# The mechanisms behind the potentiating activity of antibiotic adjuvants against *Burkholderia cenocepacia* biofilms

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ACD | Arginine decarboxylase |
| ACP | Acyl carrier protein |
| AHK | α-hydroyketon |
| AHL | Acyl homoserine lactone |
| AI | Autoinducer |
| AI-2 | Autoinducer-2 |
| AIP | Autoinducing peptides |
| AL-1 | 14-α-lipoyl andrographolide |
| AME | Aminoglycoside-modifying enzymes |
| AMR | Antimicrobial resistance |
| Bcc | *Burkholderia cepacia* complex |
| BDSF | *Burkholderia* diffusible signalling factor |
| BH | Baicalin Hydrate |
| CAI-1 | Cholera autoinducer-1 |
| c-di-GMP | cyclic dimeric guanosine monophosphate |
| CEF | Ceftazidime |
| CF | Cystic Fibrosis |
| CFTR | Cystic fibrosis transmembrane conductance regulator |
| CFU | Colony forming units |
| CIP | Ciprofloxacin |
| DMSO | Dimethyl sulfoxide |
| DNase | Deoxyribonuclease |
| DPD | 4,5-dihydroxy-2,3-pentanedione |
| DSF | Diffusible signal factor |
| ECO | Econazole |
| eDNA | Extracellular deoxyribonucleic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| EPS | Exopolysaccharides |
| ET12 | Electrophoretic type 12 |
| ETC | Electron transport chain |
| EUCAST | European Committee on Antimicrobial Susceptibility Testing |
| GN | Gentamicin |
| $H_2$DCFDA | 2',7'-dichlorodihydrofluorescein diacetate |
| HAM | Hamamelitannin |
| HGT | Horizontal gene transfer |
| HHQ | 2-heptyl-4-quinolone |
| HppD | 4-hydroxyphenylpyruvic acid dioxygenase |
| HSL | Homoserine lactone |
| HTS | High-throughput screening |
| IQS | Integrated quorum sensing |
| Kb | Kilobase |
| KIE | Kinetic isotope effects |
| KN | Kanamycin |

| | |
|---|---|
| LBA | Luria Bertoni agar |
| LBB | Luria Bertoni broth |
| LMEM | Linear mixed effect model |
| LPS | Lipopolysaccharide |
| MB | Marine Broth |
| mBTL | meta-bromo-thiolactone |
| MDH | Malate dehydrogenase |
| MDR | Multidrug resistant |
| MEM | Meropenem |
| MHB | Mueller Hinton broth |
| MIC | Minimal inhibitory concentration |
| MICO | Miconazole |
| MIN | Minocycline |
| MQ | MilliQ water |
| MTA | Methylthioadenosine |
| MTAN | 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase |
| MTP | Microtiter plate |
| MTR | 5-methylthioribose |
| NADH | Nicotinamide adenine dinucleotide |
| $NaN_3$ | Sodium azide |
| ND | Not determined |
| NEO | Neomycin |
| NP | Nucleotide polymorphism |
| NR | No reduction observed |
| ODC | Ornithine decarboxylase |
| OMP | Outer membrane protein |
| PBP | Penicillin-binding protein |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PD | Pharmacodynamics |
| PEP | Phosphoenolpyruvate |
| PK | Pharmacokinetics |
| PMNL | Polymorphonuclear leukocyte |
| PQS | *Pseudomonas* quinolone signal |
| PS | Physiological saline |
| qPCR | Quantitative polymerase chain reaction |
| QQ | Quorum quenching |
| QS | Quorum sensing |
| QSI | Quorum sensing inhibitor |
| RAP | RNAIII-activating protein |
| RIP | RNAIII-inhibiting protein |
| RNAseq | RNA sequencing |
| ROS | Reactive oxygen species |
| RPKM | Reads per kilobase per million |
| rRNA | Ribosomal RNA |

| | |
|---|---|
| SAH | S-adenosylhomocysteine |
| SAM | S-adenosylmethionine |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulphate |
| ShvR | Shiny colony variant regulator |
| SNP | Single nucleotide polymorphism |
| SOD | Superoxide dismutase |
| SRH | S-ribosylhomocysteine |
| sRNA | Small regulatory RNA |
| SXT | Sulfamethoxazole/Trimethoprim |
| TB | Tobramycin + Baicalin hydrate |
| TCA | Tricarboxylic acid |
| TE | Tobramycin + Econazole |
| TM | Tobramycin + Miconazole |
| TOB | Tobramycin |
| TRAP | Target RNAIII activating protein |
| TSA | Trypton soy agar |
| TTS | Transition state |
| VAP | Ventilator associated pneumonia |
| VBNC | Viable but not culturable |
| WGS | Whole genome sequencing |
| WHO | World Health organization |
| WT | Wild type |

“He that will not apply new remedies must expect new evils; for time is the greatest innovator.”
- Francis Bacon

# CHAPTER I
# INTRODUCTION

# 1 Bacterial resistance: a never-ending battle?

## 1.1 From golden age to post-antibiotic era

Treatment of infectious diseases has become a serious challenge since we have entered a post-antibiotic era. This post-antibiotic era threatens achievements of modern medicine, because common infections and infections following minor injuries will be able to kill patients. The World Health Organisation (WHO) has recognized antimicrobial resistance (AMR) as one of the most important health threats of this century [1].

The discovery of penicillin in 1929 was a milestone in the history of antibiotic development. Although penicillin was the first microbial-derived antibiotic, it was the discovery of streptomycin that spiked the interest of pharmaceutical companies. This led to an era of whole-cell screening of soil micro-organism extracts for their activity against pathogenic bacteria [2]. Many natural and semisynthetic analogues with antibiotic activity were discovered, leading to the golden ages of antibiotic discovery [3]. In the golden ages, there was a serious decrease in the risk of infections. This allowed important medical achievements in surgery and cancer therapy. However, the emergence and spread of antibacterial resistance started to jeopardize the effectiveness of antibiotics [1]. The rise of antibiotic resistance is both natural and inevitable. Moreover, malpractices in antibiotic use (i.e. underuse, overuse or misuse) have speeded up the process [4]. After the golden ages, the medicinal chemistry era followed. In this era synthetic versions of natural scaffolds were produced. These derivatives had both an enhanced antibacterial efficacy and a broader antimicrobial spectrum. Also resistance that already had emerged against the natural scaffolds was circumvented [3].

From the mid-1980's the discovery rate of new antimicrobial agents dropped and the strategy for antibiotic development shifted towards target-based screening to find broad-spectrum antibiotics [5]. This era is called the resistance era. As the name suggests, the approach in this era failed to provide novel antibiotics [3,6]. The reasons for the lack of success were multifactorial; some of them are commonly encountered in high-throughput screening (HTS) projects, not only in antibacterial drug discovery, such as the lack of dose-response or interference in assay-detection methods. Other reasons were specific to antibacterial drug discovery, such as the therapeutic profile at that time, which pursued broad-spectrum activity against both Gram-positive and Gram-negative organisms. HTS projects required compounds with antibacterial activity against relevant targets of both Gram-positive and Gram-negative organisms in order to progress [7].

Currently, a few antimicrobial agents (antibiotics or biologicals) are in the pipeline. However, these potential novel treatments have little added value compared to the currently available ones and will not be sufficient to tackle the threat of AMR [1,8]. In general, novel antibiotics alone will not suffice to circumvent this threat and worldwide measures are necessary to combat AMR [9]. If this problem remains unaddressed, this will have serious consequences on human health in the upcoming years (Figure 1) [5].

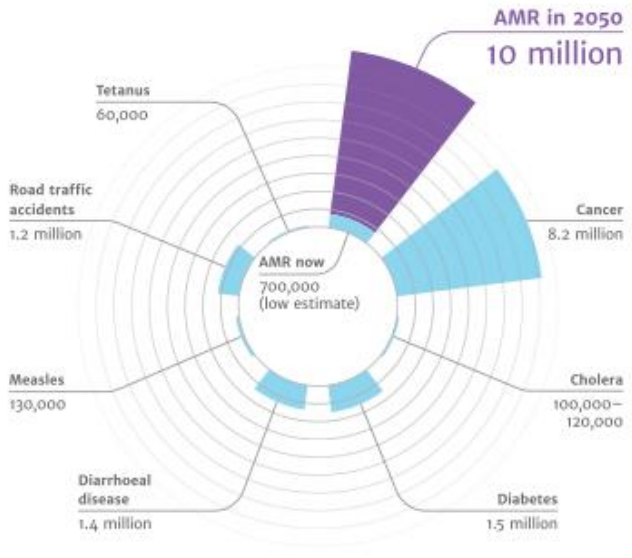



*Figure 1:* ***Deaths attributable to major illnesses in 2016 and deaths attributable to AMR predicted in 2050*** [5].

Measures could be taken on multiple levels. Firstly, the use of antibiotics should be reduced. This can be achieved by increasing global awareness of AMR, reducing unnecessary use of antimicrobials (for example in agriculture and for the treatment of viral infections) and improving hygiene which will prevent the spread of infections. Secondly, the number of antibiotics that can tackle resistant pathogens should increase. This can be addressed at a global level by increasing funding to tackle AMR, but also on a national level where the government can promote investment in new drugs and improvement of existing ones. Finally, an international coalition for global action should be in place to support previously mentioned measures, like the UN, otherwise all attempts to reduce AMR are idle [1,5].

## 1.2 Types of resistance

There are three types of antibiotic resistance; intrinsic, adaptive and acquired resistance [10]. Intrinsic resistance comprises all inherently available mechanisms that nullify the effects of antimicrobials. A good example is the outer membrane of Gram-negative bacteria that prevents the entry of many antimicrobials into the cell [11].

In the discussion of AMR inherently resistant bacteria are not the real problem, but rather bacterial populations that were originally susceptible but acquired resistance [12]. Bacteria naturally adapt to their environment, like in the presence of antibiotics. In a first scenario, mutational changes spontaneously occur in the genes that are affected by an antimicrobial agent. After emergence of a resistant mutant, the susceptible population will be eliminated by the antibiotic and a resistant population will arise. The most common mutational changes cause a modification of the target, decreased drug uptake, increased efflux, or changes in metabolic pathways [10,12]. In a second scenario resistance genes are acquired from other organisms through horizontal gene transfer (HGT). The transfer of DNA can occur via three mechanisms; transformation, transduction or conjugation. The most common mechanism in hospital environment is through conjugation, which involves cell-to-cell contact [12].

Intrinsic and acquired resistance can be transmitted vertically to following generations. This is in contrast with adaptive resistance, which is of transient nature and can be reverted when the inducing condition is eliminated. Bacteria can rapidly alter their transcriptome in response to environmental changes like the presence of (non-)lethal concentrations of antibiotics, in order to increase their chances of survival [10].

## 1.3 Resistance mechanisms

Bacteria have several mechanisms to avoid being killed by antimicrobial agents. These mechanisms can generally be divided into three main groups (Figure 2). The first group minimizes the intracellular drug concentration by reduced permeability and/or an increased efflux. Decreased uptake is achieved by changes in the outer membrane permeability, such as downregulation of porins that allow antibiotics to diffuse through the membrane [13]. Increased efflux is a major contributor to resistance in Gram-negative bacteria. This efflux is established by the overexpression of MDR efflux pumps, which are able to eliminate a wide variety of substrates from the periplasm to the cell exterior [11]. An example is the MexAB-OprM efflux pump in *Pseudomonas aeruginosa*, that not only contributes to its resistance, but also excretes other molecules such as biocides and signalling molecules [12,14].

A second group inactivates the antibiotic by hydrolysis or modification. Bacterial enzymes can hydrolyse an antibiotic, rendering it inactive. For example, the β-lactamase enzymes hydrolyse the amide bond of the β-lactam ring of penicillins and cephalosporins [15]. Bacterial enzymes can also modify the antibiotic by adding chemical groups to vulnerable sites on the antibiotic molecule, preventing the antibiotic from binding to its target due to steric hindrance. Especially aminoglycosides are prone to this resistance mechanism, because of their large amount of exposed hydroxyl and amino groups. There are three main classes of aminoglycoside-modifying enzymes (AME): acetyltransferases, phosphotransferases and nucleotidyltransferases [16,17].

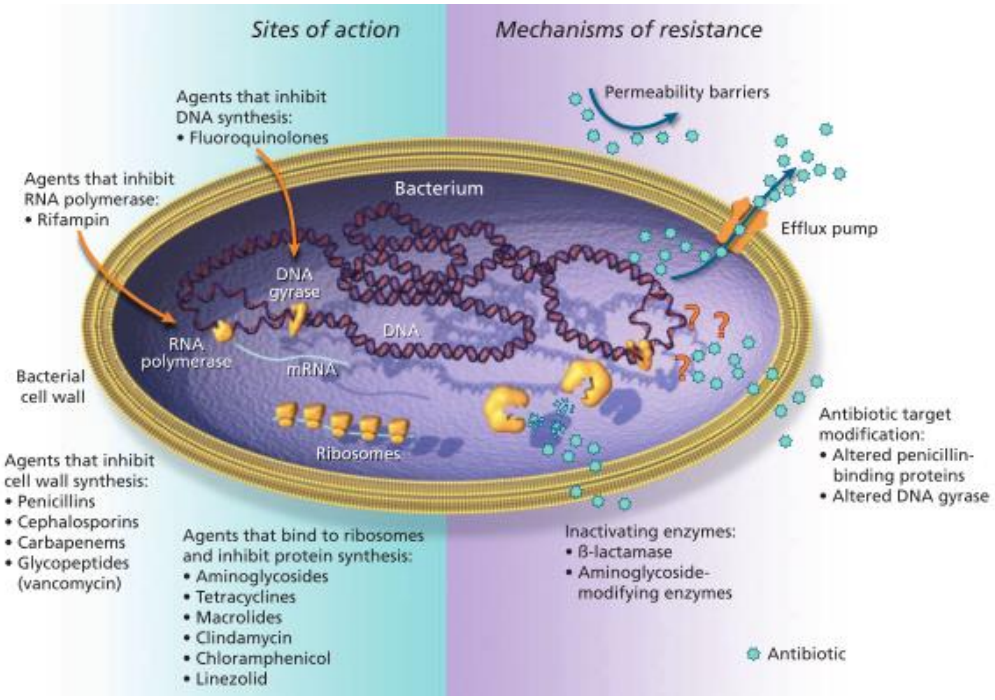



*Figure 2:* ***Target sites of commonly used antibiotics and resistance mechanisms*** [18]

Finally, the antibiotic target site can be modified by genetic mutations or by enzymatic changes. Mutational changes include point mutations in genetic regions coding for the active site of the target or the acquisition of foreign DNA. A clinically important example is the alteration in penicillin-binding protein (PBP) that results in a reduced affinity of *Streptococcus pneumoniae* towards β-lactam antibiotics [11]. Enzymatic changes can protect the antibiotic target site, by addition of chemical groups, without altering the proteins activity. For example, ribosomal RNA (rRNA) methyltransferases methylate a nucleotide in the aminoglycoside-binding site of 16S rRNA using S-adenosyl-L-methionine (SAM) as a co-substrate [17,19]. All these changes of the target site impair the binding efficiency of the antibiotic, thus limiting its potency [12].

## 1.4 Role of sociomicrobiology in resistance/tolerance

The term sociomicrobiology was first introduced by Parsek and Greenberg [20] and refers to the investigation of microbial group-behaviour. Examples of such group behaviour in bacteria are quorum sensing (QS) and biofilm formation.

### 1.4.1 Quorum sensing

QS is cell-to-cell communication that regulates gene expression in response to fluctuations in population density. Gram-positive and Gram-negative bacteria use these communication circuits to regulate several physiological activities, such as virulence, antibiotic production, swarming and biofilm formation [21]. Bacteria employ small, secreted signalling molecules, also known as autoinducers (AIs), that increase in concentration with increasing population density [21–23]. The most commonly studied AIs belong to three main categories: (i) AHLs, that are used by Gram-negative bacteria and are also referred to as autoinducer-1 (AI-1), (ii) peptide signals, that are used by Gram-positive bacteria, and (iii) AI-2, that can be used by both Gram-positive as Gram-negative bacteria and serves as an intraspecies communication tool [24] (Figure 3).

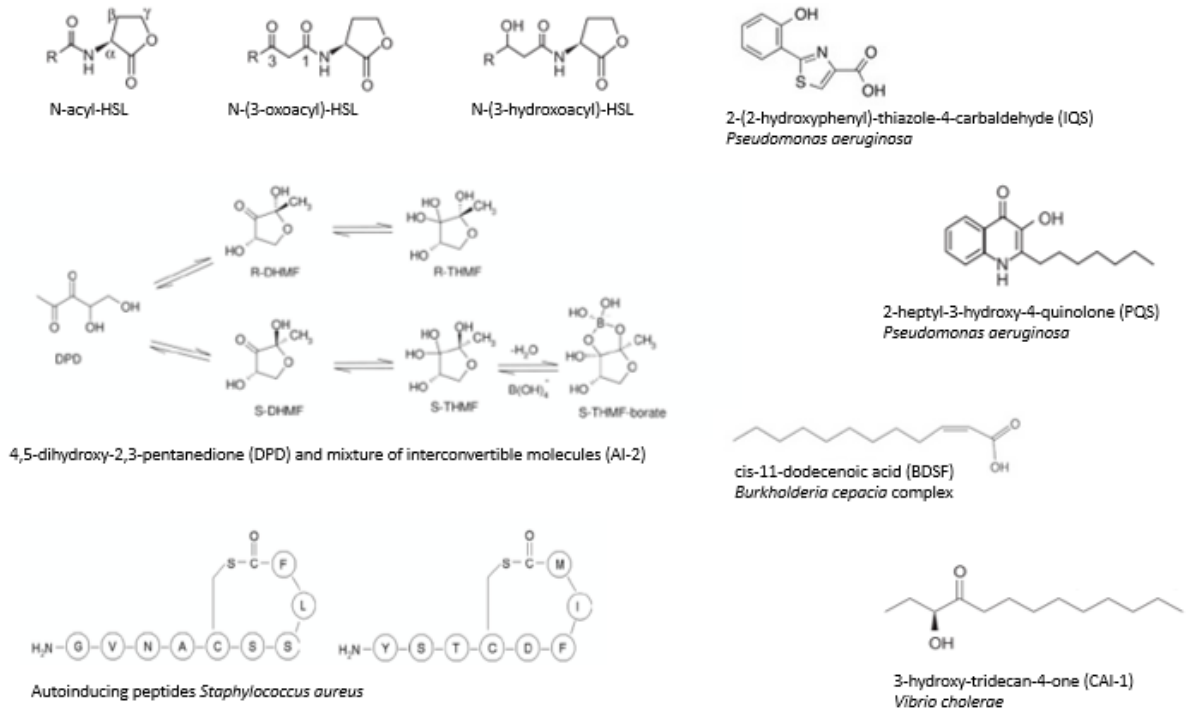



*Figure 3:* ***QS signalling molecules used by various bacteria****.*

#### *1.4.1.1 N-Acyl homoserine lactone based quorum sensing*

AHL molecules are the most commonly used signalling molecules in Gram-negative bacteria. These molecules consist of a homoserine lactone (HSL) ring attached to an acyl chain. The latter can vary in length (4-18 carbons), saturation and the oxidation state at position 3 (Figure 3). Generally, AHLs are synthesized by synthases of the LuxI family, using SAM and an acylated acyl carrier protein (ACP) as substrates [21,25]. An exception is LuxM, the AHL synthase of *Vibrio harveyi*, which catalyses the same reaction but has no shared homology with LuxI-type proteins [26]. AHL synthases predominantly produce a single type AHL, but it is not uncommon that other types of AHLs are produced in smaller amounts by the same synthase [25,27]. Small AHLs diffuse passively across the cell wall, while AHLs with longer acyl side chains require active transport [28]. When a critical threshold concentration is reached, the AHLs bind to their cognate LuxR-type receptor protein in the cytoplasm. LuxR-type proteins are transcriptional regulators whose DNA-binding activity change upon interaction with AHL. This AHL-LuxR interaction results in the modulation of target gene regulation (Figure 4) [21]. Alternatively in *V. harveyi*, AHLs synthesized by LuxM are detected by the transmembrane sensor kinase, LuxN, which initiates a phosphorelay cascade involving LuxU and LuxO upon ligand binding [26]. Overall, each QS network has a cognate synthase/receptor pair that synthesizes and responds to a specific AHL molecule (based on the differences in their acyl side chain). There are also species that harbour multiple AHL-based QS networks that generate (and respond to) distinct AHL molecules, as is the case for *Burkholderia cenocepacia* [29]. In addition, "orphan" or "solo" LuxR-type receptors (i.e. receptors that have no corresponding synthase) have been described. Examples include QscR in *P. aeruginosa* [30] and CepR2 in *B. cenocepacia* [31].

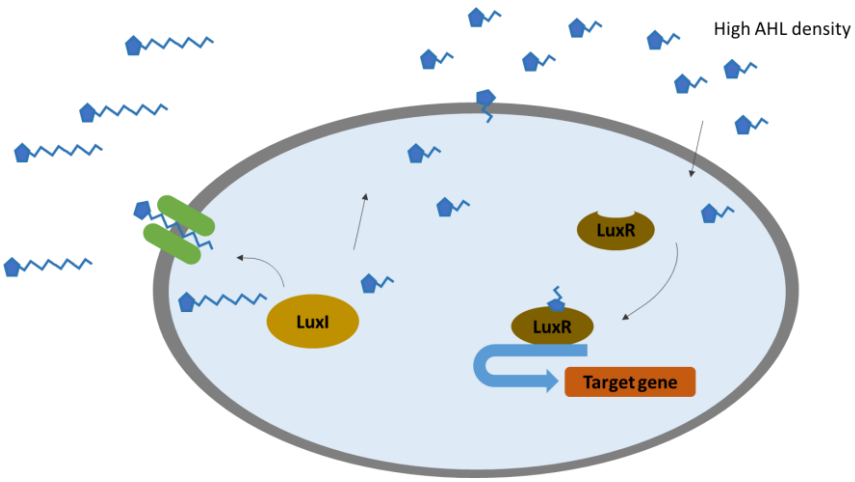



*Figure 4:* ***Schematic overview of AHL-based QS***

### 1.4.1.2 *Autoinducer-2 based quorum sensing*

AI-2 molecules can be used for interspecies communication between Gram-positive and Gram-negative organisms. AI-2 is synthesized from the substrate SAM via sequential enzymatic reactions. SAM is converted to S-adenosylhomocysteine (SAH) via methyltransferases. Subsequently, 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase (MTAN) catalyses the hydrolytic adenylation from SAH to adenine and S-ribosylhomocysteine (SRH). The latter is then converted by the metalloenzyme, LuxS, to homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD). DPD is highly unstable and spontaneously rearranges to multiple interconvertible cyclic furanone compounds, collectively designated as AI-2 (Figure 3) [32]. Bacterial species detect different forms of DPD as their AI-2 signals, e.g. *V. harveyi* synthesizes borated AI-2, which is detected via the two-component sensor kinase LuxPQ (Figure 5), whereas *Salmonella* spp. and *Escherichia coli* produce non-borated AI-2, which is detected via the Lsr ABC-transporter [33]. As DPD derivatives easily interconvert, AI-2 molecules can be used for interspecies communication [34].

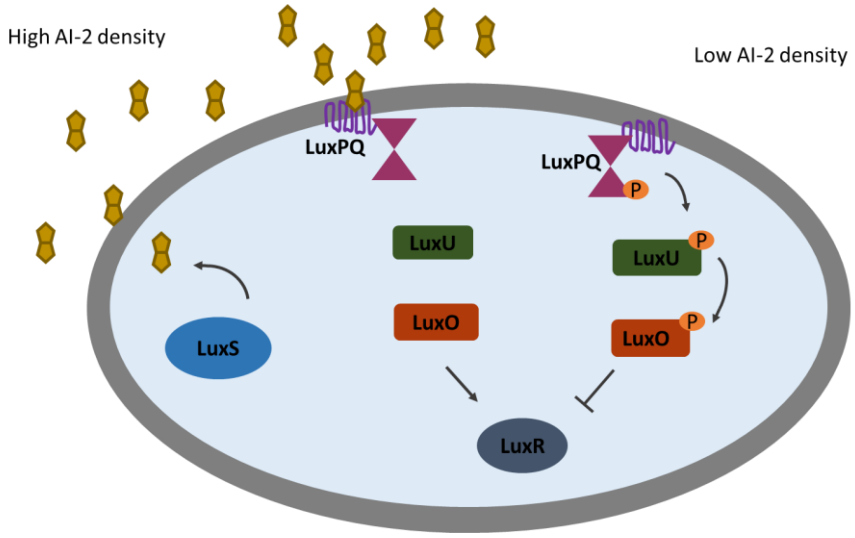



*Figure 5:* ***Schematic overview AI-2 based QS in V. harveyi***

### 1.4.1.3 *Peptide based quorum sensing*

Autoinducer molecules in Gram-positive bacteria are modified oligopeptides (Figure 3). These peptides are genetically encoded and can be the subject of several modifications, such as processing and/or cyclization, after translation [21,24]. The peptides are unable to permeate the cell membrane and are actively exported via an ABC transporter. After reaching a critical threshold concentration,

the autoinducer peptides are detected by a histidine sensor kinase of a two-component signalling system. Upon interaction with the peptide, the phosphoryl group is transferred to a cognate response regulator protein. The phosphorylated response regulator can then bind to DNA and regulates the transcription of QS-controlled target genes [21,24] (Figure 6). An example of such a peptide based QS system is the *agr* system in *Staphylococcus aureus*, which is based on cyclic autoinducing peptides (AIPs) that interact with a cognate AgrC sensor kinase to regulate exotoxin production and biofilm dispersal, via the regulatory RNA molecule (RNAIII) [35]. *S. aureus* also possesses the RAP/TRAP QS system, which consists of an RNAIII-activating protein (RAP), a QS activator, and a target RNAIII activating protein (TRAP). Phosphorylation of TRAP is induced when a certain threshold level of RAP is reached, which induces RNAIII production [36].

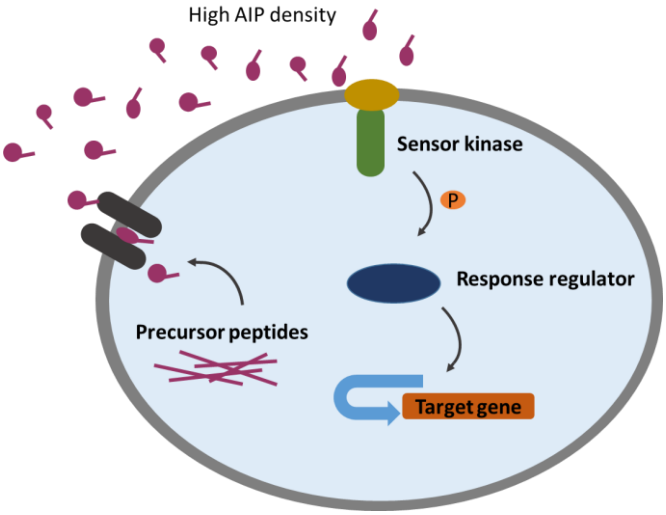



*Figure 6:* ***Schematic overview of AIP-based QS***

#### *1.4.1.4 Quorum sensing systems based on other autoinducers*

Other autoinducers can also contribute to QS regulation.

*P. aeruginosa* has four connected QS systems that are organized in a hierarchical manner [37]. The LasIR and RhlIR are both AHL-based QS systems, that use N-3-oxo-dodecanoyl HSL (3-O-$C_{12}$-HSL) and N-butyryl-HSL ($C_4$-HSL), respectively [21]. A third QS system uses the *Pseudomonas* quinolone signal (PQS) (2-heptyl-3-hydroxy-4-quinolone) as a signalling molecule (Figure 3). PQS and its precursor 2-heptyl-4-quinolone (HHQ) are biosynthesized by enzymes encoded by the *pqsABCDE* operon, and act as autoinducers through interaction with the transcriptional regulator PqsR, also known as multiple virulence factor regulator (MvfR) [38]. The production of PQS is restricted to *P. aeruginosa*, but other *Pseudomonas* species as well as some *Burkholderia* spp. can utilize HHQ as QS signal [24].

Recently, a fourth communication system has been discovered. This system is known as the integrated QS system (IQS) and is capable of integrating environmental stress signals with the other QS networks. The signalling molecules are 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS) and their synthesis involves the *ambBCDE* gene cluster. Disruption of IQS causes a decrease in the production of $C_4$-HSL, PQS and virulence factors such as elastase and pyocyanin [37].

Another example is the signalling molecule cis-11-methyl-dodecenoic acid, also known as diffusible signal factor (DSF). DSF modulates the transition between planktonic and biofilm-lifestyle. It was originally identified in *Xanthomonas campestris* [39] but structural homologues have also been found in the human opportunistic pathogen *B. cenocepacia*, in which it is called *Burkholderia* Diffusible signal factor (BDSF) [40–42] (Figure 3). BDSF is synthesized by RpfF from the ACP thioester of 3-hydroxydecanoic acid. This enzyme is a bifunctional crotonase that both dehydrates and cleaves the acyl thioester bound in order to yield the final product, BDSF [40]. The mechanism for the detection of DSF family signalling molecules differs among species, but generally leads to an alteration of intracellular cyclic dimeric guanosine monophosphate (c-di-GMP) levels [32]. In *X. campestris*, an accumulation of DSF results in the interaction with the sensor kinase RpfC, which induces a phosphorelay cascade to ultimately activate the response regulator RpfG. Activated RpfG causes a decrease of intracellular c-di-GMP concentration, which results in the activation of Clp regulators that regulate QS target gene expression [24]. In *B. cenocepacia*, however, BDSF is sensed by the receptor protein RpfR, that contains a PAS-GGDEF-EAL domain [41,43]. Binding of BDSF to RpfR leads to the induction of the c-di-GMP phosphodiesterase activity of the protein, hence lowering the intracellular c-di-GMP levels [40,41].

Furthermore, various *Vibrio* spp. use α-hydroyketons (AHK) as signalling molecules. The most common AHK is 3-hydroxy-tridecan-4-one, also known as cholera autoinducer 1 (CAI-1) (Figure 3), predominantly utilized in *Vibrio cholerae* and to a lesser extent in *V. harveyi* [44]. Amino-CAI-1 is synthesized by CqsA and amino-CAI-1 is subsequently converted to CAI-1 in a CqsA-independent step [45]. Both CAI-1 and amino-CAI-1 are sensed by the sensor kinase CqsS, resulting in a phosphorelay cascade involving LuxU and LuxO. These two proteins are also involved in AI-2 signalling in *V. cholerae* [34].

### 1.4.2 Biofilms

Biofilms are defined as communities of microbial cells embedded in a self-produced matrix that, compared to their planktonic counterparts, show reduced susceptibility towards antimicrobial therapy [46]. Biofilms are usually responsible for chronic infections, meaning that the infection persists despite antibiotic treatments and host defence responses. Biofilm infection sites are characterized by inflammation surrounding the biofilm, resulting in a persistent infection [47].

Biofilm formation is a multistep process that is initialized with a reversible attachment of microbial cells to a surface, which can be biotic or abiotic, or to each other (forming free-floating aggregates). In a next step, microbial cells proliferate and produce a matrix, containing polysaccharides, proteins and extracellular DNA (eDNA), which leads to an irreversible attachment [46,48]. Further maturation to a complex three-dimensional structure is accomplished with the build-up of biomass by increasing the production of the matrix. The matrix of the biofilm provides structural stability and protection against a hostile environment. To complete the cycle, cells can actively or passively detach from the biofilm, to either disperse and colonize other interfaces or to return to a planktonic state [48].

Although QS is not necessary for biofilm formation, there is a link between QS and biofilms. This link strongly depends on the microorganism and the environmental conditions [20,49–52]. This is shown by following examples. In *P. aeruginosa*, *E. coli* and *Salmonella enterica* serovar Typhimurium, c-di-GMP plays a role in the transition from planktonic to biofilm mode of growth. This occurs by downregulating expression of motility genes and upregulating expression of biofilm genes [53]. In *B. cenocepacia*, the regulation of BapA expression, a large secreted protein required for biofilm formation, is dependent on QS [54]. Furthermore, QS also regulates swarming motility, and production of siderophores and rhamnolipids, which contribute to *P. aeruginosa* and *B. cenocepacia* biofilm formation [49,55]. However, in *S. aureus*, the presence of active QS molecules interferes with attachment and biofilm development and contributes to dispersal [56].

Biofilms are characterized by physiological and biochemical gradients that are established from the surface to the deeper layers of the biofilm [50]. Gradients of nutrient and oxygen availability develop throughout the biofilm, and as a consequence sessile cells in the core of the biofilm have reduced access to nutrients and oxygen. This results in a reduced growth rate of these cells, leading to structurally intact cells with a very low metabolic activity, also known as viable but not culturable (VBNC) cells [57]. As bacteria require energy to sustain their metabolic activity, a gradient of activity develops throughout the biofilm. This activity gradient is exhibited in the development of subpopulations. Cells at the outer layers of the biofilm have a considerably higher growth rate than cells in the core of the biofilm [57]. The presence of this gradient creates microenvironments in the

biofilm, such as anaerobic regions or regions with low pH due to the accumulation of acidic waste products [58]. Some matrix components, such as amyloid fibres, exhibit weak binding affinity for QS signalling molecules, thus creating regions with higher concentrations of signalling molecules [59]. All these factors cause heterogeneity, which enables spatial organization of biofilms [60].

### 1.4.3 Mechanisms for tolerance/persistence/resistance

The link between virulence and antibiotic resistance often resides within a biofilm structure. Its formation is therefore an essential part of pathogenicity (i.e. the combination of virulence and antibiotic resistance). The regulation of virulence and antibiotic resistance genes is very complex and is strongly connected [61].

Cells in a biofilm are typically much less susceptible to antimicrobial agents than their planktonic counterparts. As freshly formed biofilms are also more susceptible to antibiotics than mature biofilms, the development of tolerance in biofilms is a time-requiring process [50]. Several features contribute to tolerance. The first tolerance mechanism is caused by the barrier properties of the biofilm matrix. The matrix limits the diffusion of antibiotics through the biofilm (Figure 7). However, this is only a short-term protective effect that has no substantial impact during long-term antibiotic exposure [62]. Also, some matrix compounds are known to chelate antibiotics (e.g. eDNA), or scavenge reactive oxygen species (ROS) (e.g. alginate) [50,63].

The reduced metabolic activity leads to the occurrence of subpopulations in the biofilm that are more tolerant towards various stresses. Biofilms consist of at least two subpopulations: a growing aerobic population and a dormant anaerobic population [64]. These variations in growth rate are not only the result of nutrient limitation, but can also be caused by a general stress response to protect cells from stress. This general stress response can be triggered by the central regulator RpoS [65]. Since most antibiotics target processes of metabolically active cells, dormant cells display increased antimicrobial tolerance [64] (Figure 7).

Persister cells are a small subpopulation in both sessile and planktonic cultures that are able to survive repeated antimicrobial treatments without being genetically resistant. Many studies have investigated this phenomenon and dedicated the extreme tolerance observed in persisters to slow growth or even growth arrest. However, persisters are not necessarily metabolic inactive and more research is necessary on the formation and regrowth of these persister cells in infections [66].

Also adaptive resistance (i.e. transient tolerance) can be acquired by subpopulations of the biofilm upon antibiotic exposure. This type of tolerance can be nonspecific or specific for a class of antibiotics [10,67]. An antibiotic-specific form of tolerance is the induction of β-lactamase

transcription in response to the presence of β-lactam antibiotics in *P. aeruginosa* [68]. An example of a nonspecific form of tolerance is the upregulation of efflux pumps. This has been shown in multiple species and affects their susceptibility towards several compounds, such as the antibiotics azithromycin and colistin and the biocides EDTA and chlorhexidine. This resistance mechanism in biofilms can be triggered by the presence of antibiotics or are a part of the bacterial stress response [50,69].

Furthermore, QS is partially responsible for the regulation of the oxidative stress response [70]. Several bactericidal antibiotics have a common mechanism to induce cellular death that involves the production of ROS [71] (Explained in detail in Chapter I.3.4). Induction of ROS protecting enzymes by QS can thus result in an increased resistance.

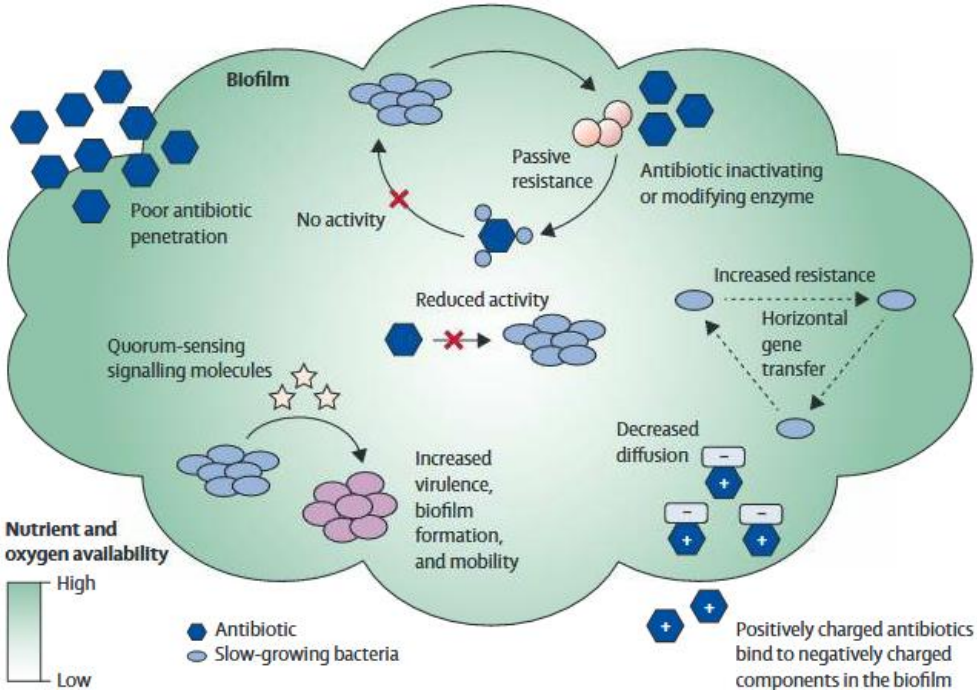



*Figure 7:* ***Antibiotic tolerance and resistance mechanisms in bacterial biofilms*** [72]

Cells in a biofilm can easily acquire resistance by hypermutability or increased HGT. Alterations in genes of DNA repair systems and/or endogenous oxidative stress have been proposed as triggers for increased genetic adaptation and evolutionary changes [47,73]. These triggers are more present in biofilms than in planktonic cultures and result in hypermutability (i.e. a higher mutation rate), which can result in genetic mutations [73]. Moreover, biofilm cells have an increased HGT due to their close proximity to each other, which enables them to spread resistance mechanisms [74] (Figure 7).

In addition to tolerance/resistance mechanisms caused by sociomicrobiology, all conventional resistance mechanisms of bacterial cells still apply on sessile cells.

# 2 Quorum sensing interfering agents as antibiotic adjuvants

## 2.1 Antibiotic adjuvants

As multidrug resistant (MDR) pathogens rapidly develop clinical resistance towards almost every bactericidal/bacteriostatic agent brought to the market [75], alternative approaches to tackle these infections are necessary. The occurrence of specific biofilm-related tolerance/resistance mechanisms further complicates treatment [76]. Treatment of biofilm-related infections using antibiotics in monotherapy is difficult and requires high concentrations and long-term exposure. This will suppress growth and spread of the biofilm but will still fail to eradicate the infection. Also, the antibiotic concentration gradient through the biofilm causes zones exposed only to sub-lethal concentrations of the antibiotic. These low concentrations induce the development of resistance without eradicating the population [50,77]. Therefore, alternative therapies, like the combination of an antibiotic with non-lethal adjuvants that increase the former's activity, are being explored as potential approaches to eradicate biofilm-related infections and decrease the emergence of resistance [78–80].

Adjuvants are active compounds that enhance antimicrobial activity by increasing the susceptibility of bacteria/biofilms towards antibiotics or the host immune response, or by reducing bacterial virulence [81,82].

An example of clinically used adjuvants that overcome resistance are the β-lactamase inhibitors. These inhibit bacterial β-lactamases and so protect β-lactam antibiotics from hydrolysis. Currently, there are five inhibitors clinically registered: clavulanic acid, sulbactam, tazobactam, avibactam and vaborbactam [83]. Other adjuvants that block or bypass AMR mechanisms are efflux pump inhibitors and outer membrane permeabilizers [84].

However, resistance is rising quickly and the road from developing a compound to bringing it to the market is long. Therefore, an interim solution can be sought in repurposed drugs. These are existing pharmaceuticals, not necessarily antimicrobials, that have a synergistic effect on bacterial survival in combination with conventional antibiotics, by increasing the antimicrobials effect and/or modulating the host defence response [85–87]. An example is the use of loperamide in combination with tetracyclines in *P. aeruginosa*. Loperamide (Imodium; Janssen Pharmaceutica) is an anti-motility drug used for the treatment of diarrhoeal diseases and has no antibiotic activity. When combined with an antibiotic treatment, it causes a decrease in the electrical component of the proton motive force. To maintain their ATP synthesis level, bacteria increase the pH gradient (chemical component) across

the inner membrane. This increased pH allows uptake of tetracycline antibiotics. As a consequence, the intrinsic resistance towards tetracyclines is circumvented [88].

Other adjuvants can interfere with bacterial virulence and/or QS pathways. These adjuvants disarm bacteria by inhibiting (the production of) virulence factors. The latter are bacterial products that promote diseases by either damaging the host and/or evading the host immune system [89]. Antivirulence adjuvants can target various virulence factors such as toxins, adhesins or secretion systems [90]. In addition, regulatory factors such as QS can be targeted. This is especially interesting since many genes encoding virulence factors are QS-regulated [91,92]. Overall, adjuvants that target virulence have some advantages compared to conventional antibiotics. They are thought to evoke less evolutionary pressure since they generally do not target essential pathways. They also increase the arsenal of pharmacological targets [93,94].

This dissertation focuses on antibiotic adjuvants that target QS.

## 2.2 Quorum sensing and quorum sensing interference

### 2.2.1 Quorum sensing

QS is the process of cell-density based communication between bacteria, which promotes group-behaviour when a certain population threshold is reached. This group behaviour includes the promotion of virulence factor production and biofilm formation. Bacterial communication is established by the production, release, detection and ultimately the response to signal molecules, called autoinducers [95]. The most commonly studied autoinducers belong to three main categories: AHLs, used by many Gram-negative bacteria and also referred to as autoinducer-1 (AI-1), peptide signals, used by Gram-positive bacteria, and AI-2 that can be used by both Gram-positive as Gram-negative bacteria and serves as an intraspecies communication tool [24].

### 2.2.2 Mode of action of quorum sensing interfering agents

Agents that interfere with QS can disrupt QS-regulated phenotypes like virulence. Those agents can be classified into two groups according to their molecular weight; small molecular and macromolecular agents, also referred to as QS inhibitors (QSI) and QQ enzymes, respectively [96]. As QS regulates the expression of many virulence factors, including biofilm formation, compounds that interfere with these communication circuits are promising antibiotic adjuvants. All QS systems share a conceptually similar signalling cascade starting with the production of AIs, which accumulate extracellular. When reaching a certain threshold, these AIs bind to a transcriptional regulator that subsequently regulates the expression of target genes [21]. This general pattern creates the opportunity to interfere with QS circuits on four different levels [97].

A first method to inhibit QS is the deactivation of the produced AIs by QQ enzymes. AHL degrading enzymes can have four different modes of action. AHL lactonases and AHL acylases degrade AHLs by hydrolysing the HSL ring and the amide bond, respectively. AHL oxidase and AHL reductase do not degrade AHLs but change their activity by modifying the AHLs [98]. Interestingly, some bacteria such as *Pseudomonas* and *Agrobacterium*, are able to cleave their own AHL signal [24]. Most of the identified QQ enzymes target AHL-based QS, although enzymatic degradation of DSF, PQS and AI-2 has also been reported [96]. Several bacterial strains belonging to *Bacillus*, *Pseudomonas* and *Staphylococcus* are capable to rapidly degrade DSF. The exact mechanism by which this occurs is unknown, although inactivation by a *Pseudomonas* spp. strain G requires *carAB*. CarAB is a heterodimeric complex responsible for carbamoylphosphate synthesis, a precursor for arginine and pyrimidine [99]. Degradation of PQS is catalysed by 2,4-dioxygenase, Hod. This enzyme is naturally involved in the quinaldine utilization pathway in *Arthrobacter nitroguajacolicus* where it cleaves 3-

hydroxy-2-methyl-4(1H)-quinolone which is structurally similar to PQS [100]. Degradation of AI-2 is catalysed by LsrK. This is a cytoplasmic AI-2 kinase that phosphorylates AI-2 to the unstable phospho-AI-2 in order to initiate the Lsr-type transduction cascade. When LsrK is added to *E. coli* cultures, the extracellular phospho-AI-2 cannot be transported across the membrane and is quickly degraded. Although AI-2 signal transduction pathways are species-specific, LsrK-mediated degradation of AI-2 also inhibits QS responses in *V. harveyi* and *S.* Typhimurium cultures [101].

An alternative method to limit extracellular signal accumulation is the disruption of AI synthesis with QSI. AHL signal molecules are produced by LuxI family proteins that utilize the substrates SAM and acyl-ACP. Therefore, QSI can target SAM or acyl-ACP biosynthesis. SAM analogues (S-adenosylhomocysteine (SAH), S-adenosylcysteine and sinefungin) and the reaction product methylthioadenosine (MTA) were found to inhibit $C_4$-HSL production in *P. aeruginosa*, demonstrating the potential of inhibiting LuxI-type substrates by end products, reaction intermediates or substrate analogues [102].

Disruption of both AHL and AI-2 synthesis can be achieved by targeting MTAN. This enzyme is responsible for the recycling of SAM in AHL-synthesizing bacteria, while producing SRH which is a precursor of AI-2 [103]. Three analogues of the transition state structure of MTAN appeared the most potent *in vitro*: 5'-methyltio- (MT-), 5'-ethylthio- (ET-) and 5'-butylthio- (BuT) DADMe-ImmucillinA [104]. These analogues inhibit MTAN activity with $IC_{50}$ values at nM level and successfully inhibit biofilm formation and AI-2 production in *E. coli* and *V. cholerae* [104–106].

A third method to inhibit QS is to directly target the transcriptional regulator by using AI-analogues as antagonists. An example is meta-bromo-thiolactone (mBTL), a partial antagonist of both LasR and RhlR, two LuxR type regulators in *P. aeruginosa*, that successfully inhibits the production of pyocyanin and biofilm formation *in vitro* and *in vivo* [107]. In another example the LuxP-AI-2 binding was impaired by several diol-containing compounds, boronic acids and sulfones [98,108]. In Gram-positive organisms, the transcriptional regulators can be targeted by the RNAIII inhibiting peptide (RIP). RIP, RIP analogues and hamamelitannin (2',5-di-O-galloyl-d-hamamelose; HAM) interfere with the RAP/TRAP QS system [36], resulting in a decreased biofilm formation and increased susceptibility towards antibiotics [52,109].

At the end of the signal transduction cascade, QSI can interfere with binding of LuxR to DNA. This is the case for the halogenated furanones, also known as fimbrolides, such as the natural furanone compound (5Z)-4-bromo-5- (bromomethylene)3-butyryl-2(5H)-furanone [110]. These furanones disrupt AI-2 QS in *V. harveyi* by decreasing the DNA binding ability of LuxR to the promoter sequences of QS-regulated genes [111]. Furthermore, furanones also accelerate LuxR turnover in

*Vibrio fischeri* [112]. QSI activity of furonanes was also examined in *P. aeruginosa*. Natural furanones showed limited QS-inhibitory effect, but their synthetic counterparts “C-30” and “C-56” showed high QSI properties [113].

While intensively studied, the exact mechanisms by which the majority of QSI exert their effect is still unknown [114].

### 2.2.3 Quorum sensing interfering agents as antibiotic adjuvants

Agents that interfere with QS are often described as potential antibiotic adjuvants, however, the amount of research of those agents in combination with antibiotics is limited. In the following section an overview is presented of the studies in which the anti-biofilm activity of the combination of antibiotics and QS interfering agents was investigated (Table 1).

#### *2.2.3.1 Quorum sensing inhibitors as antibiotic adjuvants*

##### 2.2.3.1.1 In Gram-positive bacteria

The QS system of *S. aureus* is targeted by HAM, which is a non-peptide analogue of RIP that targets the TRAP receptor [36]. RIP is an interesting QSI, since it is able to inhibit RAP and thus inhibit *in vivo* biofilm formation and agr-regulated toxin production [109]. In addition, RIP has been shown to have synergistic effects in combination with multiple antibiotics on *S. aureus* biofilms [115,116]. HAM increases *S. aureus* biofilm susceptibility towards various classes of antibiotics by affecting cell wall synthesis and eDNA release. Antibiotics potentiated by HAM were vancomycin, daptomycin, linezolid, tobramycin and various cephalosporins. The antibiotic-potentiating activity of HAM on mature biofilms was characterized *in vitro* as well as *in vivo* models [117].

The prevention of *S. aureus* biofilm formation on medical devices was also described. Since RIP was reported as an interesting QSI, characterization of the structure activity relationship was performed to synthesize potent RIP analogues, such as FS3 and FS8. Both compounds were used to prevent staphylococcal biofilm formation in combination with antibiotics on prostheses in animal models [118,119]. Prevention of biofilm formation was evaluated by comparing the microbial load on FS3-coated grafts and non-coated grafts in rats treated with daptomycin. The FS3-coated grafts prevented biofilm formation compared to the non-coated grafts [119]. Similar results were obtained for grafts coated with FS8 in combination with tigecycline [118].

Fimbrolides were also described to inhibit *S. aureus* biofilms *in vivo*. Fimbrolides show structural similarities with AHLs, and AHLs downregulate the expression of *agr* in *S. aureus* [120]. Thus fimbrolides reduce pathogenicity caused by virulence factors regulated by the *agr* QS system. This

was investigated in *S. aureus* biofilms formed on mice corneas, by applying ciprofloxacin alone or in combination with non-toxic concentrations of fimbrolides. The addition of fimbrolides to the ciprofloxacin treatment reduced the bacterial cell count and improved the clinical outcome of *S. aureus* keratitis significantly [121].

#### 2.2.3.1.2 In Gram-negative bacteria

Most QSI research in Gram-negative bacteria has been conducted on *P. aeruginosa* biofilms. QSI can directly influence the QS signalling transduction cascade resulting in an increased biofilm susceptibility to antibiotics. For example, N-(2-pyrimidyl)butanamide (called C11) mimics the $C_4$-HSL signal and is able to significantly inhibit *P. aeruginosa* biofilm formation. C11 showed a synergistic anti-biofilm activity in combination with tobramycin, ciprofloxacin or colistin [122]. 14-α-lipoyl andrographolide (AL-1) has also QSI activities in *P. aeruginosa*, where it inhibits LasR-3-oxo-$C_{12}$-HSL interaction and represses the transcriptional level of QS-regulated genes. AL-1 potentiated biofilm susceptibility in combination with azithromycin, ciprofloxacin, fosfomycin, streptomycin, or gentamicin [123].

Patulin and ajoene are both naturally occurring QSI. Patulin is a secondary metabolite produced by members of the fungal genus *Penicillium* [124], and ajoene is the most potent compound of garlic extract [125]. In *P. aeruginosa* biofilms, QS regulates rhamnolipid production, which inhibits the function of host polymorphonuclear leukocytes (PMNLs) by blocking the oxidative burst. This results in biofilms that are resistant to the actions of PMNL [81]. So, by inhibiting QS-regulated rhamnolipid production, patulin and garlic extract promote the clearance of *P. aeruginosa* biofilms by PMNLs from a chronic pulmonary infection model in mice. Furthermore, patulin and garlic extract increase *P. aeruginosa* biofilm susceptibility towards tobramycin *in vitro* [124,126]. The synergistic antimicrobial effect of QSI in combination with tobramycin was also evaluated *in vivo*. An intraperitoneal foreign-body infection mouse model was used to evaluate the potentiating activity of furanone C-30, ajoene and horseradish extract. Silicone tubes, that were pre-colonized with *P. aeruginosa*, were inserted in mice. Mice that were treated with the combination of one of these QSI and tobramycin showed significantly more clearance of *P. aeruginosa* biofilms than mice treated with tobramycin alone [127]. The potentiating activity of C-30 on tobramycin treated *P. aeruginosa* biofilms was also demonstrated in pulmonary mice models [110].

*Table 1: **Examples of QS interfering agents as antibiotic adjuvants of published data**. The downstream effect is the effect of the combination treatment (QSI + antibiotic) compared to the antibiotic alone*

| QS interfering agent | Target QS system | Pathogen | Co-administered antibiotic | Mechanism of inhibition | Downstream effects | Refs |
|---|---|---|---|---|---|---|
| **HAM** | RAP/TRAP | *S. aureus* | Vancomycin, daptomycin, linezolid, tobramycin, various cephalosporines | Potentially inhibiting RAP | Alteration cell wall synthesis and eDNA release<br><br>Increased biofilm susceptibility | [117] |
| **FS3** | RAP/TRAP | *S. aureus* | Daptomycin | Potentially inhibiting RAP | Prevention of biofilm formation | [119] |
| **FS8** | RAP/TRAP | *S. aureus* | Tigecyclin | Potentially inhibiting RAP | Prevention of biofilm formation | [118] |
| **Fimbrolides** | *agr* | *S. aureus* | Ciprofloxacin | Downregulation *agr* expression | Increased biofilm susceptibility | [121] |
| **C11** | AHL-based | *P. aeruginosa* | Tobramycin, cirprofloxacin, colistin | Potentially inhibiting RhlR | Increased biofilm susceptibility | [122] |
| **AL-1** | AHL-based | *P. aeruginosa* | Azithromycin, ciprofloxacin, fosfomycin, streptomycin, gentamicin | Inhibits interaction LasR to signal molecule | Increased biofilm susceptibility | [123] |
| **Patulin** | AHL-based | *P. aeruginosa* | Tobramycin | Potential antagonist properties due to structural similarities AHLs | Increased biofilm susceptibility | [124] |
| **Ajoene** | AHL-based | *P. aeruginosa* | Tobramycin | Attenuation $C_4$-HSL and rhamnolipid production | Increased biofilm susceptibility | [125] |
| **C-30** | AHL-based | *P. aeruginosa* | Tobramycin | Antagonist properties due to structural similarities AHLs | Increased biofilm susceptibility | [110] |
| **BH** | AHL-based | *P. aeruginosa*<br>*B. cenocepacia* | Tobramycin | Downregulation QS-regulated genes | Increased biofilm susceptibility | [128,129] |
| **Compound 6** | PQS | *P. aeruginosa* | Ciprofloxacin | Dual inhibitor of PqsR and PqsD | Reduced production pyocyanin and eDNA<br>Increased biofilm susceptibility | [130] |
| **MomL** | AHL-based | *P. aeruginosa*<br>*A. baumanii* | Ciprofloxacin, tobramycin, meropenem, colistin | Degrading extracellular AHL molecules | Increased biofilm susceptibility *in vitro*, but not *in vivo* | [131] |
| **AiiA** | AHL-based | *P. aeruginosa* | Ciprofloxacin, gentamicin | Degrading extracellular AHL molecules | Increased biofilm susceptibility | [132] |
| **Lactonase** | AHL-based | *P. aeruginosa* | Ciprofloxacin | Degrading extracellular AHL molecules | Increased biofilm susceptibility | [133] |

Another natural QSI is baicalein, which is traditionally used in Chinese Traditional Medicine. It has synergistic effects with ampicillin on *P. aeruginosa*, resulting in an increased biofilm eradication [134]. Baicalin hydrate (BH), the glucuronide of baicalein and also an AHL-QS inhibitor, showed tobramycin-potentiating activity in *B. cenocepacia* biofilms *in vitro* as well as *in vivo* models [128]. This was also observed in *P. aeruginosa* biofilms, where BH showed a dose-dependent inhibitory effect on QS-regulated phenotypes as well as a reduced expression of QS core genes such as *lasIR*, *rhlIR*, *pqsR* and *pqsA*. Moreover, the addition of BH to antibiotic treatment resulted in an increased clearance of the infection in mice [129].

The PQS network in *P. aeruginosa* is also an appealing target for QSI. Thomann et al. developed a dual-inhibitor (compound 6) that targets PqsR, the PQS transcription regulator, and PqsD, a key enzyme in PQS biosynthesis. This compound reduced the production of pyocyanin and eDNA in biofilms. Co-administration of this compound with ciprofloxacin resulted in an increased biofilm susceptibility of *P. aeruginosa* PA14 *in vitro* as well as *in vivo* [130]

Also some antibiotics like azithromycin, ciprofloxacin and ceftazidime in *P. aeruginosa* have been described to have QSI activity at sub-MIC levels. These compounds supposedly decrease the expression of QS-regulated virulence factors by modulating membrane permeability, hereby influencing the flux of 3-oxo-$C_{12}$-HSL [135]. However, some caution with these compounds is necessary, as Köhler et al. described the emergence of more virulent mutants upon treatment with sub-MIC levels of azithromycin [136].

The exact mode of action of QSI is not always clear. Therefore, it is important to distinguish between “true” QSI and compounds that affect virulence in other ways. QSI are typically small molecules that directly influence QS by interfering with AI production, detection and/or response, which ultimately leads to a differential expression of genes encoding QS-regulated virulence factors. However, virulence can be regulated by many factors independent and up- or downstream of QS [137]. So small molecules that regulate a virulent phenotype, although the phenotype is associated with QS, are not necessarily QSI [138]. Some recent reports showed that alleged QSI do not actually target QS, and are therefore now classified as antivirulence agents [114,139].

#### *2.2.3.2 Quorum quenching enzymes as antibiotic adjuvants*

The amount of research of QQ enzymes as antibiotic adjuvants is relatively limited. To our knowledge, only three different lactonases have been investigated as antibiotic adjuvants. MomL is an AHL lactonase belonging to the metallo-β-lactamase superfamily. It degrades AHL molecules produced by multiple Gram-negative pathogens [140]. The effect of MomL on biofilm susceptibility to several antibiotics was tested *in vitro* and *in vivo* on *P. aeruginosa* and *Acinetobacter baumanii* biofilms. MomL increased antibiotic-susceptibility in *in vitro* models, however failed to have an impact on biofilm susceptibility *in vivo* [131]. A second study used AiiA lactonase from *Bacillus thuringiensis 4A3* to investigate its synergistic effect with ciprofloxacin or gentamicin on *P. aeruginosa* biofilms. The addition of the lactonase to the antibiotic treatment caused an increased biofilm susceptibility [132]. In a third study, a lactonase, obtained from Bacillus sp ZA12, was tested in combination with ciprofloxacin on *P. aeruginosa* murine burn wound infections. The addition of the purified lactonase resulted in an improved efficacy of ciprofloxacin [133].

Some caution with QQ enzymes is also necessary, since these agents can have multiple functions. Kusada et al. isolated a novel AHL acylase, designated MacQ, that degrades a wide variety of AHLs. However, this enzyme appeared to be bifunctional and is also capable of degrading a wide variety of β-lactam antibiotics by deacylation. This overlooked resistance mechanism mediated by the QQ enzyme can exist in the environment and its resistance genes can be transferred to pathogenic bacteria [141].

## 2.3 Can resistance emerge towards quorum sensing inhibitors?

### 2.3.1 Are quorum sensing inhibitors evolutionarily robust?

It is generally believed that QSI exert little or no selective pressure on bacteria, since they do not target pathways that are essential for bacterial growth. However, this has not yet been investigated in a systematic way.

Since resistance can develop against all compounds that cause stress, development of resistance against QSI seems inevitable [4]. Generally, natural selection for a certain trait can only occur if there is a heritable variation that is associated with a difference in fitness. So, the risk of resistance to QSI increases when there are variations in QS genes that favour insensitivity towards QS inhibition. These heritable changes can be promoted by the natural variation among QS genes. First, QS genes and their expression vary among strains of the same species. Variation in signal molecule levels has been reported and can be important for QSI that competitively bind to the receptor protein [142]. Variation also occurs at receptor level, where overexpression of signal molecule receptor genes can neutralize QS disruption [143]. This variation among QS genes may induce fitness differences when treated with QSI, which may increase the risk of resistance [142]. Moreover, small changes (point mutations) in *luxR*-like genes can modify the ligand-binding site without influencing sensitivity towards the natural signal. These modifications can render the receptor insensitive towards the QSI, or turn the QS inhibitor into an activator of QS-regulated genes [144].

Maeda et al. [145] investigated the development of resistance towards QSI at the molecular level. The effect of a QSI was evaluated when *P. aeruginosa* was cultivated under QS-requiring growth conditions (i.e. adenosine as sole carbon source). The addition of the QSI (a brominated furanone C-30) impaired growth of *P. aeruginosa*, resulting in selective pressure. Indeed, the presence of C-30 led to the occurrence of mutations in repressor genes of the multidrug resistance efflux pump MexAB-OrpM, resulting in an increased resistance towards C-30 [145]. Noteworthy, mutations in the same genes were found in clinical isolates of CF patients never treated with C-30; also these mutants showed resistance towards C-30 [145,146]. Maeda et al. propose that any strong selective pressure, not only growth with adenosine as sole carbon source but also antibiotic treatments, could induce resistance to QSI [145,147].

In light of this evidence, a second question became relevant: how fast will mutations spread throughout the population? Gerdt and Blackwell [148] suggested that the spread of resistance would be limited, since QSI resistant mutants would be less fit than QSI sensitive strains. Upon treatment with a QSI, the small fraction of QSI resistant mutants would not be able to produce enough AIs to reach the threshold for activating the QS system. Furthermore, signal-independent

resistant cells would be less fit than QSI sensitive ones when growth was dependent on group-behaviour [148].

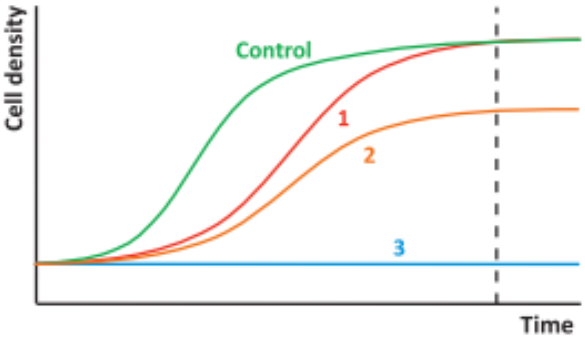
The rate of resistance development depends to a large extent on the culture conditions. When there is no selective pressure (e.g. no antibiotics or an active QS system is not required for bacterial growth) then development of resistance occurs at a slow rate. This was also confirmed by Sully et al. in Gram-positive organisms [149]. However, when selective pressure is present it is unlikely that resistance only develops at a slow rate [145]. To accurately evaluate the risk of resistance development, a correct assessment of the effect of QSI on bacterial fitness is required. This should be measured in clinically relevant conditions [142]. Therefore, it might be interesting to include antibiotics (or any other selective pressure) in the growth medium when putative QSI are assessed as antibiotic.

### 2.3.2 Natural rise of social cheaters

QS is a social system in which an entire bacterial population releases signalling molecules and bioactive molecules in the environment. These bioactive molecules (e.g. proteases for which the production is QS-regulated) are available to all cells of the population, and can easily be exploited by cheaters. These are individuals that avoid the cost of producing the bioactive molecules but still harvest the benefits [150,151]. Cheaters can be either “signal negative”, like synthase mutants, or “signal blind”, like receptor mutants [152]. There is a clear distinction in cheating when QS regulates private goods or public goods. An example of a private good is the production of the cellular enzyme required for growth on adenosine in *P. aeruginosa* [51]. In rich media, “private good cheaters” grow to a higher cell density than wild type cells, while their fitness advantage disappears completely in QS-requiring growth conditions [150]. An example of public goods is the QS-regulated production of extracellular proteases. Social cheaters of public goods can emerge quickly in a population cultivated under QS-requiring growth conditions. Sandoz et al. [151] performed an evolution experiment on *P. aeruginosa* cells and detected the emergence of a *lasR* mutant subpopulation after ca. 100 generations [151]. Despite the immediate growth advantage of cheaters over the wild type, they will be quickly eliminated in natural populations. This is due to the negative effect of cheaters on the average fitness. The organization of the community also contributes to elimination of cheaters. For example, the spatial structure of biofilms allows the co-operators to keep close together and restrict the invasion of cheaters [51,150,153].

### 2.3.3 Other physiological effects of quorum sensing inhibitors

Currently, there are three approaches to assess the toxicity of putative QSI. These are here described in order of sensitivity. In a first approach, the MIC in bacterial cells can be determined to confirm that QSI operate on sub-lethal levels. Secondly, cell density can be measured after a certain cultivation time in the presence and absence of a QSI, i.e. end-point measurement. Lastly, growth kinetics can be assessed by evaluating the growth curve of bacteria in the presence or absence of a QSI. The last approach is the most sensitive one, but can still miss significant toxic effects (Figure 8) [154]. For example, pyrogallol did not influence growth of *V. harveyi* in a complex medium at concentrations that inhibit QS-regulated bioluminescence [108]. However, the influence on bioluminescence was assessed under nutrient-poor conditions, in which pyrogallol killed *V. harveyi* due to its toxicity (i.e. peroxide production) [155]. This example highlights the importance of the full understanding of a putative QSI, in order to eliminate "false QSI" due to their hidden toxicity.




| Method | Inhibition detected for |
|---|---|
| Kinetics | 1, 2 and 3 |
| End point | 2 and 3 |
| MIC | 3 |

*Figure 8:* ***Power of different growth inhibition experiments****. The graph shows hypothetical growth curves of a strain without and with three putative QSI agents. The table shows the methods which are able to detect a growth inhibitory effect of the compounds* [154].

A full understanding of the mechanism of a putative QSI can aid to predict the emergence of resistance mechanisms caused by other physiological roles than QS inhibition. For example, furanone C-30 has a variety of QS inhibiting effects. Over time, it evokes resistance in *P. aeruginosa* cultivated in QS-requiring conditions [145]. Hentzer et al. [110] performed transcriptomic analysis on planktonically grown *P. aeruginosa* treated with C-30 and discovered that 85 genes were repressed and 8 genes were activated in response to C-30. Interestingly, expression of the AHL-based QS core genes (*lasIR* and *rhlIR*) was not affected by the addition of C-30, but expression of genes involved in the PQS biosynthesis was downregulated. Furthermore, C-30 activated *mexR* (i.e. gene encoding a multidrug resistance operon repressor), oxidoreductases, ABC transporters and MFS transporters [110].

In conclusion, QS inhibition is a promising strategy to slow the emerging bacterial resistance towards conventional antibiotics. However, there is a need for reliable identification of these QSI agents that is not biased by culture conditions, toxicity and/or other physiological effects.

# 3 *Burkholderia cenocepacia*

## 3.1 The *Burkholderia cepacia* complex

The *Burkholderia cepacia* complex (Bcc) is a group of at least 23 Gram-negative genetically distinct but phenotypically similar species [156,157] (Table 2). They were first identified by W. H. Burkholder in 1950 as the causative agent of soft onion rot, and were designated as *Pseudomonas cepacia* [158]. In 1992 they were transferred to the new genus *Burkholderia* [159]. Further taxonomic research revealed that *B. cepacia* was a group of genetically distinct species. This led to a first subdivision in five genomovars (I to V) [160]. Over the following 20 years, numerous new Bcc species have been identified [161–163].

*Table 2: An overview of all currently known Bcc species [157,163–169]*

| Species | Genomovar | Natural habitat | Clinical habitat |
|---|---|---|---|
| ***B. ambifaria*** | VII | Soil, rhizosphere | CF, non-CF |
| ***B. anthina*** | VIII | Soil, rhizosphere, water, plant | CF, hospital materials |
| ***B. arboris*** | | Soil, rhizosphere, water | CF, non-CF, industrial equipment |
| ***B. catarensis*** | | Soil | / |
| ***B. cenocepacia*** | III | Soil, rhizosphere, water, plant, animal | CF, non-CF, industrial equipment |
| ***B. cepacia*** | I | Soil, rhizosphere, water, plant | CF, non-CF, medical solutions |
| ***B. contaminans*** | | Sheep, plant | CF, non-CF, hospital materials |
| ***B. diffusa*** | | Soil, water | CF, non-CF, hospital materials |
| ***B. dolosa*** | VI | Maize, rhizosphere | CF |
| ***B. latens*** | | / | CF |
| ***B. lata*** | | Soil, rhizosphere, water, flower | CF, non-CF, industrial equipment |
| ***B. metallica*** | | / | CF |
| ***B. multivorans*** | II | Soil, rhizosphere, water, plant | CF, non-CF, CGD |

| | | | |
|---|---|---|---|
| ***B. paludis*** | | Soil | / |
| ***B. puraquae*** | | Soil | Hospital materials |
| ***B. pyrrocinia*** | IX | Soil, rhizosphere, water, plant | CF, non-CF |
| ***B. pseudomultivorans*** | | Rhizosphere | CF, non-CF |
| ***B. seminalis*** | | Soil, rhizosphere, rice | CF, non-CF, nosocomial infection |
| ***B. stabilis*** | IV | Plant, rhizosphere | CF, non-CF, hospital materials |
| ***B. stagnalis*** | | Soil | CF, non-CF |
| ***B. territorii*** | | Water | / |
| ***B. ubonensis*** | | Soil | Non-CF, nosocomial infection |
| ***B. vietnamiensis*** | V | Soil, rhizosphere, water, plant, animal | CF, non-CF, industrial equipment |

Bcc species are versatile species. They are able to protect commercially useful crops against fungal diseases. They can also utilize a wide variety of complex carbon sources which gives them the capacity to detoxify pollutants in pesticides and herbicides [161]. At the same time, Bcc species are opportunistic pathogens that can cause severe lung infections in immunocompromised people, like cystic fibrosis (CF) patients [170].

CF is the most common autosomal recessive disease in the Caucasian population. It is a multi-organ disease that arises from a mutation in the CF transmembrane conductance regulator (CFTR) gene, resulting in impaired chloride channels in epithelial cell membranes. In the lungs, this chloride channel contributes to mucocilliary clearance as it helps to control the volume of airway surface liquid. A defective CFTR results in thickened and viscous mucus, that cannot easily be cleared from the lungs. The subsequent accumulation of mucus in the respiratory tract creates an ideal environment for pathogens to thrive, causing respiratory infections [170–172]. Bcc infections are predominantly acquired from the environment, but can also be acquired nosocomially or by patient-to-patient transmission [161]. Only a small portion of CF patients is infected with Bcc (approximately 5%), but these lung infections are often associated with a progressive decline in lung function. Approximately 90% of all Bcc infections in CF are caused by *B. cenocepacia* and *B. multivorans* [27,170,173–175]. Moreover infections with *B. cenocepacia* are associated with the

development of cepacia syndrome, a progressive pneumonic illness with reduced survival and high risk to the development of fatal bacteraemia [176].

## 3.2 Epidemiology and genomics

Bcc infection outbreaks during the 1980s and 1990s in the United Kingdom (UK) and in the United States of America (USA) were mainly caused by transmissible *B. cenocepacia* strains belonging to the ET12, PHDC or Midwest clones [161]. The highly epidemic transmissible ET12 clone (electrophoretic type 12) infected CF patients in Canada, the UK and Europe [161,177]. Isolates belonging to the ET12 clone, like *B. cenocepacia* J2315, were characterized by the presence of the *cblA* gene, encoding the cable pilus, a 22kDa adhesin and the *Burkholderia cepacia* epidemic strain marker (BCESM) [161]. Isolates from the PHDC and Midwest lineages were predominantly found in North America [176]. However, some epidemic lineages are associated genetic markers, others are not. Therefore, genotyping studies are still required for a full understanding of epidemiology within the Bcc complex [178]. Measures for infection prevention and control led to a reduction in *B. cenocepacia* prevalence [179].

*B. cenocepacia* J2315 has a large genome (8.06Mb) with a high GC content (66.9%) and harbours three chromosomes and a plasmid [27]. Comparison between J2315 and other *B. cenocepacia* strains shows that approximately 21% of the genome is acquired through HGT, which contributes to the diversity of the genome [27]. Nearly 10% of the genome is made up by genomic islands. These are of key importance in microbial genome evolution; as they provide a single step acquisition of genes related to complex adaptive functions such as antibiotic resistance, fitness and virulence [180,181]. The most studied genomic island in *B. cenocepacia* is the genomic island 11 (BcenGI11), formerly designated as *c*enoc*e*pacia *i*sland (*cci*) [27,176]. BcenGI11 contains genes involved in both metabolism and in virulence [27,182].

## 3.3 Social behaviour in *B. cenocepacia*

### 3.3.1 Quorum sensing

*B. cenocepacia* comprises multiple QS networks. The CepIR system is widespread among Bcc bacteria [183,184], and was first identified in *B. cenocepacia* [185]. The AHL synthase, CepI, produces N-octanoyl-HSL ($C_8$-HSL) and in minor quantities N-hexanoyl-HSL ($C_6$-HSL) [183,186]. When the concentration threshold of AHLs is reached, AHLs bind specifically to the transcriptional regulator CepR. This interaction causes a conformational change in CepR and results in the induction or repression of target genes. Also, CepR tightly controls the expression of *cepI*. This positive feedback loop ensures a rapid increase in target gene expression once the system is triggered. CepR also negatively regulates its own expression, i.e. autoregulation [183,186]. A second AHL-based network is CciIR, which is only present in *B. cenocepacia* strains because *cciIR* genes are located on the BcenGI11 genomic island [176,187]. The AHL synthase CciI synthesizes $C_6$-HSL and in minor amounts $C_8$-HSL. Likewise as CepR, CciR also negatively regulates its own expression [187].

The CciIR and CepIR systems interact with each other (Figure 9). CciR negatively regulates the expression of *cepI*. CepR positively regulates the expression of the *cciIR* operon. Furthermore, high quantities of $C_6$-HSL inhibits CepR activity. The two systems exert a reciprocal regulation of many genes. While CepR is generally responsible for the positive regulation of gene expression, CciR mainly affects gene regulation negatively [29,187]. The CepIR and CciIR systems regulate expression of genes involved in many virulence traits such as motility, biofilm formation, secretion and antimicrobial drug resistance [29,188].

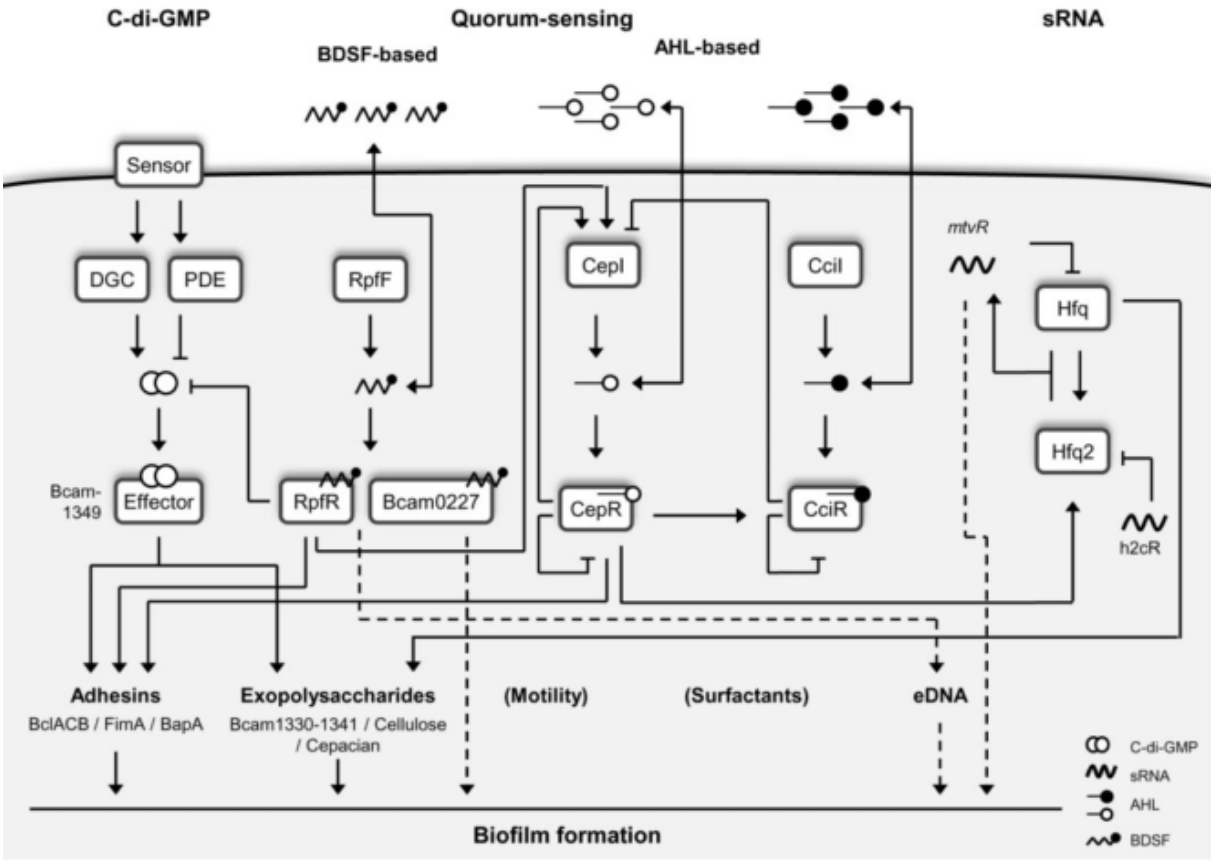



*Figure 9:* ***Schematic overview of QS in B. cenocepacia and their regulation of biofilm formation.*** *The punctuated lines are suggestive regulatory networks that lack detailed understanding of the target compounds involved in biofilm formation* [189]

Another signal molecule identified in *B. cenocepacia* J2315 is cis-2-dodecenoic acid, also known as the BDSF [42]. This signal molecule is used by the RpfFR QS system, which is highly conserved in Bcc bacteria [41]. BDSF is synthesized by RpfF, a bifunctional crotonase, and sensed by the receptor protein RpfR, that contains PAS-GGDEF-EAL domains [41,43]. Binding of BDSF to RpfR leads to the induction of the c-di-GMP phosphodiesterase activity of the protein, hence lowering the intracellular c-di-GMP levels [40,41]. This QS system affects cell motility, biofilm formation, proteolytic activity, and virulence [43,54]. The BDSF-based and AHL-based system interact with each other in *B. cenocepacia* J2315, where both systems co-regulate the transcription of several virulence genes. Moreover, BDSF can regulate AHL production via CepI [190]. However, the exact interaction between both systems appears to be strain-dependent since there are profound differences in QS regulation among *B. cenocepacia* strains [29,31,191,192].

Additional regulators also affect the *B. cenocepacia* QS network. CepR2 is an orphan LuxR homologue, so it does not require an AHL for proper folding. CepR2 negatively regulates its own expression, but does not influence expression of *cciIR* or *cepIR* [31]. Another regulator is the LysR-

type transcriptional regulator ShvR (Shiny colony variants Regulator), that regulates the expression of *cepIR* and *cciIR* negatively, while positively affecting biofilm formation [193,194]. Also the global regulator AtsR negatively regulates the AHL-based QS systems and negatively affects biofilm formation [195]. Several other regulators (CepS, SuhB, YciL, BCAM1871, BCAM0227) have been shown to influence QS regulation [182,196–198].

### 3.3.2 Biofilm formation

Like many other bacteria *B. cenocepacia* is able to form biofilms. These are complex multicellular communities that protect bacteria against antimicrobials [161,199] by reduced drug penetration, reduced growth rate, and the presence of persister cells (detailed explanation in Chapter I.4.2).

Many factors are involved in the establishment of a mature biofilm. The initial adhesion by surfaces structures, including fimbriae, pili and flagella is regulated by RpoN, a regulator of flagellar motility [49]. BapA, a large surface protein, also plays a role in biofilm formation. Lectins, encoded by *bclACB*, are also involved, especially when *bapA* expression is reduced. Moreover, regulatory factors such as QS, ShvR and AtsR, are also involved in biofilm formation [54,189].

Exopolysaccharides (EPS) are an important part of the biofilm matrix. EPS are branched, repeating polysaccharide units that are excreted by the bacteria. Five different EPS have been identified in Bcc, with cepacian as the most abundant one [49]. The subunits of cepacian are acetylated, which can contribute to their virulence by providing protection against ROS and preventing polymer cleavage due to steric hindrance [200]. Also, as other EPS, cepacian interferes with phagocytosis by PMNLs and scavenges ROS [201]. The type of EPS produced is strain-specific. Strains lacking the production of cepacian are not necessarily less virulent e.g. *B. cenocepacia* J2315 is not able to produce cepacian due to a frameshift mutation in *bceB* [49]. The regulation for the biosynthesis of EPS remains to be completely elucidated. However, Fazli et al. have provided evidence that the regulation occurs via high intracellular c-di-GMP levels that induce the production of EPS via the transcriptional regulator BCAM1349 [202,203].

Although QS is not necessarily required for biofilm formation, many genes involved in biofilm formation are QS-regulated [52].

## 3.4 Oxidative stress protection

Oxidative stress protection is inevitably linked with social behaviour in *B. cenocepacia*, since there is a high ROS generation in biofilms and the oxidative stress response is partially QS regulated.

### 3.4.1 Oxidative stress

In a biological context, ROS are typically formed as a by-product of aerobic respiration. ROS can be formed endogenously by the reaction of oxygen ($O_2$) with univalent electron donors, such as metal centres, dihydroflavin cofactors ($FADH_2$) and quinones [204]. The produced ROS are superoxide ($O_2^{\cdot-}$), hydrogen peroxide ($H_2O_2$) and hydroxyl radicals ($OH^{\cdot}$) (Figure 10). All three are cytotoxic, however the effects of hydroxyl radicals are more severe since these cannot be enzymatically degraded. Superoxide and hydrogen peroxide can be enzymatically degraded by superoxide dismutase (SOD) and catalases, respectively [205]. Enzymatic or spontaneous dismutation of superoxide radicals leads to the formation of peroxides. Those can either be detoxified by catalases to water and oxygen or they react with ferrous iron producing highly toxic hydroxyl radicals in the Fenton reaction [205]. Those hydroxyl radicals can directly damage macromolecules such as DNA, lipids and proteins [206] or indirectly damage DNA by oxidizing the deoxynucleotide pool [207]. The available amount of ferrous iron for the Fenton reaction depends on the Haber-Weiss reaction, in which superoxide radicals reduce ferric iron to ferrous iron [205].

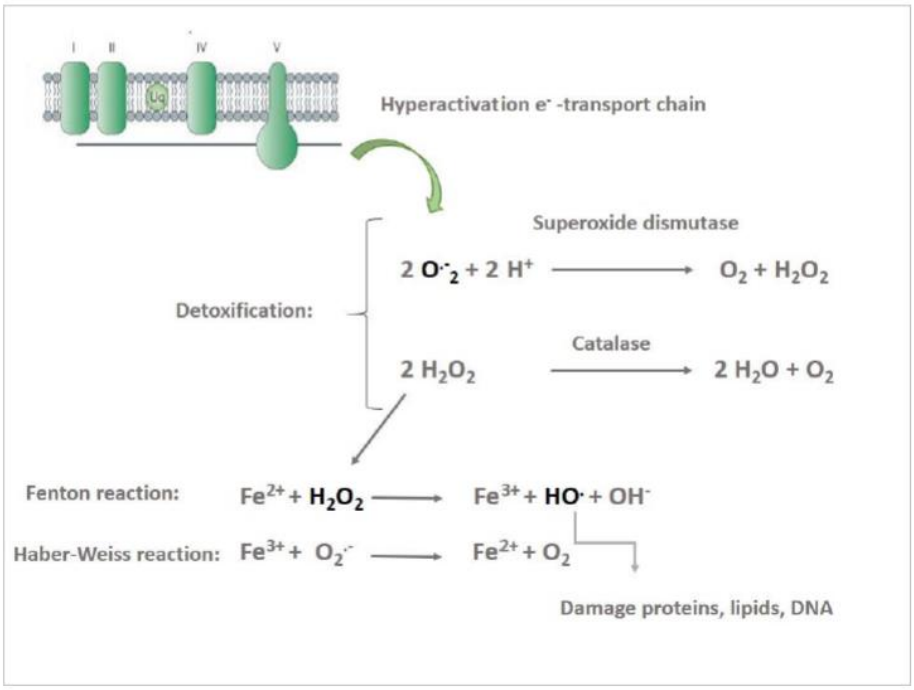



*Figure 10:* ***Generation of ROS*** [208]

*B. cenocepacia* can protect itself towards oxidative stress at several occasions. First, *B. cenocepacia* can survive in the CF lung. ROS are highly abundant in the CF sputum due to the chronic lung inflammation, and due to bacterial respiration and metabolism [170]. *B. cenocepacia* has also the ability to survive within macrophages, that usually destroy trapped pathogens with an oxidative burst [27]. Secondly, *B. cenocepacia* is an MDR pathogen that can survive high concentrations of bactericidal antibiotics [209]. A common mechanism of bactericidal antibiotics involves the production of ROS to induce cellular death. This was first described in *E. coli* by the group of Collins [71,210] and later confirmed by Van Acker et al. [211] for *B. cenocepacia*. The primary drug-target interactions are thought to stimulate the oxidation of nicotinamide adenine dinucleotide (NADH) through the electron transport chain (ETC), which depends on the tricarboxylic acid cycle (TCA). This causes the formation of superoxide radicals that can damage iron-sulphur clusters. The released ferrous iron can participate in the Fenton reaction and lead to generation of hydroxyl radicals [71,212]. The fact that *B. cenocepacia* can survive high doses of ROS, suggests that this pathogen has multiple protective mechanisms involved in scavenging and neutralizing ROS.

### 3.4.2 Oxidative stress response

The oxidative stress response in *B. cenocepacia* includes the production of oxidoreductases, cytochrome b, hydroperoxide resistance protein, alkyl hydroperoxide reductases, SODs and catalases [176].

Antioxidant enzymes in *B. cenocepacia* include SODs, catalases, catalase-peroxidases and alkyl-hydroperoxidase [213]. SODs detoxify superoxide to hydrogen peroxide which is then converted to water and oxygen by catalases. In *B. cenocepacia*, there are two SODs; cytoplasmic SodB and periplasmic SodC [170,214]. Two different catalase/peroxidase systems were identified; KatA and KatB. However, these two enzymes play a different functional role. KatB is the major catalase/peroxidase system that has a global role in the cellular protection against oxidative stress. KatA on the other hand, has negligible catalase activity but rather aids in maintaining the normal activity of the TCA cycle [215].

Besides the antioxidant enzymes, there are also other compounds involved in ROS detoxification such as thioredoxin, peroxiredoxin and gluthatione peroxidase. Thioredoxins are capable of reducing oxidized proteins and scavenging hydroxyl radicals [216]. Peroxiredoxins use their redox-active cysteine residues to reduce their substrates [217]. Glutathione peroxidases reduce peroxides while

converting glutathione to oxidized glutathione. The latter is then reduced by glutathione reductase and NADPH as a cofactor [218].

Another compound involved in the ROS detoxification is the brown pigment produced by *B. cenocepacia*. This is a pyomelanin with antioxidant properties. It is synthesized by the enzyme 4-hydroxyphenylpyruvic acid dioxygenase (HppD) from a homogentisate (HGA) precursor. Disruption in the gene encoding HppD results in a non-pigmented strain that is more sensitive to oxidative stress [219].

# CHAPTER II
# OBJECTIVES

The World Health Organisation (WHO) has recognized antimicrobial resistance as one of the most important health threats of our century [1]. The emergence of resistant bacteria drastically reduces the efficacy of available antibiotics and as only few novel antibiotics are in the pipeline, alternative approaches are needed [88]. An alternative approach is the use of antibiotic adjuvants (also known as potentiators), i.e. non-lethal compounds that enhance antibiotic activity. This approach has advantages such as the decrease in the required dose of an antibiotic and the ability to avoid bacterial resistance mechanisms [6,88]. An adjuvant approach that targets quorum sensing (i.e. the communication system between bacteria; QS), is potentially interesting as QS is involved in the regulation of many virulence factors including biofilm formation [21]. Biofilms are consortia of bacteria attached to an interface that are less susceptible towards conventional antibiotics than their planktonic counterparts. This adjuvant approach can be especially useful to treat multidrug resistant pathogens such as members of the *Burkholderia cepacia* complex (Bcc), including *Burkholderia cenocepacia* [27]. The main objective of this dissertation is to evaluate the activity and mode of action of known QS inhibitors and their analogues, and to investigate whether antibiotic adjuvants induce resistance over time.

First, the QS inhibitory activity of several transition state analogues for MTAN will be tested in *Vibrio harveyi* strains. To this end, *V. harveyi* test strains (i.e. producing signal molecules, without sensing them) and *V. harveyi* sensor strains (i.e. sensing signal molecules without producing them) will be used to measure the QS inhibitory activity of the MTAN transition state analogues.

The second goal is based on the observation that baicalin hydrate (BH) is a QSI and potentiates tobramycin susceptibility in *B. cenocepacia* [128]. However, the broader applicability of this QSI and its exact mode of action are still unknown. First, we will evaluate the potentiating effect of BH and various structural analogues of BH. This will be carried out with different antibiotics and multiple Bcc strains. Subsequently, the mechanism by which BH exerts its tobramycin-potentiating activity in *B. cenocepacia* biofilms will be determined using RNA sequencing. Putative mechanisms will be evaluated by performing relevant phenotypic assays.

Finally, we will investigate the possibility of antibiotic adjuvants to induce resistance in tobramycin-treated *B. cenocepacia* biofilms. The adjuvants that will be included in these experiments are BH (a QSI), and econazole and miconazole (repurposed antimycotics), which potentiate the activity of tobramycin towards *B. cenocepacia* J2315 *in vitro* [86,128]. To evaluate the development of resistance, an evolution experiment will be set up. Afterwards whole genome sequencing will be performed to obtain a clear picture of the evolutionary trajectories that could lead to development of resistance.

# CHAPTER III
# EXPERIMENTAL WORK

# Paper 1: Azanucleosides as 5'-methylthioadenosine / S-adenosylhomocysteine nucleosidase inhibitors in *Vibrio harveyi*

Lisa Slachmuylders and Tom Coenye

## Abstract

A collection of azanucleosides were screened for their ability to inhibit 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase (MTAN). MTAN is responsible for the synthesis of signalling molecules like autoinducer-1 and -2. So it is a crucial enzyme in the communication system of bacteria, also known as quorum sensing (QS). The screening was conducted with *Vibrio harveyi* mutant strains, impaired in QS. No QS inhibiting activity was observed for any of the azanucleosides tested. Possible explanations are insufficient uptake into the cell and/or a lack of effect on MTAN.

## Introduction

Quorum sensing is the process of cell-density based communication between bacteria, which promotes group-behaviour when a certain population threshold is reached. This group behaviour includes the promotion of virulence factor production, biofilm formation and bioluminescence. Bacterial communication is established by the production, release, detection and ultimately the response to signal molecules, called autoinducers [95]. Three types of quorum sensing systems are described in *V. harveyi* [26,220] (Figure 1). The first system produces acylhomoserine lactones (AHLs) as autoinducer via the LuxM/N QS system [21]. The CqsS/A system uses the cholera autoinducer-1 (CAI-1) signalling molecule [44]. The third QS system uses autoinducer-2 (AI-2), which is a mixture of interconvertible molecules, via the LuxS/PQ system. AI-2 is shared by many Gram-positive and Gram-negative bacteria, and is therefore considered to be a universal QS signal [221]. Although the AHL and AI-2 signalling molecules are widespread, the signal transduction pathway found in *V. harveyi* (multichannel two-component phosphorelay signal transduction pathway [Figure 1]) is lacking in most other organisms [34].

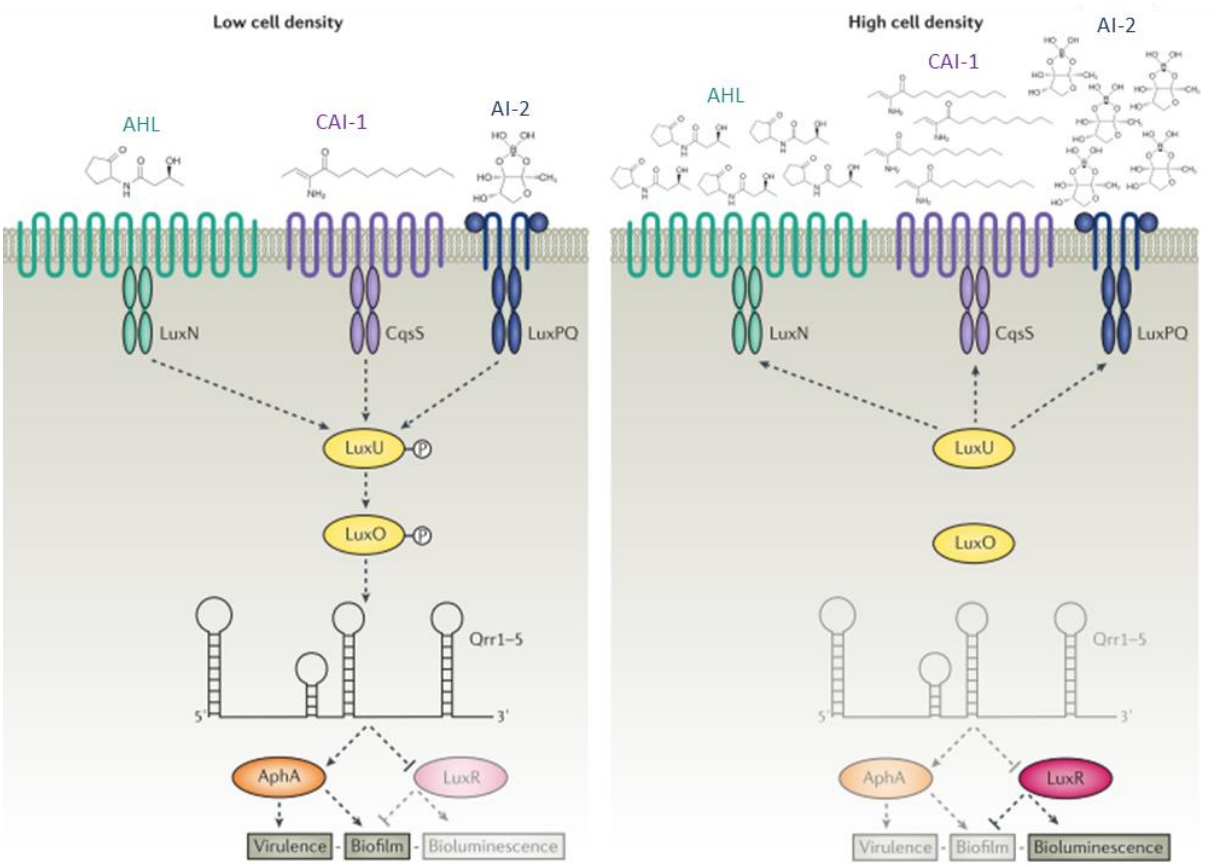



*Figure 1:* ***Quorum sensing systems in V. harveyi strains****. Three sensory systems converge to control levels of the master regulator, LuxR. At low cell density (left), the receptors autophosphorylate and pass phosphate to the phosphorelay protein LuxU, which shuttles phosphate to the response regulator LuxO. Phosphorylated LuxO activates transcription of genes encoding five small quorum regulatory RNAs (qrr). These activate translation of mRNA encoding AhpA, a low cell-density master regulator, and they repress the transcription of the master quorum sensing regulator LuxR. At high cell densities (right), autophosphorylation is inhibited by autoinducer binding, which encourages the phosphatase activities of the receptors. Expression of qrr genes is terminated due to an inactive dephosphorylated LuxO, which results in the production of LuxR* [26,34].

An important enzyme in autoinducer biosynthesis is 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase (MTAN). This is a dual-substrate bacterial enzyme that is a key player in the activated methyl cycle, where it recycles adenine and methionine through S-adenosylmethionine (SAM)-mediated reactions, producing AI-2. SAM is also essential in the biosynthesis of polyamines, AHL, and other biomolecules [106] (Figure 2). In most species, MTAN is not essential for bacterial growth but is involved in QS [104]. The inhibition of this enzyme limits biosynthesis of both autoinducers, hence causing a disruption in QS [104,106].

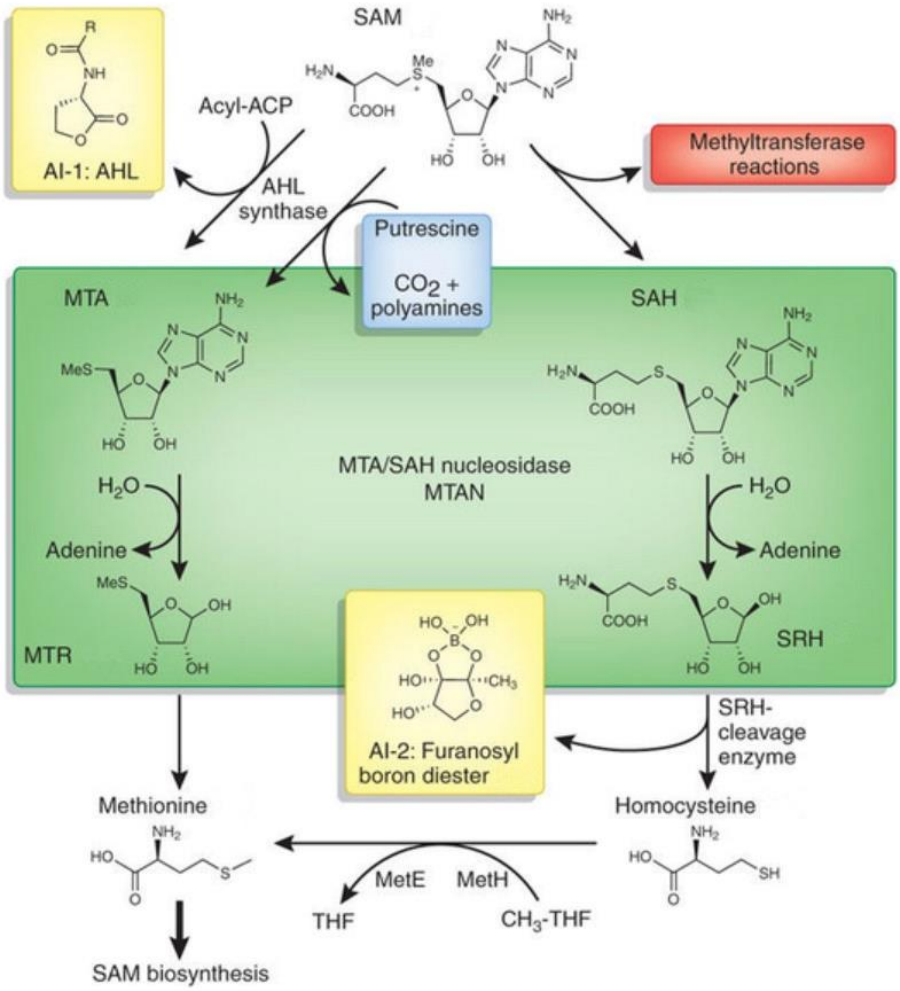



*Figure 2:* ***Role of MTAN in the biosynthesis of AI-2 and AHL****. MTAN catalyses the hydrolytic deadenylation of its substrates to form adenine and 5-methylthioribose (MTR) or S-ribosylhomocysteine (SRH). SRH is a precursor of tetrahydrofurans (AI-2 molecules). AHL molecules are synthesized from SAM by AHL synthase* [104]*. Methylthioadenosine (MTA) is a product of the AHL synthase reaction and is known to inhibit AHL synthase activity* [106]*. This schematic overview is adapted from Gutierrez et al.* [104].

Schramm et al. described that transition state analogues are powerful enzymatic inhibitors. These analogues resemble to the high-energy intermediate state of the reactants formed at the enzymatic catalytic site. So, these analogues can bind very strongly to the active site of the enzyme, without undergoing the reaction [222]. Studies utilizing intrinsic kinetic isotope effects (KIEs) and crystal structures of MTANs in complex with transition state analogues have allowed to model transition state of MTANs from *Escherichia coli*, *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Staphylococcus aureus*, and *Vibrio cholerae* and subsequently the design of analogue inhibitors [104,223–225]. Transition state analogues have been developed with high affinity ($IC_{50}$ in nM range) to disrupt bacterial functions like QS [104,226]. Immucilin A and immucilin analogues (Figure 3) were found to be extremely potent MTAN inhibitors *in vitro*. The immucilins belong to the azanucleosides, a class of sugar-modified nucleoside analogues in which the 4'-oxygen is replaced by

a nitrogen atom [227]. The methylthio (MT)-DADMe-Immucilin analogue was the most potent competitive inhibitors for MTAN in both *E. coli* and *V. cholerae*, and their activity was confirmed on the purified enzyme as well as in biofilms [104].

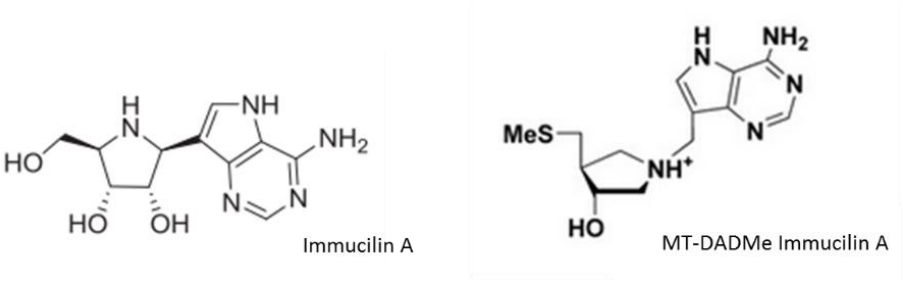



*Figure 3:* ***Structures of immucilin A and its MT-DADMe analogue***

The goal of this study was to evaluate the inhibitory activity of azanucleosides synthesized by J. Bouton in the Laboratory of Medicinal Chemistry (prof. S. Van Calenbergh).

## Materials and methods

### Bacterial strains and growth conditions

The strains used in this study are shown in Table 1. Strains were stored in Microbanks vials (Prolab Diagnostics, Richmond Hill, ON, Canada) at - 80°C, and subcultured aerobically on trypton soy agar (TSA; Lab M, Lancashire, UK) with 2 % sodium chloride (NaCl) (Applichem, Darmstadt, Germany) at 30°C. Liquid cultures were prepared in marine broth (MB) (BD, Sparks, MD, USA).

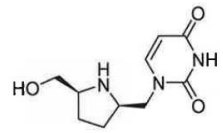
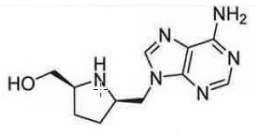
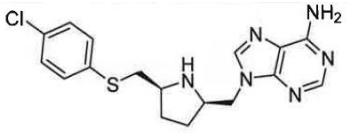
*Table 1:* ***Strains used in this study***

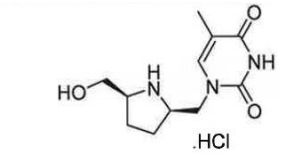
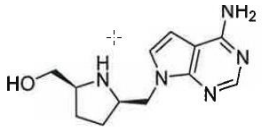
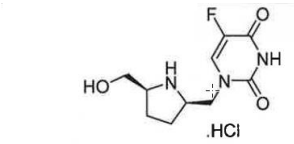
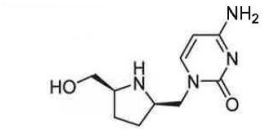
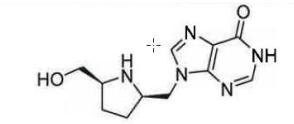
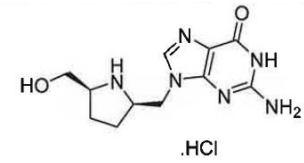
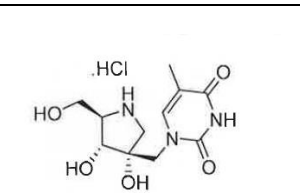
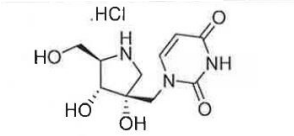
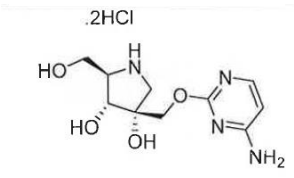
| ***V. harveyi* strain** | **Description** | **Relevant feature** | **Reference** |
|---|---|---|---|
| **BB120** | Wild type | - | B. Bassler [221] |
| **BB152** | *luxM*::Tn5 | No AHL production | B. Bassler [228] |
| **MM30** | *luxS*::Tn5 | No AI-2 production | B. Bassler [229] |
| **BB886** | *luxPQ*::Tn5 $Kan^r$ | No response to AI-2 | B. Bassler [228] |
| **JMH597** | *luxN*::Tn5 *cqsS*::$Cm^r$ | No response to AHL/CAI-1 | P. Bossier [230] |

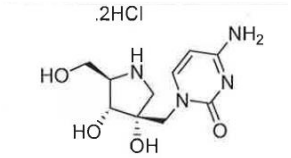
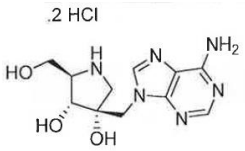
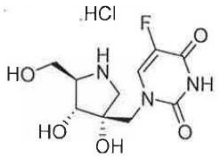
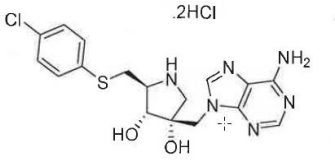
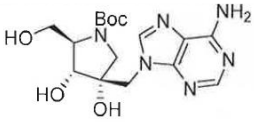
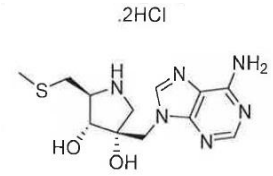
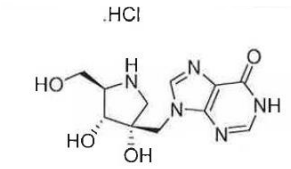
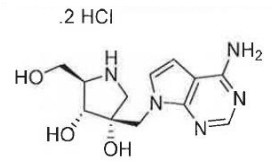
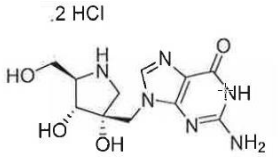
### Azanucleosides

The azanucleosides used in the present study are shown in Table 2. The azanucleosides were synthesized according to the method described by Bouton et al. [227]. The compounds were dissolved in MilliQ water (MQ) (Millipore, Billerce, MA, US) at a concentration of 1 mM, as a stock solution, and stored at - 20°C.

*Table 2:* ***Azanucleosides included in the present study***

| **Code** | **Structural formula** | **Chemical formula** |
|---|---|---|
| JB024 |  | $C_{10}H_{15}N_3O_3$ |
| JB037 |  | $C_{11}H_{16}N_6O$ |
| JB040 |  | $C_{17}H_{19}ClN_6S$ |

| | | |
|---|---|---|
| JB043 |  | $C_{11}H_{18}ClN_3O_3$ |
| JB056 |  | $C_{12}H_{17}N_5O$ |
| JB058 |  | $C_{10}H_{15}ClFN_3O_3$ |
| JB062 |  | $C_{10}H_{16}N_4O_2$ |
| JB070 |  | $C_{11}H_{15}N_5O_2$ |
| JB073 |  | $C_{11}H_{17}ClN_6O_2$ |
| JB094 |  | $C_{11}H_{18}ClN_3O_3$ |
| JB098 |  | $C_{10}H_{16}ClN_3O_5$ |
| JB101 |  | $C_{10}H_{18}Cl_2N_4O_4$ |

| | | |
|---|---|---|
| JB104 |  | $C_{10}H_{18}Cl_2N_4O_4$ |
| JB105 |  | $C_{11}H_{18}Cl_2N_6O_3$ |
| JB110 |  | $C_{10}H_{15}ClFN_3O_5$ |
| JB112 |  | $C_{17}H_{21}Cl_3N_6O_2S$ |
| JB113 |  | $C_{16}H_{24}N_6O_5$ |
| JB116 |  | $C_{12}H_{20}Cl_2N_6O_2S$ |
| JB119 |  | $C_{11}H_{16}ClN_5O_4$ |
| JB125 |  | $C_{12}H_{19}Cl_2N_5O_3$ |
| JB127 |  | $C_{11}H_{18}Cl_2N_6O_4$ |

## Evaluation of the antimicrobial effect of azanucleosides

### *Determination of minimal inhibitory concentrations*

Minimal inhibitory concentrations (MIC) of all azanucleosides were determined for both *V. harveyi* BB886 and JMH597, following the EUCAST protocol [231]. Briefly, clear flat-bottomed 96-well microtiter plates (MTP; SPL Lifescience, Korea) were filled with the different azanucleosides (concentration range: 3 µM to 400 µM, in two-fold dilutions in MB). Approximately $5x10^5$ CFU/ml of the test strains was then added to each well. After 24 hours at 30°C, growth in the plate was assessed with a multilabel microtiter plate reader (EnVision, Perkin Elmer LAS, Waltham, MA) at a wavelength of 590 nm. Growth was expressed relatively to an untreated control. The lowest concentration of the component that had a similar optical density as an un-inoculated control was considered as the MIC. All determinations of the MIC were performed in duplicate.

### *Effect of azanucleosides on bacterial growth*

To evaluate the effect of the azanucleosides on bacterial growth, the test strains were incubated with 100 µM of the compounds. An untreated control was also included. After 24 hours, growth was measured with the EnVision multilabel MTP reader at 590 nm. Growth was expressed relatively to the untreated control. Each biological replicate included four technical replicates. The experiment was performed in triplicate for components JB024, JB037, JB040, JB043, JB056, JB058, JB062, JB070 and JB073 (n = 4 x 3). Other components (JB094, JB098, JB101, JB104, JB105, JB110, JB112, JB113, JB116, JB119, JB125 and JB127) were tested once (n = 4 x 1).

## Bioassay for determination quorum sensing inhibiting activity

### *Inhibition of AI-2 production*

The effect of the azanucleosides on the production of AI-2 was measured in supernatants of *V. harveyi* BB152. This strain is incapable of producing AHLs. Liquid cultures were incubated with 100 µM of the appropriate azanucleoside at 30°C: untreated controls of both the wild type and the test strain were also included. After 18 hours, the liquid cultures were centrifuged (5000 rpm, 5 min, room temperature) and filter sterilized (0.22 µm Whatman, Dassel, Germany). Supernatants were used immediately because of the unstable character of AI-2 molecules. To determine AI-2 levels, the reporter strain *V. harveyi* JMH597 was used. In this strain both *luxN* and *cqsS* are inactivated, rendering it incapable of responding to AHL and CAI-1. The AI-2 levels were determined as described previously [232]. Briefly, an overnight culture of the reporter strains was 1:5000 diluted into fresh sterile MB medium. Ninety µl of this cell suspension was added to the wells of a black flat-bottomed 96-well plate (Perkin Elmer) and 10 µl of the appropriate supernatants was added to the wells. The MTP was incubated at 30°C and bioluminescence was measured every 15 minutes for 6 hours with an

EnVision multilabel MTP reader (emission: λ = 700nm). As the reporter strain produces (and senses) its own autoinducers over time, the time point to measure the effect of azanucleosides on autoinducer production had to be chosen carefully. In this study the selected time point was at 225 min. Bioluminescence was expressed relatively to an untreated control. Each biological replicate included four technical replicates. The experiment was performed in triplicate for components JB024, JB037, JB040, JB043, JB056, JB058, JB062, JB070 and JB073 (n = 4 x 3). Other components (JB094, JB098, JB101, JB104, JB105, JB110, JB112, JB113, JB116, JB119, JB125 and JB127) were only tested once (n = 4 x 1).

#### *Inhibition of AHL and/or CAI-1 production*

Azanucleosides were also investigated on their ability to block AHL/CAI-1 QS. The procedure is similar to the one described above, but other strains are used. The test strain is *V. harveyi* MM30, which is unable to produce AI-2, and the reporter strain is *V. harveyi* BB886, which responds to both AHL and CAI-1. In both assays for the measurement of QSI activity, the wild type was included as a control.

### Statistical analysis

Statistical analysis was performed using SPSS version 25 software (SPSS, Chicago, IL, USA). The Shapiro-Wilk test was used to verify the normal distribution of the data. Normally distributed data were analysed using a Dunnett's test. P-values smaller than 0.05 were considered significant. Statistical analysis was not performed when only one biological replicate was available.

## Results and discussion

### The azanucleosides tested do not affect bacterial growth

The effect of azanucleosides on bacterial growth of the reporter and test strains was tested with two different procedures; determination of the MIC and evaluation of 100 µM of the azanucleosides on growth. First, we selected a concentration well below the MIC for the reporter strains (JMH597 and BB886), 100 µM (Figure 4). Subsequently, the effect on growth of the compounds was also evaluated on the test strains (MM30 and BB152) and the wild type (BB120). None of the azanucleosides affected growth of the test strains at 100 µM (Figure 5).

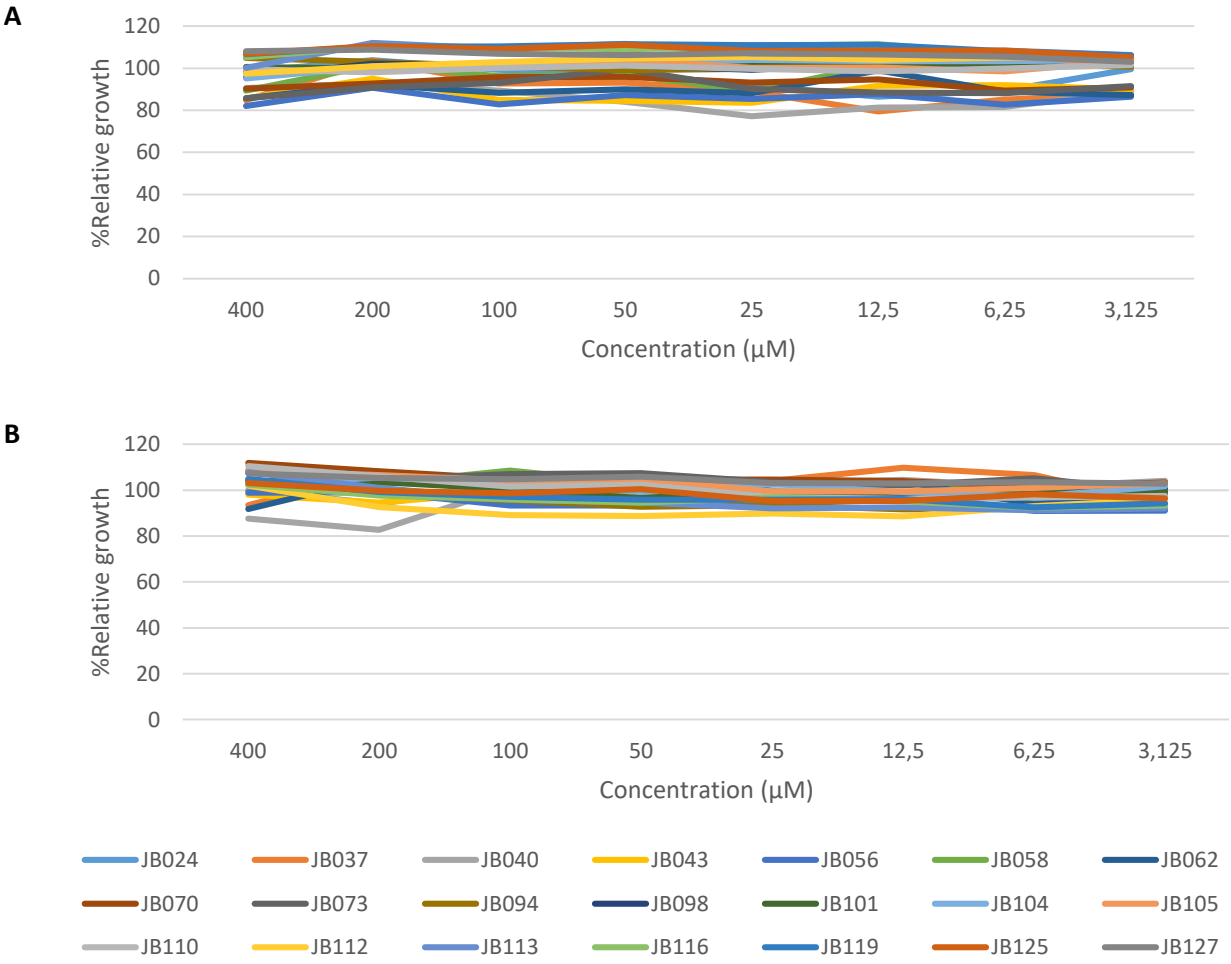



*Figure 4:* ***The effect of multiple concentrations of the azanucleosides on growth of V. harveyi reporter strains****. A concentration range of 3 µM to 400 µM of azanucleosides was evaluated on their inhibiting capacity of both V. harveyi JMH597 (A) and V. harveyi BB886 (B). Growth is expressed relatively to an untreated control. None of the azanucleosides have an inhibitory effect on growth of both strains. The experiment was performed in duplicate (n=2).*

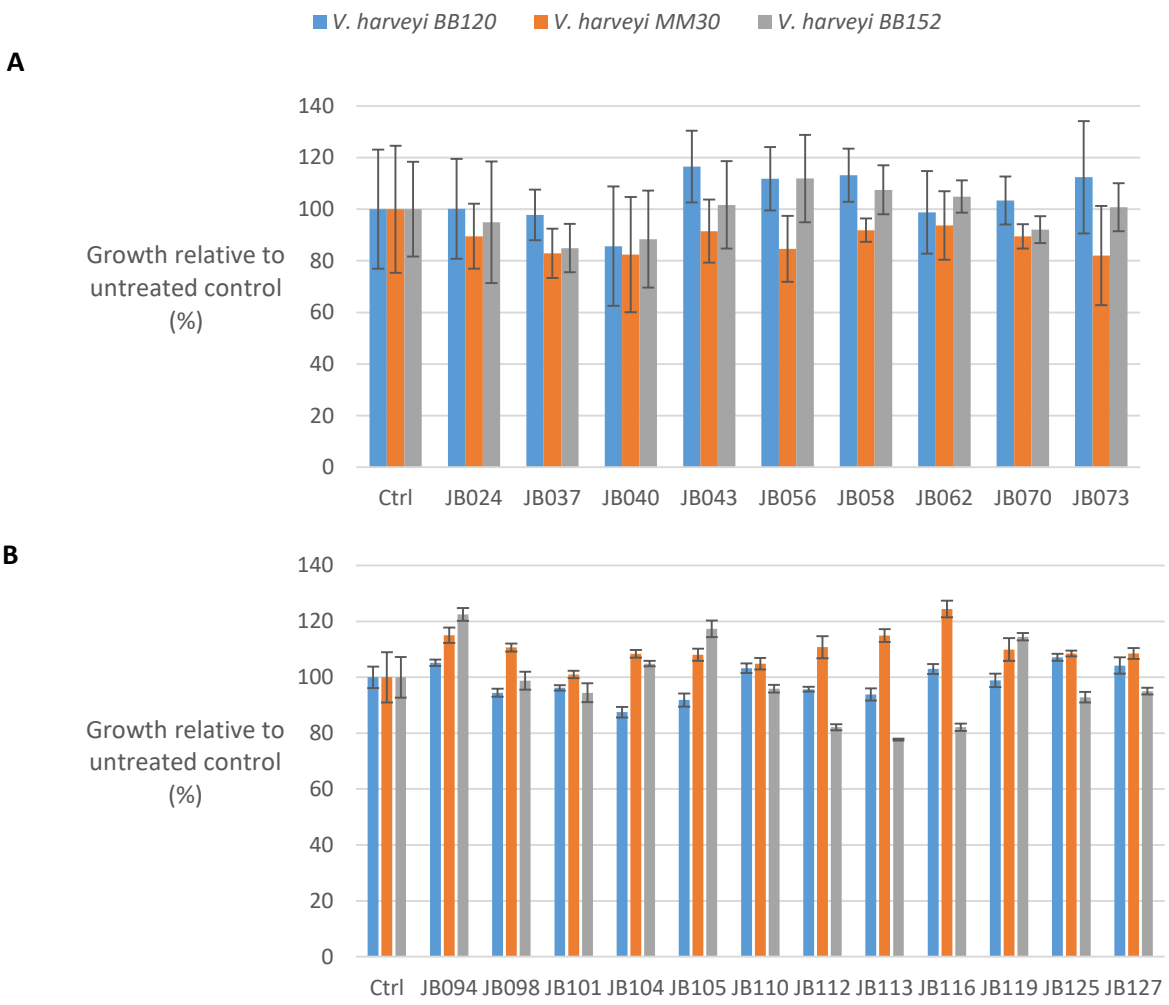



*Figure 5:* ***The effect of 100 µM azanucleosides on growth of V. harveyi BB120 (wt), MM30 and BB152****. Azanucleosides are incubated with each strain for 24 hours, growth is relatively expressed to an untreated control of the respective strain. A. Azanucleosides were tested in triplicate (n = 3 x 3). Error bars are standard deviations of the biological replicates. There is no significant (p > 0.05) difference in growth compared to the respective controls. B. These azanucleosides were tested once (n = 1 x 3). Error bars are standard deviations of the technical replicates.*

## Effect of azanucleosides on signalling molecule production

Since bioluminescence is a QS-regulated phenotype in *V. harveyi*, we evaluated the effect of azanucleosides on bioluminescence using the wild type strain (BB120) and several QS mutants (Table 1). To measure AI-2 levels, *V. harveyi* BB152 was used as a test strain and JMH597 as a reporter strain. Supernatant of an untreated control of BB152 was added to the test strain and the obtained bioluminescence was set as the maximal obtainable level. A reporter strain supplemented with MB was considered as a negative control. When the azanucleosides were able to inhibit MTAN activity, and thus decrease the production of AI-2, significantly less bioluminescence would be

detected after 225 min. The same applies to the measurement of AHL/CAI-1 levels with MM30 as a test strain and BB886 as the reporter strain.

The data obtained for the test strains are shown in Figure 6. There is no significant ($p > 0.05$) decrease in bioluminescence when any of the azanucleosides were added to the test strains. This indicates no decrease of both AI-2 and (AHL/CAI-1) production (Figure 6A). A similar observation was made for a number of other azanucleosides (these were only tested once and are shown separately in Figure 6B).

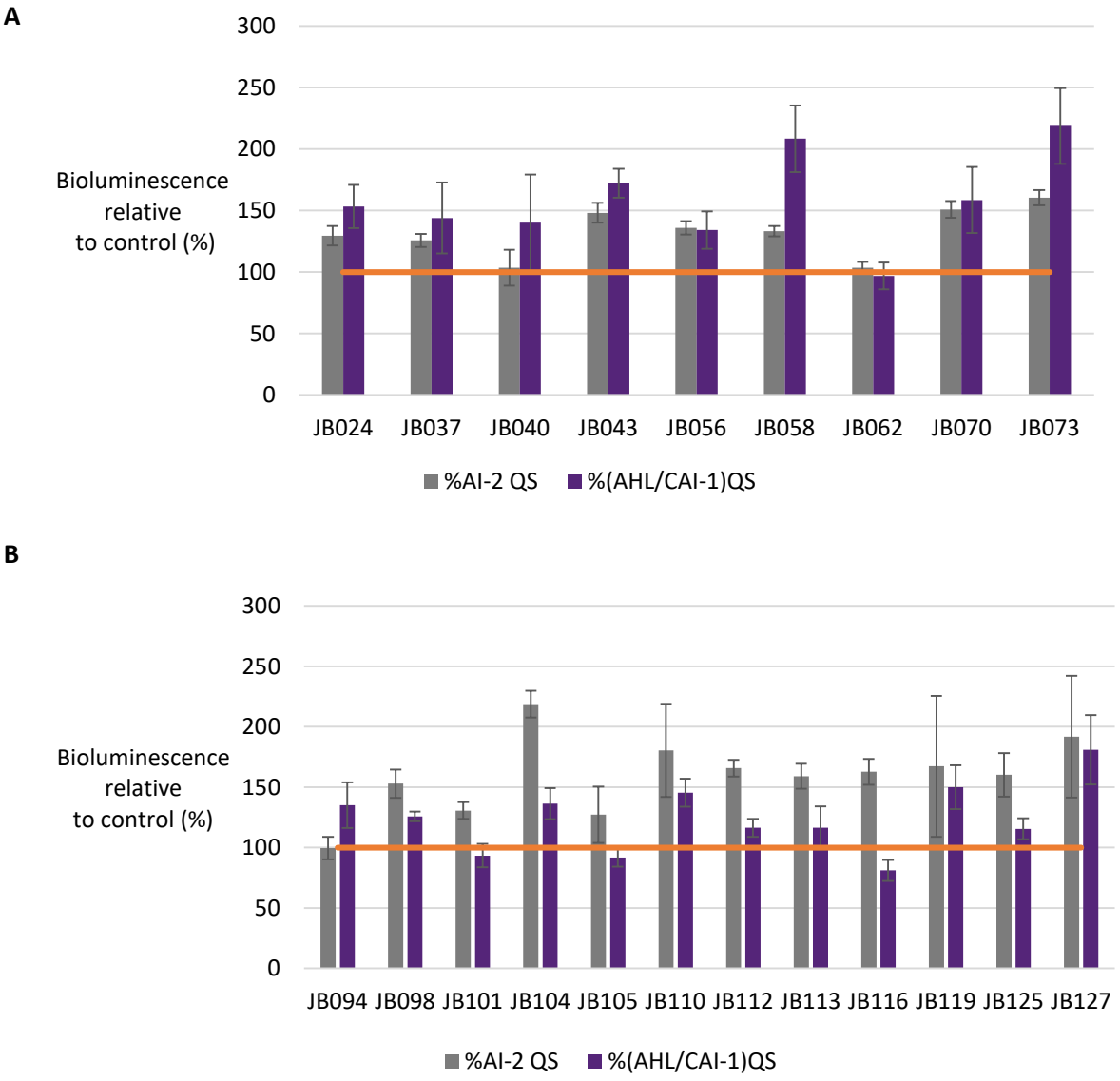



*Figure 6: **QS inhibiting capacity of the azanucleosides in V. harveyi**. QS was measured by the amount of bioluminescence detected after 225min, which is a direct measurement for QS in V. harveyi. Percentage of bioluminescence is expressed relatively to an untreated control. **A**. First set of azanucleosides tested in triplicate (n=4x3). There is no significant ($p > 0.05$) difference in bioluminescence compared to the respective controls. Error bars are standard deviations of the biological replicates. **B**. Second set of azanucleosides tested once (n = 4x 1). Error bars are standard deviations of the technical replicates.*

The lack of QS inhibition in *V. harveyi* biosensors can be caused by a lack of activity on MTAN or by difficulties of the azanucleosides to reach the target site. The latter might be due to a lack of uptake, possibly because of the hydrophilic character of the azanucleosides, metabolic degradation, and/or an increased efflux. Guttierez showed some discrepancies between the affinity for the purified enzyme and the inhibition activity in the cell, showing that there is a significant barrier for immucilin analogues to cross the membrane [104].

To ensure the potency of the azanucleosides towards MTAN, the activity can be evaluated on the purified enzyme prior to screening the activity in the cell [106]. However, since it is equally important for azanucleosides to reach the target as to inhibit the enzyme, the bioassays used in the present study probably suffice to evaluate whether the tested compounds have a QS inhibiting activity or not.

## Conclusion

The azanucleosides investigated did not lead to decreased signal molecule production in *V. harveyi*. This can be explained by an insufficient concentration that reaches the target enzyme and/or lack of activity. Further research is necessary to find appropriate compounds to inhibit MTAN.

# Paper 2: Elucidation of the mechanism behind the potentiating activity of baicalin against *Burkholderia cenocepacia* biofilms

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## Abstract

Reduced antimicrobial susceptibility due to resistance and tolerance has become a serious threat to human health. An approach to overcome this reduced susceptibility is the use of antibiotic adjuvants, also known as potentiators. These are compounds that have little or no antibacterial effect on their own but increase the susceptibility of bacterial cells towards antimicrobial agents. Baicalin hydrate, described as a quorum sensing inhibitor, is such a potentiator that increases the susceptibility of *Burkholderia cenocepacia* J2315 biofilms towards tobramycin. The goal of the present study is to elucidate the molecular mechanisms behind the potentiating activity of baicalin hydrate and related flavonoids. We first determined the effect of multiple flavonoids on susceptibility of *B. cenocepacia* J2315 towards tobramycin. Increased antibiotic susceptibility was most pronounced in combination with apigenin 7-O-glucoside and baicalin hydrate. For baicalin hydrate, also other *B. cepacia* complex strains and other antibiotics were tested. The potentiating effect was only observed for aminoglycosides and was both strain- and aminoglycoside-dependent. Subsequently, gene expression was compared between baicalin hydrate treated and untreated cells, in the presence and absence of tobramycin. This revealed that baicalin hydrate affected cellular respiration, resulting in increased reactive oxygen species production in the presence of tobramycin. We subsequently showed that baicalin hydrate has an impact on oxidative stress via several pathways including oxidative phosphorylation, glucarate metabolism and by modulating biosynthesis of putrescine. Furthermore, our data strongly suggest that the influence of baicalin hydrate on oxidative stress is likely unrelated to quorum sensing. Our data indicate that the potentiating effect of baicalin hydrate is due to modulating the oxidative stress response, which in turn leads to increased tobramycin-mediated killing.

## Introduction

*Burkholderia cepacia* complex (Bcc) bacteria are opportunistic pathogens, which cause severe lung infections in immunocompromised persons, such as cystic fibrosis (CF) patients [165]. The most frequently isolated Bcc species from these patients are *Burkholderia cenocepacia* and *Burkholderia multivorans* [173]. Infections with these pathogens are particularly difficult to treat due to their ability to form biofilms [233]. Biofilms are defined as communities of microbial cells embedded in a self-produced matrix that, compared to their planktonic counterparts, show reduced susceptibility towards antimicrobial therapy [46]. The process of biofilm formation is partially controlled by quorum sensing (QS), a cell-density-dependent communication system, that coordinates expression of various virulence factors [49,234]. *B. cenocepacia* has two acylhomoserine lactone (AHL) based systems, namely CepIR and CciIR. The CepIR system is present in all Bcc strains, while the CciIR

system is only present in highly transmissible ET12 strains containing the cci genomic island. The CepIR system is generally responsible for positive regulation of QS-regulated genes while CciIR mainly acts as a negative regulator [29]. Another QS system in *B. cenocepacia* uses cis-2-dodecenoic acid, also referred to as BDSF (*Burkholderia* Diffusible Signal Factor), as signalling molecule. BDSF is synthesized by RpfF and sensed by RpfR [54]. There is a complex interplay between the AHL- and BDSF-based QS systems [189].

One of the mechanisms contributing to biofilm tolerance is the protection against oxidative stress [76]. These responses to oxidative stress are controlled by two major transcriptional regulators, OxyR and SoxRS [213], and include the production of polyamines, such as putrescine, which reduce intracellular reactive oxygen species (ROS) levels and protect membranes from lipid peroxidation [235,236].

It was previously described [71,210] that antibiotics also induce intracellular ROS production and it was shown that this also occurs in Bcc strains [211]. The primary drug-target interactions are thought to stimulate the oxidation of nicotinamide adenine dinucleotide (NADH) through the electron transport chain (ETC), which depends on the tricarboxylic acid cycle (TCA) [71,212]. Hyperactivation of the ETC generates an increased superoxide ($O_2^-$) production. These highly toxic species damage iron-sulphur clusters in proteins, making ferrous iron available for the Fenton reaction [71]. In this reaction, ferrous iron ($Fe^{2+}$) will be oxidized by hydrogen peroxide ($H_2O_2$) to produce ferric iron ($Fe^{3+}$) and deleterious hydroxyl radicals ($^{\cdot}OH$). ROS can directly damage macromolecules such as DNA, lipids and proteins [206] or indirectly damage DNA by oxidizing the deoxynucleotide pool [207].

A decreased activity of the tricarboxylic acid (TCA) cycle leads to a larger fraction of metabolically less active cells, in which endogenous ROS production is reduced [237]. This leads to increased tolerance towards antibiotics [238,239]. A lower activity of the TCA cycle is typically associated with an induction of the glyoxylate shunt. This shunt allows the cells to avoid NADH formation in the TCA cycle and thus avoid ROS production. This was already described for *P. aeruginosa* and *B. cenocepacia* strains exposed to lethal doses of aminoglycosides [211,213,237].

A promising approach to overcome tolerance and/or resistance is the use of antibiotic adjuvants, also described as potentiators. These are compounds with little or no intrinsic antibiotic activity that increase the susceptibility of bacterial cells towards antimicrobial therapy [88]. Brackman et al. [128] already demonstrated an increased susceptibility of *B. cenocepacia* biofilms towards tobramycin (TOB) when it was combined with the potentiator baicalin hydrate (BH). Baicalin (5,6-dihydroxy-7-O-glucuronide flavone), a flavonoid isolated from the roots of *Scutellaria baicalensis*, was described as an inhibitor of QS [128] and has a long history of use in Chinese medicine [240]. The goal of the present research is to elucidate the molecular mechanism behind the potentiating activity of BH and other flavonoids.

## Materials and methods

### Strains and culture conditions

The strains used in the present study are listed in Table 1. The strains were stored at -80°C using Microbank vials (Prolab Diagnostics, Richmond Hill, ON, Canada) and subcultured at 37°C on Trypton Soy agar (TSA; Lab M, Lancashire, UK) or TSA supplemented with 800 µg/ml trimethoprim (Ludeco, Brussels, Belgium) for MDL2. Overnight cultures were grown aerobically in Mueller Hinton broth (MHB; Lab M) at 37°C. Except for cultures on which the $H_2$DCFDA assay was performed, Luria Bertoni agar (LBA; Lab M) and Luria Bertoni broth (LBB; Lab M) were used.

*Table 1:* ***Strains used in the present study***

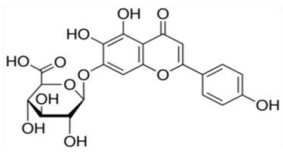
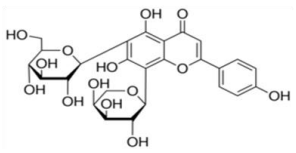
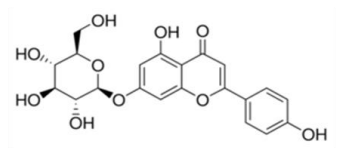
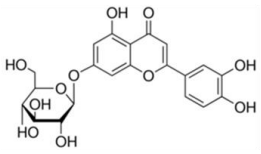
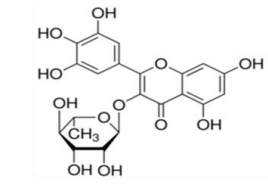
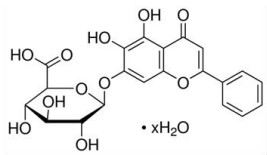
| Strain | Strain info | Source and/or reference |
|---|---|---|
| ***B. cenocepacia* strains** | | |
| **J2315 (LMG 16656$^T$)** | CF patient, UK, ET12 strain | BCCM/LMG bacteria collection (Ghent, University, Belgium) |
| **Triple QS deletion mutant** | J2315 *ΔcepIΔcciIΔrpfF* | G. Riccardi [190] |
| **C5424 (LMG 18827)** | CF patient, Canada, ET12 strain | BCCM/LMG bacteria collection |
| **MDL2** | C5424 *ΔkatB* | M. Valvano [215] |
| **K56-2 (LMG 18863)** | CF patient, Canada, ET12 strain | BCCM/LMG bacteria collection |
| **OME11** | K56-2 ΔBCAL2641 | M. Valvano [241] |
| **HI2424 (LMG 24507)** | Soil, USA, PHDC strain | BCCM/LMG bacteria collection |
| **AU1054 (LMG 24506)** | CF patient, USA, PHDC strain | BCCM/LMG bacteria collection |
| **C6433 (LMG 18828)** | CF patient, Canada | BCCM/LMG bacteria collection |
| **PC184 (LMG 18829)** | CF patient, USA | BCCM/LMG bacteria collection |
| ***B. multivorans* strains** | | |
| **LMG 13010$^T$** | CF patient, Belgium | BCCM/LMG bacteria collection |
| **LMG 18825** | CF patient, UK | BCCM/LMG bacteria collection |
| | | |
| ***B. ambifaria* LMG 19182$^T$** | Pea rhizosphere, USA | BCCM/LMG bacteria collection |

## Reagents

The following antibiotics were tested during the present study: tobramycin (TOB; TCI Europe, Zwijndrecht, Belgium), gentamicin (GN; Sigma-Aldrich), kanamycin (KN; Sigma-Aldrich), neomycin (NEO; Sigma-Aldrich), ceftazidime (CEF; Sigma-Aldrich), meropenem (MEM; Fresenius Kabi, Schelle, Belgium), minocycline (MIN; Sigma-Aldrich), ciprofloxacin (CIP; Sigma-Aldrich) and trimethoprim/sulfamethoxazole (Sigma-Aldrich) (co-trimoxazole, SXT). All antibiotics were dissolved in either MilliQ water (MQ water) (Millipore, Billerce, MA, US) to determine the minimal inhibitory concentration (MIC) or in physiological saline (PS) (0.9% w/v NaCl) (Applichem, Darmstadt, Germany) to treat biofilms. Stock solutions were filter sterilized (0.22 µm Whatman, Dassel, Germany) and stored at 4°C until use.

Structural derivatives of BH (Sigma-Aldrich, Bornem, Belgium) were selected to determine their potentiating activity in combination with TOB. These derivatives were scutellarin (Sigma-Aldrich), luteolin 7-O-glucoside (Sigma-Aldrich), schaftoside (Extrasynthese, Genay Cedex, France), myricitrin (Sigma-Aldrich) and apigenin 7-O-glucoside (Sigma-Aldrich) (Table 2). Stock solutions of the flavonoids were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and diluted to a final solution of 1% with MQ water to determine the MIC or with PS to treat biofilms. A control with the same percentage of DMSO was included. A stock solution of sodium azide ($NaN_3$) (Sigma-Aldrich) was prepared in MQ and further diluted in MHB prior to use.

*Table 2: Structural derivatives of BH*

| | | | |
|---|---|---|---|
| Scutellarin |  | Schaftoside |  |
| Apigenin 7-O-glucoside |  | Luteolin 7-O-glucoside |  |
| Myricitrin |  | Baicalin hydrate |  |

### Determination of the minimal inhibitory concentration

MICs were determined according to the EUCAST broth microdilution assay using flat-bottom 96-well microtiter plates (MTP; SPL Lifescience, Korea) [231]. The flavonoid concentrations ranged from 4 µM to 500 µM. CEF, MEM, MIN and CIP concentrations tested ranged from 0.5 µg/ml to 512 µg/ml; SXT concentrations tested ranged from 0.25/4 µg/ml to 256/4864 µg/ml. All aminoglycosides (TOB, GN, KN and NEO) were tested in a concentration range from 0.5 µg/ml to 4096 µg/ml. The MIC was defined as the lowest concentration with a similar optical density as un-inoculated growth medium. Absorbance was measured at 590 nm with a multilabel MTP reader (EnVision, Perkin Elmer LAS, Waltham, MA). All MIC determinations were performed in triplicate.

### Biofilm formation

Biofilms were grown in clear round-bottomed 96-well plates (SPL) to evaluate their survival after treatment, or in black flat-bottomed 96-well plates (Perkin Elmer) for measuring fluorescence. An inoculum of approximately $5x10^7$ CFU/ml was prepared in fresh medium from an overnight culture. 100 µl of this inoculum was added to the wells of a MTP. After 4 hours of adhesion the supernatant was removed and the wells were rinsed with PS. Subsequently, fresh medium was added to the wells and the MTP was further incubated for 20 hours at 37°C.

### Biofilm treatment

To evaluate the effect of flavonoids on the susceptibility of biofilms towards antibiotics, biofilms were treated with following components: the antibiotic alone, the flavonoid alone, a combination of both, or PS as a control. All antibiotics were tested at concentrations of 4xMIC. The concentration of flavonoids was 100 µM to initially detect their potentiating activity. In subsequent experiments, a concentration of 250 µM was used for BH. All solutions were diluted in PS. When a stock solution was prepared in DMSO, a control with the same percentage DMSO was included. Biofilms were grown as described above. After 24 hours of biofilm formation the supernatant was removed and the wells were rinsed with PS. Subsequently, PS (= control), the antibiotic alone, the flavonoid alone or a combination of both was added to the wells. After 24 hours at 37°C, the supernatant was removed and the wells were rinsed with PS. Sessile cells were harvested from the MTP by two cycles of shaking (5 min, 900 rpm; Titramax 1000, Heidolph Instruments, Schwabach, Germany) and sonicating (5 min; Branson 3510, Branson Ultrasonics Corp, Danbury, CT, USA). The number of surviving cells (CFU/ml) was determined by plating the resulting bacterial suspension.

### Transcriptomic analysis

To elucidate the molecular mechanism by which BH affects biofilm susceptibility towards TOB, transcriptomes of treated and untreated *B. cenocepacia* J2315 biofilm cells were compared using

RNA sequencing. Gene expression was determined in 24 hour-old *B. cenocepacia* J2315 biofilms that were exposed to TOB alone (3 x MIC), BH alone (250 µM), a combination of both, or PS (= control) for 24 hours. These concentrations were selected because sufficient living cells remained for the RNA extraction, while a significant difference between TOB and TOB+BH could be observed. For each treatment, three biological replicates were included. Biofilm cells were harvested as described above with two cycles of vortexing and sonicating. Total RNA was extracted using Ambion RiboPure Bacteria Kit (Ambion, Austin, TX) according to the manufacturers' instructions, including DNAse treatment for 1 hour at 37°C. The concentration and quality of the total extracted RNA was determined by using the Quant-it ribogreen RNA assay (Life Technologies, Grand Island, NY, USA) and the RNA 6000 pico chip (Agilent Technologies, Santa Clara, CA, USA), respectively. Subsequently, 200 ng of RNA was depleted for rRNA using the Ribo-Zero Magnetic Kit for Gram-negative Bacteria (Epicentre, Madison, WI, USA). Library preparation was performed using the Truseq stranded Total RNA library prep (Illumina, San Diego, CA, USA) according to manufacturer's instructions. Libraries were quantified by qPCR, according to Illumina's Sequencing Library qPCR Quantification protocol guide, version February 2011. A DNA 1000 chip (Agilent Technologies, Santa Clara, CA, US) was used to verify the library's size distribution and quality. Sequencing was performed on a high throughput Illumina NextSeq 500 flow cell generating 75 bp single reads. After an initial quality control using CLC Genomics Workbench version 8.5.1 (Qiagen, Venlo, Netherlands), the reads for each condition were mapped to the reference genome sequences (accession numbers AM747720, AM747721, AM747722, and AM747723) [27] (Cut-offs: 90% length and 80% similarity). The number of reads per transcript were divided by the transcript length and then normalized to the total amount of reads, obtaining reads per kb per million (RPKM) expression values. Statistical analysis was performed using Empirical DGE test in CLC genomics Workbench version 8.5.1. The effect of the addition of BH to treated cells (TOB) or untreated cells (PS) on gene expression was evaluated. The combination of TOB+BH was compared to treatment with TOB alone, and treatment with BH alone was compared to an untreated control (PS) to analyse the effect of BH on both treated and untreated cells. Only genes that were significantly differentially expressed ($p$-value < 0.05) and with at least a 1.5-fold change were considered. Results were evaluated using the KEGG Pathway Database [242] and *Burkholderia* Genome Database [243]. The experimental protocols and the raw sequencing data can be found in ArrayExpress under the accession number E-MTAB-6099.

### Fluorometric determination of reactive oxygen species

To evaluate endogenous ROS production, a 2',7'-dichlorodihydrofluorescein diacetate ($H_2$DCFDA)-based assay was used. $H_2$DCFDA is a colourless, non-fluorescent compound that passively diffuses into the cell, where non-specific intracellular esterases cleave the acetate groups and so trap the compound in the cell. The cleaved product will be easily oxidized by intracellular ROS, yielding highly fluorescent 2',7'-dichlorofluorescein (DCF) [237]. Cells were pre-incubated with the dye before treatment to exclude differences in fluorescence due to an altered uptake by treated cells. Since this assay has been described as highly pH dependent, a pH-matched control was included [211]. For this assay, biofilms were cultivated as described above, while planktonic cultures were grown aerobically for 24 hours and were standardized to an optical density of 1 (λ = 590 nm). Biofilms and planktonic cultures were grown in LBB. After 24 hours the cells were rinsed with PS and incubated with 10 µM $H_2$DCFDA in LBB shielded from light at 37°C. After 45 minutes the cells were rinsed with phosphate buffered saline (PBS) and treated with TOB (4xMIC), BH (250 µM) or a combination of both. A pH-matched control in PBS was included as a control for each condition. Fluorescence (λ excitation = 485 nm, λ emission = 535 nm) was measured using an Envision multilabel MTP reader. Net fluorescence was calculated by subtracting autofluorescence of bacterial cells incubated under the same conditions without $H_2$DCFDA. Each experiment included at least three biological replicates.

### Statistical data analysis

Statistical analysis was performed using SPSS version 24 software (SPSS, Chicago, IL, USA). The Shapiro-Wilk test was used to verify the normal distribution of the data. Normally distributed data were analysed using an ANOVA or an independent sample T-test. Non-normally distributed data were analysed using a Kruskal-Wallis test or a Mann-Whitney test. P-values smaller than 0.05 were considered significant.

## Results and discussion

### Determination of the potentiating effect of flavonoids on the antibiotic susceptibility of Bcc species

The ability of several structural analogues of BH to increase the susceptibility of *B. cenocepacia* J2315 towards TOB was evaluated. The analogues tested were scutellarin, luteolin 7-O-glucoside, schaftoside, myricitrin and apigenin 7-O-glucoside. First, the MIC on *B. cenocepacia* was determined in order to select a flavonoid concentration which did not inhibit growth of the bacterial cells (sub-MIC). For all flavonoids the MIC values were >500 µM for *B. cenocepacia* J2315. To limit the amount of DMSO in the final solution to 1%, a concentration of 100 µM was selected. When biofilms were treated with BH or apigenin 7-O-glucoside, an increased killing was observed compared to treatment with TOB alone (Figure 1). No potentiating effect was observed with any of the other flavonoids tested. These results were not surprising since small structural differences in flavonoids can influence their antimicrobial activity [244,245].

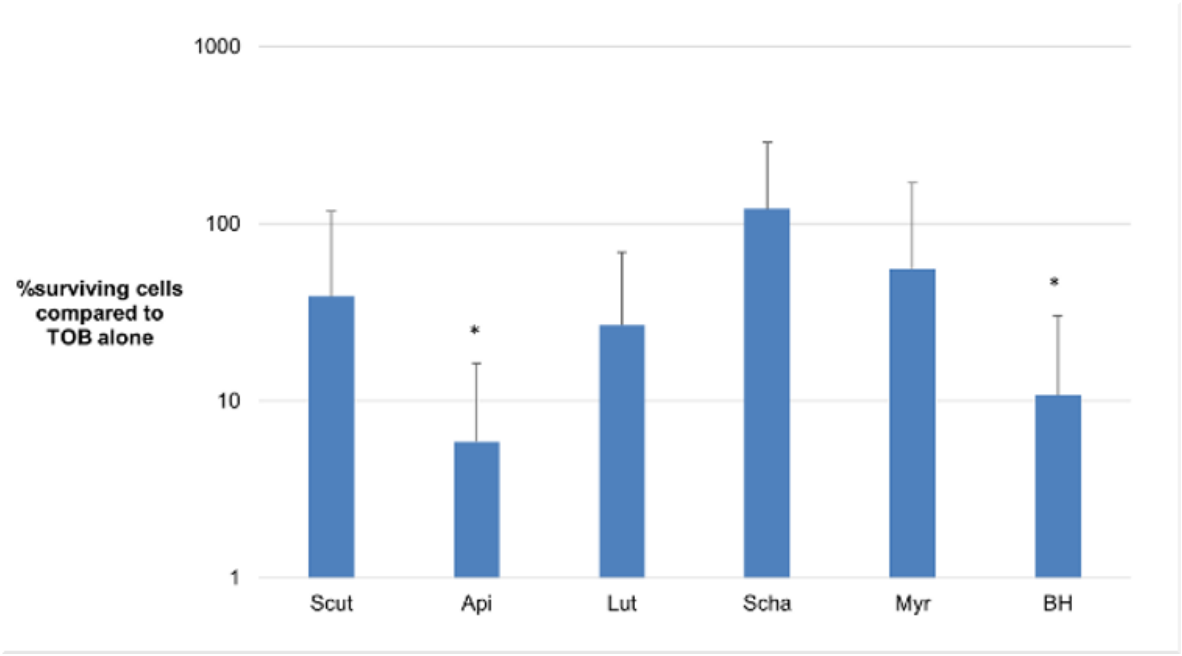



*Figure 1:* ***Potentiating effect of BH and other flavonoids****. Data shown are percentage survival of B. cenocepacia J2315 biofilm cells treated with the combination of a flavonoid (100 µM) and TOB (4 x MIC) compared to TOB alone. The tested flavonoids were scutellarin (scut), apigenin 7-O-glucoside (api), luteolin 7-O-glucoside (lut), schaftoside (scha), myricitrin (myr) and baicalin hydrate (BH). *: statistically significant ($p < 0.05$) less survival compared to TOB alone. Error bars are standard deviations (SD) (n = 4).*

The effect of BH in combination with TOB on the susceptibility of *B. cenocepacia* J2315 was already established [128]. This raised the question if BH could increase the susceptibility of *B. cenocepacia* J2315 biofilms towards other antibiotics. Therefore, several antibiotics (CEF, CIP, MIN, MEM and SXT) belonging to different classes were tested in combination with BH. However, the addition of BH did not lead to a significantly increased susceptibility towards any of the antibiotics tested (Figure S1 and Table S1). This suggests that the increased susceptibility towards TOB is specific to aminoglycosides. To test this hypothesis, other aminoglycosides (GN, KN and NEO) were tested in combination with BH against *B. cenocepacia* J2315 and other Bcc strains (Table 1). Since BH has no antibacterial effect, a significant antibacterial effect of the antibiotic alone is required in order to observe the potentiating influence of BH. Therefore, strains with a high innate resistance towards aminoglycosides (MIC ≥ 1024 µg/ml) were not included (Table S2). The reduction in surviving cells after treatment with the combination compared to the aminoglycoside alone is shown in Table 3.

*Table 3: **Potentiating effect of BH in Bcc biofilms**. Data shown are percentage reduction in CFU/ml (±SD) when combination treatment is compared to the antibiotic alone. NR, no significant reduction in CFU/ml when BH is added to the antibiotic treatment ($p > 0.05$). ND, not determined because MIC > 1024 µg/ml. Tobramycin (TOB) + BH (TOB+BH), gentamicin (GN) + BH (GN+BH), kanamycin (KN) + BH (KN+BH) and neomycin (NEO) + BH (NEO+BH) (n = 3).*

| Strain | TOB+BH vs. TOB | GN+BH vs. GN | KN+BH vs. KN | NEO+BH vs. NEO |
|---|---|---|---|---|
| *B. cenocepacia* J2315$^T$ | 88.9 (± 10.3) | 80.6 (± 14.6) | NR | NR |
| *B. cenocepacia* K56-2 | 81.3 (± 40.0) | 96.7 (± 11.5) | NR | 51.5 (± 44.1) |
| *B. cenocepacia* C5424 | NR | ND | ND | ND |
| *B. cenocepacia* AU1054 | 97.4 (± 10.4) | ND | 98.2 (± 1.4) | ND |
| *B. cenocepacia* LMG18828 | 75.8 (± 51.1) | NR | ND | ND |
| *B. cenocepacia* LMG18829 | 95.3 (± 5.7) | 69.9 (± 30.5) | NR | 97.2 (± 6.2) |
| *B. multivorans* LMG13010$^T$ | NR | NR | NR | 98.1 (± 3.2) |
| *B. multivorans* LMG18825 | NR | NR | NR | NR |
| *B. ambifaria* LMG19182$^T$ | NR | 76.7 (± 32.2) | NR | 97.0 (± 7.6) |

All *B. cenocepacia* strains, except *B. cenocepacia* C5424, showed an increased susceptibility towards TOB in combination with BH. For GN, KN and NEO, the potentiating effect of BH was strain-dependent. For *B. ambifaria* LMG 19182, an increased susceptibility was observed towards GN and NEO in combination with BH. For *B. multivorans* strains, the addition of BH only caused an increased susceptibility for *B. multivorans* LMG 13010 in combination with NEO (Table 3). The findings for this strain are in contrast with previously obtained data by Brackman et al. [128], where BH did show a

TOB-potentiating activity. However, the experimental setup of biofilm formation differs in both studies. Brackman et al. [128] used medical-grade silicone disks placed in 24-well plates, while 96-well microtiter plates were used in this study. These results indicate that the potentiating effect of BH is not only strain- and aminoglycoside-dependent, but also model-system dependent. For subsequent experiments we used *B. cenocepacia* J2315 as the test strain and TOB as the aminoglycoside.

### Effect of baicalin hydrate on gene expression in *B. cenocepacia* J2315 biofilms

To discover the molecular mechanism by which BH affects biofilm susceptibility towards TOB, transcriptomes of treated and untreated *B. cenocepacia* J2315 biofilm cells were compared using RNA sequencing. Results show that the addition of BH had a small but significant impact on gene expression, both for TOB treated and untreated cells (Figure 2). Major differences in gene expression were observed in pathways related to cellular respiration and QS. The genes significantly differentially expressed in these pathways are shown in Table 4. Genes responsible for the electron transport chain and TCA were upregulated, while the expression for genes encoding enzymes of the glyoxylate shunt showed a significant downregulation. These results point to a potential increase in intracellular oxidative stress, as Van Acker et al. [76] previously described an upregulation of glyoxylate shunt-related genes and a downregulation of genes related to the TCA cycle in *B. cenocepacia* biofilm cells after treatment with high concentrations of TOB. These cells were likely metabolically less active which leads to reduced ROS production [76]. We hypothesized that BH could stimulate cellular respiration, which subsequently would induce the production of ROS and lead to increased killing. As the oxidative stress response is partially controlled by QS [29] and as BH has already been described as a QS inhibitor [128,246] the focus in the search for the molecular mechanism of BH on the increase of the antibiotic susceptibility of *B. cenocepacia* biofilms was directed toward both QS and oxidative stress.

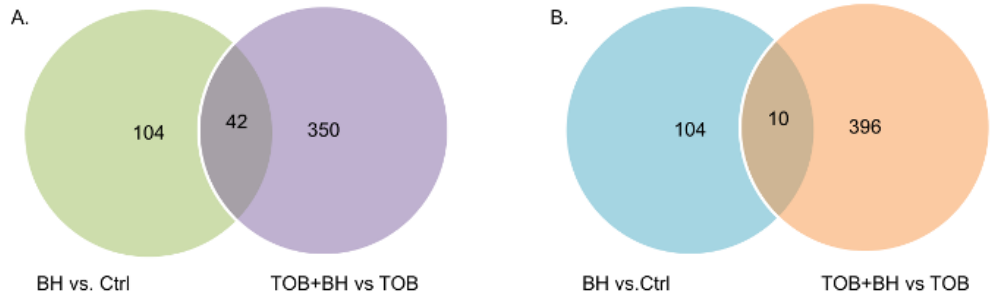



*Figure 2:* ***Differentially expressed genes in B. cenocepacia J2315 biofilms exposed to different treatments.*** *A. Upregulated genes. B. Downregulated genes.*

*Table 4:* ***Differences in gene expression expressed as fold change (p < 0.05) caused by BH (compared to TOB or to an untreated control) in B. cenocepacia J2315***

| Gene number | Annotation | BH vs. ctrl | TOB+BH vs. TOB |
|---|---|---|---|
| **Glyoxylate shunt** | | | |
| BCAL2122 (*aceB*) | Malate synthase | - | -1.4 |
| BCAL2118 (*aceA)* | Isocitrate lyase AceA | - | -1.5 |
| BCAM1588 | Isocitrate lyase | - | -1.9 |
| **TCA cycle** | | | |
| BCAM0972 (*gltA*) | Type II citrate synthase | - | 1.8 |
| BCAM0961 (*acnA*) | Aconitate hydratase | - | 1.7 |
| BCAL1215 (*lpdV*) | Dihydrolipoamide dehydrogenase | - | 1.5 |
| BCAL1517 (*odhL*) | Dihydrolipoamide dehydrogenase | - | 1.7 |
| BCAM1250 | Putative acetyl-CoA hydrolase/transferase | 1.6 | 1.5 |
| BCAM0970 (*sdhB*) | Succinate dehydrogenase iron-sulfur protein | - | 1.6 |
| **Pyruvate metabolism** | | | |
| BCAM1581 (*pckG*) | Phosphoenolpyruvate carboxykinase | - | 2.0 |
| BCAL1910 | Acetoin:2,6-dichlorophenolindophenol oxidoreductase beta subunit | 1.6 | - |
| **Oxidative phosphorylation** | | | |
| BCAL2337 | NADH dehydrogenase I chain H | - | 1.5 |
| BCAL2336 | NADH dehydrogenase I chain I | - | 1.8 |
| BCAL2335 (*nuoJ*) | NADH dehydrogenase I chain J | - | 1.5 |
| BCAL2334 (*nuoK*) | NADH-ubiquinone oxidoreductase I chain K | - | 1.8 |
| BCAL2333 (*nuoL*) | NADH-ubiquinone oxidoreductase I chain L | - | 1.5 |
| BCAL2332 (*nuoM*) | NADH-ubiquinone oxidoreductase I chain M | - | 1.6 |
| BCAL2331 (*nuoN*) | NADH dehydrogenase I chain N | - | 1.6 |
| BCAM0905 (*ndh*) | Putative NADH dehydrogenase | - | -1.4 |
| BCAM0166 (*ndh*) | NADH dehydrogenase | -2.6 | - |
| BCAM0970 (*sdhB*) | Succinate dehydrogenase iron-sulfur protein | - | 1.6 |

| | | | |
|---|---|---|---|
| BCAL0759 (*ubiA*) | Prenyltransferase family protein | - | 1.4 |
| BCAL2141 (*cyoD*) | Cytochrome O ubiquinol oxidase protein | - | 1.6 |
| BCAL0752 | Putative cytochrome c oxidase assembly protein | - | 1.6 |
| BCAM1734 | Putative cytochrome C | - | 1.7 |
| BCAL2142 (*cyoC*) | Cytochrome o ubiquinol oxidase subunit III | - | 2.0 |
| BCAL2143 (*cyoB*) | Ubiquinol oxidase polypeptide I | - | 1.5 |
| BCAM2674 | Putative cytochrome oxidase subunit I | -1.6 | - |
| BCAL0784 (*cydB*) | Cytochrome d ubiquinol oxidase subunit II | - | 1.5 |
| BCAL0034 (*atpA*) | ATP synthase alpha chain | - | 1.7 |
| BCAL0031 (*atpE*) | ATP synthase C chain | - | 1.7 |
| BCAL2622 (*ppa*) | Polyphosphate kinase | - | -1.5 |
| **Glucarate/galactarate metabolism to 2-oxo-glutarate** | | | |
| BCAL1043 (*gudD*) | Glucarate dehydratase | 2.6 | 1.5 |
| BCAM2511 (*garD*) | Putative galactarate dehydratase | 2.3 | 1.6 |
| BCAM2512 | 5-dehydro-4-deoxyglucarate dehydratase | 2.2 | 2.9 |
| BCAM2514 | Putative fatty aldehyde dehydrogenase | 2.0 | 1.6 |
| **Quorum sensing** | | | |
| BCAM1870 (*cepI*) | N-acylhomoserine lactone synthase | - | 1.5 |
| BCAM0239a (*cciI*) | N-acylhomoserine lactone synthase | - | -1.6 |
| BCAM0240 (*cciR*) | N-acylhomoserine lactone dependent regulatory protein | - | -2.3 |
| **Oxidative stress response** | | | |
| BCAS0085 (*ohr*) | Organic hydroperoxide resistance protein | - | -1.7 |
| BCAL3477 | Putative catalase | - | -1.5 |
| BCAL3301 (*oxyR*) | Oxidative stress regulatory protein | - | -1.8 |
| BCAL2643 (*sodC*) | Superoxide Dismutase SodC | - | -1.5 |
| BCAL2641 | Putative ornithine decarboxylase | - | -2.1 |
| BCAM1812 | Agmatinase | -1.8 | -1.4 |

*Effect of baicalin hydrate on oxidative stress*

The effect of BH on oxidative stress was evaluated by testing the susceptibility of a catalase deletion mutant (Δ*katB*) and the corresponding wild type strain (*B. cenocepacia* C5424) towards the combination BH+TOB. We hypothesised that if BH increases ROS-mediated killing by antibiotics, a mutant that lacks protection against oxidative stress would be more sensitive towards the potentiating effect of BH than the wild type. As shown in Figure 3, there was no increase in susceptibility for the wild type after combining BH with TOB. As previously described [211], TOB treatment of the *katB* deletion mutant resulted in more killing than in the wild type. Furthermore, addition of BH led to a significant further increase in susceptibility of the biofilm cells to TOB in the mutant (but not in the WT). In addition, the effect was more pronounced using higher concentrations of BH, suggesting a dose-dependent effect (Figure 3).

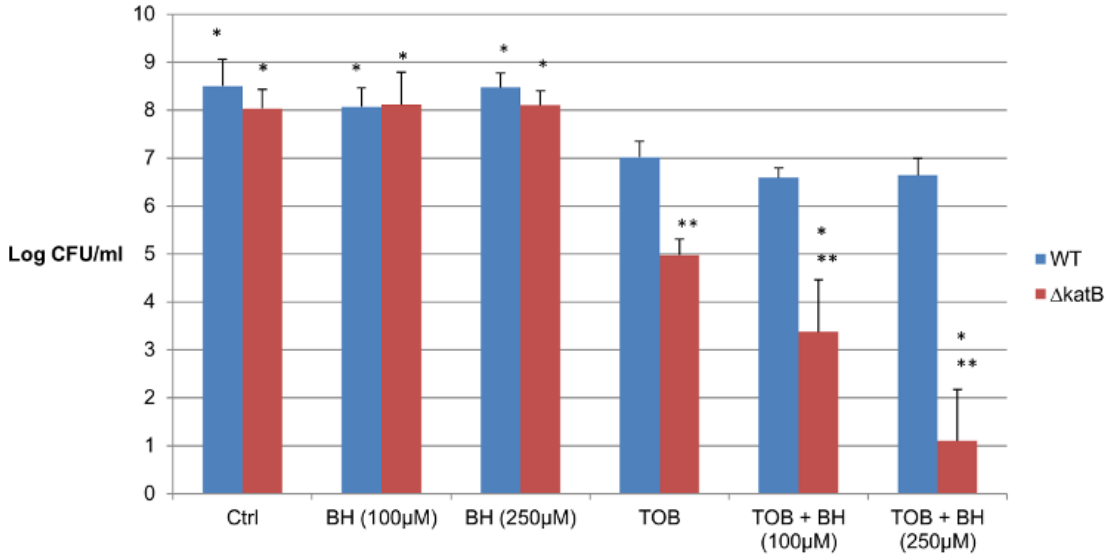



*Figure 3:* ***Potentiating effect of BH in B. cenocepacia ΔkatB****. Data shown are the average log(CFU/ml) recovered after 24h treatment of mature biofilms of B. cenocepacia C5424 (WT) and its catalase deletion mutant (ΔkatB) with 4 x MIC TOB (MIC for both strains = 128 µg/ml), and TOB in combination with BH (100 µM and 250 µM). *: significant difference ($p < 0.05$) compared to TOB alone **: significant difference ($p < 0.05$) compared to the wild type. Error bars represents SD (n = 3).*

From the transcriptomic analysis we learned that no changes in expression were observed for respiration-related genes upon exposure to BH alone, suggesting the effect of BH on biofilm susceptibility is antibiotic-mediated. This was confirmed by the lack of an effect by BH alone on WT or Δ*katB* biofilms (Figure 3).

To confirm the role of BH in promoting ROS-mediated killing, endogenous ROS accumulation was measured using the $H_2$DCFDA assay. In this assay, fluorescence generated is a measure for the amount of ROS present in the cell. Almost a 2-fold increase in fluorescence is observed when *B. cenocepacia* J2315 biofilms were treated with TOB compared to the untreated control. Another 2-fold increase is observed when BH is combined with TOB, compared to TOB alone (Figure 4). This confirms an increased production of ROS in the cells treated with TOB+BH.

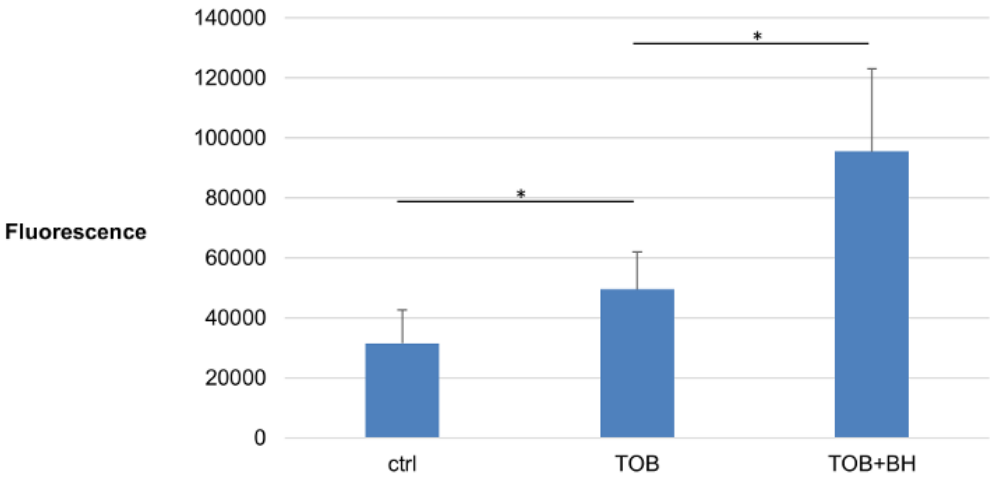



*Figure 4:* ***ROS production in B. cenocepacia J2315 biofilms after treatment with TOB alone or in combination with BH****. Accumulation of ROS in B. cenocepacia J2315 biofilms, expressed as fluorescence generated after incubation with $H_2$DCFDA, after 24 hours treatment with TOB (4 x MIC), TOB in combination with BH (250 µM) or an untreated pH-matched control. Data presented are means, error bars are standard deviations. The experiment was conducted six times. *: Significant difference ($p < 0.05$) compared to treatment with TOB alone.*

### *Baicalin hydrate as a quorum sensing inhibitor*

As the oxidative stress response is co-regulated by QS [29,211] and as BH has been described as a QS inhibitor [128], we hypothesized that BH inhibits QS and as a result increases ROS production in *B. cenocepacia*. To test this hypothesis, ROS production in a triple QS mutant (Δ*cepI*Δ*cciI*Δ*rpfF*) was compared to ROS production in the wild type after treatment with TOB and BH. A triple QS mutant was chosen over single Δ*cepI* or Δ*cciI* mutants in order to avoid biased results caused by the complex interaction between the three QS networks in *B. cenocepacia* J2315 [188,190]. The $H_2$DCFDA assay was carried out on planktonic cells to eliminate nonspecific effects due to the reduced biofilm formation of the triple QS mutant [190]. We observed a significant increase in the amount of ROS in the triple QS mutant compared to the wild type for the control and TOB treatments (Figure 5). The increased amount of ROS is probably due to a lack of oxidative stress response in the triple QS mutant, as previously described [211]. Surprisingly, the addition of BH resulted in an increased ROS

production in the triple QS mutant compared to TOB alone. Also, no difference was observed between the triple QS mutant and the wild type for the combination treatment. Whether these findings mean that the effect of BH on oxidative stress is unrelated to QS or whether the maximal amount of ROS has been reached (and cannot further be increased by addition of BH) remains to be determined.

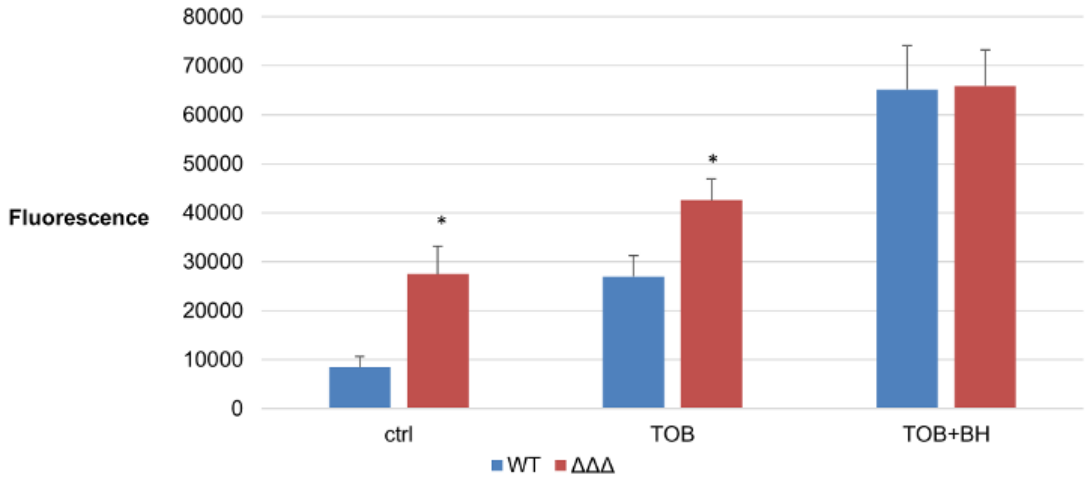



*Figure 5:* ***ROS production in B. cenocepacia J2315 and its triple QS mutant after treatment with TOB alone or in combination with BH.*** *Accumulation of ROS, expressed as fluorescence (average±SD) generated after incubation with $H_2$DCFDA, in planktonic cultures of B. cenocepacia J2315 and its triple QS mutant treated with TOB (4 x MIC) or the combination with BH (250 µM) and a pH-matching control after 16 hours. MIC for TOB was 256 µg/ml and 128 µg/ml for the wild type and its triple QS mutant respectively. The experiment was conducted using six biological replicates. *: statistically significant difference compared to the wild type ($p < 0.05$).*

Remarkably, the data obtained from RNA sequencing revealed an upregulation of genes involved in the main QS systems when BH and TOB were combined (Table 4). *cciR* (BCAM0240) and *cciI* (BCAM0239A) are both located on chromosome 2, and are co-transcribed [182]. They encode CciR and CciI, which are mainly negative regulators of QS-related genes, and showed a significant downregulation of 2.3 and 1.6 fold, respectively. *cepI* (BCAM1870) and *cepR* (BCAM1868) are also located on chromosome 2, but are divergently transcribed [182]. CepI, the synthase of the CepIR system which is mainly a positive regulator, was 1.5 fold upregulated. These results are in accordance with results from a previous study in which an upregulation of *cepI* was observed in several stress conditions (including low oxygen and high temperature) [236]. It is conceivable that the upregulation of these QS systems is not a direct result of the presence of BH, but rather an indirect effect, possibly due to differences in growth stages after both treatments. This is in agreement with observations by

Brackman et al. [247], where the addition of BH to biofilms at the same growth stage resulted in a downregulation in expression of QS-regulated genes.

Based on these results, we could not confirm a direct link between QS and the effect of BH on oxidative stress. Therefore other mechanisms were considered in the search of a mode of action for BH.

### *Influence of baicalin hydrate on cellular respiration*

An upregulation of the expression of genes involving the oxidative phosphorylation and TCA cycle was observed upon the addition of BH to TOB treatment (Table 4). This suggests that BH increases respiration, which could increase TOB-mediated killing.

To evaluate the influence of BH on oxidative phosphorylation, the effect of a cytochrome c oxidase inhibitor (sodium azide, $NaN_3$) on the potentiation of TOB by BH was investigated. Biofilms were pre-treated with $NaN_3$, BH, or a combination of both. After 4 hours, TOB was added to the pre-treated cells for an additional 20 hours. Data in Figure 6 depict the percentage of surviving cells compared to their respective controls. There is no increase in surviving cells between sessile cells treated with TOB and $NaN_3$ compared to TOB alone. However, when $NaN_3$ was combined with TOB+BH, a significant increase in surviving cells could be observed compared to TOB+BH alone, showing that the addition of $NaN_3$ suppressed the potentiating effect of BH. These results are in accordance with data showing increased production of ROS (Figure 4), since an increased activity of the electron transport chain will result in an increased production of ROS [213]. Together our data suggest an influence of BH on the proton motive force, leading to potentiation of the activity of TOB. It was previously shown that metabolic stimulation of the TCA cycle can increase susceptibility towards aminoglycosides, but not to other classes of antibiotics [248]. This is in line with the findings in the present study.

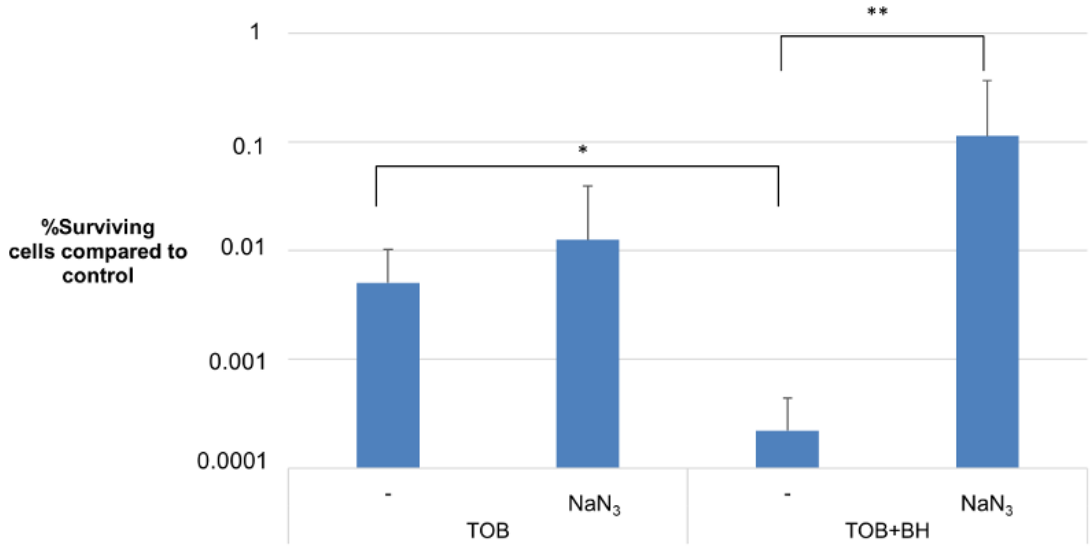



*Figure 6: **Effect of electron transport chain inhibition by $NaN_3$ on BH-mediated TOB potentiation**. Percentage of surviving cells (±SD) after treatment compared to their respective controls (which received a pre-treatment but no antibiotic). Final concentrations of $NaN_3$, BH and TOB were 150 µM, 250 µM and 1024 µg/ml (4 x MIC) respectively. Pre-treated cells received BH, $NaN_3$, a combination of both or MHB for 4 hours. The experiment was conducted in triplicate. *: significant difference ($p < 0.05$) between sessile cells not treated with $NaN_3$. **: significant difference ($p < 0.05$) between sessile cells when $NaN_3$ is included in the treatment.*

### *Influence of baicalin hydrate on glucarate metabolism*

As RNAseq data revealed an upregulation of genes involved in cellular respiration, we looked for changes in the expression of genes involved in turnover of compounds feeding into the TCA cycle.

The only pathway with a direct link to the TCA cycle that showed upregulation of multiple genes was that for glucarate utilisation (Figure 7). D-glucarate, the dicarboxylic acid analogue of glucose, can serve as a growth substrate in many bacteria [249]. According to the biochemical pathways in the KEGG database, *B. cenocepacia* J2315 is able to use two pathways for the utilization of D-glucarate [242]. In the first pathway D-glucarate is converted to D-glycerate and finally to 2-phosphoglycerate, which is a metabolite in the glycolytic pathway [250]. In the second pathway three enzymatic steps lead to the generation of α-ketoglutarate as an end product, which is a key substrate in the TCA cycle [251].

The expression of genes involved in the pathway generating D-glycerate was unaffected by addition of BH. However, in the other pathway, a significant upregulation (2.6-fold) was observed for glucarate dehydratase (*gudD*) upon the addition of BH. Also for 5-keto-4-deoxyglutarate dehydratase (BCAM2512) and α-ketoglutarate semialdehyde dehydrogenase (BCAM2514), genes coding for

enzymes involved in generating α-ketoglutarate [252], a significant upregulation could be observed (2.2-fold and 2.0-fold, respectively). This was also the case when gene expression was compared between cells exposed to the combination of TOB and BH, and those exposed to TOB alone: *gudD,* BCAM2512 and BCAM2514 showed a 1.5-fold, 2.9-fold, and 1.6-fold increased expression, respectively (Figure 7).

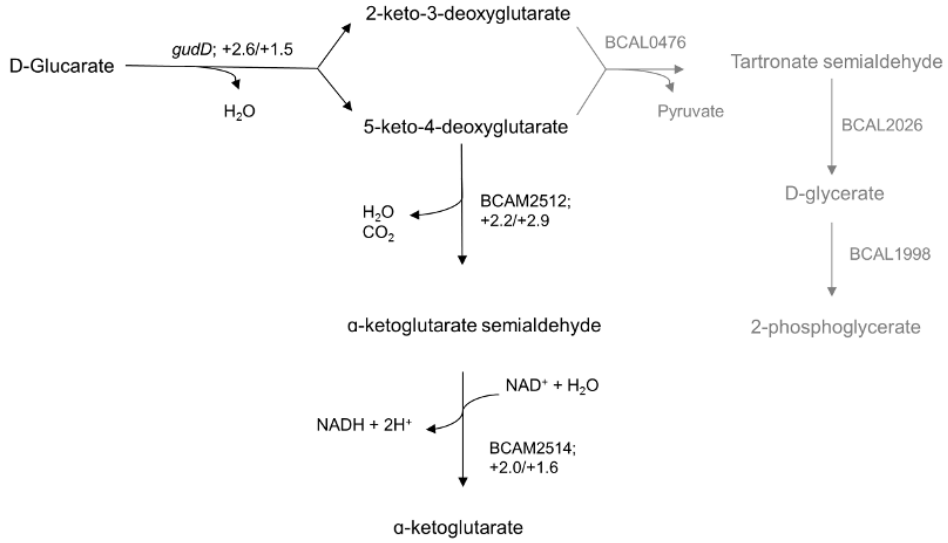



*Figure 7:* ***BH affects regulation of genes involved in glucarate metabolism of B. cenocepacia J2315.*** *The reactions depicted in black are significantly ($p < 0.05$) upregulated (fold changes of "BH vs. Ctrl" / "TOB+BH vs TOB"). The involved enzymes are gudD (glucarate dehydratase), BCAM2512 (5-keto-4-deoxyglutarate dehydratase) and BCAM2514 (α-ketoglutarate semialdehyde dehydrogenase). For the reactions depicted in grey no significant differential expression in either "BH vs. Ctrl" or "TOB+BH vs TOB" was observed.*

To further investigate the involvement of glucarate metabolism in the potentiating activity of BH, glucarate was added to sessile cells treated with TOB and TOB+BH. The glucarate+TOB treatment caused a significant reduction in the number of surviving cells compared to TOB alone. This reduction was similar to that observed for the combinations TOB+BH and TOB+BH+glucarate (Figure 8). Our data suggest that stimulation of the glucarate degradation pathway (by adding glucarate or BH) increases cellular metabolism and increases susceptibility to TOB. When both compounds are added simultaneously, the pathway is not more stimulated than it is by either one of the compounds, resulting in a similar reduction in surviving cells.

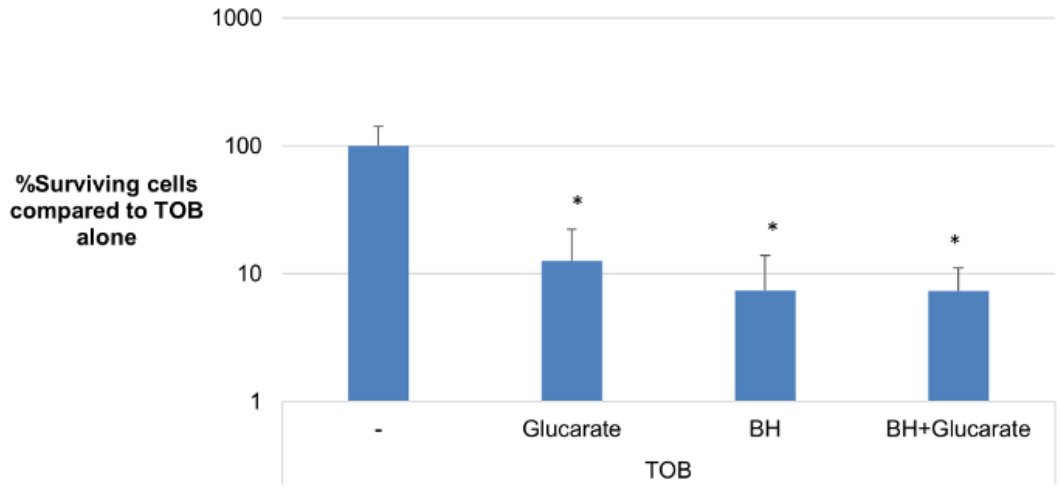



*Figure 8:* ***Impact of glucarate and BH on the susceptibility of B. cenocepacia J2315 biofilms towards TOB****. Data shown are the percentage surviving cells compared to TOB treatment alone. *: significantly less surviving cells compared to TOB alone ($p < 0.05$). Error bars show SD ($n = 3$).*

### *Influence of baicalin hydrate on putrescine biosynthesis*

Bacteria can produce polyamines that quench ROS and protect membranes against lipid peroxidation [213]. Polyamines are small aliphatic molecules with multiple amino groups, which are protonated at physiological pH. The most common cellular polyamines are putrescine, spermidine, spermine and cadaverine [253]. The most abundant one in *B. cenocepacia* is putrescine, whereas spermidine and cadaverine are produced in lower amounts [254]. *B. cenocepacia* can produce putrescine via two different pathways. In the first pathway ornithine decarboxylase (ODC) converts ornithine to putrescine. The second pathway uses arginine as a start product, which is decarboxylated to agmatine by arginine decarboxylase (ADC). In a following step, agmatine is converted to putrescine by agmatinase, releasing urea [241]. *B. cenocepacia* has two ODC homologues (BCAM1111 and BCAL2641) and one ADC homologue (BCAM1112) (Figure 9). El-Halfawy et al. [241] demonstrated that these three genes are the only contributors to putrescine production in *B. cenocepacia*, and that BCAL2641 is the key enzyme in protection against antibiotic-mediated oxidative stress. They also showed that the ODC BCAL2641 responds to antibiotic stress by increasing putrescine levels. The other ODC BCAM1111 and ADC BCAM1112 were not affected by exogenous stress and their expression appeared to be regulated by BCAL2641. This suggests that the increased levels of putrescine upon antibiotic stress depend on the activity of BCAL2641. Increased putrescine levels can induce expression of *oxyR* which activates oxidative stress response mechanisms, whereas a reduced putrescine biosynthesis resulted in an increased ROS generation [241].

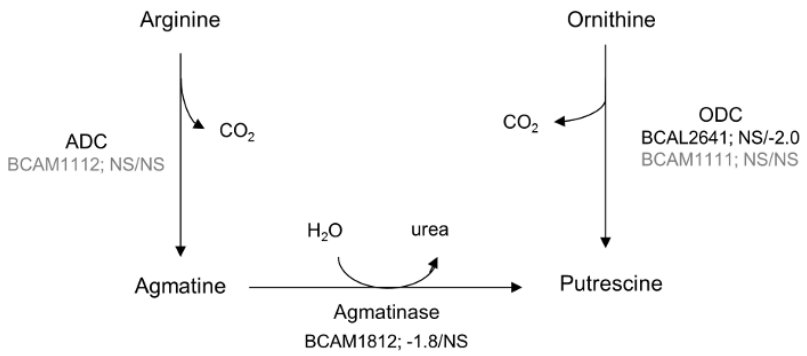



*Figure 9:* ***BH affects regulation of genes involved in putrescine biosynthesis of B. cenocepacia J2315.*** *The reactions depicted in black are significantly ($p < 0.05$) differentially regulated (fold changes of "BH vs. Ctrl" / "TOB+BH vs TOB"). The enzymes involved the putrescine synthesis pathway are ornithine decarboxylase (ODC), arginine decarboxylase (ADC) and agmatinase. NS: no significant change in gene expression ($p > 0.05$).*

The key enzyme in putrescine biosynthesis (ODC, BCAL2641) was significantly downregulated (-2.1-fold) in cells treated with TOB+BH compared to treatment with TOB alone. Since this enzyme protects against oxidative stress in *B. cenocepacia* [241], we hypothesized that BH causes a downregulation of BCAL2641 which would lead to an inhibition of putrescine synthesis, resulting in impaired oxidative stress response leading to increased biofilm susceptibility towards TOB. To test this hypothesis, we investigated the potentiating effect of BH in a ΔBCAL2641 mutant and the corresponding WT strain (*B. cenocepacia* K56-2) [241]. Biofilms were treated with TOB alone (8 x MIC) and a combination of TOB (8 x MIC) and BH (250 µM) (Figure 10). The ΔBCAL2641 deletion mutant is more susceptible to TOB than the wild type, indicating that putrescine protects against oxidative stress, as previously described [235,241]. Furthermore, there is no difference between wild type and mutant when cells were treated with TOB + BH. This could indicate that the potentiating effect of BH is indeed linked to regulation of BCAL2641 expression by BH. Together, these results suggest that BH affects putrescine biosynthesis, and by doing so affects the oxidative stress response, leading to an increased biofilm susceptibility.

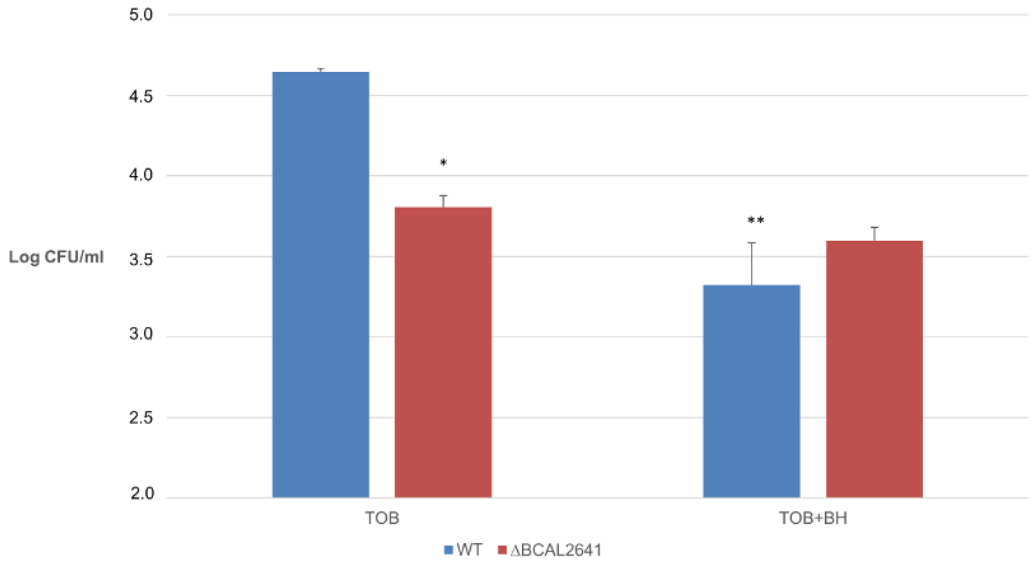



*Figure 10:* ***Influence of BH on TOB susceptibility of B. cenocepacia K56-2 biofilms and its ΔBCAL2641 mutant****. Data are averages of log(CFU/ml) surviving cells after treatment with TOB (8 x MIC) alone or in combination with BH (250 µM). Influence of BH on biofilm susceptibility was evaluated in B. cenocepacia K56-2 (wild type) and its ΔBCAL2641 deletion mutant. The MIC for TOB in both strains was 128 µg/ml. The experiment was conducted in triplicate. *: significantly different compared to the wild type (p < 0.05). **: significant difference compared to TOB alone (p < 0.05). Error bars are SD*.

## Concluding remarks

Several studies already indicated that changes in metabolism upon antibiotic treatment play an important role in the effect of antibiotics [71,76,207,237,239]. These metabolic shifts allow the bacteria to enter a protective state by reducing cellular growth, by limiting ROS production [255] and/or by reducing antibiotic uptake [248]. Especially aminoglycosides can be affected by the latter, since their uptake is an energy-requiring process [248].

In conclusion, the addition of BH to TOB treatment increases oxidative stress in *B. cenocepacia* J2315 biofilms compared to treatment with TOB alone. The potentiating activity of BH appears to be strain-, aminoglycoside- and model-system dependent. While the exact mode of action is still not entirely clear, we have shown that BH has an impact on oxidative stress by influencing oxidative phosphorylation, glucarate metabolism and the protective response by putrescin. Combined, these factors cause an increased ROS production and increased killing upon exposure to TOB.

# Supporting information

## Supporting figure

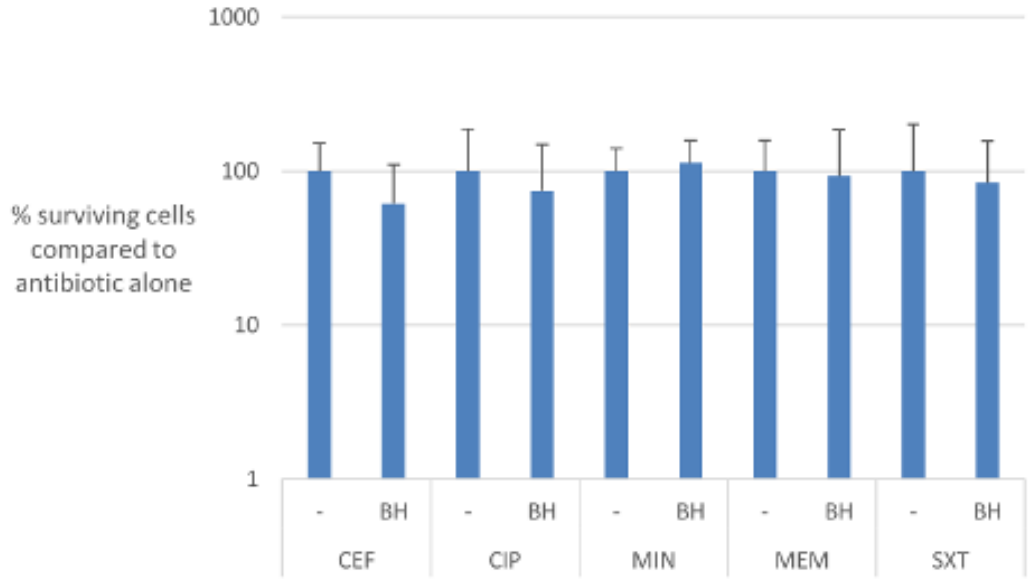



*Figure S1:* ***Potentiating effect of BH in combination with several antibiotics on B. cenocepacia J2315 biofilms****. Data shown are percentage survival of B. cenocepacia J2315 biofilm cells treated with the combination of BH (250 µM) with antibiotic compared to the antibiotic alone (4 x MIC) (MICs are shown in Table S1). The antibiotics are ceftazidime (CEF), ciprofloxacin (CIP), minocycline (MIN), meropenem (MEM) and co-trimoxazole (SXT). None of the combination treatments were significantly different ($p > 0.05$) compared to the antibiotic alone ($n = 3$).*

Supporting tables

*Table S1:* ***MICs of B. cenocepacia J2315 for several antimicrobial agents***

| **Antimicrobial agent** | **MIC (µg/ml)** |
|---|---|
| Ciprofloxacin | 8 |
| Ceftazidim | 128 |
| Meropenem | 32 |
| Minocycline | 16 |
| Co-trimoxazole | 128/2432 |
| Tobramycin | 256 |
| Kanamycin | 1024 |
| Gentamicin | 512 |
| Neomycin | 1024 |

*Table S2:* ***MIC (µg/ml) of other tested Bcc species for several aminoglycosides, including tobramycin (TOB), kanamycin (KN), neomycin (NEO) and gentamicin (GN)***

| **Bcc strain** | **TOB** | **KN** | **NEO** | **GN** |
|---|---|---|---|---|
| *B. cenocepacia* K56-2 | 128 | 512 | 512 | 256 |
| *B. cenocepacia* C5424 | 256 | 1024 | 2048 | >4096 |
| *B. cenocepacia* AU1054 | 512 | 256 | 2048 | 1024 |
| *B. cenocepacia* LMG18828 | 256 | 1024 | 1024 | 512 |
| *B. cenocepacia* LMG18829 | 128 | 64 | 256 | 128 |
| *B. multivorans* LMG13010$^T$ | 64 | 32 | 128 | 64 |
| *B. multivorans* LMG18825 | 128 | 64 | 128 | 256 |
| *B. ambifaria* LMG19182$^T$ | 16 | 16 | 32 | 32 |

# Paper 3: Various evolutionary trajectories lead to loss of susceptibility to tobramycin-potentiating compounds in *Burkholderia cenocepacia* biofilms

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Manuscript in preparation, 2018

## Abstract

Combining antibiotics with potentiators (also known as antibiotic adjuvants) that increase the former's activity is considered as a promising strategy to tackle infections caused by antibiotic-resistant bacteria. As these potentiators typically do not interfere with essential processes of bacteria, it has been hypothesized that they are less likely to induce resistance than conventional antibiotics. However, evidence supporting this hypothesis is lacking. In the present study, we investigated whether *Burkholderia cenocepacia* J2315 biofilms develop resistance towards antibiotic adjuvants. The antibiotic adjuvants used were baicalin hydrate (a quorum sensing inhibitor), and econazole and miconazole, two repurposed antimycotics. The adjuvants were added in combination with tobramycin and biofilms were repeatedly treated for 24h. After each cycle of treatment, the remaining cells were quantified using plate counting. After 15 cycles, biofilm cells were generally less susceptible to tobramycin in combination with the potentiating compounds compared to the start population. Interestingly, the minimal inhibitory concentration for tobramycin remained the same for the start population as for the evolved populations. Subsequently, whole genome sequencing was performed to probe which changes were involved in the reduced effect of the potentiators and several nucleotide polymorphisms were discovered in the evolved populations. For baicalin hydrate, a quorum sensing inhibitor found to increase-antibiotic-induced oxidative stress, we observed several mutations related to metabolism in the evolved populations. Our results indicate that tobramycin-potentiating compounds quickly lose their activity in *B. cenocepacia* biofilms. This potentially limits the clinical applicability of such potentiators.

## Introduction

Due to increasing levels of antimicrobial resistance, novel strategies to tackle bacterial infections are needed. An interesting approach is the use of antibiotic adjuvants, also known as potentiators. Potentiators are compounds with little or no antibacterial activity that interfere with bacterial resistance mechanisms and/or increase antimicrobial activity when co-administered with an antibiotic [3,82,87,88,256]. A well-known class of antibiotic adjuvants are quorum sensing inhibitors (QSI) [128]. These potentiators target the cell-density based bacterial communication network that regulates the expression of multiple virulence factors [142,257]. Some repurposed drugs (i.e. registered pharmaceuticals that are used for new indications) can also increase antibiotic efficacy, rendering the combination more effective than the antibiotic alone [87].

However, whether resistance would develop towards these adjuvants is currently unknown. QSI have long been accepted as 'evolution-proof': as QSI do not target pathways essential for growth, it has

been hypothesized that development of resistance would not occur (or at least would occur less frequently), due to the lack of selective pressure favouring resistant mutants [6,110,148,258,259].

Generally, natural selection occurs when a heritable variation provides a fitness advantage and QS disruption can affect bacterial fitness in conditions in which a functional QS system is essential [142]. This was shown by Maeda et al. [145], by cultivating *Pseudomonas aeruginosa* in medium with adenosine as a sole carbon source. Growth on adenosine depends on the production of a nucleoside hydrolase, which is positively regulated by LasR, a key QS signal receptor, meaning a functional QS system is required for the growth of *P. aeruginosa* [260]. After addition of the brominated furanone C-30 (a known QSI), growth of *P. aeruginosa* was impaired, resulting in selective pressure and the occurrence of resistant mutants. The presence of C-30 caused mutations in repressor genes of the multidrug resistance efflux pump MexAB-OrpM, which resulted in an increased resistance towards C-30 [145]. In clinical isolates of cystic fibrosis (CF) patients never exposed to C-30, mutations in the same genes were found, leading to reduced susceptibility to C-30 [145,146]. Based on these results, Maeda et al. speculated that any strong selective pressure can induce resistance to antivirulence compound [145,147].

In clinical practice, these adjuvants would be co-administered with an antibiotic. This means selective pressure imposed by this antibiotic needs to be included in the experimental set up when investigating possible development of resistance towards the adjuvants [82,256]. In addition, while most evolutionary studies on the development of resistance are carried out with planktonic cells [261,262], 65-80% of all infections are thought to be biofilm-related, and biofilm-associated bacteria typically show a reduced susceptibility towards antimicrobial agents [76].

*Burkholderia cenocepacia* is an opportunistic pathogen that causes severe lung infections in CF patients, which can further develop into a life-threatening systemic infection known as the cepacia syndrome [161]. Antimicrobial therapy often fails due to high innate resistance of *B. cenocepacia* towards many antibacterial agents and high tolerance associated with its biofilm-lifestyle [49,161]. Previously, several adjuvants were identified that increased the activity of tobramycin (TOB) - an aminoglycoside antibiotic frequently used in CF lung infections - towards *B. cenocepacia* biofilms; these include the QSI baicalin hydrate (BH) [128,129] and the repurposed antifungal drugs econazole (ECO) and miconazole (MICO) [86].

The goal of present study is to evaluate whether (and how) *B. cenocepacia* J2315 biofilm cells can develop resistance towards the TOB-potentiating activity of BH, ECO and MICO.

## Materials and methods

### Strains and culture conditions

*B. cenocepacia* J2315 was stored at -80°C using Microbank vials (Prolab Diagnostics, Richmond Hill, ON, Canada) and subcultured at 37°C on Trypton Soy agar (TSA; Lab M, Lancashire, UK). Overnight cultures were grown aerobically in Mueller Hinton broth (MHB; Lab M) at 37°C.

### Reagents

Tobramycin (TOB; TCI Europe, Zwijndrecht, Belgium) was dissolved in physiological saline (PS) (0.9 % w/v NaCl) (Applichem, Darmstadt, Germany), filter sterilized (0.22 µm Whatman, Dassel, Germany) and stored at 4°C until use. Following components were used as antibiotic adjuvants: baicalin hydrate (BH, Sigma-Aldrich, Bornem, Belgium), miconazole (MICO; Certa, Waregem, Belgium) and econazole (ECO; Sigma-Aldrich). Stock solutions of the adjuvants were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and diluted in PS.

### Biofilm formation on beads

Cryobeads from Microbank vials (Prolab Diagnostics) were used as substrates for biofilm formation. The beads were rinsed with PS prior to use in order to remove the medium present in the Microbank vials. This was done by adding 1 ml PS, vortexing the vial, removing the PS and repeating this three times. Six beads were then transferred to the wells of a 24-well microtiter plate (MTP, SPL Lifescience, Korea) and one ml of a diluted overnight culture of *B. cenocepacia* J2315 (containing approximately $5x10^7$ CFU/ml) was used as inoculum. The MTP was statically incubated at 37°C for 24 hours. To evaluate the ability of *B. cenocepacia* J2315 cells to form mature biofilms on the beads, Live/Dead staining (LIVE/DEAD BacLight bacterial viability kit, Thermo Fischer Scientific, Invitrogen, Carlsbad, CA, USA) was performed after 24 hours of biofilm formation. The biofilms on the beads were visualized using an EVOS FL Auto Cell Imaging System (Thermo Fischer Scientific, Waltham, MA, USA) (Syto9: $\lambda_{ex}$ = 470/22 nm, $\lambda_{em}$ = 510/42 nm; propidium iodide: $\lambda_{ex}$ = 531/40 nm; $\lambda_{em}$ = 593/40 nm).

### Evolution experiment

To evaluate the influence of repeated treatments on biofilm susceptibility, cells were exposed to 15 cycles of biofilm formation (24 h), treatment (24 h), and planktonic regrowth (48 h) (Figure 1). The planktonic regrowth step was included to generate a sufficiently high number of cells to set up a new biofilm for the next cycle.

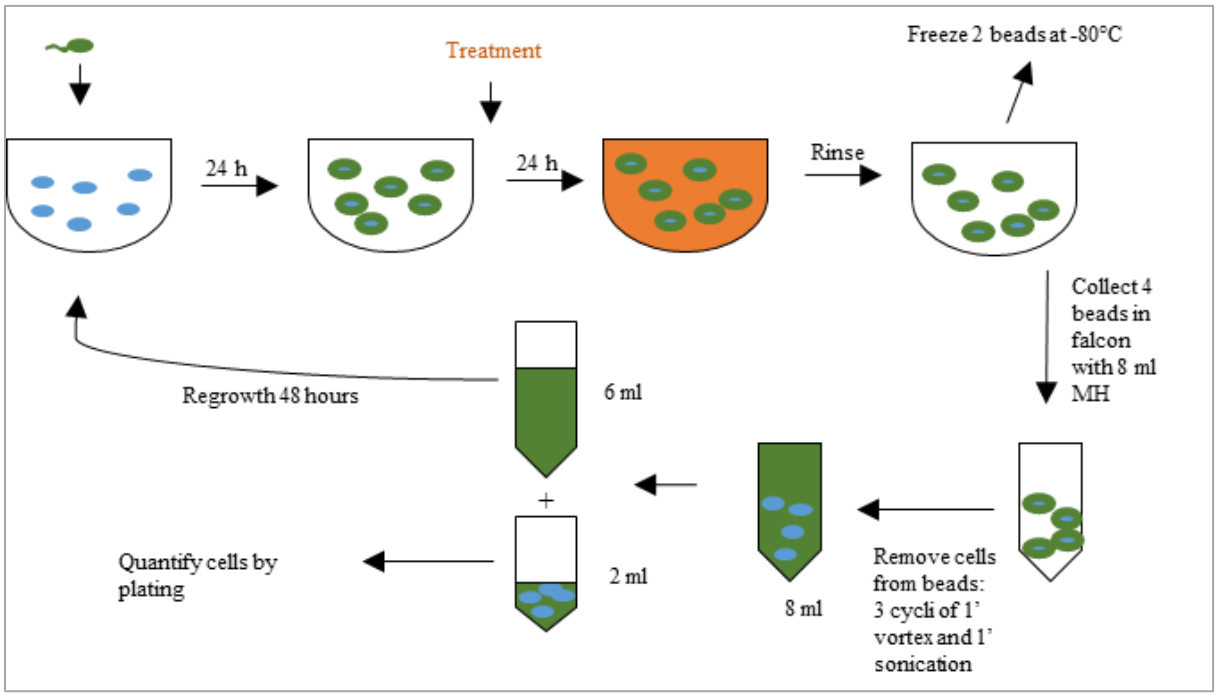



*Figure 1:* ***Experimental set up.*** *Fresh inoculum (green) is added to six cryobeads (blue full circles) in each well of a 24-well microtiter plate. After 24 hours, mature biofilms (green circles) are formed on the surface of the beads. These biofilms are treated (orange) for 24 hours. Afterwards, the supernatant is removed and the beads are rinsed with PS. Two beads, containing a mature biofilm, are stored at -80°C. The four other beads are transferred to a falcon tube, in which the sessile cells from the beads are harvested. A part of these cells is used for quantification, while another part is used for planktonic regrowth of the cells (48 hours).*

Biofilms were treated with PS (untreated control), TOB alone (at a concentration of 768 µg/ml which equals 3 times the minimal inhibitory concentration [MIC]), and TOB in combination with BH (250 µM), ECO (1 µM) or MICO (1 µM). The concentration of each component was selected based on preliminary experiments: the concentrations used in the present study lead to a significant reduction in cell numbers compared to the untreated control, but not complete eradication, so that regrowth in following cycles can occur. Three independent experiments (designated as lineages) were set up for each condition, i.e. TOB (tobramycin), TB (tobramycin + baicalin hydrate), TE (tobramycin + econazole), TM (tobramycin + miconazole) and an untreated control (Ctrl). In what follows, this designation is followed by a code indicating cycle number and lineage (e.g. TOB 8.2 refers to the tobramycin-treated biofilm after 8 cycles of biofilm formation and treatment from lineage 2). The three lineages were started from three independent pre-cultures. Biofilms were grown as described above and after 24 hours the beads were rinsed with PS and treated with TOB or a combination of TOB + potentiator. After 24 hours of treatment at 37°C, the supernatant was removed and the beads were rinsed with PS. Each well contained 6 beads: two beads were transferred to Eppendorf tubes containing 8% dimethyl sulfoxide (DMSO; Sigma-Aldrich) in MH for storage at -20°C, while the four

remaining beads were transferred to a Falcon tube containing 8 ml MH medium. Sessile cells were detached from the beads by three cycles of vortexing (1 min, Vortex-Genie 2, Scientific Industries Inc., Bohemia, NY, USA) and sonicating (1 min; Branson 3510, Branson Ultrasonics Corp, Danbury, CT, USA). Six ml of this bacterial suspension was transferred to another tube and was incubated for 48 hours, while shaking at 250 rpm at 37°C (KS 4000i control, IKA Works, Wilmington, NC, USA). The remaining 2 ml was used to determine the number of surviving cells per bead (CFU/bead) by plating.

### Determination of the minimal inhibitory concentration

To verify if possible changes in susceptibility over time were due to increased resistance towards TOB, the MIC for TOB was determined for the start and end population. MICs were determined according to the EUCAST broth microdilution assay using flat-bottom 96-well microtiter plates (MTP; SPL Lifescience, Korea) [231]. The MIC was defined as the lowest concentration with a similar optical density as uninoculated growth medium. Absorbance was measured at 590 nm with a multilabel MTP reader (EnVision, Perkin Elmer LAS, Waltham, MA). All MIC determinations were performed in duplicate.

### Genome sequencing

After planktonic regrowth of the cells, DNA was extracted using a modified bead-beater protocol, adapted from Mahenthiralingam et al.[263]. Briefly, the pellets were resuspended in 200 µl TE-buffer (10 mM Tris-HCl [Roche Diagnostics, Mannheim, Germany]), 1 mM ethylenediaminetetraacetic acid ([EDTA; Sigma-Aldrich], pH 8.0). 100 µl of this suspension was mixed with 500 µl lysis buffer (50 mM Tris-HCl, 70 mM EDTA, 1% sodium dodecyl sulphate (SDS; Sigma-Aldrich), pH 8) containing 0.5 mg/ml pronase (Roche) and 0.1-mm-diameter glass beads (Sigma-Aldrich). All tubes were then incubated for 1 hour at 37°C. Afterwards, the tubes were briefly centrifuged and 200 µl saturated ammonium acetate was added to the lysate. After vortexing and centrifuging of the tubes, 600 µl chloroform (Sigma-Aldrich) was added and the tubes were mixed vigorously. Proteins and polysaccharides were removed by centrifugation (13000 rpm, 5 min), and nucleic acids were collected from the lysate by ethanol precipitation. After degrading the remaining RNA (with 0.5 µg/ml RNase A [Sigma-Aldrich]), DNA was quantified using the BioDrop µLITE (BioDrop, Cambridge, UK). Genomic DNA from ten samples was sequenced, i.e. untreated *B. cenocepacia* J2315 (Ctrl 0.0), five evolved populations after 15 cycles (each condition of lineage 3; Ctrl 15.3, TOB 15.3, TB 15.3, TE 15.3 and TM 15.3), and the evolved populations treated with either TOB alone (TOB 15.1 and TOB 15.2) or TOB in combination with BH (TB 15.1 and TB 15.2) from lineages 1 and 2. A library of the RNase treated samples was prepared using the NEBNext Ultra II kit from Illumina. The library was sequenced on an Illumina Nextseq 500, generating 150 bp paired-end reads. Sequenced reads were mapped to the *B. cenocepacia* J2315 reference genome [27] using CLC genomics Workbench version 10.1.1. (cut-

offs: 80% length; 80% similarity). In CLC Workbench, the InDels and Structural Variants tool was used to detect insertions and deletions (indels). The Basic Variant Detection tool was used to detect Single Nucleotide Polymorphisms (SNPs). Nucleotide polymorphisms (NPs) were considered to be real when the coverage of their mapped region was similar to the average coverage in that sample (Table S1) (coverage ± 50%) and the NP in at least 35% of the reads. This is the lowest cut-off that allows to distinguish true NPs from sequencing and mapping errors. The function of the genes that acquired mutational changes was determined using the KEGG Pathway Database and *Burkholderia* Genome Database [242,243]. The experimental protocols and the raw sequencing data can be found in ArrayExpress under the accession number E-MTAB-6236.

### Statistical analysis

To determine whether the observed variations in survival over time for the different treatments were statistically significant, a linear mixed-effect model (LMEM) was used. The model uses log(CFU/bead) as the dependent variable and cycle, treatment, lineage and their two- and three-way interactions as fixed effects and was fit using SAS version 9.4 (SAS institute, Cary, NC, USA). To account for possible correlations between the measurements over cycles, a compound symmetry variance covariance structure was used. All interaction effects that were not significant were excluded from the model. When an interaction was significant, this was considered as the fixed effect to evaluate differences in treatment effect. Per lineage, treatments were compared pairwise to TOB treatment using the Tukey adjusted p-values. Assumptions associated with the LMEM were checked based on residuals from the fitted final model (Table S2).

## Results and discussion

### Biofilm formation on beads

After 24 hours of growth on the beads, LIVE/DEAD staining was performed to evaluate biofilm formation. A dense biofilm was formed in the cavity of the doughnut-shaped bead (rather than on the exterior sides of the bead) with approximately $10^7$ CFU/bead (Figure 2).

**A** **B**

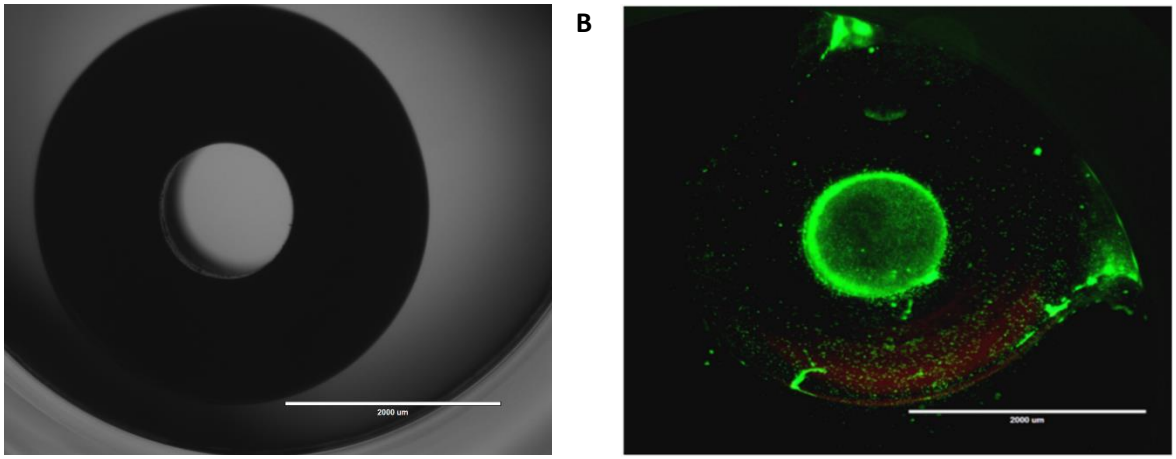



*Figure 2: **A**. Bead without biofilm. **B**. LIVE/DEAD staining of 24 hour old B. cenocepacia biofilm grown on a bead.*

## Experimental evolution

The number of log(CFU/bead) after every treatment is shown in Figure 3 and Table S3. After fitting the LMEM and plotting the residuals against the corresponding fitted values, no serious departures from the main assumptions of normality and constancy of error variance were indicated (Table 1 and S2).

*Table 1:* ***Most relevant results of the LMEM per lineage.*** *Treatments were pairwise compared to TOB over time (t-values).*

| | **Factor** | **t value** | **p value** |
|---|---|---|---|
| **Lineage 1** | Ctrl | -4.09 | <0.0001 |
| | TB | 2.71 | 0.008 |
| | TE | 1.94 | 0.05 |
| | TM | 0.57 | 0.57 |
| **Lineage 2** | Ctrl | -4.41 | <0.0001 |
| | TB | 6.13 | <0.0001 |
| | TE | 0.89 | 0.38 |
| | TM | 1.24 | 0.22 |
| **Lineage 3** | Ctrl | -2.49 | 0.014 |
| | TB | 6.08 | <0.0001 |
| | TE | 4.06 | <0.0001 |
| | TM | 4.74 | <0.0001 |

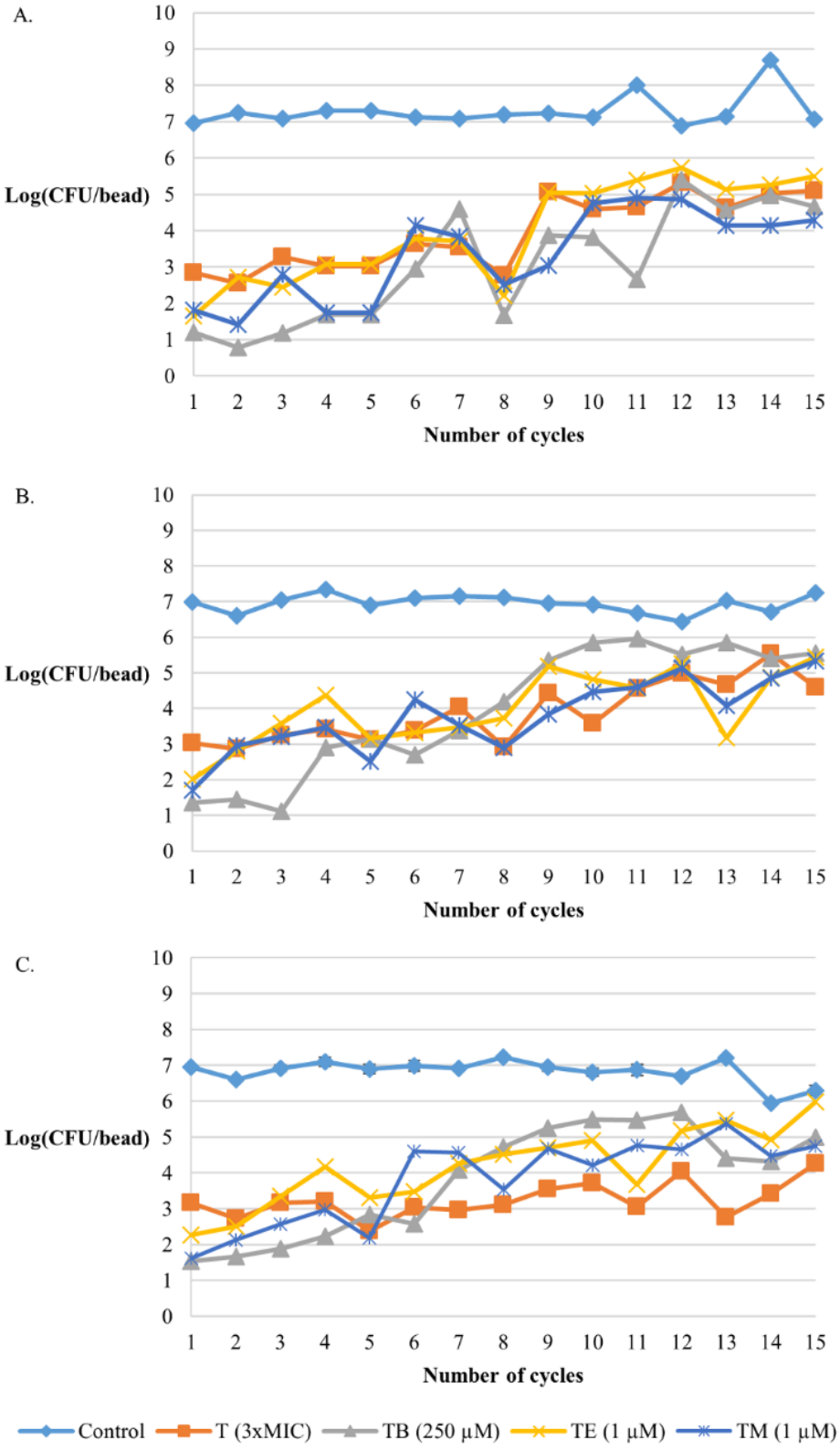



*Figure 3:* ***Number of B. cenocepacia J2315 biofilm cells****, expressed as log(CFU/bead), in the untreated control (Ctrl) and after repeated treatments with TOB, and TOB in combination with BH (TB), ECO (TE) or MICO (TM) in lineage 1 (A), lineage 2 (B) and lineage 3 (C).*

At the start of this experiment, cells were more susceptible to the different combination treatments than to TOB alone, indicating that BH, ECO and MICO potentiate the activity of TOB against biofilms, as previously shown [86,128]. Over time, biofilm-grown *B. cenocepacia* J2315 cells became gradually less susceptible to the treatment (both to treatment with TOB alone and to combination treatments); this occurred in all lineages.

Evolution towards reduced susceptibility occurred faster with the combined TB treatment, as significantly higher t-values were observed for the TB treatment, than for the TOB treatment (lineage 1: $p = 0.008$; lineage 2 and 3: $p < 0.0001$) (Table 1). In lineage 3 this was also the case for TE and TM (in both cases $p < 0.0001$); however there was no statistically significant difference between TOB and either TE or TM in lineage 1 and 2 (Table 1 and Table S2). Although there were some minor differences between the three lineages, our data indicated that in all lineages, the TOB-potentiating activity of BH, ECO and MICO was lost after 15 cycles, i.e. the combination of TOB+potentiator was not able to kill more cells than treatment with TOB alone (Figure S1).

### Genome analysis

To investigate the reason behind the decreased susceptibility, whole genome sequencing (WGS) was performed. All evolved populations treated with TOB and TB were included; as there was little variation in cell numbers in untreated control in the three lineages, only one sample was included, Ctrl 15.3. Likewise, as ECO and MICO showed overall a similar effect on TOB susceptibility in the three lineages, only the evolved cells of samples from lineage 3 were included in this experiment.

Overall, 25 variants were observed in the evolved populations compared to the start population (Table 2). These variants included one deletion and 24 NPs, affecting 22 genes. The deletion was in-frame and affected 6 amino acids. No evidence was found for transposons changing position. All NPs were found in protein-coding DNA and 23 resulted in nonsynonymous substitutions. Three genes had two NPs each. There was no overlap in affected genes between the present study and previous evolution studies in Bcc bacteria [264–266]. This is likely due to differences in experimental set-ups and strains used and suggests the observed mutations are linked to the observed phenotype. Furthermore, none of the SNPs were located in genes known to be responsible for aminoglycoside resistance such as genes encoding for aminoglycoside-modifying enzymes, efflux pumps, or ribosome methyltransferases [17,27]. This indicates that the overall gradual decrease in susceptibility of *B. cenocepacia* J2315 biofilm cells treated with TOB alone is not caused by a resistance mechanism specific for TOB, which is in line with the unchanged MIC for TOB (Table 3). This lack of mutations in resistance markers could at least be partly due to the experimental set-up as mutations which have a

positive impact on survival in the presence of TOB but a fitness cost in the absence of TOB could have been lost during the regrowth phase.

*Table 2: **Structural variants (nucleotide polymorphisms [A] and indels [B]) observed in the population**.*

*A. Nucleotide polymorphisms*

| Gene | Features | Variant | Sample | Frequency (%) | Coverage |
|---|---|---|---|---|---|
| **BCAL0296** | ABC transporter ATP-binding membrane protein | S331STOP | 15.1 TB | 98.47 | 326 |
| | | | 15.2 TOB | 98.53 | 613 |
| | | | 15.2 TB | 98.84 | 604 |
| | | I269N | 15.3 TB | 98.84 | 864 |
| | | | 15.3 TE | 98.99 | 594 |
| | | | 15.3 TM | 99.03 | 518 |
| **BCAL1723** | precorrin-3b C17-methyltransferase | A319A | 15.1 TOB | 39.48 | 385 |
| | | | 15.2 TOB | 39.36 | 404 |
| | | | 15.2 TB | 38.18 | 406 |
| | | | 15.3 TB | 40.23 | 604 |
| | | | 15.3 TE | 40.52 | 427 |
| **BCAL1736** | conserved hypothetical protein | R136G | 15.1 TB | 43.11 | 225 |
| | | | 15.2 TOB | 45.72 | 409 |
| | | | 15.2 TB | 48.56 | 416 |
| | | | 15.3 TB | 46.25 | 586 |
| | | | 15.3 TE | 50.36 | 417 |
| | | | 15.3 TM | 50.89 | 395 |
| **BCAL1937** | putative phosphorous metabolism-related protein | V485D | 15.3 TE | 81.54 | 493 |
| **BCAL1939** | putative integral membrane transport protein | A320G | 15.2 TB | 35.51 | 414 |
| **BCAL2426** | putative membrane protein | L246R | 15.3 Ctrl | 36.92 | 390 |
| **BCAL2628** | putative porphyrin biosynthesis related protein | E232STOP | 15.3 Ctrl | 87.33 | 647 |
| | | | 15.3 TOB | 77.12 | 695 |
| **BCAL2631** | phosphoenolpyruvate carboxylase | R724C | 15.2 TB | 80.00 | 640 |
| **BCAL2751** | putative ketopantoate reductase | E92A | 15.3 TOB | 36.27 | 579 |

| | | | | | |
|---|---|---|---|---|---|
| **BCAL3040** | ABC transporter, membrane permease | F51L | 15.3 TB | 99.38 | 804 |
| | | | 15.3 TE | 99.32 | 584 |
| | | | 15.3 TM | 99.52 | 631 |
| **BCAM0698** | FAD dependent oxidoreductase | D20A | 15.3 TB | 40.14 | 583 |
| **BCAM0747** | putative membrane protein | L86R | 15.1 TOB | 36.30 | 405 |
| | | | 15.2 TOB | 35.35 | 430 |
| | | | 15.2 TB | 39.96 | 453 |
| | | | 15.3 TOB | 39.08 | 435 |
| | | E89A | 15.1 TOB | 38.23 | 429 |
| | | | 15.1 TB | 40.08 | 252 |
| | | | 15.2 TB | 37.53 | 453 |
| **BCAM0821** | putative methyl-accepting chemotaxis protein | A369C | 15.3 Ctrl | 51.93 | 649 |
| **BCAM0888** | conserved hypothetical protein | P187P | 15.2 TOB | 38.26 | 413 |
| **BCAM0965** | lactate/malate dehydrogenase | W254C | 15.1 TB | 56.66 | 353 |
| | | S238STOP | 15.2 TB | 98.74 | 634 |
| **BCAM1405** | levansucrase | V40G | 15.1 TOB | 35.04 | 391 |
| | | | 15.3 Ctrl | 37.53 | 437 |
| **BCAM1677** | conserved hypothetical protein | D138G | 15.2 TOB | 35.57 | 388 |
| **BCAM1683** | putative bifunctional nitrate/sulfite reductase | V370G | 15.1 TB | 36.64 | 232 |
| | | | 15.3 Ctrl | 37.47 | 379 |
| **BCAM1870** | N-acylhomoserine lactone synthase CepI | C131W | 15.1 TB | 98.41 | 378 |
| | | | 15.2 TOB | 98.37 | 676 |
| | | | 15.2 TB | 98.82 | 680 |
| **BCAM2284** | putative mandelate racemase/muconate lactonizing enzyme | P103S | 15.3 Ctrl | 98.35 | 668 |
| | | | 15.3 TOB | 98.13 | 643 |
| **pBCA011** | putative adhesin (pseudogene) | N614H | 15.3 TE | 80.98 | 589 |

*B. Indels*

| Gene | Feature | Type | Region | Length | Sample | Variant ratio |
|---|---|---|---|---|---|---|
| **BCAL0736** | PTS system EI component | Deletion | 802697..802714 | 18 | Ctrl 15.3 | 0.98 |
| | | | | | TOB 15.3 | 0.97 |

*Table 3:* ***MIC of TOB in B. cenocepacia J2315 recovered from different samples****.*

| Sample | MIC (µg/ml) |
|---|---|
| **Ctrl 0.0 (start)** | 256 |
| **Ctrl 15.3** | 256 |
| **TOB 15.3** | 256 |
| **TB 15.3** | 256 |
| **TOB 15.2** | 128 |
| **TB 15.2** | 128 |
| **TOB 15.1** | 128 |
| **TB 15.1** | 256 |

Some NPs were common and appeared in multiple samples on the same location, whereas others were more random and only occurred in single samples. This phenomenon was also described in other evolution experiments [267,268]. Mutations appearing in multiple samples were probably already present in the start population and were enriched for during the experiment.

The mutations found in the present study could represent new mechanisms to decrease biofilm susceptibility to TOB. Seven of the affected genes also showed mutations in the untreated population, these are therefore not linked to antibiotic treatment. For six of the remaining 15 genes, mutations were found in multiple conditions, in five of these cases mutations were also found after treatment with TOB alone. Only nine mutations in total occur only in combinations treatments, five of them in TB treated lineages. Three mutations only occur in lineages treated with TOB alone. Overall the combination treatments lead only to a marginally different outcome regarding genome changes compared to treatment with TOB alone.

The mutation in BCAM1870, encoding for N-acylhomoserine lactone synthase CepI, is found in populations treated with TB, but also in populations treated with TOB alone. Therefore, although BH is a QSI, this change is not specific to treatment with BH. However, this mutation suggests that QS can play a role in TOB resistance, which is in line with the TOB-potentiating effect of BH [269].

Of the NPs occurring in only one condition, substitutions in two genes are of particular interest because they occur both only in TB treated populations and affect core metabolic functions. These genes are BCAL2631 (R724C), encoding for a phosphoenolpyruvate (PEP) carboxylase, and in BCAM0965 (W254C in TB 15.1 and S238STOP in TB 15.2), encoding a lactate/malate dehydrogenase (MDH). These genes are involved in pyruvate metabolism, more specifically in the PEP-pyruvate-oxaloacetate node [270]. This node is the metabolic link between glycolysis/gluconeogenesis and the

TCA cycle, and it directs the carbon flux in the appropriate direction. In glycolytic conditions, PEP and pyruvate are converted to acetyl-CoA (via the pyruvate dehydrogenase complex) and oxaloacetate (via PEP carboxylase), respectively, and enter the TCA cycle. In gluconeogenic conditions, the TCA intermediates oxaloacetate or malate are converted to pyruvate and PEP and serve as precursors for gluconeogenesis [270]. PEP carboxylase (BCAL2631) catalyses the carboxylation of PEP to yield oxaloacetate, whereas MDH (BCAM0965) catalyses the reversible and NAD-dependent conversion of malate to oxaloacetate [271]. MDH is involved in the conversion of malate to oxaloacetate during the TCA cycle, but it also participates in the protection against oxidative stress since oxaloacetate binds to free radicals [272]. Moreover, MDH, malic enzymes and pyruvate carboxylase have been shown to convert NADH to NADPH, which is an anti-oxidant [273]. As previous work showed that BH increased antibiotic-induced oxidative stress by altering several metabolic pathways [269], it is possible that mutations in BCAM0965 (MDH) and BCAL2631 (PEP carboxylase) lead to decreased susceptibility towards TB by modulating the oxidative stress response.

Interestingly, the potentiating agents used in the present study have all been shown to increase oxidative stress: BH in combination with TOB in *B. cenocepacia* biofilm cells [269], MICO in *Staphylococcus aureus* and in *Candida albicans* [274,275], and ECO in *Mycobacterium tuberculosis* [276]. Higher levels of oxidative stress are likely linked to an increased selective pressure, which can result in a higher rate of selection for resistant mutants [277].

## Conclusion

This study shows that under these laboratory conditions *B. cenocepacia* biofilms gradually but quickly become less susceptible to the TOB-potentiating compounds BH, MICO and ECO. Many genetic changes were observed in the evolved populations exposed to the combination of TOB and a potentiator; some of these changes point to increased efflux and changes to metabolism as mechanisms underlying the reduced susceptibility, although this remains to be confirmed. Although some genetic changes were found in multiple samples, different lineages exposed to the same treatment (TB) appeared to have used different evolutionary trajectories to counteract the potentiating activity. Our results indicate that resistance to potentiators can develop in multiple ways and this might limit the clinical applicability of such potentiators.

# Supplementary information

## Supplementary figure

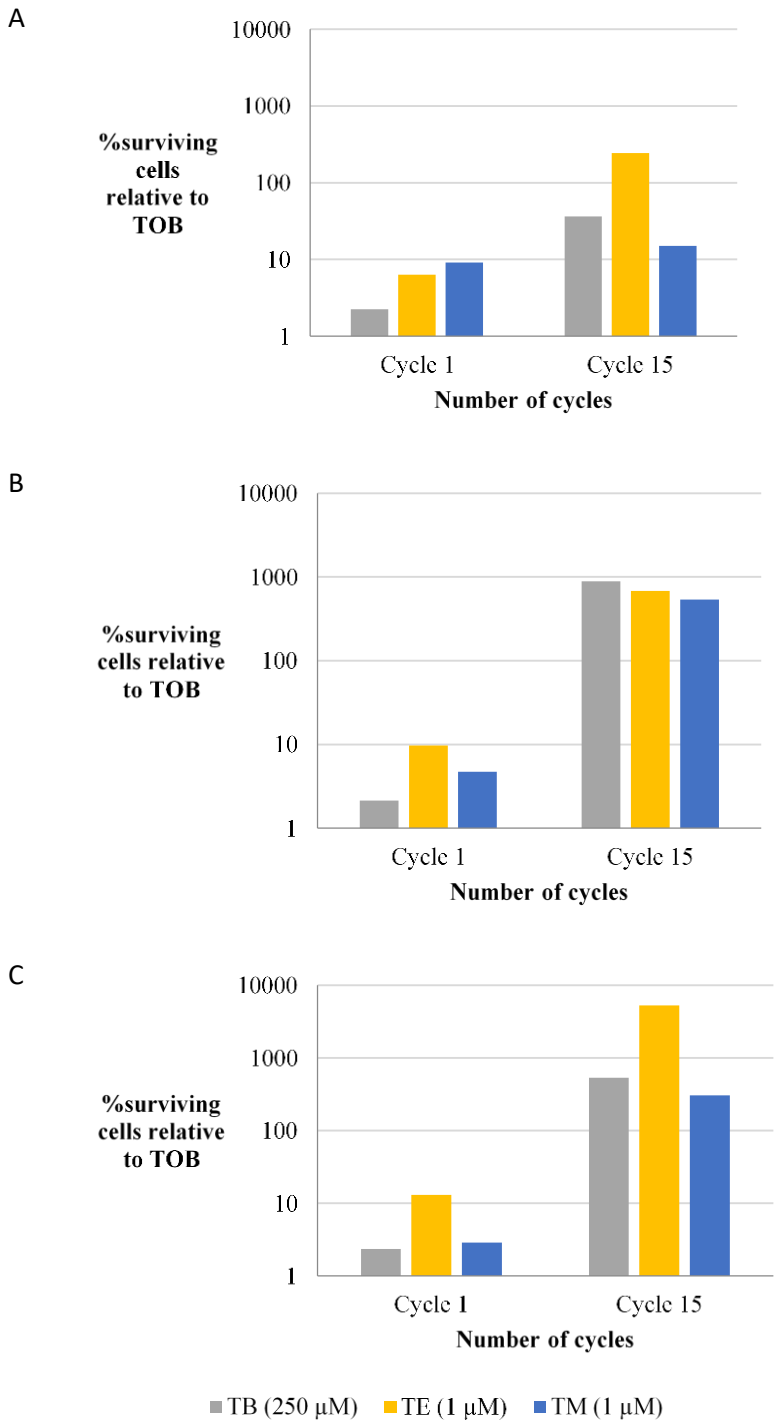



*Figure S1:* ***Percentage of surviving cells treated with a combination of TOB with BH (TB), ECO (TE) or MICO (TM) compared to treatment with TOB alone*** *(set to 100%) in lineage 1 (A), lineage 2 (B) and lineage 3 (C).*

## Supplementary tables

*Table S1:* ***Mapped reads and average coverage of each sample mapped to the reference genome*** [27].

| Sample | Mapped reads | Sample coverage |
|---|---|---|
| **0.0 Ctrl** | 41754138 | 786 |
| **15.1 TOB** | 37108378 | 698 |
| **15.1 TB** | 23340824 | 439 |
| **15.2 TOB** | 39186978 | 737 |
| **15.2 TB** | 39212192 | 738 |
| **15.3 Ctrl** | 38902414 | 732 |
| **15.3 TOB** | 42006850 | 790 |
| **15.3 TB** | 61232508 | 1152 |
| **15.3 TE** | 37870328 | 713 |
| **15.3 TM** | 39566182 | 744 |

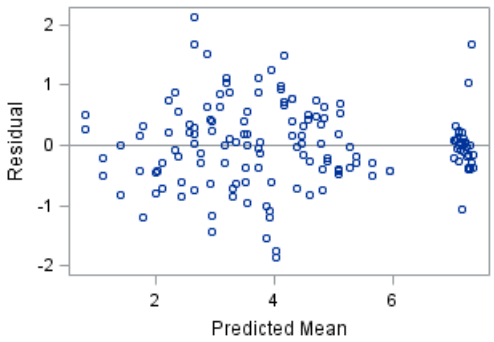
*Table S2:* ***LMEM analysis with pairwise comparisons per lineage to TOB treatments as reference****. Type 3 Tests of Fixed Effects shows that the interaction between time and treatment is significant, which means that the p-value for this interaction should be considered in the comparison between treatments per lineage.*

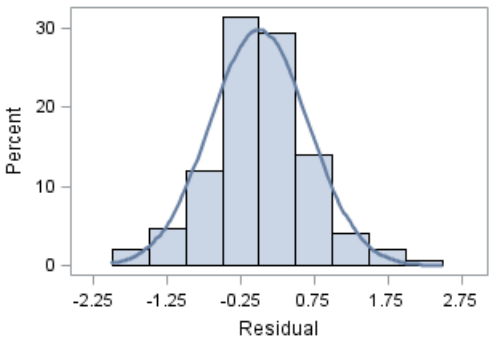
LINEAGE 1

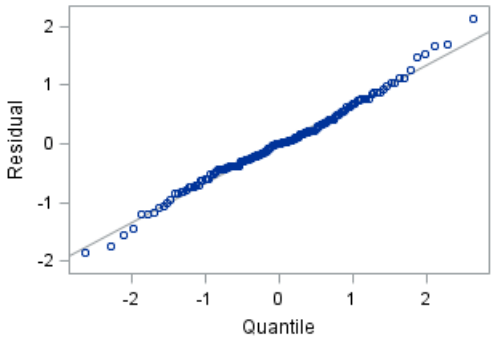
| **Type 3 Tests of Fixed Effects for lineage 1** | | | | |
|---|---|---|---|---|
| **Effect** | **Num DF** | **Den DF** | **F Value** | **Pr > F** |
| **Treatment** | 4 | 140 | 48.46 | <.0001 |
| **Timenum** | 1 | 140 | 237.70 | <.0001 |
| **Timenum*Treatment** | 4 | 140 | 13.94 | <.0001 |

| **Solution for Fixed Effects for Lineage 1** | | | | | | |
|---|---|---|---|---|---|---|
| **Effect** | **Treatment** | **Estimate** | **Standard Error** | **DF** | **t Value** | **Pr > \|t\|** |
| **Intercept** | | 2.3783 | 0.3611 | 140 | 6.59 | <.0001 |
| **Treatment** | **Control** | 4.6082 | 0.5107 | 140 | 9.02 | <.0001 |
| **Treatment** | **TB** | -1.8713 | 0.5107 | 140 | -3.66 | 0.0004 |
| **Treatment** | **TE** | -0.5404 | 0.5107 | 140 | -1.06 | 0.2918 |
| **Treatment** | **TM** | -0.7995 | 0.5107 | 140 | -1.57 | 0.1197 |
| **Treatment** | **TOB** | 0 | . | . | . | . |
| **Timenum** | | 0.1922 | 0.02923 | 140 | 6.58 | <.0001 |
| **Timenum*Treatment** | **Control** | -0.1689 | 0.04133 | 140 | -4.09 | <.0001 |
| **Timenum*Treatment** | **TB** | 0.1120 | 0.04133 | 140 | 2.71 | 0.0075 |
| **Timenum*Treatment** | **TE** | 0.08014 | 0.04133 | 140 | 1.94 | 0.0545 |
| **Timenum*Treatment** | **TM** | 0.02341 | 0.04133 | 140 | 0.57 | 0.5720 |
| **Timenum*Treatment** | **TOB** | 0 | . | . | . | . |

**Residual plots for Lineage 1**




| Residual Statistics | |
|---|---|
| Observations | 150 |
| Minimum | -1.87 |
| Mean | 18E-17 |
| Maximum | 2.1239 |
| Std Dev | 0.6704 |

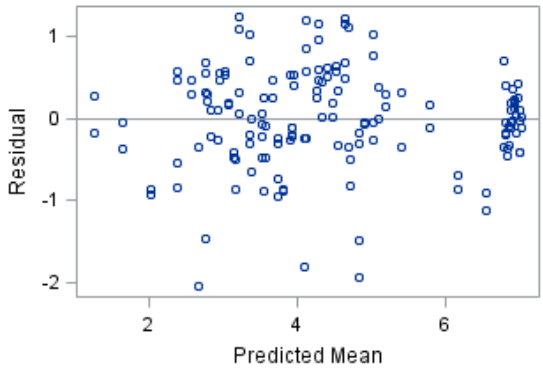
| Fit Statistics | |
|---|---|
| Objective | 342.71 |
| AIC | 346.71 |
| AICC | 346.79 |
| BIC | 345.93 |

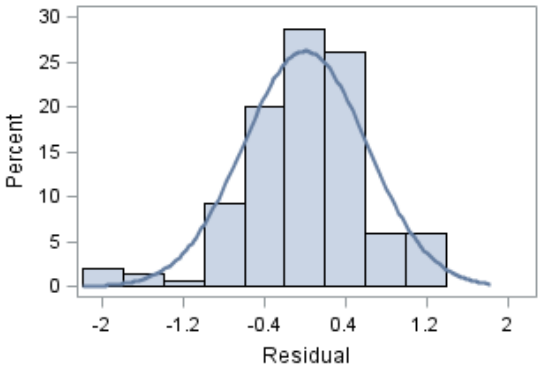
LINEAGE 2

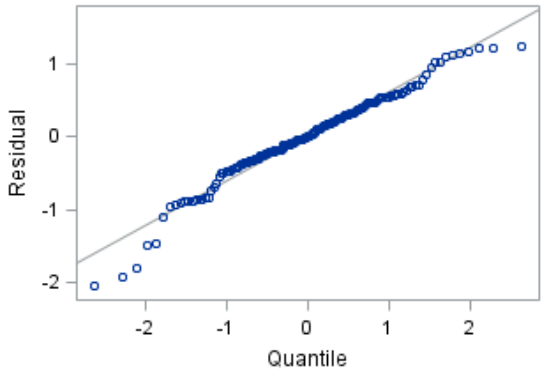
| Type 3 Tests of Fixed Effects for Lineage 2 | | | | |
|---|---|---|---|---|
| **Effect** | **Num DF** | **Den DF** | **F Value** | **Pr > F** |
| **Treatment** | 4 | 140 | 51.15 | <.0001 |
| **Timenum** | 1 | 140 | 222.43 | <.0001 |
| **Timenum*Treatment** | 4 | 140 | 28.19 | <.0001 |

| Solution for Fixed Effects for Lineage 2 | | | | | | |
|---|---|---|---|---|---|---|
| **Effect** | **Treatment** | **Estimate** | **Standard Error** | **DF** | **t Value** | **Pr > \|t\|** |
| **Intercept** | | 2.6247 | 0.3282 | 140 | 8.00 | <.0001 |
| **Treatment** | **Control** | 4.4283 | 0.4641 | 140 | 9.54 | <.0001 |
| **Treatment** | **TB** | -1.7535 | 0.4641 | 140 | -3.78 | 0.0002 |
| **Treatment** | **TE** | -0.1477 | 0.4641 | 140 | -0.32 | 0.7507 |
| **Treatment** | **TM** | -0.4426 | 0.4641 | 140 | -0.95 | 0.3419 |
| **Treatment** | **TOB** | 0 | . | . | . | . |
| **Timenum** | | 0.1482 | 0.02656 | 140 | 5.58 | <.0001 |
| **Timenum*Treatment** | **Control** | -0.1655 | 0.03756 | 140 | -4.41 | <.0001 |
| **Timenum*Treatment** | **TB** | 0.2304 | 0.03756 | 140 | 6.13 | <.0001 |
| **Timenum*Treatment** | **TE** | 0.03333 | 0.03756 | 140 | 0.89 | 0.3765 |
| **Timenum*Treatment** | **TM** | 0.04663 | 0.03756 | 140 | 1.24 | 0.2165 |
| **Timenum*Treatment** | **TOB** | 0 | . | . | . | . |

**Residual plots for Lineage 2**




| Residual Statistics | |
|---|---|
| Observations | 150 |
| Minimum | -2.056 |
| Mean | -4E-16 |
| Maximum | 1.2442 |
| Std Dev | 0.6092 |

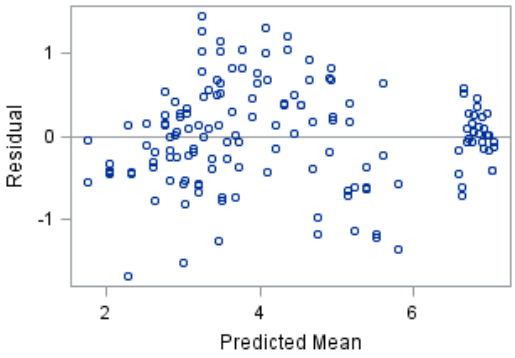
| Fit Statistics | |
|---|---|
| Objective | 315.92 |
| AIC | 319.92 |
| AICC | 320.01 |
| BIC | 319.14 |

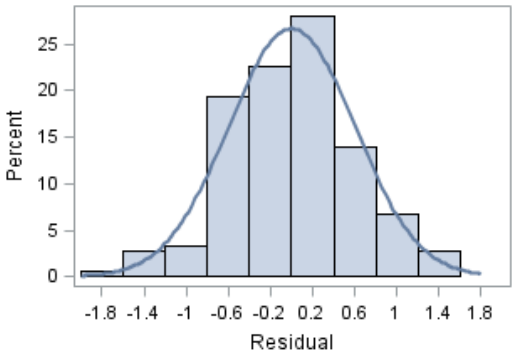
LINEAGE 3

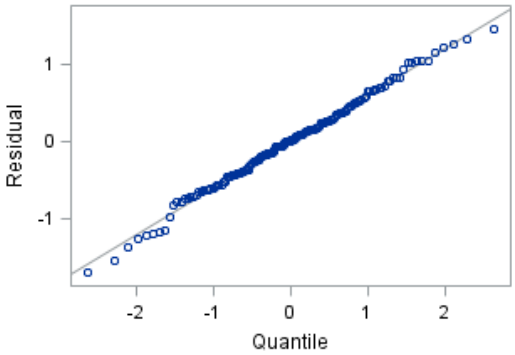
| Type 3 Tests of Fixed Effects for Lineage 3 | | | | |
|---|---|---|---|---|
| **Effect** | **Num DF** | **Den DF** | **F Value** | **Pr > F** |
| **Treatment** | 4 | 139 | 110.03 | <.0001 |
| **Timenum** | 1 | 135 | 168.55 | <.0001 |
| **Timenum*Treatment** | 4 | 135 | 25.72 | <.0001 |

| Solution for Fixed Effects for Lineage 3 | | | | | | |
|---|---|---|---|---|---|---|
| **Effect** | **Treatment** | **Estimate** | **Standard Error** | **DF** | **t Value** | **Pr > \|t\|** |
| **Intercept** | | 2.7140 | 0.2171 | 139 | 12.50 | <.0001 |
| **Treatment** | **Control** | 4.3607 | 0.3071 | 139 | 14.20 | <.0001 |
| **Treatment** | **TB** | -1.2445 | 0.3071 | 139 | -4.05 | <.0001 |
| **Treatment** | **TE** | -0.3142 | 0.3071 | 139 | -1.02 | 0.3080 |
| **Treatment** | **TM** | -0.8995 | 0.3071 | 139 | -2.93 | 0.0040 |
| **Treatment** | **TOB** | 0 | . | . | . | . |
| **Timenum** | | 0.06101 | 0.02649 | 135 | 2.30 | 0.0228 |
| **Timenum*Treatment** | **Control** | -0.09340 | 0.03746 | 135 | -2.49 | 0.0139 |
| **Timenum*Treatment** | **TB** | 0.2277 | 0.03746 | 135 | 6.08 | <.0001 |
| **Timenum*Treatment** | **TE** | 0.1521 | 0.03746 | 135 | 4.06 | <.0001 |
| **Timenum*Treatment** | **TM** | 0.1774 | 0.03746 | 135 | 4.74 | <.0001 |
| **Timenum*Treatment** | **TOB** | 0 | . | . | . | . |

**Residual plots for Lineage 3**




| Residual Statistics | |
|---|---|
| Observations | 150 |
| Minimum | -1.689 |
| Mean | 66E-17 |
| Maximum | 1.4506 |
| Std Dev | 0.5985 |

| Fit Statistics | |
|---|---|
| Objective | 306.32 |
| AIC | 310.32 |
| AICC | 310.41 |
| BIC | 310.93 |

*Table S3: Number of CFU recovered at different time points. The data shown are two technical replicates (1) and (2) expressed as log(CFU/bead) at each time point for each treatment.*

| **Lineage 1** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Treatment | | | | | | | | | |
| | Control | | TOB (3 x MIC) | | TB (250 µM) | | TE (1 µM) | | TM (1 µM) | |
| **Time (cycles)** | (1) | (2) | (1) | (2) | (1) | (2) | (1) | (2) | (1) | (2) |
| **1** | 7.08 | 6.79 | 2.78 | 2.91 | 1.30 | 1.08 | 1.82 | 1.38 | 2.10 | 0.60 |
| **2** | 7.11 | 7.35 | 2.62 | 2.48 | 0.60 | 0.90 | 2.94 | 2.19 | 1.56 | 1.20 |
| **3** | 7.15 | 7.00 | 3.34 | 3.19 | 1.41 | 0.60 | 1.91 | 2.68 | 2.97 | 2.45 |
| **4** | 7.31 | 7.30 | 2.86 | 3.15 | 1.88 | 1.30 | 3.34 | 2.29 | 1.83 | 1.60 |
| **5** | 6.85 | 7.11 | 3.38 | 2.70 | 1.60 | 1.60 | 4.30 | 4.25 | 2.94 | 2.84 |
| **6** | 7.05 | 7.18 | 3.72 | 3.53 | 2.26 | 3.20 | 3.88 | 3.64 | 4.39 | 3.51 |
| **7** | 7.37 | 6.08 | 3.68 | 3.34 | 4.30 | 4.76 | 3.60 | 3.79 | 3.92 | 3.73 |
| **8** | 7.07 | 7.28 | 2.72 | 2.83 | 1.78 | 1.51 | 2.15 | 2.26 | 2.45 | 2.58 |
| **9** | 7.26 | 7.21 | 5.03 | 5.08 | 4.11 | 3.34 | 5.05 | 5.06 | 2.90 | 3.15 |
| **10** | 7.19 | 7.03 | 4.45 | 4.70 | 4.10 | 2.60 | 4.97 | 5.07 | 4.86 | 4.60 |
| **11** | 8.28 | 7.08 | 4.33 | 4.82 | 2.30 | 2.85 | 5.46 | 5.28 | 5.20 | 3.34 |
| **12** | 6.87 | 6.89 | 5.43 | 5.17 | 4.88 | 5.64 | 5.64 | 5.80 | 4.83 | 4.89 |
| **13** | 7.30 | 6.88 | 4.66 | 4.60 | 4.62 | 4.48 | 5.09 | 5.18 | 4.36 | 3.67 |
| **14** | 7.02 | 8.99 | 4.58 | 5.25 | 4.78 | 5.11 | 5.14 | 5.35 | 4.34 | 3.78 |
| **15** | 7.16 | 6.95 | 5.25 | 4.89 | 4.68 | 4.64 | 5.49 | 5.48 | 4.08 | 4.41 |
| | | | | | | | | | | |
| **Lineage 2** | | | | | | | | | | |
| | Treatment | | | | | | | | | |
| | Control | | TOB (3 x MIC) | | TB (250 µM) | | TE (1 µM) | | TM (1 µM) | |
| **Time (cycles)** | (1) | (2) | (1) | (2) | (1) | (2) | (1) | (2) | (1) | (2) |
| **1** | 6.91 | 7.05 | 2.99 | 3.08 | 1.08 | 1.53 | 0.60 | 2.31 | 1.53 | 1.83 |
| **2** | 6.60 | 6.60 | 2.65 | 3.02 | 1.26 | 1.58 | 2.93 | 2.62 | 3.03 | 2.88 |
| **3** | 6.97 | 7.10 | 3.23 | 3.26 | 1.08 | 1.15 | 3.60 | 3.56 | 3.08 | 3.31 |
| **4** | 7.24 | 7.41 | 3.53 | 3.27 | 2.95 | 2.85 | 4.45 | 4.30 | 3.43 | 3.51 |
| **5** | 6.79 | 7.00 | 3.17 | 3.06 | 1.30 | 3.45 | 3.38 | 2.74 | 2.66 | 2.28 |
| **6** | 7.07 | 7.15 | 3.03 | 3.58 | 2.73 | 2.66 | 3.08 | 3.48 | 4.06 | 4.38 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **7** | 7.17 | 7.14 | 3.92 | 4.13 | 3.45 | 3.30 | 3.51 | 3.45 | 2.65 | 3.79 |
| **8** | 6.89 | 7.27 | 2.93 | 2.91 | 3.64 | 4.43 | 3.73 | 3.72 | 2.78 | 3.00 |
| **9** | 7.08 | 6.78 | 4.37 | 4.49 | 5.43 | 5.23 | 4.97 | 5.32 | 3.83 | 3.83 |
| **10** | 6.99 | 6.83 | 3.87 | 2.30 | 5.82 | 5.89 | 4.76 | 4.88 | 4.72 | 3.88 |
| **11** | 6.78 | 6.53 | 4.59 | 4.51 | 5.81 | 6.06 | 4.49 | 4.66 | 4.76 | 4.34 |
| **12** | 6.48 | 6.40 | 4.92 | 5.03 | 5.74 | 5.07 | 5.15 | 5.34 | 5.16 | 5.09 |
| **13** | 6.63 | 7.23 | 4.88 | 4.21 | 5.68 | 5.96 | 2.90 | 3.34 | 4.22 | 3.89 |
| **14** | 6.77 | 6.63 | 5.82 | 4.34 | 5.48 | 5.31 | 4.96 | 4.76 | 4.83 | 4.86 |
| **15** | 6.45 | 7.51 | 4.66 | 4.53 | 5.64 | 5.43 | 5.51 | 5.35 | 5.10 | 5.48 |
| | | | | | | | | | | |
| **Lineage 3** | | | | | | | | | | |
| | Treatment | | | | | | | | | |
| | Control | | TOB (3 x MIC) | | TB (250 µM) | | TE (1 µM) | | TM (1 µM) | |
| **Time (cycles)** | (1) | (2) | (1) | (2) | (1) | (2) | (1) | (2) | (1) | (2) |
| **1** | 6.97 | 6.92 | 2.92 | 3.32 | 1.20 | 1.72 | 2.30 | 2.25 | 1.64 | 1.60 |
| **2** | 6.60 | 6.60 | 2.59 | 2.83 | 1.62 | 1.72 | 2.65 | 2.30 | 2.43 | 0.60 |
| **3** | 7.00 | 6.82 | 2.90 | 3.32 | 1.88 | 1.90 | 3.32 | 3.38 | 2.68 | 2.42 |
| **4** | 6.95 | 7.21 | 3.23 | 3.19 | 1.86 | 2.44 | 4.04 | 4.28 | 2.90 | 3.03 |
| **5** | 7.01 | 6.76 | 2.20 | 2.49 | 2.97 | 2.66 | 2.21 | 3.60 | 2.44 | 1.48 |
| **6** | 7.12 | 6.81 | 2.85 | 3.18 | 2.52 | 2.62 | 3.70 | 2.95 | 4.51 | 4.70 |
| **7** | 6.96 | 6.88 | 2.95 | 2.99 | 4.13 | 4.01 | 4.35 | 4.13 | 4.51 | 4.63 |
| **8** | 7.17 | 7.27 | 3.33 | 2.60 | 4.60 | 4.82 | 3.68 | 4.80 | 3.35 | 3.66 |
| **9** | 6.84 | 7.03 | 3.73 | 3.26 | 5.38 | 5.08 | 4.71 | 4.69 | 4.73 | 4.61 |
| **10** | 6.90 | 6.69 | 3.89 | 3.41 | 5.39 | 5.57 | 4.90 | 4.90 | 4.04 | 4.34 |
| **11** | 7.00 | 6.70 | 2.99 | 3.11 | 5.33 | 5.58 | 3.56 | 3.76 | 4.94 | 4.48 |
| **12** | 6.78 | 6.61 | 3.94 | 4.12 | 5.61 | 5.75 | 5.15 | 5.20 | 4.28 | 4.85 |
| **13** | 7.24 | 7.16 | 2.78 | 2.73 | 4.60 | 4.09 | 5.57 | 5.35 | 5.62 | 4.73 |
| **14** | 5.90 | 6.00 | 3.51 | 3.30 | 4.30 | 4.34 | 5.02 | 4.78 | 4.45 | 4.51 |
| **15** | 6.41 | 6.15 | 3.93 | 4.46 | 5.23 | 4.45 | 6.24 | 5.37 | 4.75 | 4.76 |

# CHAPTER IV:
# BROADER INTERNATIONAL CONTEXT, RELEVANCE AND FUTURE PERSPECTIVES

# 1 Antimicrobial resistance in multidrug resistant pathogens

The emergence of antibiotic resistance compromises the treatment of infectious diseases and undermines many other advances in healthcare and medicine [1]. Currently, we are living in an era in which antibiotic resistance is spreading at alarming rates, without effective antibacterial agents in the pipeline to stop this. Especially multidrug resistant (MDR) pathogens are a problem since they have developed resistance towards almost every available bactericidal/bacteriostatic agent [5]. According to the World Health Organisation (WHO), these MDR pathogens can be subdivided in categories according to the urgency of need for new antibiotics. The most critical pathogens are the ones that pose a threat in patients that require intravenous catheters and ventilators for their care. These pathogens include carbapenem-resistant *Acinetobacter baumannii*, carbapenem-resistant *Pseudomonas aeruginosa* and various carbapenem-resistant Enterobacteriaceae that produce extended spectrum β-lactamases. They can cause fatal infections such as pneumonia or sepsis [278].

Antimicrobial resistance (AMR) is a serious threat to public health. In 2015, AMR was estimated to be responsible for 25,000 deaths per year in the EU and even up to 700,000 deaths per year globally. If current infection and resistance rates are not halted or reversed, 10 million deaths per year are expected worldwide in 2050 (Figure 1). Less than 1% of these deaths would occur in North America or Europe, whereas the largest numbers are in Africa and Asia [279]. Besides the threat to public health, AMR is also an economic burden. This can be a direct cost such as healthcare costs and productivity losses, but it can also be an indirect cost such as the losses to trade and agriculture when there is an outbreak amongst animals for consumption [5,279].

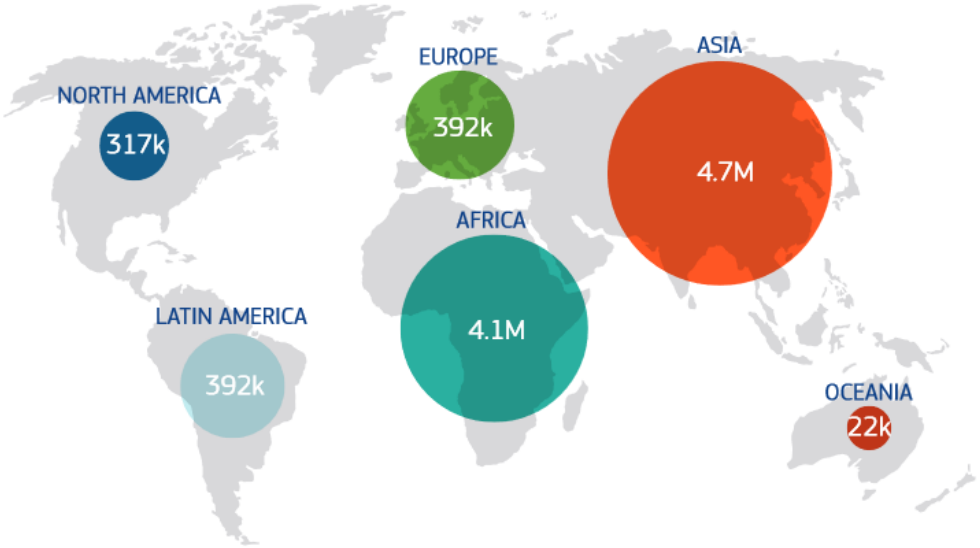



*Figure 1: Number of deaths per year attributable to AMR by 2050 if current resistance rates increased by 40% according to a factsheet of the European commission* [279]

Antimicrobial resistance is a complex issue that is driven by several interconnected factors. Hence, coordinated actions rather than isolated interventions are required to limit the emergence and spread of antimicrobial resistance. These coordinated actions are based on three pillars. Firstly, specific steps are necessary to reduce the demand of antibiotics. Secondly, the number of effective antimicrobial drugs should increase. Finally, a global coalition should be in place to obtain real action against AMR [5,279–281].

Since a "Global action plan on antimicrobial resistance" was set up in May 2015, the WHO has been leading multiple initiatives to address AMR [281]. A first initiative is the "World Antibiotic Awareness Week" themed "Antibiotics: Handle with Care". This is a global multi-year campaign that encourages best practices of antibiotics among the general public, health workers and policy makers [282]. Another initiative by the WHO is the Global Antimicrobial Resistance Surveillance System (GLASS). This system supports a standardized approach to the collection, analysis and sharing of data related to global AMR to drive local, national and regional actions [283]. Another initiative is the Global Antibiotic Research and Development Partnership (GARDP). This is a joint initiative of the WHO and Drugs for Neglected Diseases *initiative* (DND*i*) that is supported by public and private partnerships, and encourages research and development through improvement of existing antimicrobials and acceleration the development of novel antibiotic drugs [284]. Furthermore, in 2016 an Interagency Coordination Group on Antimicrobial Resistance (IACG) was set up to improve coordination between international organisations (United Nations and WHO) and to ensure effective global action against AMR. This group is a catalyst that assists in mobilizing agents across the different sectors of a multi-stakeholder process [285,286].

## 2 The high innate resistance of *Burkholderia cenocepacia*

*Burkholderia cenocepacia* is an MDR pathogen and is a member of the *Burkholderia cepacia* complex (Bcc) [157,159]. Bcc species are opportunistic pathogens that can cause severe lung infections in cystic fibrosis (CF) patients. *B. cenocepacia* and *B. multivorans* are predominantly recovered from CF lung isolates and can cause life-threatening systemic infections known as the cepacia syndrome [161,287]. The prevalence of Bcc infections in CF patients worldwide is rather low, 3 - 8% [174,175]. However, these pathogens remain problematic due to their high innate resistance towards antimicrobials, their ability of patient-to-patient transmission and the unpredictable outcome of the infection [161,287].

The high intrinsic resistance of Bcc species to most clinically available antibiotics impedes treatment. There is no straightforward optimal antibiotic regimen for patients with Bcc infection, so clinicians must assess each patient individually to determine an optimal treatment [288]. Generally, co-trimoxazole is the drug of choice to combat Bcc infections. Treatments with ceftazidime, meropenem and/or penicillins alone or in combination with other antimicrobials can also be considered [289,290]. Since Bcc species have a high intrinsic resistance towards a broad spectrum of antibiotics and have the ability to form biofilms [161], alternative therapies are necessary to eradicate biofilm-related infections and decrease the emergence of resistance.

Modern technology, like genomics, facilitates the search for novel targets to fight Bcc and related bacteria. These methods reveal resistance mechanisms and virulence factors used by the bacteria, which enables the development of novel strategies to prevent or combat infections (Figure 2) [291]. Currently, prevention of Bcc infections by immunotherapy is not yet available. Some proteins with putative immunogenic activity have been proposed as vaccine candidates, such as outer membrane proteins (OMP) or metalloproteases. However, an important aspect in vaccine design is the full knowledge of the types of host responses required for the effective clearance of the pathogen. Unfortunately this is not yet fully understood for Bcc species, since they have the ability to modulate and overcome the host immune response and to survive intracellularly in macrophages and epithelial cells [287]. Future strategies to combat Bcc infections are focused on resistance and/or virulence (e.g. quorum sensing [QS]). Genes encoding resistance or virulence factors are regulated through complex networks that are often not yet fully understood [291]. However, the addition of some non-lethal adjuvants to antibiotic therapy has been proven to increase Bcc susceptibility; such as agents that directly target resistance mechanisms like β-lactamase inhibitors [292] or quorum sensing inhibitors (QSI) like baicalin hydrate (BH) [128].

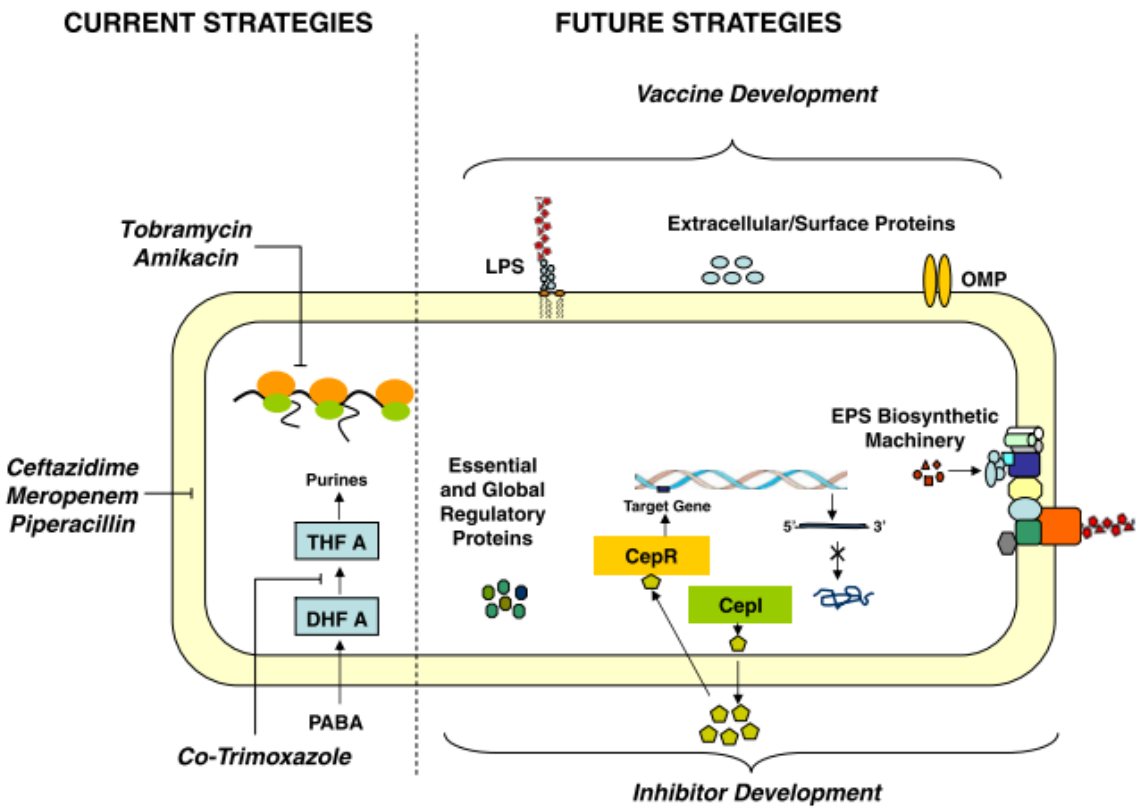



*Figure 2:* ***Current and future strategies to combat Bcc infections****. Currently used antibiotics for Bcc infections are tobramycin, amikacin, ceftazidime, meropenem, piperacillin and co-trimoxazole (PABA; p-aminobenzoic acid, DHF A; dihydrofolic acid, THF A; tetrahydrofolic acid). Future strategies can target essential and global regulatory proteins or virulence factors (e.g. QS, exopolysaccharide (EPS) biosynthesis). Structural elements such as lipopolysaccharide (LPS) or outer membrane proteins (OMP) can also be used to design immunoprotective vaccines.*

Bacterial pathogenicity can be reduced by targeting QS. This is the cell-density based communication between bacteria, that promotes group-behaviour when a certain population threshold is reached. As QS regulates the expression of many genes encoding virulence factors, agents that interfere with these communication circuits are promising antibiotic adjuvants [34]. Although QSI have been described as future antibiotic adjuvants, there is only a limited amount of research on QSI in combination with antibiotics. Research of adjuvant therapies in biofilms is also necessary since infections are predominantly biofilm-related [52].

# 3 Quorum sensing inhibitor research

## 3.1 Investigating novel quorum sensing inhibiting agents

### 3.1.1 Discovery of new agents

Current drug discovery is based on both empirical and molecular strategies. Empirical strategies are based on a quantifiable phenotypic response and are known as whole-cell screening. Molecular strategies are usually hypothesis-driven and are known as target-based [293].

In the empirical strategy, compound libraries are screened for biological activity. An important advantage of whole-cell screening is that it allows the identification of compounds that are able to cross biological barriers such as the outer membrane of Gram-negative organisms [38]. A whole-cell screening method was developed by Rasmussen et al. [294] to measure the QS inhibitory activity of novel agents using biosensors. These are genetically recombinant bacteria that harbour a QS gene circuit coupled to easy quantifiable reporter gene(s), such as *gfp*, *lacZ* or genes involved in bioluminescence. These phenotypes are expressed in response to the presence of QS signal molecules [245,295]. However, the measured phenotypes can depend on other factors and/or on the metabolic activity of the cells. Consequently, these assays are prone to errors which makes the interpretation of the results obtained difficult. To avoid these errors interesting leads are commonly verified using a second bioassay [246,296,297].

In the molecular strategy, target-driven medicinal chemistry is used to discover novel leads. Generally, target-based approaches are focused on the activity of a lead molecule towards the pharmacological target, without considering other factors such as the penetration into the bacteria, efflux issues, metabolic degradation, or ADMET (absorption, distribution, metabolism, elimination and toxicity) properties in the host. Hence, a negative outcome of follow-up biochemical assays can indicate both a lack of activity of the compound and/or difficulties to reach the target [38].

In this dissertation, we screened several azanucleosides for their QSI activity using whole cell bioassays. These azanucleosides were previously synthesized based on the structure of immucilins [227], i.e. target-based design. Immucilins inhibit MTAN, which is a bifunctional enzyme involved in the production of both AHL and AI-2, by resembling the chemical structure of the substrate transition state. Thus, immucilins can block MTAN's active site with a high affinity ($IC_{50}$ in nM range) [104]. The QSI activity was determined using *Vibrio harveyi* strains because deletion mutants were available of both the AHL and the AI-2 synthase. Therefore, potential QSI activity could be designated to the inhibition of one of the two synthases or both. *V. harveyi* strains were also selected because the biosensor system was based on *V. harveyi* strains and an optimal response to the produced signal molecule was therefore expected. The azanucleosides tested failed to inhibit QS

in *V. harveyi* strains. This could indicate a lack of activity and/or difficulties to reach the target site. Another method to assess the QSI activity of the azanucleosides is by assessing their ability to bind to the purified enzyme [104]. However, putative novel antimicrobial agents are not useful unless they can reach their target. Therefore, the cell-based bioassay used in this study is a more-appropriate method to determine the ability of the tested azanucleosides to inhibit QS, even if a lack of activity might be due to difficulties in reaching the target rather than a lack of inhibition.

### 3.1.2 Purpose of novel component

Once novel components are established as QSI by using the previously described screening methods, their potentiating activity can be evaluated. QSI can exert their potentiating activity on two levels. On the one hand they can inhibit QS and so limit the pathogenesis *in vivo* by interfering with the production of virulence factors. On the other hand they can potentiate the activity of an antibiotic by interfering with QS-related protection mechanisms of the pathogen (i.e. biofilm formation and protection against oxidative stress). The latter can easily be detected by testing antibiotic susceptibility in simple *in vitro* models. The former is more difficult to detect and requires more *in vivo*-like *in vitro* models [92].

Knowledge on how a QSI exerts its potentiating activity is essential to determine the setup of follow-up experiments. QSI that only have an impact on *in vivo* virulence are more difficult to evaluate than components that also exert an antibiotic potentiating activity *in vitro*. In Chapter III.2 and III.3, BH is used. This is an established QSI with antibiotic potentiating activity against *B. cenocepacia* and *P. aeruginosa* biofilms [128,129]. Therefore, BH's use as an antibiotic adjuvant can be assessed in *in vitro* models by evaluating the susceptibility of biofilms.

### 3.1.3 Identifying the mode of action of putative quorum sensing inhibitors

#### *3.1.3.1 Identifying and confirming putative quorum sensing inhibitors*

Cell-based QS assays are frequently used in the screening for novel QSI. These bioassays are inexpensive, convenient and allow high-throughput screening (HTS) but have one inherent limitation: they do not measure a direct interaction between the signal molecule, QS transcriptional regulator(s) and promotor DNA sequences [294,298]. Therefore, the effect of a putative QSI needs to be investigated on transcriptomic level to confirm the QSI activity and to gain more information about the exact mode of action [294]. To this end, qPCR or RNA sequencing (RNAseq) can be used [299]. The selected technique depends on the research question. When there is already a clear indication of the genes targeted by the QSI, and only a small subset of genes has to be investigated, qPCR is preferably used [300,301]. However, when there is no clear indication of the mode of action of the QSI, RNAseq is a more interesting approach, since it offers a genome-wide survey of the

transcriptome [302]. Findings on transcriptomic level are then confirmed by performing suitable phenotypic assays [303].

#### *3.1.3.2 Added value of RNA-sequencing*

RNAseq provides a complete overview of changes in expression levels caused by a putative QSI. For example, in the study by Brackman et al. [117] RNAseq was used to determine the mode of action of hamamelitannin (2',5-di-O-galloyl-D-hamamelose; HAM). HAM potentiates vancomycin-activity in *S. aureus* biofilms [128] and does so by inhibiting QS through the TraP system [36]. Brackman et al. performed a transcriptomic analysis and the RNAseq data indicated that HAM affects biofilm cells in multiple ways. HAM influences the expression of a set of genes (through the TraP system) that are involved in both cell wall thickness and amount of eDNA in the biofilm matrix. HAM also repressed vancomycin-induced bacterial virulence; enterotoxins, exotoxins and leukocidins/hemolysins [117].

In another study, the mode of action of ajoene was determined [304]. Ajoene is a small sulphur-rich molecule that is present in garlic. It was previously described as a QSI in *P. aeruginosa* where it lowered biofilm resistance to tobramycin *in vitro* and *in vivo* and blocked QS-regulated rhamnolipid production, enabling PMNLs to phagocytose biofilms more easy [127]. By performing transcriptomic analysis, the authors discovered that ajoene represses QS in both *P. aeruginosa* and *Staphylococcus aureus* by lowering the expression of small regulatory RNAs (sRNA). In *P. aeruginosa*, ajoene caused a lowered expression of two sRNAs; *rsmY* and *rsmZ*, leading to reduced *lasI* and *rhlI* expression. In *S. aureus*, ajoene caused a reduction in RNAIII transcript, resulting in a lowered expression of many QS-regulated virulence factors such as hemolysins and proteases [304]. In this case, transcriptomic analysis allowed to discover the actual target of ajoene that is located upstream of the QS core genes.

#### *3.1.3.3 Considerations while using transcriptomic analysis*

In clinical settings, QSI are likely to be used in combination with an antibiotic. So, the addition of an antibiotic can be useful, when the effect of a QSI on gene expression levels is investigated. However, the presence of the antibiotic (or other strong selective pressures) has an impact on the physiological state of the cells. This can affect the response towards the putative QSI. Therefore, when strong selective pressure is applied to the cells it is important to distinguish between changes in gene expression levels due to the QSI or due to the applied stress.

The importance of the proper evaluation of changed expression levels is shown in the study of De Cremer et al. [305]. RNA-seq was performed on *Candida albicans* biofilms treated with miconazole (MICO). After a 24 hour treatment, genes involved in sterol biosynthesis and genes encoding drug efflux pumps were highly upregulated. Also other processes were affected that were not linked to

MICO's mode of action or tolerance pathways. However, the combination of MICO with inhibitors/inducers of these pathways did not always result in a synergistic or antagonistic effect on *C. albicans* biofilms [305]. This indicates that not all identified pathways were involved in MICO's mode of action or its tolerance. Changes in expression levels can also be caused by stress response caused by MICO, which influences multiple metabolic pathways [305,306]. Therefore, some background information on gene expression levels in stress situations can be helpful to evaluate whether the observed expression levels are due to the component or due to the applied stress. However, there is not always a clear-cut distinction between mode of action, resistance/tolerance mechanisms and stress response. For example, genes controlled by starvation or stress responses contributed to tolerance of *P. aeruginosa* biofilms treated with ciprofloxacin [307].

#### *3.1.3.4 Transcriptomic analysis in this dissertation*

In this dissertation (Chapter III.2), RNAseq was performed to evaluate BH's mode of action on TOB-treated *B. cenocepacia* J2315 biofilms. The use of tobramycin at a concentration of 3 x MIC caused a difference in physiological state between treated and untreated cells. To avoid interpretation errors, we only compared expression levels between cells in a similar physiological state: i.e. untreated cells vs. cells treated with BH, and cells treated with the combination of TOB+BH vs. cells treated with TOB alone. Subsequently, we evaluated all genes that were in both cases differentially regulated. These genes were mostly hypothetical proteins or genes belonging to large families with no defined function, e.g. LysR family regulatory protein. Therefore, we focused on differences in expression levels between cells treated with the combination of TOB+BH vs. cells treated with TOB alone, since BH has no effect alone but increases susceptibility towards TOB.

We were not able to clarify a QS-related mechanism of BH. Transcriptomic analysis revealed an additional mechanism by which BH exerted a potentiating activity. We found that BH increases oxidative stress in combination with TOB, by influencing oxidative phosphorylation, glucarate metabolism and by modulating the biosynthesis of putrescine. These insights can be useful to develop other compounds that improve antibacterial treatment via similar pathways. On the other hand, the increased oxidative stress results in increased selective pressure that can favour resistant mutants. Therefore, it is important to evaluate whether adjuvants will be able to induce resistance mechanisms prior to further investigation.

### 3.1.4 Other factors influencing the potentiating effect

In the search for novel QSI, it is important to consider that the activity of a compound is context dependent (i.e. the model, strain, medium and/or the combination with an antibiotic).

The impact of the model becomes apparent when results from Chapter III.2 and results from a study performed by Brackman et al. [128] are compared. In the study by Brackman et al., the TOB-potentiating effect of BH was investigated on the same strains used in Chapter III.2 (*B. cenocepacia* J2315, *B. cenocepacia* LMG 18828 and *B. multivorans* LMG 13010) but a lower BH concentration (100 µM vs 250 µM) was used. Brackman et al. [128] found a higher TOB-potentiating activity of BH compared to what we found in Chapter III.2. For *B. cenocepacia* J2315 and LMG 18828, we found an additional reduction of approx. 90%, whereas they found an additional reduction of approx. 99%. For *B. multivorans* LMG 13010, we found no additional reduction caused by BH, whereas they found approx. 99% reduction in surviving cells. The only difference, besides the concentration of BH, between both studies is the model for biofilm formation. In Chapter III.2, biofilms were set up in a 96-well microtiter plate, whereas in the study of Brackman et al. [128] biofilms were grown on medical grade silicone disks placed in the wells of a 24-well microtiter plate. This indicates that results can vary between studies depending on the experimental conditions in which biofilms are formed. Similarly, when comparing the TOB-potentiating activity of BH on *B. cenocepacia* J2315 biofilms in Chapter III.2 and III.3, there is a difference in cell reduction. The percentages of reduction caused by TOB+BH relative to TOB alone (3 x MIC TOB and 250 µM BH) were evaluated. In the 96-well microtiter plate, BH caused an additional reduction of 91.6% (± 12.2) cells (data not shown, Chapter III.2). In the bead containing 24-well plate, BH caused an additional reduction of 97.8% (± 1.14) cells (cycle 1, Chapter III.3). The difference in cell reduction between both models is statistically significant ($p < 0.05$) and these results highlight the impact of the study design on the results.

In Chapter III.2, we observed that BH's potentiating activity was strain-dependent. The differences among strains can be due to differences in regulation of their QS systems. As previously described in *V. harveyi*, there are variations among strains in QS genes and their expression levels, which can influence the sensitivity towards QSI [142]. Also among Bcc species, variability in QS systems has been observed: e.g. *B. cenocepacia* species can be subdivided in several lineages and strains belonging to the ET-12 lineage contain an extra QS system (CciIR) enabling them to produce multiple types of signalling molecules. Also, CepIR and the BDSF circuit are omnipresent in Bcc but their configuration can be either parallel (e.g. *B. cenocepacia* H111 [308]) or hierarchical (e.g. *B. cenocepacia* J2315 [190]). This diversity among Bcc species can contribute to their differences in susceptibility towards QSI.

The type of antibiotic had also an impact on the outcome, as BH did not potentiate the activity of other antibiotics besides certain aminoglycosides. The antibiotic-dependency can be caused by BH's QSI activity and/or by its other physiological effects. Since there is no exact mechanism that causes BH's potentiating activity, we were not able to pinpoint an explanation for BH's antibiotic-dependency but its strain- and antibiotic dependency prevents general use of this adjuvant. This is not necessarily a disadvantage since narrow-spectrum agents are less likely to cause resistance than broad-spectrum agents. Also, narrow spectra are not uncommon for adjuvants, as they are also extensively described for β-lactam inhibitors [83].

## 3.2 Evolution of resistance towards quorum sensing inhibitors

### 3.2.1 Experimental study design

In experimental evolution studies, the evolutionary changes that occur in a population exposed to certain experimental conditions have been investigated [309]. Many evolutionary studies have been conducted on bacteria in the planktonic state [261,310]. Since 65-80% of all infections are thought to be biofilm-related, it is surprising that so few evolution experiments have been conducted with biofilm populations [76]. In this dissertation we wanted to evaluate the evolutionary robustness of certain antibiotic potentiators in *B. cenocepacia* biofilms.

The experimental design is important in biofilm studies, especially in long-term studies, so the model should be chosen carefully. Biofilm models vary in their complexity, from simple models to complex real-life like models, that mimic chronic biofilm infections. Simple models are used to gain a broad insight into evolutionary processes in biofilms [311,312]. More complex models, in which isolates from patients are evaluated over time, are used to gain a better understanding of the course of chronic infections and to design better therapeutics/disinfectants [261]. This variety of biofilm models includes static *in vitro* models, *in vitro* flow models, *in silico* mathematical models and *in vivo* models [261]. The simplest model is the static *in vitro* model. This model allows bacteria to form biofilms, either on the surface of the medium, on the bottom of the well or on a hard surface added to the medium (e.g. beads). Advantages of this model are its simplicity and convenience, which allows quick screenings and testing multiple conditions. This model is used to investigate biofilms that grow on surfaces, e.g. catheters [313]. In *in vitro* flow models, biofilms can receive a constant flow of nutrients or (antibiotic) treatment over time, which is in contrast to the static models. This allows unlimited growth of biofilms since there is no depletion of nutrients, and also allows variation of antibiotic treatment over time [261]. Poltak and Cooper [314] developed an *in vitro* flow model especially for biofilms. Biofilms were cultivated on plastic beads in rotating test tubes. The transfer of cells to a next cycle occurred by transferring the bead to a test tube with fresh medium and a new

bead. This technique allows the biofilm cycle (formation-maturation-dispersion) to continue, since only bacteria that can disperse from the biofilm will be able to colonize the new bead [314].

In this dissertation, a simple static *in vitro* model was used. A static model was chosen over a flow model because of its simplicity and convenience. In the selected model, we used lethal concentrations of tobramycin to treat the biofilms which resulted in a lack of sufficient biofilm regrowth on the beads in the following cycle. To overcome this lack of regrowth, we introduced a planktonic phase after each treatment which allowed the cells to grow before the following cycle. This also allowed us to standardize prior to the start of each new cycle by controlling the inoculum size.

### 3.2.2 Genomics of the experimental evolution study

#### *3.2.2.1 Genomics of this evolution study*

The evolved populations were assessed on a genomic level to clarify the decrease in biofilm susceptibility. Overall, 22 nonsynonymous substitutions occurred in 20 genes. Some nucleotide polymorphisms (NPs) were common and appeared in multiple samples on the same location, whereas others were more random and only occurred in single samples. This phenomenon was also described in other evolution experiments, in which it occurred with a greater frequency in populations exposed to a strong selective pressure than to a mild selective pressure [267,268]. Mutations appearing in multiple samples were probably already present in the start population and were enriched for during the experiment. This is concurred by the high amount of cells necessary to have all possible random non-lethal mutations present in a population [165], while there was only a limited amount of cells transferred to each new cycle.

The affected genes showed no overlap with previous evolutionary studies in Bcc. This was not surprising due to differences in experimental setup and strains [265,266,315]. In this study, the combination treatments lead only to a marginally different outcome regarding genome changes compared to treatment with TOB alone. Only nine mutations occurred in the combination treatments and not in the untreated or TOB-treated populations. Of these nine mutations, five of them occurred in TOB+BH treated lineages. Three of these mutations were found in two genes involved in the PEP-pyruvate-oxaloacetate node (BCAL2631 and BCAM0965 [*mdh*]). For *mdh*, two separate SNPs occurred in two independent lineages, suggesting the importance of this gene. The PEP-pyruvate-oxaloacetate node is a metabolic link between glycolysis/gluconeogenesis and the tricarboxylic (TCA) cycle that directs the carbon flux in the appropriate direction [270]. It is possible that mutations in *mdh* and BCAL2631 lead to decreased susceptibility towards TOB+BH by

modulating the oxidative stress response. These findings are in line with the influence of BH on oxidative stress found in ChapterIII.2.

#### *3.2.2.2 The implications of the experimental setup*

In this dissertation, no typical resistance markers/genes were affected. This could at least partly be due to the experimental set-up. The time when antibiotic treatment was applied, the cells probably did not grow (much) because they were submerged in PS. Growth occurred in a separate step, without selective pressure of the antibiotic. As a consequence, mutations which might be beneficial for the survival of the antibiotic treatment can only have been enriched if they were at least neutral with respect to growth rate. Mutations which increase the resistance and/or persistence but reduce the growth rate will have been depleted and might have been eliminated during growth.

This set-up is different from the classical evolution studies in which continuous and even rising antibiotic concentrations are used. Those evolutionary experiments found mutations in genes related to resistance mechanisms or in genes unrelated to known resistance mechanisms [267,268,316]. Mutations in the latter genes can either induce unknown resistance mechanisms, independently or in combination with other mutations, or they are compensatory mutations that limit the fitness cost of resistant mutants [317,318].

Furthermore, mutations occurred with high and low frequencies in the affected genes. This phenomenon was already described by Lenski et al. as clonal interference, i.e. the co-occurrence of multiple beneficial mutations, resulting in a temporally higher diversity [310]. In the present study, clonal interference was unsurprising since we investigated the genome of a whole population and not the genome of single cell-derived isolates. Mutations are enriched for by competition over time, therefore they do not have to reach 100% frequency to be of importance. To include all mutations, we selected the lowest cut-off possible (35%) that still enabled us to distinguish enriched mutations from sequencing and mapping errors.

#### *3.2.2.3 The implications of genome plasticity*

Genome plasticity allows adaptation to changing environments and limits the predictability of antibiotic resistance evolution [265,317]. The plasticity can result from point mutations or from genome rearrangements like deletions, insertions, duplications, amplifications, inversions or translocations [319,320]. The genome of *B. cenocepacia* has a pronounced plasticity due to its multiple replicons and large numbers of insertion sequences [321].

We observed the plasticity of the genome in our study. Compared to the reference genome of Holden et al. [27], five SNPs had occurred in all populations (start and evolved) (data not shown). After 15 cycles of biofilm formation, we also observed SNPs in the untreated evolved populations.

These mutations were obtained without applying any antibiotic selective pressure. Therefore, these mutation could have occurred due to the high plasticity of the *B. cenocepacia* genome.

While the plasticity can explain why some mutations are also present in the untreated evolved populations and the enrichment of already existing mutations can explain the prevalence of the same mutation in multiple evolved populations, there is still no explanation for the prevalence of mutations in the untreated and in the TOB-treated evolved cells but not in other evolved populations. Especially since the selective pressure in the untreated or TOB-treated populations was different than in populations treated with a combination of TOB+adjuvant.

#### *3.2.2.4 Conclusion*

Based on our results, explaining the decreased susceptibility of *B. cenocepacia* J2315 biofilms is difficult. Regardless the mechanism by which they do so, it is interesting that biofilm cells gradually become less susceptible to potentiators. This highlights the importance of investigating the potential of an adjuvant to evoke resistance prior to further investigation.

# 4 Hurdles for quorum sensing inhibitors in preclinical and clinical research

## 4.1 Manifestation of quorum sensing and its inhibition in clinical trials

Only a few clinical trials have been initiated regarding QS and its inhibition. These trials evaluated either QS in patients or the impact of QSI as sole therapeutics on the infection. A search using the key words “infection” and “quorum sensing” on ClinicalTrials.gov database [322] showed three clinical trials. One was terminated early and two were completed. In the terminated trial (NCT00610623) azithromycin, a macrolide with QSI properties, was used. The purpose of this study was to investigate the efficacy of azithromycin in preventing or delaying the occurrence of ventilator associated pneumonia (VAP) in patients colonized with *P. aeruginosa*. Van Delden et al. [323] described a trend, although not significant, towards reduced VAP in ventialted patients treated with azithromycin. Especially rhamnolipid-dependent VAP was prevented, suggesting that inhibition of virulence is a promising antimicrobial approach [323]. Nevertheless, some caution is necessary when using anti-virulence strategies as Köhler et al. [136] described an increased virulence of *P. aeruginosa* after treatment with azithromycin. In the absence of treatment, *lasR* mutants appeared spontaneously in the population. These mutants have a reduced virulence due to their inability to regulate QS-associated virulence factors. However, azithromycin treatment prevented selection for the *lasR* mutant and increased the amount of wild type cells in the population. This demonstrates that an antivirulence treatment can increase the prevalence of more virulent genotypes [136]. Although there is evidence of antivirulence activity in CF patients, it should be kept in mind that these results should be interpreted with caution since azithromycin has both anti-inflammatory activity and bactericidal effects under certain conditions [324].

A second study investigated the effect of prebiotics, probiotics and antibiotics on QS signalling molecules and on innate and adaptive immunity in healthy individuals. This study was completed in 2011, but no study results were posted (NCT01201577) [322]. The third trial (NCT01306279) was an observational study in cystic fibrosis (CF) patients. Three different aspects of infections were studied. They investigated (i) the clinical significance of the bacterial diversity in the CF lung, (ii) the role of hypermutators in infection and (iii) the inter-bacterial communication and its role in exacerbations. In this study, the role of QS in infections was investigated, rather than the impact of QSI agents. This study was completed in 2012 but again no results were posted [322].

A search in the EU Clinical Trials Register [325] revealed a trial that was started in 2005. It was a randomised controlled trial, in which the use of macerated garlic oil was investigated in CF patients

infected with *P. aeruginosa* (2005-000311-98). The objectives of this trial were to (i) proof that garlic extract can inhibit QS molecules *in vivo*, (ii) develop an assay for garlic components and (iii) determine data from a representative CF patient group for the future design of a large trial [325] Smyth et al. [326] reported that the garlic capsules were well tolerated. However, no significant effects were observed on the presence of QS molecules in sputum when garlic treated patients were compared to the placebo group [326].

Despite the extensive research on QSI, only four documented trials were initiated over the past 15 years. Therapeutic use of QSI will likely occur in combination therapy with conventional antibiotics. Therefore, the focus of clinical research of QSI should shift towards these combination treatments, which is in contrast with the previously initiated trials. Rigorous preclinical studies, including appropriate animal models and toxicology studies, are required to assess the potential of a combinatorial regimen and to set the stage for clinical trial design.

## 4.2 Considerations prior to clinical trials

### 4.2.1 Considerations regarding quorum sensing inhibitors

The discovery that QS disruption attenuates virulence, has sparked the interest in developing QSI as potential therapeutics [327]. However, there are many specific questions regarding QSI that need to be answered: At what point during the infection will QS inhibition be valuable? Will QSI function better as prophylactic agents or will they generally be useful? What pathogens should be targeted? How fast will resistance spread throughout a population? Do QSI have other mechanisms than targeting QS and how do these mechanisms contribute to the potentiating activity or toxicity? How will the impact of QSI manifest in the host? Will other (pathogenic) bacteria present in the patient get a selective advantage when a signalling system of a certain species is targeted? To answer some of these questions, *in vitro* tests can be performed. This can easily be performed in a laboratory without ethical concerns. However, experiments in the host are also necessary. Some AHLs possess immune-modulatory activities for the host, inhibiting QS can thus affect certain cells and tissues in the body [51,328,329].

Furthermore, a better understanding of QS in the targeted pathogen is also necessary. In order to implement a solid QSI therapeutic, following assumptions should be met: (i) QS does only affect the regulation of virulence genes without affecting bacterial growth, (ii) the expression of virulence factors is only positively regulated by QS, (iii) the immune system of the infected patient will be able to remove the infection, (iv) the therapy must be active towards the causative agents of the infection. Therefore, it is necessary to examine the exact role of QS in virulence and in the pathogen's behaviour during infection [330].

### 4.2.2 Considerations regarding combination treatments of biofilm-related infections

In the future, when QSI-related questions will be answered regarding target and toxicity, then QSI can be tested as an adjuvant in preclinical research. However, combination treatments give rise to new questions: Which antibiotics can be used in combination with an adjuvant? What is the spectrum of activity? What is the optimal dosing? How do the PK/PK profiles of both compounds match?

There is already a clinical example available of a non-lethal adjuvant used in combination with an antibiotic, i.e. the combination of antibiotics with β-lactamase inhibitors (BLI). As for other adjuvants, the activity of each BLI depends on the pathogen, the co-administered antibiotic and dosing regimen [83]. In BLI research, the optimal dosing regimen is determined by nonclinical pharmacokinetic and pharmacodynamics (PK/PD) analyses [331].

In PK/PD analyses, the PK/PD index and PK/PD target should be established for each compound (antibiotic and adjuvant) [331]. The PK/PD index is the quantitative relationship between a pharmacokinetic measure of exposure to the test agent (e.g. AUC) and a measure of bacterial susceptibility (e.g. MIC). The PK/PD target is the magnitude for a certain PK/PD index and can be derived from both clinical as nonclinical studies [332]. PK/PD indices are $C_{max}$/MIC ($C_{max}$ = peak concentration), AUC/MIC (AUC = area under the concentration-time over 24h at steady state) or T>MIC (cumulative percentage of a 24 h period that the drug concentration is higher than the MIC at steady state) [331–333] (Figure 3). The parameters necessary for these PK/PD indices can easily be obtained from *in vitro* models [334]. A frequently used *in vitro* technique is the two-compartment hollow-fibre infection model [335].

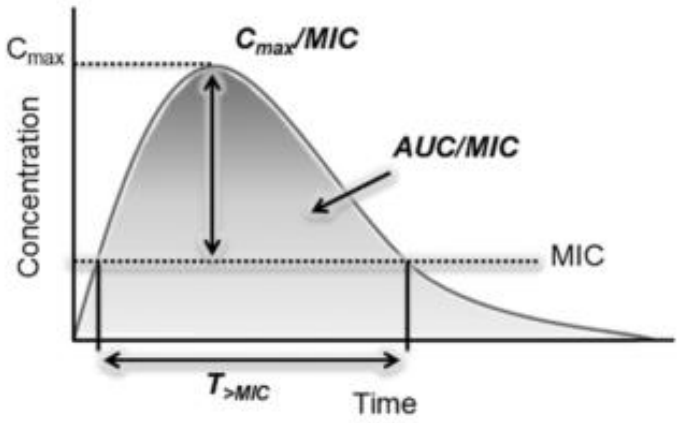



*Figure 3: **PK/PD indices associated with the efficacy of antibiotics*** [334]

The parameters in PK/PD analysis are usually based on the MIC, so they are less applicable to QSI as adjuvants. As previously mentioned, QSI can exert their antibiotic potentiating activity on two levels. Firstly, QSI can potentiate the co-administered antibiotic's activity. This is reflected in an increased susceptibility of biofilms. However, MIC results cannot be extrapolated to biofilm cells since biofilms differ from planktonic cells in growth rate, gene expression and metabolism [333,336]. Alternative parameters are necessary to evaluate the efficacy/toxicity of antibiotics in biofilms. PK/PD indices have been studied in biofilms using MBIC (minimal biofilm inhibiting concentration) and MBEC (minimal biofilm eradicating concentration) as parameters [337]. However, none of these parameters have been standardized or certified for clinical use, which limits their application in clinical settings [338,339].

Secondly, QSI reduce pathogenicity by interfering with the production of virulence factors. The impact on virulence is not necessarily reflected in a drop in MIC of the co-administered antibiotic [296], as is the case for BH in this dissertation. This is in contrast to the addition of a BLI, which inhibits a bacterial resistance mechanism, resulting in a lower MIC of the co-administered antibiotic [340,341]. Since the impact on virulence cannot be measured using MIC or MBIC/MBEC as parameters, alternative parameters that reflect the efficacy of a QSI in combination with antibiotic are necessary for future PK/PD analysis. The activity of QSI is specific to the host and to the pathogen [55], so it is less likely to find a general parameter to evaluate the efficacy of QSI. However, more specific parameters can be applied to evaluate the QSI activity. An example is the fold change in gene expression of QS genes in response to gradual increasing concentrations of a QSI. Another more specific parameter is the concentration of signalling molecules present in response to the QSI. These concentrations can be detected by biosensors or by chromatographic techniques [342]. Nevertheless, this parameter can only be implemented when the QSI inhibits the signal molecule synthase. Hence, extensive further research is necessary to find other possible parameters that can be implemented in clinical settings.

# CHAPTER V:
# SUMMARY

# Summary

Treatment of infectious diseases has become challenging due to the limited amount of novel antibiotics and the emergence of antimicrobial resistance. Since there are only a few novel antibiotics in the pipeline, alternative approaches are required to slow the spread of bacterial resistance. One such approach is the use of antibiotic adjuvants (also known as potentiators), i.e. non-lethal compounds that enhance antibiotic activity. These adjuvants can target quorum sensing (i.e. the communication system between bacteria; QS), which is potentially interesting as QS is involved in the regulation of many virulence factors including biofilm formation. Biofilms are consortia of bacteria attached to a surface that are generally less susceptible towards conventional antibiotics than their planktonic counterparts. This adjuvant approach can be especially useful to treat multidrug resistant pathogens such as members of the *Burkholderia cepacia* complex (Bcc), including *Burkholderia cenocepacia*. The latter is an opportunistic pathogen that can cause severe lung infections in cystic fibrosis (CF) patients. Such infections can escalate into the "cepacia syndrome", which is often associated with bacteraemia and a rapid decline in lung function. Treatment of Bcc infections is difficult due to the high innate resistance of Bcc species towards a broad range of antibiotics. The main objective of this thesis was to study the activity and mode of action of known QS inhibitors (QSI) and their analogues, and to investigate whether or not antibiotic adjuvants induce resistance over time.

In the first part of this dissertation, the QSI activity of several azanucleosides was investigated. These azanucleosides are putative transition state inhibitors of 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase (MTAN). MTAN is a crucial enzyme in QS and is responsible for the synthesis of the signalling molecules autoinducer-1 and -2. Screening of the azanucleosides with *Vibrio harveyi* biosensor strains showed that none of the analogues were able to inhibit QS. Possible explanations for this lack of activity are insufficient uptake into the cell and/or a lack of effect on MTAN.

In the second part, the goal was to determine the mode of action of baicalin hydrate (BH). BH is a flavonoid with QSI activity that increases the susceptibility of *B. cenocepacia* biofilms to tobramycin (TOB). We first determined the effect of multiple flavonoids on susceptibility of *B. cenocepacia* J2315 towards TOB. This was most pronounced in combination with apigenin 7-O-glucoside and BH. For BH, also other Bcc strains and other antibiotics were tested. The potentiating effect was only observed for aminoglycosides and was both strain- and aminoglycoside-dependent. Subsequently, gene expression was compared between BH treated and untreated cells, in the presence and absence of TOB. This revealed that BH affects cellular respiration, resulting in increased reactive oxygen species

(ROS) production in the presence of TOB. We subsequently demonstrated that BH has an impact on oxidative stress by influencing oxidative phosphorylation, glucarate metabolism and by modulating the biosynthesis of putrescine. Furthermore, our data suggest that the influence of BH on oxidative stress is likely unrelated to QS. Our data indicate that the potentiating effect of BH is due to modulating the oxidative stress response, which in turn leads to increased TOB-mediated killing.

Finally, we investigated whether *B. cenocepacia* J2315 biofilms develops resistance towards antibiotic adjuvants. The adjuvants used were BH (a QSI), and econazole and miconazole, two repurposed antifungal agents. To evaluate the development of resistance, cells were exposed to 15 cycles of biofilm formation (24 h), treatment (24 h) and planktonic regrowth (48 h). Over time, biofilm cells became gradually less susceptible to the potentiating activity of the adjuvants. Whole genome sequencing was performed on the start and the evolved population to investigate which changes in the genome were responsible for the decreased biofilm susceptibility. We observed several mutations in the evolved populations treated with TOB+BH. Some were related to metabolism. This is in line with the previous observation that BH increases antibiotic-induced oxidative stress by altering several metabolic pathways. Generally, our results indicate that TOB-potentiating compounds quickly lose their activity in *B. cenocepacia* biofilms.

In conclusion, our data show the importance of exploring the full mode of action of potentiating compounds. Potentiators can affect biofilms in multiple ways, causing an increase in biofilm susceptibility. Knowledge of these pathways can provide insights in potential resistance mechanisms caused by the potentiator. Furthermore, it is generally assumed that QSI have no impact on survival of the cells and will therefore not induce resistance. In this study, we showed that *B. cenocepacia* biofilms gradually become less susceptible to the potentiating activity of the QSI, BH, and two repurposed antifungal drugs. These findings potentially limit the clinical applicability of such potentiators These data also show that it is important to study the effect of potentiators over time.

## Samenvatting

Het behandelen van infectieziekten is een hele uitdaging geworden door de toenemende antimicrobiële resistentie en het beperkte aantal antibiotica op de markt. Aangezien er slechts enkele nieuwe antibiotica in de pijplijn zitten, zijn alternatieve behandelingen noodzakelijk om de verspreiding van bacteriële resistentie tegen te gaan. Een alternatieve aanpak is het gebruik van antibiotica adjuvantia (ook gekend als potentiators). Dit zijn niet-lethale componenten die de activiteit van een antibioticum verbeteren. Deze adjuvantia kunnen gericht zijn op het inhiberen van quorum sensing, (i.e. het communicatiesysteem tussen bacteriën [QS]). Dit is een interessante strategie aangezien QS betrokken is bij het reguleren van verschillende virulentiefactoren waaronder biofilmvorming. Biofilms zijn consortia van bacteriën omgeven door een laag zelfgeproduceerd slijm, vastgehecht aan een oppervlak of aan elkaar. Bacteriën in biofilms zijn algemeen minder gevoelig aan conventionele antibiotica dan bacteriën in de planktonische vorm.
Het toevoegen van adjuvantia aan antibiotica is in het bijzonder interessant voor de behandeling van multiresistente pathogenen zoals leden van het *Burkholderia cepacia* complex (Bcc), waaronder *Burkholderia cenocepacia*. Dit is een opportunistisch pathogeen dat ernstige longinfecties kan veroorzaken bij mucoviscidose patiënten. Deze infecties kunnen escaleren tot het "cepacia-syndroom", wat vaak gepaard gaat met bacteriemie en een snelle achteruitgang van de longfunctie. Behandeling van Bcc infecties is moeilijk door de hoge intrinsieke resistentie van Bcc bacteriën tegen een breed spectrum van antibiotica. Het doel van dit proefschrift was om de activiteit en werkingswijze van bekende QS inhibitoren (QSI) en hun analogen te bestuderen en om te onderzoeken of er resistentieontwikkeling tegen antibiotica adjuvantia mogelijk is.

In het eerste deel van dit proefschrift werd de QSI activiteit van verschillende azanucleosiden onderzocht. Deze azanucleosiden zijn vermeende transitietoestandinhibitoren van 5'-methylthioadenosine/S-adenosylhomocysteïne nucleosidase (MTAN). MTAN is een cruciaal enzym in QS en is verantwoordelijk voor de synthese van de signaalmoleculen autoinducer-1 en -2. Screening van deze azanucleosiden, met *Vibrio harveyi* biosensorstammen, toonde aan dat geen van de analogen QS kon inhiberen. Mogelijke verklaringen voor het gebrek aan activiteit kunnen zijn dat een onvoldoende hoeveelheid van de component zijn target bereikt en/of dat er een gebrek aan effect is tegen MTAN.

In het tweede deel zijn we op zoek gegaan naar het werkingsmechanisme van baicalin hydraat (BH). BH is een flavonoïde met QSI activiteit dat de gevoeligheid van *B. cenocepacia* biofilms voor tobramycine (TOB) verhoogt. Eerst hebben we het effect van meerdere flavonoïden op de gevoeligheid van *B. cenocepacia* J2315 voor TOB bepaald. Dit was het meest uitgesproken voor BH en

apigenine 7-O-glucoside. Voor BH werden ook andere Bcc stammen en andere antibiotica getest. Het versterkend effect werd enkel waargenomen in combinatie met aminoglycosiden, en was zowel stam- als aminoglycoside-afhankelijk. Vervolgens werd de genexpressie vergeleken tussen onbehandelde en met BH behandelde cellen, in de aan- en afwezigheid van TOB. Hieruit bleek dat BH de cellulaire respiratie beïnvloedt, wat resulteerde in een verhoogde productie van reactieve zuurstofverbindingen (ROS) in de aanwezigheid van TOB. We hebben vervolgens aangetoond dat BH een invloed heeft op oxidatieve stress via oxidatieve fosforylering, glucaraatmetabolisme en door modulatie van de biosynthese van putrescine. Bovendien suggereren onze data dat de invloed van BH op oxidatieve stress waarschijnlijk niet gerelateerd is aan QS. Deze bevindingen wijzen erop dat het versterkende effect van BH te wijten is aan het moduleren van de oxidatieve stressrespons, wat op zijn beurt leidt tot een toename in TOB-gemedieerde afdoding.

Ten slotte hebben we onderzocht of *B. cenocepacia* J2315 biofilms resistentie ontwikkelen tegen antibiotica adjuvantia. De gebruikte adjuvantia waren BH (een QSI) en econazol en miconazol, twee repurposed antimycotica. Om de ontwikkeling van resistentie te evalueren, werden cellen blootgesteld aan 15 cycli van biofilmvorming (24 uur), behandeling (24 uur) en planktonische hergroei (48 uur). De biofilmcellen werden geleidelijk minder gevoelig voor het effect van de adjuvantia. Om na te gaan welke veranderingen in het genoom verantwoordelijk waren voor de verminderde biofilmgevoeligheid werd ‘whole genome sequencing’ (WGS) uitgevoerd op de start en de geëvolueerde populaties. In de geëvolueerde populaties die behandeld waren TOB+BH, werden verschillende mutaties waargenomen, waarvan sommigen gerelateerd waren aan het metabolisme. Dit komt overeen met de vorige observatie dat BH de TOB-geïnduceerde oxidatieve stress verhoogt door verschillende metabole routes te wijzigen. Algemeen geven onze resultaten aan dat TOB-potentiërende componenten snel hun activiteit verliezen in *B. cenocepacia* biofilms.

Dit werk toont het belang aan van het onderzoek van de werkingswijze van potentiators. Deze kunnen namelijk op verschillende manieren biofilmgevoeligheid doen toenemen. Desalniettemin is er in deze studie aangetoond dat *B. cenocepacia* biofilms geleidelijk minder gevoelig worden voor de versterkende activiteit van de QSI, BH en twee repurposed antimycotica. Deze bevindingen zouden de klinische toepasbaarheid van potentiators kunnen beperken. Daarom is het dus belangrijk om de invloed van potentiators te onderzoeken in functie van de tijd.

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# CURRICULUM VITAE

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## EDUCATION

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2008 – 2013: Master in Pharmaceutical Drug Development, Ghent University

2001 – 2008: Science – Mathematics, Regina Caeli Lyceum, Dilbeek

## PUBLICATIONS

Cullen L., Weiser R., Olszak T., Maldonado R., Moreira A., **Slachmuylders L.**, Brackman G., Paunova-Krasteva T.,Zarnowiec P., Czerwonka G., Reilly J., Drevinek P., Kaca W., Melter O., De Soyza A., Perry A., Winstanley C., Stoitsova S., Lavigne R., Mahenthiralingam E., Sa´-Correia I., Coenye T., Drulis-Kawa Z., Augustyniak D., Valvano M. and McClean S. 2015. Phenotypic characterization of an international *Pseudomonas aeruginosa* reference panel: strains of cystic fibrosis (CF) origin show less *in vivo* virulence than non-CF strains. *Microbiology*, 181, 1951-1977

**Slachmuylders L.**, Van Acker H., Brackman G., Sass A., Van Nieuwerburgh F. and Coenye T. 2018. Elucidation of the mechanism behind the potentiating activity of baicalin hydrate against *Burkholderia cepacia* complex biofilms. *PLoS ONE* 13(1): e0190533

**Slachmuylders L.**, Van Acker H., Sass A., Vandenbussche I., Van Nieuwerburgh F., Abatih E., and Coenye T., Various evolutionary trajectories lead to loss of susceptibility to tobramycin-potentiating compounds in *Burkholderia cenocepacia* biofilms. *Manuscript in preparation*

**Slachmuylders L**. and Coenye T. Quorum sensing interfering agents as antibiotic adjuvants. *Manuscript in preparation*

**CONFERENCES**

| | | |
|---|---|---|
| 2016 | Antimicrobial Resistance in Microbial Biofilms and Options for Treatment, Ghent, Belgium | Poster |
| 2016 | Knowledge for Growth, Ghent, Belgium | Poster |
| 2015 | International Symposium on Quorum Sensing Inhibition, Santiago de Compostela, Spain | Oral |
| 2015 | Knowledge for Growth, Ghent, Belgium | Poster |

**TRAININGS**

Doctoral Schools UGent:

- Leadership Foundation Course
- Effective Image Editing training
- Denk over je competenties voor een niet-academische carrière
- 9th From PhD to Job Market: 'Believing in First Mover Advantages'
- Fostering Responsible Conduct of Research
- Communication skills basic
- Advanced Academic English Writing Skills