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Chemical Communication of Antibiotic Resistance by Highly Resistant Bacteria.

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Microbiology and Immunology

CHEMICAL COMMUNICATION OF ANTIBIOTIC RESISTANCE BY HIGHLY RESISTANT BACTERIA

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by

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Abstract

The overall antibiotic resistance of a bacterial population results from the combination of a wide range of susceptibilities displayed by subsets of bacterial cells. Bacterial heteroresistance to antibiotics has been documented for several opportunistic Gram-negative bacteria, but the mechanism of heteroresistance is unclear. I use *Burkholderia cenocepacia* as a model opportunistic bacterium to investigate the implications of heterogeneity in the response to the antimicrobial peptide polymyxin B (PmB) and also other bactericidal antibiotics. Here, I report that *B. cenocepacia* is heteroresistant to PmB. Population analysis profiling identified *B. cenocepacia* subpopulations arising from a seemingly homogenous culture that are resistant to higher levels of PmB than the rest of the cells in the culture, and protect the more sensitive cells from killing, as well as sensitive bacteria from other species, such as *Pseudomonas aeruginosa* and *Escherichia coli*. Communication of resistance depended on upregulation of putrescine synthesis and YceI, a widely conserved low-molecular weight secreted protein. Deletion of genes for the synthesis of putrescine and YceI abrogate protection, while pharmacologic inhibition of putrescine synthesis reduced resistance to PmB. Polyamines and YceI were also required for heteroresistance of *B. cenocepacia* to various bactericidal antibiotics. I propose that putrescine and YceI resemble "danger" infochemicals whose increased production by a bacterial subpopulation, becoming more resistant to bactericidal antibiotics, communicates higher level of resistance to more sensitive members of the population of the same or different species.

Putrescine protects from antibiotics through its ability to compete with PmB for surface binding and protection against antibiotic-induced oxidative stress. YceI proteins are conserved bacterial lipocalins or “bacteriocalins”. Bacteriocalins from different Gram-positive and Gram-negative bacteria are involved in the response to hydrophobic or amphiphilic antibiotics (PmB, rifampicin, norfloxacin and ceftazidime) but not hydrophilic ones (such as gentamicin). This effect is achieved by their preferential binding affinity to hydrophobic moieties. Together, my findings uncover a novel, non-genetic and cooperative mechanism of transient increase in resistance chemically communicated from more resistant members of heterogeneous populations to less resistant bacteria of the same or other species. This multifactorial mechanism of communication of antibiotic resistance offers novel targets for antimicrobial intervention.

Keywords

Antibiotic resistance; Heteroresistance; Communication of antibiotic resistance; Antimicrobial peptides; Polymyxin B; Bactericidal antibiotics; Polyamines; Putrescine; Population analysis profiling; YceI; Lipocalins; Reactive oxygen species; ROS; *Burkholderia cenocepacia*; *Pseudomonas aeruginosa*; *Mycobacterium tuberculosis*; *Salmonella typhi*; *Shigella flexneri*; *Escherichia coli*.

Dedication

To my father, my mother, my brother, my wife Somiraa and my daughter Nada for their unconditional support throughout my academic career.

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Introduction

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El-Halfawy OM, and Valvano MA (2013). Communication is key: do bacteria use a universal 'language' to spread resistance? Future Microbiol.; 8: 1357-9. With permission of Future Medicine Ltd.

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1.1. Introduction

Infectious diseases are among the most aggressive killers worldwide claiming the lives of millions of people annually. Microbial infections in general constitute a major burden on the society and the healthcare systems. While immunization provides a preventative approach against infection with certain bacteria; this strategy does not provide protection against the increasing variety of microbial infections. Since the middle of the twentieth century, antibiotics helped eradicate infections saving lives. However, the rapid emergence of antibiotic resistance in addition to the severe decline in development of new antibiotics following the golden era of antibiotic discovery have often put patients and healthcare practitioners in the losing side of combating microbial infections. Furthermore, the individuality of response to antibiotics within a population of bacteria also known as antimicrobial heteroresistance may further complicate the clinical picture impeding the eradication of microbial infections. On the other hand, the ability of bacteria to transfer antibiotic resistance determinants by means of horizontal gene transfer mechanisms has led to the rapid spread of multi-drug resistance across various bacterial species. The ability of bacteria to communicate antibiotic resistance among each other via small molecules has recently drawn attention to the probability of the transient increase in antibiotic resistance and protection of bacteria, normally sensitive to an antibiotic, mediated by more resistant bacteria by non-genetic mechanisms leading to therapeutic failure. In this section, I will introduce different classes of antibiotics together with the mechanisms of response and resistance of bacteria to antibiotic exposure. In addition, I will discuss the phenomenon of antimicrobial heteroresistance, which has been poorly characterized in the literature despite its clinical importance, as well as the chemical communication of antibiotic resistance among different bacteria.

1.2. Antibiotics and the dilemma of antibiotic resistance

Common usage of the term antibiotics often extends to include synthetic antimicrobial chemotherapeutic agents, such as sulfonamides and quinolones (1). Antibiotics differ markedly in physical, chemical, and pharmacological properties, in antimicrobial spectra, and in mechanisms of action (1).

1.2.1. Classes of antibiotics

Classically, antibiotics are classified into two broad classes; bacteriostatic and bactericidal agents. Bacteriostatic agents act by preventing the growth of bacteria, whereas bactericidal ones act by killing bacteria. A generally accepted definition of bactericidal activity is ~99.9% reduction in viable bacterial density in an 18–24-h period in a standard Minimum bactericidal concentration (MBC) assay according to the CLSI guidelines, whereas bacteriostatic activity has been defined as a ratio of MBC to MIC of >4 (2). Indeed, these 2 categories may overlap in that no category of antibiotics exclusively kills bacteria and another that only inhibits growth of bacteria; bacteriostatic and bactericidal categorizations in clinical practice are not absolute (2). Selected classes of antibiotics pertaining to the work of the present thesis will be discussed briefly in this section.

1.2.1.1. Antimicrobial peptides

Antimicrobial peptides (APs) are evolutionarily conserved molecules involved in the defense mechanisms of a wide range of organisms. Produced in bacteria, insects, plants and vertebrates, APs protect against a broad array of infectious agents (3). Moreover, APs are being tested in clinical trials as anti-infective agents while others are already in use, such as polymyxin B (PmB) (4). APs vary enormously in sequence and structure, but certain features are common. The natural APs are generally 12–50 amino acids in length, have a net positive charge, and contain around 50% hydrophobic amino acids. They fold into amphiphilic structures in which the positively charged and hydrophilic domain(s) are well separated from the hydrophobic domain(s) (4). Such molecules are well suited to interact with membranes, especially bacterial membranes with their negatively charged and hydrophilic head groups and hydrophobic cores (4). Based on their amino acid composition, size and conformational structures, APs can be divided into several categories, such as peptides with (i) α- helix structures, e.g. human cathelicidin; (ii) β-sheet structures stabilized by disulfide bridges, e.g. human defensins; (iii) extended structures, e.g. indolicidin, a bovine AP; and (iv) loop structures, e.g. cyclic defensins found in rhesus macaques (3), and cyclic lipopeptides like polymyxin B (PmB) (4).

The exact mechanism by which APs exert their antimicrobial properties is continuously being investigated, but it is generally accepted that cationic APs interact by electrostatic forces with the negatively charged phospholipid headgroups on the bacterial membrane and cause disruption (3). The most widely accepted mechanisms of interaction are the “barrel stave” model, in which the peptides bind to the cell membrane, then the peptides themselves insert into the hydrophobic core of the membrane forming a pore, causing leakage of cytoplasmic material and death of the cell, and the “carpet model” in which peptides bind to the phospholipids at the outer surfaces of the cell membrane, followed by the alignment of the peptide monomers, then the peptides reorient themselves towards the hydrophobic core of the membrane causing the disintegration of the lipid bilayer (3). Permeabilization of bacterial membranes is a crucial step in the antimicrobial activity of APs, but evidence shows that they also inhibit a variety of essential microbial processes, such as protein, cell wall, and nucleic acid synthesis (5).

Alteration of the surface charges by reducing the net negative charges is one of the major mechanisms that bacteria utilize to resist killing by antimicrobial peptides (5). Examples of this strategy of resistance are phosphoethanolamine or aminoarabinose modifications of lipid A, lysyl phosphatidylglycerol modification of membranes, and shielding of surface charges by capsular polysaccharides (5). Other mechanisms of resistance include degradation by extracellular proteases, reduced import and increased export by efflux pumps (5, 6).

1.2.1.2. β- lactams

Penicillin G (benzylpenicillin) was the first β-lactam antibiotic introduced into clinical practice. β-lactam antibiotics now include: penicillinase-resistant, amino-, carboxy- , indanyl-, and ureido-penicillins; first- to fifth-generation cephalosporins; monobactams; and carbapenems. The distinctive structural feature of a β-lactam is the highly reactive four-membered ring (7). All β-lactam antibiotics are bactericidal agents that inhibit cell wall synthesis through inhibition of bacterial transpeptidases known as penicillin-binding proteins (PBPs), which are essential enzymes that catalyze the final crosslinking step since the β-lactams are structurally similar to the penultimate D-Alanyl-D-Alanine of the pentapeptide that is attached to N-acetyl muramic acid. Subsequent steps in cell wall synthesis are hindered while autolysis by cell wall degrading

(autolytic) enzymes continues. Bacterial cells become permeable to water, rapidly take up fluid, and eventually lyse (7, 8).

Bacteria tend to avoid the bactericidal effect of β-lactams through: production of beta-lactamases, altered PBPs that exhibit low affinity for β-lactam antibiotics such as PBP 2' (PBP2a) of *Staphylococcus aureus* and lack or diminished expression of outer membrane proteins (OMPs) in Gram-negative bacteria which restricts the entry of certain β-lactams into the periplasmic space of Gram-negative bacteria and hence access to PBPs on the inner membrane (7). Furthermore, efflux mechanisms have been described in which bacteria pump out β-lactam antibiotics (9).

1.2.1.3. Aminoglycosides

The aminoglycosides are bactericidal antibiotics active against many Gram-negative aerobes and against some strains of staphylococci. They are taken up into sensitive bacterial cells by an active transport process that is inhibited in anaerobic, acidic, or hyperosmolar environments (10). An essential target of aminoglycoside antibiotics is the ribosome where they bind to the 16S ribosomal RNA and this binding interferes with protein synthesis. Low concentrations of aminoglycosides can cause misreading of the genetic code and higher concentrations will block translation (11). The bactericidal effect of aminoglycosides may be due to aberrant proteins resulting from misreading which form membrane channels or inhibition of other steps in metabolism (12). Aminoglycosides are also capable of directly disrupting the integrity of the outer membrane in Gram negative bacteria by displacing the divalent cations which bridge adjacent LPS molecules and are required to maintain integrity. This alteration in outer membrane results in the 'self-promoted' uptake into the cell (11, 12).

The major mechanisms of resistance to aminoglycosides in pathogenic bacteria are: (i) deactivation of the drug, e.g. by N-acetylation, O-nucleotidylation, or O-phosphorylation, (ii) reduction of the intracellular concentration of the drug by alteration of outer membrane permeability, decreased inner membrane transport, or active efflux from the cell, and (iii) alteration of the target by mutation in ribosomal proteins or in 16S rRNA (13, 14). Methylation of 16S ribosomal RNA has emerged as a mechanism of resistance against aminoglycosides among Gram-negative pathogens belonging to the family Enterobacteriaceae and glucose-

nonfermentative microbes, including *Pseudomonas aeruginosa* and *Acinetobacter* species. This event is mediated by a newly recognized group of 16S rRNA methylases, which share modest similarity to those produced by aminoglycoside-producing actinomycetes (15).

1.2.1.4. Tetracyclines

The tetracyclines are mainly bacteriostatic, with a broad spectrum of antimicrobial activity including many aerobic and anaerobic Gram-positive and Gram-negative pathogenic bacteria, Chlamydiaceae, Mycoplasma spp., Rickettsia spp., spirochaetes, and some protozoa (10). Tetracyclines are taken up into sensitive bacterial cells by an active transport process (11). Once within the cell they bind reversibly to the 30S subunit of the ribosome, preventing the binding of aminoacyl transfer RNA and inhibiting protein synthesis and hence cell growth (16). Although tetracyclines also inhibit protein synthesis in mammalian cells they are not actively taken up, permitting selective activity against the infecting organism (11).

Resistance to the tetracyclines is usually plasmid-mediated and transferable (10). It is often inducible, and appears to be associated with the ability to prevent accumulation of the antibiotic within the bacterial cell, both by decreasing active transport of the drug into the cell and by increasing tetracycline efflux most commonly through the tetracycline resistant efflux proteins which belong to the major facilitator superfamily (MFS)(16, 17).

1.2.1.5. Chloramphenicol

Chloramphenicol is a naturally occurring compound discovered in a systematic screening of Streptomyces strains following the discovery of streptomycin in the 1940s (18). Chloramphenicol acts by inhibiting the peptidyl transferase reaction at which the peptide bond is formed on 70S ribosomes (18). It possesses a broad spectrum of activity by acting against Gram-positive and Gram-negative bacteria, in addition to chlamydiae and rickettsiae; being mainly bacteriostatic in action especially against Gram-negative bacteria (18). Acetyltrasferases, bacterial enzymes that acetylate the hydroxyl groups of chloramphenicol hence inactivating it, are the most common mechanism of resistance against this antibiotic (19). In addition, efflux proteins specific for chloramphenicol has been described providing high-level resistance (19).

1.2.1.6. Rifampicin

Rifampicin (known in the US as rifampin) is a semi-synthetic derivative of rifamycin B produced by *Streptomyces mediterranei*. It interferes with mRNA formation by binding to the β-subunit of DNA-dependent RNA polymerase (RpoB) (20). It exhibits potent bactericidal activity against a range of bacteria, notably staphylococci and legionellae; it is a useful antimycobacterial drug used for tuberculosis and leprosy (20). Resistance readily arises by point mutations in the rifampicin-binding region of *rpoB* (19).

1.2.1.7. Quinolones

Fluoroquinolones are bactericidal (10, 21). They inhibit bacterial DNA synthesis by inhibiting the A subunit of DNA gyrase and topoisomerase (10). DNA gyrase, encoded by *gyrA* and *gyrB*, is the enzyme responsible for inducing negative supercoils in DNA, whereas topoisomerase IV, encoded by *parC* and *parE,* is involved in DNA relaxation and separation (22). In general, fluoroquinolones have good *in vitro* activity against many Gram-positive and Gram-negative pathogens. These agents also are active against some drug resistant pathogens, including penicillin- or macrolide-resistant *Streptococcus pneumoniae* (21).

Mutations in specific regions termed quinolone resistance-determining regions (QRDR) of the *parC* or *gyrA* genes of topoisomerase IV and DNA gyrase lead to development of resistance to quinolone antimicrobials (21). Active efflux, an alternate mechanism leading to fluoroquinolone resistance, prevents accumulation of the antimicrobial in the bacterial cell. Efflux typically results in low-level resistance and may or may not occur in conjunction with mutations in topoisomerase IV or DNA gyrase (21).There is complete cross-resistance between ciprofloxacin and the other fluoroquinolones (10, 23).

1.2.1.8. Novobiocin

Novobiocin is a bacteriostatic coumarin antibiotic that binds to the B subunit of DNA gyrase (GyrB) blocking the ATPase activity and hence inhibiting DNA supercoiling (24). It is active against Gram-positive organisms, with little activity against enteric Gram-negative bacilli (20). Resistance against novobiocin may be mediated by multi-drug efflux systems (19), or due to

mutation in *gyrB* (24). In 2009, FDA withdrew the approval for use of novobiocin capsules on the market (25).

1.2.1.9. Trimethoprim

Trimethoprim, a bacteriostatic agent (2), is a diaminopyrimidine that inhibits dihydrofolate reductase (DHFR), the enzyme that converts the metabolically inactive dihydrofolate into the active form tetrahydrofolate (20). Such inhibition leads to various effects, including failure to synthesize purine nucleotides and thymidine (20). The selective toxicity of trimethoprim stems from its greater affinity for the dihydrofolate reductase of bacteria than for the corresponding mammalian enzyme (20). Mutations of the chromosomal DHFR or plasmid-encoded, drug-insusceptible DHFRs are the most common means of resistance against trimethoprim that also led to rapid dissemination of resistance in particular the insusceptible enzyme encoded by mobile genetic elements (26).

1.2.2. Intrinsic versus acquired resistance to antibiotics

Bacterial resistance to antibiotics can be achieved through intrinsic or acquired mechanisms (19). Intrinsic resistance to antibiotics is a natural trait independent of antibiotic selective pressure; whereas acquired resistance is induced upon exposure of previously susceptible bacteria to antibiotics.

1.2.2.1. Intrinsic resistance to antibiotics

Many bacteria display intrinsic resistance to different classes of antibiotics; a trait that is genetically encoded within their genome, and not accredited to horizontal gene transfer (27). Intrinsic resistance is conventionally attributed to impermeability of the bacterial cell envelope (mainly due to the outer membrane of Gram-negative bacteria) in conjunction with multidrug efflux pumps that effectively reduce the intracellular concentration of antibiotics (28). However, recent studies have revealed that intrinsic antibiotic resistance further involves a complex network of genetic loci; deletion of such genes renders bacteria hyper-susceptible to antibiotics (27).

Outer membrane (OM) impermeability acts as a barrier in Gram-negative bacteria that overcomes the large permeability threshold of the peptidoglycan layer (which renders Gram-positive bacteria susceptible to various antibiotics). Moreover, porins, proteins channels within the OM for the uptake of key nutrients, restricts the influx of numerous antibiotics [references in (27)]. Alternatively, constitutive modification of the OM results in intrinsic resistance; for example, the decoration of *Burkholderia cenocepacia* OM with the positively charged aminoarabinose sugar prevents the essential initial binding of antimicrobial peptides to its surface rendering it highly resistant to APs (29).

Efflux as a mechanism of antibiotic resistance was first reported for tetracyclines; since then, it has been documented as a mechanism of resistance against a wide range of antibiotics in many Gram-positive and Gram-negative bacteria [references in (19)]. Efflux pumps can either be substrate specific such as the Mef efflux transporters in streptococci which transport 14- and 15-membered macrolides only, or they can be more broad-spectrum dealing with different antibiotic classes such as the *Escherichia coli* AcrAB efflux system (19). Most drug efflux proteins that span the bacterial membrane belong to five distinct families: the ATP binding cassette (ABC), the major facilitator (MF), the multidrug and toxic-compound efflux (MATE), the small multidrug resistance (SMR), and the resistance-nodulation-division family (RND) [references in (27)]. ATP hydrolysis drives efflux in the primary (ABC) transporters; whereas efflux by the other families is driven by proton (and sodium) motive force and is hence called secondary transport (19).

Synergistic relationship between OM permeability barrier and active efflux systems results in high-level of intrinsic resistance in many Gram-negative bacteria (27); conceivably, certain porins and efflux systems are co-regulated (19). However, additional chromosomally encoded elements are involved in intrinsic resistance to antibiotics; such elements act in a concerted manner towards such resistance phenotype [references in (27)]. Evidences challenging the classical definition of intrinsic resistance demonstrate that such resistance is not merely due to protective shields, but rather encompass the action of numerous proteins from all functional categories forming a complex and dynamic network including proteins involved in amino acid biosynthesis and metabolism, protein secretion and export, and transport of small molecules (30).

1.2.2.2. Acquired resistance to antibiotics

Acquired mechanisms of antibiotic resistance involve mutations in chromosomally located genes and the horizontal genetic transfer (HGT) of resistance determinants borne on plasmids, bacteriophages, transposons, and other mobile genetic material (19). HGT is generally accomplished through the processes of transduction (via bacteriophages), conjugation (via plasmids and conjugative transposons), and transformation (via incorporation of free DNA from dying organisms) (31).

Alteration of the antibiotic target sites is one mechanism of acquired resistance. This can be mediated through mutation(s) of gene encoding these targets leading to non-susceptible variants; examples are discussed in previous sections about different classes of antibiotics. Alternatively, this can occur through chemical modifications such as the *vanA* gene cluster-mediated modification of peptidoglycan in response to glycopeptide antibiotics (32). Genomic duplication of antibiotic resistance genetic determinants is another means of acquired resistance leading to overexpression of the resistance mechanism (19). Other mechanisms of acquired resistance include enzymatic chemical modification of the antibiotic itself rendering it inactive. Antibiotic-modifying enzymes may either degrade the antibiotics such as β-lactamases and extracellular proteases, or perform chemical transformations such as aminoglycoside-modifying proteins [references in (19)].

1.2.3. Antibiotic resistant bacteria

The crisis of antibiotic resistance presents a unique clinical challenge due to the widespread of multi-drug resistant (MDR) organisms.These MDR organisms may be classified into at least two groups; well-known (or primary) pathogens, and opportunistic pathogens (33).

1.2.3.1. Antibiotic resistant primary pathogens

Primary pathogens are highly virulent microorganisms capable of causing a disease in an otherwise normal or healthy individual (34). These pathogens may also cause more drastic disease in a host with compromised immunity (34). Many of these pathogens are former non-pathogenic commensal flora that acquired antibiotic resistance genes and increased virulence such as community-associated methicillin resistant *Staphylococcus aureus* (CA-MRSA), and

multi-drug resistant *Escherichia coli* (35). *Mycobacterium tuberculosis* is another serious pathogen specially that it mostly displays extensive drug-resistance (XDR) (19). Other important pathogens that may display MDR include *Shigella* spp. and *Salmonella* spp (36).

1.2.3.2. Antibiotic resistant opportunistic pathogens

Opportunistic pathogens are mostly environmental bacteria and may be normally in contact with the host; however, they only cause an infectious disease following an injury such as an open fracture; or immunosuppression whether due to a disease such as malaria or cystic fibrosis (CF), or a drug as cytotoxic chemotherapy (34). The bacterial opportunistic pathogens are frequently intrinsically resistant to multiple antibiotics (33). Examples of these pathogens include *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Acinetobacter baumannii* and *Burkholderia cepacia* complex (33).

In this thesis, I investigate novel mechanisms of high-level intrinsic multidrug resistance using *Burkholderia cenocepacia* as a model bacterium. *B. cenocepacia* is an environmental, opportunistic pathogen that belongs to the *B. cepacia* complex; it causes serious respiratory infections in CF patients and expresses high-level multidrug intrinsic resistance (37). These infections are associated with faster decline in lung function, debilitating exacerbations and ultimately death (38-40), and they also reduce the survival of CF patients after lung transplant (41). *B. cenocepacia* possesses an arsenal of virulence determinants resulting in such aggressive infections and poor prognosis of the infected patients [references in (37)].

1.3. Antimicrobial Heteroresistance: an emerging field in need of clarity

Infections by multi-drug resistant bacteria impose a serious encumber on the society and economy worldwide and account for a soaring fraction of global morbidity and mortality. Variable responses to the antibiotic from bacterial cells within the same population, a phenomenon known as heteroresistance, further complicates the problem of antibiotic resistance.Heterogeneous resistance to antibiotics was first described in 1947 for the Gram-negative bacterium *Haemophilus influenzae* (42), and almost 20 years later for Gram-positive

staphylococci (43), but the first reported use of the term ‘heteroresistance’ was in 1970 (44). Clinical laboratory standards and recommendations for practices concerning antimicrobial resistance are developed by organizations such as the Clinical and Laboratory Standards Institute (CLSI), the British Society of Antimicrobial Chemotherapy (BSAC), and others. Therefore, antimicrobial susceptibility testing methods, such as the minimum inhibitory concentration (MIC) and disc-diffusion techniques, and standard guidelines to define isolates as susceptible, resistant or intermediately resistant to any antibiotic are generally agreed upon world-wide. In contrast, heteroresistance is poorly characterized and consensus-based standards to define and identify heteroresistant bacteria are lacking.

The term ‘heteroresistance’ is indiscriminately used in the literature to describe not only population-wide variation in antibiotic resistance phenotype but also other observations. Furthermore, methods to determine heteroresistance vary significantly among laboratories. Together, this increases the confusion regarding this phenomenon, precluding establishing its clinical significance and implementing proper therapeutic interventions and guidelines. Here, I review the available literature on heteroresistance, to expose the contradictions and variations in its definition.

1.3.1. Multiple definitions of heteroresistance

Heteroresistance broadly refers to a population-wide, variable response to antibiotics. Several reports, including the earliest studies describing the phenomenon in 1964 and 1970, used this definition without specifying a particular antibiotic concentration range (43-46). Figure 1 illustrates the notion of heteroresistance (A) in contrast to a bacterial population homogeneously responding to an antibiotic (B). This phenomenon is distinct from bacterial persistence. Persisters neither die nor grow in the presence of antibiotic (Fig. 1C), suggesting they are dormant (47). These bacterial cells grow only after the antibiotic removal, and the progeny of persisters do not exhibit increased resistance to the antibiotic; they rather show the pattern of sensitivity to the antibiotic identical to that of the original bacterial population (48).

In other reports describing heteroresistance, specific concentration ranges were indicated. For example, colistin heteroresistance in *Acinetobacter baumannii* was used to describe subpopulations (less than 0.1% from 10^8 to 10^9 CFU/ml) growing in the presence of 3 to 10

µg/ml colistin, while the MIC of the culture ranged from 0.25 to 2 µg/ml (49). In another report, heteroresistance was described when a subset of the microbial population was considered resistant to an antibiotic while the rest of the population was generally considered to be susceptible to that antibiotic based on the concentration breakpoints used for traditional *in-vitro* susceptibility testing (50). This definition does not encompass cases where the bacterial culture comprises subpopulations with varying levels of resistance, and therefore the entire population including the least resistant members is considered resistant to the antibiotic (Fig. 2).

Other definitions for heteroresistance were also used, which further contributed to misconception about the nature of the phenomenon. Some of them were based on a single cut-off concentration, which did not describe the pattern of resistance among various members of a bacterial population. For example, heteroresistance was defined by growth of *A. baumannii* colonies on plates containing 8 µg/ml of colistin, with confirmation of an MIC of 8 µg/ml by subsequent broth microdilution test (51). Similarly, heterogeneously resistant staphylococci were defined as any culture containing subpopulations at a frequency of 1 in 10^6 cfu/ml or higher with MIC > 4 µg/ml for vancomycin or ≥ 16 µg/ml for teicoplanin (52) or simply above the CLSI breakpoints (53). A similar approach was adopted by setting a cut-off diameter of 10 mm in disc diffusion assays below which the strain was considered heteroresistant rather than merely resistant (54). While heteroresistance was properly recognized by population analysis profiling (PAP), the improper definition misrepresented the phenomenon, giving rise to ambiguity in its understanding at an early stage of its discovery (54). In another approach, high MIC of *Enterococcus faecium* against vancomycin (>256 mg/L) determined by broth dilution but low MIC (=1.8 mg/L) by Etest was suggestive of heteroresistance (55).

Other forms of bacterial heterogeneous behaviour against antibiotics have been reported. Certain *S. aureus* strains displayed resistance to high concentrations of methicillin (64 to 512 mg/L) and susceptibility to low concentrations of methicillin (2 to 16 mg/L) (56). This phenomenon, termed "Eagle-type" resistance, was similar to the previously described Eagle killing by penicillin, in which the bactericidal action of penicillin was paradoxically reduced at high

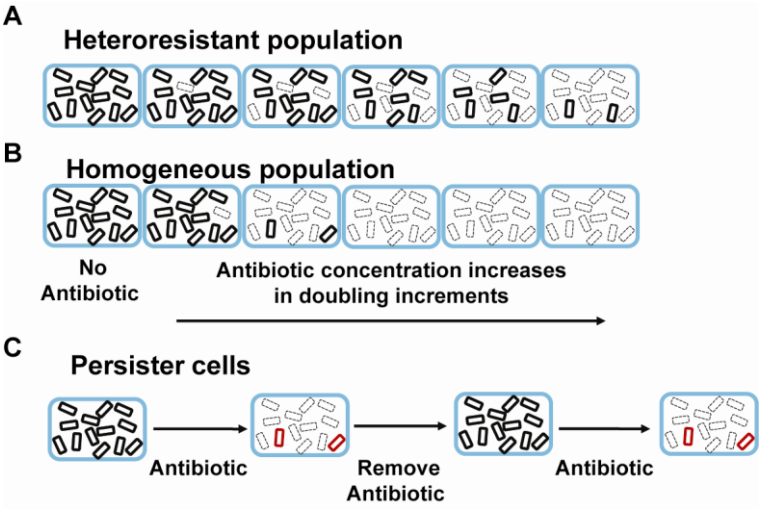

Figure 1. Illustration of the phenomenon of heteroresistance to antibiotics.

(A) heteroresistant population; (B) homogeneous population; and (C) the phenomenon of persistence. Bold cells denote alive cells, dotted cells denote dead cells, red bold cells denote dormant persisters.

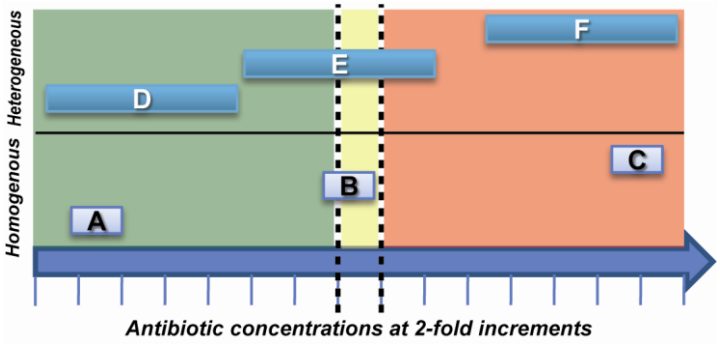

Figure 2.Heteroresistant vs. homogenous response to antibiotics.

Dotted lines represent the breakpoints for resistance. Homogenous bacterial cultures (A-C) can either be A, susceptible, B, of intermediate susceptibility, or C, resistant to an antibiotic according to the traditional *in vitro* susceptibility testing. Heteroresistant bacteria (D-F) may be: D, completely susceptible to an antibiotic, whereby all the different subpopulations respond to antibiotic concentrations extending below the breakpoints. This form is less likely to be detected and is probably the least clinically important (unless the least responsive subpopulations develop resistance to the antibiotic). E, the more classical form of heteroresistance in which the majority of the bacterial population is susceptible to an antibiotic with a highly resistant minority. Antibiotic treatment guided by the traditional susceptibility testing breakpoints would select for the resistant subpopulation, leading to therapeutic failure. F, the entire bacterial population, including the least resistant subpopulations, is resistant to the antibiotic. Chemical communication of antibiotic resistance from the more resistant members of the population protecting less resistant bacteria is the major concern of such bacterial populations.

concentrations (57). Similar pattern of 2-peaks of growth in population analysis profiles was observed in *A. baumannii* with cefepime (58). Other *S. aureus* strains displayed 'thermosensitive' heteroresistance (59), whereby cultures growing in the presence of high concentrations of methicillin at 30°C lost this ability within 30 min after shifting the growth temperature to 37°C. Shifting the temperature in the reverse direction resulted in an equally rapid expression of methicillin resistance (59).

Adding to the confusion, 'heteroresistance' was often applied to describe incidences of infection with bacterial strains exhibiting different levels of resistance to an antibiotic. Amoxicillin-resistant and -susceptible *Helicobacter pylori* isolates (with MICs of 2 mg/L and 0.06 mg/L, respectively) were observed in different biopsies from one patient, a case described as 'inter-niche' heteroresistance (60). More recently, pairs of *H. pylori* isolated from the same patients showed different levels of resistance to levofloxacin, metronidazole and in only one case to clarithromycin; the antibiotic resistant strains were mostly derived from a pre-existing sensitive strain rather than from mixed infection (61). Similar cases were reported in *Mycobacterium tuberculosis* as heteroresistance, defined as the coexistence of bacteria susceptible and resistant to anti-tuberculosis drugs in the same patient (62, 63). More recently, heteroresistance in *M. tuberculosis* was defined as the coexistence of populations with different mutations at drug resistance locus within a sample of organisms (64).Therefore, it is clear that heteroresistance does not have a uniformly consistent definition, making it difficult to compare studies retrospectively to assess its clinical significance.

1.3.2. Measuring heteroresistance

1.3.2.1. Population analysis profiling (PAP).

Population analysis profiling (PAP) is considered the gold standard method for determining heteroresistance. In this method, the bacterial population is subjected to a gradient of antibiotic concentrations and the bacterial growth at each of these concentrations is quantified. The PAP performed since the earliest description of the phenomenon adopted the format of standard MIC determination techniques with antibiotic increments following a 2-fold difference pattern. PAP

assays are mostly done by spread-plate techniques for cfu counting (43, 44, 46, 54, 56, 58, 59, 65-81). Cfu counting by dropping smaller aliquots showed similar efficiency as spread-plate (82). Turbidimetric PAP assays are also performed using 2-fold antibiotic increments (83), and antibiotic increments wider than 2-fold steps were also used (42, 84). However, in most of the studies no criteria were set to define homogeneous vs. heterogeneous resistance. The lack of a standardized method to perform PAP, in particular the selection of increments of antibiotic concentration, has led to confounding observations. For example, several studies investigating the response to glycopeptide antibiotics used PAP assays with narrow increments in antibiotic concentrations, such as 1 µg/ml steps (49, 53, 85-105) and even as low as 0.1 µg/ml steps (106). In these cases, a homogeneous strain could be inaccurately considered heteroresistant, and sometimes the same strain appeared as homogenous in one curve and heterogeneous in another (52).

A modified PAP assay comparing the area under the curve (PAP-AUC) of a given strain to that of a strain previously shown to be heterogeneous was used to determine the heterogeneous response of *S. aureus* to vancomycin (107-121). PAP-AUC ratios between test and control strain of <0.9, 0.9 to 1.3, and >1.3 were considered indicative of vancomycin susceptible *S. aureus*, heterogeneous vancomycin intermediate *S. aureus* (hVISA), and vancomycin intermediate *S. aureus* (VISA), respectively. The concern with this method is its comparative nature that relies on the response to vancomycin of the *S. aureus* control strain, whereby any instability in its antibiotic resistance phenotype would cause significant changes in the results.

Another variation of PAP was used to screen clinical isolates for heteroresistance against glycopeptides. The typical PAP method is time-consuming and labor intensive, which may not be suitable for clinical laboratories that screen hundreds of isolates for heteroresistance. Thus, screening of clinical isolates was performed on plates containing only one concentration of either vancomycin or teicoplanin, hence called glycopeptides screening plates (96, 108-110, 115, 122-124). The concentrations of glycopeptides and the medium type were slightly altered among different laboratories. However, some comparative studies proved these methods not reliable and showed poor performance in detection of heteroresistance (123, 125).

1.3.2.2. Disc diffusion and Etest assays.

Disc diffusion assays were used early on to detect heteroresistance (43, 54, 58, 95, 126-132). Later, Etest strips were similarly used at the same inoculum size as that recommended for the traditional *in vitro* susceptibility testing (55, 58, 90, 103, 104, 106, 108, 111, 113, 116, 120, 126, 128-131, 133-141). Special Etest strips were developed for glycopeptides resistance detection (GRD Etest) which are double-sided strips, one side contains vancomycin while the other contains teicoplanin (109, 114, 115, 121, 142). As with PAP, the lack of guidelines hampers the detection of heteroresistance using Etest and disc diffusion assays. The clear phenotype indicating heteroresistance is the appearance of distinct colonies growing at the otherwise clear zone of inhibition in the disc diffusion or Etest assays. However, many reports set cut-off concentrations or inhibition zone diameters to decide based on which the heterogeneity of the response of the bacterial population to antibiotics as discussed previously, but such cut-off values cannot sufficiently describe the population-wide characteristics.

1.3.2.3. Additional methods to characterize heteroresistance.

Gradient agar plates, containing a linear gradient of antibiotic, were used to determine the susceptibility of clinical isolates to antibiotics and identify antibiotic-resistant cells within bacterial populations (143). Flow cytometry using a fluorescent penicillin derivative is another approach employed to assess methicillin resistant *S. aureus* (MRSA) isolates with known heteroresistance (144). However, this method was not compared to others used for heteroresistance detection, thus its reliability cannot be assessed. Other methods to characterize heteroresistant bacteria have included bacterial re-growth at later time points in time-kill assays after an initial significant reduction in survivors (49, 80), and increase in MIC values of the same strain on prolonging the incubation time (67). In both cases, these strategies provide time to the less abundant more resistant members of the population to proliferate. On the other hand, uninterpretable and irreproducible MIC results featured mainly in the form of ‘skipwells’ (wells exhibiting no growth although growth still occurs at higher concentrations of the antibiotic) could suggest heteroresistance which was further confirmed by PAP in some of the tested isolates of *Enterobacter cloacae* and *Enterobacter aerogenes* against polymyxin B (145).

1.3.3. Reports of heteroresistance in different bacteria

Heteroresistance denotes the presence of subsets in the population of bacterial cells with higher levels of resistance to antibiotics. The more resistant bacterial cells were often isolated, but the stability of such high level of resistance differed among bacteria. After 5 to 10 serial passages in antibiotic-free medium some highly resistant cells reverted to the heterogeneous resistance phenotype displayed by their original population (43, 70, 80), whereas others retained their high level of resistance (68). On the other hand, bacteria exhibited a distinctive pattern of heteroresistance; cultures showed fixed strain-specific frequencies of more resistant subpopulations each time suggesting genetic control over this phenomenon (70).

Heteroresistance has been reported in several Gram-positive and Gram-negative bacteria. Most of the reported incidences of heteroresistance involve bactericidal antibiotics including β-lactams, glycopeptides, antimicrobial peptides, fluoroquinolones, aminoglycosides, and the nitroimidazole antibiotic metronidazole, which acts on anaerobic bacteria (Tables 1 and 2). This suggests that heteroresistance is more apparent with bactericidal antibiotics. However, no systematic comparisons of the response of heteroresistant bacteria to bacteriostatic versus bactericidal antibiotics have been reported. Only two studies report incidences of heteroresistance against bacteriostatic antibiotics. In one of them, *S. aureus* strains showing heteroresistance to fusidic acid were reported (85), but the PAP was performed using a narrow range of fusidic acid concentrations in small increments. In the other study, *Bordetella pertussis* showed heteroresistance to erythromycin (128), which could only be detected after 7 days of incubation using Etest and disc diffusion techniques, as indicated by the appearance of discrete colonies in the clear zones of inhibition.

For Gram-positive bacteria, the majority of heteroresistance cases were reported in *S. aureus* although there are several reports concerning other *Staphylococci*, *Enterococci* and *Clostridium difficile*. The earliest reports of heteroresistance in *S. aureus* were on the response to methicillin (43, 44), but this extended to other β-lactams, which accounted for the majority of research related to heteroresistance until late 1990s (Table 1).

Table 1. Heteroresistance in Gram-positive bacteria

Organism	**Antibiotic**	**Method**	**Year, Ref.**
S. aureus (MRSA isolates)	Methicillin	PAP by cfu (2 fold increments). Disc diffusion (colonies in inhibition zone)	1964(43)
S. aureus	Cephalexin& oxacillin	PAP by cfu (2-fold increments).	1970(44)
S. aureus	cephalothin, methicillin, and cephalexin	PAP by cfu (2 fold increments). Disc diffusion.	1972 (54)
Staphylococcus epidermidis and *S. haemolyticus*	methicillin	PAP by cfu (2-fold increments).	1985 (66)
S. aureus	nafcillin	PAP by cfu (2 fold increments). MIC (MICs at 48 h > at 24 h)	1985 (67)
S. aureus	Methicillin “Thermosensitive”	PAP by cfu (2 fold increments).	1986 (59)
S. aureus	Methicillin	PAP by cfu	1993 (70); 1994 (46); 1996 (71); 1996 (72); 1997 (73); 2003 (74); 2013 (146)
S. aureus	Methicillin "Eagle-type"	PAP by cfu (2 fold increments).	2001 (56)
S. epidermidis	Methicillin/ oxacillin	PAP	2005 (147)
Streptococcus pneumoniae	Penicillin	Etest (complicated by zone of hemolysis); PAP by cfu (very small increments, as low as 0.1 mg/L)	2007 (106)
S. aureus	Oxacillin		2008 (45)
S. aureus	Cefazolin; Methicillin	PAP by cfu (2 fold increments)	2008 (77)
S. aureus	Methicillin	Flow cytometry using Bocillin FL, known heteroresistant MRSA used as reference.	2009 (144)
S. aureus	Methicillin/ oxacillin	PAP & selection of high resistance by growing at subinhibitory concentration of oxacillin.	2009 (148)
S. pneumoniae	Penicillin	PAP by cfu	2014 (149)
S. aureus	Ceftaroline	PAP by cfu	2014 (150)
S. epidermidis	Methicillin; Vancomycin; Teicoplanin	PAP by cfu (2 fold increments)	1999 (86)
S. aureus	Methicillin; Vancomycin	PAP by cfu (compared spread-plate to spotting of 10 µl techniques):	2001 (82)
S. aureus	Methicillin; Vancomycin	PAP	2004 (91)
S. aureus	Vancomycin	PAP (1 mg/L increments).	1997 (53); 2000 (87); 2001 (88); 2008 (151)
S. aureus	Vancomycin	PAP Disc diffusion: (Satellitism detected)	1999 (127)
S. aureus	Vancomycin	Cfu on Vancomycin (4 mg/L) plates.	1999 (125); 2000 (122)
Enterococcus faecium	Vancomycin	E-tests (growth in zone of inhibition).	2001 (133)
S. aureus	Vancomycin	PAP by cfu (narrow increments).	2002 (89); 2005 (92); 2005 (52); 2009 (152); 2011 (102)
Coagulase negative staphylococci	Vancomycin; Teichoplanin	PAP by cfu (narrow increments).	2006 (93)
S. aureus	Vancomycin	Etest	2007 (135); 2009 (140)
Staphylococcus spp.	Vancomycin; Teichoplanin	BHI Agar screening method with 4 or 6 mg/L; PAP (narrow increments)	2007 (96)

S. aureus	Vancomycin and Teicoplanin	BHI agar + 6 mg/L Vancomycin, Mueller Hinton agar (MH) + 5 mg/L Vancomycin and MH + 5 mg/L Teicoplanin); Etest macromethod (using a 2 McFarland)	2007 (123)
Enterococcus faecium	Vancomycin	MIC by broth dilution; Etest (colonies in inhibition zone).	2008 (55)
S. aureus	Vancomycin	Modified PAP by cfu on BHI agar +0.25, 0.5, 1, 1.5, 2, 4, 6 and 8 mg/L Vancomycin. The area under the curve (AUC) was calculated.	2008 (107)
Staphylococcus capitis	Vancomycin	PAP (1 mg/L increments) and calculating (AUC test/AUC Mu3) ratios; Etest (colonies in inhibition zone); BHI agar +4 mg/L Vancomycin	2008 (108)
Enterococcus faecium	Teicoplanin	Etest	2008 (137)
S. aureus	Glycopeptides	Etest GRD strips, with one incorporated with nutrients to enhance the growth of hGISA; BHI agar + 6 mg/L Vancomycin; MH agar + 5 mg/L Teicoplanin; PAP-AUC.	2008 (109)
S. aureus	Vancomycin	PAP by cfu (2 fold increments)	2009 (78)
S. aureus	Vancomycin	PAP-AUC; Screening cascade: BHI agar +5 mg/L teicoplanin then MET for positive isolates.	2009 (110)
S. aureus	Vancomycin	MET; PAP (narrow increments)	2009 (98)
S. aureus	Vancomycin	Etest; PAP-AUC compared to Mu3.	2009 (111); 2011 (113); 2011 (116); 2012 (104); 2013 (153)
S. aureus	Vancomycin	PAP-AUC	2010 (112); 2011 (118); 2011 (119)
S. aureus	Vancomycin, but not Telavancin (bactericidal lipoglycopeptide)	PAP (narrow increments).	2010 (99)
S. aureus	Vancomycin	PAP by cfu compared to the Mu3 (hVISA) and Mu50 (VISA)	2010 (100)
S. aureus	Vancomycin/ glycopeptides	MET; PAP	2010 (101); 2011 (117)
S. aureus	Vancomycin	PAP-AUC; MET; GRD Etest; broth microdilution (MIC cutoff $\geq$ 2 mg/L); standard Vancomycin Etest (MIC cutoff $\geq$ 2 mg/L)	2011 (114)
S. aureus	Vancomycin	PAP/AUC; MET; GRD Etest; BHI agars + 3 or 4 mg/L Vancomycin.	2011 (115)
S. aureus	Vancomycin	Broth microdilution; GRD Etest on 4,210 clinically significant isolates from 43 U.S. centers; PAP-AUC for GRD-positive.	2011 (142)
S. aureus	Vancomycin	Broth microdilution; MET; Standard Etest on 220 clinical isolates (121 MSSA, 99 MRSA) from bloodstream infections. PAP-AUC; BHI agar +4 mg/L Vancomycin	2011 (124)
S. aureus	Vancomycin	PAP on 750 MRSA clinical strains isolated from Japan in 1990, before the introduction of injectable Vancomycin into clinical use in Japan in 1991.	2012 (154)
S. aureus	Vancomycin	Etest; PAP-AUC on 288 MRSA isolates from a Connecticut Veterans Hospital.	2012 (120)
S. aureus	Vancomycin	PAP on 268 MRSA isolates from Seoul, Republic of Korea	2012 (103)
S. aureus	Vancomycin	GRD Etest; PAP-AUC on 43 MRSA isolates from Malaysia.	2012 (121)

S. aureus MRSA	Glycopeptides; Daptomycin	Etest.	2009 (138)
S. aureus	Daptomycin	PAP by cfu (narrow increments)	2006 (94)
S. aureus	Daptomycin	PAP	2011 (155)
Toxigenic *Clostridium difficile*	Metronidazole	Etest and disc diffusion	2008 (129)
Clostridium difficile	Metronidazole		2010 (156)
Staphylococcus	Ciprofloxacin but not nalidixic acid	PAP & MIC	1986 (69)
Streptococcus pneumoniae	Fosfomycin	PAP (wide scale of increments higher than 2-fold)	2013 (84)
Bordetella pertussis	Erythromycin	Disc diffusion and Etest.	2002 (128)
S. aureus	Fucidic acid	PAP by cfu (narrow increments).	1998 (85)

MET (Macro-Etest): an Etest in which higher inoculum sizes are used to increase the probability of detection of more resistant members of the bacterial population.

Heteroresistance to vancomycin and other glycopeptides was first detected in Japanese vancomycin-resistant *S. aureus* (53). This also initiated a trend using the PAP format with a narrow range of antibiotic concentrations with minor increments. These findings geared heteroresistance studies towards glycopeptides and its clinical relevance and spread as vancomycin was used to eradicate the notorious MRSA infections. With focus on the clinical importance of the heterogeneous response to vancomycin, its prevalence was often assessed. However, controversial findings, originating from similar time range and geographical distribution, were reported with data showing that such heterogeneity in response to vancomycin is common among *S. aureus* strains (87, 90, 101, 103, 110, 119, 135). Others reported that heterogeneous response to vancomycin was not prevalent (91, 104, 112, 113, 120, 121, 142, 157). These studies aimed at identifying the need to assess heteroresistance in clinical laboratories as a standard procedure. However, since they adopted different guidelines for heteroresistance determination and in many cases, used improper methods to detect heterogeneity, the results were conflicting.

Much fewer reports described heteroresistance in Gram-negative bacteria. Table 2 describes the incidences of heteroresistance in *Pseudomonas aeruginosa*, *Klebsiella*, *Acinetobacter*, and *B. cenocepacia*.

While antibiotic resistance in general could either be intrinsic or acquired (19), similar observations apply to heteroresistance. Intrinsic heteroresistance occurs without the need to previous exposure to the antibiotic; however, heteroresistance may be acquired or induced as a result of initial exposure to antibiotics. For example, repeated exposure of homogenously sensitive *Staphylococci* to methicillin resulted in mixed populations resembling the intrinsically heteroresistant strains (43). A similar selection method was conducted in MRSA involving step-wise exposure to vancomycin leading to acquired heteroresistance (158).

Molecules other than antibiotics can induce heteroresistance. For example, exogenous glycine led to heterogeneous response to methicillin in the highly homogeneous MRSA COL strain (71). Increasing concentrations of glycine in the medium resulted in replacement of the D-alanyl-D-alanine terminus of the muropeptides with D-alanyl-glycine-terminating muropeptides of bacterial peptidoglycan leading to decreased methicillin resistance and the appearance of a

Table 2. Heteroresistance in Gram-negative bacteria

Organism	Antibiotic	Method	Year, Ref.
Type b *Hemophilus influenzae*	Streptomycin	PAP by cfu count (concentrations <10-1000 U/ml)	1947(42)
Enterobacter aerogenes; E. coli; other Enterobacteria	Cefamandole;Cefoxitin, Carbenicillin; nalidixic acid.	PAP by cfu (2-fold increments)	1979 (65)
E. coli	Cefamandole; Cefotaxime; Cefoxitin; imipenem	Turbidimetric PAP (2 fold increments or more)	1985 (83)
8 species of Enterobacteriaceae	Cefotaxime	PAP: *E.coli* and *Proteus mirabilis*: homogeneous; *Klebsiella oxytoca* and *Citrobacter koseri*: less homogeneous; *Enterobacter cloacae*, *Citrobacter freundii*, *Proteus vulgaris*, and *Morganella morganii*: heterogeneous.	1985 (68)
P. aeruginosa, and 7 strains from 5 genera of Enterobacteriaceae	Ciprofloxacin	PAP & MIC	1986 (69)
Helicobacter pylori	Metronidazole	Etest and disc diffusion	1996 (126)
Acinetobacter baumannii	Imipenem;Meropenem	Etest	2005 (134)
A. baumannii	Colistin	PAP by cfu (narrow increments); Time kill curves.	2006 (49)
P. aeruginosa	Carbapenems (Imipenem and Meropenem)	Disc diffusion; PAP by cfu: (narrow increments and low initial inoculum)	2007 (95)
Invasive nontypeable *H. influenzae*	Imipenem	PAP by cfu (2 fold increments); Etest (for MIC determination)	2007 (75)
Enterobacter cloacae and *A. baumannii*	Colistin	Disk diffusion; Etest; agar dilution; broth microdilution	2007 (51)
A. baumannii-calcoaceticus complex	Colistin	PAP by cfu (2 fold increments).	2008 (76)
P. aeruginosa	Carbapenems	Agar dilution according to CLSI. Increments of 2 mg/L for concentrations ranging from 2 to 32mg /L and of 8mg/L from 32 to 64 mg/L	2008 (97)
Bartonella sp.	Ciprofloxacin	Etest	2008 (136)
A. baumannii	Ampicillin/Sulbactam	Etest (incubation for ≥48 h)	2009 (139)
A. baumannii	Carbapenem	Disk-diffusion; Etest.	2009 (130)
E. aerogenes	Carbapenem	Etest; automated MicroScan WalkAway system (failed to detect heteroresistance detected by Etest)	2009 (159)
A. baumannii	Meropenem	PAP by cfu (2 fold increments).	2009 (79)
Klebsiella pneumoniae	Meropenem	MIC & PAP (2 fold increments); Time kill assays	2010 (80)
K. pneumonia	Carbapenem	Etest; PAP.	2010 (141)
A. baumannii	Imipenem	Etest; disk diffusion (colonies in the inhibition zone)	2011 (131)
Carbapenemase-producing *K. pneumoniae*	Colistin	PAP by cfu (2 fold increments) & MIC	2011 (81)
A. baumannii	Cefepime	Etest, disc diffusion, PAP by cfu (2 fold increments).	2012 (58)
A. baumannii	Carbapenems	Disc diffusion	2012 (132)
P. aeruginosa	Polymyxin B	PAP by cfu (PmB concentrations from 0 to 8 mg/L).*	2013 (105)
E. cloacae; E. aerogenes	Polymyxin B	PAP	2013 (145)
H. pylori	levofloxacin (5/19), clarithromycin (1/19) and metronidazole (16/19)	MIC by Etest and agar dilution for 19 pairs of clinical isolates. Each pair was isolated from the same patient. Heteroresistance was reported when pairs showed difference in resistance.	2014 (61)
Providencia rettgeri	Carbapenems	PAP by cfu	2014 (160)

*Isolates presenting subpopulations that exhibited growth at Polymyxin B concentrations ≥2 mg/L were considered heteroresistant. Isolates containing subpopulations that grew at Polymyxin B concentrations at least twice as high as the original MIC but <2 mg/L were considered heterogeneous.

heterogeneous resistance phenotype. On the other hand, heteroresistance may originate from genetic manipulation events such as transposition of resistance genes (161, 162) or conjugation (163). The generated progenies included cells having different MIC values due to differences in the number of copies of the inserted resistance genes or random disruption of genes involved in the bacterial response to antibiotics.

1.3.4. Mechanisms of heteroresistance

Non-genetic individuality in bacterial populations has been observed in a wide range of biological processes, including differentiation and cell division (164), chemotaxis (165), enzymatic activity (166), sporulation (167), stress response and antibiotic resistance (48, 168, 169). However, the exact mechanism of heteroresistance is not clear and appears to be multifactorial. In several cases, increased resistance was due to mutations or differential expression of key resistance genes or regulatory systems. Long term infection was proposed to result in instability of genomic DNA of bacteria potentially leading to heteroresistance; for example, mutations in gene products having metronidazole nitroreductase activities, mainly oxygen-insensitive NAD(P)H nitroreductase (RdxA) and NAD(P)H flavin oxidoreductase (FrxA) occurred in *H. pylori* heteroresistant to metronidazole (61).

1.3.5. Significance and relevance of heteroresistance

The lack of standardized definition of heteroresistance, which may lead to misidentification of homogenous strains as heteroresistant, hinders the proper assessment of the clinical relevance of heteroresistance. While certain reports argued against the clinical significance of heteroresistance observing no differences in the clinical outcome between heterogeneous populations and their respective homogenously sensitive cultures (91, 103, 116, 152), others showed clear deterioration in the clinical outcome due to bacteria displaying a heterogeneous phenotype of resistance (86, 90, 104, 111, 117, 118, 170-173). Indeed, this controversy might be influenced by the lack of standardized definition of heteroresistance as outlined in a previous section, thus the lack of influence on the clinical outcome might reflect non-truly heteroresistant bacteria.

Heteroresistance presents a risk of possible misinterpretations when only a single colony, picked from the primary bacterial populations isolated from patients, is analyzed for its susceptibility to antibiotics (126). The clinical impact of heteroresistance range between recurrence of infections (86, 111), chronic infections (118) to increased mortality rates (104, 117, 171, 172). The perceived main cause of such deterioration of the clinical picture and therapeutic failure is the speculated selection of more resistant cells in the bacterial population by antimicrobial therapy; this was directly detected in one study (170). However, the chemical communication and transfer of resistance from the more resistant subpopulations to sensitive cells is another aspect that would impede the therapeutic efficiency of antibiotics.

1.3.5.1. Selection of more resistant cells of the population

Therapeutic dosing of antibiotics determined without taking into consideration the highly resistant subpopulations of a heteroresistant bacterial isolate would result in selection of such subpopulations. This is particularly the case when the majority of the population is sensitive to antibiotics while only a small subset, undetectable through the criteria set for the traditional *in vitro* antibiotic susceptibility testing, display resistance to the antibiotic above the clinical breakpoint (Fig. 2). In these situations, the net outcome of antibiotic therapy would be the eradication of the more sensitive members of the bacterial population while the more resistant cells proliferate and become predominant in the infection.

Colistin treatment of a patient with postneurosurgical meningitis harboring a colistin-heteroresistant *A. baumannii* isolate resulted in the selection of colistin-resistant strains (170). Moreover, *A. baumannii* isolates transitioned *in vivo* from susceptibility to full-resistance to carbapenems, with heteroresistance as an intermediate stage in the same intensive care unit due to administration of meropenem (130). Meropenem pressure can produce meropenem-heteroresistant subpopulations of *A. baumannii* that could be selected for by the use of suboptimal therapeutic drug dosages giving rise to highly resistant strains (79). Similarly, evidence of *in vivo* development of heteroresistance as a result of antibiotic therapy was shown in a patient with MRSA (138). Initial treatment with glycopeptides led to the development of heterogeneous glycopeptide resistance, which transformed to full resistance following

daptomycin treatment. A similar observation of switching from susceptibility to heteroresistance occurred in *A. baumannii* in patients after prolonged exposure to imipenem (131).

1.3.5.2. Chemical communication of antibiotic resistance

Highly resistant subpopulations of heteroresistant bacteria could further complicate the clinical picture of polymicrobial infections by providing protection to normally sensitive bacteria through chemical signals. Although not truly heteroresistant owing to the lack of significant variation in concentrations tolerated by the members of an *E. coli* bacterial population, more resistant mutants arising from the continuous antibiotic treatment protected less resistant cells of the same population from norfloxacin and gentamicin (174). Such mutants could maintain the same level of indole production in the presence of antibiotic treatment, which could protect less resistant cells that produced lower concentration of indole under antibiotic stress. These mutants, although more resistant relative to the rest of the population, cannot be considered absolutely highly resistant as their MIC is at or slightly above the MIC breakpoint for sensitive bacteria especially for norfloxacin, hence questioning their survival *in vivo* at therapeutic doses of antibiotics. Interestingly, indole production is not common among bacteria (175); nevertheless, indole produced by *E. coli* was shown to confer antibiotic resistance to the indole-negative intestinal pathogen *Salmonella enterica* serovar Typhimurium (176). Other chemical signals normally produced by bacteria and that may modulate the antibiotic resistance profiles of bacteria can potentially be similarly implicated in the communication of resistance among different bacteria; hence, such signals will be further discussed in following sections.

Protection from antibiotics also occurred through antibiotic degrading enzymes. Protection of sensitive bacteria was mediated by beta-lactamases produced from resistant *E. coli* cells against beta-lactamase sensitive agents as cefamandole, but not cefotaxime, cefoxitin or imipenem which are more resistant to beta-lactamases (83).

1.4. Non-genetic mechanisms communicating antibiotic resistance

The alarming emergence of outbreaks by multidrug resistant clinical isolates pose a serious challenge to the treatment of infections, often turning a mild infection into a life threat, and has led to extensive worldwide studies on the mechanisms of antibiotic resistance and its genetic transfer across bacterial populations (177). Unveiling the various mechanisms of genetic transfer that contribute to the spread of antibiotic resistance has been a major breakthrough. However, these findings have overshadowed other potentially important mechanisms of resistance mediated by non-genetic factors. Indeed, the horizontal transfer of genetic determinants encoding antibiotic resistance markers has been considered as the only mechanism of transfer of antibiotic resistance across bacterial populations (178). However, bacteria possess signal transduction machineries that rely on chemical signals enabling cell-to-cell communication and coordinating multicellular behavior. The best examples are N-acyl-homoserine lactone and non-N-acyl-homoserine lactone-based quorum sensing systems (179), which have been extensively reviewed and will not be discussed here.

In this section, I will further challenge the traditional view of intrinsic resistance by focusing on the current understanding of the small molecules that are capable of altering the antibiotic susceptibility of bacterial cells by modulating cellular responses towards antibiotic stress. This does not include mechanisms involving physical elimination of the antibiotics through neutralization, or degradation, which would eventually lead to protection of other cells from the antibiotic effect. The small molecules discussed in this section can be secreted by bacterial cells or alternatively, by host cells in body fluids and tissues. They may also be secreted from plants into the soil and thus present in the bacterial milieu. The interactions of bacteria with these molecules occur in the context of intra-species, interspecies or interkingdom communication, and contribute to the mechanism of intrinsic resistance to antibiotics. A better understanding of how the small molecule-mediated interactions influence antibiotic resistance would eventually lead to designing more effective inhibitors of such mechanisms and provide better therapeutic solutions for combating multidrug resistant microbial infections.

1.4.1. Chemical signals modulating antibiotic resistance

In this section, I will discuss various small molecules that act as infochemicals modulating the susceptibility of bacteria to antibiotics (Table 3) and their potential mechanisms of action (Fig. 3). I will also attempt to expose new targets for developing lead compounds that could act as inhibitors of the action of infochemicals to prevent increased resistance to antibiotics.

1.4.1.1. Indole

Indole is an aromatic heterocyclic organic compound derived from the amino acid tryptophan in a reaction mediated by the TnaA tryptophanase. It is produced by around 85 species of Gram-positive and Gram-negative bacteria (175). Indole has been recently recognized as an intercellular signal molecule that controls diverse aspects of bacterial physiology including biofilm formation (175).

Lee *et al.*revealed that few antibiotic resistant mutant cells that arise in an *Escherichia coli* population in response to norfloxacin or gentamicin improved the overall response of the bacterial population towards the antibiotics in part due to indole production (174). Indole production was not induced in the more resistant mutant cells by the antibiotics but rather its level was unchanged regardless of antibiotic exposure, as opposed to wild type bacteria in which indole production was suppressed in response to the antibiotics (174).

Exogenous indole conferred resistance against norfloxacin and gentamicin to the less resistant isolates (Table 3) (174). Moreover, *E. coli* cells treated with indole became rhodamine 6G and sodium dodecylsulfate (SDS) resistant (180). Similar findings were reported in *E. coli* cells exposed to ampicillin and kanamycin, in which a higher level of extracellular indole production protected bacterial cells from antibiotic damage (181). Indole was also shown to influence persister formation (182). Persisters neither die nor grow in the presence of antibiotic, as they are dormant during antibiotic exposure (47). These persister bacterial cells grow after the antibiotic removal, and their progeny does not exhibit increased resistance to the antibiotic. Therefore, their pattern of sensitivity to the antibiotic remains identical to that of the original bacterial population (48).

Table 3.Effect of different small molecules on the activity of antibiotics.

Organism	Molecule	Conc.	Antibiotic	Method	Effect	Ref.
Indole						
E. coli MG1655	Indole	300 µM	Norfloxacin and gentamicin	MBC	Increased resistance	(174)
E. coli MC4100		2 mM	Rhodamine 6G and SDS	Survival rate assay	Increased resistance	(180)
E. coli K-12 BW25113		1 mM	Ampicillin and kanamycin	OD growth assay	Increased resistance	(181)
Polyamines						
S. aureus strains Mu50 and N315, *S. aureus* strain ATCC 35556.	Spn	1mM	15 different β-lactams, chloramphenicol, polymyxin B, and tetracycline	MIC	Increased sensitivity	(183)
			Vancomycin, ciprofloxacin, and gentamicin	MIC	No effect	(183)
P. aeruginosa PAO1and 10 clinical isolates	Spn Spd	1 mM 20 mM	Carbenicillin, chloramphenicol	MIC	Increased Sensitivity (with few exceptions)	(183)
			Imipenem, ciprofloxacin, and Polymyxin B	MIC	Increased resistance (with few exceptions)	(183)
E. coli K-10, K-12, C921-61 and *S. enterica* serovar Typhimurium LT2	Spn Spd	1 mM 20 mM	Chloramphenicol and 8 different β-lactams	MIC	Increased sensitivity	(183)
P. aeruginosa PAO1	Spn Spd Put Cad	1 mM 20 mM 20 mM 20 mM	14 β-lactams, chloramphenicol, nalidixic acid and trimethoprim	MIC	Increased sensitivity	(184)
			Erythromycin, novobiocin and fusidic acid	MIC	No effect	(184)
P. aeruginosa PAO1	Spn Spd Put* Cad*	1 mM 20 mM 20 mM 20 mM	Polymyxin B*, colistin*, kanamycin, gentamicin, ciprofloxacin, norfloxacin	MIC	Increased resistance (* combinations: no effect)	(185)
			Tetracycline	MIC	No effect	(185)
E. coli GGB2600	Spd, Put, or their equimolar mix	0.1, 1 or 10 mM	Levofloxacin, netilmicin, cefotaxime	MIC	Increased resistance	(186)
E. coli RO91	Put	10 mM		CFU assay		(186)
E. coli BL21, *P.*	Spd	2 mM	Tetracycline,	Growth	Increased	(187)

aeruginosa Lm1, *B. subtilis* and *S. aureus* Xen36	Put		kanamycin	on antibiotic-containing agar	resistance to Tet, Increased sensitivity to Kan	
Volatile signals						
E. coli BL21, *P. aeruginosa* Lm1, *B. subtilis* and *S. aureus* Xen36	Volatile ammonia	From 5-50 mM NH_4^+ sol.	Tetracycline	Growth on antibiotic-containing agar	Increased resistance	(187)
			Kanamycin		Increased sensitivity	
Serratia rubidaea and *S. marcescens*	Volatile ammonia	From 0.1% sol.	Ampicillin		Increased resistance	(188)
E. coli	TMA	From 0.5% Sol.	Tetracycline		Increased resistance	(189)
			Aminoglycosodes ; chloramphenicol		Increased sensitivity	
B. subtilis	2,3-BD and glyoxylic acid		Ampicillin and tetracycline		Increased resistance	(190)
Burkholderia ambifaria	sulfur compounds, ketones, aromatic compounds		Gentamicin and kanamycin	Disc-diffusion assay	Increased resistance	(191)
Pseudomonas Quinolone Signal (PQS)						
P. aeruginosa PAO1	PQS	60 µM	Tetracycline, chloramphenicol, carbenicillin, spectinomycin and to a lesser extent kanamycin	Filter-disk assay	Increased sensitivity	(192)
Indole Acetic Acid (IAA)						
E. coli K-12	IAA	0.5 mM	Erythromycin, rifampicin, penicillin and novobiocin	CFU assay	Increased resistance	(193)

TMA: trimethylamine; 2,3-BD: 2,3-butanebione; Spn: spermine; Spd: spermidine; Put: putrescine; Cad: cadaverine; PQS: Pseudomonas quinolone signal; IAA: indole acetic acid.

The improved response to antibiotics mediated by indole was attributed to the stimulation of certain drug efflux pumps (174, 180, 194) and oxidative stress protective mechanisms (174) (Fig. 3). Furthermore, indole is sensed in a heterogeneous manner across the bacterial population (182), causing induction of OxyR and phage-shock pathways via a periplasmic or membrane component, and triggering protective responses that result in the appearance of a persistent subpopulation.

1.4.1.2. Polyamines

Natural polyamines consist of diamines (putrescine and cadaverine) and oligoamines (spermidine and spermine) (195). The first description of a natural polyamine dates from more than 300 years ago when spermine crystals were discovered in human semen (196). Since then, natural polyamines were found in almost all living organisms. In plants, polyamines are involved in growth, development, and modulation of defense responses to diverse environmental stresses. In addition, polyamines have acid neutralizing and antioxidant properties, as well as membrane and cell wall stabilizing abilities (197). Like in plants, polyamines also regulate cell growth and proliferation in humans, as well as stabilize negative charges of DNA influencing RNA transcription, protein synthesis, apoptosis, and immune responses (198). Spermidine enhances autophagy, thus suppressing necrosis and enhancing cell longevity (199, 200). Spermine is produced at higher levels by regenerating tissues, while injured or dying cells release spermine into the extracellular milieu, so that tissue levels of spermine significantly increase at inflammatory sites of infection or injury (201). Also, polyamines have anti-inflammatory and immunosuppressive properties similar to those of glucocorticoid hormones (202). Spermine accumulates at sites of infections such as mycobacteria-infected sites and pneumonia (201, 203).

Nearly all bacteria produce polyamines, with rare exceptions such as most *Staphylococcus aureus* strains; polyamines, namely spermidine and spermine, are toxic to such strains as they lack polyamines detoxifying enzymes (204). While polyamines play roles in growth and other physiological processes in bacteria including incorporation into the cell wall, biosynthesis of siderophores, acid resistance, scavenging free radical ion, signaling cellular differentiation and biofilm formation (205), they also contribute to the bacterial responses to antibiotics

demonstrated by the response of polyamine-deprived mutants and bacteria exposed to exogenous polyamines to antibiotics.The two most common bacterial polyamines are putrescine and spermidine (205). Cadaverine is usually synthesized only when putrescine synthesis is blocked or in cases where there is excess lysine, its amino acid precursor, under anaerobic conditions at low pH (206).

Both the inhibition of polyamine biosynthesis (207-210) and excess of exogenous polyamines prevent bacterial growth (183, 211), as shown with certain strains of *Staphylococcus aureus*, *E. coli* and *Salmonella enterica* (Table 4). These effects vary for the same strain under different experimental conditions (183). On the other hand, *Pseudomonas aeruginosa* isolates are resistant to the effects of the tested polyamines (183). These opposite effects of polyamines on bacterial growth might indicate that a fine balance in their concentration within the cells is needed for the proper homeostasis of the bacteria. This is supported by the observation that the polyamine content of cells is highly regulated by biosynthesis, modification, uptake and excretion (212).

1.4.1.2.1. Effect of polyamines on bacterial susceptibility to antibiotics

Few studies reporting the effects of exogenous polyamines on the antibacterial activity of different antibiotics against a variety of organisms (183-187) are summarized in Table 3. Results vary from study to study depending on the polyamine used, its concentration, the strain tested, and the test conditions. Despite some contradictory observations and the lack of systematic studies on polyamine-antibiotic interactions, it appears that high concentrations of polyamines approaching their inhibitory range (Table 4) increase the sensitivity to antibiotics, whereas lower concentrations of polyamines increase resistance to antibiotics. Conversely, organisms that have higher level of resistance to polyamines, such as *P. aeruginosa*, usually show increased resistance to antibiotics in response to polyamines, whether at high or low concentration.

1.4.1.2.2. The mechanism of alteration of antibiotic response by polyamines

It is not clear how polyamines mediate increased sensitivity to antibiotics. Polyamines do not apparently affect the outer membrane as their effects are not abolished by divalent ions like magnesium or calcium in contrast to the membrane-destabilizers polymyxin B and EDTA (184), and they do not increase outer membrane permeability (213). Moreover, polyamines do not inhibit efflux pumps, as for example they exert the same synergistic effects in AcrAB efflux pump defective mutants and wild-type *E. coli* (183). On the other hand, the induced sensitivity to aminoglycosides as a result of increased levels of intracellular polyamines is due to higher translation of the oligopeptide binding protein OppA (Fig. 3) (187). OppA is a periplasmic protein involved in aminoglycoside uptake and reduction in its level leads to aminoglycosides resistance (214).

On the other hand, polyamines contribute to increasing the resistance to various antibiotics in different bacterial species through multiple mechanisms (Fig.3). One mechanism involves preventing the uptake of antibiotics by either blocking certain outer membrane porin channels or competing with antibiotics for uptake through certain porins. For example, spermine can block the porin OprD of *P. aeruginosa* resulting in increased resistance to imipenem (183, 215). Similarly, the interaction of polyamines with the porin OmpF, and to a lesser extent OmpC, resulted in reduced permeability to various antimicrobials (216-221). Tkachenko et al. (222) demonstrated that polyamines increased the resistance of *E. coli* to antibiotics transported through porin channels by decreasing the outer membrane permeability, and suggested three mechanisms: (i) direct inhibition of the transport activity of porin channels, (ii) activation of the transcription of *micF* whose product is an antisense RNA that inhibits the translation of porins, and (iii) increase in the cell content of the stress resistance regulator σ^S that suppresses the transcription of *ompF* and induces cadaverine synthesis, thus leading to a decrease in the porin transport.

Spermidine and other polyamines may also modulate efflux pump activity. In *Burkholderia pseudomallei*, spermidine upregulates efflux pumps such as BpeAB-OprB, AmrAB-OprB and BpeEF-OprC, contributing to aminoglycoside and macrolide resistance as well as biofilm formation through the increased efflux of N-acyl homoserine lactones (223). Furthermore, GeneChip experiments and promoter fusion studies have shown that spermidine induces the expression of the *P. aeruginosa oprH-phoPQ* and *PA3552-PA3559* operons encoding enzymes

Table 4.Inhibitory effects of Polyamines.

Organism	Molecule	Conc.	Method	Effect	Ref.
E. coli strain B	Spd	390 µg/ml	MIC	Inhibition	(211)
	Spn	17 µg/ml	MIC	Inhibition	(211)
S. aureus	Spd	12 µg/ml	MIC	Inhibition	(211)
	Spn	2 µg/ml	MIC	Inhibition	(211)
S. aureus strains Mu50 and N315, *S. aureus* ATCC 35556 *E. coli* K-10 and K-12 and enterotoxigenic strain C912-61 10 clinical isolates of *P. aeruginosa, P. aeruginosa* PAO1. *S. enteric* serovar Typhimurium LT2	Spd	Up to 16 mM	MIC	No inhibition	(183)
P. aeruginosa PAO1, *E. coli K-12*, and *E. coli* C921-16	Spn	Up to 16 mM	MIC	No inhibition	(183)
E. coli K-10 and *Salmonella enterica* serovar Typhimurium LT2	Spn	1 to 2 mM	MIC	Inhibition	(183)
S. aureus ATCC 35556 (MSSA/VSSA), N315, (MRSA/VSSA), and Mu50, (MRSA/VISA)	Spn	4 mM	MIC	Inhibition	(183)
P. aeruginosa PAO1	Spn	10 mM	OD growth curve	No effect	(183)
S. aureus Mu50	Spn	1 mM	OD growth curve	Doubling time increased from 39 min to 62 min	(183)
*E. coli*K-12, *E. coli* K-10 and *S. enterica* serovar Typhimurium LT2	Spn	1, 2 and 4 mM	OD growth curve	Concentration-dependent inhibition	(183)

Spn: spermine; Spd: spermidine

for LPS modification and resulting in PhoPQ-mediated spermidine-induced resistance to cationic antimicrobial peptides and quinolones (185). This study also suggests a possible spermidine-responsive sensor residing in the cytoplasmic membrane and modulating the phosphorylation status of PhoP (185).

Another mechanism of polyamines to protect bacteria from antibiotic damage is preventing oxidative stress. *E. coli* cells respond to oxidative stress induction by sublethal concentrations of fluoroquinolones, aminoglycosides and cephalosporins with a 2-3 fold increase in cell polyamine content (putrescine, and spermidine) due to upregulation of the ornithine decarboxylase (186). Moreover, exogenous polyamines reduced intracellular reactive oxygen species production, thereby preventing the damage to proteins and DNA, eventually increasing cell viability, growth recovery and antibiotic resistance (186). This agrees with other studies showing the induction of polyamines biosynthesis by reactive oxygen species and the role of polyamines in modulating the cellular response to counter such stress (224, 225). Also, surface-localized spermidine is produced under Mg^{2+}-limiting conditions as an organic polycation and is proposed to bind lipopolysaccharide (LPS) and to stabilize and protect the outer membrane against antibiotic and oxidative damage (226).

1.4.1.3. Volatile signals

Bacteria can produce various volatile compounds as complex as those of plants and fungi, but the ecological function of these compounds remains unknown (227). Volatile compounds may modulate interactions within the microbial communities that can potentially result in the creation of a favorable niche for some community members (228). The profiles of volatile compounds produced by microorganisms are consistent, within the same cultural conditions, environment and inputs (228), and have led to attempts to identify bacteria based on their volatile fingerprints (229).While volatile compounds constitute a large class of potential infochemicals, their role in bacteria–bacteria interactions remains unexplored. However, recent investigations have clearly demonstrated that bacteria employ their volatiles during interactions with other organisms to influence populations and communities (230). This phenomenon was considered to resemble olfaction, or the sensing of airborne volatile compounds, a property of higher eukaryotes (231).

Volatile-mediated transfer of antibiotic resistance to ampicillin in *E. coli* was first reported in 2002; however, the nature of the airborne signal was unknown (232). A more recent study showed that exposure to gaseous ammonia, a catabolic product of L-aspartate, released from stationary phase *E. coli* K12 cultures alters the antibiotic resistance profile of several Gram-negative and Gram-positive bacteria (187). Ammonia increased resistance to tetracycline in *E. coli* BL21, *P. aeruginosa* Lm1, *Bacillus subtilis*, and *Staphylococcus aureus* Xen36 whereas it increased sensitivity to the aminoglycoside kanamycin (Table 3). These effects resulted from ammonia-dependent increase in polyamine levels,which altered the membrane permeability to antibiotics and increased the resistance to oxidative stress (Fig.3); but whether or not the ammonia release was induced in response to antibiotics was not determined (187). Similar ammonia-mediated protection from ampicillin in *Serratia rubidaea* and *S. marcescens* was attributed to antibiotic inactivation by alkalinization of the medium (188). Interestingly, volatiles emitted from the tested Gram-positive and Gram-negative bacteria increased resistance of *E. coli* to tetracycline; however, volatiles emitted from *E. coli* did not alter resistance to ticarcillin, chloramphenicol, ofloxacin and vancomycin (187).

Trimethylamine (TMA) is another volatile compound produced by *E. coli* that can alter antibiotic resistance patterns of *E. coli*, *P. aeruginosa*, *S. aureus*, and *B. subtilis* as a result of TMA-mediated alkalinization of extracellular medium which would increase uptake of aminoglycosides and chloramphenicol and reduce uptake of tetracycline (189). On the other hand, 2,3-butanedione and glyoxylic acid emitted from *B. subtilis* increased resistance of *E. coli* to ampicillin and tetracycline, a phenotype regulated by the previously uncharacterized *ypdB* gene product through the downstream transcription factors *soxS*, *rpoS* or *yjhU*(190).

Burkholderia ambifaria emitted highly bioactive volatile blend (not regulated by quorum-sensing systems) containing predominantly sulfur compounds, ketones, and aromatic compounds with dimethyl disulfide being the most abundant compound. These volatile blends, and their individual components, increased resistance to the aminoglycoside antibiotics gentamicin and kanamycin in *E. coli* and induced significant biomass increase in the model plant *Arabidopsis thaliana* as well as growth inhibition of two phytopathogenic fungi (*Rhizoctonia solani* and *Alternaria alternata*) (191).

1.4.1.4. Pseudomonas quinolone signal (PQS)

The *Pseudomonas* quinolone signal (PQS; 2-heptyl-3-hydroxy-4-quinolone), like the well-studied N-acyl homoserine lactones, functions as a quorum-sensing signal that controls genes required for virulence factor expression and biofilm formation (233, 234). Being hydrophobic, it is difficult to explain how the PQS acts as an extracellular signal. However, outer membrane vesicles can transport the PQS signal among *P. aeruginosa* cells (235). Furthermore, many of the *P. aeruginosa* quinolones/quinolines packed into these vesicles have antibiotic activity against Gram-positive cells such as *S. aureus* and *B. subtilis*, suggesting that the production of such molecules might provide *P. aeruginosa* with an advantage to gain a niche by inhibiting the growth of competing microorganisms (236).

Indirect evidences suggest the possibility that PQS-dependent cell-to-cell communication in *P. aeruginosa* may be involved in controlling susceptibility to antimicrobial agents. For example, exogenous PQS increased the susceptibility of the wild type *P. aeruginosa* PAO1 strain to tetracycline, chloramphenicol, carbenicillin, spectinomycin, and to a lesser extent kanamycin (Table 3). This was suggested to be a consequence of the PQS-dependent repression of other multidrug efflux pumps, or direct regulation of genes involved in controlling cell envelope permeability by PQS (Fig.3) (192). Overexpression of the MexEF-OprN multidrug efflux system results in a delay in PQS production due to efflux of kynurenine, a PQS precursor, thus increased antibiotic resistance was accompanied with lower intracellular levels of PQS (237).Other studies also showed that overproduction of the MexEF-OprN pump results in increased resistance to quinolone antibiotics and chloramphenicol, but hypersusceptibility to most β lactams (238). Further work is still required to demonstrate a direct effect of PQS in modulation of antibiotic susceptibility and its mechanism.

1.4.1.5. The phytohormone Indole-3-acetic acid

Indole-3-acetic acid (IAA) is the primary auxin in plants that regulates many plant developmental and cellular processes and is capable of inducing changes in gene and protein

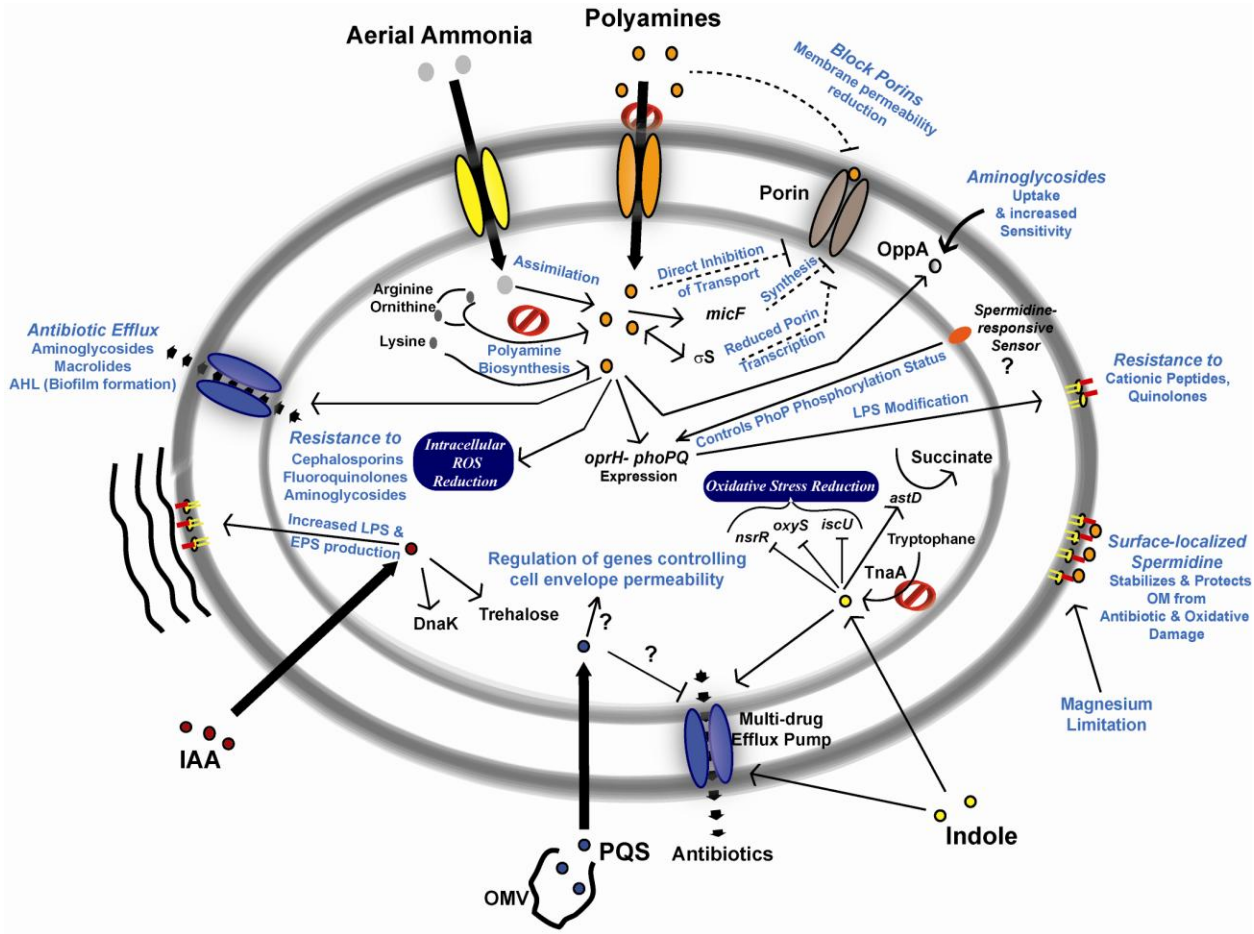

Figure 3. Mechanisms of alteration of antibiotic susceptibility mediated by small molecules and potential targets for drug design.

For detailed mechanisms of action of each molecule and their corresponding references, please refer to the text. The potential targets for the design of novel therapeutics are marked by this sign (⊘). AHL: N-acyl-homoserine lactone; EPS: exopolysaccharide; IAA: Indole-3-acetic acid; LPS: lipopolysaccharide; OM: outer membrane; OMV: outer membrane vesicles; PQS: *Pseudomonas* quinolone signal; ROS: reactive oxygen species.

expressions, in prokaryotic and eukaryotic organisms, leading to different physiological alterations (239). Auxin biosynthesis is also widespread among soil- and plant-associated bacteria such as *Streptomyces, Bacillus, Pseudomonas, Burkholderia, Erwinia, Flavobacterium,* and *Stenotrophomonas* as part of a system to communicate with their plant host (240). IAA treatment of *E. coli* K-12 cells enhanced their resistance to various stress conditions including exposure to antibiotics such as erythromycin, rifampicin, penicillin and novobiocin (Table 3) (193). It seemed that IAA activates different protective pathways to synergistically enhance stress tolerance. Treatment with IAA resulted in increased lipopolysaccharide (LPS) and exopolysaccharide production, and enhanced synthesis of the chemical and molecular chaperones, trehalose and DnaK respectively, which correlated with the higher resistance to stress conditions (Fig.3) (193).

1.4.2. New targets for drug discovery

The successful therapeutic outcome of bacterial infections is impeded by the continuous emergence of antibiotic resistant bacteria. This represents a major challenge that aggravates the problems posed by microbial infections, especially when these infections further complicate existing health-deteriorating conditions. The concept of using helper compounds that inhibit certain features of pathogenic bacteria provides an appealing approach to reverse bacterial resistance to antibiotics by targeting the bacterial membrane permeability to enhance penetration of antibiotics or by inhibition of efflux pumps (241-244). Given the advances in our understanding of the mechanisms of intrinsic resistance involving small molecular cues modulating resistance to antibiotics, I propose that another target for potential inhibitors is extracellular signaling, and various potential targets of this type for drug design are outlined in Fig. 3. Interfering with biosynthetic pathways of the signaling molecules could not only overcome increased antibiotic resistance (245), but also aid preventing the potential communication and spread of antibiotic resistance to other bacteria mediated by these small molecules. Such treatments would neither stop cellular division directly nor be toxic to the cells, thus reducing the selective pressure to evolve mechanisms of resistance. In addition, targeting small-molecule signaling pathways ensures that treatments will be directed specifically at the pathogenic organism, rather than the entire microbiome (245).Overall, this direction for drug

discovery could potentially expand further as our understanding of the mechanisms, biosynthetic pathways and uptake of the different small molecules continues to increase.

1.5. Hypothesis and general objectives

Microbial infections are becoming more refractory to antibiotic therapy. The clinical outcome of antibiotic treatment does not always correlate with the expectations based on *in vitro* susceptibility testing performed on individual clinical isolates (246). Owing to the polymicrobial nature of many infections (247), cross talk between the different bacterial species is probable during infection. Hence, *I hypothesize that bacteria displaying high-level intrinsic antibiotic resistance, especially the more resistant members within a heteroresistant population, can communicate such high level of resistance to other less resistant bacteria through chemical cues, protecting them from the lethal action of antibiotics.* To address this hypothesis, I will use *B. cenocepacia* as a model organism to determine:

1. The population-wide response of *B. cenocepacia* to antibiotics whether it is a homogeneous response or it displays heteroresistance.

2. If *B. cenocepacia* (the more resistant members of its population in case of heteroresistance) can communicate its high level of resistance to less resistant bacteria.

3. The signals involved in the chemical communication of antibiotic resistance, in case *B. cenocepacia* cells are capable of protecting other less resistant cells.

4. The mechanism by which the chemical signals involved in the phenomenon protects against the action of antibiotics.

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Chapter 2

Chemical communication of antibiotic resistance by a highly resistant subpopulation of bacterial cells

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2.1. Introduction

Treating infection caused by multidrug resistant bacteria is challenging, especially in immunocompromised patients. These individuals often succumb from infections by opportunistic bacteria that display intrinsic, high-level resistance to virtually all antimicrobials available for clinical use. Reduced permeability of the bacterial cell envelope in conjunction with multidrug efflux pumps are considered major determinants of intrinsic multidrug resistance (1). However, the overall resistance of a bacterial population results from the combination of a wide range of susceptibilities displayed by subsets of bacterial cells. Bacterial heteroresistance to antibiotics has been documented for several pathogenic bacteria, but the mechanism of heteroresistance is not always clear. Here, I use *Burkholderia cenocepacia* as a model opportunistic bacterium to investigate the implications of heterogeneity in the response to the antimicrobial peptide polymyxin B (PmB) and also other antibiotics. *B. cenocepacia* is an environmental, opportunistic pathogen that causes serious infections in patients with cystic fibrosis and expresses high-level multidrug resistance (2). Using the prototypic *B. cenocepacia* K56-2 strain, I observed a population-wide variation in the response to PmB and more importantly, that the more resistant members communicate higher level of resistance to less resistant members of the same population, and to other bacterial species in co-culture, such as *Pseudomonas aeruginosa* and *Escherichia coli*. Communication of increased resistance depended on overproduction by the more resistant subpopulations of the polyamine putrescine and increased secretion of YceI, a highly conserved small protein of unknown function. This rather general multifactorial mechanism of communication of antibiotic resistance is distinct from previously reported population-based resistance involving production of indole (3, 4), biogenic ammonia (5), and intercellular nanotubes (6). My findings uncover a novel, non-genetic and cooperative mechanism of transient increase in resistance that can be chemically communicated from more resistant members of a heterogeneous population to less resistant bacterial cells of the same or other species.

2.2. Materials and Methods

2.2.1. Strains and reagents

Table 5 lists bacteria and plasmids used in this work. Bacteria grew in LB at 37°C. Antibiotics (Sigma, St Louis, MO, USA) were diluted in water except for PmB, which was diluted in 0.2% bovine serum albumin/0.01% glacial acetic acid buffer. For growth analyses, overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.0008 and incubated at 37°C with medium continuous shaking in a Bioscreen C automated growth curve analyzer (MTX Lab Systems, Vienna, VA, USA). Medium 121 containing 83 μM phosphate was used to test low phosphate conditions (7). Extracellular protease production was determined on dialyzed Brain-Heart infusion milk agar plates (8). Lipopolysaccharide was extracted and visualized by silver staining (9). Etest strips (AB bioMérieux, Solna, Sweden) were applied to agar plates inoculated with test bacteria by swabbing overnight cultures diluted to OD_{600} of 0.04; plates were then incubated at 37^{o}C for 24 h. Unmarked non-polar deletions were performed as described previously (10). Unmarked chromosomal single copy complementation of *BCAL2641* was performed using pMH447 (11). Complementation of *yceI* (*BCAL3310* and *BCAL3311*) was performed using pSCrhaB2 (12).

2.2.2. Population analysis profiling (PAP)

This involved treating bacterial cultures with doubling increments of antibiotic concentrations and determining growth at each concentration by turbidimetry in LB broth (PAP by broth dilution) or by cfu counting on agar plates (PAP by agar dilution). Heteroresistance was considered when the antibiotic concentration exhibiting the highest inhibitory effects was 8-fold or more higher than the highest non-inhibitory concentration.

Table 5. Strains and plasmids used in Chapter 2

Strain or plasmid	Relevant characteristics[a]	Source and/or reference
Strains		
Burkholderia cenocepacia		
CP706-J	CF clinical isolate	Cleveland
K56-2	ET12 clone related to J2315, CF clinical Isolate	[b]BCRRC,(13)
Δ*cciI*	Deletion of *cciI* in K56-2	(14)
Δ*cepI*	Deletion of *cepI* in K56-2	(14)
Δ*rpfF*	Deletion of *rpfF* in K56-2	(14)
Δ*rpoE*	SAL65, Deletion of *rpoE* in K56-2	S. Loutet
Δ*hldA*	Deletion of *hldA* in K56-2	(10)
Δ*arnBC*	K56-2*Prha-arnT*Δ*arnBC*Δ*amrAB*, suppressor strain	(11)
Δ*BCAL3390*	OME2, Deletion of *BCAL3390* in K56-2	This study
Δ*BCAM2086*	OME3, Deletion of *BCAM2086* in K56-2	This study
Δ*yceI*	OME4, Deletion of *yceI* (*BCAL3310* and *BCAL3311*) in K56-2	This study
Δ*BCAM1679*	OME5, Deletion of *BCAM1679* in K56-2	This study
Δ*BCAL3390*Δ*BCAM2086*	OME7, Deletion of *BCAM2086* in K56-2 Δ*BCAL3390*	This study
Δ*BCAL1281*	OME8, Deletion of *BCAL1281* in K56-2	This study
Δ*BCAL2641*	OME11, Deletion of *BCAL2641* in K56-2	This study
Δ*BCAM1111*Δ*BCAM1112*	OME12, Deletion of *BCAM1111* and *BCAM1112* in K56-2	This study
K56-2 pSCrhaB2	OME19, K56-2 carrying pSCrhaB2, Tp^R	This study
Δ*yceI* pSCrhaB2	OME20, K56-2 Δ*yceI* carrying pSCrhaB2, Tp^R	This study
Δ*yceI*p*yceI*	OME21, K56-2 Δ*yceI* carrying *yceI* cloned into pSCrhaB2, Tp^R	This study
Δ*amrAB*	OME29, Deletion of *amrAB* in K56-2	This study
Δ*BCAL2641*Δ*amrAB*	OME30, Deletion of *amrAB* in K56-2Δ*BCAL2641*	This study
Δ*amrAB::BCAL2641*$^+$	OME31, Chromosomal *BCAL2641* integration in *amrAB* locus in K56-2Δ*BCAL2641*	This study
Escherichia coli		
DH5α	F^-ϕ80*lacZ* M15 *endA1 recA1 supE44 hsdR*17(r_K^- m_K^+) *deoR thi-1 nupG supE44 gyrA96relA1* Δ(*lacZYA-argF*)*U169*, λ–	Laboratory stock
GT115	F^- *mcrA*Δ(*mrr-hsdRMS-mcrBC*) ϕ80Δ*lacZ*ΔM15 Δ*lacX74 recA1rpsL* (StrA) *endA1*Δ*dcm uidA*(ΔMluI)::*pir-116* Δ*sbcC-sbcD*	Invivogen, San Diego, CA
HB101	F^-*mcrBmrrhsd*S20(rB-mB-) *rec*A13 *leu*B6 *ara*-14 *proA2 lacY1 gal*K2*xyl*-5 *mtl*-1 *rpsL*20(Sm^R) *glnV*44 λ -	Laboratory Stock
SY327	*araD* Δ(*lac pro*) *argE*(*Am*) *recA56 rifr nalA*, λ *pir*	(15)
BL21	F^-*dcm ompT hsdS*(r_B^- m_B^-) *gal*	Novagen
Pseudomonas aeruginosa		
PAO1	Non-CF clinical isolate	(16)
Plasmids		
pDAI-SceI-SacB	ori_{pBBR1}, Tet^R, P_{dhfr}, *mob*$^+$, expressing I-SceI, SacB	(17)
pGPI-SceI	$ori_{R6K,}$ ΩTp^R , *mob*$^+$, including an I-SceI restriction site	(10)
pMH447	pGPI-SceI derivative used for chromosomal complementation allowing gene integration in the gentamicin efflux pump	(11)
pRK2013	$ori_{colE1,}$ RK2 derivative, Kan^R, *mob*$^+$, *tra*$^+$	(18)
pDel*BCAL3390*	pOE2, pGPI-SceI with fragments flanking *BCAL3390*	This study
pDel*BCAM2086*	pOE3, pGPI-SceI with fragments flanking *BCAM2086*	This study
pDel*yceI*	pOE4, pGPI-SceI with fragments flanking *BCAL3310* and *BCAL3311*	This study
pDel*BCAL1281*	pOE5, pGPI-SceI with fragments flanking *BCAL1281*	This study
pDel*BCAM1679*	pGPI-SceI with fragments flanking *BCAM1679*	(19)
pDel*BCAL2641*	pOE6, pGPI-SceI with fragments flanking *BCAL2641*	This study
pDel*BCAM1111*	pOE7, pGPI-SceI with fragments flanking *BCAM1111* and *BCAM112*	This study

pSCrhaB2	ori$_{pBBR1}$*rhaR*, *rhaS*, P_{rhaB}Tp^R*mob*$^+$	(12)
p*yceI*	pOE8, *yceI* cloned in pSCRha-B2	This study
p*BCAL2641*	pOE9, *BCAL2641* cloned in pMH447 for chromosomal complementation	This study
pET28a(+)		Novagen
pExp*BCAL3310*	pOE15, *BCAL3310* without signal peptide encoding sequence cloned in pET28a(+)	This study
pExp*BCAL3311*	pOE16, *BCAL3311* without signal peptide encoding sequence cloned in pET28a(+)	This study

[a]Tp^R, trimethoprim resistance, Kan^R, kanamycin resistance, Tet^R, tetracycline resistance.
[b]BCRRC, *B. cepacia* Research and Referral Repository for Canadian CF Clinics.

2.2.3. Co-culture

Co-culture was performed by mixing overnight cultures of *P. aeruginosa* PAO1 and *B. cenocepacia* (treated with 500 μg/ml PmB) diluted to OD_{600} of 0.004 at ratio 100:1 in LB broth with or without PmB. Controls with the pure cultures at the same inoculum size were included in the experiment. The mixtures were incubated at 37^{o}C at 200 rpm and cfu of each species was determined by using differential antibiotic selection on LB agar plates at 6 and 24 h. *B. cenocepacia* was selected with PmB (50 μg/ml) and PAO1 was selected with trimethoprim (100 μg/ml). The total count was determined on LB agar plates.

2.2.4. Volatile-mediated protection

Overnight culture of *B. cenocepacia* was diluted 1 in 200 in LB containing 500 μg/ml PmB and incubated at 37^{o}C for 17 h at 200 rpm. The supernatant was collected at 4^{o}C, filtered using 0.2 μm nylon membrane filters, and 10 ml aliquots were placed at one side of the septum in septate Petri dishes. MIC by agar dilution was performed on test bacteria (*B. cenocepacia* K56-2Δ*arnBC, E. coli* DH5α, HB101, and GT115) at the other side of the septum by spotting (10 μl) of their overnight cultures diluted to OD_{600} of 0.004 on LB agar containing PmB at doubling increments. The plates were then incubated at 37^{o}C for 24 h.

2.2.5. RNA extraction

Δ*rpoE*/500 and Δ*rpoE* cells were grown overnight and then diluted to OD_{600} of 0.05 in 50 ml of LB with 500 μg/ml PmB or vehicle control respectively. Cells were grown at 37^{o}C for 30 min at 200 rpm then collected by centrifugation at 39,000 Xg for 30 min at 4^{o}C. RNA was prepared from approximately $5X10^{8}$ cfu using the RiboPure-Bacteria kit (Ambion, Inc., Austin, TX, USA) and treated with DNAse 1 (Ambion), followed by treatment with DNAse 1 (Qiagen Inc., Mississauga, ON, Canada) following the manufacturer's protocol. Integrity of the RNA was

assessed by agarose gel electrophoresis and by measuring the ratio of absorbance at 260 nm to 280 nm (values obtained between 2.0 and 2.2).

2.2.6. qRT-PCR

RNA was converted to cDNA and real-time PCR was performed as previously described (20). Fold changes in gene expression were calculated using the Pfaffl Method (21) relative to *BCAS0175*, an internal control used for microarray and real-time PCR analysis (22). Data were calculated from 3 independent experiments each done in triplicate.

2.2.7. Ornithine decarboxylase (ODC) assay

Overnight cultures in LB broth were diluted to OD_{600} of 0.004 in the rapid ornithine broth medium described by Fay and Barry (23) with or without PmB or the polyamine synthesis inhibitors adjusted at pH 5.5. Aliquots (300 µl each) were transferred to 100-well Bioscreen C plates and overlaid with 100 µl of mineral oil. The plates were incubated in the Bioscreen C automated growth curve analyzer at 37^{o}C without shaking and the color was monitored at 420 nm.

2.2.8. Thin-layer chromatography analyses of polyamines

Polyamine analysis was performed as previously described (24). Overnight cultures (~20 h) in M9 medium with or without PmB were used. M9 medium was used to eliminate potential polyamine contamination in complex media such as LB medium. Supernatants, collected by centrifugation at 16,100*g* for 5 min, corresponding to cultures of OD_{600} of 0.1 were used. $HClO_4$ (4 N) was added to supernatants to reach a final normality of 0.4 N and incubated at 37^{o}C for 1 h with shaking. $HClO_4$ extracts were centrifuged at 16,100*g* for 5 min. Fifty microlitres of the supernatants were mixed with 50 µl of 2M Na_2CO_3 and 100 µl of 2.7 mg/ml dansyl chloride (Sigma, St Louis, MO, USA) solution in acetone and incubated in the dark at 37^{o}C for 2h with

shaking. Standard solutions of putrescine, cadaverine, spermidine and spermine (0.2 mM each) were treated similarly. The mixtures were evaporated to dryness under Nitrogen gas and extracted with 200 µl benzene at 4^{o}C for ~18 h with shaking. Fifty microlitres of the benzene extracts of each of the samples and 5 µl of each of the standards were applied onto TLC silica gel plates (20 × 20 cm, Merck, Darmstadt, Germany) and sequentially separated in two systems: I) benzene–triethylamine (20 : 2 v/v); II) benzene–methanol (10 : 0.45 v/v). The dried plates were photographed in ultraviolet light, which excites the green-blue fluorescence of dansyl polyamine spots. The size and intensity of these spots were proportional to the polyamine concentration, which was quantified using Image J 1.46r software.

2.2.9. Competition between putrescine and fluorescent PmB on surface binding

Overnight culture of *B. cenocepacia* K56-2 was centrifuged at 16,100 *g* for 1 min, and cells were washed with PBS (3X) followed by dilution to OD_{600} of 1 in PBS. Polymyxin B Oregon Green 514 conjugate, PmB-OG (Invitrogen) was added to 100 µl diluted cells at final concentration of 25 µg/ml and incubated at 37^{o}C for 10 min. Then, cells were washed with PBS (3X), resuspended in 100 µl of PBS, and placed into 96-well white plates. Fluorescence was measured at λ_{ex} of 480 nm and λ_{em} of 535 nm. Data was reported as a ratio of Fluorescence to OD_{600}.

2.2.10. Cloning, expression, and purification of YceI

Genes encoding the 2 YceI homologues (BCAL3310 and BCAL3311) were individually amplified by PCR from K56-2 genomic DNA without the sequences encoding the signal peptides. The constructs were cloned into the pET28a expression vector. The positive pET28a–BCAL3310 or 3311 clones were verified by sequencing. The two YceI homologues were overexpressed in *E. coli* (BL21 strain) using 0.05 mM isopropyl thio-β-D-galactoside, and the expression was prolonged for 3 h at 25 ^{o}C. Bacterial cells were harvested and the cell pellet was resuspended in 50 mM phosphate buffer pH 7.8 and lysis was achieved using one shot cell disrupter (Constant Systems Ltd., Northants, UK) at 27 KPSI. The resulting supernatant was

isolated from the insoluble fraction by centrifugation at 16,100 *g* for 60 min at 4 ^{o}C. His-tag batch purification was performed using Ni^{++} coated beads. The purified proteins were detected by Coomassie blue staining following 16% SDS-PAGE and quantified by Bradford assay using bovine serum albumin (BSA) as standard.

2.2.11. Binding assay of YceI to PmB

Purified BCAL3310 and BCAL3311 were diluted to 10 µg/ml concentration, treated with PmB-OG at final concentration of 1 µg/ml in a total volume of 100 µl and incubated at 37^{o}C for 10 min with rotation. The fluorescence was measured at λ_{ex} of 480 nm and λ_{em} of 535 nm. Background fluorescence of PmB-OG with the buffer control was subtracted. BSA was used as a control for non-specific binding.

2.2.12. Statistical analyses

Unpaired student's t-tests were conducted with GraphPad Prism 5.0.

2.3. Results and Discussion

2.3.1. Heteroresistance of *B. cenocepacia* to PmB

The prototypic *B. cenocepacia* clinical strain K56-2 was assessed for heteroresistance by performing population analysis profiling (PAP) of cultures exposed to serial dilutions of PmB. The percent growth inhibition increased gradually at high concentrations of PmB but without reaching complete bacterial inhibition, revealing residual subpopulations of more resistant cells (Fig. 4A) and suggesting heteroresistance. However, the limited solubility of PmB in the culture medium at concentrations higher than 2,048 µg/ml precluded the determination of the exact

minimal inhibitory concentration (MIC) for PmB against K56-2. To investigate this phenomenon in more detail, I performed PAP in isogenic mutants with intermediate sensitivity to PmB. K56-2Δ*rpoE*, which lacks an extracytoplasmic stress response regulator (25), showed evident heteroresistance to PmB (Fig. 4A). A fraction of bacteria from the same culture was inhibited at 64 µg/ml despite that the MIC of PmB against the entire bacterial population was higher than 1,024 µg/ml. Gradual reduction in the resistant subpopulation was observed upon increasing PmB concentrations over a 16-fold range. Heteroresistance to PmB was confirmed by E-test, which demonstrated small colonies growing within the zone of inhibition surrounding the highest concentrations of PmB on the E-test strips, both in K56-2 and K56-2Δ*rpoE* (Fig. 4B and C respectively). A similar pattern of heteroresistance was also previously observed for the K56-2Δ*suhB* (26), which lacks an inositol monophosphatase and like K56-2Δ*rpoE*, has intermediate sensitivity to PmB. Heteroresistance to PmB was also observed in the *B. cenocepacia* clinical isolate CP706-J, indicating that it is not a phenomenon unique to a single strain (Fig. 4D). In contrast, *P. aeruginosa* PAO1 did not show heteroresistance to PmB, as demonstrated by the abrupt drop in the bacterial growth on a two-fold increase of PmB concentration to reach complete growth inhibition (Fig. 4E).

Heteroresistance to PmB was not related to the level of PmB resistance since mutants displaying high sensitivity to PmB were also heteroresistant. K56-2Δ*hldA*, a strain lacking the ability to produce a complete lipopolysaccharide (LPS) molecule as a result of the loss of the *hldA* gene (27), demonstrated heteroresistance to PmB at concentrations ranging from 32 µg/ml to 256 µg/ml (Fig. 4F). Furthermore, K56-2Δ*arnBC* carrying a deletion of genes required for 4-amino-4-deoxy-L-arabinose (Ara4N) synthesis displayed similar heterogeneity in the response to PmB despite its exquisite sensitivity to PmB (Fig. 4G). Since *B. cenocepacia* LPS modification with Ara4N is the major determinant for the extreme resistance to PmB (11) my results suggest that the heteroresistance of *B. cenocepacia* to PmB is not associated to LPS modifications and therefore depends on a different mechanism.

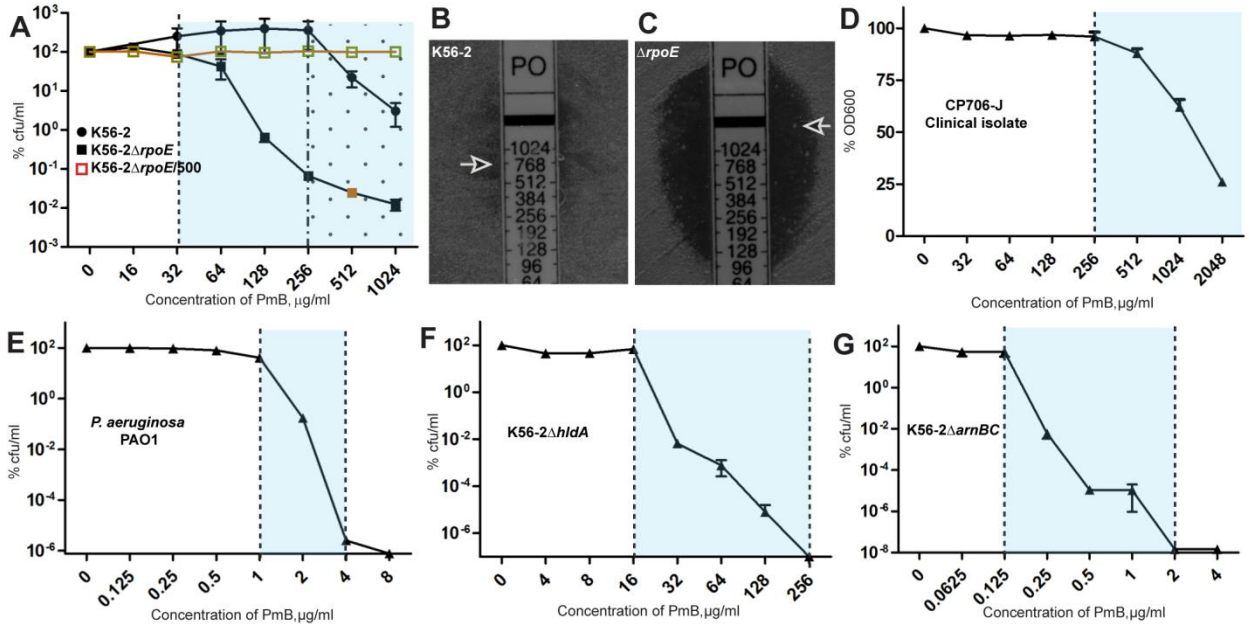

Figure 4.Heterogeneous response of *B. cenocepacia* to PmB.

(A) Population analysis profiling (PAP) of *B. cenocepacia* strains K56-2, K56-2Δ*rpoE*, K56-2Δ*rpoE*/500 by agar dilution at 24 h. (B) E-test of K56-2; and (C) E-test of K56-2Δ*rpoE* showing discrete colonies at otherwise clear zones of inhibition, indicating heterogeneous response to PmB. (D) PAP of *B. cenocepacia* CF clinical isolate CP706-J by broth dilution at 18 h. (E) PAP of *P. aeruginosa* PAO1; (F) PAP of K56-2Δ*hldA*; and (G) PAP of K56-2Δ*arnBC* by agar dilution at 24 h. n = 6. The shaded regions on the PAP graphs indicate ranges of antibiotic concentrations over which the bacterial population transitions from lack of inhibition to maximal inhibition by the antibiotic under the test conditions.

2.3.2. A more resistant subpopulation of *B. cenocepacia* protects naïve bacteria from PmB

I investigated whether the more resistant subpopulations of *B. cenocepacia* could influence the overall level of antibiotic resistance of naïve cells in mixed cultures. To test this hypothesis I chose to focus on the K56-2Δ*rpoE* mutant, as this bacterium has a PmB resistance profile that is similar to the parental strain but sufficiently less resistant to reach higher levels of growth inhibition at testable concentrations of PmB (Fig. 4A). Based on the K56-2Δ*rpoE* PAP, I selected the subpopulation of K56-2Δ*rpoE* exposed to 500 µg/ml (Fig. 4A, Δ*rpoE*/500), which arose at a frequency of 2.48×10^{-4} and demonstrated uniform high-level resistance when re-exposed to PmB (Fig. 4A). Δ*rpoE*/500 cells passaged for up to five days in the absence of PmB displayed PAP identical to that of cells grown overnight in the presence of 500 µg/ml PmB, indicating that the high-level resistance of Δ*rpoE*/500 was stable without selective pressure, likely as a result of one or more mutations that confer increased PmB resistance. No differences were found between Δ*rpoE*/500 and naïve Δ*rpoE* cells in LPS electrophoretic profiles (Fig. 5A). Furthermore, the increased resistance of Δ*rpoE*/500 was not due to an increase in the Ara4N LPS modification, since the differential expression of *arnT* and *arnB* genes, representing the 2 transcriptional units of the *arn* cluster (28), was 1.08 (+/-0.09) and -1.73 (+/-0.04) respectively, as determined by qRT-PCR. This was expected since it was previously shown that the *arn* cluster in *B. cenocepacia* is not regulated by PmB challenge (28). Δ*rpoE*/500 cells treated with PmB also displayed reduced metabolic activity at 24 h relative to naïve Δ*rpoE* and K56-2 with or without exposure to PmB, as revealed in the resazurin metabolic assay (Fig. 5B), suggesting that increased PmB resistance in the Δ*rpoE*/500 subpopulation is associated with reduced metabolic fitness.

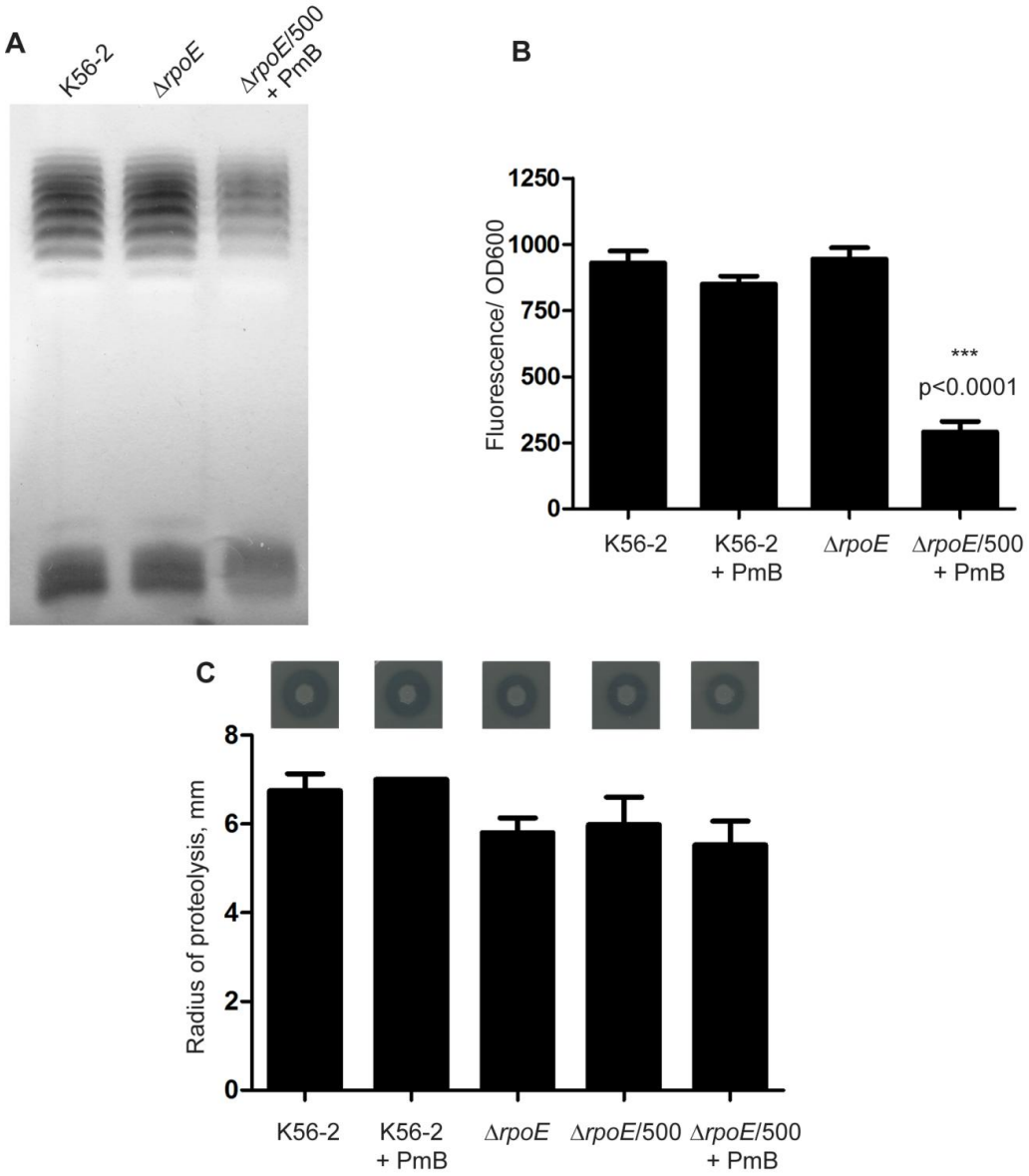

Figure 5.Characterization of the more resistant subpopulation Δ*rpoE*/500.

(A) LPS profiles; (B) Metabolic activity. Overnight cultures were diluted to OD_{600} of 0.02, treated with PmB or vehicle control, incubated at 37°C with continuous medium shaking for 24 h in a Bioscreen C automated growth curve analyzer. Cells were then collected, washed, resuspended in PBS, transferred to white 96-well plate, and treated with resazurin at final concentration 2.5 µg/ml. The plates were incubated in the dark at 37°C for 90 min, and the fluorescence was measured at λ_{ex} of 485 nm and λ_{em} of 600 nm, unpaired student's t-tests were conducted comparing each condition to the control K56-2; (C) Secreted protease activity.

Since Δ*rpoE*/500 represents ~1% of the Δ*rpoE* population in the turbidimetric PAP experiments (not shown), Δ*rpoE*/500 was co-cultured in a 1:100 ratio with *P. aeruginosa* in the presence of 2 μg/ml of PmB. This concentration of PmB was based on the current clinical guidelines for *P. aeruginosa* therapeutic breakpoints of the closely related antibiotic polymyxin E (colistin), which is set at 2 μg/ml (29) and is equivalent to the MIC of PmB against *P. aeruginosa*. Co-culture under these conditions resulted in more than a 3- to 5-log survival of *P. aeruginosa* at 6 and 24 h, respectively, compared to *P. aeruginosa* grown alone (Fig. 6). There was no effect of *P. aeruginosa* on the growth of *B. cenocepacia* cells in co-culture (Fig. 7). Protection by Δ*rpoE*/500 did not depend on secreted extracellular proteases since no differences were found between Δ*rpoE*/500 and naïve Δ*rpoE* cells in the amount of these proteases (Fig. 5C). Similarly, protection did not depend on quorum sensing molecules, as mutants defective in the various quorum systems of *B. cenocepacia* also showed heteroresistance to PmB and could protect *P. aeruginosa* from PmB (Fig. 8A and B). Also, it could not be due to production of indole (3, 4) since *B. cenocepacia* and *Burkholderia* in general are indole negative (30).

Furthermore, the filtered supernatant of an overnight culture of Δ*rpoE*/500 in PmB communicated higher-level resistance in a volatile-mediated manner to physically separated K56-2Δ*arnBC* and several *E. coli* strains. The MIC of the PmB-sensitive strains doubled due to volatiles emitted from the supernatant of Δ*rpoE*/500 (Table 6), with the exception of *E. coli* GT115, which only showed slight enhancement in the growth in the presence of PmB (not shown). These results were consistent and reproducible. The protective effect of Δ*rpoE*/500 was therefore not limited to the same species and could be communicated by one or more volatile compounds in the bacterial supernatant.

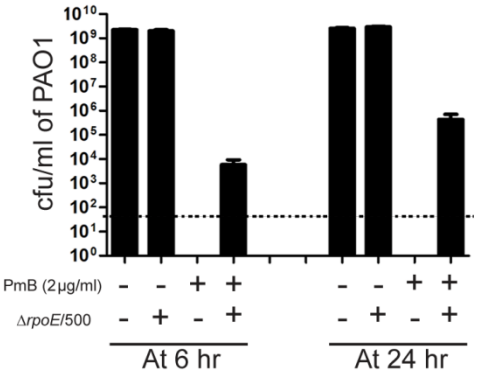

Figure 6. Protective effects of *B. cenocepacia* Δ*rpoE*/500 on *P. aeruginosa* PAO1, exposed to PmB, in co-culture.

The dotted line represents the limit of detection (50 cfu/ml). Three independent experiments each done in duplicate.

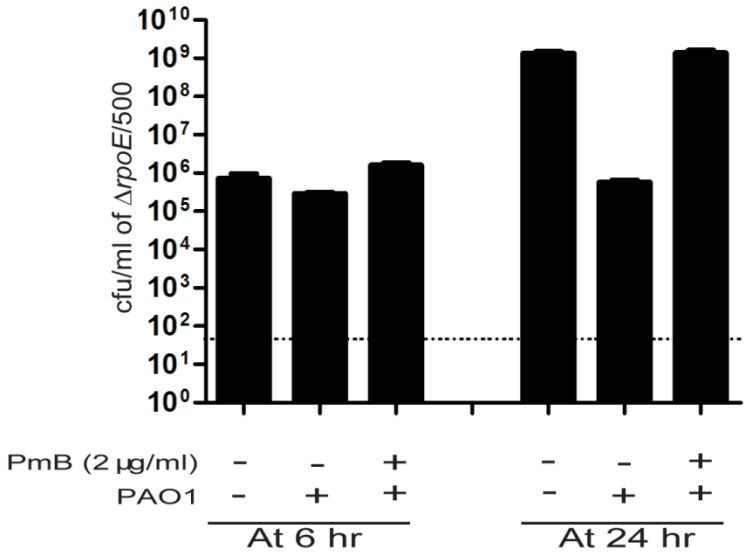

Figure 7. The growth of *B. cenocepacia*Δ*rpoE*/500 subpopulation was not impaired in co-culture with *P. aeruginosa* PAO1except at 24 h in co-culture without PmB where its ratio relative to PAO1 dropped 10 fold probably due to limiting nutrients as a result of the increased biomass of both bacteria in the absence of PmB.

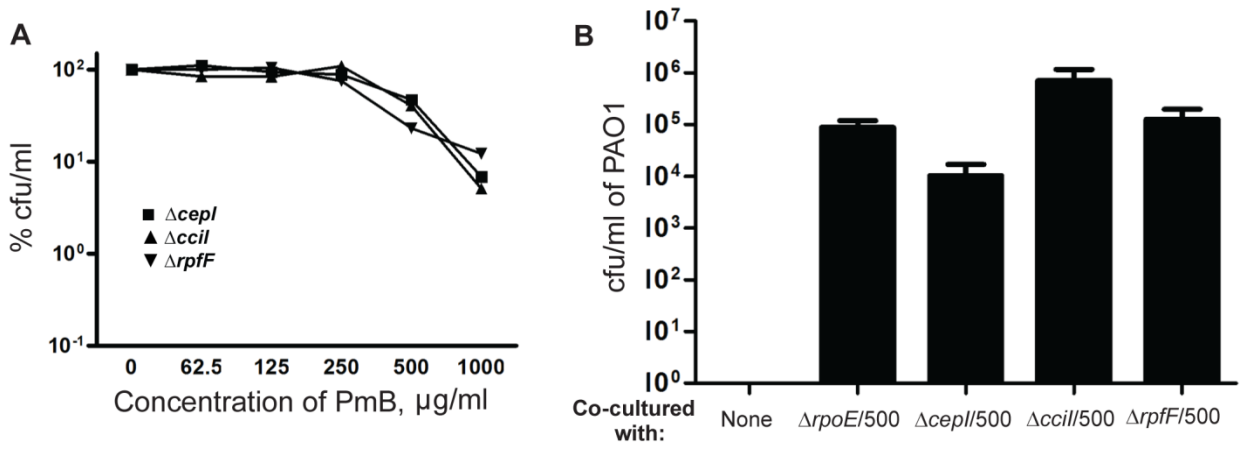

Figure 8. Quorum sensing systems of *B. cenocepacia* are neither involved in the heterogeneity of response to PmB nor in protection to naïve populations.

(A) PAP by agar dilution of the quorum-sensing mutants. (B) Direct co-culture of *P. aeruginosa* PAO1 with subpopulations of the quorum-sensing mutants growing at 500 µg/ml in comparison to Δ*rpoE*/500 subpopulation. The co-cultures were treated with 2 µg/ml PmB for 24 h; the differences are not statistically significant based on unpaired student's t-tests.

Table 6.MIC by agar dilution technique to determine the volatile-mediated protective effect of the supernatant of Δ*rpoE*/500 from the effects of PmB on sensitive bacteria.

	Sterile LB Control (n)	Supernatant of Δ*rpoE*/500 (n)
Sensitive bacteria	MIC, µg/ml	
B. cenocepacia K56-2Δ*arnBC*	0.25 (4)	0.5 (4)
E. coli DH5α	0.25 (4)	0.5 (4)
E. coli HB101	0.25 (3)/ 0.5 (1)	0.5 (4)
E. coli GT115	0.25 (4)	0.25 (4)

2.3.3. The more resistant subpopulation releases higher amounts of a subset of proteins upon exposure to PmB

To gain clues on the secreted molecules mediating the protective effects of *B. cenocepacia* from PmB, I compared the profile of proteins released into the supernatant of PmB-treated Δ*rpoE*/500 with naïve K56-2Δ*rpoE* and parental K56-2 treated or untreated with PmB.The Δ*rpoE*/500 subpopulation and K56-2 treated with PmB showed a similar pattern of overexpression of several polypeptide bands (Fig. 9), which were identified by mass spectrometry. One of these bands corresponded to BCAM2827, which is a predicted periplasmic component of an ABC transporter involved in the biosynthesis of hopanoids. Hopanoids, bacterial substitutes of eukaryotic cholesterol that stabilize membranes and regulate membrane fluidity and permeability, have been recently shown to be required for PmB resistance in *B. cenocepacia* (31). Another protein band was identified as YceI, a conserved protein of unknown function proposed to bind amphiphilic molecules and sequester toxic fatty acids or amides (32). Two highly related YceI homologues, BCAL3310 and BCAL3311, are present in K56-2. Other polypeptides were identified as flagellin, in agreement with the reported effects of PmB on the flagellar assembly apparatus at the transcriptional level (33), and with the reduced motility in parental K56-2 and Δ*rpoE*/500 upon exposure to PmB (not shown).

Three other polypeptide bands were identified as lysine-arginine-ornithine-binding periplasmic proteins, which are involved in the import of these amino acids. I hypothesized that increased import of lysine, arginine and ornithine could be utilized in the modification of the membranes through the formation of lysylphosphatidylglycerol and ornithine-lipid derivatives, since modification of bacterial membranes with cationic molecules reducing their overall negative charge is one of the common mechanisms of increasing resistance to antimicrobial peptides (34). However, deletion of *BCAM1679* encoding a putative lysylphosphatidylglycerol synthetase and *olsB* (*BCAL1281*), previously shown to render the cells incapable of synthesizing the ornithine-lipid under low phosphate conditions (35), did not affect the resistance to PmB when tested in LB medium or in low-phosphate containing medium in K56-2 background (not shown).

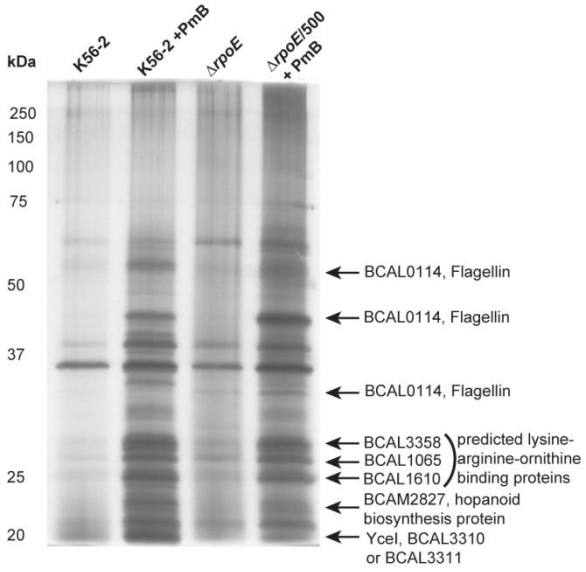

Figure 9. Proteins released into the supernatant of *B. cenocepacia* K56-2 and Δ*rpoE*/500 treated with 500 µg/ml of PmB compared to those released from untreated K56-2 and naïve Δ*rpoE*.

Proteins were run on 14% SDS-PAGE and detected by silver staining and those that were differentially expressed were further identified by LC-MS.

2.3.4. A role for putrescine in PmB resistance

The increased import of lysine, arginine and ornithine suggested by the overexpression of their periplasmic binding proteins in Δ*rpoE*/500 exposed to PmB could be also utilized for synthesis of polyamines (Fig. 10A). Therefore, I tested the involvement of polyamines as possible candidate molecules conferring increased PmB resistance. Spermidine, at concentrations ranging from submicromolar to millimolar levels, had negligible effect on resistance of *B. cenocepacia* to PmB (not shown). However, treatment of the parental K56-2 with 50 mM putrescine increased the resistance to PmB since putrescine-treated cells survived better at 2,048 µg/ml PmB compared to control cells (Fig. 10B). Putrescine treatment of K56-2Δ*arnBC* also resulted in a 2-fold increase in the MIC of PmB, suggesting that putrescine plays a role in the increased resistance to PmB and its transfer among the bacterial population.

To test this notion, I deleted the genes encoding key enzymes for polyamines biosynthesis (Fig. 10A). Mutants with double deletions of both genes encoding spermidine synthases (BCAL3390 and BCAM2086) showed a slight reduction in resistance to PmB and no changes in growth rate (Fig. 11). However, the mutant lacking *BCAL2641*, which encodes an ornithine decarboxylase, had a marked reduction in resistance to PmB and no growth rate defects (Fig. 10C). PmB resistance was restored to parental level by single-copy complementation of *BCAL2641* (Fig. 10D). To further confirm these findings I used two polyamines synthesis inhibitors, dicyclohexylamine and 3-(Methylthio)propylamine (Fig. 10E and Fig. 12). Dicyclohexylamine, originally reported as a spermidine synthase inhibitor but also capable of inhibiting the ornithine decarboxylase enzyme (Fig. 13), reduced resistance to PmB in K56-2 and Δ*rpoE*/500 cells in a concentration dependent manner (Fig. 10E and Fig. 12, respectively). However, 3-(Methylthio)propylamine, which is more specific for spermidine synthase and lacks any detectable inhibitory effect on the ornithine decarboxylase reaction (Fig. 13) had no effect on resistance of Δ*rpoE*/500 (Fig. 12) and only caused a lower reduction of resistance of K56-2 to PmB (Fig. 10E). Exogenous putrescine increased the resistance of K56-2 Δ*BCAL2641* to PmB in a concentration-dependent manner; full restoration of the level of resistance of the parental strain was achieved at 50 mM putrescine (Fig. 10B). A comparison of the level of transcription of *BCAL2641* in both naïve Δ*rpoE* and Δ*rpoE*/500

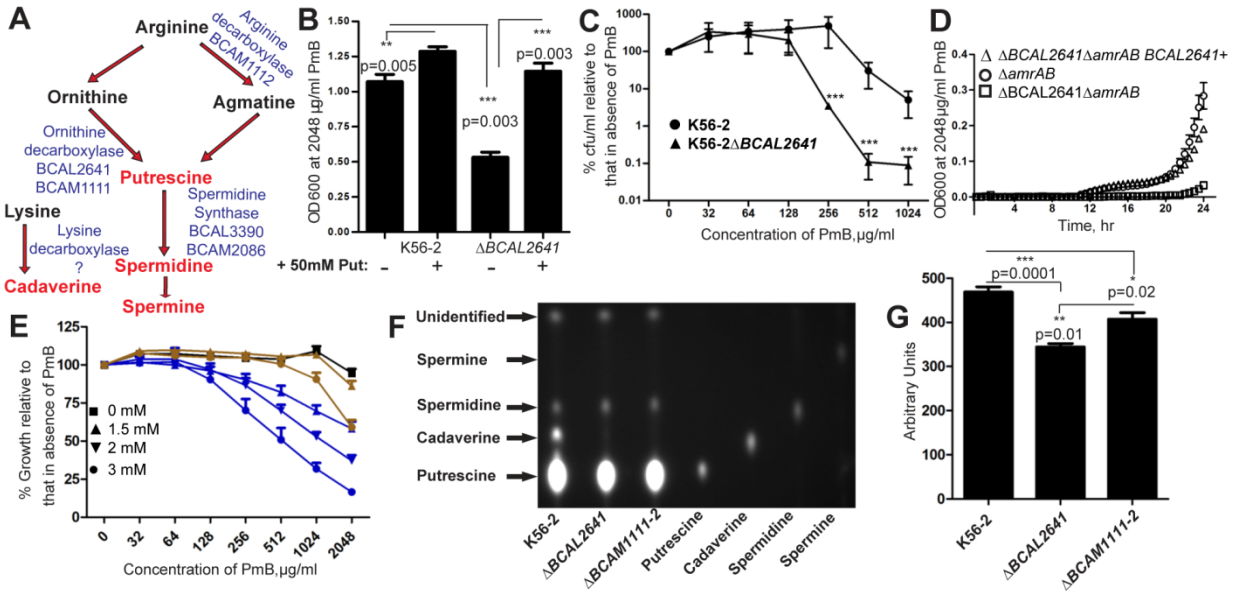

Figure 10.Contribution of the polyamine putrescine in the response to PmB.

(A) Polyamines biosynthetic pathway. (B) Exogenous putrescine increases the resistance of the parental K56-2 to PmB and Δ*BCAL2641* shows significant reduction in resistance to PmB which was restored to the parental level using 50mM exogenous putrescine; n=6 (C) The deletion of *BCAL2641* leads to reduced resistance to PmB relative to the parental K56-2. (D) Single-copy complementation of Δ*BCAL2641*. (E) The polyamine synthesis inhibitor dicyclohexylamine (blue) reduces the resistance of *B. cenocepacia* K56-2 to PmB, with little to no effect of 3-(methylthio)propylamine (red), shown in a turbidimetric PAP at 24 h; n=5. (F) TLC analysis of polyamines released in the supernatants of 20 h old M9 cultures compared to standards visualized under UV after derivatization to their dansylated derivatives. (G) Relative amounts of putrescine released from the wild-type and mutants, n=4. Unpaired student's t-tests were conducted to determine significance of differences among different test conditions.

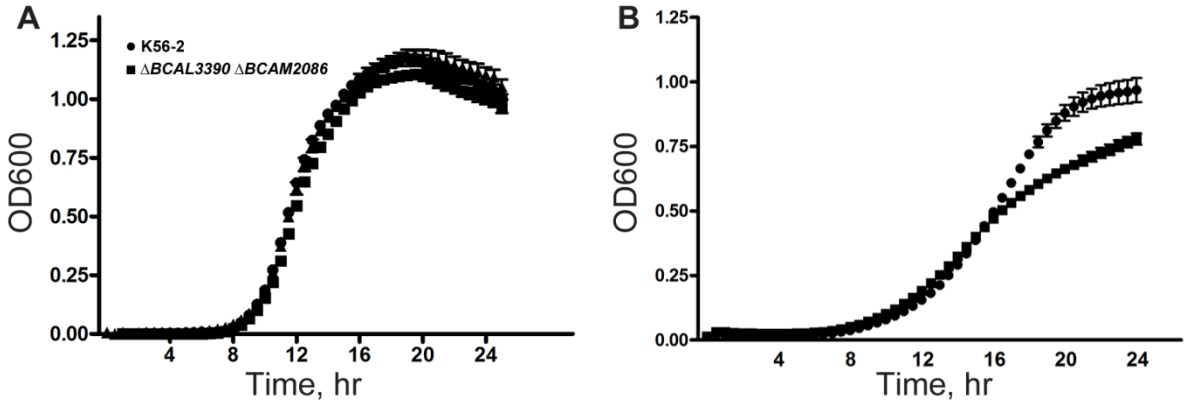

Figure 11.PmB resistance of the spermidine synthase double mutant, K56-2 ΔBCAL3390ΔBCAM2086.

(A) Growth curves in absence of PmB; (B) Effect of 2048 µg/ml PmB on growth.

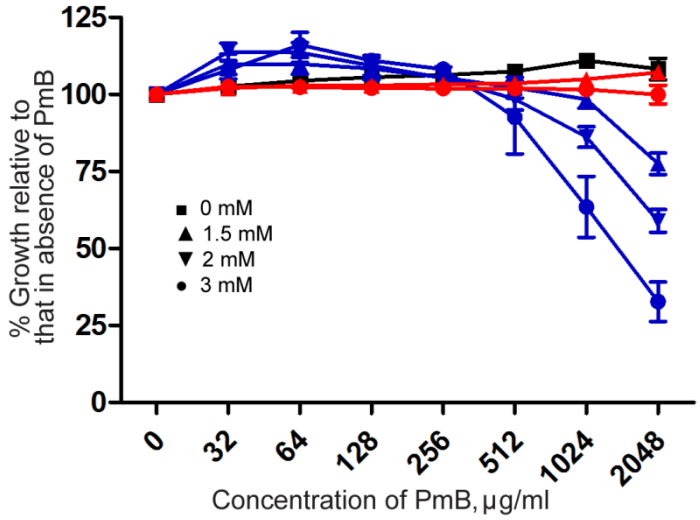

Figure 12. The polyamine synthesis inhibitor dicyclohexylamine (blue) reduces the resistance of *B. cenocepacia* Δ*rpoE*/500 subpopulation to PmB, with no effect of 3-(methylthio)propylamine (red), shown in a turbidimetric PAP at 18 h; n=5.

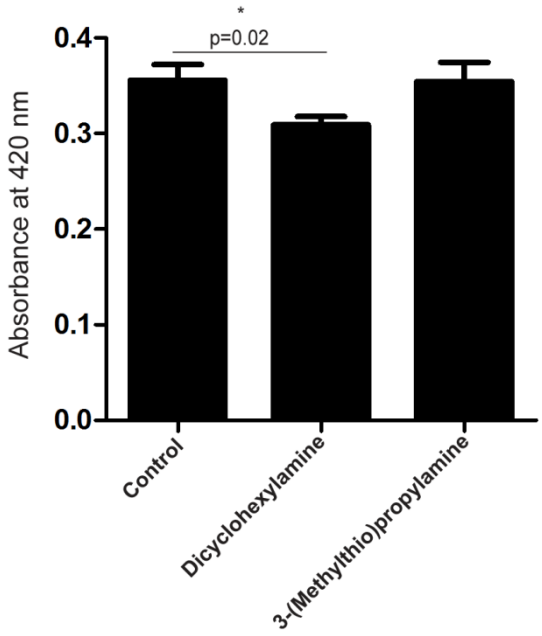

Figure 13. Ornithine decarboxylase (ODC) activity of *B. cenocepacia* K56-2 either untreated or treated with 1 mM of dicyclohexylamine or 3-(methylthio)propylamine at 24 h.

This concentration of the polyamine synthesis inhibitors did not affect the growth of the bacteria. n=9. Unpaired student's t-tests were conducted comparing each condition with the control untreated group.

bacteria treated with PmB, demonstrated that the expression of this gene is upregulated by 2.9 (+/- 0.9) in Δ*rpoE*/500 in response to PmB. Moreover, higher levels of putrescine released in the supernatant of Δ*rpoE*/500 treated with PmB were observed relative to Δ*rpoE* naïve population (Fig. 14).

Putrescine is the most abundant polyamine secreted from *B. cenocepacia*, while much less amounts of spermidine and cadaverine are secreted from K56-2 (Fig. 10F). The release of putrescine was significantly reduced in the Δ*BCAL2641* compared to the wild type K56-2 (Fig. 10G). However, *B. cenocepacia* possesses another predicted ornithine decarboxylase, BCAM1111 and a putative arginine decarboxylase, BCAM1112. Deletion of genes encoding both enzymes did not have an effect on resistance to PmB in K56-2 (not shown), and only a small effect in the release of putrescine (Fig. 10G). In contrast, cadaverine was not detected in the supernatant of Δ*BCAL2641* and Δ*BCAM1111*Δ*BCAM1112* precluding the involvement of cadaverine in increased PmB resistance (Fig. 10F). By qRT-PCR, *BCAM1111* and *BCAM1112* were 2000-fold less transcribed relative to *BCAL2641* in naïve Δ*rpoE* and Δ*rpoE*/500 bacteria; they were also not differentially transcribed in the more resistant subpopulation (Δ*rpoE*/500) relative to the naïve population, suggesting that their gene products are not preferentially used in polyamines biosynthesis. In agreement, the ornithine decarboxylase (ODC) activity of Δ*BCAL2641* was much more reduced relative to Δ*BCAM1111*Δ*BCAM1112* (Fig. 15). The pattern of ODC activity corresponded to the levels of secretion of putrescine in the different mutants relative to the wild type (Fig. 10G). Together, this shows that BCAL2641 is the primary contributor of putrescine in *B. cenocepacia* explaining the phenotype observed upon its deletion. K56-2 Δ*BCAL2641* also lost the protective effects from PmB in co-culture with *P. aeruginosa* PAO1 (Fig. 16A).These results implicated putrescine as a critical polyamine conferring protection from PmB and communicating resistance to neighbouring bacterial cells.

The initial binding of antimicrobial peptides to the bacterial surfaces is crucial for their subsequent antibacterial effects (34). Putrescine competed with PmB for binding to the surface of *B. cenocepacia* K56-2, where treatment of cells with both putrescine and the fluorescent PmB-Oregon green 514 conjugate showed reduced binding of the fluorescent PmB derivative relative to control cells (Fig. 16B). Putrescine also replaced already bound fluorescent PmB conjugate (Fig. 16B). This agrees with previous findings showing that polyamines provide protection of

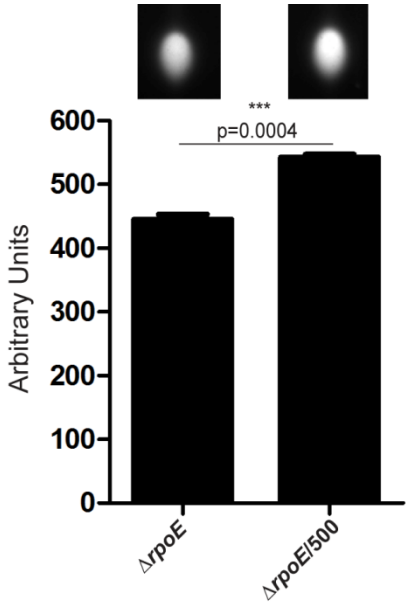

Figure 14. Increased release of putrescine in the supernatant of Δ*rpoE*/500 subpopulation treated with 500 µg/ml PmB relative to naïve Δ*rpoE* determined at 20 h from M9 cultures by TLC analysis. n=6. Unpaired student's t-test was conducted.

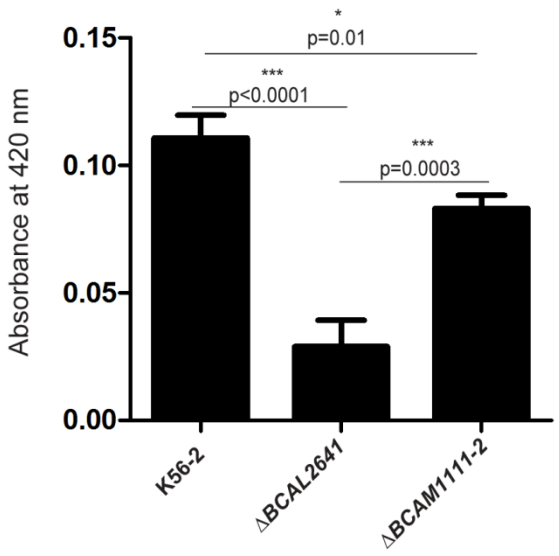

Figure 15. ODC assay of the parental strain K56-2 and different PAs biosynthetic mutants at 6 h. n=9. Unpaired student's t-tests were conducted.

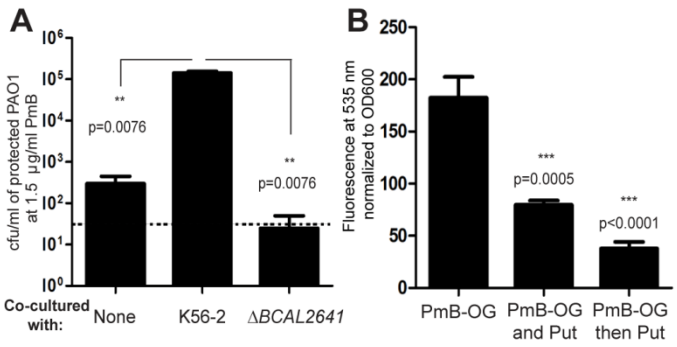

Figure 16.Role of putrescine in the protective effects of *B. cenocepacia* against PmB.

(A) Involvement of putrescine in the protective effects of *B. cenocepacia* on *P. aeruginosa* PAO1 shown by performing direct co-culture between PAO1 and K56-2 wild type or ΔBCAL2641 mutant at 24 h. The dotted line represents the limit of detection (50 cfu/ml). Three independent experiments each done in duplicate. (B) Putrescine protects the bacterial surface from binding to PmB; 50 mM of putrescine reduced binding of PmB-Oregon green 514 conjugate (25 µg/ml) when both added together, whereas it could replace already bound PmB; n=6.

the outer membrane of *P. aeruginosa* from PmB damage (36). However, this does not preclude other mechanisms mediated by putrescine to protect against the effects of PmB. For example, polyamines can reduce oxidative stress in *E. coli* exposed to bactericidal antibiotics (37) and protect from membrane lipid peroxidation in *P. aeruginosa* (36). These additional mechanisms of protection by polyamines are consistent with the notion that bactericidal antibiotics at sublethal concentrations stimulate the production of hydroxyl radicals, which in turn may induce mutations leading to various levels of antibiotic resistance (38).

2.3.5. The role of YceI protein

I also tested the involvement of YceI in heteroresistance. Mutants with a double deletion of *BCAL3310* and *BCAL3311* had increased sensitivity to PmB, but no differences in growth rate relative to K56-2 (Fig. 17A). Complementing the double deletion mutant Δ*BCAL3310*Δ*BCAL3311* (K56-2Δ*yceI*) with both genes restored resistance to PmB to the parental level (Fig. 17B). Moreover, YceI contributed to the protective effects of *B. cenocepacia* towards *P. aeruginosa* PAO1 cells exposed to 1.5 µg/ml PmB (Fig. 17C). The level of transcription of BCAL3310, determined by qRT-PCR in both naïve Δ*rpoE* and the more resistant subpopulation Δ*rpoE*/500 treated with PmB, indicated that this gene was upregulated by 2.5 (+/- 0.6) in the more resistant subpopulation in response to PmB. Together, these experiments reveal that the YceI homologues contribute to the increased resistance to PmB in Δ*rpoE*/500 and the protective effects on other cells against PmB. Purified YceI BCAL3310 and BCAL3311 (Fig. 17D), were both capable of binding PmB-Oregon green 514 conjugate, although BCAL3311 being more potent than BCAL3310 (Fig. 17E). This supports their role in sequestering PmB thus protecting other cells from the toxic effects of the antibiotic.

2.3.6. *B. cenocepacia* is heteroresistant to other bactericidal antibiotics

I determined whether heteroresistance in K56-2 is exclusive to PmB. Turbidimetric PAP using various antibiotics indicated that K56-2 is heteroresistant to gentamicin (protein synthesis inhibitor), norfloxacin (DNA replication inhibitor), rifampicin (mRNA transcription inhibitor)

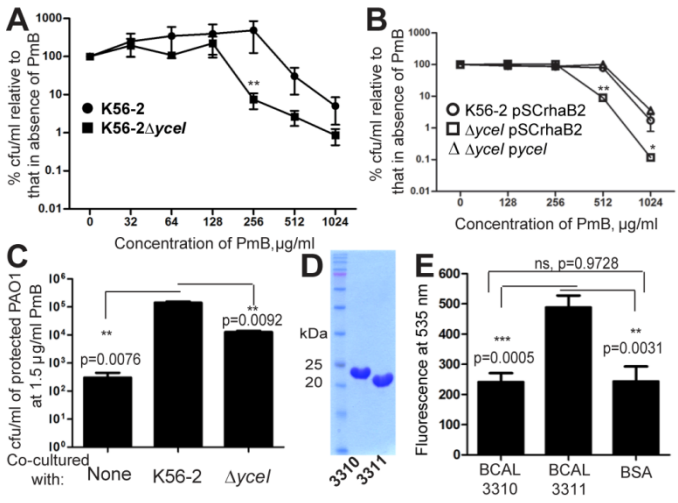

Figure 17.Contribution of YceI in the response to PmB and its role in protection against PmB.

(A) The deletion of *BCAL3310* and *BCAL3311* (Δ*yceI*) leads to reduced resistance to PmB relative to the parental K56-2. (B) PAP by agar dilution showing complementation of the reduced resistance in Δ*yceI* mutant by *yceI* (using both *BCAL3310* and *BCAL3311*) under the control of the rhamnose promoter on pSCrhaB2 to the parental level at 0.4% rhamnose. (C) Involvement of YceI in the protective effects of *B. cenocepacia* on *P. aeruginosa* PAO1 shown after 24 h of direct co-culture of PAO1 and K56-2 wild type or Δ*yceI*. Three independent experiments each done in duplicate. (D) Purified YceI homologues, BCAL3310 and BCAL3311. (E) Binding of BCAL3310 and BCAL3311 to PmB-Oregon green 514 conjugate. BSA was used as a control for binding. n=6.

and ceftazidime (cell wall peptidoglycan synthesis inhibitor), all of which belong to different classes of bactericidal antibiotics (Fig. 18). In contrast, the response of K56-2 was homogeneous to tetracycline, chloramphenicol, novobiocin, trimethoprim, which are all bacteriostatic antibiotics (Fig. 19). Polyamines play a role in the heterogeneity of response to the bactericidal antibiotics. Δ*BCAL2641* displayed a more homogeneous response to the different bactericidal antibiotics, except for gentamicin (Fig. 18). Similarly, YceI was involved in the heterogeneous response to the amphiphilic bactericidal antibiotics rifampicin and norfloxacin; however, the Δ*yceI* mutant only showed minor reduction in the percentage of the more resistant fractions of the population in response to ceftazidime (Fig. 18).

2.4. Conclusions

I show that (i) *B. cenocepacia* is heteroresistant to PmB and different classes of bactericidal antibiotics; (ii) a more resistant subpopulation of *B. cenocepacia* communicates high-level resistance to less resistant cells; (iii) the protection extends to other bacterial species and is chemically mediated by putrescine, a polyamine, and the secretion of YceI. Since putrescine is volatile (39), resistance can also be communicated to physically separated bacteria in a volatile-mediated manner. Natural polyamines, discovered more than 300 years ago, occur in almost all living organisms; they are involved in growth, development, and other important functions related to modulation of defence responses to diverse environmental stresses and modulation of immune responses in plants and humans respectively (40, 41). Polyamines are significantly increased at inflammatory sites of infection or injury (42, 43); they are also produced by a wide range of bacteria, playing roles in growth and other functions including incorporation into the cell wall, biosynthesis of siderophores, acid resistance, scavenging free radical ion, signaling cellular differentiation and biofilm formation (44). The two most common bacterial polyamines are putrescine and spermidine (44). I show here that the most abundant polyamine in *B. cenocepacia* is putrescine, while spermidine and cadaverine are produced in much lower amounts. Polyamines were previously shown to increase the resistance of *P. aeruginosa* to antimicrobial peptides (36, 45, 46). Heteroresistance of *B. cenocepacia* K56-2 was common to bactericidal antibiotics regardless of their site of action. I speculate that bacterial

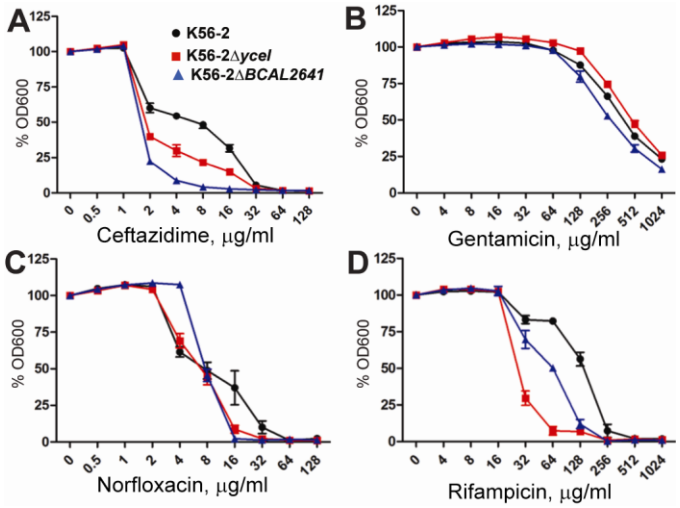

Figure 18.Heterogeneous response of *B. cenocepacia* K56-2 to bactericidal antibiotics.n = 6.

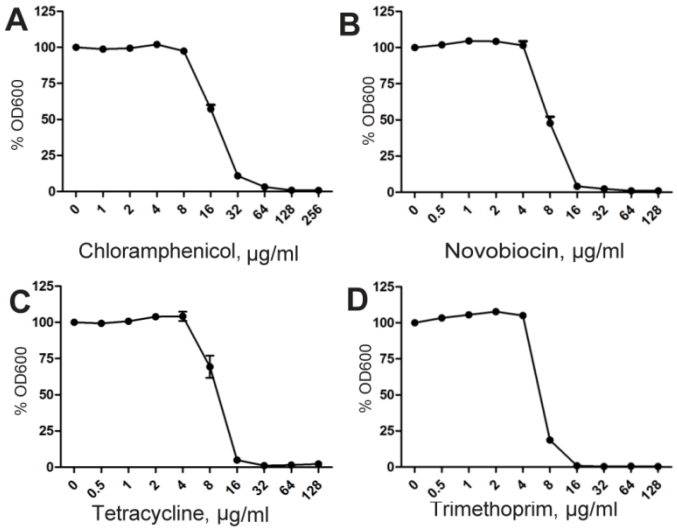

Figure 19.Homogenous response of *B. cenocepacia* K56-2 to bacteriostatic antibiotics.n = 6.

cells may be exposed to greater stress in case of bactericidal agents, relative to bacteriostatic antibiotics, which might result in variation across the bacterial population in their capabilities to withstand and respond to such hostile insult. The involvement of polyamines in heteroresistance to the different classes of bactericidal antibiotics and of YceI in the response to amphiphilic bactericidal antimicrobials leads me to propose that these mediators serve as "danger" infochemicals. These chemical signals may be employed in the non-genetic communication of resistance among members of heteroresistant bacterial populations against the different bactericidal antibiotics. The action of YceI on amphiphilic antibiotics fits with its proposed mechanism sequestering toxic amphiphiles with acyl fatty chains, such as PmB, as I have shown in this study. However, this does not preclude other mechanisms in the response of *B. cenocepacia* to bactericidal antibiotics, especially the aminoglycoside gentamicin, which still requires further investigation.

The proposed danger infochemicals can serve as a general mechanism of protection of other bacterial species in a polymicrobial infection such as that found in patients with cystic fibrosis. YceI would reduce available amphiphilic antibiotics from the medium thus protecting any organism; whereas putrescine could interact with most of the bacterial species, since polyamines are produced by most bacteria, with rare exceptions such as *Staphylococcus aureus* strains, which do not tolerate polyamines as they lack the necessary detoxifying enzymes (47).

In conclusion, I show that antibiotic heteroresistance leads to a cooperative behaviour such that the more antibiotic-resistant members of the population protect the less resistant ones as well as less resistant members of other species. A similar observation has been made previously with indole production by *E. coli* strains (3). However, indole production in the more resistant cells was at the exact same level as in naive cells with no antibiotic treatment and unlike putrescine, indole was neither induced by antibiotics nor over-secreted by the more resistant cells. I believe my findings are relevant in the clinical setting, particularly for intrinsically resistant opportunistic Gram-negative bacteria. Attempts to modulate these interactions using polyamine synthesis inhibitors may contribute to disrupting heteroresistance so the bacterial population will have a more uniform response to the antibiotic, reducing the window of therapeutic failure.

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Chapter 3

Putrescine reduces antibiotic-induced oxidative stress as a mechanism of modulation of antibiotic resistance in *Burkholderia cenocepacia*

A version of this chapter has been published:

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3.1. Introduction

The relentless increase in multidrug resistance, particularly intrinsic, high-level resistance, undermines new treatments improving health and extending the life of patients especially of those with chronic conditions (1). For example, respiratory failure secondary to chronic pulmonary bacterial infection in patients with cystic fibrosis hinders the dramatic improvements in survival achieved over the last several decades and remains the primary cause of death (2). The emergence of growing numbers of cystic fibrosis pathogens with intrinsic, multidrug resistance such as *Burkholderia cepacia* complex, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, and nontuberculous mycobacteria creates a further need for novel therapies (2). I investigate the mechanisms of high-level intrinsic multidrug resistance using *Burkholderia cenocepacia* as a model bacterium. *B. cenocepacia* is an environmental, opportunistic pathogen that belongs to the *B. cepacia* complex and causes serious respiratory infections in CF patients (3). These infections are associated with faster decline in lung function, debilitating exacerbations and ultimately death (4-6), and they also reduce the survival of CF patients after lung transplant (7).

While genetic mechanisms are considered the quintessential means of transfer of antibiotic resistance traits among bacteria, small molecules are also capable of modulating the antibiotic response of bacteria (8). The clinical outcome of antibiotic treatment does not always correlate with the expectations based on *in vitro* susceptibility testing performed on individual clinical isolates (9). Owing to the polymicrobial nature of many infections (10), cross-talk between the different bacterial species is likely to occur during infection. Such chemical communication of antibiotic resistance among bacteria may aggravate the problem of antibiotic resistance by potentially causing transient reduction in the susceptibility to antibiotics, potentially leading to therapeutic failures. For example, a transient increase in resistance to antimicrobial peptides by exposure to host polyamines was shown for the urogenital pathogen *Neisseria gonorrhoeae* (11). Identifying chemical communicators of antibiotic resistance and their mechanism of protection would provide another avenue for intervention to combat the increase and spread of antimicrobial resistance. Recently, we demonstrated that *B. cenocepacia* exhibits a non-genetic mechanism to reduce antibiotic susceptibility that is chemically mediated

by putrescine and YceI, a small secreted protein of unknown function that is highly conserved in bacteria (12). Putrescine is a polyamine produced by almost all living organisms (13). When released from *B. cenocepacia*, putrescine protects less resistant cells from the same and different species from the antimicrobial peptide polymyxin B (PmB) (12).

The mechanism of protection is partly due to the ability of putrescine to compete with PmB for binding to the surface of *B. cenocepacia* (12). However, polyamines can also quench oxidative species (14) and protect membranes from lipid peroxidation (15). Various classes of antibiotics induce oxidative stress and increased production of reactive oxygen species (ROS) (16-19). Although the specific lethal role of ROS generated in response to antibiotics remains under discussion (16, 20, 21), oxidative stress constitutes a burden on the bacterial cells (22). Therefore, it is conceivable that protection from oxidative stress accompanying antibiotic exposure would improve the bacterial response to antibiotics, thus increasing resistance.

Here I show that when present at sub-lethal concentrations, PmB and other bactericidal antibiotics induce oxidative stress in *B. cenocepacia*. My findings revealed that exogenous and endogenous putrescine protects against antibiotic-mediated oxidative stress. This work exposes another mechanism of putrescine-mediated protection from antibiotics alongside with protection of cell surface from binding of PmB previously described (12). By examining the expression patterns of the different putrescine synthesizing enzymes in response to antibiotics, I discovered that the ornithine decarboxylase BCAL2641 is a plausible target for designing inhibitors that would block putrescine-mediated communication of antibiotic resistance among different bacteria, ultimately reducing the window of therapeutic failure in treating bacterial infections.

3.2. Materials and Methods

3.2.1. Strains and reagents.

Table 7 lists bacteria and plasmids used in this study. Bacteria grew in LB at 37°C. Antibiotics (Sigma, St Louis, MO, USA) were diluted in water except for PmB, which was diluted in 0.2% bovine serum albumin/0.01% glacial acetic acid buffer. Rifampicin was dissolved in dimethyl sulphoxide (DMSO).

3.2.2. General molecular techniques.

DNA manipulations were performed as previously described (23). T4 DNA ligase (Roche Diagnostics, Laval, Quebec, Canada), Antarctic phosphatase (New England Biolabs, Pickering, Ontario, Canada) and restriction endonucleases (Roche or New England Biolabs) were used as recommended by the manufacturers. Transformation of *Escherichia coli* GT115 was performed using the calcium chloride method (24). Mobilization of plasmids into *B. cenocepacia* was conducted by triparental mating (25) using *E. coli* DH5α carrying the helper plasmid pRK2013 (26). DNA amplification by polymerase chain reaction (PCR) was performed using a C1000 Thermal cycler (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada) with Taq or HotStar HiFidelity DNA polymerases (Qiagen, Mississauga, Ontario, Canada) and optimized for each primer pair. DNA sequencing was carried out at the DNA sequencing Facility of York University, Toronto, Canada or at Eurofins MWG Operon, Huntsville, Alabama, USA. The DNA sequences were analyzed with the BLAST computer program and compared to the sequenced genome of *B. cenocepacia* strain J2315.

3.2.3. Fluorometric determination of ROS.

Overnight cultures of the parental *B. cenocepacia* K56-2 and the appropriate mutants in LB medium were diluted to an optical density at 600 nm (OD_{600}) of 0.1 in fresh medium. Five-ml aliquots were incubated at 37^{o}C for 3 h at 200 rpm. Antibiotics and/or putrescine were added at the specified concentrations and the cultures were further incubated at 37^{o}C for 2 h at 200 rpm. After incubation, the OD_{600} was measured and aliquots containing cells equivalent to an OD_{600} of 0.4 were pelleted, washed with phosphate buffered saline (PBS), and resuspended in 1 ml of

PBS. Superoxide radicals and other ROS were determined by diluting the obtained suspension 100 fold in 1 ml PBS and adding 2',7'-dichlorofluorescein diacetate (DCF) to a final concentration of 2 µM. The reaction mixture was then incubated at 37^{o}C for 30 min with rotation. After incubation, the fluorescence was measured in 200-µl aliquots placed into 96-well white plates (Microfluor-2 White, Thermo Scientific) at λ_{ex}= 480 nm and λ_{em}= 521 nm, using Cary Eclipse fluorescence spectrophotometer (Varian, Inc., Mississauga, Ontario, Canada). In addition, the OD_{600} of the same suspensions were measured and used to normalize the fluorescence values. Hydroxyl radical production was determined in 600 µl bacterial suspensions without dilution using 3'-(*p*-hydroxyphenyl) fluorescein (HPF) at a final concentration of 5 µM. Fluorescence was measured at λ_{ex}= 495 nm and λ_{em}= 530 nm in 200 µl aliquots placed into 96-well white plates. Background fluorescence of each probe in buffer control was subtracted. Autofluorescence of the bacterial suspensions, without adding the probes, was measured and corrected for by subtraction from the fluorescence signals. Data were normalized to the OD_{600} of the bacterial suspensions. The suspensions were protected from light throughout the assays to avoid photo-oxidation.

3.2.4. Antibiotic susceptibility testing.

Overnight cultures of the parental *B. cenocepacia* K56-2 and the appropriate mutants in LB medium were diluted to an optical density at 600 nm (OD_{600}) of 0.0008 (low inoculum) or 0.04 (high inoculum) in fresh LB medium and 0.04 in fresh M9 minimal medium with or without the antibiotic and incubated at 37°C with medium continuous shaking in a Bioscreen C automated growth curve analyzer (MTX Lab Systems, Vienna, VA, USA). Bacterial growth was assessed turbidimetrically at 600 nm.

3.2.5. *In vitro* antioxidant activity assay.

The ability of putrescine to scavenge free radicals was determined using a system of *in vitro* generation of superoxide radicals containing phenazine methosulfate (PMS)-NADH as previously described (27). Briefly, the reaction mixture consisted of 21 mM phosphate buffer (pH 8.3), 0.7 mM NADH, 17 µM nitro blue tetrazolium, and the corresponding quantity of putrescine.The reaction was initiated by adding 4 µM PMS. The reaction mixtures were mixed and the amount of formazan formation was measured immediately using the spectrophotometer

Table 7. Strains and Plasmids used in Chapter 3

Strain or plasmid	Relevant characteristics[a]	Source and/or reference
Strains		
Burkholderia cenocepacia		
K56-2	ET12 clone related to J2315, CF clinical Isolate, [b]BCRRC	(28)
OME11	K56-2, ΔBCAL2641	(12)
OME12	K56-2, ΔBCAM1111-ΔBCAM1112	(12)
OME49	OME12 P_{rha}::BCAL2641	This study
OME50	K56-2, $P_{BCAL2641}$::pGSVTp-*luxCDABE*; Tp^R	This study
OME51	OME12, $P_{BCAL2641}$::pGSVTp-*luxCDABE*; Tp^R	This study
OME52	K56-2, $P_{BCAM1111}$::pGSVTp-*luxCDABE*; Tp^R	This study
OME53	OME11, $P_{BCAM1111}$::pGSVTp-*luxCDABE*; Tp^R	This study
OME54	K56-2, $P_{BCAM1112}$::pGSVTp-*luxCDABE*; Tp^R	This study
OME55	OME11, $P_{BCAM1112}$::pGSVTp-*luxCDABE*; Tp^R	This study
OME56	K56-2, P_{oxyR}::pGSVTp-*luxCDABE*; Tp^R	This study
OME57	OME11, P_{oxyR}::pGSVTp-*luxCDABE*; Tp^R	This study
OME58	OME12, P_{oxyR}::pGSVTp-*luxCDABE*; Tp^R	This study
Escherichia coli		
DH5α	F^-ϕ80*lacZ* M15 *endA1 recA1 supE44 hsdR*17(r_K^- m_K^+)*deoR thi-1 nupG supE44 gyrA96relA1* Δ(*lacZYA-argF*)*U169,* λ^-	Laboratory stock
GT115	F^- *mcrA*Δ(*mrr-hsdRMS-mcrBC*) ϕ80Δ*lacZ*ΔM15 Δ*lacX74 recA1rpsL*(StrA) *endA1*Δ*dcm uidA*(ΔMluI)::*pir-116* Δ*sbcC-sbcD*	Invivogen
Plasmids		
pRK2013	$ori_{colE1,}$ RK2 derivative, Kan^R, mob^+, tra^+	(26)
pGSVTp-*lux*	Mobilizable suicide vector containing the *lux* operon, derivative from pGSV3-*lux*(29); OriT; Tp^R	(30)
pSC200	oriR6K, P_{RhaB} rhamnose-inducible promoter, Tp^R, mob^+	(31)
pOE14	$P_{BCAL2641}$::*luxCDABE* transcriptional fusion in pGSVTp-*lux*	This study
pOE17	P_{rha}::BCAL2641 in pSC200	This study
pOE18	$P_{BCAM1111}$::*luxCDABE* transcriptional fusion in pGSVTp-*lux*	This study
pOE19	$P_{BCAM1112}$::*luxCDABE* transcriptional fusion in pGSVTp-*lux*	This study
pOE20	P_{oxyR}::*luxCDABE* transcriptional fusion in pGSVTp-*lux*	This study

[a]Tp^R, trimethoprim resistance, Kan^R, kanamycin resistance, Tet^R, tetracycline resistance.
[b]BCRRC, *B. cepacia* Research and Referral Repository for Canadian CF Clinics.

at 560 nm. The percentage of inhibition of formazan formation by putrescine was calculated relative to the control lacking putrescine.

3.2.6. Transcriptional fusions to *luxCDABE*.

The promoter regions from BCAL2641, BCAM1111, BCAM1112 and OxyR were PCR amplified. The PCR products were digested with *Eco*RI and cloned into the *Eco*RI digested and dephosphorylated pGSVTp-*lux* plasmid. The orientation of the promoter region was checked by PCR and luminescence of *E. coli* GT115 colonies carrying the plasmids. The resulting plasmids contained the promoter region of the genes of interest fused to the *luxCDABE* reporter system. The plasmids were mobilized into K56-2 and the appropriate mutants by triparental mating. Transconjugants (carrying the chromosomal promoter-reporter fusions) were selected on LB agar plates containing 100 µg/ml of trimethoprim (Tp), 200 µg/ml ampicillin and 10 µg/ml gentamicin.

3.2.7. Luminescence expression assays.

Overnight cultures in LB containing 100 µg/ml Tp were diluted into fresh LB medium to OD_{600}=0.04. After addition of the antibiotics and/or putrescine, 300 µl of sample were loaded in triplicate, for each time-point, in a 100-well honeycomb microtitre plate. The plates were incubated at 37°C with medium continuous shaking in a Bioscreen C automated growth curve analyzer (MTX Lab Systems, Vienna, VA, USA). Growth was followed by measuring the OD_{600} at 37°C every 30 min. At pre-determined time points post-inoculation, the Bioscreen was paused and three 200 µl aliquots for each condition tested were transferred into a flat bottom 96-well microtiter plate (Microfluor-2 White, Thermo Scientific) and luminescence (in relative light units, RLU) was measured using a Fluoroskan Ascent FL Microplate Fluorometer and Luminometer (Thermo Scientific, Ottawa, Ontario, Canada). Expression levels of each gene of interest in the different strain backgrounds were calculated as RLU/OD_{600} for each time-point.

3.2.8. Construction of a conditional mutant.

A fragment (~300-bp) spanning the 5′ region of BCAL2641 was PCR amplified, digested by *NdeI* and *XbaI* and cloned into the *NdeI* and *XbaI* digested and dephosphorylated pSC200

plasmid. The plasmids were mobilized into OME12 (ΔBCAM1111-1112) by triparental mating. Transconjugants were selected on LB agar plates containing 100 µg/ml of trimethoprim (Tp), 200 µg/ml ampicillin, 10 µg/ml gentamicin and 0.5% (wt/vol) rhamnose. This strategy creates conditional mutants in which the expression of the targeted gene depended on the rhamnose concentration in the medium (31).

3.2.9. Thin-layer chromatography analyses of polyamines.

The conditional mutant and the wild type were grown at 37°C in M9 minimal medium supplemented with final concentrations of Tp 100 µg/ml and rhamnose 0.4% (wt/vol), permissive condition of expression. An aliquot of an overnight culture in M9 medium with rhamnose was spun down and washed three times with sterile phosphate-buffered saline (PBS), resuspended in PBS, and adjusted to an OD_{600} of 1. Drops (10 µl) of undiluted suspension and 10-fold serial dilutions were plated onto M9 agar plates supplemented with 0.4% (wt/vol) glucose and incubated at 37°C (non-permissive condition of expression). Bacteria growing on the plates were collected, suspended in sterile PBS, and the OD_{600} was adjusted to 0.1. Polyamines were extracted, derivatized to their dansyl derivatives, sequentially separated on TLC silica gel plates (20×20 cm, Merck, Darmstadt, Germany) in two solvent systems: I) benzene–triethylamine (20:2 v/v); II) benzene–methanol (10:0.45 v/v) and visualized under ultraviolet light as previously described (12). Standard solutions of putrescine, cadaverine, spermidine and spermine (0.2 mM each) were treated similarly and included as controls.

3.2.10. Catalase enzyme activity assay.

Overnight cultures of the wild type *B. cenocepacia* K56-2 in LB were diluted to OD_{600}=0.04 into 30 ml fresh LB medium, with or without antibiotics, and incubated at 37^{o}C, 200 rpm for 16 h. Bacterial cells were pelleted, washed with sterile PBS and resuspended in 300 µl (or less if necessary depending on bacterial inhibition of growth by antibiotics) of PBS. The OD_{600} of the bacterial suspensions was measured. The catalase enzyme activity was evaluated using the method described by Iwase et al. (32). Briefly, 100 µl of bacterial suspension or bovine liver catalase solution at different concentrations were added in a glass tube followed by the addition of 100 µl of 1% Triton X-100. Finally, 100 µl of undiluted hydrogen peroxide (30%) were added to the solutions, mixed thoroughly and incubated at room temperature. The height of O_2-forming

foam that remained constant for 15 min in the test tube was finally measured using a ruler. The catalase activity of bacterial suspensions was determined using calibration curves constructed using the standard catalase solutions with different concentrations and normalized to the OD_{600} of the tested suspensions.

3.2.11. Statistical Analyses.

Unpaired student's t-tests were conducted with GraphPad Prism 5.0.

3.3. Results And Discussion

3.3.1. Putrescine reduces ROS production induced by PmB.

Treatment of *B. cenocepacia* K56-2 with 1 mg/ml PmB led to significantly increased production of intracellular ROS, as detected by 2',7'-dichlorofluorescein diacetate (DCF) (Fig. 20). DCF is a colorless, nonfluorescent fluorescein derivative which passively diffuses into cells where the two acetate groups are cleaved by intracellular esterases to yield the non-cell permeable 2', 7'-dichlorofluorescein (33). This cleaved product becomes trapped within the cells and becomes oxidized by intracellular ROS resulting in the formation of a highly fluorescent product; hence it is a measure of generalized oxidant production rather than that of any particular reactive species (33). Lower concentrations of PmB (0.5 mg/ml or less) did not alter the intracellular DCF-detectable ROS pool (data not shown), whereas due to its reduced solubility in the culture medium higher concentrations of PmB could not be reliably tested. Since putrescine protects *B. cenocepacia* from PmB (12), I assessed whether it also alleviates PmB-induced ROS production. Compared to control cells, exogenous putrescine reduced DCF-detectable ROS generation in PmB-treated bacteria (Fig. 20). This effect was assessed at 2 h incubation with PmB and/or putrescine to avoid potential interference from putrescine degradation or metabolic by-products at prolonged incubation times. It should be noted that putrescine did not decrease the background ROS levels produced by bacterial cells not exposed to PmB, but rather caused a slight but significant increase in DCF-detected ROS levels compared to control cells at 20 mM (Fig. 20, white bars). I attributed these results to polyamines catabolism, which also generate ROS (34).

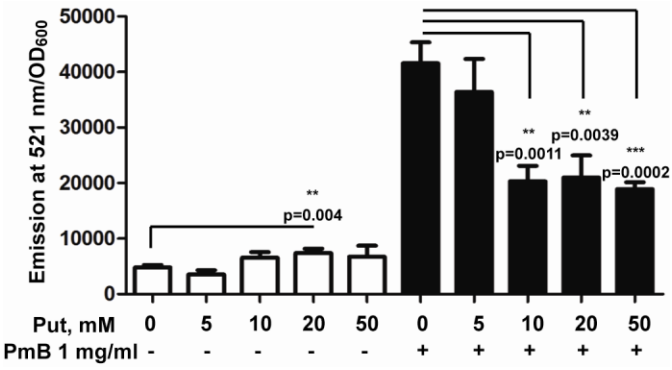

Figure 20. Putrescine reduces ROS production induced by PmB in *B. cenocepacia* K56-2.

ROS were detected by DCF.n= 6 from 2 independent experiments. Unpaired student's t-tests were conducted between each condition and its respective control.

To assess whether endogenous putrescine also has the ability to reduce ROS levels in PmB-treated *B. cenocepacia*, I employed deletion mutants in the putrescine biosynthesis pathway. Putrescine can arise through the action of either ornithine decarboxylase or arginine decarboxylase (12). *B. cenocepacia* has two ornithine decarboxylase homologues, BCAL2641 and BCAM1111, and one arginine decarboxylase protein, BCAM1112 (Fig. 21A). The ornithine decarboxylase BCAL2641 is encoded by a gene located on chromosome 1 of *B. cenocepacia*; whereas both the ornithine decarboxylase BCAM1111 and the arginine decarboxylase BCAM1112 are encoded by genes located adjacent to each other, but in opposite orientation, on chromosome 2. In a previous study, we have shown that ΔBCAL2641 had a greater reduction in the amount of secreted putrescine compared to wild type than ΔBCAM1111-BCAM1112 (12). Here, I confirmed that these three enzymes are the only contributors to putrescine production in *B. cenocepacia*. A conditional mutant of BCAL2641 in the ΔBCAM1111-BCAM1112 background did not produce detectable levels of putrescine at the non-permissive conditions of expression compared to the wild type strain (Fig. 21B). With respect to the response to PmB, the ornithine decarboxylase BCAL2641 was the only enzyme, among the 3 putrescine synthesis enzymes, involved in resistance against PmB. ΔBCAL2641, but not ΔBCAM1111-BCAM1112, had increased susceptibility to PmB compared to wild type when tested in LB medium (Fig. 21C and 21D) or M9 medium (Fig. 21E). Although the growth of ΔBCAM1111-BCAM1112 was not impaired in LB medium regardless of the initial inoculum size (Fig. 21C and 21D), it exhibited significant reduction in growth compared to the wild type cells in M9 medium (Fig. 21E). Nevertheless, this mutant did not show increased susceptibility to PmB in M9 medium in which its growth was retarded (Fig. 21E). On the contrary, ΔBCAL2641 showed slight reduction in growth in LB medium only at low inoculum size (Fig. 21C) but not at high inoculum size (Fig. 21D) or in M9 medium (Fig. 21E). This suggests that these genes involved in putrescine synthesis are not functionally redundant; they seem to be stimulated under different conditions and regulated differently with BCAL2641 only involved in resistance to antibiotics. Next, detection of ROS by DCF was assessed after incubation of ΔBCAL2641 and ΔBCAM1111-BCAM1112 mutants with PmB for 16 h to allow the different enzymes to reach their maximum expression levels which occurred at about 12 h in the luminescence expression assays (not shown). No differences were observed in PmB-untreated cells between the wild type and the deletion mutants (Fig. 22, white bars). In contrast,

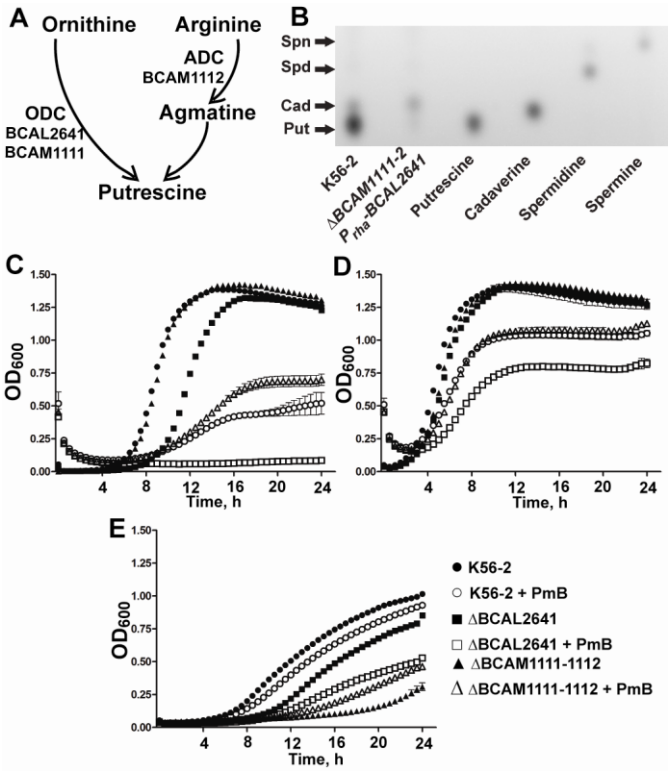

Figure 21. BCAL2641 is the only putrescine synthesis enzyme in *B. cenocepacia* involved in reduced susceptibility to PmB.

A. Putrescine synthesis pathway in *B. cenocepacia* K56-2 together with the enzymes involved. ADC, arginine decarboxylase; ODC, ornithine decarboxylase. B. TLC plate showing the lack of production of putrescine in ΔBCAM1111-1112P_{rha}-BCAL2641conditional mutant under non-permissive conditions. Put, putrescine; Cad, cadaverine; Spd, spermidine; Spn, spermine. C-E. Sensitivity of wild type and putrescine synthesis mutants ΔBCAL2641 (OME11) and ΔBCAM1111-1112 (OME12) to 2048 µg/ml PmB determined turbidimetrically. n=3 from a representative experiment. C, low initial inoculum in LB medium; D, high initial inoculum in LB medium; D, in M9 minimal medium.

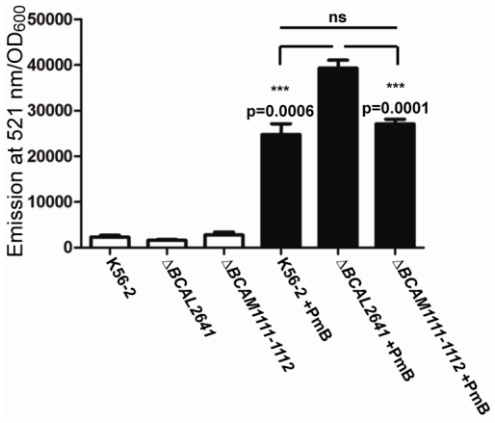

Figure 22. BCAL2641 is the main ornithine decarboxylase responsible for reduction of ROS accumulation.

ROS production in response to 1 mg/ml PmB in wild type K56-2, compared to putrescine synthesis mutants ΔBCAL2641 (OME11) and ΔBCAM1111-1112 (OME12) detected by DCF.n=6 from 2 independent experiments. Unpaired student's t-tests were conducted.

ΔBCAL2641 exhibited a significant increase in levels of superoxide and other ROS detected by DCF in response to PmB compared to wild type, whereas ΔBCAM1111-BCAM1112 produced the same level as that in the parental strain (Fig. 22). Together, these results support the notion that putrescine reduces the level of PmB-induced ROS production and this reduction contributes to protection of bacteria from the bactericidal effects of PmB.

Hydroxyl radical is another ROS that may be produced upon oxidative stress. Others have used hydroxyphenyl fluorescein (HPF) to fluorometrically detect hydroxyl radicals upon antibiotic stress (16). Using HPF in similar experiments as above, I found a comparable pattern of reduction of PmB-induced ROS by putrescine (data not shown). However, the fluorescence signal detected by HPF was too low compared to that detected by DCF, and required 100-fold higher inoculum than that for the DCF experiments to detect signal above the background noise of fluorescence. Such high inoculum of cells led to high autofluorescence compared to the actual fluorescence signal detected upon adding HPF, which was not the case with the DCF assays (Fig. 23). Thus, I disregarded the results of HPF assays. Similar criticism to the use of HPF was raised recently concerning the interference between the autofluorescence of cells with the actual fluorescence in the presence of the probe especially upon antibiotic treatment (35).

Although the DCF fluorometric assay is a well established method and has many advantages over other techniques developed for measurement of intracellular ROS (33), the probe may be nonselective reacting with other oxidants such as hydroxyl radicals and lipid peroxides (36). Hence, to provide additional evidence supporting the DCF fluorometric assays results, I measured the expression of OxyR as an independent indicator of oxidative stress. OxyR belongs to the LysR family of transcription factors whose regulon is involved in the cellular response to oxidative stress (22). OxyR is very sensitive to ROS, and is activated at very low hydrogen peroxide concentrations, leading to upregulation to its regulon (37). Moreover, an *oxyR::lacZ* promoter fusion is also upregulated in response to hydrogen peroxide (38). Similarly, another LysR-type transcription regulator involved in the response to oxidative stress is also overexpressed in response to ROS (39). Therefore, I constructed derivatives of wild type and mutant strains carrying an *oxyR*::*lux* promoter fusion to measure *oxyR* gene expression at chromosomal levels. PmB stimulated the *oxyR* expression (Fig. 24A), which was consistent with the induction of intracellular ROS detected by DCF (Fig. 20 and 22). Likewise, catalase

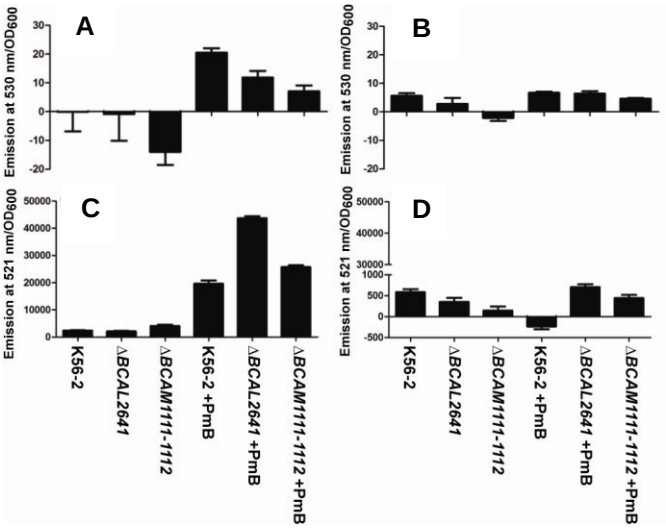

Figure 23. Comparison of the autofluorescence of cells relative to fluorescence signals of fluorescent probes detecting reactive oxygen species in *B. cenocepacia* K56-2.

(A) Emission signal following treatment with HPF without correction for autofluorescence background; (B) Autofluorescence of cells at the same inoculum size and under the same conditions used for HPF assay; (C) Emission signal following treatment with DCF without correction for autofluorescence background; (D) Autofluorescence of cells at the same inoculum size and under the same conditions used for DCF assay. n=3 from one representative experiment.

activity, regulated by OxyR (22), increased in response to PmB (Table 8). This further confirms the induction of intracellular ROS in response to PmB and validates the findings of DCF fluorometric and *oxyR* expression assays as measures of intracellular ROS. *oxyR* expression was significantly higher in ΔBCAL2641 compared to the parental strain both in the presence or absence of PmB. In contrast, no difference in *oxyR* expression between the wild type and ΔBCAM1111-BCAM1112 was detected in response to PmB (Fig. 24A). No differences in the growth rate of the different strains were noted in absence of PmB; whereas ΔBCAL2641 was more susceptible to PmB compared to the wild type and ΔBCAM1111-BCAM1112 (Fig. 25). These results follow the same pattern of ROS generated in response to PmB in the mutants compared to the wild type strain (Fig. 22).

Next, I investigated the mechanism by which putrescine protects from oxidative stress. Putrescine stimulated the expression of *oxyR* (Fig. 24A), probably as a result of a slight induction of ROS accumulation as detected by DCF (Fig. 20). However, putrescine alleviated the increase in *oxyR* expression in response to PmB (Fig. 24A), suggesting a protective effect against ROS. Nevertheless, putrescine did not induce a statistically significant difference in growth of the wild type in the presence or absence of PmB at this early time point of incubation (3 h) under the conditions of this test (Fig. 25). Supporting the protective role of putrescine from oxidative stress, I confirmed the antioxidant properties of putrescine by demonstrating that it could scavenge superoxide radicals generated *in vitro* from a phenazine methosulfate-NADH system in a concentration dependent manner (Fig. 24B). Together, the results of this section reveal a link between reduced susceptibility to PmB, induction of ROS production, and expression of OxyR with the intracellular level of putrescine, which can be attributed to the antioxidant properties of this polyamine.

3.3.2. Expression of the putrescine synthesis enzymes in response to PmB.

To better understand the role of the different putrescine synthesizing enzymes in response to oxidative stress and consequently to PmB, I investigated the expression profiles of their corresponding genes also using *lux* promoter fusions as before. BCAL2641::*lux* gene expression was stimulated by exposure to PmB (Fig. 26A); whereas neither BCAM1111::*lux* nor BCAM1112::*lux* fusions were responsive to PmB (Fig. 26B and 26C respectively). This agrees

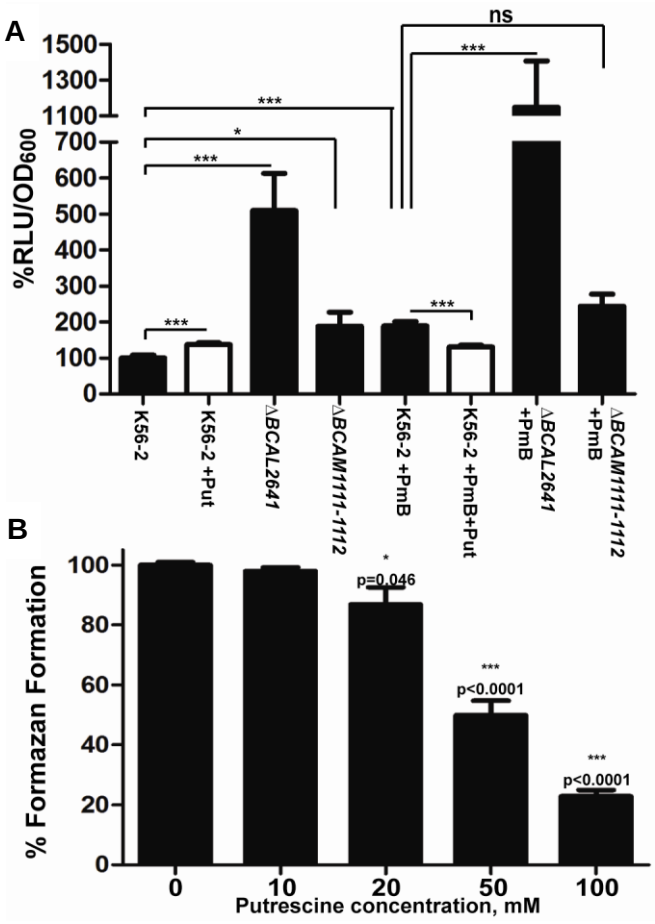

Figure 24. A. Induction of OxyR expression as an indicator of ROS accumulation in the wild type (OME56) compared to putrescine synthesis mutants ΔBCAL2641 (OME57) and ΔBCAM1111-1112 (OME58) in response to 500 µg/ml PmB with or without 10 mM Put determined by luciferase expression assay at 3 h. Results are shown as percentage of relative light units RLU/OD_{600} relative to the OME56 control (K56-2 background). The mean RLU/OD_{600} of the control is 0.09567. The percentages of OD_{600} are shown in Fig. 25. n=9 from 3 different clones. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ from unpaired student's t-tests. B. *In vitro* antioxidant activity of putrescine. n=6 from 2 independent experiments.

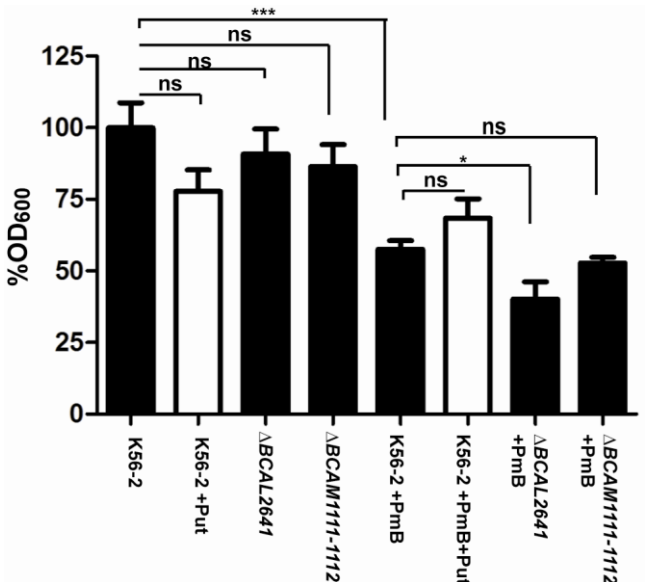

Figure 25. The relative growth of cells in the luminescence expression assay for *oxyR* expression in the wild type (OME56) compared to putrescine synthesis mutants (ΔBCAL2641 background, OME57; and ΔBCAM1111-1112 background, OME58) at 3 h shown in Figure 24A. Results are shown as percentage of OD_{600} relative to the control (untreated K56-2 background). The mean OD_{600} of the control is 0.1663. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ from unpaired student's t-tests.

Table 8. Catalase enzyme activities.

Antibiotic Concentration (μg/ml)	**Catalase Activity*** % Units/OD_{600} (SEM)	**Difference from control** (P-value)
None	100 (1.3)	Not applicable
Polymyxin B (500)	120.1 (4.2)	0.0002
Norfloxacin (8)	110.6 (1.2)	0.0012
Rifampicin (16)	134.5 (5.2)	<0.0001
Ceftazidime (32)	99.8 (12.4)	0.978 (Not significant)
Gentamicin (1000)	48.0 (7.1)	<0.0001

* Results from 2 independent experiments, n=6. r^2 of calibration curves was: 0.9644 and 0.9544.
Significance of differences from control was determined using unpaired student's t-tests.

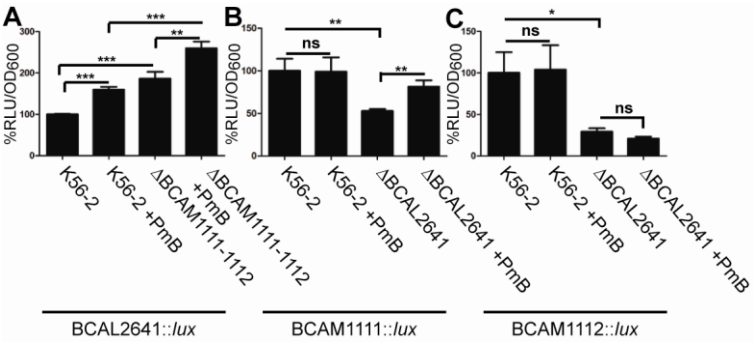

Figure 26. Luciferase expression assay of the different putrescine synthesizing enzymes in response to 500 µg/ml PmB at 3 h.

Results are shown as percentage of relative light units RLU/OD_{600} relative to the control (untreated K56-2 background). The percentages of OD_{600} are shown in Fig. 27. A, Expression of BCAL2641 in the wild type (OME50) and ΔBCAM1111-1112 (OME51) backgrounds.n=6 from 2 different clones. The mean RLU/OD_{600} of the control is 1.4829. B, Expression of BCAM1111 in the wild type (OME52) and ΔBCAL2641 (OME53) backgrounds.n= 6 from 2 different clones. The mean RLU/OD_{600} of the control is 1.5585. C, Expression of BCAM1112 in the wild type (OME54) and ΔBCAL2641 (OME55) backgrounds.n= 7 from 2 different clones. The mean RLU/OD_{600} of the control is 0.2423. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ from unpaired student's t-tests.

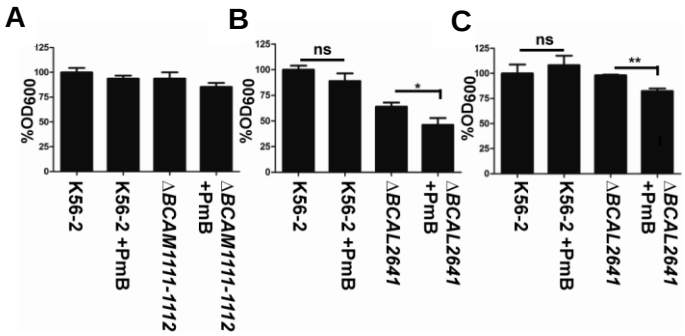

Figure 27.The relative growth of cells in the luminescence expression assay for the different putrescine synthesizing enzymes in response to 500 μg/ml PmB at 3 h shown in Figure 26.

Results are shown as percentage of OD_{600} relative to the control (untreated K56-2 background). (A) Expression of BCAL2641 in the wild type (OME50) and ΔBCAM1111-1112 (OME51) backgrounds. n=6 from 2 different clones. The mean OD_{600} of the control is 0.1422. (B) Expression of BCAM1111 in the wild type (OME52) and ΔBCAL2641 (OME53) backgrounds. n= 6 from 2 different clones. The mean OD_{600} of the control is 0.1523. (C) Expression of BCAM1112 in the wild type (OME54) and ΔBCAL2641 (OME55) backgrounds. n= 7 from 2 different clones. The mean OD_{600} of the control is 0.1017. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ from unpaired student's t-tests.

with the behaviour of ΔBCAL2641 and ΔBCAM1111-BCAM1112 mutants to PmB in terms of antimicrobial resistance (Fig. 21C-21E) and ROS production (Fig. 22). Moreover, this is consistent with our previous data showing increased transcription of BCAL2641, but not BCAM1111 or BCAM1112, in response to PmB (12). BCAL2641 also appears to regulate by an unknown mechanism the gene expression of BCAM1111 and BCAM1112 putrescine synthesis enzymes, since the expression of both genes was significantly reduced in the ΔBCAL2641 background (Fig. 26B and 26C, respectively). This regulation is not mediated through the action of putrescine since 10 mM of putrescine did not stimulate the gene expression of BCAM1111 or BCAM1112 (not shown). Other indirect regulatory pathways may be involved which will require further investigation. On the other hand, the gene expression of BCAL2641 increased in the absence of BCAM1111 and BCAM1112 (Fig. 26A), which may explain the slight increase in survival of the ΔBCAM1111-BCAM1112 when exposed to PmB shown in Fig. 21E. This might be due to compensation of the reduced synthesis of putrescine by these enzymes being normally stimulated by BCAL2641. Alternatively, BCAM1111 and BCAM1112 might provide feedback inhibition to BCAL2641; thus their absence would lead to increased BCAL2641 gene expression. Notably, the expression of BCAM1112 (RLU/OD_{600} 0.2423) is much lower than that of the other 2 enzymes (RLU/OD_{600} 1.4829 and 1.5585 for BCAL2641 and BCAM1111 respectively). This suggests that *B. cenocepacia* does not preferentially utilize the arginine decarboxylase BCAM1112. This agrees with the fact that *B. cepacia* can degrade arginine only through the use of the succinyl transferase pathway, despite the possession of an arginine decarboxylase homologue (40, 41). Except for ΔBCAL2641, which exhibited reduced growth in the presence of PmB, no differences in growth were observed in the other strains tested regardless of PmB exposure (Fig. 27). Together, these findings expose BCAL2641 as a crucial contributor of putrescine synthesis in the response against antibiotics.

3.3.3. ROS production in response to other bactericidal antibiotics.

To evaluate whether the induction of oxidative stress and its amelioration by putrescine is a general phenomenon, I tested other bactericidal antibiotics. Exposure of *B. cenocepacia* to gentamicin, norfloxacin, ceftazidime and rifampicin led to increased ROS production as detected by DCF (Fig. 28) at sub-lethal concentrations; i.e. concentrations below but more specifically near the MIC of these antibiotics (Fig. 29). Putrescine reduced the antibiotic-induced elevation

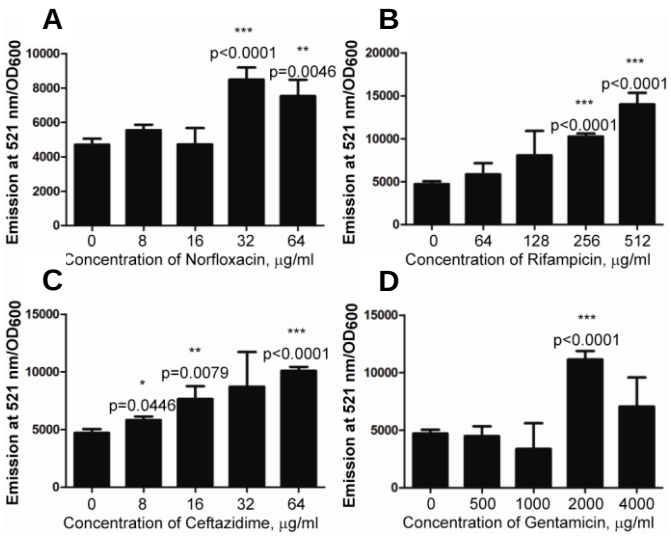

Figure 28. Effect of different bactericidal antibiotics on superoxide radical at different concentrations determined using DCF.

n=6 from 2 independent experiments.

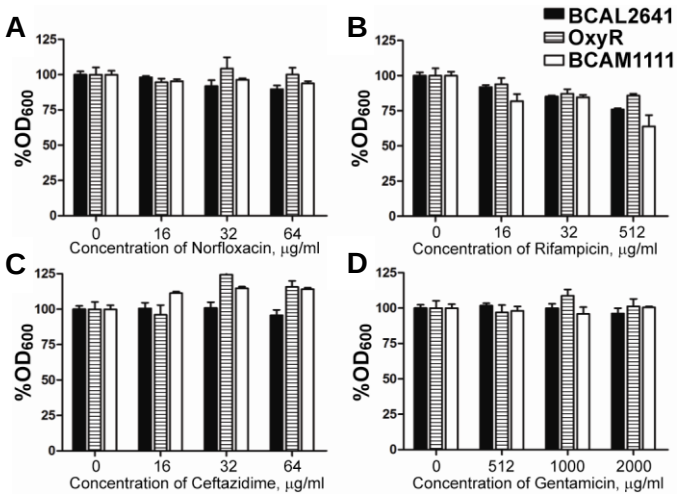

Figure 29.The relative growth of cells in the luminescence expression assay for BCAL2641, (in OME50), *oxyR* (in OME56), and BCAM1111 (in OME52) in response to different bactericidal antibiotics at 3 h shown in Fig. 31.

Results are shown as percentage of OD_{600} relative to the control (untreated K56-2 background). n= a minimum of 6 from at least 2 different clones. The mean OD_{600} of the control is 0.1943 for BCAL2641; 0.1816 for OxyR and 0.2166 for BCAM1111. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

in ROS levels only for norfloxacin and rifampicin (Fig. 30), and this correlated with induction of BCAL2641 gene expression (Fig. 31A and 31B, respectively). This agrees with the contribution of BCAL2641 in resistance to both antibiotics that we have previously reported (12). Moreover, *oxyR* transcription was also upregulated in response to both norfloxacin and rifampicin (Fig. 31A and 31B, respectively), which was reflected in an increase in the catalase activity (Table 8), supporting the notion that both antibiotics lead to increased ROS production (Fig. 30 and Fig. 28). In contrast, neither antibiotic affected BCAM1111 gene expression (Fig. 31), indicating that this gene and its product are not directly involved in the response to antibiotic-mediated oxidative stress. It should be noted that higher rifampicin concentrations resulted in great reduction in the expression of BCAL2641, *oxyR*, and BCAM1111 (Fig. 31B), which might be attributed to non-specific inhibition of transcription by rifampicin, especially at 512 µg/ml where expression from these genes was almost completely inhibited.

Putrescine did not reduce ROS production generated in response to ceftazidime, but rather further increased the generated ROS at 10 mM but not at 20 mM concentration of putrescine (Fig. 30). Ceftazidime did not affect the expression of BCAL2641, *oxyR* or BCAM1111 (Fig. 31C), and did not alter the catalase enzyme activity (Table 8). However, in a previous study we reported that BCAL2641 is involved in the response of *B. cenocepacia* to ceftazidime (12). This may suggest another role of BCAL2641 in the protective actions against ceftazidime not related to the oxidative stress.

Concerning the response to gentamicin, exogenous putrescine did not affect the level of gentamicin-induced superoxide anion (Fig. 30). Moreover, gentamicin did not alter the expression of BCAL2641 (Fig. 31D). This agrees with the previously reported lack of involvement of this enzyme in the response to gentamicin in *B. cenocepacia* (12). Furthermore, gentamicin did not affect the expression of *oxyR* (Fig. 31D). However, the highest tested concentrations of gentamicin did reduce the expression of both BCAL2641 and *oxyR* (Fig. 31D). Similarly, gentamicin reduced the catalase enzyme activity (Table 8). Such inhibition might be due to the mechanism of action of the aminoglycoside inhibiting translation and protein synthesis in general, since it also inhibited the expression of BCAM1111, which consequently might have led to increased ROS levels at high concentration (Fig. 30).

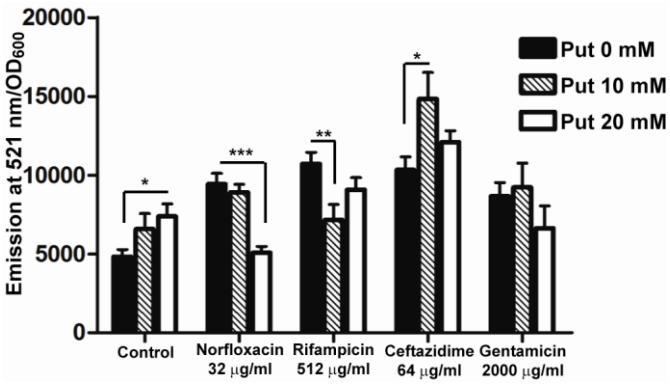

Figure 30.The role of putrescine in the bactericidal antibiotics-mediated ROS accumulation in *B. cenocepacia* K56-2.

n= 9 from 3 independent experiments. The 4 tested antibiotics alone significantly ($p<0.001$) induced the accumulation of ROS compared to control cells. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ from unpaired student's t-tests compared to the respective control conditions.

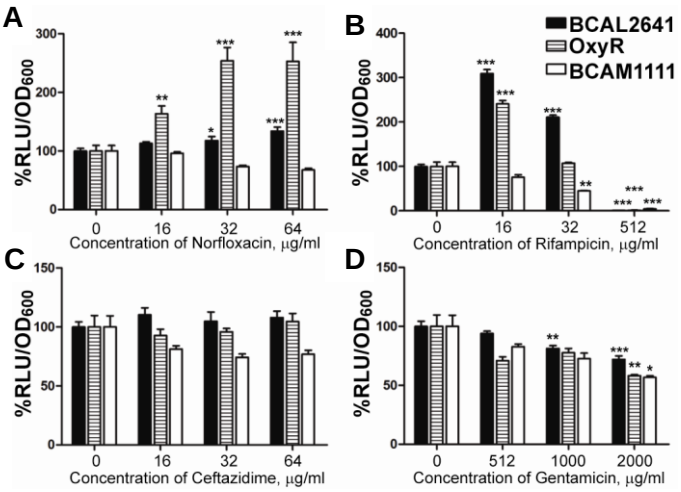

Figure 31. Effect of different antibiotics on the expression of BCAL2641 (in OME50), *oxyR* (in OME56), and BCAM1111 (in OME52) determined using a luciferase expression assay at 3 h.

Results are shown as percentage of relative light units RLU/OD_{600} relative to the control (untreated K56-2 background). The percentages of OD_{600} are shown in Fig. 29. n= a minimum of 6 from at least 2 different clones. The mean RLU/OD_{600} of the control is 1.0759 for BCAL2641; 0.1087 for *oxyR* and 1.4723 for BCAM1111. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ from unpaired student's t-tests compared to the respective control conditions.

3.4. Conclusions

In this Chapter, I show the following: (i) Sub-lethal concentrations of different bactericidal antibiotics (PmB at 1 mg/ml, rifampicin at 256 and 512 µg/ml and norfloxacin at 32 and 64 µg/ml) induce oxidative stress in *B. cenocepacia* that is manifested as induction of ROS formation as detected by DCF, stimulation of expression of the transcription regulator OxyR involved in response to oxidative stress (at antibiotic concentrations similar to or even lower than those inducing ROS formation; PmB at 0.5 mg/ml, rifampicin at 16 and 32 µg/ml and norfloxacin at 16-64 µg/ml), and increased catalase enzyme activity (PmB at 0.5 mg/ml, rifampicin at 16 µg/ml and norfloxacin at 8 µg/ml). (ii) This response does not apply to gentamicin and ceftazidime which do not induce OxyR expression or increase catalase enzyme activity, suggesting that not all bactericidal antibiotics induce oxidative stress. (iii) Putrescine protects against oxidative stress induced by several bactericidal antibiotics (PmB, norfloxacin and rifampicin). (iv) Protection by putrescine correlates with increased BCAL2641 gene expression. (v) BCAL2641, in addition to synthesizing putrescine, regulates the other putrescine biosynthetic enzymes BCAM1111 and BCAM1112 by an unknown mechanism that does not directly involve putrescine. Together, these observations suggest a model (Fig. 32) by which *B. cenocepacia* responds to antibiotic stress by overproducing putrescine and in turn, this polyamine protects bacterial cells by a surface effect blocking antibiotic binding (12) as well as by reducing oxidative damage.

Putrescine was previously shown to communicate antibiotic resistance among different bacteria (12). Its increased production in *B. cenocepacia* occurs in response to a subset of bactericidal antibiotics (12), which induce oxidative stress in bacterial cells at near lethal concentration ranges. It is still controversial whether the generation of ROS is the cause of lethality of antimicrobial agents or a consequence of antibiotic stress (16, 20, 21). However, it is conceivable that the oxidative stress accompanying antibiotic treatment imposes a metabolic burden on the bacterial cells at near death conditions. Thus, my results demonstrating a protective role for putrescine in the response to the oxidative stress generated in *B. cenocepacia* during antibiotic exposure represent another mechanism of protection from the antibacterial effects of bactericidal antibiotics. This agrees with previous reports on the antioxidant properties and protective effects of putrescine against antibiotic induced ROS formation in *E. coli* (17).

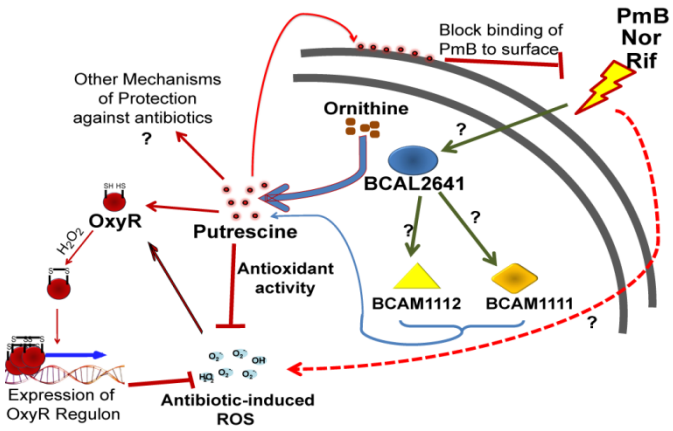

Figure 32. Model summarizing the role of putrescine in protecting *B. cenocepacia* from antibiotic-induced stress.

While little is known about the physiological levels of putrescine, it seems that its level varies in different body sites. For example, putrescine concentration was reported to be 3 mM in urine (11), but was no greater than 0.2 mM in sputum samples from CF patients (42, 43). However, it is difficult to predict the local concentration of putrescine and other polyamines in the lung of CF patients, as infection alters the rheology of the mucus and the lung environment (44). Moreover, putrescine levels increase dramatically (by 10 fold or more) during exacerbations of bacterial infections in CF patients (42, 43). Hence, the concentrations used in this study could potentially resemble the physiological situation in certain body compartments. Furthermore, a direct relationship exists between increased putrescine concentration during infection and the proliferation of lung microbiota and specific pathogens such as *P. aeruginosa* in the lungs of CF patients (43). Also, putrescine and other polyamines in genital mucosal fluids increase the resistance of *N. gonorrhoeae* to antimicrobial peptides (PmB and LL-37), possibly enhancing its survival during infection by reducing bacterial susceptibility to host-derived antimicrobials (11). Interestingly, the expression of the ornithine decarboxylase BCAL2641 is induced in *B. cenocepacia* in CF conditions compared to soil environmental like conditions shown by comparative transcriptomics, underscoring the importance of putrescine, and this enzyme in particular, during infection (45).

This study also provides new information on the regulation of the putrescine synthesis enzymes. The ornithine decarboxylase BCAL2641 gene responds to the external antibiotic signals, while the other ornithine decarboxylase BCAM1111 or the arginine decarboxylase BCAM1112 do not. Also, BCAL2641 regulates the expression of BCAM1111 and BCAM1112 since their expression depends on the presence of BCAL2641. This suggests that upon antibiotic stress maximal production of putrescine is required, which arises from the upregulation of BCAL2641 and by maintaining the expression of the other two enzymes in a BCAL2641-dependent manner. The molecular mechanism of this regulation awaits further investigation.

In conclusion, this study broadens our understanding on the mechanism of chemical communication of antibiotic resistance mediated by putrescine. In addition, it provides a clear target for the design of inhibitors targeting the ornithine decarboxylase BCAL2641 that is critically implicated in this phenomenon. Such inhibitors would not only reduce the resistance to

antibiotics in *B. cenocepacia* but also would reduce its ability to communicate high-level resistance to other less resistant bacteria.

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Chapter 4

A novel mechanism of resistance and protection from the action of hydrophobic antibiotics mediated by secreted bacterial lipocalins

4.1. Introduction

Communication among bacteria via small molecules is implicated in the transient increase of bacterial resistance to antibiotics, which could lead to therapeutic failures, thereby aggravating the problem of antibiotic resistance. The extremely antibiotic resistant bacterium *Burkholderia cenocepacia* protects *Pseudomonas aeruginosa*, in direct co-culture, from the lethal action of polymyxin B (PmB) (1).YceI is a small, secreted protein implicated in this protection (1).YceI constitutes a large family of conserved bacterial small proteins that share a common tertiary fold, similar to lipocalin proteins found in many prokaryotic and eukaryotic organisms, including humans. Bacterial lipocalin or "bacteriocalin" genes are present in 1524 bacterial species both Gram-positive and negative (according to SMART research tool (2)). The bacteriocalin structure has been elucidated in a few cases and consists of an extended, eight-stranded, antiparallel beta-barrel that resembles the lipocalin fold, although no sequence homology exists with lipocalins (3).

Bacteriocalin gene expression was induced in response to bases in *Escherichia coli* (4), and oxidative stress in *Pseudomonas aeruginosa* (5), and the protein was predicted to bind polyisoprenoid chain within the pore of the barrel via hydrophobic interactions in *Thermus thermophilus* based on its crystal structure (3). More recently, I have shown that YceI is involved in the bacterial response to several amphiphilic bactericidal antibiotics; the transcription of YceI was upregulated in *B. cenocepacia* in response to PmB and the purified YceI proteins were able to bind PmB (1). Interestingly, the structure of bacteriocalins resembles that of human α-1-acid glycoprotein (AGP), which was shown to bind polymyxin B in serum (6). However, until now, there has been no direct demonstration of bacteriocalin function. Here I hypothesize that bacteriocalins are involved in the bacterial response to stress conditions, including exposure to antibiotics and oxidative stress by binding toxic and undesired compounds. *B. cenocepacia* possesses 2 bacteriocalin homologues, BCAL3310 and BCAL3311. In this study, I characterized their individual roles in antibiotic resistance, their binding affinity to compounds of different chemical characteristics, and their expression profiles in response to antibiotic stress. I further investigated the functional conservation of bacteriocalins among different bacterial species and whether bacteriocalins secreted from one bacterium can protect other bacterial species *in vitro*

and *in vivo*. Herein, I present the first report of a defined function for bacteriocalin proteins in the communication of transient antibiotic resistance in *B. cenocepacia*.

4.2. Materials and methods

4.2.1. Strains and reagents

Table 9 lists bacteria and plasmids used in this study. Bacteria grew in LB (supplemented with 0.4% rhamnose when required) at 37°C. *Escherichia coli* cultures were supplemented as required with the following antibiotics (final concentrations): tetracycline (30µg/ml), kanamycin (40 µg/ml), and trimethoprim (50 µg/ml). *B. cenocepacia* cultures were supplemented as required with trimethoprim (100 µg/ml), and tetracycline (100 µg/ml). Antibiotics (Sigma, St Louis, MO, USA) were diluted in water except for PmB, which was diluted in 0.2% bovine serum albumin/0.01% glacial acetic acid buffer. Rifampicin was dissolved in dimethyl sulphoxide (DMSO).

4.2.2. General molecular techniques

DNA manipulations were performed as previously described (7). T4 DNA ligase (Roche Diagnostics, Laval, Quebec, Canada), Antarctic phosphatase (New England Biolabs, Pickering, Ontario, Canada) and restriction endonucleases (Roche or New England Biolabs) were used as recommended by the manufacturers. Transformation of *Escherichia coli* GT115 and DH5α was performed using the calcium chloride method (8). Mobilization of plasmids into *B. cenocepacia* was conducted by triparental mating (9) using *E. coli* DH5α carrying the helper plasmid pRK2013 (10). DNA amplification by polymerase chain reaction (PCR) was performed using a C1000 Thermal cycler (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada) with Taq or HotStar HiFidelity DNA polymerases (Qiagen, Mississauga, Ontario, Canada) and optimized for each primer pair. DNA sequencing was carried out at Eurofins MWG Operon, Huntsville, Alabama, USA. The DNA sequences were analyzed with the BLAST computer program and compared to the sequenced genome of *B. cenocepacia* strain J2315. Cloning, expression, and purification of *B. cenocepacia* bacteriocalins was performed as previously described (1). Transcriptional fusions to *luxCDABE* and the subsequent luminescence expression assays were performed as previously described (11).

Table 9. Strains and Plasmids used in Chapter 4

Strain or plasmid	Relevant characteristics[a]	Source and/or reference
Strains		
Burkholderia cenocepacia		
K56-2	ET12 clone related to J2315, CF clinical Isolate	[b]BCRRC,(12)
OME19	K56-2 pSCrhaB2; Tp^R	(1)
OME37	K56-2 pOE12; BCAL3310 with C-terminus FLAG tag; Tet^R	This study
OME40	K56-2 pOE13; BCAL3311 with C-terminus FLAG tag; Tet^R	This study
OME59	K56-2, $P_{BCAL3309}$::pGSVTp-*luxCDABE*; Tp^R	This study
OME60	K56-2, $P_{BCAL3310}$::pGSVTp-*luxCDABE*; Tp^R	This study
OME61	K56-2, $P_{BCAL3312\text{-}3311}$::pGSVTp-*luxCDABE*; Tp^R	This study
OME62	K56-2, ΔBCAL3311	This study
OME63	K56-2, ΔBCAL3312	This study
OME65	K56-2, ΔBCAL3310	This study
OME66	K56-2 pDA17; Tet^R	This study
OME67	OME62 pDA17; Tet^R	This study
OME68	OME62 pOE13 (BCAL3311); Tet^R	This study
OME69	OME62 pOE31 (PA0423); Tet^R	This study
OME70	OME62 pOE32 (PA4340); Tet^R	This study
OME71	OME62 pSCrhaB2; Tp^R	This study
OME72	OME62 pOE33 (BCAL3311); Tp^R	This study
OME73	OME62 pOE34 (PA0423); Tp^R	This study
OME74	OME62 pOE35 (PA4340); Tp^R	This study
OME75	OME62 pOE36 (PA4345); Tp^R	This study
OME76	OME62 pOE37 (Rv1890c); Tp^R	This study
Escherichia coli		
DH5α	F^-ϕ80*lacZ* M15 *endA1 recA1 supE44 hsdR*17($r_K^- m_K^+$)*deoR thi-1 nupG supE44 gyrA96relA1* Δ(*lacZYA-argF*)*U169,* λ–	Laboratory stock
GT115	F^- *mcrA*Δ(*mrr-hsdRMS-mcrBC*) ϕ80Δ*lacZ*ΔM15 Δ*lacX74 recA1 rpsL* (StrA) *endA1*Δ*dcm uidA*(ΔMluI)::*pir-116* Δ*sbcC-sbcD*	Invivogen, San Diego, CA
BL21	F^-*dcm ompT hsdS*($r_B^- m_B^-$) *gal*	Novagen
Pseudomonas aeruginosa		
PAO1	Non-CF clinical isolate	(13)
Salmonella typhi		
SARB63		(14)
Shigella flexneri		
SF51571	Serotype 1a, antigenic formula 1:4	
Acinetobacter species		

A. baumannii (AB1)	Clinical isolate	LHSC[c]
A. lwoffi (AB2)	Clinical isolate	LHSC[c]
A. junni (AB3)	Clinical isolate	LHSC[c]
Klebsiella pneumoniae		
Kpn18		
Staphylococcus aureus		
USA300	Community acquired MRSA	
Plasmids		
pRK2013	$ori_{colE1,}$ RK2 derivative, Kan^R, mob^+, tra^+	(10)
pGSVTp-*lux*	Mobilizable suicide vector containing lux operon, derivative from pGSV3-*lux*(15); OriT; Tp^R	(16)
pSCrhaB2	ori_{pBBR1}*rhaR*, *rhaS*, $P_{rhaB}Tp^R mob^+$	(17)
pDAI-SceI-SacB	ori_{pBBR1}, Tet^R, P_{dhfr}, mob^+, expressing I-SceI, SacB	(18)
pGPI-SceI	$ori_{R6K,}$ ΩTp^R , mob^+, including an I-SceI restriction site	(19)
pDA17	ori_{pBBR1}, Tet^R, mob^+, P_{dhfr}, FLAG epitope	D. Aubert, unpublised
pOE12	pDA17, BCAL3310, C-terminus FLAG, Tet^R	This study
pOE13	pDA17, BCAL3311, C-terminus FLAG, Tet^R	This study
pOE15	BCAL3310 without signal peptide encoding sequence cloned in pET28a(+)	(1)
pOE16	BCAL3311 without signal peptide encoding sequence cloned in pET28a(+)	(1)
pOE22	$P_{BCAL3310}$::*luxCDABE* transcriptional fusion in pGSVTp-*lux*, Tp^R	This study
pOE23	$P_{BCAL3312-3311}$::*luxCDABE* transcriptional fusion in pGSVTp-*lux*, Tp^R	This study
pOE25	pGPI-SceI with fragments flanking BCAL3310, Tp^R	This study
pOE26	pGPI-SceI with fragments flanking BCAL3311, Tp^R	This study
pOE27	pGPI-SceI with fragments flanking BCAL3312, Tp^R	This study
pOE31	pDA17, PA0423, C-terminus FLAG, Tet^R	This study
pOE32	pDA17, PA4340, C-terminus FLAG, Tet^R	This study
pOE33	pSCrhaB2, BCAL3311, Tp^R	This study
pOE34	pSCrhaB2, PA0423, Tp^R	This study
pOE35	pSCrhaB2, PA4340, Tp^R	This study
pOE36	pSCrhaB2, PA4345, Tp^R	This study
pOE37	pSCrhaB2, Rv1890c, Tp^R	This study

[a]Tp^R, trimethoprim resistance, Kan^R, kanamycin resistance, Tet^R, tetracycline resistance.
[b]BCRRC, *B. cepacia* Research and Referral Repository for Canadian CF Clinics.
[c]LHSC, London Health Science Centre, London, Ontario, Canada.

4.2.3. Protein analysis and Western Blotting

Overnight cultures were diluted to OD_{600} 0.03 in 30 ml fresh LB medium with or without PmB and incubated for 3.5 h at 37^{o}C, 200 rpm. Following incubation, cells equivalent to OD_{600} ~0.2 were pelleted, resuspended in 30 µl SDS-PAGE protein loading dye, and boiled to obtain whole cell lysates. Secreted proteins were precipitated from the supernatant of the rest of the cultures using 10% trichloroacetic acid as previously described (20). The precipitated proteins were resuspended by Tris buffer, 1M, pH 7.5. The volume of protein samples loaded to the 16% SDS-polyacrylamide gel was normalized to the OD_{600} value. After SDS–PAGE, proteins were transferred onto nitrocellulose membranes and the membranes were blocked overnight at 4°C with Western blocking reagent (Roche Diagnostics, Laval, QC, Canada) in TBST (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1 % Tween-20). The primary antibodies, anti-FLAG M2 monoclonal antibody (Sigma) or anti-α-subunit RNA Polymerase (*E. coli*) (Neoclone, Madison, WI, USA), were diluted to 1:15,000 in TBST and applied for 1.5 h. Secondary antibody, goat anti-mouse Alexa Fluor 680 IgG antibodies (Invitrogen), was diluted to 1:15,000 and applied for 1 h. Western blots were developed using LI-COR Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA)

4.2.4. Antibiotic susceptibility testing.

Overnight cultures of the parental *B. cenocepacia* K56-2 and the appropriate mutants in LB medium were diluted to an optical density at 600 nm (OD_{600}) of 0.0008 in fresh LB with or without the antibiotic and incubated at 37°C with medium continuous shaking in a Bioscreen C automated growth curve analyzer (MTX Lab Systems, Vienna, VA, USA). Bacterial growth was assessed turbidimetrically at 600 nm.

Etest strips (AB bioMérieux, Solna, Sweden) were applied to agar plates (17 ml agar in 85 mm Petri dish) inoculated with test bacteria by swabbing overnight cultures diluted to OD_{600} of 0.04; plates were then incubated at 37^{o}C for 24 h. Alternatively, population analysis profiling (PAP) was performed turbidimetrically or by cfu counting as previously described (1). For *in*

vitro protection assays, *B. cenocepacia* bacteriocalins were added to LB broth at a final concentration of 1.5 µM.

4.2.5. Fluorometric binding assays

These assays were performed as previously described (6) with few modifications. Purified bacteriocalins, 8-Anilino-1-naphthalenesulfonic acid (ANS) and Auramine O were prepared in phosphate buffered saline (PBS, pH 7.4). Phospholipids and Nile Red were prepared in DMSO. The binding of each fluorescent probe to bacteriocalins was measured by titrating 100 µl of bacteriocalins (1.5 µM) in a flat bottom 96-well microtiter plate (LUMITRAC 200 White, Greiner bio-one, Monroe, North Carolina, United States) with aliquots of increasing concentrations of probe until fluorescence intensity reached plateau. All spectra were corrected for background fluorescence determined from probe into buffer titrations. Fluorescence was measured using a Cary Eclipse Fluorescence spectrophotometer (Varian**,** Mississauga, ON, Canada) set at an excitation wavelength (λ_{ex}) specific for each probe, as follows: ANS (400 nm), Auramine O (428 nm), Nile Red (550 nm), and BODIPY phospholipids (500 nm for fatty acyl BODIPY labeled phosphocholine and 505 nm for head group BODIPY labeled phosphoethanolamine). The emission spectrum for each probe was collected across the following wavelengths (λ_{em}): ANS (420–600 nm), Auramine O (460–660 nm), Nile Red (590–750 nm), and BODIPY phospholipids (510–665 nm). The background-corrected binding fluorescence with each probe was fitted to a one-site binding model. The dissociation constant for the probe–bacteriocalin complex at a probe concentration equivalent to half the saturation concentration at which the maximum specific fluorescence enhancement occurs (K_d), was determined by non-linear least square regression analysis of the binding isotherms using GraphPad Prism V5.0 software (GraphPad software, San Diego, CA, USA).

For probe displacement experiments, antibiotic solutions diluted in PBS, pH 7.4 were titrated against bacteriocalin–probe complex at a saturating concentration necessary to obtain the maximum fluorescence when bound. Displacement of probe was measured as the corresponding decrease in fluorescence upon the progressive increase of antibiotic concentration.

4.2.6. *Galleria mellonella* larvae *in vivo* infection models

These assays were performed as described in Harding et al. (21). Overnight cultures were diluted to OD_{600} in PBS, pH 7.4 with or without *B. cenocepacia* bacteriocalins at 1.5 µM final concentration as follows: *P. aeruginosa* PAO1 to 0.00004, *K. pneumoniae* Kpn18 to 0.04, *A. baumannii* AB1 to 0.4 and *S. aureus* USA300 to 0.004. The larvae were injected with 10 µl of the bacterial suspensions or sterile PBS (10 larvae/group in each experiment) using 10 µl Microliter syringes (Hamilton Company, Reno, Nevada, USA). The larvae were incubated at 30^{o}C and their viability was checked at regular time intervals. In similar assays, 5 larvae/group were sacrificed at 200 min post-infection and the hemolymph was extracted as previously described (21). The hemolymph was immediately serially diluted in PBS, plated on LB agar supplemented with 0.3% cetrimide to quantify the cfu of *P. aeruginisa* PAO1 recovered from the infected larvae.

4.2.7. Statistical Analyses

Unpaired student's t-tests and other statistical analyses were conducted with GraphPad Prism 5.0.

4.3. Results and discussion

4.3.1. Secretion of *B. cenocepacia* bacteriocalins

To confirm the release of bacteriocalins into the extracellular milieu from *B. cencocepacia*, plasmid-encoded Flag-tagged versions of each bacteriocalin homologue were used. This revealed that BCAL3311, but not BCAL3310, was only secreted from the wild type K56-2 irrespective of exposure to PmB (Fig. 33). BCAL3310 was not detected in the supernatant even upon treatment with PmB at 2 µg/ml (Fig. 33) or 500 µg/ml (not shown).

4.3.2. The role of the different *B. cenocepacia* bacteriocalins in response to antibiotics

The function of the individual bacteriocalin homologues (BCAL3310 and BCAL3311), and the associated cytochrome b561 (BCAL3312) was assessed by performing individual deletions.

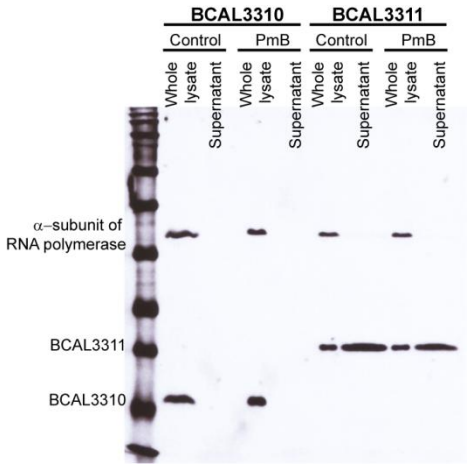


Figure 33. BCAL3311 is the only secreted *B. cenocepacia* bacteriocalin.

Proteins (carrying a C-terminal Flag-tag) were detected in whole cell lysates and supernatants of control untreated cultures or cultures treated with 2 μg/ml PmB by Western blot using anti-Flag antibody. The α-subunit of the RNA polymerase was used as a control for cell lysis.

The deletion mutant of only BCAL3311 showed increased susceptibility to PmB, rifampicin, norfloxacin and ceftazidime, but not the hydrophilic antibiotic gentamicin (Fig. 34). Etest revealed that ΔBCAL3311 has more homogenous response to ceftazidime observed as fewer discrete colonies at the otherwise clear zone of inhibition relative to the parental strain (Fig. 34F). This matches with previously reported antibiotic susceptibility phenotypes of double deletion mutant of YceI homologues (1). However, BCAL3310 deletion did not affect the susceptibility to antibiotics (Fig. 34), which is consistent with being not secreted (Fig. 33). Similarly, the cytochrome b561 BCAL3312 was not involved in the response to any of the tested antibiotics (Fig. 34).

4.3.3. Fluorometric assays of binding interaction of *B. cenocepacia* bacteriocalins

To test the binding preference of bacteriocalins, I used fluorescent compounds that probe binding sites of proteins. These fluorophores have different chemical features enabling them to probe different binding sites of proteins. I used Nile Red (consisting of a hydrophobic multi-cyclic structure and a tertiary amine) which tests hydrophobic binding sites, Auramine O (consisting of a bridged bi-phenyl structure with a tertiary amine on each phenyl and a central basic group), which tests basic binding sites, and 8-Anilino-1-naphthalenesulfonic acid, ANS (consisting of three hydrophobic phenyl groups, a secondary amine and an acidic sulfonate group) which tests acidic binding sites (6). I determined the binding affinity of purified recombinant bacteriocalin homologues for each of the 3 probes in PBS buffer by measuring the increase in fluorescence intensity upon probe–protein complex formation (Fig. 35A-F). The rise in fluorescence at the specific emission wavelengths of each probe was monitored with a series of concentrations of probes until no further increase in the fluorescence intensity was detected, indicating all binding sites were occupied. A one-site binding model was fit to the binding isotherms to derive the dissociation constant for each probe–BCAL3311 complex as previously described for human AGP (6). Auramine O and Nile Red exhibited higher binding affinity for BCAL3311 relative to ANS (Fig. 35). This suggests that BCAL3311 binds with higher affinity to hydrophobic and basic molecules, whereas it binds acidic compounds with lower affinity. However, Auramine O interaction with BCAL3311 yielded fluorescence emission with low signal to noise ratio, and hence a low correlation coefficient compared to that of Nile Red-BCAL3311 interaction. The interaction of BCAL3310 with the same fluorophores was compared

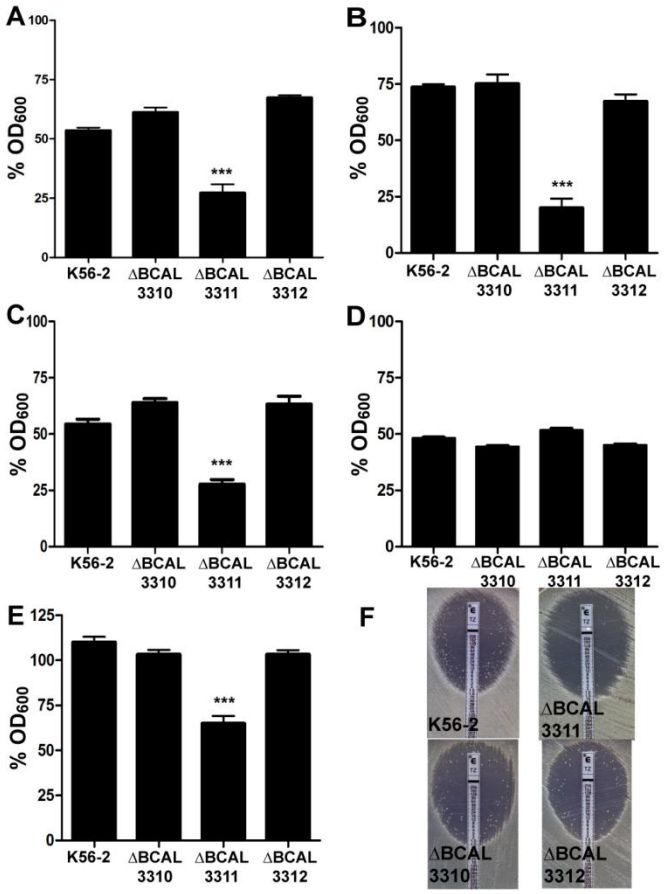


Figure 34. BCAL3311 is the only *B. cenocepacia* bacteriocalin involved in resistance to hydrophobic but not hydrophilic antibiotics.

Mutants carrying markerless deletions of individual genes encoding the 2 bacteriocalins BCAL3310 (OME65) and BCAL3311 (OME62) and the associated cytochrome b561 BCAL3312 (OME63) were compared to the parental strain K56-2 in turbidimetric assays the results of which are expressed as $\%OD_{600}$ relative to control untreated culture of the corresponding mutant (A-E) and by Etest (F) in their response to the antibiotics: A, PmB 1 mg/ml at 18 h, from 3 independent experiments, n=9; B, rifampicin 16 µg/ml at 18 h, from 2 independent experiments, n=6; C, norfloxacin 4 µg/ml at 24 h, from 3 independent experiments, n=9; D, gentamicin 512 µg/ml at 24 h, from 2 independent experiments, n=6; E, ceftazidime 16 µg/ml at 24 h, n=3; F, ceftazidime Etest. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ from unpaired student's t-tests compared to the respective control conditions.

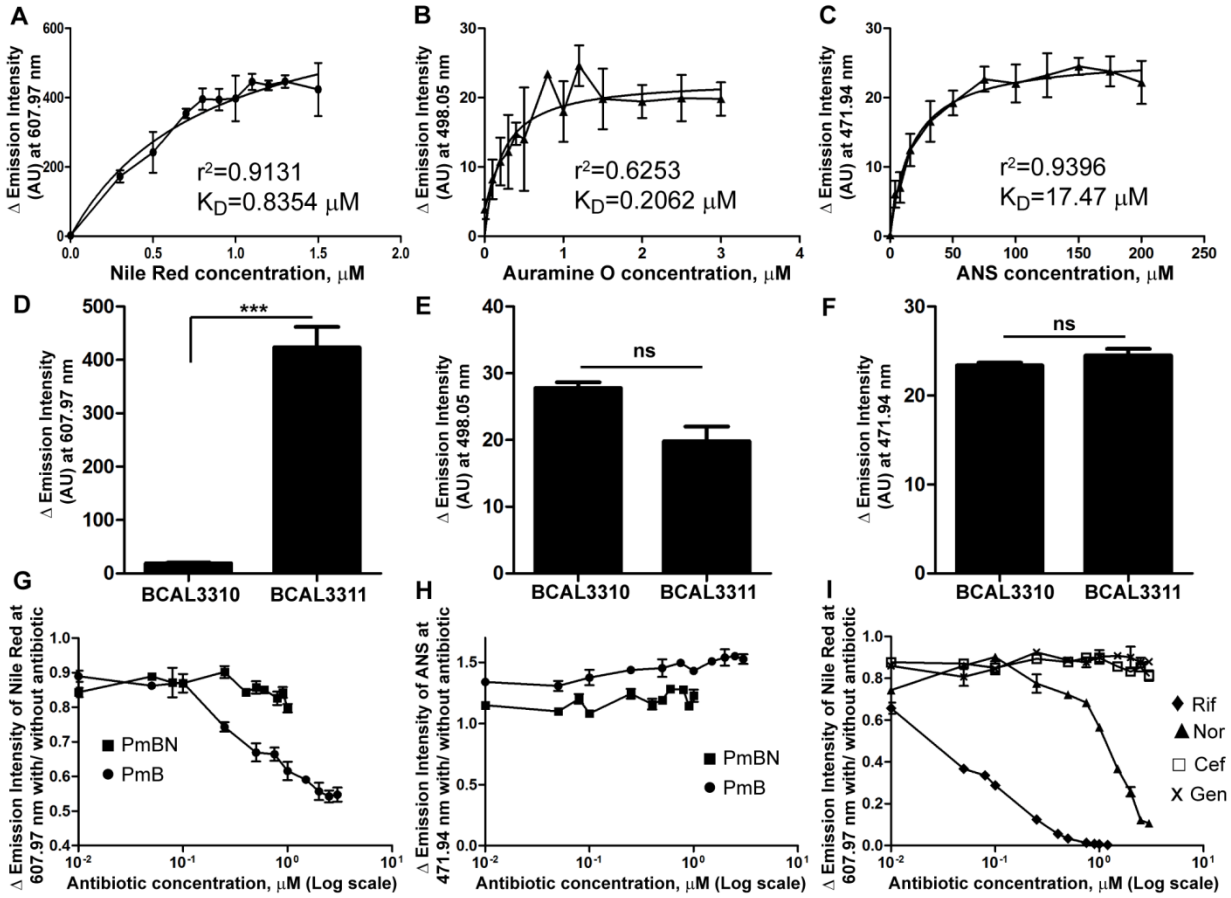


Figure 35. Bacteriocalins bind with higher affinity to hydrophobic molecules; BCAL3311 shows superior binding affinity relative to BCAL3310.

Fluorometric assays were used to determine the binding affinity of 1.5 μM recombinant *B. cenocepacia* bacteriocalins lacking their signal peptide sequences to fluorophores having different chemical characteristics in PBS. Binding affinity of BCAL3311 to Nile Red (A), Auramine O (B), and ANS (C) was determined from 3 independent experiments, n=5. The binding affinity of BCAL3310 was compared to that of BCAL3311 with 1.5 μM Nile Red (D), 1.5 μM Auramine O (E), and 150 μM ANS (F). Displacement assays of the fluorophores from BCAL3311-fluorophore complex by antibiotics were performed with PmB and PmBN against 1.5 μM Nile Red (G) and 150 μM ANS (H) and with rifampicin, norfloxacin, ceftazidime and gentamicin against 1.5 μM Nile Red (I); n=2. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ from unpaired student's t-tests.

to that with BCAL3311 (Fig. 35D-F). While there was no difference in the interaction at basic or acidic binding sites, BCAL3310 bound Nile Red with much lower affinity than BCAL3311 (Fig. 35D). This suggests that binding hydrophobic moieties is critical to the function of BCAL3311. This agrees with my previous study showing that BCAL3310 binds with lower affinity to a fluorescent derivative of PmB compared to BCAL3311 (1). To further test the binding preference of BCAL3311 to hydrophobic moieties as opposed to hydrophilic ones, I measured the binding of two fluorescent phospholipid analogs labeled with the BODIPY fluorophore on the head group (BODIPY-phosphoethanolamine) and fatty acyl chain (BODIPY-phosphocholine). There was no fluorescence emission observed upon titration of BCAL3311 with BODIPY-phosphoethanolamine, whereas titration of BCAL3311 with BODIPY-phosphocholine resulted in high fluorescence emission suggesting high affinity interaction comparable to that with Nile Red (Not shown). This indicates that the hydrophilic head group segment of the molecule with the fluorescent label does not bind to BCAL3311 whereas the fatty acyl segment of the molecule with the fluorescent label is responsible for binding.

Next, the ability of different antibiotics to compete with each probe in complex with BCAL3311 was examined by incremental titration with each antibiotic to gain insights on the basis of interaction between BCAL3311 and the different antibiotics (Fig. 35G-I). Probe displacement assay for Auramine O was not feasible due to the low signal-to-noise ratio. Initially, I compared the probe displacement ability of PmB to its nonapeptide derivative (PmBN). PmBN only lacks the fatty acyl tail of PmB, which results in a significant loss of its bactericidal activity (22). The fatty acyl tail of PmB seemed critical for binding to hydrophobic binding sites of BCAL3311 where its loss led to significant reduction of its Nile Red displacement ability (Fig. 35G). Moreover, neither PmB nor PmBN could displace ANS (Fig. 35H), further supporting the notion of hydrophobic interaction between PmB and BCAL3311. Similarly, rifampicin and, to a lower extent, norfloxacin could displace Nile Red (Fig. 35I). Slight Nile Red displacement was observed only at the highest tested concentrations of ceftazidime, whereas no displacement of Nile Red from its interaction with BCAL3311 was observed with any of the tested gentamicin concentrations (Fig. 35I).

4.3.4. Luciferase expression assays of *B. cenocepacia* bacteriocalins

The role of the two *B. cenocepacia* bacteriocalin homologues in response to antibiotics was further assessed by testing the expression of these proteins. I created chromosomal promoter-*luxCDABE* transcriptional fusions for BCAL3310 (Fig. 36A) and the transcriptional unit BCAL3311-BCAL3312 (Fig. 36B). The expression of BCAL3311 and the associated cytochrome b561 (BCAL3312) was upregulated in response to PmB, rifampicin, and norfloxacin (Fig. 36B). The expression was not altered in response to ceftazidime whereas it was slightly reduced in response to gentamicin probably due to the mode of action of this aminoglycoside targeting protein expression (Fig. 36B). This agrees, in the most part, with the antibiotic susceptibility phenotypes observed for the ΔBCAL3311 mutant. Conversely, BCAL3310 expression was overexpressed by norfloxacin and ceftazidime only whereas it was slightly inhibited by rifampicin and gentamicin probably due to their general mechanism of action targeting transcription and translation respectively (Fig. 36A). Interestingly, the expression of BCAL3311 only was upregulated by paraquat (Fig. 36), which is an inducer of the superoxide anion. This suggests that BCAL3311 is also involved in the response to oxidative stress. A similar link between the response to antibiotics and oxidative stress was recently shown in case of putrescine released from *B. cenocepacia* (11).

4.3.5. Bacteriocalins from different bacterial species are involved in antibiotic resistance

As bacteriocalins are conserved among bacteria, I sought to determine if bacteriocalins from the YceI family of proteins from different bacteria are also involved in response to antibiotics. To address this, I attempted to complement ΔBCAL3311 with bacteriocalin homologues from different bacterial species. I tested the *B. cenocepacia* K56-2 $yceI_{Bc}$ BCAL3311, *P. aeruginosa* PAO1 $yceI_{Pa}$ PA0423, PA4340 and PA4345, the *Mycobacterium tuberculosis* H37Rv $yceI_{Mtb}$ Rv1890c and the *S. aureus* USA300 SAUSA300_2620. Cloning genes encoding BCAL3311, PA0423 and PA4340 with C-terminal Flag-tag did not complement the ΔBCAL3311 phenotype, potentially due to interference of the tag at this position with function of the proteins (Not shown). Next, I cloned all tested genes from the different species under the control of the rhamnose-inducible promoter in pSCRhaB2. PAP by agar dilution against PmB revealed that the

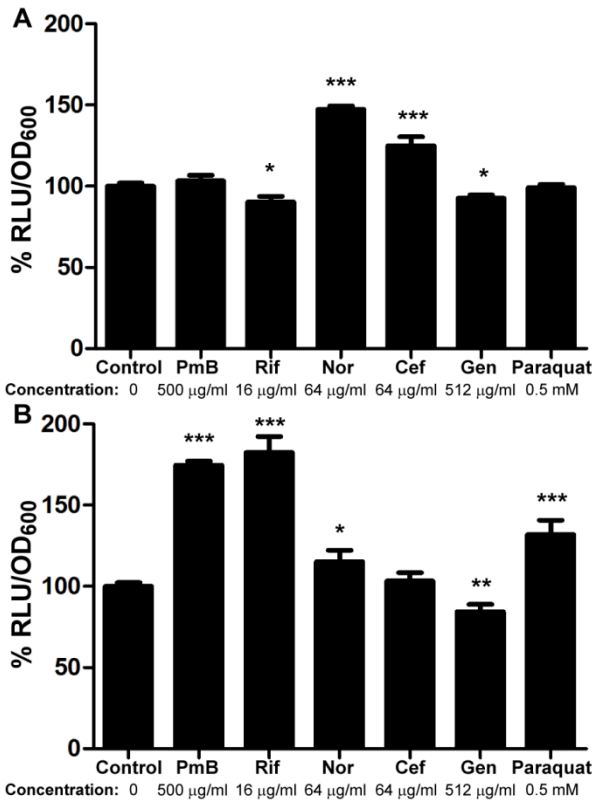


Figure 36. Luciferase expression assay of the different *B. cenocepacia* bacteriocalins in response to antibiotics at 3 h.

Results are shown as percentage of relative light units RLU/OD_{600} relative to the control (untreated K56-2 background). The percentages of OD_{600} are shown in Fig. 37. A, Expression of BCAL3310 (OME60).n=6 from 2 different clones. The mean RLU/OD_{600} of the control is 0.5531. B, Expression of BCAL3311 and the associated cytochrome b561 BCAL3312 (OME61).n= 6 from 2 different clones. The mean RLU/OD_{600} of the control is 1.3464. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ from unpaired student's t-tests compared to the respective control conditions.

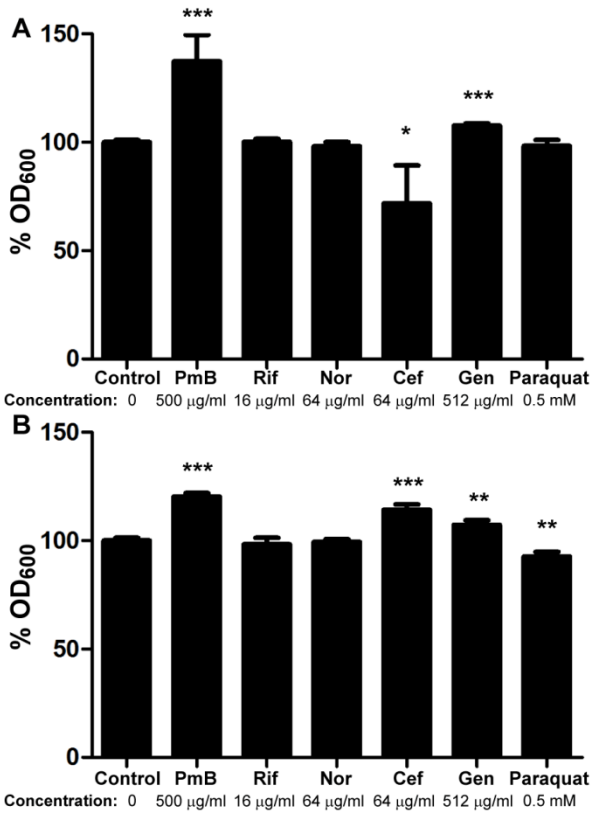


Figure 37. The relative growth of cells in the luminescence expression assay of the different *B. cenocepacia* bacteriocalins in response to antibiotics at 3 h shown in Fig. 36.

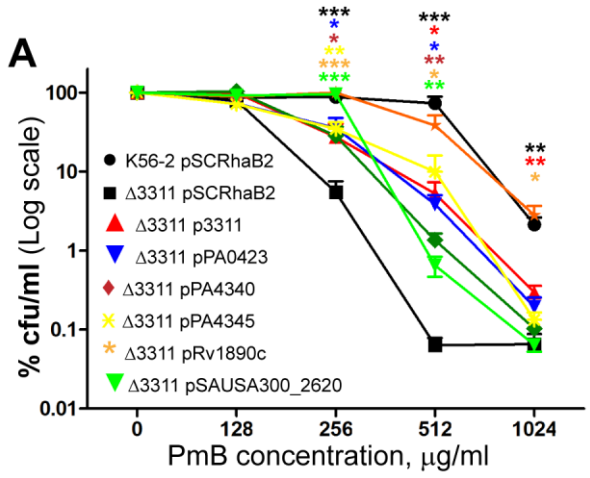
Results are shown as percentage of OD_{600} relative to the control (untreated K56-2 background). A, Growth in the expression assay of BCAL3310 (OME60).n=6 from 2 different clones. The mean OD_{600} of the control is 0.0963. B, Growth in the expression assay of BCAL3311 and the associated cytochrome b561 BCAL3312 (OME61).n= 6 from 2 different clones. The mean OD_{600} of the control is 0.0998. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ from unpaired student's t-tests compared to the respective control conditions.

B. cenocepacia BCAL3311 significantly increased the resistance of ΔBCAL3311 to PmB (Fig. 38A). Moreover, the *M. tuberculosis* $yceI_{Mtb}$ fully complemented the deletion of BCAL3311, whereas the 3 *P. aeruginosa* $yceI_{Pa}$ homologues and the *S. aureus* USA300 bacteriocalin homologue significantly increased resistance to PmB in the ΔBCAL3311 relative to the control vector (Fig. 38A). MIC determined by Etest showed that all tested bacteriocalin homologues from *B. cenocepacia*, *P. aeruginosa*, *M. tuberculosis* and *S. aureus* increased resistance to Rifampicin in the ΔBCAL3311 to the wild type level (Fig. 38B). Together, this shows that bacteriocalins from different pathogens are involved in antibiotic resistance and suggests that the function of bacteriocalins is conserved among bacteria.

4.3.6. Bacteriocalins protect different bacterial species *in vitro* and *in vivo*

I sought to determine if bacteriocalins secreted from one bacterial species can protect other bacteria from the action of antibiotics. *In vitro* assays showed that *P. aeruginosa* PAO1 treated with purified recombinant $yceI_{Bc}$ BCAL3311 had reduced sensitivity to PmB, an effect that was not observed with the less active BCAL3310 (Fig. 39A). However, both proteins protected *Salmonella typhi* SARB63, *Shigella flexneri* SF51571, *Acinetobacter baumanni* AB1, *Acinetobacter lwoffi* AB2, and *Acinetobacter junni* AB3 (Fig. 39B-F) at concentrations ~10-20 folds lower than that where protection was observed in case of *P. aeruginosa* (Fig. 39A). This difference in concentrations at which protection is observed for the different bacteria corresponds to difference in magnitude of affinity to Nile Red between BCAL3310 and BCAL3311 (Fig. 35D), which could explain the protective effect of BCAL3310 in case of the different bacterial species and its lack with *P. aeruginosa* PAO1.

Next, I used *Galleria mellonella* larvae infection model. BCAL3311-treated *P. aeruginosa* PAO1 cells were more virulent than the control PAO1 cells or BCAL3310-treated cells (Fig. 39G). After 200 minutes post-infection with ~700-900 cfu of *P. aeruginosa*, I recovered significantly higher numbers of BCAL3311-treated cells compared to the control untreated cells or the BCAL3310-treated group from the hemolymph of *G. mellonella* larvae plated on LB agar supplemented with 0.3% cetrimide (Fig. 39H). This suggests that the increased virulence due to



B

Mutant	MIC of Rifampicin, μg/ml
K56-2 pSCRhaB2	>32
Δ3311 pSCRhaB2	16
Δ3311 p3311	>32
Δ3311 pPA0423	>32
Δ3311 pPA4340	32
Δ3311 pPA4345	>32
Δ3311 pRv1890c	>32
Δ3311 pSAUSA300_2620	>32

Figure 38. The function of bacteriocalins in the response to hydrophobic antibiotics are conserved among different bacteria.

Bacteriocalins from *B. cenocepacia* K56-2 (BCAL3311), *P. aeruginosa* PAO1 (PA0423, PA4340, PA4345), *M. tuberculosis* H37Rv (Rv1890c) and *S. aureus* USA300 (SAUSA300_2620) were cloned in pSCRhaB2 under the control of the rhamnose promoter and used to complement the ΔBCAL3311 mutant in the presence of 0.4% rhamnose. A, PAP by agar dilution against PmB, from 3 independent experiments, n=6, asterisks are color coded and denotes difference from ΔBCAL3311 pSCRhaB2 mutant. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ determined by unpaired student's t-tests. B, MIC determined by Etest against rifampicin, a representative of 3 independent experiments. Discrete colonies in the otherwise clear zone of inhibition indicating heteroresistance similar to those in Fig. 34 panel F were not taken into consideration for MIC determination.

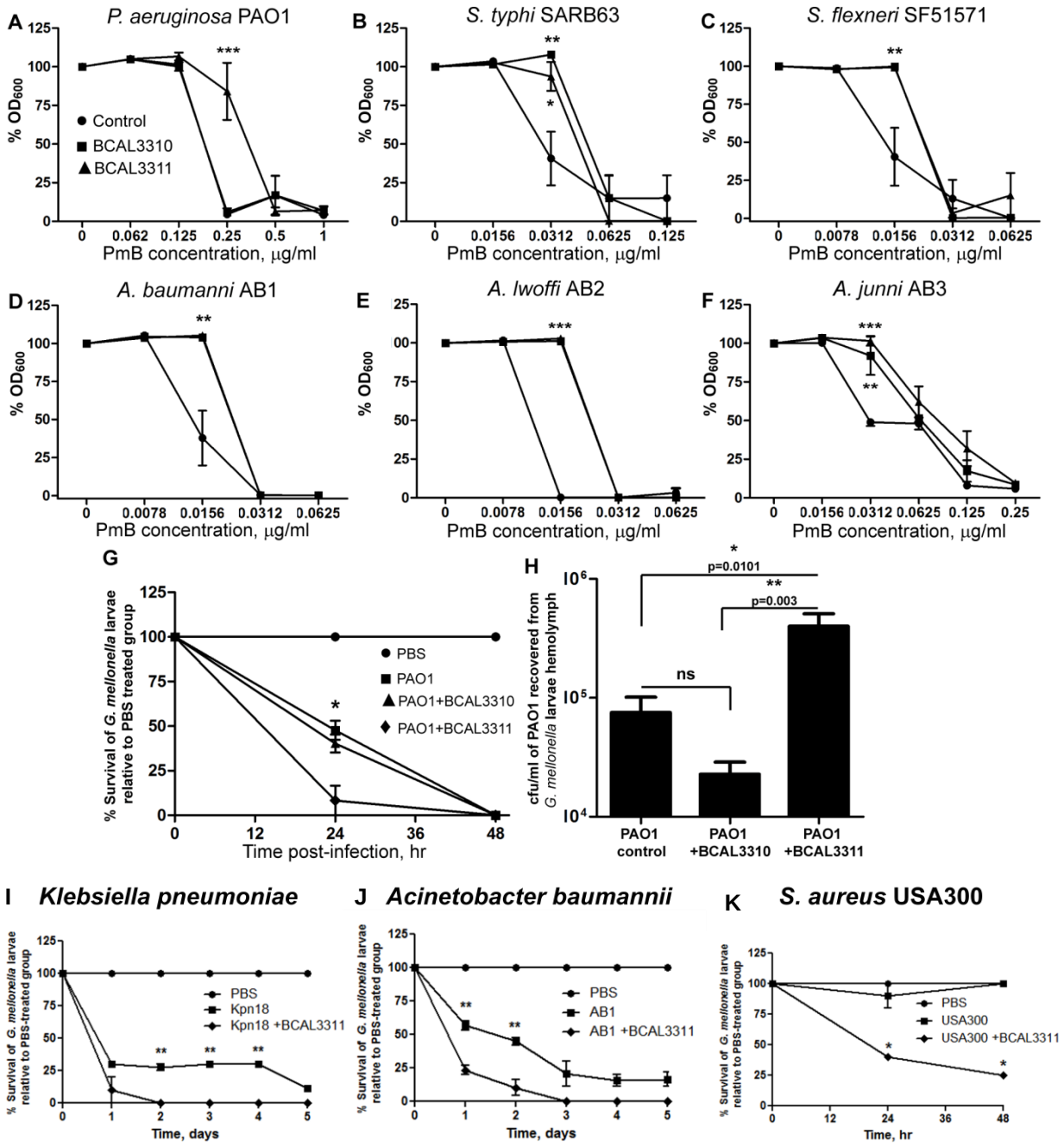


Figure 39. *B. cenocepacia* bacteriocalins, mainly the secreted BCAL3311, protect different bacterial species *in vitro* and *in vivo*.

(A-F) *In vitro* protection assays against PmB with 1.5 µM of BCAL3310 or BCAL3311: A, *P. aeruginosa* PAO1, n=8 from 4 independent experiments; B, *S. typhi* SARB63, n=7 from 3 independent experiments; C, *S. flexneri* SF51571, n=7 from 3 independent experiments; D, *A. baumanni* AB1, n=6 from 2 independent experiments; E, *A. lwoffi* AB2, n=5 from 2 independent experiments; F, *A. junni* AB3, n=5 from 2 independent experiments.

(G-K) *In vivo* protection assay using *G. mellonella* infection model. Each larva was injected with 10 µl of suspensions of different bacteria in PBS with or without *B. cenocepacia* bacteriocalins. The survival was monitored over time and compared to control group injected with sterile PBS. Each group included 10 larvae. (G) *P. aeruginosa* PAO1, the results are obtained from 3 independent experiments; the survival of both PAO1 and PAO1-BCAL3310 treated larvae is significantly different from that of PAO1-BCAL3311 treated group at p= 0.0165 and 0.0303 respectively. (H) In an independent experiment, larvae were sacrificed 200 min post-infection and the hemolymph was collected and plated on 0.3% cetrimide agar to quantify the recovered *P. aeruginosa* PAO1; n=10 from 2 independent experiments. (I) *K. pneumoniae* Kpn18, from 2 independent experiments; (J) *A. baumannii* AB1 from 3 independent experiments; and (K) *S. aureus* USA300, from 2 independent experiments. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ from unpaired student's t-tests compared to the respective control conditions.

treatment with BCAL3311 is a result of protection from killing of *P. aeruginosa* cells inside the host. *G. mellonella* larvae possess a humoral immune response where the hemolymph of bacteria-challenged larvae contains several antimicrobial peptides among them are members of linear α-helical peptides (cecropins and moricin-like peptides), cysteine-stabilized peptides (defensins), proline-rich peptides, and glycine-rich peptides in addition to lysozyme (23). Similar phenotype of increased virulence upon treatment with BCAL3311 was observed in *K. pneumoniea* Kpn18, *A. baumannii* AB1, and *S. aureus* USA300 (Fig. 39 I-K).

4.4. Conclusions

I show that bacteriocalins are involved in the bacterial response to hydrophobic or amphiphilic antibiotics (PmB, rifampicin, norfloxacin and ceftazidime), but not hydrophilic ones (such as gentamicin). This effect is attained by their preferential binding affinity to hydrophobic moieties. Bacteriocalins are functionally conserved among different bacteria and those secreted from one bacterial species can protect bacteria from other species from the effects of antibiotics whether *in vitro* or *in vivo*. Interestingly, the expression of both *B. cenocepacia* bacteriocalins is induced in CF conditions compared to soil environmental like conditions shown by comparative transcriptomics, underscoring the importance of bacteriocalins during infection (24). On the other hand, bacteriocalins could be involved in the response to oxidative stress. BCAL3311 expression was induced in response to paraquat. This is consistent with the overexpression of *P. aeruginosa* PAO1 bacteriocalin PA0423 in response to hydrogen peroxide and paraquat (5). Furthermore, Mammalian Odorant binding protein (OBP), a soluble lipocalin, when overexpressed in *E. coli*, protected the bacterial cells from oxidative stress induced by hydrogen peroxide (25). In conclusion, I report for the first time a novel mechanism of antibiotic resistance, based on physical binding of antibiotics, that is conserved among large number of bacteria whether Gram-positive or Gram-negative and that can non-specifically protect various clinically relevant pathogens from the action of antibiotics. These findings offer a new avenue for intervention against antibiotic resistance and its spread among different bacteria by developing inhibitors against bacteriocalins.

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Chapter 5

General Discussion

5.1. General Overview

At the present time, existing pipelines of novel antibiotic drug development are insufficient to bridge the widening gap that is inherent in the global dissemination of multi-drug resistant bacteria versus the effectiveness of available antibiotic therapy to treat microbial infections. This dilemma is further complicated by confusion regarding a closely related phenomenon, namely heteroresistance, which would complicate the overall therapeutic outcome. Despite the recognition of this phenomenon since 1947, the field is plagued with misconceptions and confusion about heteroresistance. In this thesis, I aimed at providing better characterization of heteroresistance and one of its major implications, which is the possibility of chemical communication of antibiotic resistance.

5.2. Heteroresistance: the current understanding

The term ‘heteroresistance’ is sometimes used indiscriminately to describe other observations not related to population-wide response to antibiotics. Moreover, no clear definition or globally standardized methods to determine heteroresistance are available. The lack of standardization of test formats and the guidelines to decide heteroresistance led to the lack of agreement between outcomes of different methods and between different laboratories (1-3). Various studies showed that heteroresistance could have serious implications in therapy of microbial infections as discussed in Chapter 1. Hence, the harmonization and standardization of definitions and methods to describe heteroresistance is of utmost importance.

Based on my study (Chapter 2) and upon extensive and critical review of the available literature (Chapter 1), I recommend defining heteroresistance as the population-wide variation of antibiotic resistance whereby different subpopulations within an isolate exhibit varying susceptibilities to a particular antimicrobial agent. With respect to the methods of detection, PAP remains the gold standard for detection of heteroresistance preferably by cfu counts, while turbidimetric PAP is an acceptable alternative, provided that the antibiotic increments are set at 2-fold. A strain can be considered heteroresistant when the lowest antibiotic concentration exhibiting maximum inhibition of the bacterial population is equal to or greater than 8-fold

higher than the highest non-inhibitory concentration. A 4-fold difference may be regarded as intermediate heterogeneity while a lower difference indicates homogenous response to an antibiotic. An alternative to PAP would be disc diffusion or Etest assays, whereby the growth of discrete colonies at the otherwise clear zone of inhibition is indicative of heteroresistance. This method can be used for faster screening of clinical isolates rather than using the laborious PAP assays. With standard criteria to define and assess heteroresistance world-wide, the prevalence of heteroresistant bacteria as well as their clinical relevance and impact on healthcare can be better assessed. Consequently, effective therapeutic strategies should be explored to counteract heteroresistance. This may include testing synergistic combinations of antibiotics (4), or using antibiotic adjuvants inhibiting key pathways involved in antibiotic resistance in conjunction with front-line antibiotics (5).

On the other hand, elucidation of the mechanisms of heteroresistance when this phenomenon is properly defined will help understand whether a common mechanism exists among the different bacteria and against the different antibiotics or these mechanisms are antibiotic-specific, species-specific or both. In *B. cenocepacia*, heteroresistance to polymyxin B depends on putrescine and YceI secretion, being differentially expressed across the different subpopulations as shown in Chapter 2. Moreover, a periplasmic component of an ABC transporter involved in the biosynthesis of hopanoids was overexpressed in the more resistant subpopulation exposed to polymyxin B. While the role of this transporter in heteroresistance was not directly evaluated, hopanoids have been shown to contribute to polymyxin B resistance in *B. cenocepacia* (6). Putrescine and YceI were similarly implicated in heteroresistance to rifampicin, norfloxacin and ceftazidime to various extents but not gentamicin. Identifying the mechanisms of heteroresistance would potentially aid in finding new targets for disruption of this phenomenon, thus reducing the window of therapeutic failure.

It is also essential to determine thoroughly whether heteroresistance is only restricted to the response to bactericidal antibiotics, or bacteria can display heteroresistance to bacteriostatic antibiotics as well. No systematic comparisons of the response of heteroresistant bacteria to bacteriostatic versus bactericidal antibiotics have been reported. Here I showed that *B. cenocepacia* exhibited heteroresistance to bactericidal antibiotics from different classes and homogenous responses to bacteriostatic antibiotics.

Interestingly, the same strain can display both intrinsic and acquired heteroresistance. Here I showed that *B. cenocepacia* wild type and several less resistant isogenic mutants, including Δ*hldA*, displayed intrinsic heteroresistance to several bactericidal antibiotics including polymyxin B (5). Previously, heteroresistance was induced in the same *hldA* mutant possessing truncated LPS where it developed subpopulations resistant to higher concentrations of the antibiotic that are not tolerated by even the most resistant members of the original population after exposure to multiple rounds of selection in polymyxin B (7).

In conclusion, global organizations concerned with antimicrobial resistance are urged to advocate for harmonized recommendations and coordinate general consensus concerning the phenomenon of heteroresistance. This is of utmost importance especially in clinical practice where currently thousands of clinical isolates are screened for heteroresistance, but rather using non-standardized methods that differ from one laboratory to another, further puzzling the global picture. Such efforts can be better directed to more accurate and standardized detection of heteroresistance, leading to superior therapeutic outcomes based on improved identification of heteroresistant bacteria and optimized strategies to eradicate them.

5.3. Chemical communication of antibiotic resistance

Highly resistant subpopulations of heteroresistant bacteria could further complicate the clinical picture of polymicrobial infections by providing protection to normally sensitive bacteria through chemical signals. Here I showed in Chapter 2 an example of such chemical communication of resistance in which *P. aeruginosa* was protected from the action of the antimicrobial peptide polymyxin B by highly resistant subpopulation of the heteroresistant pathogen *B. cenocepacia*. Simultaneous infection of both organisms is not uncommon; cystic fibrosis patients are among the groups having high potential of contracting such polymicrobial infection (8). This provides high potential for clinical relevance of such interaction between microorganisms given this particular example. This chemical communication of antibiotic resistance was mediated by the release of excessive amounts of the polyamine putrescine, and YceI, a small conserved protein with a lipocalin fold, from *B. cenocepacia* and resulted in *P.*

aeruginosa surviving polymyxin B treatment equivalent to the recommended therapeutic breakpoint instead of being completely killed in its pure culture. This was shown in an *in vitro* direct co-culture experiment and awaits further *in vivo* confirmation. In chapter 4, I showed an *in vivo* evidence of protection of *P. aeruginosa* PAO1 by the bacteriocalin BCAL3311 in *G. mellonella* larvae infection model. On the other hand, putrescine and other polyamines in genital mucosal fluids increased the resistance of *N. gonorrhoeae* to antimicrobial peptides (PmB and LL-37), possibly enhancing its survival during infection by reducing bacterial susceptibility to host-derived antimicrobials (9). Together, this supports the clinical relevance of putrescine and bacteriocalins as communicators of antibiotic resistance.

Putrescine protected the surface of the bacteria from the initial binding of polymyxin B [Chapter 2 and (5)] and reduced antibiotic-induced oxidative stress [Chapter 3 and (10)]; however, this does not preclude other mechanisms by which putrescine modulate the antibiotic response (11). On the other hand, YceI could bind and sequester polymyxin B and other hydrophobic antibiotics thus potentially reducing their levels in the bacterial milieu [Chapters 2 and 4, and (5)]. Given that the mechanism of such chemical communication could be universal among bacteria; i.e. the signals involved could be sensed and benefited from by almost all bacteria, extra caution should be in effect while dealing with heteroresistant *B. cenocepacia* infections (or by other bacteria displaying similar phenotype of extreme antibiotic resistance) in particular when associated with other bacteria in mixed infection.

5.4. New targets for drug discovery

Unveiling these mechanisms contributing to intrinsic antibiotic resistance and communication of resistance among bacteria provides novel targets for therapeutic interventions.

A promising avenue for potential synergists is inhibition of biosynthesis of polyamines, putrescine in particular. In this study (Chapter 3), I identified the ornithine decarboxylase BCAL2641 as a critical player in response to antibiotics despite the presence of other putrescine synthesizing enzymes (BCAM1111 and BCAM1112), which seemed to have other physiological functions not related to antibiotic resistance. Therefore, these findings suggest BCAL2641 as a

plausible target for inhibitors that would potentiate the effects of antibiotics. Interestingly, certain inhibitors of key enzymes in the biosynthetic pathway of polyamines, such as ornithine decarboxylase and spermidine synthase, are available (12-15). Such inhibitors have been effective in the treatment of parasitic infections such as different types of trypanosomiases (16, 17). The uptake of putrescine and other polyamines may present another potential target for inhibition potentially blocking the chemical communication among bacteria. Certain anthracene- and benzene-polyamine conjugates that inhibit polyamine transport were also effective for treatment for *Pneumocystis* pneumonia (18). Together, the identification of specific bacterial targets such as BCAL2641 in addition to the clinical implementation of interfering with polyamine synthesis and uptake in parasitic infections supports the feasibility of this approach in bacterial infections.

Similarly, bacteriocalins can provide another plausible target for inhibition. One strategy for inhibition is to design or search for molecules capable of binding to bacteriocalins with higher affinity than antibiotics thus preventing sequestration of antibiotics. Overall, this direction for drug discovery could potentially expand further as our understanding of the mechanisms, biosynthetic pathways and uptake of the different small molecules continues to increase.

5.5. Ongoing and future research

Indeed this study revealed novel observations in terms of the heteroresistance of *B. cenocepacia* to different bactericidal antibiotics as well as the chemical communication of antibiotic resistance and the signals involved in this interaction. However, much remains to be learned about antimicrobial heteroresistance and new avenues for drug development has been generated.

Thorough investigation of the mechanisms of heteroresistance in *B. cenocepacia* is still required. Although I have showed the implication of putrescine and bacteriocalins in this phenomenon through differential expression among the different bacterial subpopulations; however, the stability of the high level of resistance in the more resistant subpopulation implies the presence of underlying mutations contributing to such level of antibiotic resistance. Whole genome sequencing of the more resistant subpopulations is currently underway. This would

increase our understanding of the mechanisms of heteroresistance in *B. cenocepacia* and would potentially identify novel mechanisms of antibiotic resistance in general.

To better exploit our knowledge of chemical communication of antibiotic resistance, better understanding of the mechanisms involved in the mediator infochemicals is essential. For example, while surface competition and protection from ROS has been revealed as mechanisms of protection by putrescine from the action of various antibiotics, other mechanisms of protection by putrescine needs to be identified as well. Two-dimensional gel electrophoresis is currently optimized to compare between the response of wild type and ΔBCAL2641 to antibiotics in a non-biased manner. On the other hand, since BCAL2641 was proven to be central in the putrescine-mediated response to antibiotics, it is desirable to explore its regulatory pathways, in particular those involved in its stimulation in the presence of antibiotics. This will be pursued through creating transposon libraries. Regulators important in such antibiotic response could serve as targets for inhibitors as well. Equally important, understanding the affinity of BCAL2641 to the available inhibitors of polyamines biosynthesis would aid in the rational drug design of novel specific inhibitors against this ornithine decarboxylase enzyme.

With respect to bacteriocalins, mapping of the binding sites through *in silico* binding modeling is underway. This can be validated by site-directed mutagenesis and *in vitro* binding assays. This could be coupled with determination of the 3D structure of bacteriocalins by NMR. This would ultimately aid in identifying the favorable parameters for effective inhibitors for bacteriocalins.

5.6. Significance and Concluding Remarks

Using *B. cenocepacia* as a model opportunistic bacterium, I reported heteroresistance against PmB and other bactericidal antibiotics. Population analysis profiling identified *B. cenocepacia* subpopulations arising from a seemingly homogenous culture that are resistant to higher levels of PmB than the rest of the cells in the culture, and can protect the more sensitive cells from killing, as well as sensitive bacteria from other species, such as *P. aeruginosa* and *E. coli*. Communication of resistance depended on upregulation of putrescine synthesis and YceI, a

widely conserved low-molecular weight secreted bacterial lipocalin (bacteriocalin). Polyamines and bacteriocalins were also required for heteroresistance of *B. cenocepacia* to various bactericidal antibiotics. This work proposes that putrescine and bacteriocalins resemble "danger" infochemicals whose increased production by a bacterial subpopulation, becoming more resistant to bactericidal antibiotics, communicates higher level of resistance to more sensitive members of the population of the same or different species.

Putrescine protects less resistant cells from PmB partly due to its ability to compete with PmB for binding to the surface of bacteria. In addition, it protects against oxidative stress induced by PmB and other antibiotics. On the other hand, I report for the first time a new mechanism of antibiotic resistance, based on physical binding of antibiotics, mediated by bacteriocalins. Shown in *B. cenocepacia*, *M. tuberculosis*, *P. aeruginosa*, and *S. aureus*, this mechanism can be conserved among a large number of bacteria that are predicted to possess bacteriocalins as well. Furthermore, bacteriocalins can non-specifically protect various clinically relevant pathogens from the action of antibiotics.

In conclusion, the findings of this thesis uncover a novel, non-genetic and cooperative mechanism of transient increase in resistance that can be chemically communicated from more resistant members of a heterogeneous population to less resistant bacterial cells of the same or other species. This multifactorial mechanism of communication of antibiotic resistance is distinct from previously reported population-based resistance, hence expanding our knowledge on intrinsic antibiotic resistance mechanisms and offering novel targets for antimicrobial intervention.

5.7. Chapter 5 References

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Appendix A

Characterization of regulators of polymyxin B resistance in *B. cenocepacia*

A.1. Background and rationale

Pathogens respond to various insults in the host environment for infection to be successful. Integration of the signals generated into coherent messages, which the organism can counter with both transcriptional and post-translational responses is paramount. These responses involve several global regulatory systems. This also applies to survival of pathogens in the environment (1). Several envelope stress response systems have been characterized in other Gram-negative bacteria such as *Escherichia coli*, and investigated individually and globally (2-6); however, little is known about the regulation of extracytoplasmic stress response pathways in *B. cenocepacia*. Thus, it is important to study the regulation mechanisms, which would enhance our understanding of the extreme resistance of *B. cenocepacia* to APs.

Outer membrane permeability is controlled in part by the master regulator of the extracytoplasmic stress responses, RpoE (7). RpoE has a similar role in *E. coli* in which many of the genes identified in its regulon are involved in membrane biogenesis or repair, protein folding or degradation, and they include *rpoE* itself along with its regulatory proteins (8). It has been shown that RpoE is required in *B. cenocepacia* for PmB resistance at 37^{o}C (9) but not at 30^{o}C (7). The MIC_{50} of Polymyxin B (PmB) at 37^{o}C for the Δ*rpoE* mutant is 64 µg/ml versus more than 1024 µg/ml for the wild type strain (9). Therefore, characterization of the RpoE regulon in *B. cenocepacia* is essential to understand its contribution in resistance to APs.

A relatively more recently identified two component regulatory system (BCAL2831/BCAL2830) (10) has been shown to contribute to resistance of *B. cenocepacia* to PmB (9). This two-component system might correspond to the PmrA/PmrB system in *Pseudomonas aeruginosa*. Indeed, the gene encoding the putative response regulator, *BCAL2831* encoded a protein that exhibited a certain level of similarity (53% identity) at the primary amino acid sequence level with PmrA of *P. aeruginosa* (10). Previous reports have shown that the *P. aeruginosa* PmrA/PmrB system regulates resistance to APs in part by modifications of LPS, mainly through the addition of 4-L-aminoarabinose (Ara4N) (11, 12). Interestingly, the *pmrA-pmrB* operon is activated by a number of cationic peptides (11). In striking contrast to other bacteria, the Ara4N modification of LPS in *B. cenocepacia* is essential for viability (13). This could imply that the regulation of this pathway would be different in *B. cenocepacia* than other

bacteria in which it is non-essential. Thus, I investigated whether the BCAL2831/BCAL2830 system is involved in the regulation of the modification of lipid A or not.

A.2. Characterization of the RpoE regulon in *B. cenocepacia*

Initially, I performed a bioinformatic analysis to identify genes potentially regulated by RpoE in *B. cenocepacia* by analogy to the previously characterized RpoE regulon of *Escherichia coli* K12 (3). Some predictions were tested by comparing the gene expression in wild type K56-2 and the Δ*rpoE* mutant SAL65 using real-time PCR upon subjecting the cultures to PmB stress.

The bioinformatic analysis performed by analogy to the RpoE regulon in *E. coli* K12 resulted in a list of ~ 60 genes potentially regulated by RpoE in *B. cenocepacia* J2315. Selected genes from these predictions were tested by comparing the gene expression in wild type *B. cenocepacia* K56-2, which is clonally related to J2315 and proven to be more amenable to genetic manipulation (14), and the Δ*rpoE* mutant SAL65 using real-time PCR. The preliminary results show that compared to the RpoE-regulated operons in *E. coli*, RpoE regulates some of them in *B. cenocepacia* while others are not. The differential expression of genes between wild type and SAL65 was different in the case of 30^{o}C and 37^{o}C (Table 10), which could explain the different patterns of resistance to PmB at those temperatures (7, 9). Among the tested genes, most of those that are significantly regulated by RpoE in *B. cenocepacia* at 37^{o}C are genes encoding periplasmic proteases [*BCAM1695* and *BCAL0326*], enzymes involved in lipid A synthesis [genes in the same operon with *rseP* as *lpxABD* genes], LPS transport and assembly [*lptA* and *bamA* present in the same operon with *rseP*], in addition to the *rpoE* operon itself [*rseA*] and its regulatory machinery [*rseP*]. However, previous studies showed that the periplasmic proteases are not involved in resistance to PmB mediated by RpoE (9). Furthermore, I have tested other genes of interest as those involved in Ara4N modification [*arnT* and *arnB*] and phosphoethanolamine binding [*peb*]; however, none of them was regulated by RpoE (Table 10). In the previous experiment, the cultures were exposed to PmB for 15 minutes prior to RNA extraction. However, on prolonging this period to 30 minutes, I could see differences in the

Table 10. Differential expression of selected genes in wild type K56-2 versus the Δ*rpoE* mutant at 30°C and 37°C subjected to 1 mg/ml PmB for 15 min determined by qPCR.

Gene ID	Gene name	Function	Fold change normalized to *BCAS0175*	
			37°C	30°C
BCAL0999	*rseA*	Anti-RpoE sigma factor	**13.87**	**1.69**
BCAL2084	*rseP*	Inner membrane zinc metalloprotease; activates RpoE by degrading RseA	**7.37**	**1.01**
BCAM1695	*degQ/degP*	Periplasmic, membrane-associated serine endoproteases	**10.5**	**2.8**
BCAL0326	*degQ/htrA*		**10.2**	**-2.2**
BCAL2829	*degP/htrA*		**2.56**	**2.17**
BCAL0634		Putative lipoprotein	**-2.3**	**-18.4**
BCAL1881	*yfgL*	Putative OM assembly lipoprotein	**-2.38**	**2.14**
BCAL3091	*uppP/bacA*	undecaprenyl pyrophosphate phosphatase	**-2.17**	**-1.81**
BCAL0163	*yraP*	Putative phospholipid-binding lipoprotein	**2.14**	**-1.93**
BCAL0815	*lptA*	LPS transport periplasmic protein	**3.93**	**3.21**
BCAL0203	*Peb*	Phosphatidylethanolamine-binding protein	**-2.16**	**-6.37**
BCAL1929	*arnT*	putative undecaprenyl phosphate-α-4-amino-4-deoxy-l-arabinose arabinosytransferase	**1.67**	
BCAL1931	*arnB*	putative UDP-4-amino-4-deoxy-l-arabinose-oxoglutarate aminotransferase	**1.24**	
BCAL0508	*lpxL*	lipid A biosynthesis lauroyl acyltransferase	**1.49**	
BCAL1459		Calcineurin-like phosphoesterase	**-2.83**	**-3.32**
BCAL1659		Ribose transport permease	**-17.3**	**-2.08**
BCAL2694		Putative dehydrogenase	**-2.08**	**-1.38**
BCAL1861	*phbB*	Acetyl-CoA reductase	**-4.23**	**1.26**

Table 11. Differential expression of selected genes in wild type K56-2 versus the Δ*rpoE* mutant at 37°C subjected to 1 mg/ml PmB for either 15 or 30 min determined by qPCR.

Gene	Fold Change normalized to *BCAS0175*	
	Duration of PmB Stress	
	15 min	30 min
BCAL1929 (arnT)	**1.678**	**2.08**
BCAL1931 (arnB)	**1.240**	**6.01**
BCAL0203 (peb)	**-2.158**	**1.35**
BCAL2829 (degP/htrA)	**2.563**	**-4.63**
BCAL0999 (rseA)	**13.875**	**7.16**

differential expression profiles of most of the tested genes to variable extents (Table 11). This is consistent with previous studies performed on *rpoE* in *E. coli* that showed that different genes in the regulon were upregulated at different time points along the course of stress (3). It was interesting to see that prolonging the PmB stress showed that the *arnB* gene involved in the Ara4N modification pathway was upregulated in the wild type compared to SAL65. This points out that characterizing the RpoE regulon at a single time point might be misleading as we observed in the subset of genes tested after two durations of stress.

To further investigate this, the differential expression of genes as a function of the duration of stress and the optimal stress conditions for expression of *rpoE* should be tested.This can be tested in a strain with *luxCDABE* reporter genes downstream of the promoter of the *rpoE* operon by monitoring the level of expression by determining the luciferase activity as a function of stress condition and time. Different variables should be tested such as concentration of PmB and duration of exposure to it under different conditions of culture (in nutrient rich or minimal media). Also, other conditions may be tested such as the effect of heat shock, osmotic stress or a combination of different stresses; RpoE is required by *B. cenocepacia* for survival under those stressful conditions (7). Next, whole transcriptome sequencing using mRNA-enriched RNA samples extracted from K56-2 and SAL65 after exposure to the previously determined optimal stress condition for expression of *rpoE* should be performed followed by validation of selected genes by qRT-PCR.

A.3. Study of the BCAL2831/BCAL2830 two-component system.

Due to its similarity to PmrAB system, I expected that the BCAL2831/BCAL2830 regulatory system might regulate LPS modification pathways such as those involving the addition of either Ara4N or phosphoethanolamine. It has been reported that the disruption of this system reduces the MIC_{50} of PmB by about 4 fold (9). I speculated that this low contribution to resistance to PmB may be due to the fact that a proportion of the OM in *B. cenocepacia* is already constitutively decorated with Ara4N, which is essential for viability (13), or that the

disruption of this regulatory system might stimulate RpoE thus compensating for the reduced resistance to APs.

I first tested the susceptibility to PmB in the medium 121 (15) that is reported to induce LPS modifications in *E. coli* (16). This was performed in deep rough mutants due to their higher susceptibility to PmB (17). Deep rough mutants lack O-antigen as a result of the deletion of the *hldA* gene which encodes a heptokinase involved in the modification of heptose sugars prior to their incorporation into the LPS core oligosaccharide (17). I compared the susceptibility to PmB of the deep rough mutant to that of other mutants with further disruptions in *rpoE* or *BCAL2831* genes (Table 12). It may be expected that if a system regulates the modification of LPS, the mutant with its functional gene will have increased MIC values in the medium 121 relative to LB medium, as opposed to the case in the mutant with disrupted gene of this regulator. Growth of the tested mutants was retarded in the medium 121, which was reflected in their lower MIC_{50} values. The relative susceptibilities to PmB of the different mutants were the same under the different conditions, suggesting no significant modifications in LPS profile. This might indicate that neither RpoE nor the two-component system under study regulates LPS modifications, or that the medium 121 does not have LPS modifying properties in *B. cenocepacia* as those exerted in *E. coli*.

Then I compared the expression of selected genes in wild type *B. cenocepacia* K56-2 and RSF29 by qRT-PCR after subjecting them to 1 mg/ml PmB for 15 minutes. However, under the tested condition, this system did not affect the expression of *arnB* or *arnT* involved in Ara4N modification, *peb* involved in phosphoethanolamine modification, *rseA* present in the *rpoE* operon, or even the *htrA BCAL2829* present in its own operon (Table 13). However, other conditions of stress should be tested before making a definitive conclusion on its role in resistance to PmB.

A.4. Significance

The significance of these studies is that they will advance our understanding of the resistance of *B. cenocepacia* to APs in terms of its genetic basis and regulation and will provide insights for enhanced treatment of this pathogen's severe infections.

Table 12. MIC_{50} of PmB against different *B. cenocepacia* mutants in different culture media.

Strain	Medium	MIC_{50}, μg/ml	
		24 hr	48 hr
	LB	128	256
RSF34	121 LP	<4	8
	121 XP	8	32
	LB	16	64
SAL47	121 LP	<4	≤4
	121 XP	<4	8
	LB	64	128
SAL55	121 LP	<4	4
	121 XP	<4	16

Note:

RSF34: K56-2, Δ*hldA*
SAL47: RSF34, with disrupted *rpoE*
SAL55: RSF34, with disrupted *BCAL2831*
LP: Limiting phosphate conditions (K_2HPO_4: 8.3 x $l0^{-5}$M)
XP: excess phosphate conditions (K_2HPO_4: 8.3 x $l0^{-4}$M)

Table 13. Differential expression of selected genes in wild type K56-2 versus mutant with disrupted BCAL2831, RSF29, at 37°C subjected to 1 mg/ml PmB for 15 min determined by qPCR.

Gene	Fold Change normalized to *BCAS0175*
BCAL1929 (arnT)	**1.69**
BCAL1931 (arnB)	**-1.05**
BCAL0203 (peb)	**-1.17**
BCAL2829 (degP/htrA)	**1.18**
BCAL0999 (rseA)	**-1.15**

Note:
RSF29: K56-2, with disrupted *BCAL2831*

A.5. Appendix A References

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Appendix B

Search for synergistic inhibitors potentiating the action of Polymyxin B against *B. cenocepacia*

The concept of using helper compounds that could inhibit certain features of pathogenic bacteria has been investigated as an appealing approach to reverse resistance of bacteria to antibiotics (1-3). Such attempts targeted the membrane permeability of bacteria to enhance penetration of antibiotics (1) or the inhibition of efflux pumps (2). Another target for potential inhibitors is extracellular signaling where interfering with it would prevent the release of virulence factors, the formation of biofilms or increased antibiotic resistance (4). Such treatments targeting signaling systems neither arrest cellular division directly nor are they toxic to the cells, which means the selective pressure to evolve mechanisms of resistance is likely to be substantially reduced. In addition, targeting small-molecule signaling pathways ensures that treatments will be directed specifically at the pathogenic organism, rather than the entire microbiome (4). In the largest sense, the helper compounds or antimicrobial adjuvants inhibit a bacterial resistance mechanism to an antibiotic, thus rendering the bacterium susceptible to that antibiotic (5).

In this study, I attempted to identify inhibitors of the outer membrane (OM) barrier effect, which would permeabilize the cells to APs thus reducing resistance towards them and increasing their antimicrobial effectiveness. This would provide a therapeutic solution to the extremely high level of intrinsic resistance of *B. cenocepacia* to APs.

The library consisted of 448 compounds derived from Sea life (obtained from Instituto Biomar, Leon, Spain). Solutions of these compounds were seeded in pairs of 100-well Bioscreen C plates and the solvent was evaporated. Overnight cultures of the wild type *B. cenocepacia* K56-2 diluted to OD_{600} 0.0002 in fresh LB medium were added to the plates (300 µl/well) dissolving the compounds at final concentration of 1 µg/ml. Plate pairs were incubated in the Bioscreen C automated growth curve analyzer at 37^{o}C with continuous medium shaking. After two hours, polymyxin B was added to one plate at a final concentration of 500 µg/ml and the vehicle control in which polymyxin B is dissolved (0.2% bovine serum albumin, 0.01% acetic acid) was added to the other plate. Wells that received no compounds were also included in each plate of the pairs. The plates were further incubated at 37^{o}C and OD_{600} was read every 30 minutes. This experimental design allows the identification of compounds that have direct

antibiotic activity against *B. cenocepacia* as well as those that synergize the antimicrobial activity of polymyxin B.

From this preliminary screening of compound library, 4 compounds were effective in potentiating the antimicrobial activity of PmB against the wild type K56-2 (Figure 40). These compounds alone did not possess any toxic effects on the cells at the tested concentration (1 µg/ml); this does not preclude that they may possess direct antibiotic activity at higher concentrations. These compounds were thielavin B, micacocidin B, and other 2 new compounds; the chemical structure of one of them is yet to be determined.

Thielavin B (Figure 41A) is a Prostaglandin synthesis inhibitor produced by the fungus *Thielavia terricola* (6); it inhibits glucose-6-phosphatase (G6Pase) (7), telomerase and viral reverse transcriptase (8), and interferes with cell wall transglycosylation in *Enterococcus faecalis* (9). Micacocidin B (Figure 41B) is a Copper-containing compound produced by *Pseudomonas* species previously shown to exhibit potent activity against various *Mycoplasma* species (10). Notably, 3 other compounds with similar structure but not in complex with metal were included in the compound library; however, these did not have any synergistic effects with PmB against *B. cenocepacia*. Interestingly, derivatives of Micacocidin have recently been synthesized with activity against *Mycoplasma pneumoniae* (11). The third compound was a novel cyclic peptide coded CL0231 (Figure 41C). Interestingly, a similar compound (CL0236, Figure 41D) with slight side chain modification did not have any synergistic effects with PmB. The fourth synergistic compound was also novel and its chemical structure is yet to be determined.

Follow up experiments were not possible due to inability of extraction of higher concentrations of these compounds from their marine sources. Ideally, confirming the results of the primary screening assays followed by determination of the optimal dose ratio for the combinations of these compounds with PmB would have been the next steps. However, these results offer a proof of concept for the screening of synergists for the activity of PmB. In addition, the positive hits may be worth pursing in more details in the future by fermentation from their producer organisms or their expression from an expression system for further

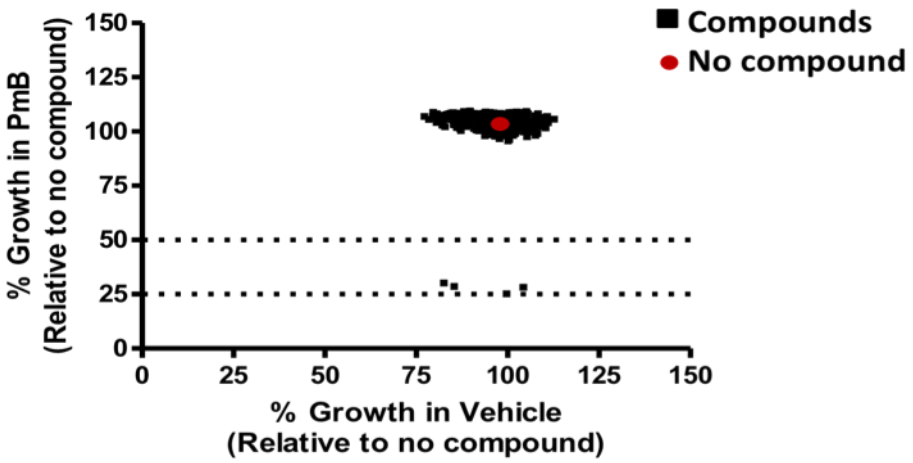

Figure 40. Screening of a library of ~450 compounds for synergists of the antimicrobial activity of PmB against *B. cenocepacia* K56-2 at 20 h.

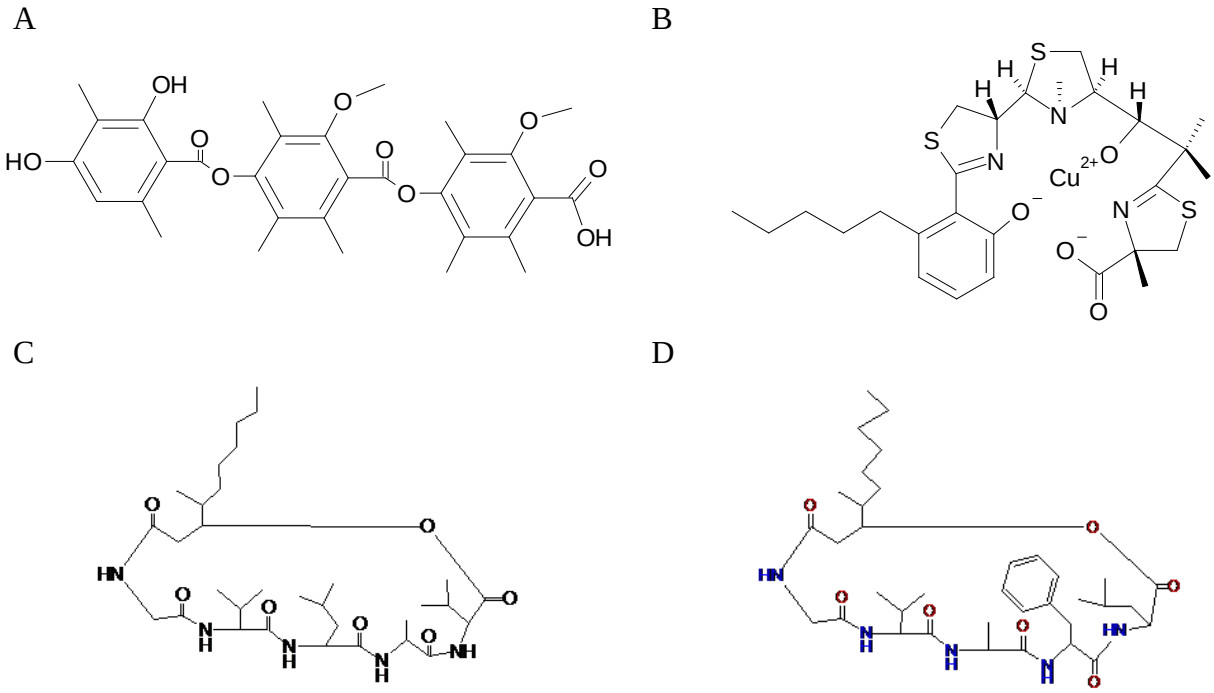

Figure 41.Chemical structures of compounds from the screened library of compounds.

A, Thielavin B. B, Micacocidin B. C, CL0231. D, CL0236.

evaluation of their antimicrobial activity. These results encourages screening larger libraries of compounds for synergists. On the other hand, future studies should aim at screening for inhibitors targeting a specific pathway or signal known to be involved in antibiotic resistance such as putrescine biosynthesis or bacteriocalins as previously discussed in the different chapters of the thesis.

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Appendix C

Characterization of the more resistant subpopulation of *B. cenocepacia* Δ*rpoE*/500

Heteroresistance complicates the problem of antibiotic resistance and hence may pose serious problems in microbial infections. Hence, determination of the mechanism of heteroresistance is required. Characterizing the more resistant subpopulation in terms of its phenotypes in response to PmB is the first step towards understanding the mechanism of heteroresistance.

For practical reasons, I used for these experiments a subpopulation isolated at 500 µg/ml in the Δ*rpoE* mutant, which displayed a stable higher level of resistance to PmB as indicated in their individual PAP (details in Chapter 2), as it was difficult to isolate the more resistant subpopulations of the parental strain K56-2 due to its extreme resistance to PmB. As discussed in Chapter 2, no differences in the secreted extracellular proteases, or LPS profiles between the Δ*rpoE*/500 resistant subpopulation, its naïve population and the wild type K56-2 (details in Chapter 2). On the other hand, experiments of microbial adherence to *n*-hexadecane (1) revealed that the Δ*rpoE*/500 subpopulation was more hydrophilic than the naïve bacteria (Figure 42A), suggesting cell surface changes in the more resistant subpopulation.

Exposure of the wild type K56-2 and Δ*rpoE*/500 to PmB led to reduction in their swimming motility and ability to form biofilm [determined as previously described in (2) and (3) respectively; Figure 42B and C respectively]. This agrees with previous findings showing that genes encoding proteins required for building and operating flagella are downregulated in *B. cenocepacia* following exposure to PmB (2), which would lead to reduction in motility. This reduced motility might, in turn, be responsible of the reduced biofilm formation, since motility and the flagellar apparatus are required in the initial steps of biofilm establishment (4). On the other hand, only Δ*rpoE*/500 treated with PmB exhibited reduced metabolic activity at 24 h relative to its naïve population and the wild type K56-2, whether treated with PmB or not, in a resazurin metabolic assay (details in Chapter 2). Thus, the increased resistance displayed by the more resistant subpopulation comes at a fitness cost.

While these phenotypic characterizations provide insights about the properties of the more resistant subpopulation, detailed analysis of this subpopulation is required to understand the mechanism of its increased resistance to PmB. Therefore, whole-genome sequencing of this subpopulation in comparison with its naïve population is underway. The significance of this study is that it would advance the understanding of the mechanism of heteroresistance providing

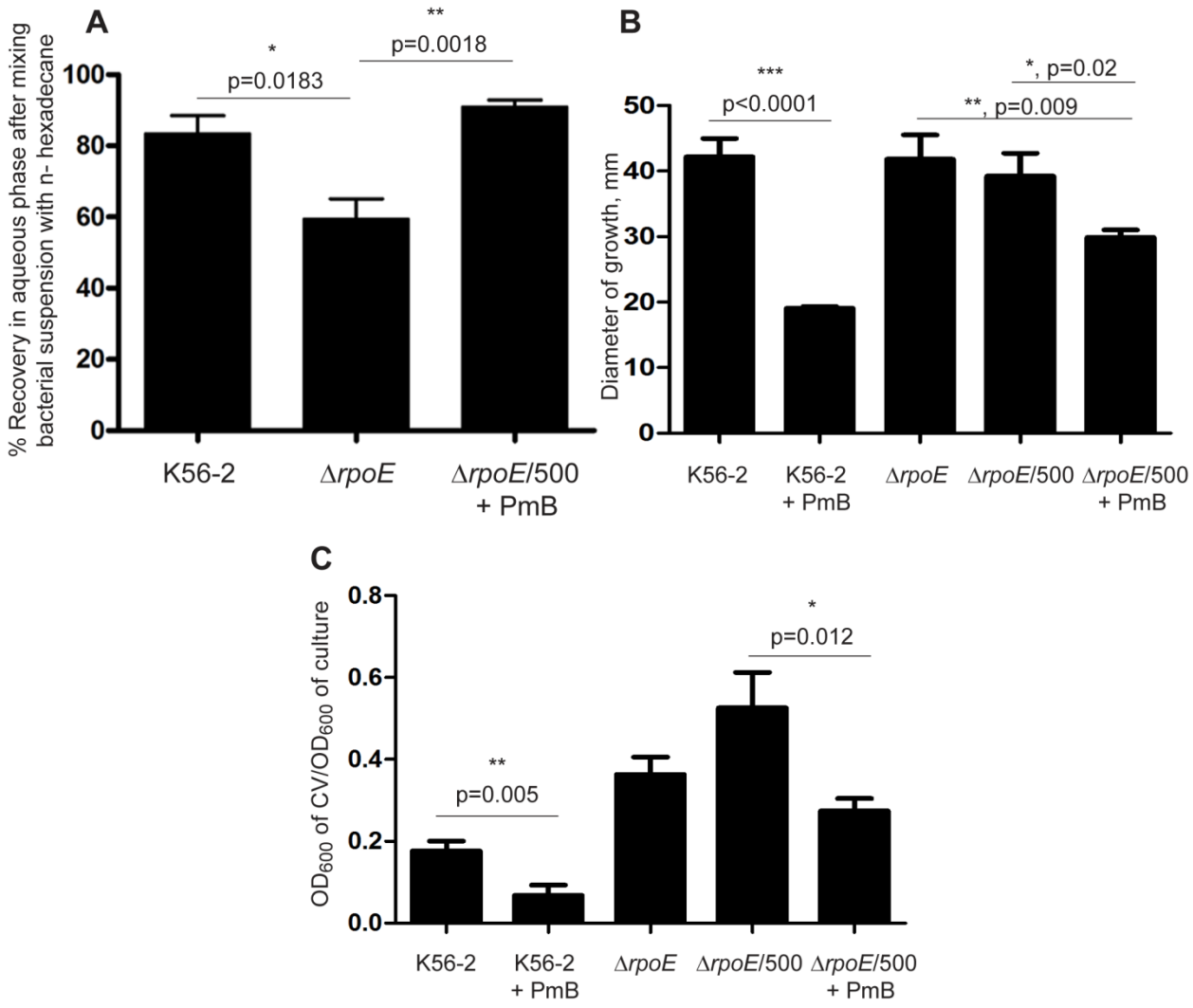

Figure 42.Characterization of the more resistant subpopulation Δ*rpoE*/500.

A. Cell-surface hydrophobicity; B. Motility assay; and C. Biofilm assay.

CV: Crystal violet.

targets for therapeutic intervention to disrupt such phenomenon, hence reducing the window for therapeutic failures.

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Appendix D

Characterization of other mechanisms of Putrescine-mediated protection from the antimicrobial activity of Polymyxin B by 2-dimensional gel electrophoresis

In Chapters 2 and 3, I have shown that putrescine protects from the antimicrobial activity of PmB through two distinct mechanisms; protection of the bacterial surface from the initial binding of PmB and reduction of PmB-induced oxidative stress. Indeed, these mechanisms of protection against PmB do not preclude other mechanisms by which putrescine modulate the response towards antibiotics and hence acting as a chemical signal communicating antibiotic resistance. To further investigate the role of putrescine in the protection against the effects of PmB, I initiated an unbiased proteomic approach. I compared the total cell lysate of cells treated with PmB (500 µg/ml), putrescine (50 mM) or both, relative to control untreated cells using 2-dimensional gel electrophoresis. Preliminary experiments were performed using 7 cm immobiline dry strips of pH range 3-10; following the first dimension of isoelectric focusing (Ettan IPGphor II, Amersham), the strips were run on 12% SDS-PAGE and stained with Sypro Ruby stain. The gels were visualized and scanned using ProXPRESS 2D Proteomics Imaging System and analyzed using Progensis SameSpots Software. Six strips were used per condition. This revealed a total of 27 differentially expressed protein spots among the different tested conditions (Figure 43). However, the amount of protein in these spots was not sufficient to be visualized by the Page Blue secondary stain and the subsequent Mass Spectrometric identification; also the resolution of the proteins at certain areas of the gel needed to be improved. Therefore, the next step is to use 13 cm strips, in which the total amount of proteins loaded can be increased (200 µg of proteins/strip instead of 75 µg) and the separation will be improved in both dimensions, to compare total cell lysates of PmB treated wild type and ornithine decarboxylase BCAL2641 mutants.

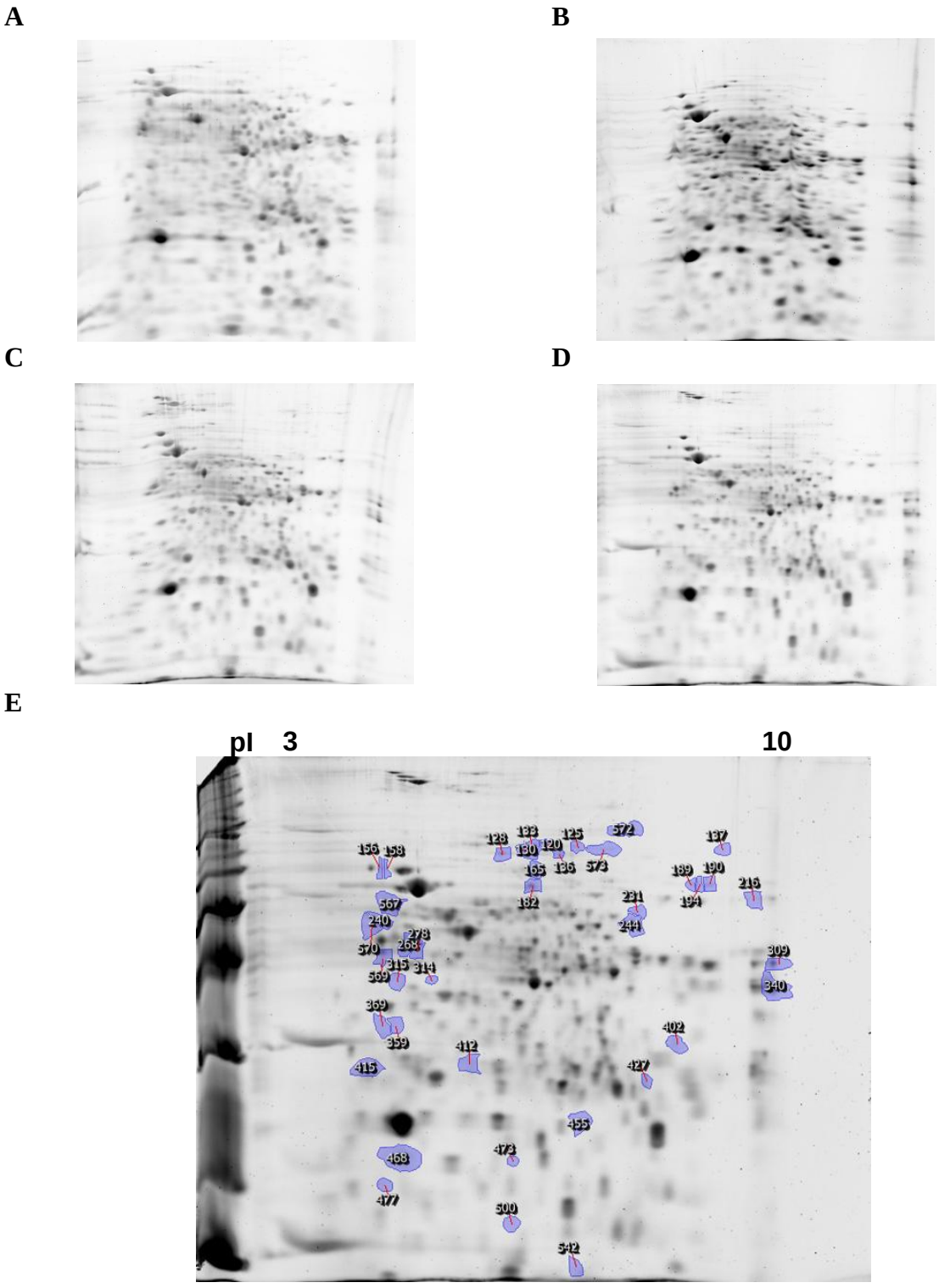


Figure 43. Two-dimensional gel electrophoresis investigation of the mechanism of putrescine to protect against PmB.

A representative gel is shown for total cell lysates obtained from the different conditions: A, Control untreated cells; B, Putrescine-treated cells; C, PmB-treated cells; and D, both PmB and Putrescine-treated cells. Panel E shows a representative gel with locations of differentially expressed protein spots under the different conditions.

Appendix E

Expression profiles of gene clusters (BCAL3309 and BCAL3313) adjacent to *B. cenocepacia* bacteriocalins clusters in response to antibiotics

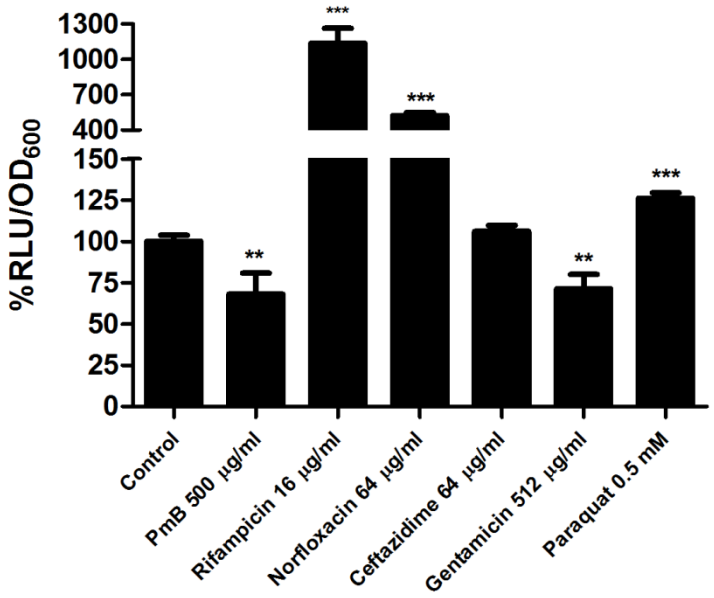
Parallel to the evaluation of the expression of the transcriptional units of *B. cenocepacia* bacteriocalins (Chapter 4), similar promoter-*luxCDABE* reporter constructs were prepared in K56-2 *B. cenocepacia* (details of construction of mutants are in Chapter 4) for downstream (BCAL3309; in OME59) and upstream (BCAL3313; in OME62) genes. The expression profiles of these genes were determined using luciferase expression assays under the same conditions described in Chapter 4.

BCAL3309 is a putative Major Facilitator Superfamily protein. Its expression was stimulated by rifampicin, norfloxacin and paraquat; whereas it was inhibited by gentamicin and PmB after 3 h of incubation at the concentrations used in this assay (Figure 44A). Ceftazidime did not alter the expression of BCAL3309.

BCAL3313 is a hypothetical protein predicted to be paraquat-inducible protein A. Its expression was stimulated by rifampicin, norfloxacin, paraquat and ceftazidime as well; whereas it was inhibited by gentamicin under the tested conditions (Figure 44B). However, PmB did not alter the expression of BCAL3313.

These results suggest the involvement of these proteins in the response to various antibiotics and potentially oxidative stress being responsive to paraquat. This warrants further characterization of their role in antibiotic resistance and stress response initially by deletion mutagenesis.

A



B

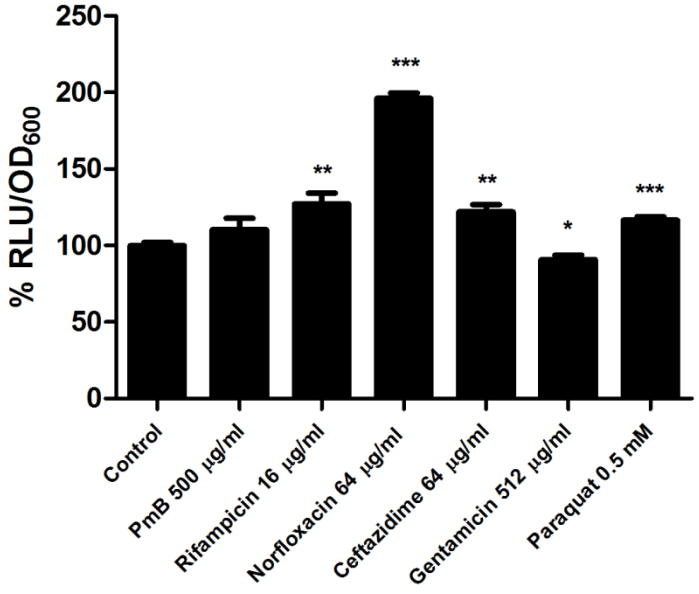


Figure 44. Luciferase expression assay of BCAL3309 in OME59 (A) and BCAL3313 in OME62 (B) in response to antibiotics at 3 h.

Results are shown as percentage of relative light units RLU/OD_{600} relative to the control (untreated K56-2 background). n=6 from 2 different clones. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Appendix F

Copyright Releases

COPYRIGHT RELEASES FOR CHAPTER 1

The following request was sent to Future Medicine Ltd.:

30 May 2014

Re: Permission to Use Copyrighted Material in a Doctoral Thesis

Dear Madame/Sir:

I am a University of Western Ontario graduate student completing my Doctoral thesis entitled "**Chemical Communication of Antibiotic Resistance by Highly Resistant Bacteria.** ".

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El-Halfawy OM, and Valvano MA (2013). Communication is key: do bacteria use a universal 'language' to spread resistance? Future Microbiology, November 2013, Vol. 8, No. 11, Pages 1357-1359.

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Omar M. El-Halfawy

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Curriculum Vitae- Omar M. El-Halfawy

EDUCATION

1. Ph. D. Candidate, Microbiology and Immunology Department, University of Western Ontario, London, Ontario, Canada. September 2010 - present.
Passed **Candidacy Exam** in August 2011 **with Distinction**.
Thesis title: "Chemical Communication of Antibiotic Resistance by Highly Resistant Bacteria."

2. M.Sc. in Pharmaceutical Microbiology, Faculty of Pharmacy, University of Alexandria, Egypt. June 2006 -January 2009.
Passed **Special graduate courses** qualifying for M Sc. Degree in Pharmaceutical Science (Pharmaceutical Microbiology) with general grade **Excellent**. November 2007.
Passed **General graduate courses** with general grade **Excellent**. November 2006.
Thesis title: "Microbiological studies on certain antihistaminic-antibiotic combinations."

3. **B.Sc. in Pharmaceutical Sciences** with general grade **Excellent with Honor**, University of Alexandria, Egypt, from September 2000 to June 2005.

HONORS AND AWARDS

1. Ontario Graduate Scholarship (OGS) for International Students. May 2013 to April 2014.

2. Travel Award from the department of Microbiology and Immunology, the University of Western Ontario. April 2012 and September 2013.

3. Microbiology & Immunology Graduate Entrance Scholarship 2010, the University of Western Ontario**.** Obtained at the entrance to the program September 2010

4. Western Graduate Research Scholarship (WGRS) from the Department of Microbiology and Immunology, University of Western Ontario. September 2010 to August 2014.

5. International Scholarship and membership in **NRW International Graduate Research School Biotech-Pharma**, **University of Bonn, Germany**. Awarded in May 2010 for 3 years to obtain Ph. D., declined.

6. First place prize for **Innovation and Entrepreneurship** from **Alexandria Univ.**, for research conducted as part of multidisciplinary Nanotechnology research team at Alexandria Univ. May 2011.

7. Certificate of merit from the **Faculty of Pharmacy, Alexandria Univ.** for being the **Top Graduate Student** in both the general and specialized **graduate courses for the M.Sc. Degree**. December 2007.

8. Plaque and Award for being the **Second top student** on my undergraduate class (~1200 students) at the cumulative total of the **B.Sc. in Pharmaceutical Sciences degree**, with cumulative grade Distinction with Honor, **Faculty of Pharmacy, Alexandria University**. September 2005.

9. Travel and Training Award from the **International Pharmaceutical Student's Federation** in cooperation with **Charles University, Hradec Králové, Czech Republic,** and Pharmacy: Lékárna U Rubínu, Brno, **Czech Republic**. July-August 2004.

WORK EXPERIENCE

Assistant Lecturer at the Pharmaceutical Microbiology Department, Faculty of Pharmacy, University of Alexandria.February 2009 -present.

Teaching assistant (**Demonstrator**) at the Pharmaceutical Microbiology Department, Faculty of Pharmacy, University of Alexandria. September 2005 - January 2009.

Community Pharmacist. Abd Allah El-Gedida Pharmacy, Alexandria, Egypt.June 2005 - September 2005.

TEACHING EXPERIENCE

Biology of Prokaryotes: Microimmun 2100, Microbiology and Immunology Dept., the University of Western Ontario. Fall semesters 2011, 2012 and 2013.

Practical Pharmaceutical Microbiology course for the second year undergraduate students. Faculty of Pharmacy, Alexandria University.September 2005-June 2010.

Practical Public health and Parasitology course for the third year students, Faculty of Pharmacy, Alexandria University. September 2005 -January 2009.

Practical microbiology course for the second year students, Faculty of Dentistry, Alexandria University.September 2008-January 2009.

Participated in the organization of workshops on "PCR Technology and its applications" held at the department of pharmaceutical microbiology, Faculty of Pharmacy, Alexandria University.

SUPERVISORY EXPERIENCE

Co-supervised second year volunteer student in a research project. October 2011 -February 2012.

SCHOLARLY AND PROFESSIONAL ACTIVITIES

Graduate students Representative in the Graduate Studies Committee for updating graduate programs and courses' curricula at the Faculty of Pharmacy, Alexandria University. May 2009 - July 2010.

Founding member of the Alumni association of the Faculty of Pharmacy -Alexandria University, Egypt.

Organization of a workshop on "PCR Technology and its applications" at the department of pharmaceutical microbiology, Faculty of Pharmacy, Alexandria University. August 2007.

PUBLICATIONS

***** *Articles in which I am the corresponding author. The work in these articles appears in my M. Sc. Thesis.*
****** *This work involved a multi-disciplinary research team in which I was the only microbiologist. I designed and executed the microbiological experiments and wrote that part of it.*

11. El-Halfawy OM, and Valvano MA (2014). Putrescine reduces antibiotic-induced oxidative stress as a mechanism of modulation of antibiotic resistance in *Burkholderia cenocepacia*. Antimicrob Agents Chemother. 58(7):4162-4171, AAC.02649-14.

10. El-Halfawy OM, and Valvano MA (2013). Communication is key: do bacteria use a universal 'language' to spread resistance? Future Microbiol.;8: 1357-9.

9. El-Halfawy OM and Valvano MA (2013). Chemical Communication of Antibiotic Resistance by a Highly Resistant Subpopulation of Bacterial Cells. PLOSOne Jul 3;8(7):e68874.

This article was featured by PlosOne *("Resisting Antibiotics: Some Bacteria Get By With a Little Help From Their Friends; http://blogs.plos.org/everyone/2013/07/03/resisting-antibiotics-some-bacteria-get-by-with-a-little-help-from-their-friends/).*
This article was highlighted by the media *such as CTV-London news, the London free press, Toronto Sun, and others.*

8. El-Halfawy OM and Valvano MA (2012). Non-genetic mechanisms communicating antibiotic resistance: rethinking strategies for antimicrobial drug design. Expert Opin Drug Discov;7(10):923-33.

7. Rosales-Reyes R, Saldías MS, Aubert DF, **El-Halfawy OM**, Valvano MA (2012). The *suhB* gene of *Burkholderia cenocepacia* is required for protein secretion, biofilm formation, motility and polymyxin B resistance. Microbiology. Sep;158:2315-24.

***6.** El-Nakeeb MA, Abou-Shleib HM, Khalil AM, Omar HG and **El-Halfawy OM** (2012). Reversal of antibiotic resistance in Gram-positive bacteria by the antihistaminic azelastine. APMIS;120(3):215-20.

****5.** Said SS, **El-Halfawy OM**, El-Gowelli HM, Aloufy AK, Boraei NA, El-Khordagui LK (2012). Bioburden-responsive antimicrobial PLGA ultrafine fibers for wound healing. European J. of Pharmaceutics and Biopharmaceutics, 80(1):85-94.

This article received the A.C.D.I.M.A. award for the best scientific publication *in the Arab countries in 2012 (Value of the award: **$7,200**).*

4. El-Halfawy OM, Valvano MA (2011). Heteroresistance of opportunistic bacteria to antimicrobial peptides: A new challenge to antimicrobial therapy. Therapy 8, (6), 591-595.

***3.** El-Nakeeb MA, Abou-Shleib HM, Khalil AM, Omar HG and **El-Halfawy OM** (2011). *In vitro* antibacterial activity of some antihistaminics belonging to different groups against multi-drug resistant clinical isolates. Brazilian Journal of Microbiology 42 (3): 980-991.

***2.** El-Nakeeb MA, Abou-Shleib HM, Khalil AM, Omar HG and **El-Halfawy OM** (2011). Membrane permeability alteration of some bacterial clinical isolates by selected antihistaminics. Brazilian Journal of Microbiology 42 (3): 992-1000.

1. Said SS, Aloufy AK, **El-Halfawy OM**, Boraei NA, El-Khordagui LK (2011). Antimicrobial PLGA ultrafine fibers: Interaction with wound bacteria. European J. of Pharmaceutics and Biopharmaceutics, 79 (1): 108-18.

ORAL PRESENTATIONS

3. El-Halfawy OM and Valvano MA (2014). Bacteria speak "Universal Language" to communicate antibiotic resistance. Western Research Forum, the University of Western Ontario, London, Canada. 19 March 2014.

2. El-Halfawy OM and Valvano MA (2012). Heteroresistance of *Burkholderia cenocepacia* to antimicrobial peptides. International Burkholderia cepacia working group conference, April 2012, Montreal, Canada.

1. Aloufy AK, Said SS, **El-Halfawy OM**, Boraei NA, El-Khordagui LK (2010). Interaction of Antimicrobial Nano/Ultrafine Fibers with Wound Bacteria.The 5th International Conference on Surfaces, Coatings and Nanostructured Materials (NANOSMAT-5), Reims, France. 19-21 October 2010,

POSTER PRESENTATIONS

4. El-Halfawy OM, and Valvano MA (2013). Chemical communication of antibiotic resistance by putrescine via protection against antimicrobial peptide-induced oxidative stress. London Health Research Day, London, Canada. 18 March 2014.

3. El-Halfawy OM, and Valvano MA (2013). Chemical communication of antibiotic resistance by putrescine via protection against antimicrobial peptide-induced oxidative stress. Infection and Immunity Research Forum, London, Canada. 1 November 2013.

2. El-Halfawy OM, and Valvano MA (2013). Chemical communication of antibiotic resistance by putrescine via protection against antimicrobial peptide-induced oxidative stress. International Meeting on Antimicrobial Peptides, London, United Kingdom. 23-24 September 2013.

1. El-Halfawy OM, EL-Nakeeb MA, Abou-Shleib HM, Khalil AM, Omar HG (2010). Effect of the antihistaminic azelastine on the antibacterial activity of selected antibiotics. Biovision Alexandria Conference 2010, Bibliotheca Alexandrina, Alexandria, Egypt. 11-15 April 2010.

PATENTS

****1.** Said SS, **El-Halfawy OM**, Aloufy AK, Boraei NA, El-Khordagui LK. Smart antimicrobial polymeric ultrafine fibres for the preparation of novel wound dressings. Patent Pending applied for to the Patent office, Egyptian Ministry of Scientific Research, Application no. 2010060999 submitted on 14 June 2010.

AD HOC REVIEWER ACTIVITIES

African Journal of Microbiology Research
Brazilian Journal of Microbiology

Bentham Science Publishers: ebooks.
BioMed Research International

TEACHING TRAINING

Future Professor Workshop Series, Teaching Support Centre, the University of Western Ontario:

Preparing an Effective Course Syllabus, July 2014.
The Concept of Universal Design for Learning: Reaching a Diverse Group of Learners, July 2014.
Using Social Media Effectively in the University Classroom, June 2014.
A Look Back: Leading Effective Exam Review Sessions, March 2014.
Strategies that Work: Teaching in the Sciences, March 2014.
Getting Feedback on Your Teaching, March 2014.
Putting Together a Teaching Dossier, October 2013.
Writing A Teaching Philosophy Statement, October 2013.

Teaching Assistant Training Program (TATP), at the University of Western Ontario, September 2011.

Three-day workshops at the **Faculty and Leadership Development Center** (FLDC), Alexandria University, Alexandria, Egypt:

Communication skills, June 2006.
Thinking skills, June 2006.
Effective teaching, May 2007.
Financial Affairs at the university, May 2007.
Credit hours, May 2007.
Research Methodology, May 2007.
Ethics of scientific research, June 2010.
International publishing of scientific papers, June 2010.
Organization of scientific conferences, July 2010.
University administration, July 2010.
Examination and students' assessment methods, July 2010.