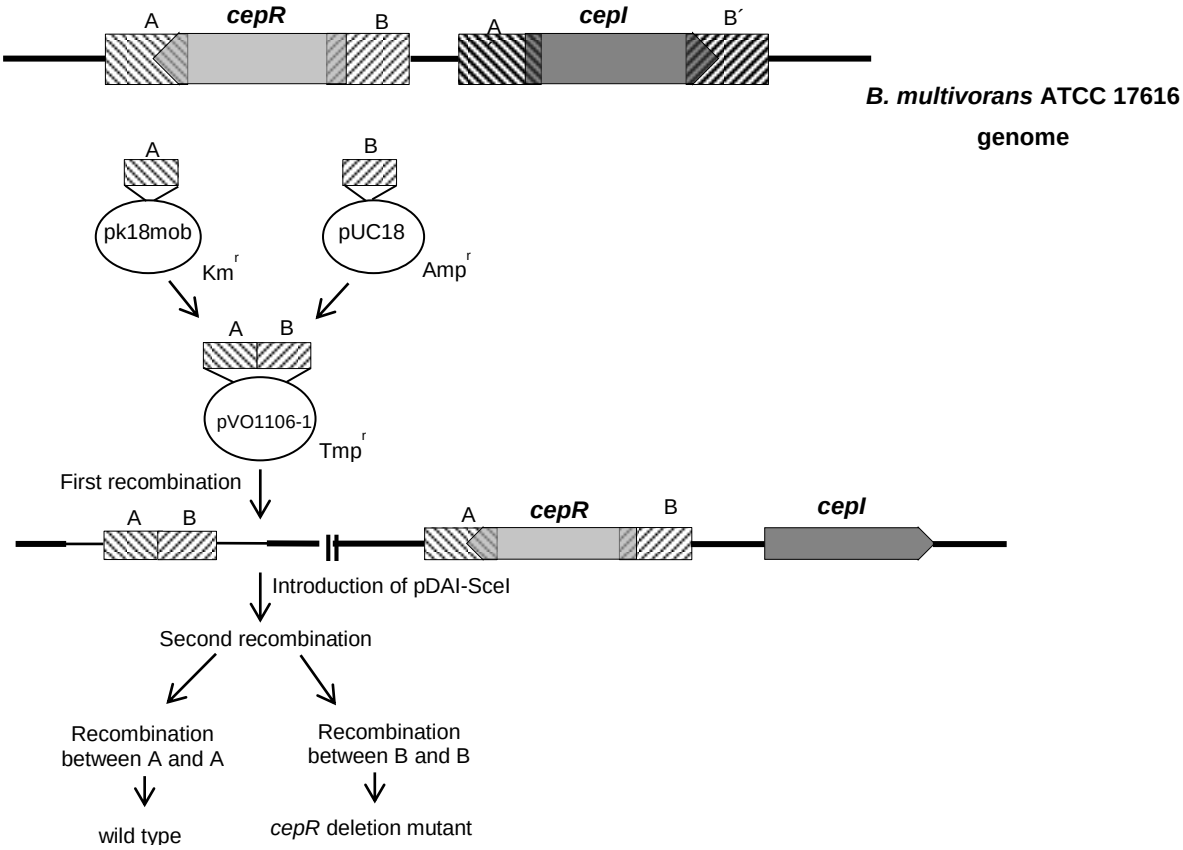
6 Supplementary data

Annex A

Schematic representation of the cloning strategy to construct unmarked deletion mutants in *cepI* and *cepR* genes (only the strategy for *cepR* is exemplified)



Annex B

AHL synthases used to search *B. multivorans* ATCC 17616 genome.

Strain	Protein name	ID number
Vibrio fischeri ES114	LuxI	VF_AO924
Erwinia amylovora CFBP1430	ExpI	YP_003530770
Ralstonia solanacearum PSI07	SolI	YP_003750860
Burkholderia kururiensis	BraI	CAP91066
Bradyrhizobium sp. BTAi1	TraI	ABQ39897
Burkholderia cenocepacia J2315	CciI	YP_002232872
Burkholderia cenocepacia J2315	CepI	CAR55728
Pseudomonas aeruginosa PAO1	LasI	NP_250123
Pseudomonas aeruginosa PAO1	RhiI	PA3476