

Identification of novel antimicrobial drug targets in *Burkholderia cepacia* complex bacteria and evaluation of treatment approaches

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LIST OF ABBREVIATIONS

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ABC	ATP-binding cassette family
acetyl-CoA	Acetyl-coenzyme A
AHL	Acyl homoserine lactone
AMOX	Amoxicillin
AVI	Avibactam
AZT	Aztreonam
Bcc	<i>Burkholderia cepacia</i> complex
BCESM	<i>Burkholderia cepacia</i> epidemic strain marker
BDSF	<i>Burkholderia</i> diffusible signal factor
BHIA	Brain Heart infusion agar
BHIB	Brain Heart infusion broth
CA	Community-acquired
CAZ	Ceftazidime
CCCP	Carbonyl-cyanide-3-chlorophenyl hydrazine
CDP-ME	4-diphosphocytidyl-2-C-methyl-D-erythritol
CF	Cystic fibrosis
CFP	Cefepime
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming units
CFX	Cefoxitin
CGD	Chronic granulomatous disease
cIAIs	Complicated intra-abdominal infections
CLA	Clavulanic acid
CMY	Cephameycins
CPE	Carbapenemase-producing <i>Enterobacteriaceae</i>
Cph	Carbapenem hydrolyzing capabilities
CPPs	Cell-penetrating peptides
CTX-M	Cefotaxime hydrolyzing capabilities
cUTIs	Complicated urinary tract infections
DBO	diazabicyclooctane
DD pathway	De Ley-Doudoroff pathway
<i>dhfr</i>	dihydrofolate reductase
DMAPP	dimethylallyl diphosphate
DOXP	1-deoxy-D-xylulose-5-phosphate
DXR	DOXP reductoisomerase
DXS	DOXP-synthase
EDTA	Ethylenediaminetetraacetic acid
<i>e-gfp</i>	Enhanced green fluorescent protein gene

LIST OF ABBREVIATIONS

EPS	Exopolysaccharides
EPs	Efflux pumps
ESBL	Extended-spectrum β -lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FIC	Fractional inhibitory concentration
<i>fsr</i>	Fosmidomycin resistance gene
G3P	D-glyceraldehyde-3-phosphate
GlpT	glycerol-3-phosphate transporter
HA	Hospital-acquired
HINT-1	Histidine triad nucleotide-binding protein 1
HMG-CoA	3-hydroxy-3-methylglutaryl CoA
HMGCR	HMG-CoA reductase
IL-8	Interleukin-8
IM	Intramuscular
IMP	Imipenem
IPP	isopentenyl diphosphate
IspD	CDP-ME synthase
IspE	CDP-ME kinase
IspF	MER-cPP synthase
IV	Intravenous
KPC	<i>Klebsiella pneumoniae</i> carbapenemase
LBA	Luria-Bertani agar
LBB	Luria-Bertani broth
LPS	Lipopolysaccharides
MAM	N-acetyl muramic acid
MATE	Multidrug and toxic efflux family
MBA	Middlebrook 7H10 agar
MBB	Middlebrook 7H9 broth
MBL	Metallo- β -lactamase
MDR	Multidrug-resistant
MEM	Meropenem
MEP	2-C-methyl-D-erythritol-4-phosphate
MER-cPP	2-C-methyl-D-erythritol-2,4-cyclodiphosphate
MF	Major facilitator superfamily
MHB	Mueller-Hinton Broth
MIC	Minimal Inhibitory Concentration
MM9	M9 minimal medium
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MVA	Mevalonate pathway
NAG	N-acetylglucosamine
NCBI	National Center for Biotechnology Information
NDM	New Delhi metallo- β -lactamase
NGM	Nematode Growth Medium

LIST OF ABBREVIATIONS

ORF	Open reading frame
OXA	Oxacillin hydrolyzing capabilities
PaβN	Phenylalanine-arginine-β-naphthylamide
PBPs	Penicillin-binding proteins
PC	Penicillin
PCR	Polymerase Chain Reaction
PDR	Pandrug-resistant
PER	<i>Pseudomonas</i> extended resistant
<i>Pf</i> DXR	DXR enzyme from <i>Plasmodium falciparum</i>
PMBN	Polymyxin B nonapeptide
PMF	Proton motive force
PMOs	Phosphorodiamidate-morpholino oligomers
<i>P_{rhaB}</i>	rhamnose-inducible promotor
PS	Physiological saline
qPCR	Quantative real-time Polymerase Chain Reaction
QS	Quorum sensing
RND	Resistance-nodulation division family
ROS	Reactive oxygen species
RTG	Arginine, threonine, glycine
SCFM	Synthetic Cystic Fibrosis Medium
SHV	Sylfhydryl variable
SMR	Small-drug resistance family
SOD	Superoxide dismutases
SUL	Sulbactam
TAZ	Tazobactam
TB	Tuberculosis
TEM	Temoneira
TNF	Tumor necrosis factor
TraDIS	transposon directed insertion-site sequencing
TSB	Tryptic Soy Broth
VEB	Vietnam extended-spectrum β-lactamase
VIM	Verona integron-encoded metallo-β-lactamase
WHO	World Health Organization
XDR	Extensively drug-resistant

CHAPTER I. INTRODUCTION

Burkholderia cepacia complex: multifarious opportunistic pathogens with important natural biology

Eshwar Mahenthiralingam

1. *BURKHOLDERIA CEPACIA* COMPLEX

1.1. Background

The *Burkholderia cepacia* complex or Bcc is a complex of 20 closely related opportunistic, Gram-negative bacterial species.[1] An overview of all currently described Bcc species is shown in Table 1. Thus far, the diversity of the Bcc is not completely mapped and additional Bcc species will likely be described in the future. Bcc species most frequently occur in association with a variety of hosts (including humans, animals, plants and fungi) in which they may cause severe infections. Bcc members possess bioremediation and pesticidal properties when occurring in soil, water, plants or fungi.[2, 3] In 1950, these obligate aerobic, non-spore-forming bacilli were first identified by Walter Burkholder as the causative agent of onion rot.[4, 5] They were then referred to as *Pseudomonas cepacia*, until their transfer to the genus *Burkholderia* in 1992 on the basis of 16S rRNA sequences and DNA-DNA homology analyses.[6, 7] In 1997, Vandamme and colleagues proposed a subdivision of *Burkholderia cepacia* in five genomovars (I – V), referring to phenotypically similar but genotypically different species.[8] In the next 20 years, numerous new Bcc species and genomovars have been identified. More accurate identification and novel insights into the natural diversity of this group of organisms will likely reveal many more.[7, 9]

Table 1. Overview of *Burkholderia cepacia* complex species and their isolation sources.[1, 10-12]

Species	Genomovar	Natural habitat	Clinical habitat
<i>B. ambifaria</i>	VII	Soil, rhizosphere	CF, non-CF
<i>B. anthina</i>	VIII	Soil, rhizosphere, water, plant	CF, hospital materials
<i>B. arboris</i>		Soil, rhizosphere, water	CF, non-CF, industrial equipment
<i>B. cenocepacia</i>	III	Soil, rhizosphere, water, plant, animal	CF, non-CF, industrial equipment
<i>B. cepacia</i>	I	Soil, rhizosphere, water, plant	CF, non-CF, medical solutions
<i>B. contaminans</i>		Sheep, plant	CF, non-CF, hospital materials
<i>B. diffusa</i>		Soil, water	CF, non-CF, hospital materials
<i>B. dolosa</i>	VI	Maize, rhizosphere	CF
<i>B. latens</i>		/	CF
<i>B. lata</i>		Soil, rhizosphere, water, flower	CF, non-CF, industrial equipment
<i>B. metallica</i>		/	CF
<i>B. multivorans</i>	II	Soil, rhizosphere, water, plant	CF, non-CF, CGD
<i>B. pyrrocinia</i>	IX	Soil, rhizosphere, water, plant	CF, non-CF
<i>B. pseudomultivorans</i>		Rhizosphere	CF & non-CF sputum
<i>B. seminalis</i>		Soil, rhizosphere, rice	CF, non-CF, nosocomial infection
<i>B. stabilis</i>	IV	Plant, rhizosphere	CF, non-CF, hospital materials

<i>B. stagnalis</i>		Soil	CF sputum, non-CF tracheal aspirate
<i>B. territorii</i>		Water	/
<i>B. ubonensis</i>		Soil	non-CF, nosocomial infection
<i>B. vietnamiensis</i>	V	Soil, rhizosphere, water, plant, animal	CF, non-CF, industrial equipment

CF; Cystic Fibrosis. CGD; Chronic granulomatous disease

1.2. Infections with Bcc bacteria

Members of the Bcc are well-known as rare but potentially life-threatening opportunistic pathogens in immunocompromised patients, such as subjects with cystic fibrosis (CF) or chronic granulomatous disease (CGD). *Burkholderia multivorans* and *Burkholderia cenocepacia* are currently the most frequently encountered Bcc species in CF patients and patients with CGD.[7]

CF is an autosomal, recessive disease causing a rapid decrease in lung function and low life expectancy, and is most common among Caucasian people.[13] Mutations in the gene that encodes CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) are the main cause of the development of CF.[13, 14] CFTR mainly functions as a chloride channel but has also many regulatory roles including regulation of ATP transport, sodium transport and intracellular transport of vesicles.[15] Mutations of the CFTR gene disrupt the function of CFTR thereby leading to clinical defects in the lungs, pancreas and gastrointestinal tract and to a lesser extent in sweat glands, reproductive tracts and submucosal glands.[16] The most common mutation is a single deletion of phenylalanine at position 508 in NBD1 ($\Delta F508$).[13, 17] Impaired chloride transport leads to the production of a sticky, dehydrated mucus in the respiratory tract, causing a decrease in mucociliary and alveolar clearing. Consequently, colonization of the lungs by bacterial pathogens is facilitated, leading to airway infections.[18, 19] In young children (<10 years old) with CF, the bacterial pathogens most frequently recovered from the airways are *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Haemophilus influenzae*. *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, *Aspergillus fumigatus*, nontuberculous mycobacteria and species belonging to the Bcc are also present in CF lungs but are found less frequently.[20] Although Bcc species are less commonly found in CF airways (prevalence in the USA is approximately 3%), their impact on pathogenesis and prognosis can be considerable.[7] Acute bacterial infections lead to increased cough and sputum production, weight loss, fever and pulmonary exacerbations, requiring antibacterial chemotherapy. Eventually, CF patients can suffer from chronic respiratory infections which are the main cause of the decline in lung function and ultimate mortality.[6, 21] Furthermore, an acute infection

known as ‘cepacia syndrome’ characterized by necrotizing pneumonia, bacteremia and sepsis can cause a rapid clinical deterioration leading to an early death.[6]

CGD is a hereditary immune disease where autosomal, recessive mutations on the X-chromosome lead to defects in polymorphonuclear leukocytes, causing difficulty in forming reactive oxygen species (ROS).[22, 23] Due to this defective oxidative killing, these immune cells are not able to kill certain ingested bacteria.[7] The most commonly isolated microorganisms from CGD patients are *S. aureus*, *Escherichia coli*, *Aspergillus* species, *Klebsiella* species and Bcc species.[24] Bcc infections leading to sepsis and pneumonia are the second most common cause of death in CGD patients.[24]

Therapeutic options for treatment of Bcc infections are limited because of their innate resistance to a variety of antibiotics. This is further discussed in 1.3.3. Therefore, elucidation of novel targets for anti-Bcc chemotherapy is indispensable to conquer infections with Bcc bacteria. For this dissertation we focused on two strategies to overcome Bcc infections, including the nonmevalonate pathway for isoprenoid biosynthesis as an antibacterial target and combination therapy of β -lactam antibiotics with β -lactamase inhibitors which are further discussed in Chapter I.2 and I.3, respectively.

1.3. Virulence factors

1.3.1. *Quorum sensing*

Bcc bacteria possess a comprehensive, density-dependent, cell-to-cell communication system, also known as quorum sensing (QS).[25] QS systems are triggered when a bacterial population reaches a critical density, enabling bacterial cells to release signal molecules which are able to modify gene expression in adjacent bacterial cells. Bacteria use QS systems to regulate certain processes including biofilm formation, antibiotic resistance, virulence, secretion systems and stress response.[26, 27]

The first QS system in Bcc was discovered in *B. cenocepacia* K56-2 and designated as CepIR.[7] CepIR consists of two components: CepI which is an acyl homoserine lactone (AHL) synthase and CepR which detects AHLs and regulates transcription. In 2005, CciIR was discovered as a second AHL-controlled QS system in *B. cenocepacia* strains belonging to the epidemic ET12 lineage. CciIR is encoded in the genomic region surrounding the *B. cenocepacia* epidemic strain marker (BCESM).[28] CepR primarily functions as a positive regulator while CciR globally induces negative gene regulation. Both AHL-based QS systems were found to reciprocally regulate the expression of multiple genes. Homologous systems have been

described in all Bcc species, but most QS systems in Bcc are controlled by CepR.[27] In 2008, another QS system was described in *B. cenocepacia* which employs *cis*-2-dodecenoic acid (also known as the *Burkholderia* diffusible signal factor or BDSF) as a signal molecule.[29]

1.3.2. Biofilm formation

Biofilm formation is thought to be an important factor for chronic Bcc infections in the lungs, nevertheless, the exact mechanisms of biofilm formation remain to be determined.[30] Bcc bacteria are assumed to form biofilms on both biotic (e.g. epithelial cells) and abiotic surfaces (e.g. synthetic heart valves).[31-33] The process of biofilm formation starts with bacteria approaching closely to a surface, simultaneously decreasing their motility and associating with the surface and/or microorganisms already attached to the surface (Figure 1 [33]). This first reversible step involves intercellular signalling and is mediated by flagella and/or pili on the bacterial cell surface.[33] In the second step, the transient

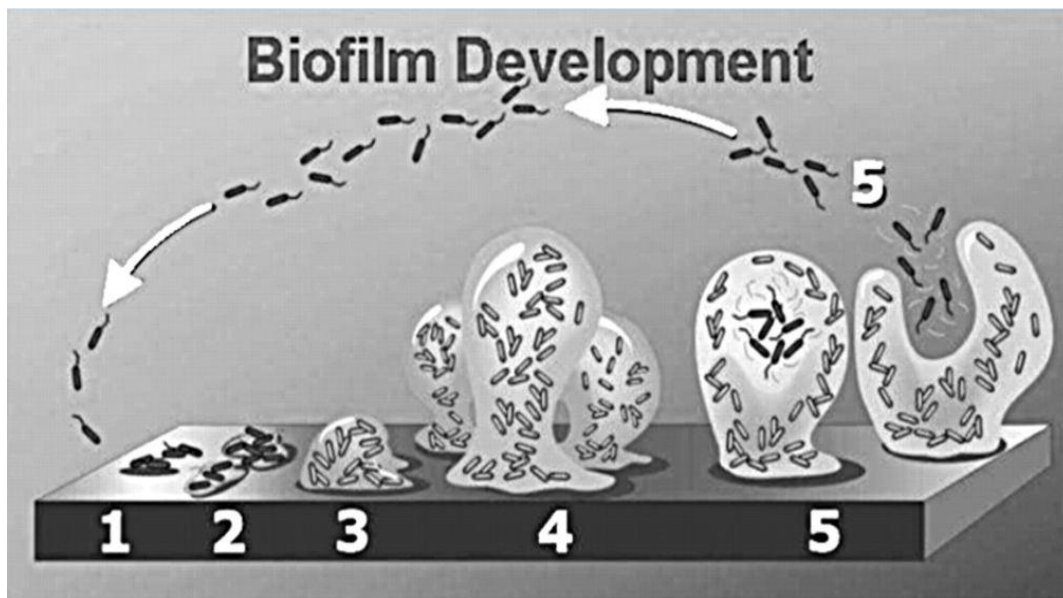


Figure 1. Schematic overview of biofilm formation. Step 1 involves the approach of bacterial cells to a surface, decrease in cellular motility and attachment to the surface. In step 2 the cells start to form microcolonies, in step 3 and 4 bacteria start to divide, produce an extracellular matrix and form a three-dimensional biofilm structure. In step 5, bacterial cells can become motile again and spread to other locations.[33]

attachment stabilizes and bacteria start to form microcolonies. Consequently, bacteria start to divide and produce an extracellular matrix generally consisting of extracellular polymeric substances composed of exopolysaccharides (EPS), extracellular DNA, proteins and lipids.[32] Biofilms are provided with nutrients and signalling molecules by a network of open water channels, which separate bacterial

microcolonies. Mature biofilms may adopt a flat and undifferentiated form or can adopt a more differentiated (sometimes mushroom-shaped) form, depending on the environmental conditions and available nutrients. In the fifth and last step, sessile bacterial cells can be released from a microcolony, become motile again and disperse to other locations.[34] Dispersal of biofilms enables bacteria to spread to other organs or even through the whole body, where they can lead to several threatening clinical implications.[32]

Bacterial biofilms can move in numerous ways on their trajectory to infect new tissues. They can move cooperatively, by detaching in clumps, or by rolling across the attached surface.[35] The ability of Bcc bacteria to form biofilms *in vitro* and *in vivo* contributes to reduced antimicrobial susceptibility, treatment failure and persistent infection.[36, 37]

1.3.3. *Antimicrobial uptake mechanisms and resistance*

The uptake of antibiotics in Gram-negative bacterial cells is significantly slowed by the presence of an outer membrane consisting of lipopolysaccharides (LPS) and phospholipids, which functions as a permeability barrier. Nonspecific porins and specific uptake channels are the most important factors accounting for active uptake of antimicrobial compounds through the membrane.[38, 39] Passive diffusion of hydrophobic compounds is slower due to the high rigidity of the LPS-containing bilayers and narrow porins limit penetration of large hydrophilic drugs.[40] There is an urgent need for quantitative methods to measure and image the localisation of antibiotics and to understand penetration in bacteria cells. Several research groups are currently focusing on different methods to address this query; label-free 3D imaging of antibiotics in bacteria, real-time quantitative measurement of drug-uptake in bacteria and biofilms and traceable quantification of vertical concentration profiles of antibacterial agents in bacteria.[41-43]

Members of the Bcc show a high level of intrinsic resistance towards a variety of antibiotics including aminoglycosides, polymyxins, first and second generation cephalosporins, carboxypenicillins and other β -lactam antibiotics. Bcc species are also able to develop *in vivo* resistance towards almost any class of antibiotics currently available.[7, 44, 45] Various mechanisms are responsible for this multidrug-resistance in Bcc bacteria, including reduced membrane permeability, modified target sites (e.g. penicillin binding proteins (PBPs)), drug elimination by efflux pumps (EPs), and/or production of

antibiotic-modifying and inactivating enzymes (e.g. β -lactamases and aminoglycoside-inactivating enzymes) (schematically depicted in Figure 2).[45-47] Antimicrobial resistance mechanisms can be divided into two stages: a first and fast response with changes in membrane organization and permeability and a second and slower response in which genetic changes are responsible for the acquisition of long-term antibiotic resistance.[39, 48, 49]

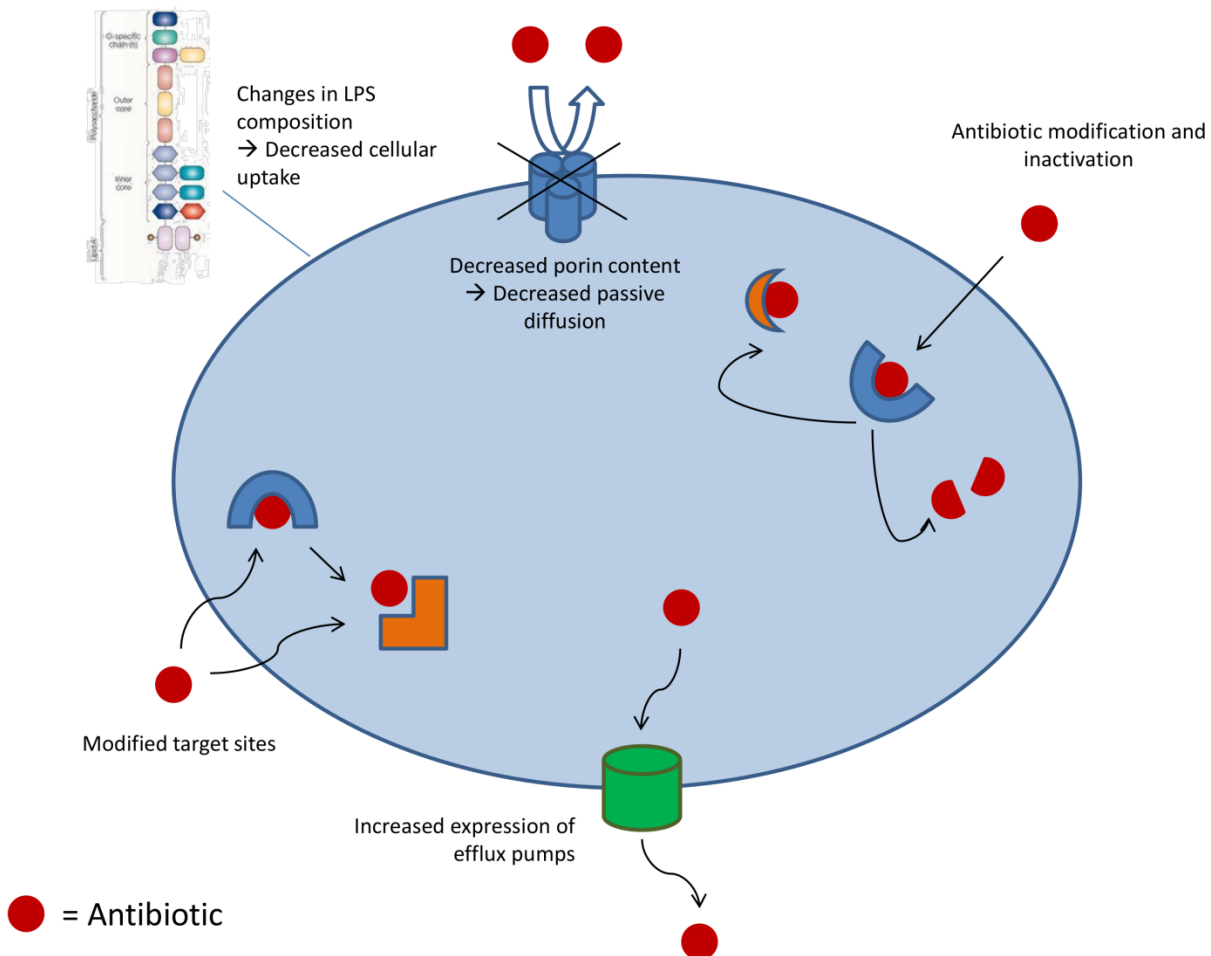


Figure 2. Schematic overview of antimicrobial resistance mechanisms in Bcc species.

1.3.3.1. Reorganization of the cell membrane

The permeability of bacterial cells can be changed due to a reorganization of different compounds of the cell membrane including decrease of porin content, alterations in composition of LPS and/or overexpression of EPs.[48]

Porins are multimers of identical subunits creating a pore, and are found in Gram-negative bacteria and mycobacteria. Porins form channels of sufficient size to enable passive diffusion through the outer membrane and serve as the main entrance into the periplasm for antibiotics (including β -lactams and fluoroquinolones) and a variety of small hydrophilic molecules.[48] Mutations in the genes encoding porins or decreased porin content can cause resistance against several antibacterial agents, as passive diffusion through the outer membrane becomes difficult or impossible.[50]

LPS consist of a core polysaccharide, a lipid A compound, and an O-antigen, shown in Figure 3 [7]). LPS form a lipophilic layer in the outer membrane of Gram-negative bacteria and thereby contribute to the protection and structural integrity of the bacterial cells.[51] LPS do not only form a simple barrier; they play an essential role in the response of bacteria to their environment.[52] The properties of LPS from Bcc species are different from those of other Gram-negative bacteria as they are less negatively charged

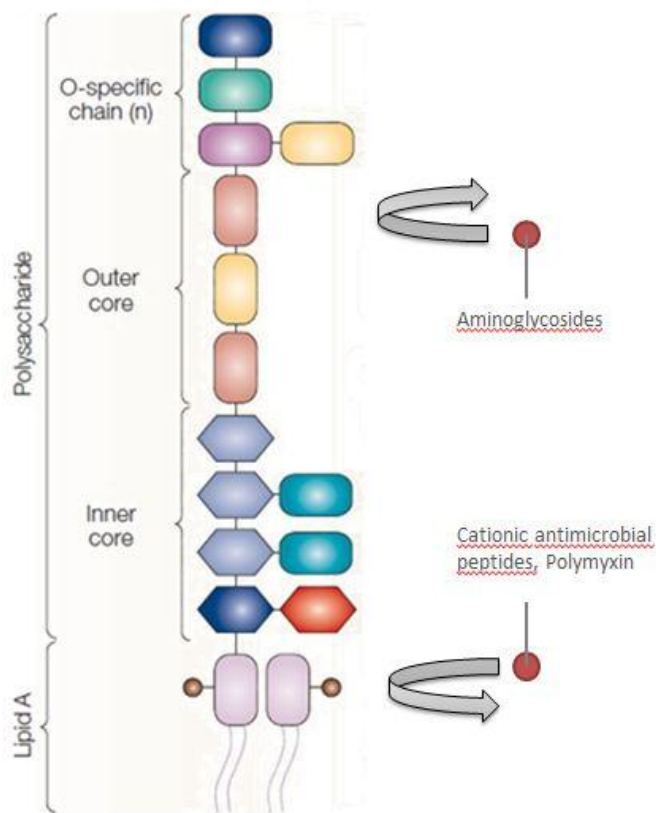


Figure 3. LPS from the outer membrane of *Burkholderia cepacia* complex bacteria.[7]

due to a lower content of phosphate and/or 3-deoxy-D-manno-oct-2-ulosonic acid (KDO) residues on their core oligosaccharide.[53] This characteristic confers resistance to aminoglycosides due to an inability of these antibiotics to bind to Bcc LPS.[7, 54] Additionally, 4-deoxyarabinose groups are attached to the lipid A backbone, further reducing the negative charge of the LPS and decreasing the cellular uptake of cationic antibiotics such as polymyxin B and colistin.[7] LPS from Bcc species are more endotoxic than *P. aeruginosa* LPS, and lead to a pronounced cytokine production, including tumor necrosis factor (TNF) and interleukin-8 (IL-8), that leads to inflammatory reactions.[7, 55]

Transport of antibiotic molecules out of the cell by overexpression of EPs is one of the most

commonly encountered resistance mechanisms in clinical isolates.[56, 57] These cellular pumps play an

important role in the physiology and homeostasis of the cell as they are responsible for the extrusion of both endogenous and exogenous toxic compounds.[48] EPs also play a significant role in QS signalling systems and biofilm formation in some bacteria.[58, 59] Five different transporter families have been identified: major facilitator superfamily (MF), multidrug and toxic efflux (MATE), resistance-nodulation-division (RND), small multidrug resistance (SMR) and ATP-binding cassette (ABC). All transporters systems, except for the ABC family, use the proton motive force (PMF) to drive the export of substrates. The ABC transporters use ATP hydrolysis as an energy source for export.[60]

1.3.3.2. Genetic acquisition of antimicrobial resistance

Susceptible bacteria can acquire resistance through mutations in their DNA (that may be promoted by activation of certain “mutator genes”) and/or by horizontal gene transfer between organisms.[48, 61]

Mutations can occur in genes involved in normal physiological processes and cellular structures, for example in the gene encoding dihydrofolate reductase (*dhfr*) which is essential for amino acid synthesis and can be inhibited by trimethoprim. A point mutation in *dhfr* is responsible for trimethoprim-resistant strains of Bcc and other Gram-negative bacteria (*E. coli* and *H. influenzae*).[62]

Horizontal gene transfer between bacterial species can happen through transformation, transduction or conjugation of transferable resistance genes. Transformation involves direct uptake and incorporation of DNA (mostly carried on plasmids, transposons or integrons) by competent bacterial cells.[63] Transduction comprises transfer of DNA via bacteriophages from one bacterium to another and conjugation involves transfer of DNA between bacterial cells in direct cell-to-cell contact.[64] Plasmid-mediated transfer of genes encoding AmpC β -lactamases is an example of horizontal gene transfer and has been described in *E. coli*, *Klebsiella pneumoniae* and other Gram-negative species.[65, 66]

1.3.4. Cell envelope structures

Besides LPS, located in the outer membrane of Gram-negative bacteria, two other structures of the cell envelope play a significant role in the virulence of Bcc species. The flagellum enables bacterial strains to invade lung epithelial cells by making bacteria motile and serving as an adhesin, and cable pili contribute to binding and killing of human airway epithelial cells.[67, 68]

1.3.5. *Synthesis of exopolysaccharides (EPS)*

Bcc species have the ability to produce EPS which play a role in stability, adherence and protection against environmental factors.[69] EPS are essential for biofilm formation and/or biofilm maintenance in many bacteria. Five different EPS have been identified in members of the Bcc including PS-I, PS-II (also called cepacian) and levan, with cepacian being the most frequently encountered EPS.[70] Cepacian is a heteropolysaccharide with a branched heptasaccharide as the basic unit with D-glucose, D-rhamnose, D-mannose, D-galactose and D-glucuronic acid. Cepacian has been found in both mucoid and non-mucoid Bcc strains where it provides multiple functions.[71, 72] In addition to its importance in biofilm formation, cepacian plays a role in the inhibition of neutrophil chemotaxis, interference with neutrophilic phagocytosis and the scavenging of reactive oxygen species (ROS).[73] Some Bcc strains exclusively produce cepacian as EPS, other strains also produce other EPS, while some strains do not produce any cepacian at all.[36, 74] *B. cenocepacia* LMG 16656, for example, lacks cepacian production due to a frameshift mutation (11-bp deletion) in BCAM0856, a coding DNA sequence in the gene cluster associated with cepacian production.[45, 73]

1.3.6. *Resistance to oxidative stress*

In mammalian hosts, phagocytic cells produce ROS to help eliminate bacterial species.[75] High concentrations of ROS (including peroxides, superoxide, hydroxyl radicals and singlet oxygen) can cause significant damage to cells, ultimately leading to cell death.[76] However, Bcc bacteria are able to resist oxidative stress by producing catalases, peroxidases and superoxide dismutases (SOD). In *B. cenocepacia* two different catalase/peroxidase systems were identified; KatB as the major catalase/peroxidase system and KatA required for resistance to hydrogen peroxide.[77] SODs convert superoxide to hydrogen peroxide which is further degraded to water and oxygen by catalases.[78, 79] The production of catalases, peroxidases and SODs by Bcc species play an important role in their protection against ROS and therefore also for intracellular survival and virulence.[78]

The gene *hppD*, encoding an enzyme required for pigment production, is suggested to protect *B. cenocepacia* from both ROS and reactive nitrogen species. Disruption of this gene leads to a

nonpigmented *B. cenocepacia* strain which has increased sensitivity to hydrogen peroxide and superoxide.[80]

1.3.7. Iron acquisition

Iron is an essential component for important bacterial processes including DNA synthesis and respiration, and siderophores are essential for Bcc bacteria to scavenge iron ions (Fe^{3+}).[81] The solubility of the resulting Fe^{3+} -complexes is higher than the Fe^{3+} ion alone and they can therefore be taken up by active transport.[82] Bcc bacteria produce four different siderophores (pyochelin, ornibactin, cepaciachelin, and cepabactin), with ornibactin and pyochelin being the most predominant siderophores in *B. cenocepacia*. [83, 84] *B. cenocepacia* can also use heme as an iron source and can obtain iron from the iron-binding protein ferritin, which is found at up to 100-fold higher concentrations in the lungs of CF patients compared to healthy individuals.[82, 85]

To prevent iron toxicity, the host sequesters free iron ions in iron binding proteins (e.g. transferrin, lactoferrin or ferritin), in heme and hemoproteins. Therefore, the amount of freely available iron in host cells and/or animal tissues is extremely limited and the ability of bacterial pathogens to grow under limited iron conditions is an important virulence factor.[82, 86]

1.4. Treatment of Bcc infections

Anti-infective therapy can be subdivided into prevention and infection control, early antibiotic treatment, maintenance therapy as suppression of chronic infections, and acute exacerbation therapy.

Most commonly used antibiotics to treat Bcc infections act by inhibition of cell wall synthesis (β -lactams), inhibition of cell membrane function (polymyxins, taurolidine), inhibition of nucleic acid synthesis (fluoroquinolones) or inhibition of protein synthesis (macrolides, aminoglycosides). Trimethoprim interferes with the folic acid pathway by binding to dihydrofolate reductase and sulfamethoxazole inhibits an enzyme further upstream in the same pathway. Most compounds are bactericidal, except for macrolides, trimethoprim and sulfamethoxazole which have bacteriostatic activity.[87]

1.4.1. *Infection control*

Guidelines for preventing transmission and controlling spread of an infection among CF patients have been established to reduce the impact of bacterial infections (especially Bcc) on morbidity and mortality. These guidelines recommend segregation of CF patients infected with Bcc species from other patients with CF and taking precautions when caring for a CF patient with Bcc infections in hospital, ambulant and social settings.[88]

1.4.2. *Early antibiotic treatment*

Bcc isolates are recovered in respiratory secretions of 3% of all US CF patients, whom are almost all previously colonized with mucoid *P. aeruginosa*. [88] Data from clinical studies concerning the treatment of Bcc infections in CF patients suggest that therapy of serious infections should include at least two parenteral antibiotics at a standard dose.[89, 90] For susceptible strains, parenteral trimethoprim-sulfamethoxazole (co-trimoxazole) should be administered in combination with a β -lactam (e.g. ceftazidime, meropenem, piperacillin), a fluoroquinolone (e.g. ciprofloxacin) or chloramphenicol. For Bcc strains resistant to co-trimoxazole, combination therapy guided by antibiotic sensitivity patterns should be used.[90-92]

1.4.3. *Maintenance therapy*

Chronic infections should be treated with maintenance therapy in order to reduce progressive lung damage and to lower the frequency of pulmonary exacerbations. Because systemically administered antibiotics may lead to sub-therapeutic concentrations in the lungs and because their long-term systemic use may lead to side-effects, much research has been focused on inhalation therapy.[93] Guidelines from the UK CF Trust recommend the use of inhaled ceftazidime, colistin or taurolidine for the treatment of chronic Bcc infections.[94, 95] Antibiotic agents that are currently being investigated as inhalation therapy are aztreonam lysine, macrolides (erythromycin and azithromycin) or tobramycin in high concentrations.[96-99] Moreover, combination therapy with nebulized tobramycin and amiloride has been used in two case reports as eradication therapy for Bcc strains.[100, 101]

The role of oral maintenance therapy for chronic Bcc infections in CF has not yet been defined. When oral therapy is used, co-trimoxazole is recommended for susceptible strains and oral minocycline or doxycycline for resistant strains.[90, 102] However, the development of resistance and toxicity, including skin and dental discoloration, are of great concern.[102]

1.4.4. Treatment of pulmonary exacerbations

Therapy for acute pulmonary exacerbations is commonly based on intravenous administration of minimum two antibiotics for 14 – 21 days, chest physiotherapy to improve airway clearance and bronchodilator administration.[103]

Recommended treatment for exacerbations associated with Bcc strains is a combination of meropenem plus one of the following: tobramycin, amikacin, ceftazidime or co-trimoxazole.[104] When *P. aeruginosa* and Bcc are both identified in sputum cultures, combination therapy with an aminoglycoside and a β -lactam antibiotic is often suggested.[105]

A systematic review in 2011 concludes that there is a lack of randomized trials and therapies for the treatment of pulmonary exacerbations associated with Bcc strains. Therefore, clinicians must assess treatment for every CF patient individually guided by *in vitro* susceptibility results, previous clinical response and clinical experience.[106]

1.4.5. Treatment of Bcc infections in CGD patients

Bcc species are also an important cause of infections in patients suffering from CGD, mostly due to the ability of these microorganisms to resist neutrophil-mediated bacterial killing. The antimicrobial regimen in CGD patients should include co-trimoxazole combined with ceftazidime or a similar broad-spectrum antimicrobial agent.[107, 108]

2. NONMEVALONATE PATHWAY

2.1. Introduction

Animals (including humans), fungi, *Archaea*, insects and some bacteria use the mevalonate pathway for isoprenoid biosynthesis.[109-112] Isoprenoids play important roles in all living organisms. Most bacteria use an alternative pathway to synthesize isoprenoids; the 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway or nonmevalonate pathway and some plant species (e.g. *Glycine max*, *Zea mays*) are able to use both pathways.[113, 114] The DOXP pathway is present in several important pathogenic microorganisms including *Acinetobacter*, *Klebsiella*, *Mycobacterium*, *Pseudomonas* and Bcc species.[115] Since this pathway is not present in human cells, it is possibly an interesting target for the development of antibacterial chemotherapy. In 1998, fosmidomycin was discovered as an inhibitor of DXR, the second enzyme in the DOXP pathway, of *E. coli* K-12 and was also shown to completely inhibit bacterial growth.[116, 117] Consequently, several studies described inhibitory activity of fosmidomycin and its acetyl analogue against *P. falciparum*, *P. aeruginosa* and *K. pneumonia*.[118, 119] Jomaa and colleagues also confirmed that inhibitors of this pathway can be used for the treatment of the malaria parasite *Plasmodium falciparum*.[120] Nevertheless, few studies have explored the nonmevalonate pathway in bacteria and investigation of the potential of this pathway as antibacterial target was mainly restricted to *E. coli* and *Mycobacterium tuberculosis*.[118, 121] Fosmidomycin is currently clinically used in combination with clindamycin for the treatment of malaria caused by *P. falciparum*. However, difficulties with toxicity, short half-life and low bioavailability limit its potential as an antimalarial drug.[122-124]

In the further chapters, several bacteria were studied (including *P. aeruginosa*, *P. acnes*, *M. smegmatis* as a model for *M. tuberculosis* and Bcc species) for which analysis of the genome sequence demonstrated that the nonmevalonate pathway is the sole pathway for isoprenoid biosynthesis.[115, 125-127]

2.2. Isoprenoids

Isoprenoids (also called terpenoids) form one of the largest classes of natural compounds, with over 30,000 compounds identified. They play a significant role in essential physiological processes in all living organisms including regulation of growth (steroid hormones), electron transport (menaquinone), cell wall and membrane biosynthesis, and conversion of light in chemical energy (carotenoids).[119, 128] All

isoprenoids consist of a sequence of the common isoprene units, i.e. five-carbon units, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Figure 4; adapted from [129]).[128]

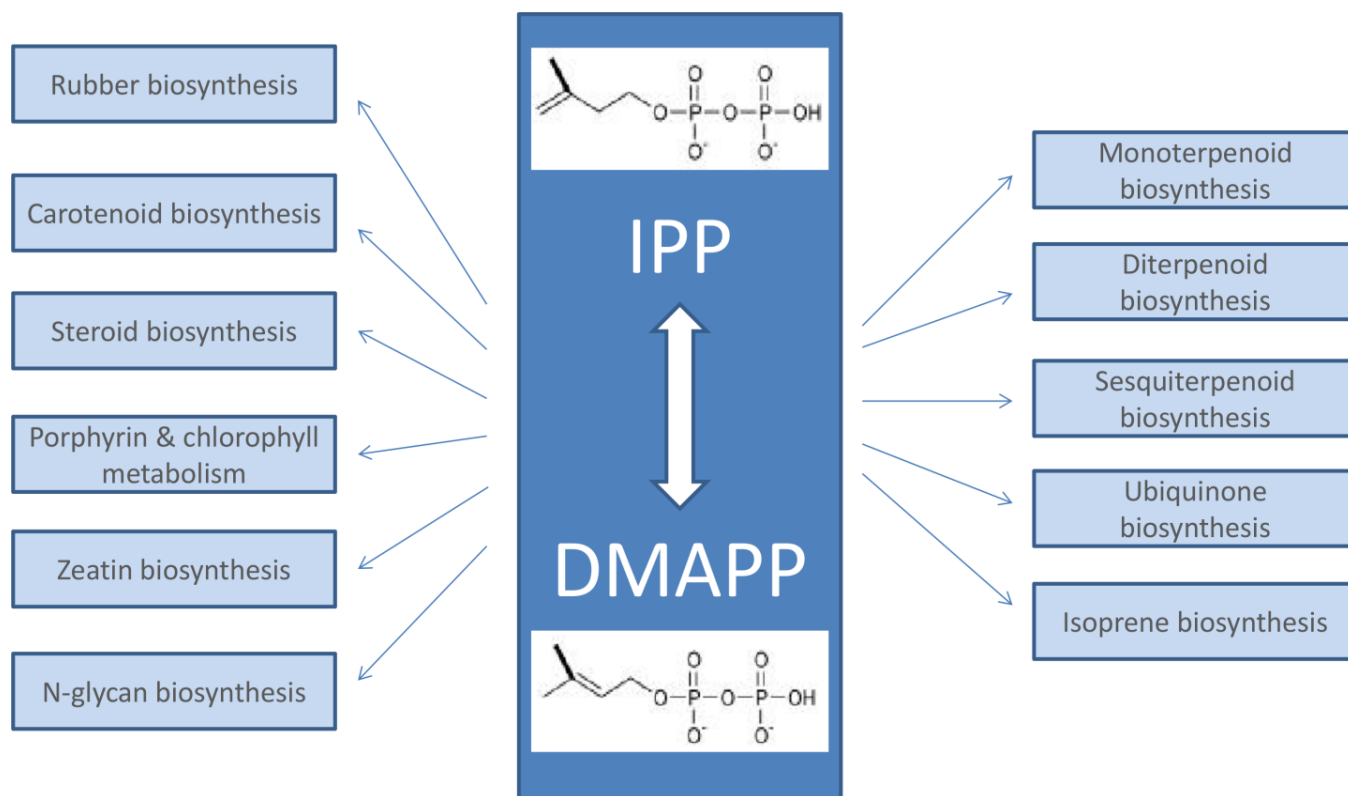


Figure 4. Biosynthesis of different isoprenoid classes, all derived from isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Figure adapted from [129].

2.3. Mevalonate versus nonmevalonate pathway

In the 1990s, the nonmevalonate pathway was identified as an alternative pathway for isoprenoid biosynthesis (Figure 5; adapted from [130]).[131] The final products of both biochemical pathways are IPP and its isomer DMAPP, the building blocks for further synthesis of isoprenoid compounds.[128, 132]

The mevalonate pathway starts with the condensation of three acetyl-coenzyme A (acetyl-CoA) molecules to 3-hydroxy-3-methylglutaryl CoA (HMG-CoA). Consequently, HMG-CoA is reduced by HMG-CoA reductase (HMGCR) to mevalonate, which is in turn converted to IPP and its isomer DMAPP.[133]

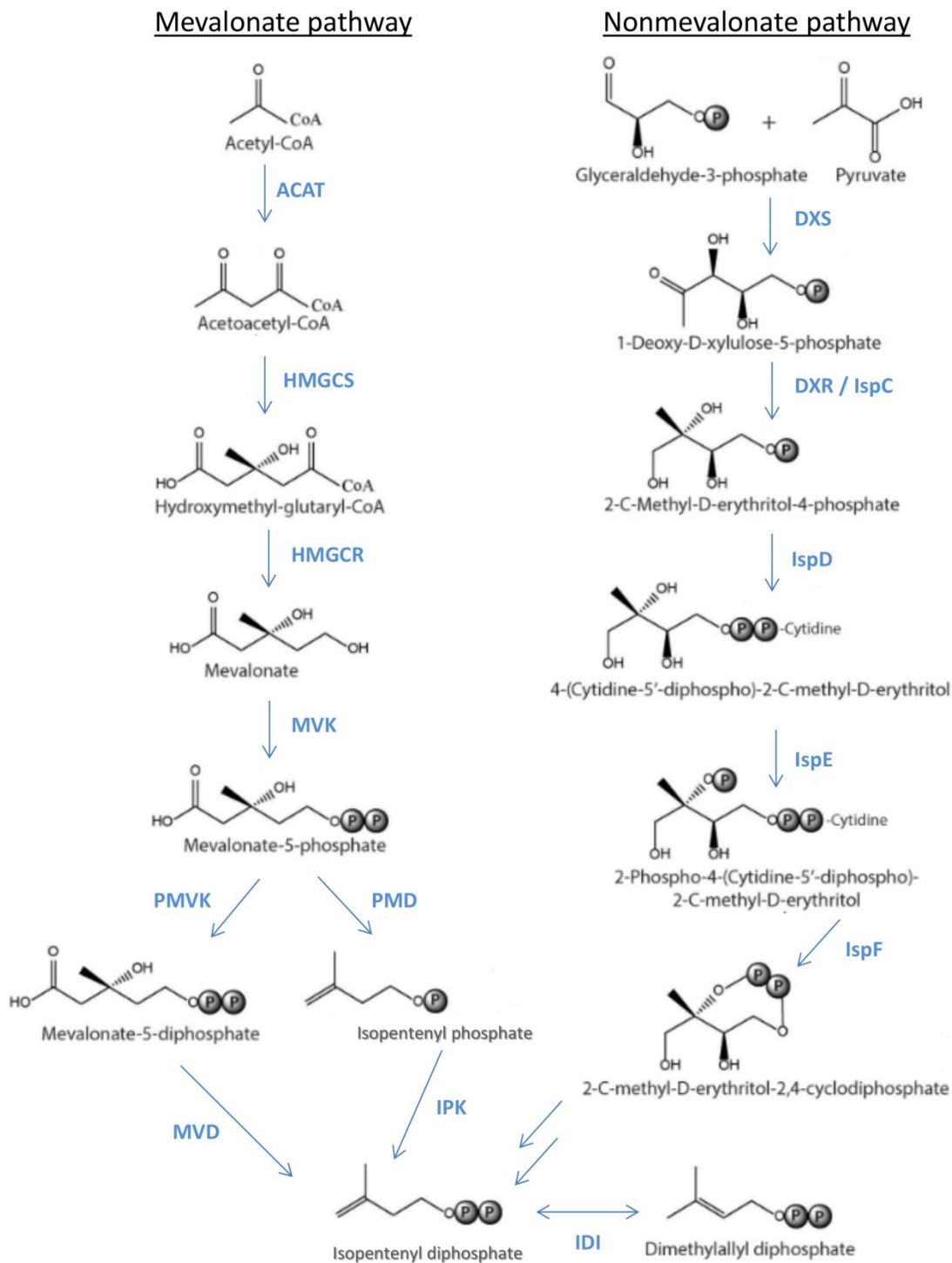


Figure 5. Schematic overview of mevalonate and nonmevalonate (MEP) pathways. ACAT; Acetyl-CoA acetyltransferase, HMGCS; HMG-CoA synthase, HMGCR; HMG-CoA reductase, MVK; mevalonate kinase, PMVK; Phosphomevalonate kinase, PMD; Phosphomevalonate decarboxylase, MVD; diphosphomevalonate decarboxylase, IPK; Isopentenyl phosphate kinase, DXS; DOXP-synthase, DXR/IspC; DOXP-reductase, IspD; CDP-ME synthase, IspE; CDP-ME kinase, IspF; MER-cPP synthase and IDI; isopentenyl diphosphate isomerase. Adapted from [130]

The nonmevalonate pathway (DOXP or MEP-pathway) starts with the condensation of D-glyceraldehyde-3-phosphate and pyruvate to DOXP, catalysed by the enzyme DOXP synthase (DXS). Next, DOXP is converted to 2-C-methyl-D-erythritol-4-phosphate (MEP) by DOXP reductoisomerase (DXR). Consequently, 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME) is formed in a reaction mediated by CDP-ME synthase (IspD). CDP-ME is then phosphorylated by CDP-ME kinase (IspE), with the formation of CDP-ME-2-phosphate. This diphosphate is converted by MER-cPP synthase (IspF) to 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (MER-cPP) and this cyclic diphosphate molecule gives rise to the synthesis of IPP and DMAPP.[113]

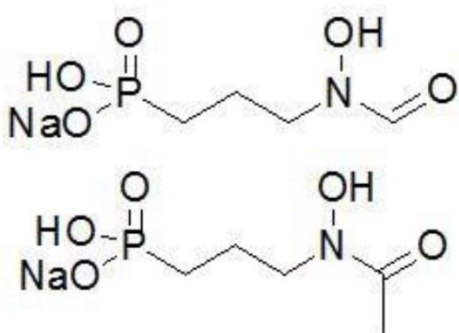


Figure 6. Structures of fosmidomycin (top) and FR900098 (bottom)

The rate-limiting step in the nonmevalonate pathway is the conversion of DOXP to MEP, catalysed by DXR, the second enzyme in the pathway. Fosmidomycin and FR900098 (Figure 6), the N-acetyl derivative of fosmidomycin, are effective inhibitors of purified DXR from several bacteria but have limited effect on whole bacterial cells.[116, 117] A possible explanation

for this limited antibacterial effect is the low bioavailability and poor cellular uptake by passive diffusion into bacterial cells due to the high polarity and negative charge of these molecules at physiological pH.[134, 135]

2.4. Nonmevalonate pathway as a target for antibacterial chemotherapy

Fosmidomycin and FR900098 are natural products isolated from *Streptomyces lavendulae* and *Streptomyces rubellomurinus* and mimic DOXP, the substrate of DXR.[118, 119, 136, 137] The activity of FR900098 is twice as high as that of fosmidomycin against *P. falciparum* *in vitro* and against *Plasmodium vinckei* in a mouse infection model.[120] Both fosmidomycin and FR900098 require hexose-phosphate or glycerol-3-phosphate transporters (GlpT) for accurate uptake into bacterial cells, just as their structural analogue fosfomycin.[119, 138]

P. falciparum and *M. tuberculosis* are the causative agents of malaria and human tuberculosis (TB), respectively, two infectious diseases which require urgent attention according to the World Health Organization (WHO).[139-141] For both organisms, most of the available antibiotic treatments lost their efficacy because of the development of multi-drug-resistant (MDR) strains and also extensively-drug-resistant (XDR) strains of *M. tuberculosis* have been described.[140, 141] Fosmidomycin and FR900098 show potent inhibition of isolated DXR from *E.coli*, *P. falciparum* and *M. tuberculosis* and demonstrate growth inhibition of *P. falciparum* cultures. Both compounds are inactive against *M. tuberculosis*, due to the lack of a GlpT transporter required for fosmidomycin uptake. Additionally, the highly lipophilic nature of the *M. tuberculosis* membrane structure prevents fosmidomycin and FR900098 (strongly polar compounds) to penetrate the mycobacterial cell. *E. coli* does possess GlpT transporters, making it possible for fosmidomycin to traverse into the cell and inhibit bacterial growth.[118, 119, 139]

Fosmidomycin and FR900098 lack activity against species of the Bcc, although GlpT transporters are encoded in their genome. Unfortunately, during treatment with fosmidomycin and FR900098, the upregulation of a fosmidomycin resistance gene (*fsr*), encoding an efflux pump, is observed. This finding helps to explain the limited antibacterial activity against Bcc strains.[127]

2.5. Molecular basis of DXR inhibition by fosmidomycin

The DXR enzyme from *P. falciparum* (*PfDXR*) is a homo-dimer with each subunit consisting of a NADPH-binding domain and a divalent metal ion (Mn^{2+} or Mg^{2+}). Both NADPH and a divalent cation are required for the DXR-mediated transformation of DOXP to MEP. A similar organization of DXR has been observed in other organisms.[142-144]

Each subunit of DXR comprises two large domains separated by a large gap where a deep pocket, a linker region and a small C-terminal domain are located (Figure 7 [142]). Both large domains have a specific function; one domain is responsible for NADPH binding and the second domain provides catalytic groups for metal and substrate binding. Fosmidomycin binds in the catalytic domain forming an inhibitor-bound quaternary complex.[142]

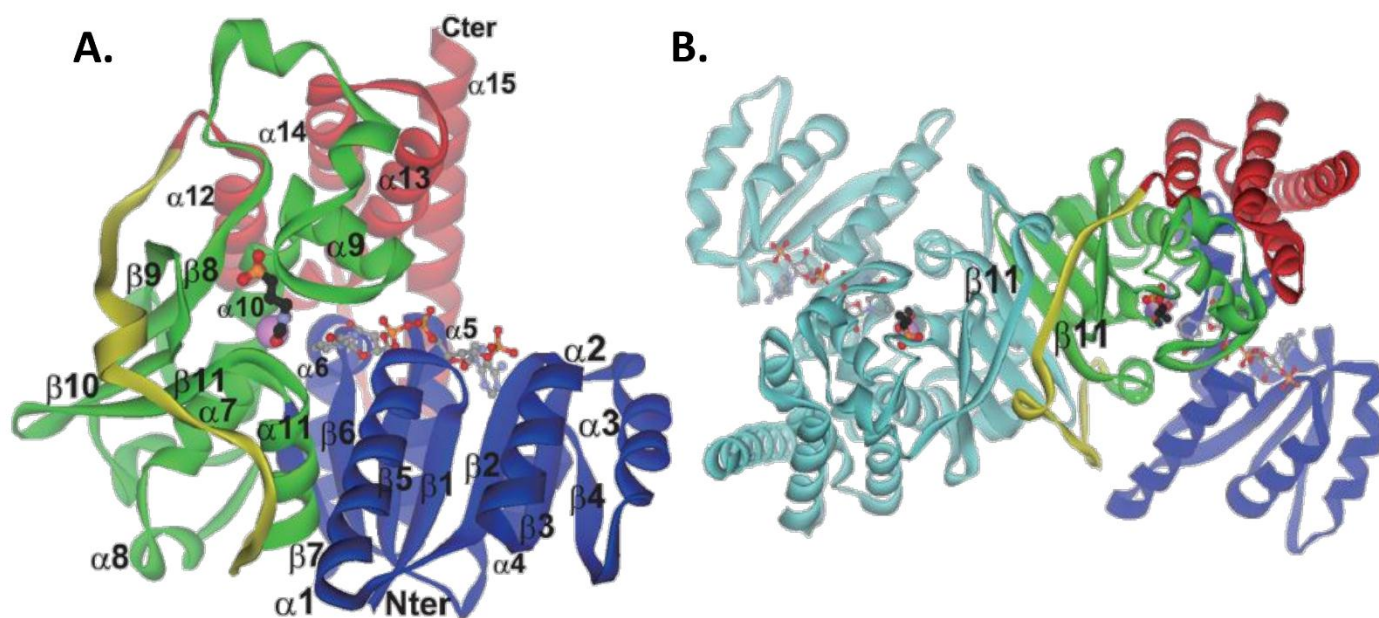


Figure 7. The three-dimensional structure of *Pf*DXR. The structure of one subunit is depicted in A., with the NADPH-binding domain coloured in blue, the catalytic domain in green, the linker region in yellow and the C-terminal domains in red. Fosmidomycin and NADPH are represented in a ball-and-stick model, with the carbon atoms coloured in black and grey, respectively. Structure B. shows the overall DXR-structure, with one subunit shown in identical colours as A. and the other subunit coloured in cyan.[142]

2.6. Fosmidomycin derivatives

Limited antibacterial activities of fosmidomycin and FR900098 can be attributed to poor cellular uptake by passive diffusion due to their high polarity. Attempts have been made to improve this cellular uptake by synthesizing more lipophilic inhibitors of the nonmevalonate pathway. First, fosmidomycin derivatives with modified physicochemical properties (e.g. introduction of an aromatic functional group) were synthesized.[133, 145, 146] Both the hydroxamate and phosphonate group of fosmidomycin are essential for their activity, but modifications to the three-carbon spacer linking these moieties have shown to lead to potent analogues. Substitutions at the α -position of fosmidomycin have been widely

explored in the past, but β -modifications have only recently received more attention (Figure 8 [139]).[133, 139]

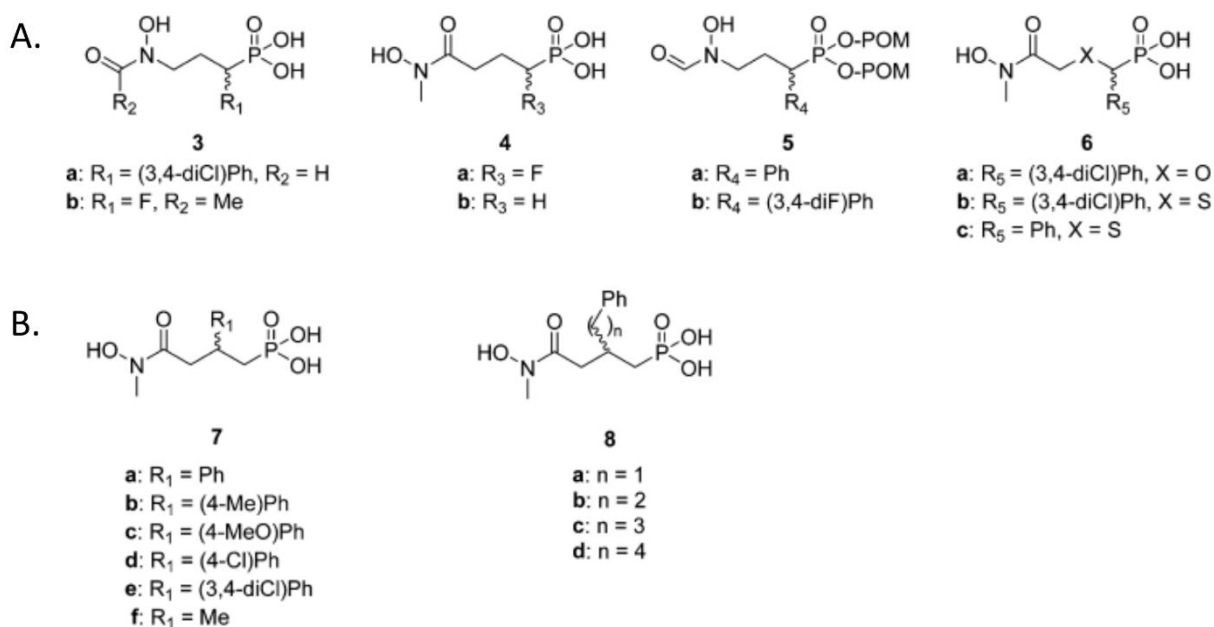


Figure 8. Fosmidomycin analogues with A. α -modifications and B. β -modifications at the three-carbon spacer.[139]

Secondly, attempts have been made to improve cellular uptake and bioavailability (currently <30%) by synthesizing prodrugs of fosmidomycin and its analogues (Figure 9 [137]), for example through the ProTide approach.[134, 135] The principle of synthesizing prodrugs is masking the charged phosphonate or hydroxamate functional groups with variable aryl or amino ester groups to increase cellular uptake. Once inside the bacterial cell, the prodrugs are hydrolysed by non-specific esterases, releasing the functional product.[119, 135, 137]

A.

Compound Name	Structure
Fosmidomycin	
FR900098	
Compound 1	
Compound 2	
Compound 3	

B.

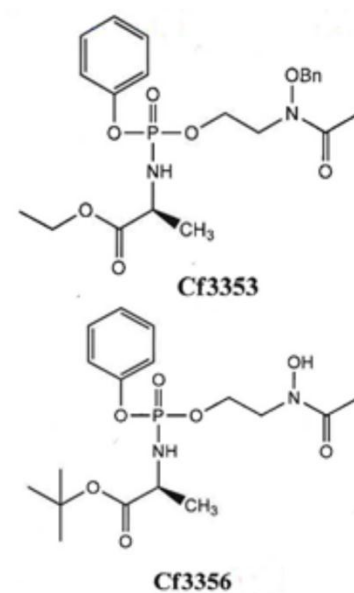


Figure 9. A. Lipophilic prodrugs of fosmidomycin and FR900098 [137] and B. ProTide prodrugs of fosfoxacin (Chofo R., Van Calenbergh S., unpublished data).

One sometimes finds what one is not looking for

Sir Alexander Fleming

3. β -LACTAM RESISTANCE

3.1. Introduction

The first β -lactam antibiotic was discovered in 1921, when Alexander Fleming found a mould (*Penicillium rubrum*) producing a substance with antimicrobial activity against *Staphylococcus*, later identified as penicillin.[147]

β -lactam antibiotics are one of the major classes of drugs available to treat bacterial infections. This class of antibiotics contains several families, including penicillins, cephalosporins, carbapenems and monobactams, all characterized by a four-membered lactam ring within their structure, as shown in Figure 10.[148, 149] β -lactams irreversibly inhibit the action of penicillin-binding proteins (PBPs) involved in cell wall synthesis by binding and inactivation of an essential serine residue in the enzyme's active site.[147, 150] The bacterial cell wall consists of alternating N-acetyl muramic acid (NAM) and N-acetylglucosamine (NAG) units, linked by transglycosidases, as shown in Figure 11 ([151]). Each NAM unit is attached to a pentapeptide by PBPs which catalyse the cross-linking of two D-alanine-D-alanine-NAM pentapeptides. This cross-linking is responsible for the structure and rigidity of the cell wall. As β -lactam rings are sterically similar to D-alanine-D-alanine of the NAM pentapeptide, the PBPs use the β -lactams as building blocks during synthesis of the cell wall. Consequently, the PBP is acylated, rendering it unable to catalyse further cross-linking reactions and ultimately leading to cell lysis, as shown in Figure 12.[65, 152, 153]

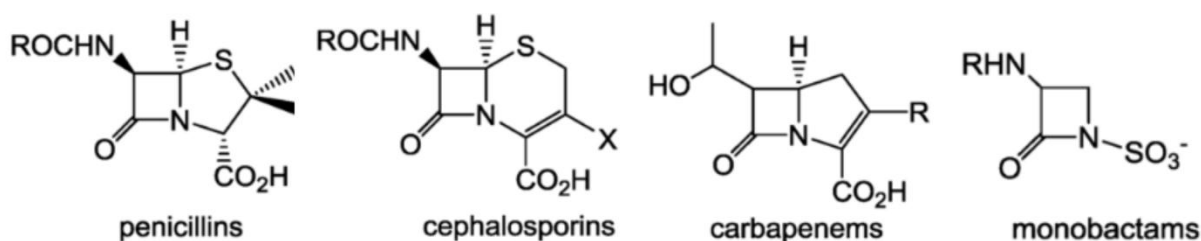


Figure 10. Structure of different β -lactam antibiotic families.[148,149]

The frequent use of β -lactam antibiotics has led to the development of broad resistance to most β -lactams, for example in the well-known methicillin-resistant *S. aureus* (MRSA). Resistance to β -lactams is most commonly mediated by expression of β -lactamases, efflux pumps or modifications of PBPs.[46, 47]

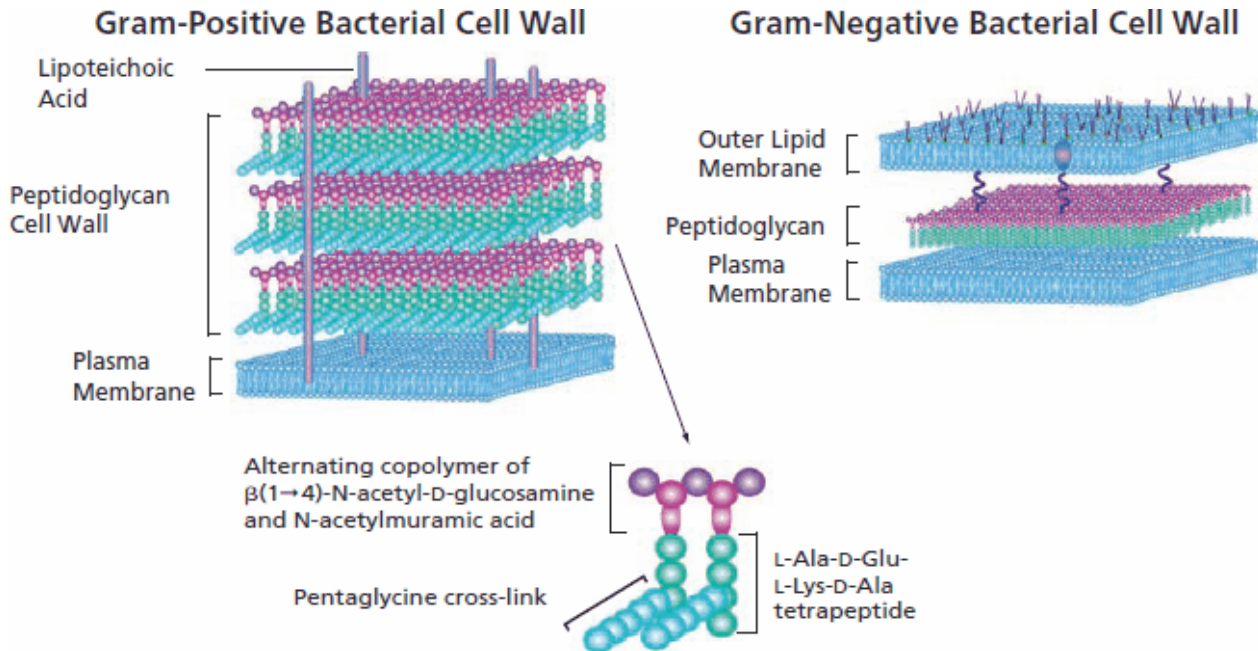


Figure 11. Organization of the cell wall of Gram-positive and Gram-negative bacteria.[151]

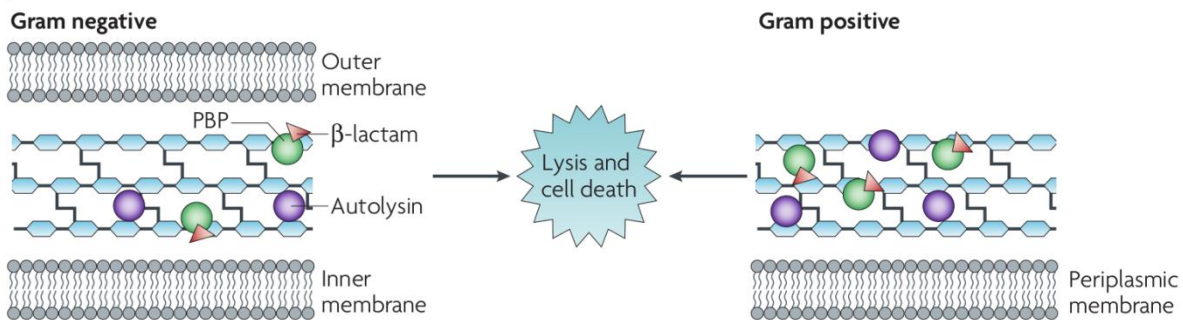


Figure 12. Mechanism of action of β -lactam antibiotics in Gram-negative and Gram-positive bacterial cells. β -lactams inhibit transpeptidation by binding to penicillin-binding proteins (PBPs) on maturing peptidoglycan strands. This decrease in peptidoglycan leads to cell wall defects and eventually cell lysis and cell death.[153]

3.2. Classification of β -lactam antibiotics

Penicillins are the most widely prescribed antibiotics worldwide, both for community-acquired (CA) infections, and for hospital-acquired (HA) infections. Commercially available penicillins (including

amoxicillin, piperacillin and ticarcillin), are usually prescribed for treatment of Gram-positive infections.[154]

Members of a second β -lactam family, the cephalosporins, are used both in outpatient settings (for treatment of Gram-positive and Gram-negative infections) and in hospital settings for prophylaxis of infection during surgery. The cephalosporin family includes cephalexin, ceftriaxone, ceftazidime, cefdinir and cefepime as most frequently prescribed antibiotics.[150]

Carbapenems, e.g. meropenem, imipenem-cilastatin, ertapenem and doripenem, are “last resort antibiotics” for many serious infections and can only be used by parenteral administration.[155, 156]

The fourth and last group of β -lactam antibiotics, monobactams, are only active against Gram-negative bacteria, with aztreonam as the commonly used antibiotic for *Pseudomonas* and other Gram-negative infections. Only intravenous (IV) or intramuscular (IM) administration is used in hospital environments.[150]

β -lactamase inhibitors are regularly prescribed in combination with β -lactam antibiotics, to protect the latter against hydrolysis by β -lactamase enzymes, one of the most common mechanism of resistance against β -lactam antibiotics. Currently available inhibitors are clavulanic acid, tazobactam, sulbactam and the recently discovered avibactam (structures shown in Figure 13).[65, 157]

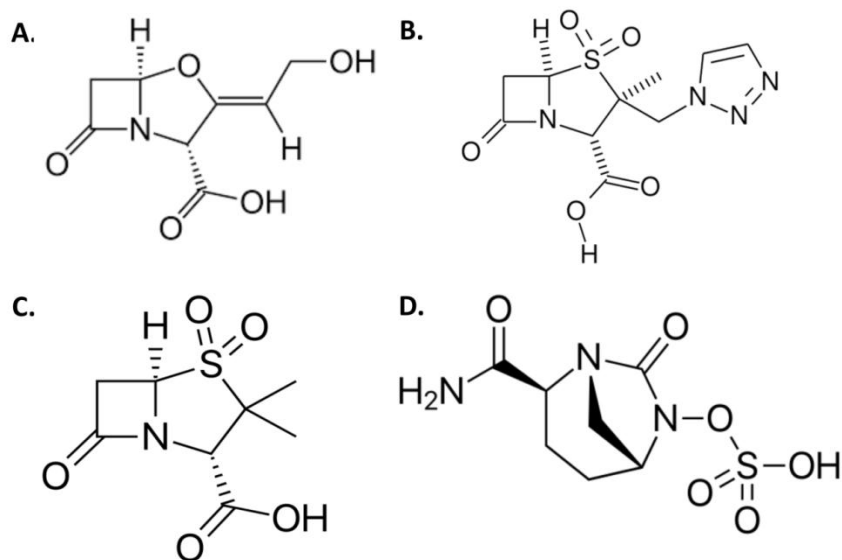


Figure 13. Chemical structures of β -lactamase inhibitors; A. clavulanic acid, B. tazobactam, C. sulbactam and D. avibactam.[65,157]

3.3. Classification of β -lactamases

The first β -lactamase enzyme was identified in 1940 (even before the clinical use of penicillin) as penicillinase in *E. coli*, and similar enzymes have since been identified in a variety of other bacteria, including species of the Bcc.[65, 158] β -lactamases are able to inactivate β -lactam antibiotics by hydrolysing the β -lactam ring.

Two molecular classification schemes for β -lactamases are currently used: the Ambler molecular classification scheme (based on amino acid sequences and structural similarities) and the Bush-Jacoby-Medeiros scheme (according to functionality or activity against β -lactam antibiotics).[157, 159, 160]

In the Ambler scheme (Table 2), four classes of β -lactamases are recognized; class A through D.[65] Enzymes belonging to class A, C and D have a serine active site while class B enzymes are metallo- β -lactamases (MBLs) that require Zn^{2+} as a cofactor.[65, 161] The most commonly encountered β -lactamases are class A enzymes with TEM and SHV enzymes regularly found in Gram-negative bacteria (e.g. *E. coli* and *Klebsiella* spp.), as well as CTX-M extended-spectrum β -lactamases (ESBLs) and *K. pneumoniae* carbapenemases (KPCs) which are now also frequently encountered. Class B β -lactamases (MBLs) pose a particular challenge for medicinal chemists and clinicians because they can hydrolyse carbapenems and none of the available inhibitors can effectively inhibit members of this class.[162, 163] The recently discovered New-Delhi metallo β -lactamase (NDM) causes resistance to almost every antibiotic (except colistin and tigecycline), including imipenem and meropenem, and is one of the major reasons for antibacterial resistance in *Enterobacteriaceae* (e.g. *E. coli* and *K. pneumoniae*).[164] NDM-1-carrying bacteria were first described in New Delhi, India in 2009 but have since mid-2010 also been introduced in other countries including the United States and UK.[165] Class C or AmpC β -lactamases are mostly chromosomally encoded, although the prevalence of plasmid-mediated AmpC-enzymes is also increasing. Oxacillinases or class D β -lactamases are active against a broad range of β -lactam antibiotics, besides carbapenems.[46, 65, 161]

Table 2. Classification of β -lactamases according to the Ambler molecular classification scheme.[65]

Molecular class	Main substrates	Active β -lactamase inhibitors?	Examples
A Serine penicillinases	penicillins cephalosporins monobactams carbapenems	Clavulanic acid Tazobactam Sulbactam Avibactam	TEM ESBL SHV CTX
B Metallo- β -lactamases	Most β -lactams (incl. carbapenems)	/	NDM
C Cephalosporinases	penicillins cephalosporins monobactams	Avibactam	AmpC
D Oxacillinases	penicillins oxacillins	Avibactam (inhibits some class D enzymes)	OXA

The β -lactamase classification scheme proposed by Bush, Jacoby and Medeiros mostly correlates with the Ambler molecular classification scheme. This functional classification takes substrate and inhibitor profiles into account and groups β -lactamase enzymes according to phenotypic characteristics in clinical isolates.[160] In 2010, Bush and Jacoby published an updated classification system (Table 3) including group 1 (Ambler class C) cephalosporinases, group 2 (Ambler classes A and D) broad-spectrum, inhibitor-resistant and extended-spectrum β -lactamases and serine carbapenemases, and group 3 (Ambler class 3) metallo- β -lactamases.[159]

Table 3. Updated functional β -lactamase classification scheme according to Bush and Jacoby and correlating Ambler classes.[159]

Bush-Jacoby class	Ambler class	Main substrates	Examples
1	C	cephalosporins	<i>E. coli</i> AmpC
1e	C	cephalosporins	CMY-37
2a	A	penicillins	PC-1
2b	A	penicillins early cephalosporins	TEM-1, TEM-2, SHV-1
2be	A	extended-spectrum cephalosporins monobactams	TEM-3, SHV-2 PER-1, VEB-1
2br	A	penicillins	TEM-30, SHV-10
2ber	A	extended-spectrum cephalosporins monobactams	TEM-50
2c	A	carbenicillin	PSE-1
2ce	A	carbenicillin, cefepime	RTG-4
2d	D	cloxacillin	OXA-1, OXA-10

2de	D	extended-spectrum cephalosporins	OXA-11, OXA-15
2df	D	carbapenems	OXA-23, OXA-48
2e	A	extended-spectrum cephalosporins	CepA
2f	A	carbapenems	KPC-2
3a	B	carbapenems	IMP-1, VIM-1
3b	B	carbapenems	CphA

3.4. β -lactam resistance in Bcc species

Resistance against β -lactam antibiotics in Bcc bacteria appears to be the result of two synergistic methods; decreased drug access caused by decreased porin content and the induction of chromosomal β -lactamases.[166-169] Porins play a fundamental role in antibiotic uptake into Bcc species and β -lactam resistant *B. cenocepacia* isolates of CF patients were shown to have a decreased porin content.[168, 169] Resistance to β -lactam antibiotics such as ceftazidime in Bcc species mostly caused by class A β -lactamases, first described in 1997 and now named PenB and PenR.[167, 170] However, a recent study in *B. cenocepacia* also identified ceftazidime-driven mutations caused by AmpC β -lactamases.[171] Elucidation of the whole genome sequence of *B. cenocepacia* LMG 16656 (J2315) led to the identification of at least 21 different β -lactamases encoded in this Bcc strain (shown in Table 4).[45, 65] These enzymes were shown to confer resistance against ceftazidime, clavulanic acid and a variety of other β -lactams.[45, 172] Interestingly, all classes of β -lactamases are represented in this *B. cenocepacia* strain but most enzymes (15 out of 21) belong to the metallo β -lactamase (MBL) family. As mentioned before, MBL enzymes pose particular challenges since none of the available β -lactamase inhibitors is able to inhibit members of this β -lactamase class.[162]

In conclusion, bacterial resistance occurs as a consequence of various different mechanisms and constitutes a continuous challenge for researchers, clinicians and pharmaceutical companies to provide counter-acting agents in the fight against infections.

Table 4. β -lactamases encoded on the genome of *B. cenocepacia* LMG 16656 (J2315); the locus tag, replicon (Chr. Indicates 'chromosome') and description of the gene product.[65]

Locus Tag	Replicon	Product description
BCAS0156	Chr. 3	AmpC; class C beta-lactamase/ family S12 serine peptidase
BCAM0393	Chr. 2	class D putative acetyltransferase/putative beta-lactamase
BCAM2596	Chr. 2	beta-lactamase family protein/ family S12 serine peptidase
BCAM2643	Chr. 2	beta-lactamase family protein/ family S12 serine peptidase
BCAM2165	Chr. 2	PenA; putative beta-lactamase/ family S11 serine peptidase
BCAM1779	Chr. 2	class A putative beta-lactamase
BCAS0557	Chr. 3	metallo β L superfamily protein
BCAL0673	Chr. 1	metallo β L superfamily protein
BCAM0300	Chr. 2	metallo β L superfamily protein
BCAL0158	Chr. 1	metallo β L superfamily protein
BCAL2183	Chr. 1	metallo β L superfamily membrane protein
BCAM2668	Chr. 2	metallo β L superfamily protein
BCAS0689	Chr. 3	metallo β L superfamily protein
BCAL1818	Chr. 1	metallo β L superfamily protein
BCAL2584	Chr. 1	metallo β L superfamily protein
BCAM2120	Chr. 2	metallo β L superfamily protein
BCAS0034	Chr. 3	metallo β L superfamily protein
BCAL2165	Chr. 1	metallo β L superfamily protein
BCAM1430	Chr. 2	metallo β L superfamily protein
BCAS0179	Chr. 3	metallo β L superfamily protein
BCAL0386	Chr. 1	putative exported metallo β L superfamily protein

CHAPTER II. OBJECTIVES

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Infectious diseases are still one of the main sources of mortality with respiratory infections, malaria, tuberculosis and HIV/AIDS belonging to the top 10 causes of death worldwide.[173, 174] The introduction of antibiotics since the 1930s provided us with a weapon to fight microbial infections, but after the golden era of antibiotic drug discovery there has been a 'discovery void'. [175] Furthermore, the utility of currently available antibiotics has become increasingly compromised due to the development of antimicrobial resistance.[87, 176] Therefore, research should focus with high priority on the development of antibiotics with a novel mechanism of action.[176, 177] Bacteria belonging to the *Burkholderia cepacia* complex or Bcc are important human pathogens in patients with cystic fibrosis and especially require attention because of their innate intrinsic resistance against many antimicrobial drugs.[7, 73] The main objective of my work was to explore two novel strategies to tackle Bcc infections, including the nonmevalonate pathway for isoprenoid biosynthesis and combination therapy of β -lactam antibiotics with β -lactamase inhibitors.

First, the *in vitro* antibacterial activity of potential inhibitors of the nonmevalonate pathway was determined against Bcc species, *P. aeruginosa*, *Propionibacterium acnes* and *Mycobacterium smegmatis*. Furthermore, different resistance mechanisms against these inhibitors were identified and methods to circumvent them were examined.

Because of the low *in vitro* activity of the compounds tested in this first part, the essentiality of different genes of the nonmevalonate pathway in *B. cenocepacia* was determined. Therefore, we constructed conditional knock-down mutants of *B. cenocepacia* where a rhamnose-inducible promoter controlled the expression of the candidate essential genes. Consequently, the expression kinetics, phenotypic characteristics and virulence of these conditional mutants were explored in appropriate *in vitro* and *in vivo* models.

Finally, we investigated the ability of β -lactamase inhibitors to restore the antibacterial activity of β -lactam antibiotics against members of the Bcc. Moreover, the β -lactamase activity after different treatment conditions was determined to validate the role of β -lactamase production in resistance against β -lactams. Both older and newer inhibitors were included in this study, including clavulanic acid, sulbactam, tazobactam and avibactam.

CHAPTER III. EXPERIMENTAL WORK

1. THE NONMEVALONATE PATHWAY AS NOVEL TARGET FOR CHEMOTHERAPY AGAINST MULTIDRUG-RESISTANT BACTERIA

1.1. Introduction

In contrast to the more common mevalonate pathway, some bacteria use an alternative pathway for isoprenoid biosynthesis, the 1-deoxy-D-xylulose-5-phosphate (DOXP) or nonmevalonate pathway. Since this pathway is not present in human cells, it is a potential target for the development of antibacterial agents against multidrug-resistant bacteria. The rate-limiting step in this metabolic pathway is the conversion of 1-deoxy-D-xylulose-5-phosphate (DOXP) to 2-C-methyl-D-erythritol-4-phosphate (MEP), catalyzed by the enzyme DOXP-reductoisomerase (DXR). Previous studies confirmed that fosmidomycin and its acetylated analogue FR900098 are active inhibitors of DXR from *E. coli*, *M. tuberculosis* and *P. falciparum*. [118, 121, 139] Fosmidomycin and FR900098 can be used for the treatment of malaria caused by *P. falciparum*, but relatively few studies have explored the use of inhibitors of the nonmevalonate pathway in bacteria. [120] So far, investigation of the DOXP pathway as a potential antibacterial target was mainly restricted to *E. coli* and *M. tuberculosis*. Fosmidomycin and FR900098 are effective inhibitors of purified DXR from several bacteria but have limited effect on whole bacterial cells. [116, 117] A possible explanation for this limited antibacterial effect is the poor cellular uptake by passive diffusion into bacterial cells due to the high polarity of these molecules. Several attempts have been made to improve limited cellular uptake, by modification of physicochemical properties or the synthesis of prodrugs of fosmidomycin. [133-135, 145, 146]

1.2. Objectives

A first goal of the present study was to evaluate the activity of potential new inhibitors of the nonmevalonate pathway against various bacterial pathogens. The second goal was to identify different resistance mechanisms against fosmidomycin analogues and other DOXP inhibitors and to examine how to circumvent these mechanisms.

1.3. Materials and methods

1.3.1. Bacterial strains and culture conditions

The following bacterial strains were used for MIC determination: *P. aeruginosa* PAO1, several members of the Bcc (*B. cenocepacia* LMG 16656, *B. cepacia* LMG 1222 and *B. multivorans* LMG 13010), *P. acnes* LMG 16711, *M. smegmatis* ATCC 607 and ATCC 700084. *E. coli* ATCC 8739, ATCC 25922, and K-12 were included as control strains. In further experiments, additional Bcc strains were included: *Burkholderia ambifaria* LMG 19467 and LMG 19182, and *B. multivorans* LMG 18825 and LMG 18822. One additional *P. acnes* strain (*P. acnes* LMG 16712) and two additional mycobacterial strains (*M. smegmatis* LMG 8190 and *Mycobacterium phlei* LMG 4056) were also included. All strains were obtained from the BCCM/LMG Bacteria collection (Ghent, Belgium) or from the ATCC collection (Manassas, VA, USA). Bacterial cultures were stored at -80°C in Microbank vials (Prolab Diagnostics, Richmond Hill, ON, Canada). *P. aeruginosa*, Bcc and *E. coli* strains were subcultured twice on Luria-Bertani agar (LBA; LabM Limited, Heywood, UK) before use and incubated aerobically at 37°C. *P. acnes* strains were subcultured on Brain Heart Infusion agar (BHIA; Sigma-Aldrich, Saint-Louis, USA) and anaerobically incubated at 37°C. Strains of *M. smegmatis* were subcultured on Middlebrook 7H10 agar (MBA) supplemented with 10% OADC enrichment (BD, Erembodegem, Belgium) and aerobically incubated at 37°C.

1.3.2. Antibacterial compounds

The DXR-inhibitors fosmidomycin (Invitrogen, Thermo Fisher Scientific, Merelbeke, Belgium) and FR900098 (Sigma-Aldrich) were tested together with a selection of β -substituted analogues and nucleoside phosphoramidate prodrugs (ProTides), synthesized as previously described and provided by Prof S. Van Calenbergh (Ghent University, Belgium).[119, 139, 178, 179] The DXS-inhibitors clomazone and 4(3H)-pyrimidinone were obtained from Sigma-Aldrich and 5-ketoclomazone was purchased from Tebu-Bio (Le Perray-en-Yvelines, France). The thiazolopyrimidines ritanserine and ketanserine (both purchased from Sigma-Aldrich) were included as IspF-inhibitors, together with the structurally related molecules altanserine, setoperone (both obtained from ABX Chemicals, Radeberg, Germany) and IspF-inhibitor-1 (obtained from Echelon-Biosciences, Salt Lake City, UT, USA). All chemical structures are shown in Figure 14, Figure 15 and Figure 16.[180-182] Control antibiotics tobramycin, kanamycin, isoniazid and rifampicin were obtained from Sigma-Aldrich and fosfoxacin was kindly provided by Prof. S. Van Calenbergh.

Efflux pump inhibitors carbonyl-cyanide-3-chlorophenyl hydrazine (CCCP) and phenylalanine-arginine- β -naphthylamide (Pa β N) were both purchased from Sigma-Aldrich. The cell-penetrating peptides colistin, polymyxin B and polymyxin B nonapeptide (PMBN) were also obtained from Sigma-Aldrich and octaarginine was purchased from Eurogentec (Seraing, Belgium).

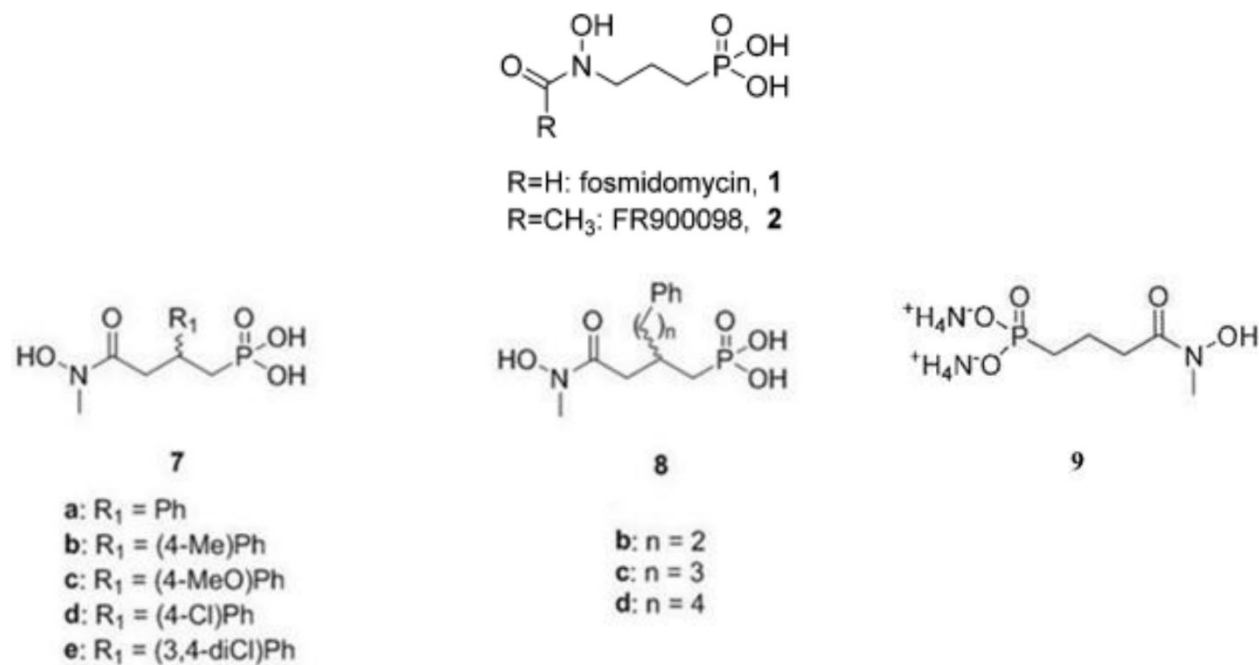


Figure 14. Fosmidomycin, FR900098 and β -substituted fosmidomycin analogues.[139]

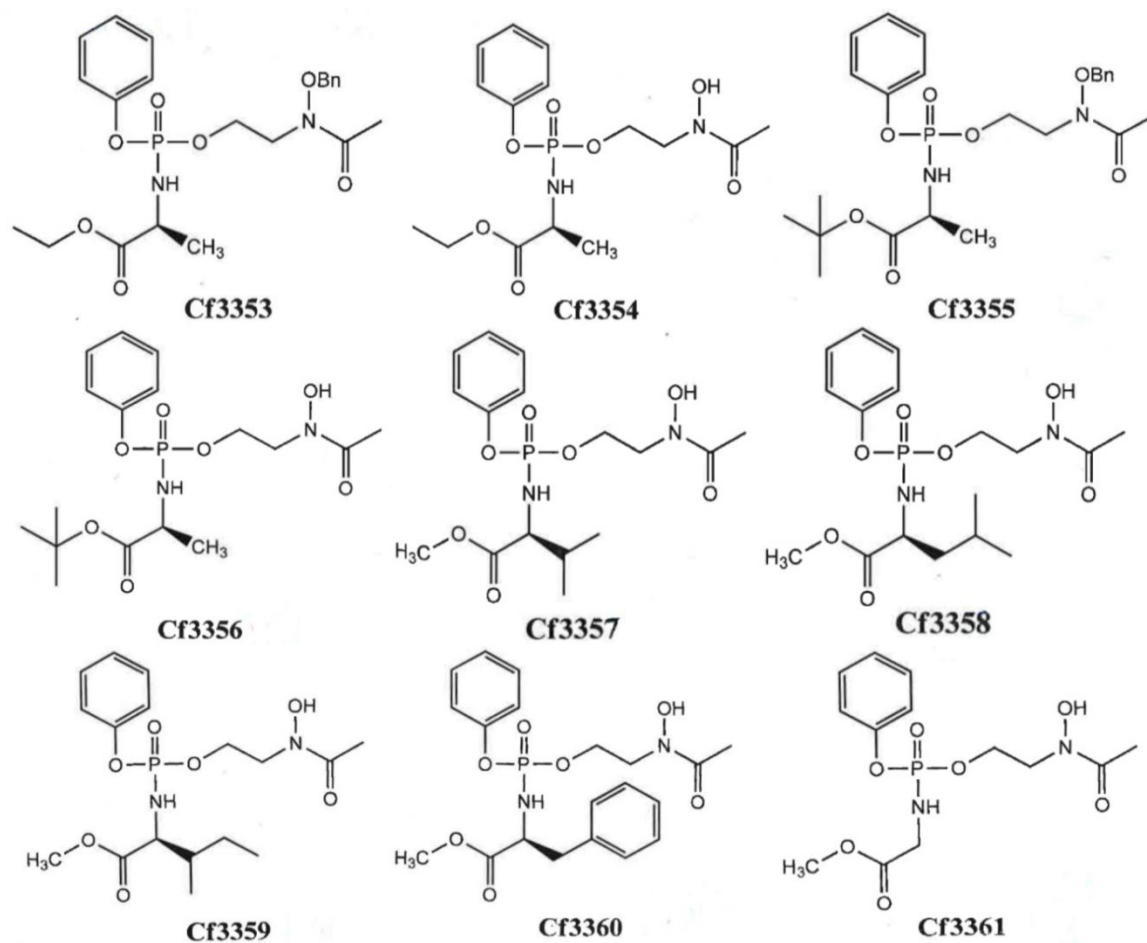


Figure 15. ProTide prodrugs of fosmidomycin, synthesized by Prof. S. Van Calenbergh and Dr. R. Chofoor (Ghent University, Belgium). [Chofoor R. & Van Calenbergh S., data not published]

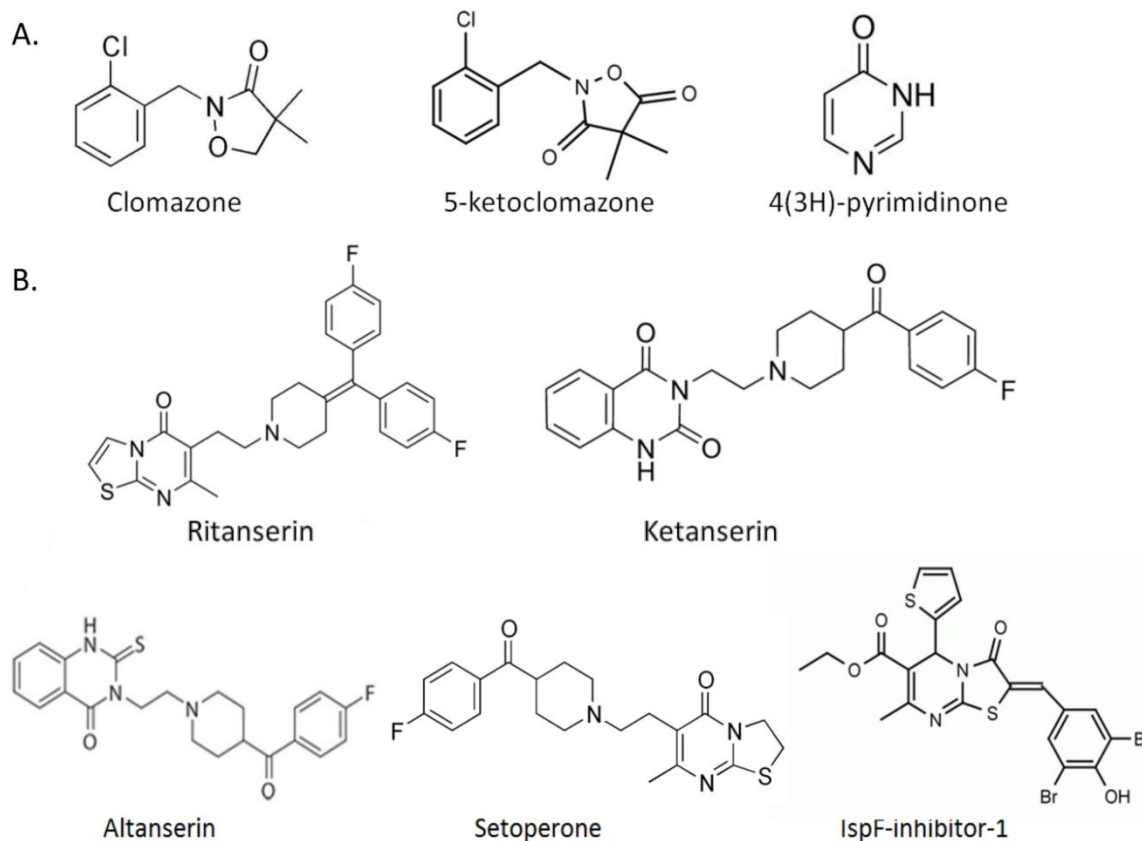


Figure 16. Chemical structures of A. DXS-inhibitors and B. IspF-inhibitors.[180-182]

1.3.3. Determination of the minimal inhibitory concentration (MIC)

Susceptibility screening of the test panel towards the nonmevalonate inhibitors was performed according to the EUCAST broth dilution guidelines.[183] MICs were determined in triplicate, using flat-bottomed 96-well microtitre plates (TPP, Trasadingen, Switzerland), with concentrations ranging from 0.49 μM to 250 μM . Mueller-Hinton broth (MHB) (BD) was used as growth medium for *P. aeruginosa*, *B. cenocepacia* and *E. coli*. Brain heart infusion broth (BHIB) (Sigma-Aldrich) was used for the growth of *P. acnes*, and *M. smegmatis* was grown in Middlebrook 7H9 broth (MBB) supplemented with 10% ADC enrichment (BD). For the susceptibility screening of ProTides, standard media were enriched with 5% horse blood (Biotrading, Keerbergen, Belgium) according to the EUCAST agar dilution guidelines.[184] Horse blood contains histidine triad nucleotide-binding protein 1 (HINT-1), which is essential for the intracellular enzymatic conversion of ProTides to their active form.[185]

Planktonic bacterial cultures were grown overnight in liquid medium at 37°C, adjusted with double-concentrated medium to obtain a final inoculum of approximately 5×10^5 colony forming units/ml (CFU/ml) and added to the 96-well plates. Plates were incubated for 24h at 37°C and optical density was determined at 590 nm using an Envision multilable plate reader (Perkin Elmer, Waltham, MA, USA). The MIC value is the lowest concentration of the antibiotic that completely inhibits bacterial growth. The MIC₅₀ is the lowest concentration that reduces bacterial growth to 50% of the untreated control.[183, 186]

In every experiment, fosmidomycin and FR900098 were included as controls. Kanamycin was added as additional control antibiotic for *E. coli*, *P. aeruginosa* and *P. acnes* strains, tobramycin for Bcc species, and isoniazid and rifampicin for *M. smegmatis* strains.

1.3.4. Evaluation of the bactericidal effect on mature biofilms

Biofilm cultures were grown in U-bottomed 96-well microtitre plates for 4h at 37°C, then washed twice with physiological saline (PS) and incubated for another 20h. Subsequently, mature biofilm cells were washed twice and treated with antibiotic solutions at 37°C for 24h. The bactericidal effect was determined by quantification of the percentage of bacterial survival after antibiotic treatment by resazurin staining or plating. Resazurin (CellTiter-Blue, Promega, Madison, WI, USA) is a non-fluorescent dye which can be converted to the fluorescent resorufin by metabolically active cells. The Envision multilable plate reader was used to determine the amount of formed resorufin, which is an indication for the amount of metabolically active cells present in the wells.[187] Quantification of the CFU was done using conventional plating on standard growth media, described above.[188, 189]

1.3.5. Determining the role of efflux pumps in resistance

To determine the role of efflux pumps in resistance, we determined MIC values of DOXP-inhibitors in the presence of efflux pump inhibitors CCCP and PaβN. First, we determined the intrinsic inhibitory effect of CCCP or PAβN on planktonic bacterial cultures. Therefore, MIC values were determined (with concentrations ranging from 0.195 μM to 100 μM) for *E. coli* ATCC 25922 and ATCC 8739, *P. aeruginosa* PAO1, *B. cenocepacia* LMG 16656, *B. cepacia* LMG 1222 and *B. multivorans* LMG 13010. Consequently, different concentrations (5 μM, 50 μM and 100 μM) of CCCP or PAβN were added to treatment with

fosmidomycin or β -substituted fosmidomycin-analogues. Finally, the differences in MIC values of the antibacterial agents in the presence or absence of an efflux pump inhibitor were determined.

1.3.6. Effect of increasing cellular permeability

Two different strategies were tested to enhance the cell penetration of our set of analogues. First, MIC values of the nonmevalonate inhibitors in CDM-G were determined against *B. cenocepacia* LMG 16656, *B. cepacia* LMG 1222, *B. multivorans* LMG 13010, *E. coli* ATCC 8739 and K-12. CDM-G is a Chemically Defined Medium in which the carbon source glucose is replaced by equimolar concentrations of glucose-6-phosphate, which increases cellular uptake, and glucose-1-phosphate, which has no effect on MIC values but is able to improve bacterial growth.

Second, the effect of the addition of cell-penetrating peptides (CPPs) was explored by performing checkerboard synergy assays in combination with the nonmevalonate inhibitors. Planktonic cells of *E. coli* K-12 and *M. smegmatis* ATCC 700084 were treated with fosmidomycin (concentration range 3.9 μ M – 250 μ M) in combination with several peptides; colistin (0.625 μ g/mL – 40 μ g/mL), polymyxin B (8 μ M – 512 μ M), octaarginin (1.25 μ g/mL – 80 μ g/mL) and polymyxin B nonapeptide (PMBN; 0.78 μ g/mL – 50 μ g/mL) (all purchased from Sigma-Aldrich). After addition of the bacterial suspension (50 μ l in a total volume of 100 μ l), the plate was incubated at 37°C for 24h and the absorbance was measured at 590 nm using an Envision multilable plate reader. Finally, the fractional inhibitory concentration (FIC) index was calculated.

$$\text{FIC index} = (A/\text{MIC}_A) + (B/\text{MIC}_B)$$

$$A = \text{MIC}_{\text{fosmidomycin} + \text{CPP}}$$

$$B = \text{MIC}_{\text{CPP} + \text{fosmidomycin}}$$

$$\text{MIC}_A = \text{MIC}_{\text{fosmidomycin}}$$

$$\text{MIC}_B = \text{MIC}_{\text{CPP}}$$

A FIC index smaller than 0.5 indicates a synergistic effect of the combination and a FIC greater than 4 indicates an antagonistic combination.[190-192]

1.3.7. Statistical analysis

Statistical analysis was performed by comparison of mean values through an independent samples t-test using SPSS 23.0 software (SPSS, Chicago, IL, USA).

1.4. Results and discussion

1.4.1. Determination of the MIC

1.4.1.1. β -substituted fosmidomycin analogues

The results for the susceptibility screening of our test panel in standard bacterial growth media are shown in Table 5. All MIC values of the β -substituted fosmidomycin analogues are $>250\ \mu\text{M}$, for each bacterial strain examined. These results indicate that the *in vitro* activity of the β -substituted fosmidomycin analogues is at best equal to the activity of fosmidomycin and FR900098. Chofo and colleagues investigated the inhibitory activity of these β -substituted fosmidomycin analogues on recombinant DXR-enzymes from *E. coli*, *M. tuberculosis* and *P. falciparum*. [139] In their research is stated that the 8-series compounds have better inhibitory activity on DXR than the 7-series compounds (see Figure 14), but fosmidomycin itself has a higher inhibitory activity on recombinant DXR, which could be a possible explanation for the lack of *in vitro* antibacterial activity up to $250\ \mu\text{M}$.

Table 5. MIC values (μM) of β -substituted fosmidomycin analogues and several control antibiotics (fosmidomycin, FR900098, kanamycin, tobramycin, isoniazid and rifampicin) in standard growth media.

Compound	MIC (μM)									
	GRAM (-)							GRAM (+)	MYCOBACTERIA	
	<i>E. coli</i>			<i>P. aeruginosa</i>	<i>B. cenocepacia</i>	<i>B. cepacia</i>	<i>B. multivorans</i>	<i>P. acnes</i>	<i>M. smegmatis</i>	
	ATCC 8739	ATCC 25922	K-12	PAO1	LMG 16656	LMG 1222	LMG 13010	LMG 16711	ATCC 607	ATCC 700084
7a	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
7b	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
7c	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
7d	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
7e	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
8b	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
8c	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
8d	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
9	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
FOS	0.98	250	7.8	62.5	250	>250	>250	>250	>250	>250
FR900098	7.8	62.5	15.6	>250	>250	125	>250	>250	>250	>250
Kanamycin	3.9	3.9	3.9	62.5	-	-	-	31.25	62.5	62.5
Tobramycin	3.9	3.9	-	1.95	0.98	1.95	3.9	-	-	-
Isoniazid	-	-	-	-	-	-	-	-	62.5	62.5
Rifampicin	-	-	-	-	-	-	-	-	31.25	31.25

1. 7a – 9; β -substituted fosmidomycin analogues, FOS; fosmidomycin. “-”; not determined.

1.4.1.2. ProTide prodrugs of fosmidomycin

ProTide prodrugs of fosmidomycin are expected to have higher *in vitro* antibacterial activity than fosmidomycin itself, due to their masked polarity. Unfortunately, MIC values of all ProTides tested are >250 μM for *E. coli* ATCC 8739, *P. aeruginosa* PAO1, *B. cenocepacia* LMG 16656, *B. cepacia* LMG 1222 and *B. multivorans* LMG 13010 (see Table 6) and thus higher (for *E. coli*, *P. aeruginosa* and *B. cenocepacia*) or equal (for *B. cepacia* and *B. multivorans*) than the MIC values of fosmidomycin and FR900098. The activity of these ProTide prodrugs against recombinant DXR-enzymes has not yet been investigated, which is purposeless because prodrugs are inactive molecules and have to be intracellularly converted to their active compounds. Several potential explanations exist regarding the lack of antibacterial activity of the prodrugs, including limited inhibitory activity against the target enzyme DXR, export of the prodrugs by efflux pumps and/or low membrane permeability.

Table 6. MIC values (μM) of ProTide fosmidomycin prodrugs (Cf3353 – Cf3361) and control antibiotics.

Compound	MIC (μM)				
	GRAM (-)				
	<i>E. coli</i> ATCC 8739	<i>P. aeruginosa</i> PAO1	<i>B. cenocepacia</i> LMG 16656	<i>B. cepacia</i> LMG 1222	<i>B. multivorans</i> LMG 13010
Cf3353	>250	>250	>250	>250	>250
Cf3354	>250	>250	>250	>250	>250
Cf3355	>250	>250	>250	>250	>250
Cf3356	>250	>250	>250	>250	>250
Cf3357	>250	>250	>250	>250	>250
Cf3358	>250	>250	>250	>250	>250
Cf3359	>250	>250	>250	>250	>250
Cf3360	>250	>250	>250	>250	>250
Cf3361	>250	>250	>250	>250	>250
FOS	0.98	62.5	250	>250	>250
Fosfoxacin	>250	>250	>250	>250	>250
Tobramycin	3.9	0.98	0.98	1.95	3.9

Cf3353 – Cf 3361; ProTide fosmidomycin prodrugs, FOS; Fosmidomycin.

The membrane permeability of fosmidomycin is expected to be low due to its high polarity, but cellular uptake of ProTide prodrugs would be facilitated by temporarily “masking” the polar functional groups of fosmidomycin.[134, 135] Unfortunately, the ProTide strategy does not increase *in vitro* antibacterial activity of fosmidomycin against the strains tested in this study.

1.4.1.3. DXS- and IspF-inhibitors

The susceptibility of our test panel towards DXS- and IspF-inhibitors is displayed in Table 7. Because no complete growth reduction was observed after treatment, susceptibility is expressed as MIC₅₀ which is the lowest concentration that reduces bacterial growth to 50% of the untreated control. Most inhibitors tested show MIC₅₀ values >250 µM. To our knowledge, the inhibitory activity of the tested compounds on recombinant enzymes has not yet been described. Only the thiazolypyrimidines ritanserine and ketanserine and their structural analogue altanserine show some *in vitro* antibacterial activity. Ritanserine shows a MIC₅₀ of 125 µM against *P. acnes* LMG 16711 and *M. smegmatis* ATCC 700084, MIC₅₀ of 62.5 µM against *P. aeruginosa* PAO1 and a MIC₅₀ of 31.25 µM against *B. cenocepacia* LMG 16656. Ketanserine is able to inhibit *in vitro* growth of *M. smegmatis* ATCC 700084 in concentrations starting from 250 µM.

Table 7. MIC₅₀ values (µM) of DXS-inhibitors (clomazone, 5-ketoclomazone and 4(3H)-pyrimidinone) and IspF-inhibitors (ritanserine, ketanserine, altanserine, setoperone and IspF-inhibitor-1).

Compound	MIC ₅₀ (µM)				
	GRAM (-)			GRAM (+)	MYCOBACTERIA
	<i>E. coli</i> K-12	<i>P. aeruginosa</i> PAO1	<i>B. cenocepacia</i> LMG 16656	<i>P. acnes</i> LMG 16711	<i>M. smegmatis</i> ATCC 700084
Clomazone	>250	>250	>250	>250	>250
Ketoclomazone	>250	>250	>250	>250	>250
Pyrimidinone	>250	>250	>250	>250	>250
Ritanserine	>250	62.5	31.25	125	125
Ketanserine	>250	>250	>250	>250	250
Altanserine	>250	>250	>250	>250	250
Setoperone	>250	>250	>250	>250	>250
Isp-inhibitor-1	>250	>250	>250	>250	>250

As ritanserine is able to inhibit *in vitro* planktonic growth of *B. cenocepacia*, *P. acnes* and *M. smegmatis*, and ketanserine can also inhibit *M. smegmatis* growth, we expanded our test panel of bacterial strains. A number of additional Bcc strains were included (*B. multivorans* LMG 18825, *B. ambifaria* LMG 19182 and *B. ambifaria* LMG 19467), together with one additional *P. acnes* strain (*P. acnes* LMG 16712) and three mycobacterial strains (*M. smegmatis* ATCC 607, *M. smegmatis* LMG 8190 and *M. phlei* LMG 4056).

The results for this extended susceptibility screening are presented in Table 8. The antibacterial activity of ketanserine and altanserine is limited, as concentrations of ketanserine as high as 250 µM are required to inhibit growth of *M. smegmatis* ATCC 700084. Ritanserine, however, does show a greater capacity to inhibit planktonic growth of strains in our test panel, as shown in Table 8.

Table 8. MIC₅₀ values (μM) of ritanserine, ketanserine, altanserine and control antibiotics (FOS; fosmidomycin and FR900098) for an expanded test panel of Gram-negative, Gram-positive and mycobacterial strains.

Compound	MIC ₅₀ (μM)					
	GRAM (-)					
	<i>B. cenocepacia</i> LMG 16656	<i>B. cepacia</i> LMG 1222	<i>B. multivorans</i> LMG 13010	<i>B. multivorans</i> LMG 18825	<i>B. ambifaria</i> LMG 19182	<i>B. ambifaria</i> LMG 19467
Ritanserine	31.25	62.5	>250	250	62.5	62.5
Ketanserine	>250	>250	>250	>250	>250	>250
Altanserine	>250	>250	>250	>250	>250	>250
FOS	250	>250	>250	>250	>250	>250
FR900098	>250	125	>250	>250	>250	>250
	GRAM (+)		MYCOBACTERIA			
	<i>P. acnes</i> LMG 16711	<i>P. acnes</i> LMG 16712	<i>M. smegmatis</i> ATCC 700084	<i>M. smegmatis</i> ATCC 607	<i>M. smegmatis</i> LMG 8190	<i>M. phlei</i> LMG 4056
Ritanserine	125	125	125	>250	250	250
Ketanserine	>250	>250	250	>250	>250	>250
Altanserine	>250	>250	>250	>250	>250	>250
FOS	>250	>250	>250	>250	>250	>250
FR900098	>250	>250	>250	>250	>250	>250

1.4.1.4. Survival of planktonic organisms after ritanserine treatment

Ritanserine inhibits planktonic growth of some strains of our test panel, as described above. However, except for *P. acnes* LMG 16711, it seemed that treatment with ritanserine does not lead to complete inhibition of planktonic growth. We determined the growth reduction of *B. cenocepacia* LMG 16656, *B. ambifaria* LMG 19467, *P. acnes* LMG 16711, *M. smegmatis* ATCC 700084 and *M. smegmatis* LMG 8190 by cultivating these in the presence of ritanserine (125 μM, 250 μM or 500 μM) and quantifying the percentage survival after treatment with conventional plating. The results for this experiment are shown in Figure 17.

Ritanserine caused complete growth inhibition of *P. acnes* LMG 16711 in concentrations starting from 125 μM. For *B. cenocepacia* LMG 16656, *B. ambifaria* LMG 19467, *M. smegmatis* ATCC 700084 and *M. smegmatis* LMG 8190 ritanserine caused significant growth inhibition of planktonic cells, but no complete growth reduction, up to 500 μM. These results were in line with the expectations from the susceptibility screening.

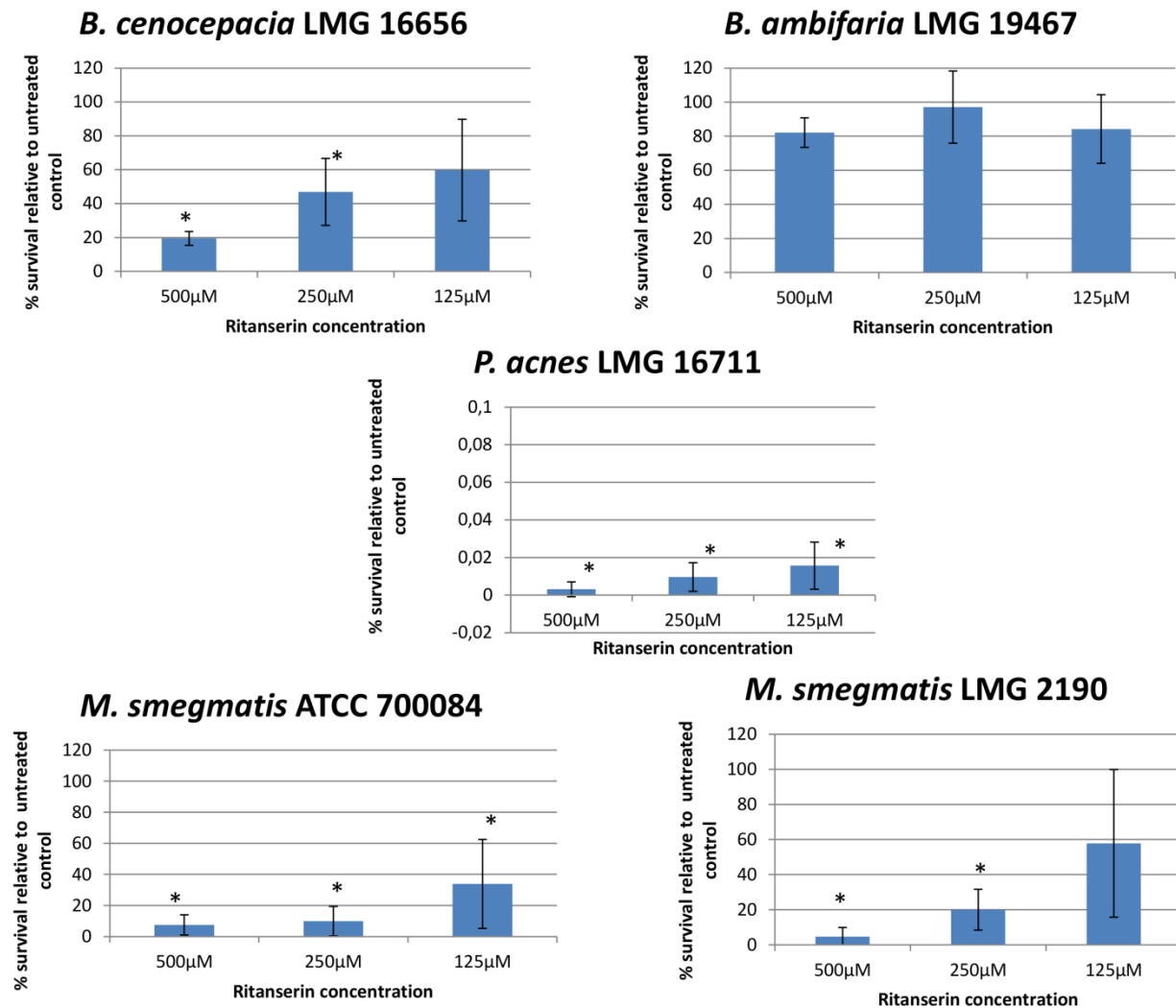


Figure 17. Percentage survival of planktonic bacterial cultures, relative to untreated controls, of *B. cenocepacia* LMG 16656, *B. ambifaria* LMG 19467, *P. acnes* LMG 16711, *M. smegmatis* ATCC 700084 and *M. smegmatis* LMG 8190 after ritanserin treatment. Error bars represent standard deviations. ' * ' represents statistically significant differences compared to untreated controls ($p < 0.05$, $n = 3$).

1.4.1.5. Survival of sessile organisms after ritanserin treatment

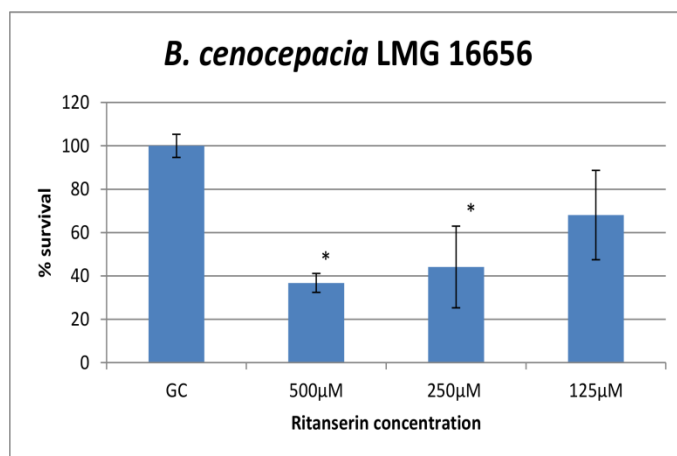


Figure 18. Percentage bacterial survival, relative to untreated control, of sessile cells of *B. cenocepacia* LMG 16656 after ritanserin treatment. GC; untreated control. Error bars represent STDEV. ' * ' indicates statistically significant differences compared to untreated control ($p < 0.05$, $n=3$).

To determine the growth inhibition of biofilms of *B. cenocepacia* LMG 16656, we cultivated sessile cells in the presence of ritanserin (125 μM, 250 μM and 500 μM) and quantified the percentage survival after ritanserin treatment by conventional plating. As shown in Figure 18, ritanserin induced a significant growth inhibition of biofilm cells of *B. cenocepacia* LMG 16656, but no complete growth reduction, up to 500 μM.

1.4.2. Determining the role of efflux pumps in resistance

One of the main resistance mechanisms in bacterial cells is efflux of antibacterial compounds by the action of efflux pumps.[193-195] Due to the low *in vitro* activity of the β-substituted derivatives and ProTide prodrugs of fosmidomycin, we wanted to determine the importance of efflux in this antimicrobial resistance. Therefore, we investigated the *in vitro* activity of the DXR-inhibitors in combination with broad-spectrum efflux pump inhibitors CCCP and

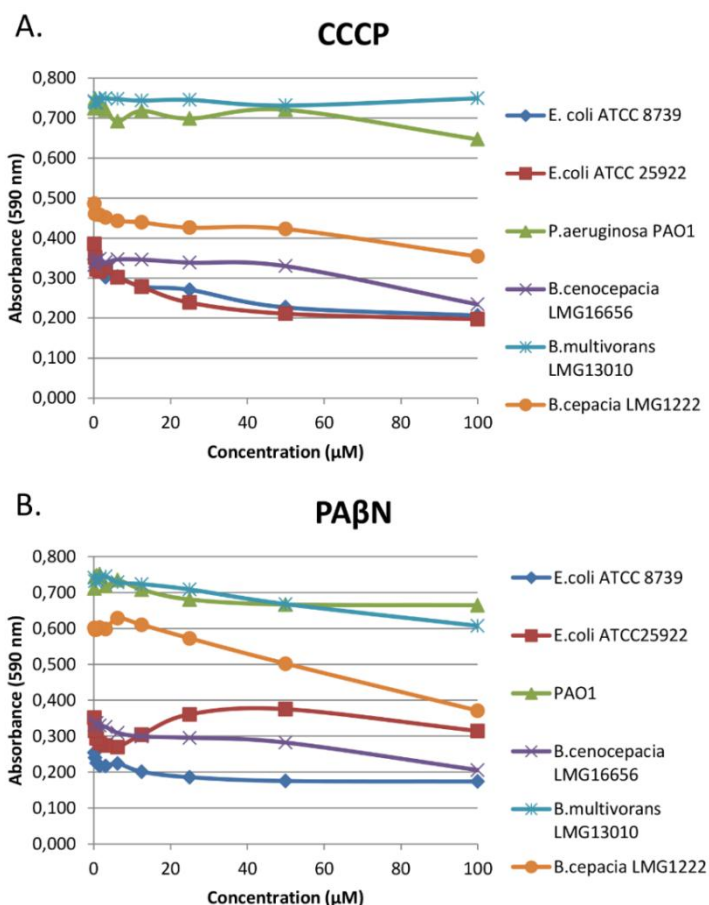


Figure 19. Intrinsic antibacterial activity of A. CCCP and B. PAβN. 56

PA β N. CCCP acts as a PMF uncoupler, inhibiting the energy source of RND efflux pumps [193, 196] and PA β N is able to inhibit efflux pumps in Gram-negatives belonging to the RND family.[197]

First, the intrinsic antibacterial activity of both efflux pump inhibitors alone was determined. CCCP caused no growth inhibition of *B. multivorans* LMG 13010. Minimum 10% growth inhibition of *E. coli* ATCC 25922 was caused by 6.25 μ M CCCP, of *E. coli* ATCC 8739 by 12.5 μ M CCCP, of *B. cepacia* LMG 1222 by 25 μ M CCCP, and of *P. aeruginosa* PAO1 or *B. cenocepacia* LMG 16656 by 100 μ M CCCP (see Figure 19A). PA β N did not cause growth inhibition of *E. coli* ATCC 25922 or *P. aeruginosa* PAO1. Minimum 10% growth inhibition of *E. coli* ATCC 8739 was caused by 12.5 μ M PA β N, of *B. cenocepacia* LMG 16656 or *B. cepacia* LMG 1222 by 50 μ M PA β N, and of *B. multivorans* LMG 13010 by 100 μ M PA β N (see Figure 19B).

To explore the role of efflux pumps in resistance against fosmidomycin analogues, we determined the susceptibility of our test panel towards the combination of these analogues with CCCP or PA β N. Fixed concentrations (5 μ M, 50 μ M and 100 μ M) of CCCP and PA β N were tested, based on results obtained in previous studies and the intrinsic antibacterial activity determined above.[196] The results are shown in Table 9. The results in the presence of 5 μ M or 50 μ M efflux pump inhibitor were similar and therefore not presented.

Table 9. MIC₅₀ values (μ M) for β -substituted fosmidomycin analogues (7a-9), fosmidomycin (FOS) and tobramycin in combination with 100 μ M CCCP or PA β N.

Compound	MIC ₅₀ (μ M)					
			<i>E. coli</i> ATCC 8739		<i>E. coli</i> ATCC 25922	
			+ CCCP	+ PA β N	+ CCCP	+ PA β N
					<i>P. aeruginosa</i> PAO1	
			+ CCCP	+ PA β N	+ CCCP	+ PA β N
7a	>250	>250	>250	>250	>250	>250
7b	>250	>250	>250	>250	>250	>250
8b	>250	>250	>250	>250	>250	>250
8c	>250	>250	>250	>250	>250	>250
8d	>250	>250	>250	>250	>250	>250
9	>250	>250	>250	>250	>250	>250
FOS	0.98	0.98	7.8	7.8	125	125
Tobramycin	3.9	3.9	3.9	3.9	0.98	1.95
			<i>B. cenocepacia</i> LMG 16656		<i>B. cepacia</i> LMG 1222	
			+ CCCP	+ PA β N	+ CCCP	+ PA β N
					<i>B. multivorans</i> LMG 13010	
			+ CCCP	+ PA β N	+ CCCP	+ PA β N
7a	>250	>250	>250	>250	>250	>250
7b	>250	>250	>250	>250	>250	>250
8b	>250	>250	>250	>250	>250	>250
8c	>250	>250	>250	>250	>250	>250
8d	>250	>250	>250	>250	>250	>250

9	>250	>250	>250	>250	>250	>250
FOS	250	250	>250	>250	>250	>250
Tobramycin	0.98	0.98	1.95	3.9	3.9	3.9

For none of the strains tested, changes in *in vitro* antibacterial activity of fosmidomycin and its analogues were observed upon addition of CCCP or PA β N. These results suggest that resistance of the strains tested against fosmidomycin and its analogues is not related to efflux. However, Messiaen and colleagues described a significant upregulation of a fosmidomycin resistance gene (*fsr*) encoding an efflux pump after fosmidomycin or FR900098 treatment in Bcc species.[127] In *E. coli* a similar *fsr* gene was identified which is involved in efflux of fosmidomycin from bacterial cells.[198]

Table 10. MIC₅₀ values (μ M) of ritanserin against *B. cenocepacia* LMG 16656 and RND-efflux pump mutants.

Strain	MIC ₅₀ (μ M)
LMG 16656	31.25
Δ RND1	62.5
Δ RND2	62.5
Δ RND3	62.5
Δ RND4	62.5
Δ RND5	>250
Δ RND6-7	62.5
Δ RND8	62.5
Δ RND9	125
Δ RND4-9	31.25
Δ RND10	125
Δ RND11	125
Δ RND12	125
Δ RND13	62.5
Δ RND14	125
Δ RND15	>250
Δ RND16	62.5

To expand our insight in the role of efflux pumps in resistance of *B. cenocepacia* LMG 16656, the susceptibility of RND-efflux pump mutants was tested. This collection of mutants was previously constructed by Silvia Buroni and Giovanna Riccardi from Pavia University.[199] MIC₅₀ values of fosmidomycin, FR900098, ritanserin, ketanserin and the β -substituted fosmidomycin analogues 7a, 8b and 9 were determined. The results for ritanserin are shown in Table 10, MIC₅₀ values for the other nonmevalonate inhibitors were all >250 μ M (results not shown). These data indicate no clear differences between the wild-type strain of *B. cenocepacia* LMG 16656 and its RND-mutants, except for Δ RND5 and Δ RND15 which, unexpectedly, showed a reduced susceptibility towards ritanserin.

The results from this study suggest that resistance of *E. coli* ATCC 8739 and ATCC 25922, *P. aeruginosa* PAO1, *B. cenocepacia* LMG 16656, *B. cepacia* LMG 1222 and *B. multivorans* LMG 13010 against nonmevalonate inhibitors is not related to efflux. Nevertheless, the comprehensiveness of this efflux-related study is rather limited and other methods may be used to further investigate the role of efflux pumps. Blair and Piddock describe a fluorometric determination of efflux by measuring the direct efflux of a fluorescent dye (e.g. ethidium bromide) in cells that are preloaded with high concentrations of antibiotics in the presence of an efflux inhibitor (e.g. CCCP).[200,

201] Quantitative PCR experiments (qPCR) could also offer an interesting approach to quantify the efflux pump RNA expression in bacterial cells after treatment with antibiotics.[202-204]

1.4.3. Effect of increasing cellular permeability

A possible explanation for the lack of antibacterial efficacy of fosmidomycin analogues and other nonmevalonate inhibitors could be the low cellular uptake by passive diffusion due to the polarity of these molecules, with a low bioavailability as a consequence.[126] Also, in several studies deficiencies in fosmidomycin uptake were characterized in various pathogens, due to the lack of a glpT-transport system or the acquisition of mutations (shown in Table 11).[205]

Table 11. Fosmidomycin resistance mechanisms characterized in various pathogens, retrieved from [205].

Organism	Resistance classification	Resistance description
<i>Brucella abortus</i>	Lack of fosmidomycin-sensitive DXR	Encodes a fosmidomycin insensitive DXR-like enzyme [206]
Bcc	Insufficient uptake and efflux stimulation	Enhanced expression of <i>fsr</i> when treated with fosmidomycin [127]
<i>E. coli</i>	Expression of a fosmidomycin export protein	Overexpression of a drug export protein [198]
<i>E. coli</i>	Lack of fosmidomycin uptake	Adenylate cyclase deficient mutant: glpT function is cAMP-dependent [207]
<i>Francisella novicida</i>	Lack of fosmidomycin uptake	Deletions in <i>glpT</i> encoding the L- α -glycerophosphate transport system [208]
<i>M. tuberculosis</i>	Lack of fosmidomycin uptake	Lacks the L- α -glycerophosphate transport system [126]
<i>P. falciparum</i>	Gene amplification	<i>dxr</i> gene duplication [209]
<i>P. aeruginosa</i>	Lack of fosmidomycin uptake	Lacks the L- α -glycerophosphate transport system [210]
<i>Toxoplasma gondii</i>	Lack of fosmidomycin uptake	Lacks the L- α -glycerophosphate transport system [211]

Two different approaches to enhance the cell penetration of fosmidomycin and its analogues have been investigated. First, Messiaen and colleagues described that the MIC values of FR900098 for *B. cenocepacia* LMG 16656, *B. cepacia* LMG 1222 and *B. multivorans* LMG 13010 decreased at least 4-fold in CDM-G medium, compared to MICs in MHB. This phenomenon was clarified by qPCR; confirming a

significantly increased gene expression in *B. cenocepacia* of three genes involved in glycerol-3-phosphate transport (BCAL0282, BCAL0284 and BCAL0285) in the presence of glucose-6-phosphate (CDM-G) compared to controls without glucose-6-phosphate.[127] Therefore, the hypothesis was formulated that the susceptibility of our test panel towards fosmidomycin and its β -analogues would also increase in CDM-G. Unfortunately, the MIC-value of every β -substituted fosmidomycin analogue was $> 250 \mu\text{M}$ (as illustrated in Table 12), for every bacterial strain examined in this experiment.

Table 12. MIC values (μM) of β -substituted fosmidomycin analogues (7a – 9), FOS; fosmidomycin and FR900098 in CDM-G.

Compound	MIC (μM)				
	GRAM (-)				
	<i>E. coli</i> ATCC 8739	<i>E. coli</i> K-12	<i>B. cenocepacia</i> LMG 16656	<i>B. cepacia</i> LMG 1222	<i>B. multivorans</i> LMG 13010
7a	>250	>250	>250	>250	>250
7b	>250	>250	>250	>250	>250
7c	>250	>250	>250	>250	>250
7d	>250	>250	>250	>250	>250
7e	>250	>250	>250	>250	>250
8b	>250	>250	>250	>250	>250
8c	>250	>250	>250	>250	>250
8d	>250	>250	>250	>250	>250
9	>250	>250	>250	>250	>250
FOS	0.98	7.8	250	>250	>250
FR900098	7.8	15.6	>250	125	>250

Second, combination therapy of antibiotics and the CPPs colistin, polymyxin, octaarginin and PMBN was tested. This experiment was performed with fosmidomycin in combination with a CPP, for treatment of *E. coli* K-12 and *M. smegmatis* ATCC 700084; FIC indexes are shown in Table 13.

Table 13. FIC indexes for fosmidomycin treatment combined with a CPP. FIC < 0.5 indicates synergistic effect, FIC > 4 indicates antagonistic effect. FOS; fosmidomycin, COL; colistin, POLY; polymyxin, OCTA; octaarginin and PMBN, polymyxin B nonapeptide.

Combination	FIC index	
	<i>E. coli</i> K-12	<i>M. smegmatis</i> ATCC 700084
FOS + COL	0.53	1
FOS + POLY	0.74	0.75
FOS + OCTA	2	2
FOS + PMBN	1.5	2

All FIC indexes are between 0.5 and 4, indicating that neither a synergistic nor an antagonistic effect exists when combining fosmidomycin with colistin, polymyxin, octaarginin or PMBN to treat *E. coli* K-12 or *M. smegmatis* ATCC 700084.

The results of both experiments concerning enhancement of membrane permeability showed negative results. No differences in MIC values were observed in a chemically defined medium by which cellular uptake should be increased and the addition of CPPs also failed to increase the antibacterial activity of fosmidomycin. Nevertheless, it may be interesting to explore other approaches to enhance membrane permeability of fosmidomycin and its analogues (e.g. investigation of the effect of CPPs covalently bound to fosmidomycin).

1.5. Conclusions

The nonmevalonate or DOXP pathway is potentially an interesting target for the development of new antibacterial chemotherapeutics. In the present study, the antimicrobial activity of a set of new nonmevalonate inhibitors was evaluated by testing their *in vitro* activity on planktonic and sessile cells of Gram-negative, Gram-positive and mycobacterial species. Susceptibility screening of our test panel towards DXS-, IspF- and DXR-inhibitors (fosmidomycin and derivatives) showed that the *in vitro* activity of most compounds is low, with MIC values >250 μ M. Only the thiazolopyrimidine ritanserine does partially inhibit planktonic and sessile growth of several Bcc species, two *P. acnes* strains and three mycobacterial strains. Subsequently, several experiments were performed to elaborate the mechanisms of resistance against fosmidomycin, fosmidomycin analogues, DXS- and IspF-inhibitors. Our data suggest that the role of efflux in resistance towards these compounds is limited and that the major issue is low membrane permeability leading to limited uptake into the bacterial cell. However, the results obtained in this study did not prove this hypothesis and further research upon resistance against DOXP-inhibitors therefore is essential.

2. IS THE NONMEVALONATE PATHWAY AN ESSENTIAL BIOCHEMICAL PATHWAY IN *BURKHOLDERIA CENOCEPACIA*?

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2.1. Abstract

The nonmevalonate pathway is the sole pathway for isoprenoid biosynthesis in *B. cenocepacia* and possibly a novel target for the development of antibacterial chemotherapy. The goal of the present study was to evaluate the essentiality of *dxr* and *ispD*, the second and third gene of the nonmevalonate pathway, in *B. cenocepacia*. We constructed rhamnose-inducible conditional knock-down mutants of *B. cenocepacia* K56-2 by using plasmid pSC200, which enables the delivery of a rhamnose-inducible promoter in the chromosome, to drive the expression of a candidate essential gene. A rhamnose-inducible *dxr* mutant of *B. cenocepacia* K56-2 was successfully constructed, but we were unable to insert a correct *ispD* construct in K56-2. Growth experiments of planktonic and biofilm cultures of *B. cenocepacia* K56-2 showed that expression of *dxr* is important for growth, but not essential. MIC-values of ceftazidime and aztreonam decreased 16- and 8-fold, respectively, upon disruption of *dxr* in *B. cenocepacia* K56-2. Data obtained in a *Caenorhabditis elegans* infection model suggest that sufficiently high expression levels of *dxr* are required for full virulence of *B. cenocepacia* K56-2. Moreover, *dxr* seems to be involved in the ability of *B. cenocepacia* to metabolize several carbon sources, including D-galactonate which is required for biosynthesis of D-glyceraldehyde-3-P, a start product of the nonmevalonate pathway. To conclude, although not essential, DXR appears to play an important role in different physiological processes of *B. cenocepacia*. Further research into inhibitors of DXR and other enzymes of the nonmevalonate pathway could lead to new treatment approaches for *B. cenocepacia* infections.

2.2. Introduction

Isoprenoids are a diverse class of naturally occurring compounds with widely varying roles in physiological processes of all living organisms. Animals (including humans), fungi, *Archaea* and some bacteria use the mevalonate pathway for isoprenoid biosynthesis.[133] An alternative metabolic pathway for the synthesis of isoprenoids, the 1-deoxy-D-xylulose-5-phosphate (DOXP)-pathway or nonmevalonate pathway, was discovered in the late 1980s.[212] This pathway starts with the condensation of D-glyceraldehyde-3-phosphate and pyruvate to DOXP, catalyzed by the enzyme DOXP-synthase (DXS). Next, DOXP is converted to 2-C-methyl-D-erythritol-4-phosphate (MEP) by DOXP-reductoisomerase (DXR). Subsequently, 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME) is formed with CDP-ME-synthase (*IspD*) as catalyst. CDP-ME is then phosphorylated by CDP-ME kinase (*IspE*), with

the formation of CDP-ME-2-phosphate. This diphosphate is converted to 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (MER-cPP) by MER-cPP synthase (IspF) and finally, this cyclic diphosphate molecule gives rise to the synthesis of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP).[113, 114] The rate-limiting step in the nonmevalonate pathway is the conversion of DOXP to MEP, catalyzed by DXR. Fosmidomycin, a natural phosphonic acid antibiotic, and FR900098, the acetyl derivative of fosmidomycin, are effective inhibitors of purified DXR from several bacteria but have limited effects on whole bacterial cells.[116, 117, 139]

The Bcc is a group of 20 closely related opportunistic, Gram-negative species, which present particular challenges because of their innate resistance to a variety of antibiotics.[1] Since the early 1980s, members of the Bcc are well-known as rare but potentially life-threatening respiratory pathogens in patients with cystic fibrosis (CF), with *B. multivorans* and *B. cenocepacia* being the most frequently isolated Bcc species.[7] Therapeutic options to treat Bcc infections are very limited because of their intrinsic resistance to a wide range of antimicrobial agents[90, 171], and the ability of Bcc strains to form biofilms *in vitro* and *in vivo* contributes to reduced antimicrobial susceptibility, treatment failure and persistent infection.[36, 37]

Bcc bacteria use the nonmevalonate pathway as sole pathway for isoprenoid biosynthesis and since this pathway is not present in human cells, it is possibly a novel target for the development of antibacterial chemotherapy against Bcc infections.[213, 214] A study from Messiaen et al. describes the *in vitro* susceptibility of 40 Bcc strains against fosmidomycin, FR900098 and several fosmidomycin derivatives. Resistance against all derivatives was observed for all Bcc strains, which could partly be explained by the lack of a glycerol-3-phosphate transporter (GlpT) system required for import of fosmidomycin and FR900098. However, Bcc species possess an alternative GlpT system which can be induced by addition of glucose-6-phosphate, leading to increased uptake of fosmidomycin. Unfortunately, a fosmidomycin resistance gene (*fsr*), involved in efflux of fosmidomycin and FR900098, is upregulated in treated cultures and is able to compensate for the increased fosmidomycin uptake in the presence of glucose-6-phosphate.[127] Other research groups have also studied the potential of fosmidomycin and its analogues to combat Bcc species and observed Bcc species to be highly resistant to fosmidomycin.[136, 215] Both studies state that this resistance is probably due to insufficient cellular uptake and the presence of a fosmidomycin efflux pump. Shtannikov et al. did not observe higher susceptibility of Bcc species to lipophilic fosmidomycin analogues and concluded that while these derivatives show higher cell penetration they also have lower affinity for DXR.[136] Isoprenoids are precursors for the biosynthesis of

hopanoids which are involved in regulation of membrane stability, permeability and fluidity, and contribute to the intrinsic resistance of Bcc bacteria against various antibiotics, including polymyxins, erythromycin and chlorhexidine.[215-217] Deletion of genes (BCAM2831 and BCAS0167) involved in hopanoid biosynthesis resulted in loss of swarming and swimming motility, suggesting that hopanoids play an important role in the physiology of *B. cenocepacia*. [216] Several studies provide evidence that reducing the amount of hopanoids in the Bcc membrane can increase susceptibility to antibiotics. [215-219] Malott et al. described resistance of *B. multivorans* isolates to fosmidomycin and colistin when used alone, but they observed antimicrobial synergy when both antibiotics were used in combination. [215]

The goal of the present study was to determine the essentiality of different genes of the nonmevalonate pathway in *B. cenocepacia*, because previous research upon promising nonmevalonate inhibitors did not show any *in vitro* activity against species of the Bcc (Chapter III.1). Identifying essential genes is important because the products of these essential genes are potential novel antibacterial targets for the treatment of multidrug-resistant bacteria. A number of techniques have been used in the past to identify and study candidate essential genes in *B. cenocepacia*, including antisense overexpression mutants [220], unmarked deletion mutants [221-223], transposon directed insertion-site sequencing (TraDIS) [224] and rhamnose-inducible conditional expression mutants. [225-228] In the present study, we used plasmid pSC200 to construct rhamnose-inducible conditional mutants of *B. cenocepacia*. [226-228] pSC200 enables the replacement of the native promoter of a gene by a rhamnose-inducible promoter to drive the expression of the targeted candidate essential gene. This procedure gives rise to conditional mutants in which the expression of the target gene is highly dependent on the rhamnose concentration in the growth medium. [226-228] The second and third enzyme (DXR and IspD, respectively) were chosen as targets of the nonmevalonate pathway. DXR catalyzes the rate-limiting step of this pathway namely the conversion of DOXP to MEP. [116, 117] IspD was also included because growth inhibition of *B. cenocepacia* strains was observed in previous experiments (Chapter III.1) by an inhibitor of IspF, which is located in one operon with IspD. Both genes were identified as essential in *Escherichia coli*, *Mycobacterium tuberculosis* and *Plasmodium falciparum* [119, 126] and recent TraDIS analysis by Wong *et al.* also showed that all five genes of the nonmevalonate pathway are essential in *B. cenocepacia* J2315. [224]

2.3. Materials and methods

2.3.1. Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 14. Cultures *E. coli* and *B. cenocepacia* strains were grown in Luria-Bertani agar or broth (LBA or LBB; LabM, Lancashire, UK) or in a synthetic medium described by Ortega *et al.*[226], further referred to as ‘pSC200 broth’. pSC200 broth contains M9 minimal medium (42mM Na₂HPO₄, 22 mM KH₂PO₄, 8 mM NaCl and 10 mM NH₄Cl) supplemented with yeast extract (5 g/L), casamino acids (2 g/L), vitamin B1 (2 mg/L), tryptophan (20 mg/L), CaCl₂ (1μM) and 0.5% (wt/vol) glycerol. These media were supplemented with 0.5% (wt/vol) rhamnose or glucose, 50 μg/mL gentamicin, 40 μg/mL kanamycin or trimethoprim (50 μg/mL or 800 μg/mL) when required (all purchased from Sigma-Aldrich, St-Louis, MO, USA). Liquid cultures were grown in a shaking hot water bath at 37°C.

Pure cultures of mutant *B. cenocepacia* K56-2 strains were grown on LB agar supplemented with 800 μg/mL trimethoprim and 0.5% rhamnose. Liquid cultures for phenotypic and genotypic experiments were grown in LBB or pSC200 broth, without addition of rhamnose or antibiotics.

Table 14. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference
<u><i>B. cenocepacia</i></u>		
K56-2	Wild type (wt), ET-12 lineage, CF isolate	Lab. Collection
K56-2 <i>dxr</i>	<i>dxr</i> knock-down mutant of K56-2	This study
<u><i>E. coli</i></u>		
DH5α	λpir	Lab. Collection
HB101	pRK2013	Lab. Collection
DH5α <i>dxr</i>	λpir, pSC200 <i>dxr</i>	This study
DH5α <i>ispD</i>	λpir, pSC200 <i>ispD</i>	This study
<u>Plasmids</u>		
pRK2013	<i>ori_{colE1}</i> , RK2 derivative Km ^R , <i>mob</i> ⁺ , <i>tra</i> ⁺	[229]
pSC200	pGpΩTp derivative	[230]
	<i>ori_{R6K}</i> , <i>rhaR</i> , <i>rhaS</i> , <i>P_{rhaB}</i> , <i>e-gfp</i> , <i>dhfr</i>	[226]
pSC200 <i>dxr</i>	pSC200 carrying fragment of <i>dxr</i>	This study
pSC200 <i>ispD</i>	pSC200 carrying fragment of <i>ispD</i>	This study

2.3.2. Construction of conditional mutants of *B. cenocepacia* K56-2

To confirm the essentiality of DOXP genes in *B. cenocepacia* K56-2, conditional knock-down mutants were constructed by replacing the native promoters of the target genes *dxr* (BCAL2085) and *ispD* (BCAL2016) with the rhamnose-inducible promoter P_{rhaB} (Figure 20 [227, 228]). Primers used in this study are listed in Table 15. The genome sequence of *B. cenocepacia* K56-2 was obtained from the website of the *Burkholderia* Genome Database.[231]

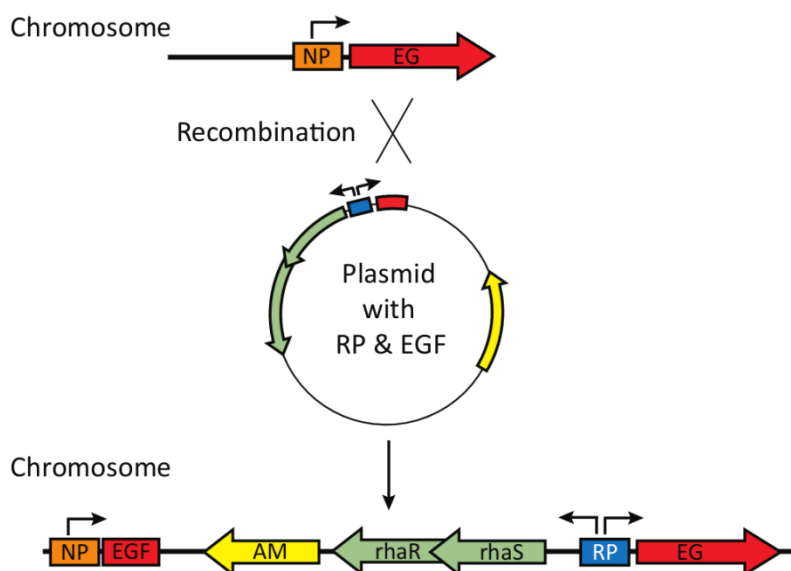


Figure 20. Illustration of the strategy used to construct conditional mutants. NP; native promotor, EG; essential gene, RP; rhamnose-inducible promotor, EGF; essential gene fragment, AM; antibiotic resistance marker, rhaR and rhaS; activators of rhamnose catabolism.[227]

Primers were designed to amplify *dxr* and *ispD* fragments of 283 bp and 115 bp, respectively, spanning the 5' region of each targeted gene, using the website of the National Center for Biotechnology Information (NCBI) and their specificity was evaluated using BLAST.[42, 43] The essential gene fragments were amplified by PCR with primers DXR F1/DXR R2 and IspD F1/IspD R5, digested with restriction enzymes *Xba*I and *Nde*I (Promega, Madison, WI, USA) and ligated into digested pSC200 downstream of the plasmid-borne P_{rhaB} , resulting in plasmids pSC200*dxr* and pSC200*ispD*. Then, each of the recombinant plasmids was transformed into *E. coli* DH5α λpir by CaCl₂ transformation resulting in *E. coli* DH5α*dxr* and DH5α*ispD* which were subcultured on LB agar supplemented with 50 µg/mL trimethoprim and 0.5% glucose. Subsequently, recombinant plasmids were transferred into *B. cenocepacia* K56-2 by triparental mating, using the method described by Ortega et al. ([226]) with the following modifications. Overnight cultures of *B. cenocepacia* K56-2, *E. coli* HB101 (pRK2013) and *E. coli* DH5α*dxr* or DH5α*ispD* were mixed in equal volumes, 300 µL was spotted on LB agar + 0.5 % rhamnose and incubated at 37°C for six hours. Afterwards, cells were washed with physiological saline (PS) and released from the agar surface, and 1

mL of each cell solution was spotted on LB agar plus 50 µg/mL gentamicin (to select against *E. coli*), 800 µg/mL trimethoprim, and 0.5% rhamnose. Plates were incubated at 37°C for about 48 hours and successful exconjugants (further referred to as K56-2*dxr* and K56-2*ispD*) were subcultured on LB agar plus 800 µg/mL trimethoprim and 0.5% rhamnose.

Table 15. Primer sequences used in this study

Name	Sequence	Purpose
DXR F1	CAAAAACGTCTGACATTGCTC	Amplification of <i>dxr</i> fragment
DXR R2	AGCCGTCGCTCTTCGACAC	Amplification of <i>dxr</i> fragment
IspD F1	ACTCCCCGACTTTTCGCC	Amplification of <i>ispD</i> fragment
IspD R5	CGAGCGTGTAGTGCAGCAG	Amplification of <i>ispD</i> fragment
rhaF	CACGTTTCATCTTCCCTGGT	Confirmation of transformation and triparental mating
200-MCS-R	AACACTTAACGGCTGACATGG	Confirmation of transformation
DXR R4	AGGCCCTTGTTTCATCATCG	Confirmation triparental mating K56-2 <i>dxr</i>
ispD R4	GAGCTTCTCGTTGGTCTTCG	Confirmation triparental mating K56-2 <i>ispD</i>

2.3.3. Chelex DNA-extraction and confirmation of transformation/triparental mating

Chelex DNA-extractions were performed on colonies of *E. coli* DH5α λpir and *B. cenocepacia* K56-2 after transformation and triparental mating, respectively. A 5% Chelex suspension was prepared (200-400 Mesh, Bio-rad Laboratories NV, Nazareth, Belgium) in DEPC-treated water (Invitrogen, ThermoFisher Scientific, Merelbeke, Belgium) and used for DNA-extraction. Subsequently, 2 µL of each supernatant was used as template DNA for PCR reactions using a C1000 Touch Thermal Cycler (Bio-rad). The cycling conditions included an initial denaturation at 95°C for 5 min, followed by 40 amplification cycles consisting of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C and terminated with a final extension of 2 min at 72°C. PCR primers rhaF/200-MCS-R were used to confirm transformation and primers rhaF/DXR R4 and rhaF/ispD R4 were used to confirm triparental mating. Purified PCR products were separated on a 1% agarose gel and sent for Sanger sequencing (LightRun sequencing, GATC Biotech AG, Constance, Germany) to confirm the correct insertion of the desired construct into *E. coli* DH5α λpir or *B. cenocepacia* K56-2. Sequences obtained are presented in Supplementary file S1.

2.3.4. RNA-extraction and cDNA synthesis

Cells of wild-type and mutant *B. cenocepacia* K56-2 were cultured in either pSC200 broth or in LBB. Both media were used without addition of a carbon source or were supplemented with 0.5% rhamnose or glucose. After 24 hours incubation at 37°C, cells were collected and RNA was extracted using the RiboPure Bacteria Kit (Ambion, Austin, TX) according to the manufacturer's guidelines including a 1 h DNase treatment. Subsequently, RNA concentrations were determined using BioDrop μ Lite (Isogen Life Science, Utrecht, The Netherlands) and 500 ng of each RNA sample was used to synthesize cDNA using qScript cDNA Synthesis kit (QuantaBio, Beverly, MA, USA).

2.3.5. Quantitative real-time polymerase chain reaction (qPCR)

qPCR experiments were performed on a Bio-Rad CFX96 Real-Time PCR Detection System using a Perfecta SYBR Green Fastmix (QuantaBio), according to the manufacturer's instructions. The PCR cycling protocol included an initial denaturation at 95°C for 3 min, followed by 40 amplification cycles consisting of 15 s at 95°C and 1 min at 60°C; a melting curve analysis was performed at the end of each run. All PCR data were normalized to the mean expression value of three reference genes (BCAS0175, BCAM2784 and BCAL2694) which were stably expressed in all conditions.

2.3.6. Evaluation of differences in planktonic growth

To evaluate differences in planktonic growth between different *B. cenocepacia* strains, growth rates were measured. To this end bacterial cultures were grown overnight in pSC200 broth, and subsequently diluted in a variety of bacterial growth media, including LBB, pSC200 broth, M9 minimal medium (MM9), Tryptic Soy Broth (TSB, LabM), Mueller-Hinton broth (MHB, BD) and Synthetic Cystic Fibrosis Medium (SCFM, [232]) to obtain a final inoculum of approx. 5×10^5 colony forming units/mL (CFU/mL). 200 μ l of each bacterial suspension was added in duplicate to a flat-bottomed 96-well microtiter plate (TPP, Trasadingen, Switzerland) and bacterial growth was monitored by measuring absorbance at 590 nm in an Envision multilable plate reader (Perkin Elmer, Waltham, MA, USA), every 30 min for 48-72 hours at 37°C, until stationary phase was reached.

2.3.7. Evaluation of biofilm formation

Wild-type and mutant strains of *B. cenocepacia* K56-2 were cultured in pSC200 broth. After overnight incubation, cell densities were again adjusted to obtain a final inoculum of approx. 5×10^5 CFU/mL, using six different growth media (LBB, LBB plus 0.5% rhamnose, LBB plus 0.5% glucose, pSC200, pSC200 plus 0.5% rhamnose and pSC200 plus 0.5% glucose). Biofilms were grown in U-bottomed 96-well microtiter plates for 4 h at 37°C, washed with PS and incubated at 37°C for another 20 h. Biofilm formation was quantified by resazurin staining and conventional plating. After 24 h of biofilm formation at 37°C, a resazurin solution (CellTiter-Blue, Promega) was added to the sessile cultures followed by 2h incubation at 37°C; fluorescence at 535 nm was measured in an Envision plate reader. [187] Quantification of the CFU per biofilm was performed through plating on LBA or LBA supplemented with 0.5% rhamnose.

2.3.8. Evaluation of in vitro antimicrobial susceptibility

Antimicrobial susceptibility was investigated by determination of the minimal inhibitory concentration (MIC) according to the EUCAST broth microdilution guidelines.[183] Antibiotics tested included ceftazidime, ciprofloxacin, meropenem, minocycline, tobramycin, aztreonam, erythromycin, chloramphenicol, rifampicin, tetracycline, fosmidomycin, FR900098 and ritanserin. Ceftazidime, ciprofloxacin, minocycline, erythromycin, chloramphenicol, rifampicin, tetracycline, FR900098 and ritanserin were all purchased from Sigma-Aldrich, meropenem was obtained from Hospira Benelux (Antwerp, Belgium), tobramycin and aztreonam were purchased from TCI Europe (Zwijndrecht, Belgium), and fosmidomycin was obtained from Invitrogen. MICs were determined in triplicate using flat-bottomed 96-well microtiter plates, with concentrations ranging from 1 to 512 µg/mL except for fosmidomycin, FR900098 and ritanserin, where concentrations of 0.98 to 500 µM were tested. Planktonic bacterial cultures were grown overnight in pSC200 broth, adjusted to obtain a final inoculum of approx. 5×10^5 CFU/mL and added to the 96-well plates. Plates were incubated for 24h at 37°C and absorbance was measured at 590 nm using an Envision plate reader.

2.3.9. Checkerboard assays

The combinatorial effect of DXR-inhibitors and certain β -lactam antibiotics on wild-type and mutant *B. cenocepacia* K56-2 strains was investigated by performing checkerboard synergy assays. Planktonic cells of K56-2 and K56-2*dxr* were treated with fosmidomycin or FR900098 (concentration range 3.9 μ M – 500 μ M) in combination with ceftazidime or aztreonam (0.06 – 64 μ g/mL). After addition of the bacterial suspension (100 μ L in a total volume of 200 μ L), the plate is incubated at 37°C for 24h and the absorbance is measured at 590 nm using an Envision multilable plate reader. Experiments were performed in triplicate.

2.3.10. *Caenorhabditis elegans* infection assay

C. elegans was maintained under standard conditions, cleaned and synchronized by sodium hypochlorite bleaching, and subcultured on nematode growth medium (NGM) agar using *E. coli* OP50 as a nutrient source.[233] Synchronized worms (L4 stage worms) were suspended in OGM medium consisting of 95% M9 buffer (3g/L KH_2PO_4 , 6 g/L Na_2HPO_4 , 5g/L NaCl and 1mL of 1M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), 5% Brain Heart Infusion broth (BHI, Oxoid, Aalst, Belgium) and 10 μ g/mL cholesterol. Cultures of wild-type and mutant *B. cenocepacia* were grown overnight in pSC200 broth, centrifuged and resuspended to OD 2 in OGM medium. A flat-bottomed 96-well plate was used for the *C. elegans* infection assay. Uninfected controls contained 25 μ L nematode suspension (approx. 15 worms) and 75 μ L OGM, while other wells contained 25 μ L nematode suspension, 25 μ L bacterial suspension (approx. 10^8 CFU/mL) and 50 μ L OGM. Experiments were carried out in the presence or absence of 0.5% rhamnose or 0.5% glucose. Plates were incubated at 25°C for up to 72h and the ratio living/dead nematodes was determined every 24h using an EVOS microscope (Thermo Fisher Scientific, Waltham, MA, USA). After 72h incubation, the nematodes were collected (content of four wells was pooled) and rinsed three times with M9 buffer supplemented with 1 mM NaN_3 (Sigma-Aldrich). Then, the nematodes were mechanically disrupted using sterile silicon carbide beads, vortexed for 10 min and a dilution series of every supernatant was prepared. The dilutions were plated on LB agar supplemented with 600 μ g/mL polymyxin (Sigma-Aldrich) and 50 μ g/mL gentamicin (to select against growth of *E. coli* OP50) and for plating of the mutant strains we also added 0.5% rhamnose. After 24h incubation at 37°C the number of CFU/nematode was determined. Every

condition was tested at least four times in each assay and the assay was performed in triplicate (interday replicates, $n=3 \times 4$).

2.3.11. Evaluation of differences in carbon metabolism

Biolog Phenotype Microarrays (Labconsult, Brussels, Belgium) were used to explore differences in carbon metabolism between wild-type and mutant *B. cenocepacia* strains. Bacterial cultures were grown at 37°C on LB agar. Inoculation fluids and cell suspensions were prepared according to the manufacturer's guidelines. PM1 and PM2A Microplate Carbon Sources (Biolog) were inoculated with 100 µL cell suspension/well and incubated at 37°C for up to 72 hours. Every 24h, absorbance was measured at 590 nm using an Envision plate reader. Experiments were performed in triplicate ($n= 3$). Carbon sources were defined as 'not metabolized' when the absorbance was equal to the negative control. For carbon sources that showed differences in metabolism between wild-type and mutant strains, the biochemical pathways in which they participate were identified using the KEGG Pathway Database and the *Burkholderia* Genome Database.[231, 234]

2.3.12. Statistical analysis

Statistical analysis was performed by comparison of mean values through an independent samples t-test or nonparametric test for independent samples, using SPSS 23.0 software (SPSS, Chicago, IL, USA).

2.4. Results

2.4.1. Construction of conditional mutants of *B. cenocepacia* K56-2

Using the approach described above, we were able to construct *B. cenocepacia* K56-2*dxr*, a mutant strain in which the expression of *dxr* is controlled by a rhamnose-inducible promoter. However, we were unable to insert the correct *ispD* construct in *B. cenocepacia* K56-2. qPCR was used to determine differences in mRNA expression of *dxr* between K56-2 and K56-2*dxr* in the presence or absence of rhamnose or glucose, in both LB and pSC200 broth. In the absence of rhamnose, *dxr* mRNA expression in K56-2*dxr* is very low; while in the presence of rhamnose, the mRNA expression level was similar to that observed in the wild type strain (Fout! Verwijzingsbron niet gevonden.1). However, gene expression

of *dxr* is not completely switched off in the absence of rhamnose; the small amounts of mRNA can be enough to produce DXR.

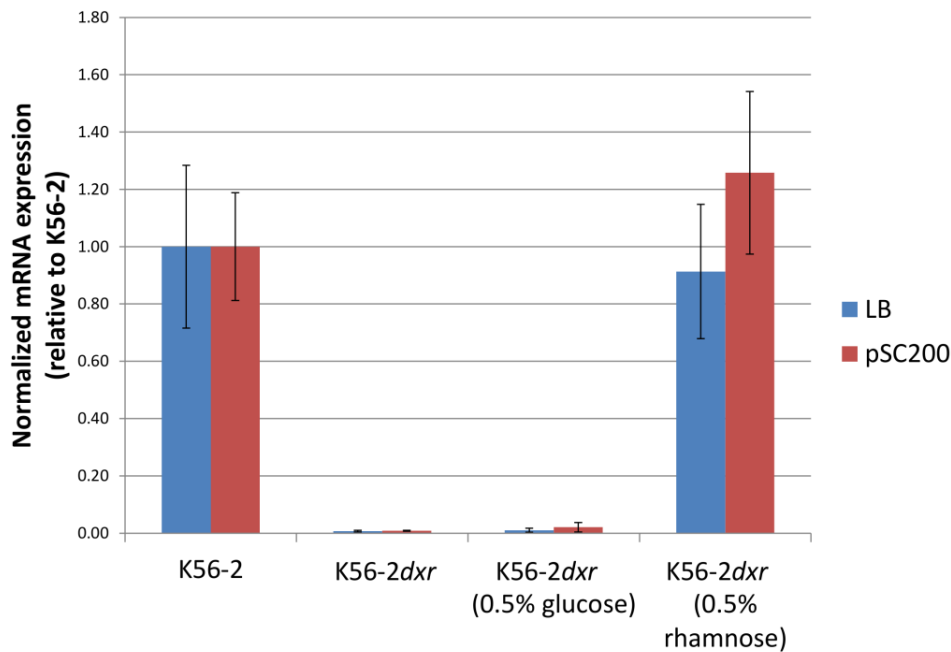


Figure 21. Relative expression of *dxr* measured by qPCR. Expression levels are normalized to the expression observed in *B. cenocepacia* K56-2. Error bars represent min-max, n=2.

2.4.2. Differences in planktonic growth between *B. cenocepacia* K56-2 and K56-2dxr

Growth curves of K56-2 and K56-2dxr in different culture media are shown in Figure 22. Planktonic growth in all six media tested shows two remarkable trends: (i) growth of K56-2dxr in the presence of 0.5% rhamnose is similar to that of the wild type strain, and (ii) K56-2dxr requires more time to reach stationary phase when grown in the absence of rhamnose. Nevertheless, K56-2dxr is still able to grow without addition of rhamnose, suggesting that *dxr* is essential for planktonic growth of *B. cenocepacia* K56-2, but that low levels of *dxr* are still expressed (in accordance with qPCR results).

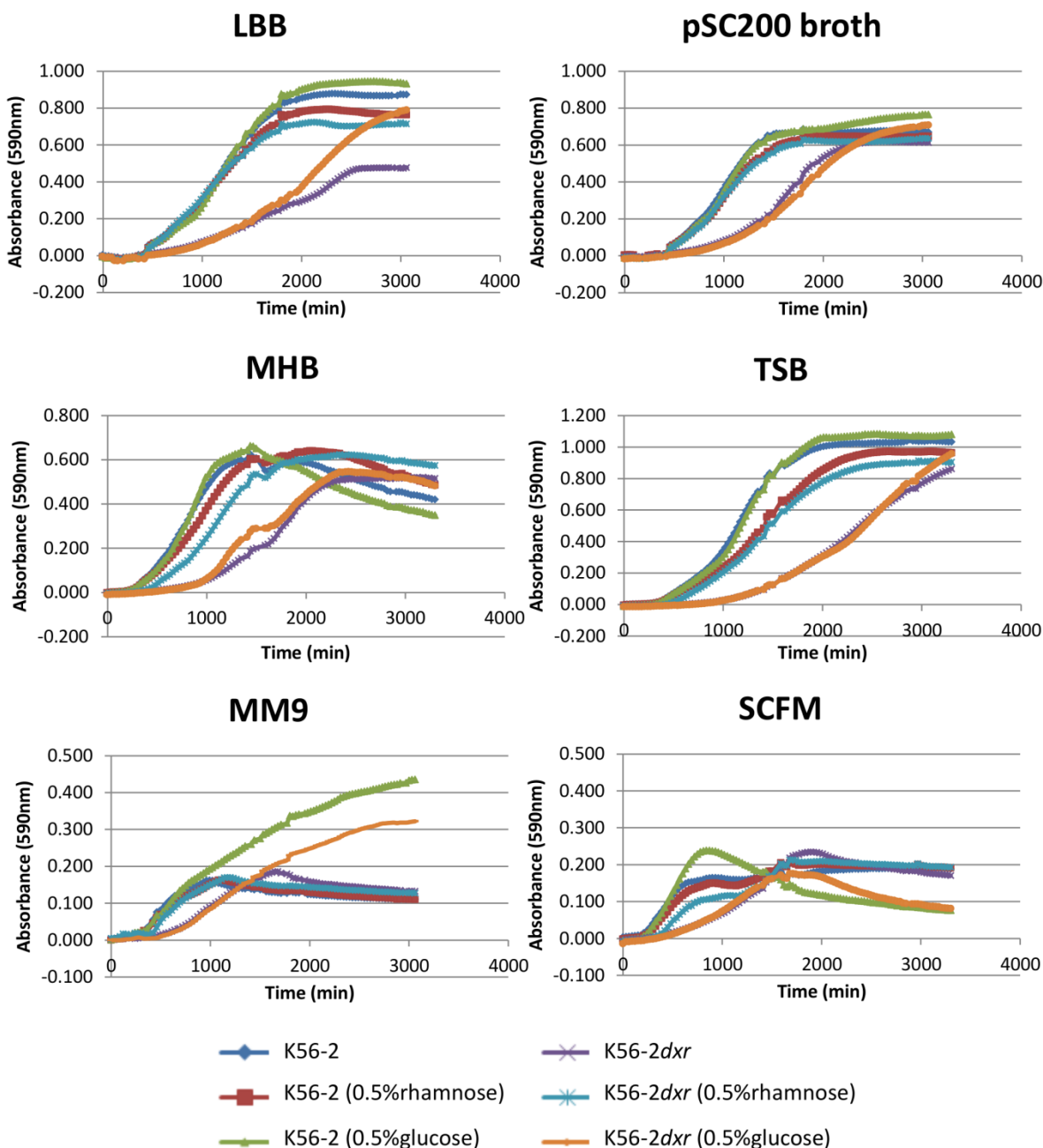


Figure 22. Growth curves of planktonic cultures of K56-2 and K56-2dxr in different growth media (LBB, pSC200 broth, MM9, TSB, MHB and SCFM).

2.4.3. Biofilm formation

Biofilm formation of *B. cenocepacia* K56-2 and K56-2dxr in LBB and pSC200 was evaluated and quantified by resazurin staining (Figure 23). When grown in LBB without rhamnose, biofilm formation of K56-2dxr is

significantly lower ($p < 0.05$, $n=3$) than that observed for the wild type, but no significant differences in biofilm formation are observed in pSC200 medium.

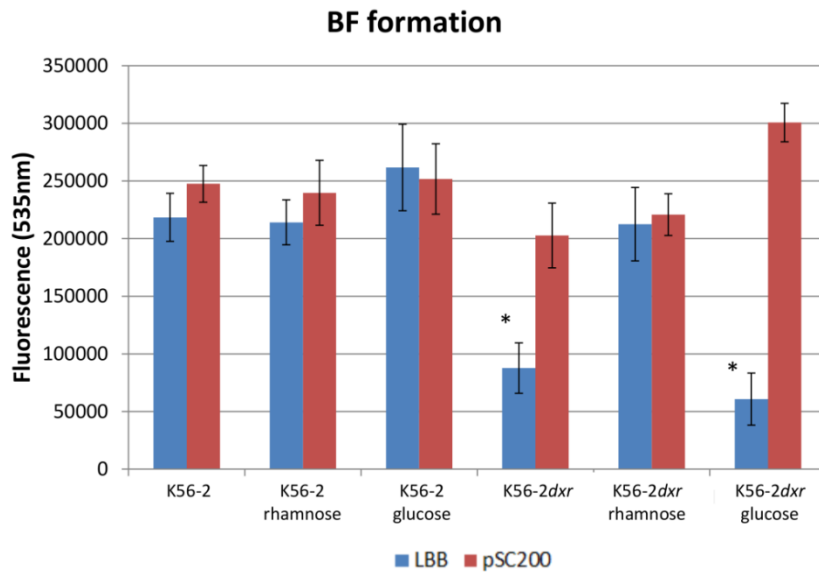


Figure 23. Biofilm formation (24h) of *B. cenocepacia* K56-2 and K56-2dxr in LBB and pSC200 broth, quantified by resazurin staining. Concentration of rhamnose and glucose is 0.5% (wt/vol). Error bars represent standard deviation. ' * ' indicates statistically significant differences with respect to K56-2 under identical growth conditions ($p < 0.05$, $n=12$).

To confirm these observations, we determined the number of CFU/biofilm by conventional plating (Figure 24). A small, but statistically significant decrease ($p < 0.05$, $n=9$) in biofilm formation of K56-2dxr was observed when grown in LBB or pSC200 without rhamnose, compared to K56-2 cultured under identical conditions. No significant differences in CFU/biofilm are observed in LBB or pSC200 with 0.5% rhamnose.

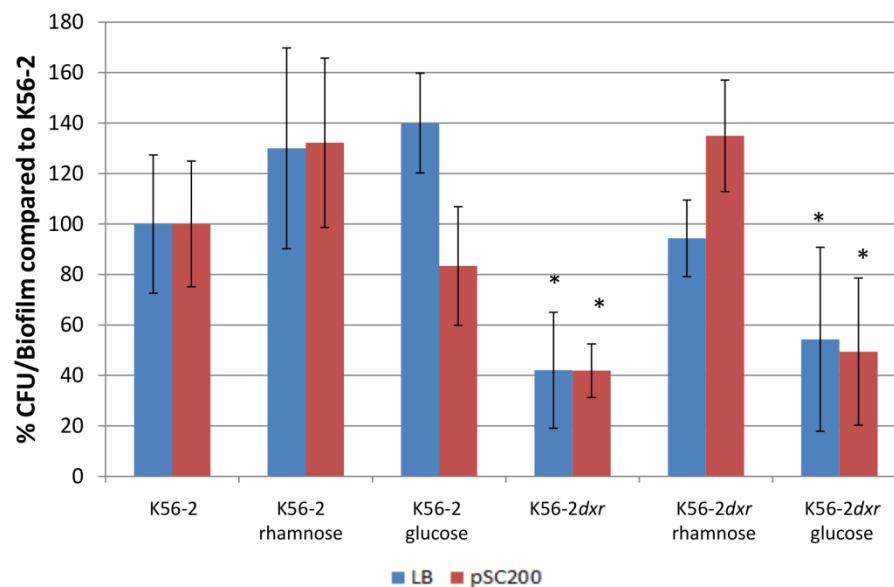


Figure 24. CFU/Biofilm of *B. cenocepacia* K56-2 and K56-2dxr in LB and pSC200, compared to K56-2. Error bars represent STDEV. Concentration of rhamnose and glucose is 0.5% (wt/vol). ' * ' indicates statistically significant differences with respect to K56-2 under identical growth conditions ($p < 0.05$, $n=3 \times 3$).

2.4.4. Differences in *in vitro* antimicrobial susceptibility

We evaluated whether there are differences in antimicrobial susceptibility between *B. cenocepacia* K56-2 and K56-2*dxr* (Table 16). These experiments were carried out in the absence of rhamnose (i.e. very low levels of *dxr* expression). Relevant (i.e. more than two-fold)

MIC (µg/ml)	K56-2	K56-2 <i>dxr</i>
Ceftazidime	64	4
Ciprofloxacin	8	8
Meropenem	16	8
Minocycline	8	4
Tobramycin	128	256
Aztreonam	>512	64
Erythromycin	256	256
Chloramphenicol	16	16
Rifampicin	64	64
Tetracyclin	32	32
MIC (µM)	K56-2	K56-2 <i>dxr</i>
Ritanserin	>500	>500
Fosmidomycin	>500	>500
FR900098	>500	250

differences in MIC-values between K56-2 and K56-2*dxr* were only observed for ceftazidime and aztreonam. The MIC of ceftazidime decreased 16-fold (from 64 µg/mL for K56-2 to 4 µg/mL for K56-2*dxr*) and the MIC of aztreonam decreased at least 8-fold (from > 512 µg/mL for K56-2 to 64 µg/mL for K56-2*dxr*).

Table 16. MIC-values (in µg/mL or µM) of 13 different antibiotics for *B. cenocepacia* K56-2 and K56-2*dxr*.

The increased susceptibility of *B. cenocepacia* K56-2*dxr* for aztreonam and ceftazidime raised the possibility of specific DXR-inhibitors to potentiate the antibacterial activity of both antibiotics. Therefore, checkerboards synergy assays were performed with DXR-inhibitors fosmidomycin or FR900098 in combination with ceftazidime or aztreonam on planktonic cultures of K56-2 and K56-2*dxr*. Results for ceftazidime-FR900098, which was shown to be the most effective combination, are shown in Figure 25 and results for the remaining combinations are depicted in Supplementary file S2. *In vitro* checkerboard assays with *B. cenocepacia* K56-2*dxr* in the presence of rhamnose showed that the susceptibility for ceftazidime is reversed to wild-type levels, with an MIC-value of 32 µg/mL without addition of FR900098 (graphs presented in Supplementary file S3).

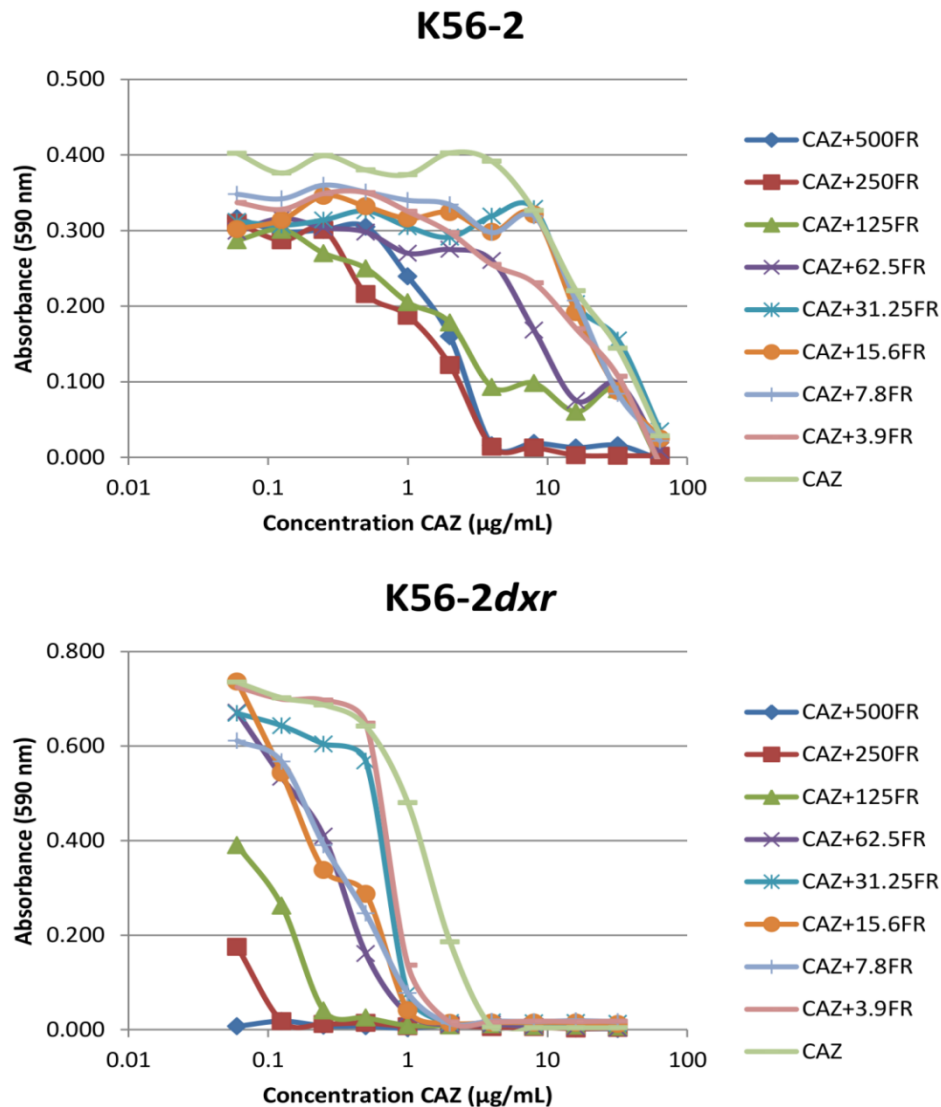


Figure 25. *In vitro* activity of ceftazidime (CAZ) combined with FR900098 (FR) against *B. cenocepacia* K56-2 and K56-2dxr. '500FR', '250FR', etc. indicates the concentration of FR900098 (in μM).

2.4.5. Survival of *C. elegans* infected with *B. cenocepacia* K56-2 or K56-2dxr

The virulence of *B. cenocepacia* K56-2 and K56-2dxr was evaluated using a *C. elegans* *in vivo* model system. Percentage survival of *C. elegans* after 24, 48 and 72 hours of infection is shown in Figure 26. Percentage survival of the conditional mutant K56-2dxr was compared to wild type K56-2 cultured under the same conditions. In the absence of rhamnose, significantly more ($p < 0.05$) worms survived infection with the conditional K56-2dxr mutant than with the wild type strain, at all time points. When *C. elegans*

was infected with K56-2dxr in the presence of 0.5% rhamnose, significantly ($p < 0.05$) more worms survived infection at 24 and 48 hours, compared to wild type K56-2. But, no significant ($p > 0.05$) difference in survival percentage was observed between mutant and wild type K56-2 after 72 h infection.

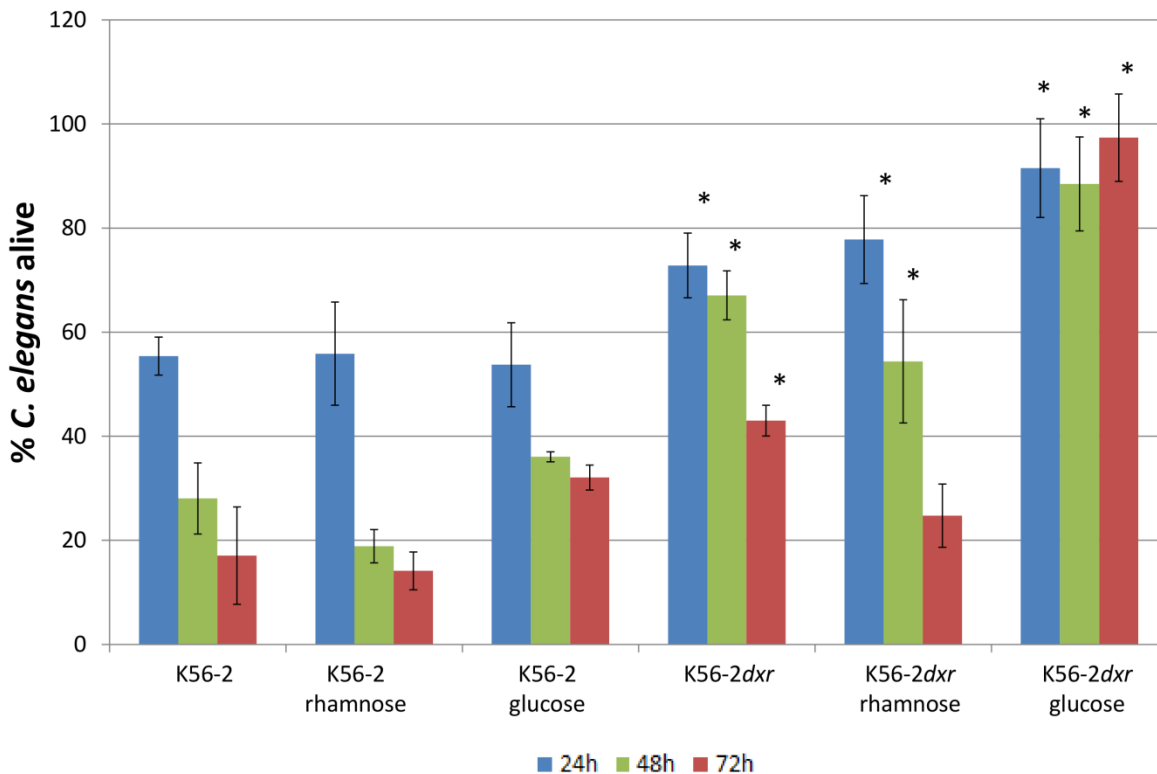
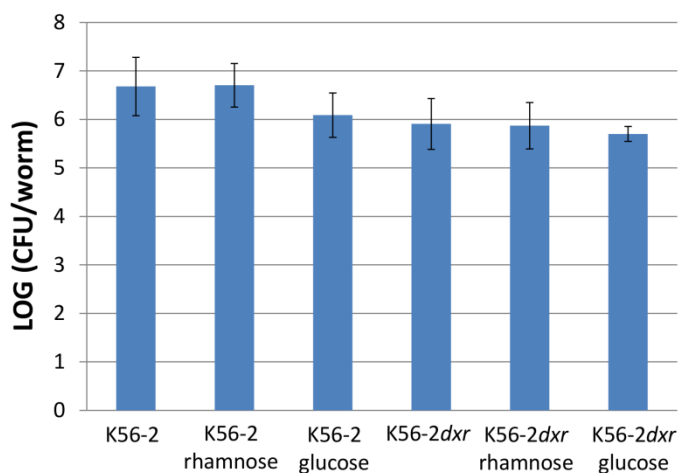


Figure 26. Percentage survival of *C. elegans*; uninfected (+) or infected with K56-2 or K56-2dxr. The results are expressed as percentage survival after 24, 48 and 72 hours of infection, relative to uninfected controls with identical culture conditions. Rhamnose and glucose are added at 0.5% (wt/vol). Error bars represent STDEV ($n=12$), ' * ' indicates statistically significant differences compared to K56-2 cultured under the same conditions ($p < 0.05$, $n=12$).

Additionally, we quantified the number of CFU/worm by conventional plating after 72h of infection. No significant differences in the number of bacteria per nematode were observed, confirming that the



difference shown in Figure 26 are not due to differences in bacterial growth (Figure 27).

Figure 27. LOG CFU/worm determined by conventional plating after 72 hours infection of *C. elegans* ($n=3$). Concentration of rhamnose and glucose is 0.5% (wt/vol).

2.4.6. Evaluation of differences in carbon metabolism

Biolog Phenotype Microarrays were used to analyze the metabolism of 190 different carbon sources by *B. cenocepacia* K56-2 and the conditional mutant K56-2*dxr*. Carbon sources for which the absorbance was equal to the negative controls were identified as 'not metabolized'. Eight compounds were identified that are metabolized by K56-2 but not by K56-2*dxr*: D-galactose, N-acetyl-D-galactosamine, D-tagatose, β -methyl-D-glucoside, salicin, lactitol, capric acid (decanoic acid) and D-galactonic acid- γ -lactone (D-galactonate).

2.5. Discussion

B. cenocepacia is one of the major pathogens causing bacterial infections in CF patients and is difficult to treat because of its high innate antimicrobial resistance. The goal of the present study was to evaluate the essentiality of the nonmevalonate pathway in *B. cenocepacia* and its potential as a novel antibacterial target. A conditional mutant of DXR was constructed in *B. cenocepacia* K56-2 and its phenotype was studied. Combined, our qPCR and sequencing data clearly indicate that we successfully constructed a rhamnose-inducible *B. cenocepacia* K56-2 *dxr* mutant. Unfortunately, we were unable to insert the correct *ispD* construct in *B. cenocepacia*, possibly because the size of the *ispD* fragment was too small or because *ispD* is expressed in one operon with *ispF*. However, disrupting *dxr* is sufficient to investigate essentiality of this pathway in *B. cenocepacia* since this enzyme catalyzes the rate-limiting step.

Growth experiments showed that DXR is essential for planktonic growth of *B. cenocepacia* in several culture media. Our data also show that biofilm growth of *B. cenocepacia* K56-2 in LBB is dependent on expression of *dxr*. In pSC200 broth, no differences of sessile growth of K56-2 are observed with resazurin staining however, a significant decrease in CFU/biofilm is observed upon disruption of *dxr*. We currently do not have an explanation for this discrepancy between both quantification methods or between LB and pSC200, although it cannot be attributed to differences in CFU/biofilm between both media. The growth inhibition of *B. cenocepacia* upon disruption of DXR is contradictory to the negative MIC results of the DXR inhibitors in Chapter III.1. Two explanations can possibly clarify this discrepancy. First, the *in vitro* activity of the DXR inhibitors has not yet been tested on purified DXR of *B. cenocepacia*, previous research has mostly been focused on inhibitory activity on the enzymes of *E. coli*, *P. falciparum* or *M.*

tuberculosis. Second, the lack of *in vitro* activity of the tested compounds against whole cells of *B. cenocepacia* is most likely related to limited cellular uptake. Therefore, it is not straightforward to correlate the MIC results in Chapter III.1 to differences in growth observed in the current chapter.

Susceptibility of *B. cenocepacia* K56-2 towards ceftazidime and aztreonam increased upon disruption of *dxr*, which can possibly be attributed to cell wall defects through a lack of isoprenoid biosynthesis. Therefore, the increased susceptibility of *B. cenocepacia* K56-2*dxr* for aztreonam and ceftazidime raised the possibility to use specific DXR-inhibitors to potentiate antibacterial activity of both antibiotics. *In vitro* checkerboard assays with fosmidomycin or FR900098 and ceftazidime or aztreonam confirmed this hypothesis. Addition of at least 250 μ M FR900098 to ceftazidime treatment against *B. cenocepacia* K56-2 reduced the MIC of ceftazidime from 64 μ g/mL to 4 μ g/mL which represents a shift from resistant to susceptible, according to EUCAST breakpoints.[235] However, FR900098 also increased the activity of ceftazidime against K56-2*dxr* in the absence of rhamnose, where the expression of *dxr* is shown to be very low. This MIC-decrease from 4 μ g/mL to less than 0.06 μ g/mL upon addition of at least 125 μ M FR900098 could be explained by the fact that this DXR-inhibitor might also target other nonmevalonate enzymes when used at high concentrations. This *in vitro* activity screening showed that combination therapy of ceftazidime with FR900098 (or other potent DXR inhibitors) could offer an approach to treat *B. cenocepacia* infections.

Results obtained in an *in vivo* *C. elegans* infection model suggest that full virulence of *B. cenocepacia* K56-2 in this model requires sufficiently high levels of expression of *dxr*. Significantly more worms survived infection with K56-2*dxr* in the absence of rhamnose (40-70% survival), compared to wild type K56-2 (15-50% survival). When glucose is added to the medium, a high survival percentage of 90-100% is observed. This can be explained by the fact that in this mutagenesis system, expression of P_{rhaB} (and thus of *dxr*) upon glucose addition is even lower than without an extra carbon-source. The fact that DXR significantly affects *in vivo* virulence of *B. cenocepacia* can be of substantial value.

Data obtained with Biolog Phenotype microarrays showed that disruption of *dxr* in *B. cenocepacia* leads to alterations in the metabolism of eight carbon sources. Investigation of all eight compounds through the KEGG Pathway Database and the *Burkholderia* Genome Database led to the following observations.[231, 234] β -D-glucoside plays a role in starch and sucrose metabolism of *B. cenocepacia*; it is converted by beta-glucosidases to D-glucose. Salicin is involved in gluconeogenesis and the phosphotransferase system through synthesis of β -D-glucose-6-P and phospho- β -glucoside, respectively.

Capric acid (decanoic acid) is a fatty acid which is involved in catabolic and anabolic reactions to generate energy and biologically important molecules. Thus, three out of eight carbon sources play an important metabolic role in *B. cenocepacia* but no clear relation with DXR or the nonmevalonate pathway could be found. For the five other compounds, an interesting connection with DXR was observed. D-galactose, N-acetyl-D-galactosamine, D-tagatose, lactitol and D-galactonic acid- γ -lactone (D-galactonate) can all be metabolized to D-glyceraldehyde-3-phosphate (G3P).[236] G3P is essential for isoprenoid biosynthesis in *B. cenocepacia* since it is, together with pyruvate, the precursor for the nonmevalonate pathway.[213] The results in this study suggest that disruption of *dxr* in *B. cenocepacia* does not only inactivate the nonmevalonate pathway for isoprenoid biosynthesis, but also has an influence on upstream metabolic pathways such as galactose metabolism and synthesis of G3P.

To conclude, the present study proves that DXR is essential for *in vitro* growth of planktonic and sessile cultures of *B. cenocepacia* K56-2. DXR also has an influence on antimicrobial resistance against ceftazidime and aztreonam and plays a key role in *in vivo* virulence in a *C. elegans* infection model. Our data indicate that the nonmevalonate pathway is an interesting target for antibacterial chemotherapy against *B. cenocepacia*.

SUPPLEMENTARY DATA**S1. Data Sanger sequencing**

DNA sequence *B. cenocepacia* K56-2*dxr*

(primers rhaF/DXR R4)

AGGTCGCGAATTCAGGCGCTTTTACTGGTCGTAATGAAATTCAGCAGGATCACATATGCAAAAACGTCTGACA
TTGCTCGGTTCCACAGGCTCGATCGGAGACAGCACGCTCGACGTGGTCGCGCGCCATCCCAGCGCTTCTCGGTCT
ATGCGCTGACCGCGCACCGCAACGGCGACAAGCTCGTCGAGCAGTGTCTGCGCTTCGCGCCTGAAGTGGCGGTGG
TCGGCGATGCCGCGACGGCCGCGCACGTGACGCCAACTGCGCGCGGCCGCGCAGCAAGACGGTCGTGCTGCAC
GGGCCGAGGCGCTCGTCGACGTGTGCAAGAGCGACGGCTGCGACACGGTGGTCGCGGCGATCGTCGGCGCGGC
CGGCCTCGCGCCGAGCCTGGCGGCCGACGCGCCGGCAAGCGGATCCTGCTCGCGAACAAGGAAGCGCTCGTGA
TGTCGGGCGCGATCTTCATGGACGCCGTACGCGACCATGGCGCGATCCTGCTGCCGGTCGACAGCGAACACAACG
CGATCTTCCAGTGCATGCCGCGCGACGCGGCCGAGCACGGCGGGATCTCGAAGATCATCTGACCGCGTCGGGCG
GCCCCGTTCCGTACGCGCGAACCGGCCACGCTGGTCGACGTACGCCGGACGAAGCGTGCAAGCACCCGAAGTGGG
TGATGGGCCGCAAGATCTCGGTGATTCCGCGACGATGATG

dxr sequence (nucleotide 1-657 of 1197)

Prha_B sequence (nucleotide 226-255)

CATATG NdeI restriction site

DNA sequence *E. coli* DH5α λpir containing pSC200 with *ispD* insert

(primers rhaF/200-MCS-R)

GGCGCTTTTACTGGTCGTAATGAAATTCAGCAGGATCACATATGACTCCCCGACTTTTCGCCCTGATTCCCTGT
GCCGGCACGGGCGAGCCGTTCCGGCTCGGCCGTGCCGAAGCAATACCGGACGCTGGCCGGCCGCGCGCTGCTGCA
CTACACGCTCGTCTAGAGGTACCGCATGCGATATCGAGCTCTCCGGGAATTCCACAAATTGTTATCCGCTCACAAT
TCCACATGTGGAATTCCACATGTGGAATTCATGTCAGCCGTTAAGTGT

Sequence *ispD* insert (amplified with primers ispD F1/ispD R5)

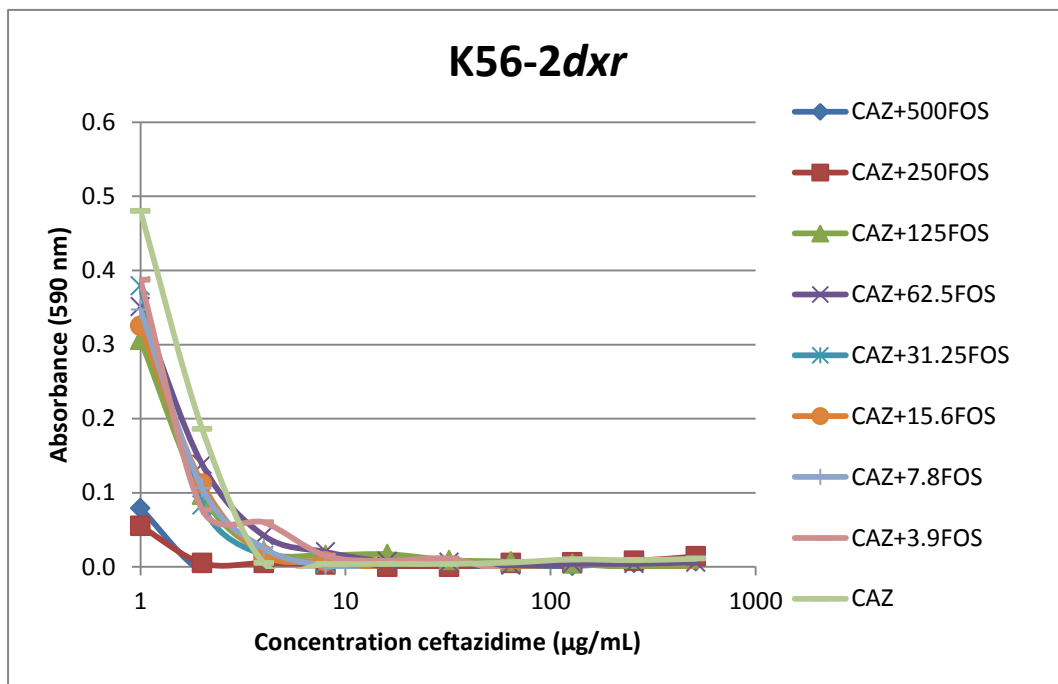
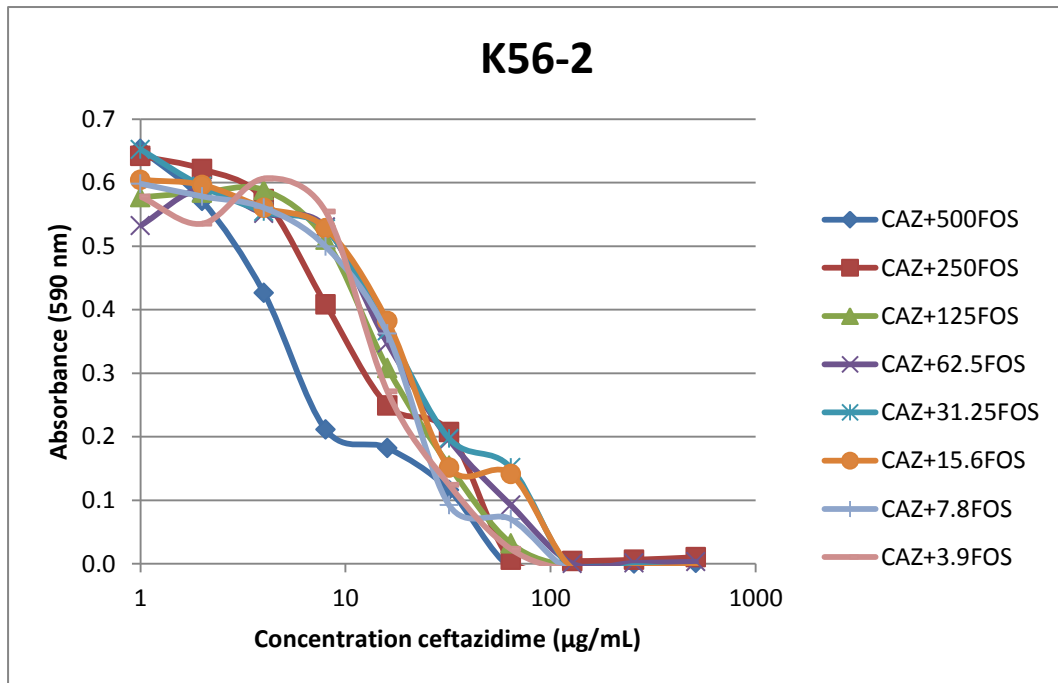
Prha_B sequence (nucleotide 226-255)

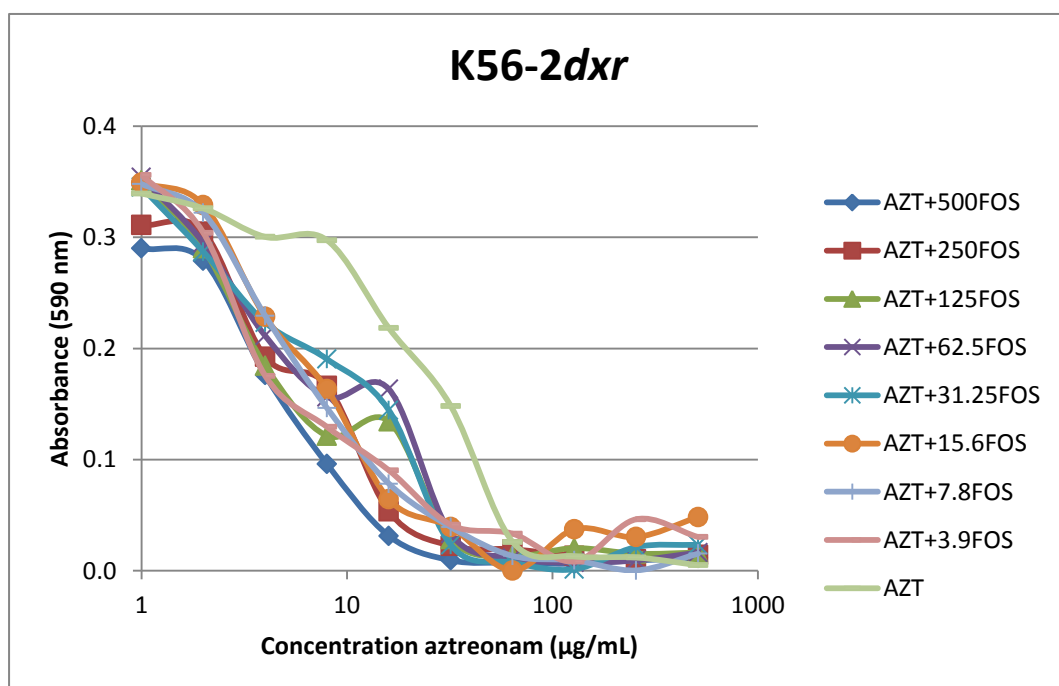
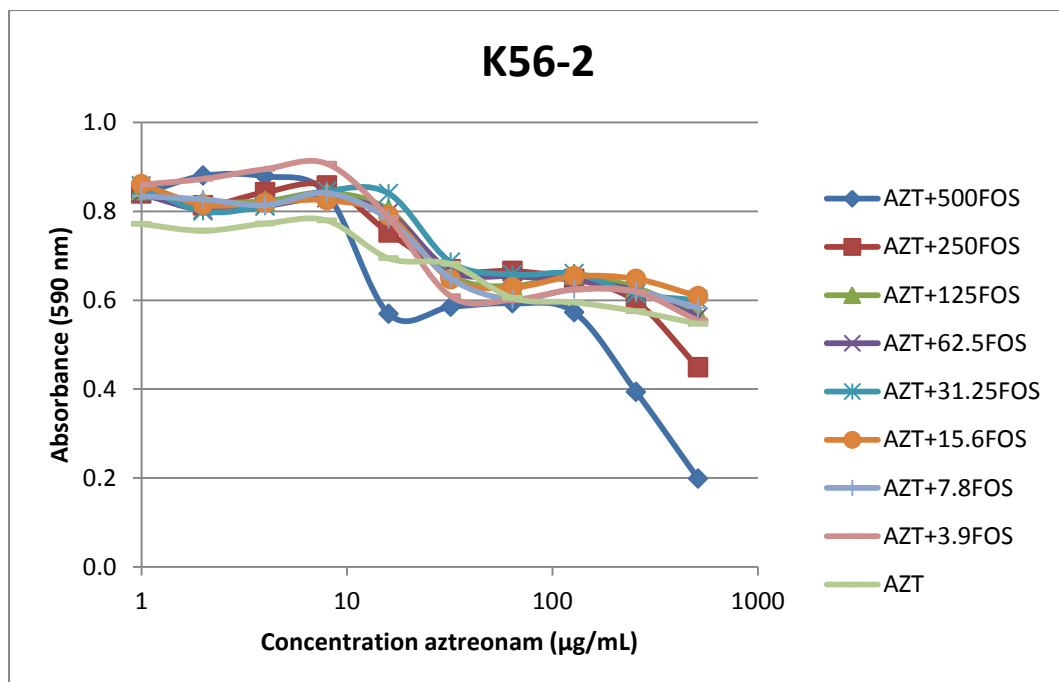
Partial MCS and *mob* sequence of pSC200

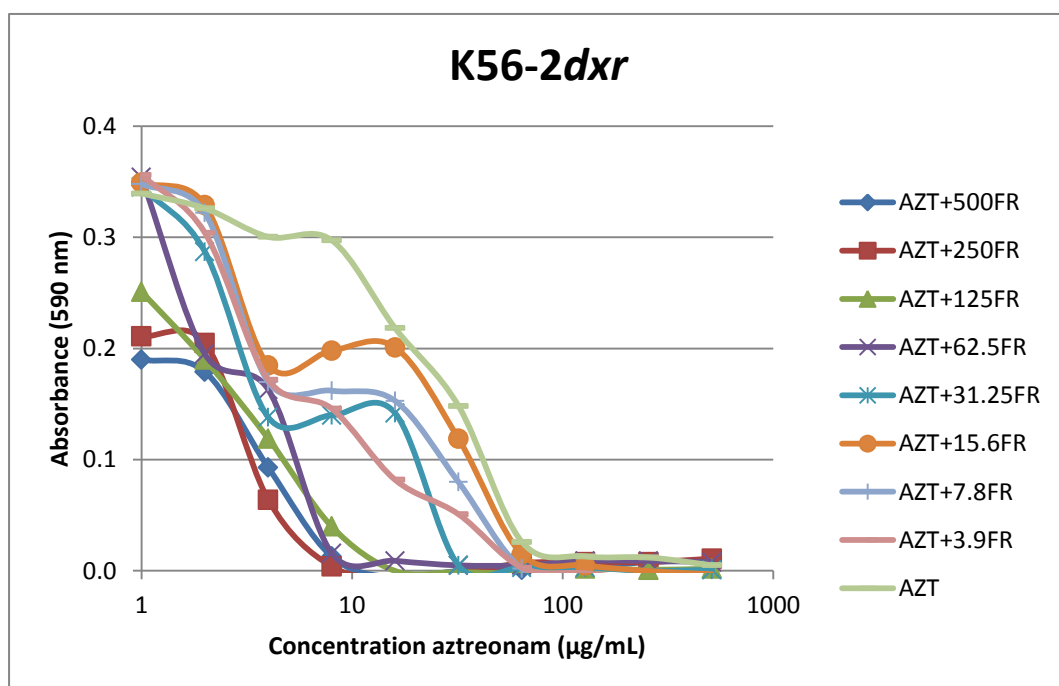
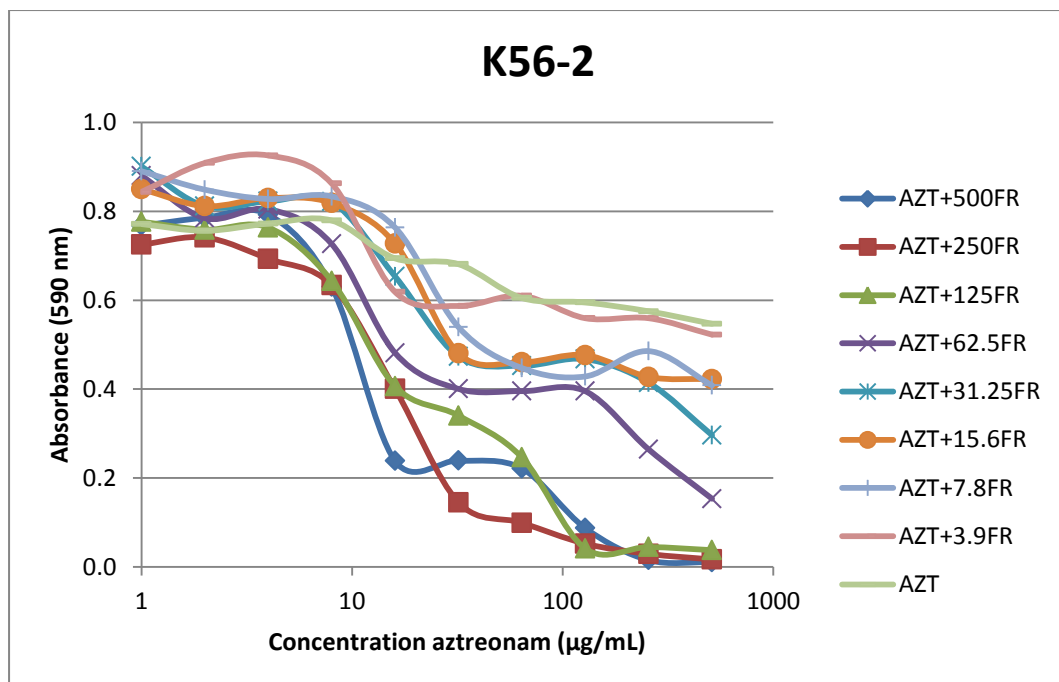
CATATG NdeI restriction site

TCTAGA XbaI restriction site

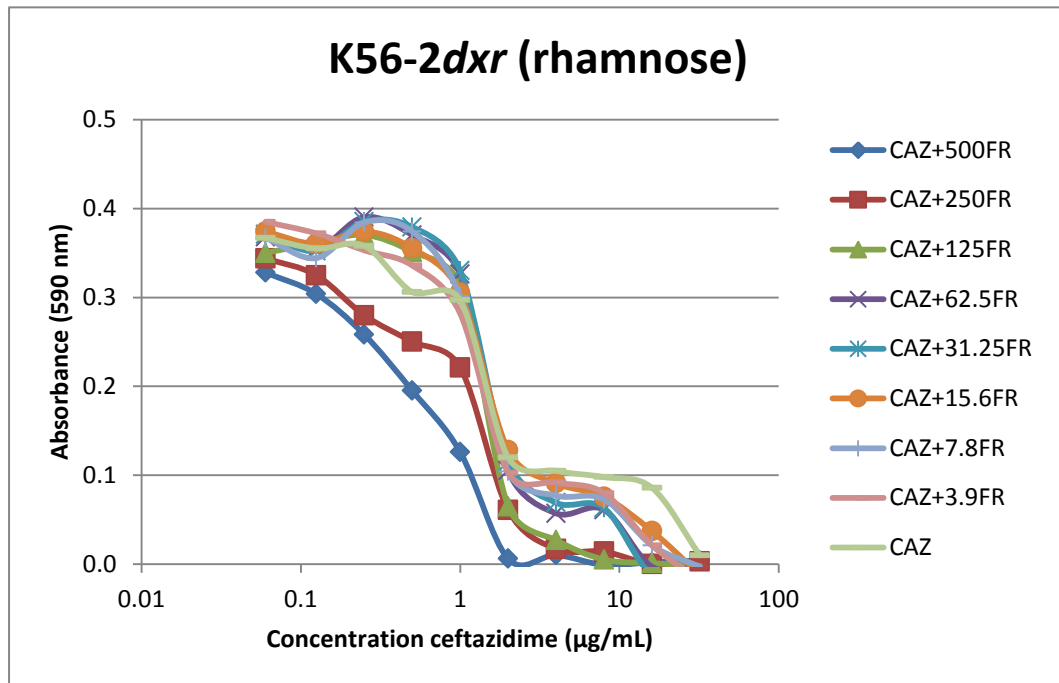
S2. Checkerboard assay on *B. cenocepacia* K56-2 and K56-2dxr with ceftazidime (CAZ) or aztreonam (AZT) in combination with fosmidomycin (FOS) or FR900098 (FR).







S3. Checkerboard assay on *B. cenocepacia* K56-2*dxr* with ceftazidime (CAZ) in combination with FR900098 (FR), in the presence of 0.5% rhamnose.



3. EFFECT OF β -LACTAMASE INHIBITORS ON *IN VITRO* ACTIVITY OF β -LACTAM ANTIBIOTICS
AGAINST *BURKHOLDERIA CEPACIA* COMPLEX SPECIES

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3.1. Abstract

BACKGROUND. Bacteria belonging to the Bcc are an important cause of chronic respiratory tract infections in cystic fibrosis patients. Intrinsic resistance to a wide range of antimicrobial agents, including a variety of β -lactam antibiotics, is frequently observed in Bcc strains. Resistance to β -lactams is most commonly mediated by efflux pumps, alterations in penicillin-binding proteins or the expression of β -lactamases. β -lactamase inhibitors are able to restore the *in vitro* activity of β -lactam molecules against a variety of Gram-negative species, but the effect of these inhibitors on the activity of β -lactam treatment against Bcc species is still poorly investigated.

METHODS. In the present study, the susceptibility of a panel of Bcc strains was determined towards the β -lactam antibiotics ceftazidime, meropenem, amoxicillin, cefoxitin, cefepime and aztreonam; alone or in combination with a β -lactamase inhibitor (clavulanic acid, sulbactam, tazobactam and avibactam). Consequently, β -lactamase activity was determined for active β -lactam/ β -lactamase inhibitor combinations.

RESULTS. Clavulanic acid had no effect on minimum inhibitory concentrations, but addition of sulbactam, tazobactam or avibactam to ceftazidime, amoxicillin, cefoxitin, cefepime or aztreonam leads to increased susceptibility (at least 4-fold MIC-decrease) in some Bcc strains. The effect of β -lactamase inhibitors on β -lactamase activity is both strain- and/or antibiotic-dependent, and other mechanisms of β -lactam resistance (besides production of β -lactamases) appear to be important.

CONCLUSIONS. Considerable differences in susceptibility of Bcc strains to β -lactam antibiotics were observed. Results obtained in the present study suggest that resistance of Bcc strains against β -lactam antibiotics is mediated by both β -lactamases and non- β -lactamase-mediated resistance mechanisms.

3.2. Background

Infections caused by antibiotic-resistant bacteria pose an increasing threat to public health, both in terms of human suffering and in economic terms. In addition to the costs associated with an extended hospital stay, the costs for treating infections caused by multidrug-resistant organisms are much higher than the costs for treating similar infections caused by sensitive organisms.[237] Despite the impact of

these infections, the number of novel antibiotics in the pipeline is small. This is partly due to the high costs and extensive research-time associated with the development of a new antibiotic.[238]

The Bcc is a group of 20 closely related opportunistic, Gram-negative pathogens.[1] Bcc species are an important cause of severe chronic respiratory infections in patients with cystic fibrosis (CF).[186] Bcc infection in CF patients often correlates with a rapid decrease in lung function leading to a poorer prognosis, longer hospital stays and an increased risk of death. The ability of Bcc strains to form biofilms *in vitro* and *in vivo* contributes to reduced antimicrobial susceptibility, treatment failure and persistent infection.[7, 36, 37] Intrinsic resistance to a wide range of antimicrobial agents, including aminoglycosides, polymyxin, first and second generation cephalosporins, carboxypenicillins and other β -lactam antibiotics is frequently observed in Bcc strains. Resistance of Gram-negative microorganisms to β -lactam molecules is most commonly mediated by inducible or constitutively expressed β -lactamases, efflux pumps or alterations in PBPs.[46, 47] β -lactamase was first identified in 1940 as penicillinase in *E. coli* even before the clinical use of penicillin, and has since been identified in a variety of other bacteria, including species of the Bcc. [65, 158, 171, 239]

β -lactam antibiotics cause cell death by inhibition of bacterial cell wall synthesis. They do so by binding to PBPs, resulting in a decreased cross-linking of peptidoglycan in the cell wall, eventually leading to cell death.[46, 161] The utility of β -lactams has become compromised through the increasing presence of both chromosomally and plasmid-encoded β -lactamase enzymes. Two classification schemes are currently used, i.e. the Ambler molecular classification (based on amino acid sequences and structural similarities) and the Bush-Jacoby-Medeiros scheme (classification according to functionality or activity against β -lactam antibiotics).[157] In the Ambler scheme, four groups of β -lactamases are recognized; class A through D. Class A, C and D have a serine active site and class B enzymes are metallo- β -lactamases (MBLs) that need Zn^{2+} as a cofactor for their activity.[65, 161] The most commonly encountered β -lactamases are class A enzymes with TEM and SHV enzymes regularly found in Gram-negative bacteria (e.g. *E. coli* and *Klebsiella* spp.), as well as CTX-M extended-spectrum β -lactamases (ESBLs) and *K. pneumoniae* carbapenemases (KPCs) which are now also frequently encountered. Most class A enzymes can be inhibited with the commercially available β -lactamase inhibitors clavulanic acid, sulbactam or tazobactam. Class B β -lactamases (MBLs) pose a particular challenge for medicinal chemists and clinicians because thus far, none of the available inhibitors can effectively inhibit members of this class.[162, 163] Currently, treatment of MBL-producing organisms is limited to relatively toxic antibiotics (e.g. colistin) and/or antimicrobials likely to cause further development of resistance (e.g. tigecycline).

EDTA, that functions as a chelator of divalent cations (including Zn^{2+}), was used earlier as an active MBL inhibitor but was withdrawn from the market in 2008 due to toxicity concerns.[152, 161] Class C or AmpC β -lactamases are encoded by genes on the bacterial chromosome, although the prevalence of plasmid-mediated AmpC-enzymes is increasing. Only the novel β -lactamase inhibitor avibactam is able to inhibit class C enzymes.[65, 161] Finally, class D β -lactamases (also known as oxacillinases), are active against a broad range of β -lactam antibiotics. Clavulanic acid, sulbactam nor tazobactam inhibit these enzymes, but avibactam inhibits some class D enzymes. [46, 65, 161]

The fact that some β -lactamase inhibitors restore the *in vitro* activity of ceftazidime against a variety of Gram-negative bacteria is known and well-established [46, 161, 240, 241], but the effect of adding these inhibitors to β -lactam antibiotics is still poorly investigated for Bcc species. Previous research on this matter led to contradictory results; Lagacé-Wiens et al. reported that avibactam does not have the ability to potentiate ceftazidime against clinical Bcc isolates, but Mushtaq et al. observed that avibactam does have variable ability to restore ceftazidime activity against Bcc isolates from patients with CF.[161, 242] These data suggest that in these Bcc isolates resistance against ceftazidime is not only mediated by expression of β -lactamases but is also due to other resistance mechanisms (efflux pumps or altered PBPs). In the present study we wanted to validate this assumptions for a larger research panel; therefore we systematically investigated the effect of β -lactamase inhibitors (clavulanic acid, sulbactam, tazobactam and the novel inhibitor avibactam) on the susceptibility of Bcc species against several β -lactam antibiotics.

3.3. Methods

3.3.1. Strains and culture conditions

The following strains were used: *B. cepacia* LMG 1222 and LMG 18821; *B. multivorans* LMG 18822, LMG 18825, LMG 13010 and LMG 17588; *B. cenocepacia* LMG 16656, LMG 18828, LMG 18829 and LMG 18830; *Burkholderia vietnamiensis* LMG 10929 and LMG 18835; *B. ambifaria* LMG 19182 and LMG 19467; *Burkholderia lata* LMG 6992 and R-9940; *Burkholderia stabilis* LMG 14294 and LMG 14086; *Burkholderia dolosa* LMG 18943 and LMG 18941; *Burkholderia anthina* LMG 20980 and LMG 20983; *Burkholderia pyrrocinia* LMG 21824; *Burkholderia ubonensis* LMG 20358 and LMG 24263; *Burkholderia latens* LMG 24064; *Burkholderia arboris* LMG 24066 and R-132; *Burkholderia seminalis* LMG 24067 and LMG 24272; *Burkholderia metallica* LMG 24068 and R-2712; and *Burkholderia contaminans* LMG 16227

and R-12710. The biological and geographic origin of every Bcc strain is presented in Supplementary data (Table S1). All strains were obtained from the BCCM/LMG Bacteria Collection (Ghent, Belgium) or were kindly provided by Prof. P. Vandamme (Ghent University, Belgium). Two control strains were included; *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922, both obtained from the ATCC collection (Manassas, VA, USA). Bacterial cultures were stored at -80°C in Microbank vials (Prolab Diagnostics, Richmond Hill, ON, Canada) and were subcultured twice on Luria-Bertani agar (LBA; LabM Limited, Heywood, UK) before use. All cultures were incubated aerobically at 37°C.

3.3.2. Antibiotics and β -lactamase inhibitors

We used several β -lactam antibiotics of different classes including amoxicillin (AMOX; aminopenicillin), cefoxitin (CFX; 2nd generation cephalosporin), ceftazidime (CAZ; 3rd generation cephalosporin), cefepime (CFP; 4th generation cephalosporin), meropenem (MEM; carbapenem) and aztreonam (AZT; monobactam). The effect of combining these antibiotics with the β -lactamase inhibitors clavulanic acid (CLA), sulbactam (SUL), tazobactam (TAZ) and avibactam (AVI) was investigated. AMOX, CFX, CAZ, CFP, CLA, SUL and TAZ were purchased from Sigma-Aldrich (St. Louis, MO, USA). MEM was obtained from Hospira Benelux (Antwerp, Belgium), AZT from TCI Europe (Zwijndrecht, Belgium) and AVI was obtained from Adooq Bioscience (Irwin, CA, USA). The concentration range tested for CAZ and MEM was 0.25 to 128 mg/L. Higher concentrations were tested for AMOX, CFX, CFP and AZT; between 1 and 512 mg/L (according to Peeters et al. [186] and CLSI guidelines). The β -lactamase inhibitors were added at fixed concentrations as mentioned in the European Committee on Antimicrobial Susceptibility testing (EUCAST) breakpoint tables; 2 mg/L for CLA and 4 mg/L for SUL, TAZ and AVI.[235]

3.3.3. MIC determination

Susceptibility of the selected Bcc strains was investigated by determining minimum inhibitory concentrations (MICs) (in triplicate) of β -lactam antibiotics in the presence or absence of β -lactamase inhibitors, according to the EUCAST broth dilution guidelines using flat-bottomed 96-well microtitre plates (TPP, Trasadingen, Switzerland).[183] Antibiotic solutions were added to the wells and two-fold dilutions were made. Planktonic cultures were grown overnight in Luria-Bertani broth (LBB; LabM, Lancashire, UK) at 37°C. The cultures were then adjusted with double-concentrated Mueller-Hinton

broth (MHB; Beckton, Dickinson & Company (BD), Erembodegem, Belgium) to obtain a final inoculum of 5×10^5 cfu/ml. Plates were incubated for 24h at 37°C and optical density was determined at 590 nm using an Envision multilable plate reader (Perkin Elmer, Waltham, MA, USA). The MIC value is the lowest concentration of the antibiotic that completely inhibits bacterial growth.[183, 186]

3.3.4. β -lactamase activity assay

We explored differences in β -lactamase activity by using a β -lactamase activity assay kit (Sigma-Aldrich, St. Louis, MO, USA). This assay is based on the hydrolysis of the chromogenic molecule nitrocefin, a non-antimicrobial cephalosporin, by β -lactamase which leads to the production of a colorimetric product. Formation of this product is monitored by measuring absorbance at 490 nm in an Envision multilable plate reader; every minute, for 60 to 90 minutes at 25°C. The amount of enzyme required to hydrolyze 1.0 μ mol of nitrocefin per minute at pH 7.0 at 25°C is equal to one unit of β -lactamase.[65, 158, 243] Bacterial cultures were grown in 96-well microtitre plates in the presence or absence of antibiotics. Antibiotics were added to the microtitre plates at concentrations of $\frac{1}{4}$ MIC and the β -lactamase inhibitors SUL, TAZ and AVI were added at 4mg/L. Planktonic cultures were grown overnight in LB broth at 37°C, then adjusted with double-concentrated MHB to obtain a final inoculum of 5×10^5 cfu/ml in the 96-well plates. For every condition 10 wells in the same plate were filled and the plates were incubated at 37°C for 24h. After incubation, the content of the 10 wells was collected in a pre-weighed plastic tube and centrifuged at 10 000 RCF for 10 minutes. Then, supernatant was discarded and the pellet was weighed and resuspended with 5 μ L of β -lactamase assay buffer per mg sample. Subsequently, samples were sonicated for 5 minutes, placed on ice for 5 min and centrifuged at 16 000 RCF at 4°C for 20 minutes. 1 – 50 μ L of the unknown samples was added to a clear flat 96-well plate and supplemented with nitrocefin and buffer to a final volume of 100 μ L. Immediately after addition of nitrocefin, absorbance at 490 nm was measured in an Envision plate reader.

3.4. Results and Discussion

3.4.1. MIC determination

For six Bcc strains (*B. cepacia* LMG 1222, *B. multivorans* LMG 18822, *B. cenocepacia* LMG 16656, *B. vietnamiensis* LMG 10929, *B. ambifaria* LMG 19182 and *B. lata* LMG 6992) and two control strains (*P.*

aeruginosa ATCC 27853 and *E. coli* ATCC 25922) MIC values of all compounds and combinations were determined (Table 17). When the MIC value for a given antibiotic decreased 4-fold or more upon addition of a β -lactamase inhibitor, the inhibitor was considered to have meaningful activity. CLA showed no effect in this initial screening and was not included in further experiments. SUL, TAZ and AVI did show some effect in the initial screening and these three inhibitors were tested against a larger Bcc strain panel (Table 18).

In general, the MICs of each β -lactam antibiotic alone varied widely; with MIC values of CAZ ranging from 0.25 to >128 mg/L, MEM from 0.25 to 64 mg/L, AMOX from 8 to >512 mg/L, CFX from 2 to >512 mg/L and of CFP and AZT ranging from 1 to >512 mg/L. Antibiotic susceptibility results for Bcc species can be expressed as susceptible (S) or resistant (R) according to the EUCAST PK/PD (non-species related) breakpoints.[235] *In vitro* activity of CAZ against some Bcc strains was increased when combined with SUL or AVI; e.g. for *B. multivorans* LMG 17588 the MIC for CAZ decreased from 16 mg/L in the absence of a β -lactamase inhibitor to 2 mg/L in the presence of SUL. Hence, addition of SUL to CAZ for treatment of *B. multivorans* LMG 17588 induces a shift from R to S. Also for AMOX and AZT an increased *in vitro* activity against some strains was observed, in combination with SUL or AVI. However, only the addition of SUL to AZT treatment leads to a change from R to S for a selection of strains (*B. anthina* LMG 20980, *B. multivorans* LMG 13010 and *B. vietnamiensis* LMG 18835). CFP showed at least 4-fold increased activity against some Bcc strains when combined with SUL, TAZ or AVI and especially addition of TAZ and SUL lead to a shift from R to S for certain Bcc strains. Resistance towards MEM due to β -lactamases has not yet been observed in the Bcc and MEM is one of the β -lactam antibiotics with the best growth-inhibitory activity against Bcc species at clinically relevant concentrations.[186, 244] We did not observe altered susceptibility to MEM when it was combined with SUL, TAZ or AVI, confirming that Bcc strains do not produce β -lactamases that can degrade MEM. For CFX only one combination with meaningful activity was observed; MIC of CFX against *B. lata* LMG 6992 decreased from 32 mg/L in the absence of a β -lactamase inhibitor to 8 mg/L in the presence of SUL. Due to the lack of PK/PD breakpoints for CFX, it is unclear whether this change is clinically relevant. These results suggest that for most Bcc strains non- β -lactamase-mediated resistance to CFX, in the form of efflux pumps, altered PBPs, and/or reduced permeability, plays a more important role than β -lactamases in β -lactam resistance. To summarize, the β -lactam resistance of 21.6% of the Bcc strains (8 out of 37) was reduced by 1 β -lactamase inhibitor, 24.3% (9/37) by 2 β -lactamase inhibitors and 8.1% (3/37) was effectively inhibited by 3 or more β -lactamase inhibitors.

Table 17. MICs of six antibiotics (in mg/L); alone or in combination with a β -lactamase inhibitor.

	CAZ					MEM					AMOX					CFX					CFP					AZT				
	-	+ Cla	+ Sul	+ Taz	+ Avi	-	+ Cla	+ Sul	+ Taz	+ Avi	-	+ Cla	+ Sul	+ Taz	+ Avi	-	+ Cla	+ Sul	+ Taz	+ Avi	-	+ Cla	+ Sul	+ Taz	+ Avi	-	+ Cla	+ Sul	+ Taz	+ Avi
<i>B. ambifaria</i> LMG 19182	4	4	2	4	1	2	2	2	2	1	>512	>512	>512	>512	>512	64	64	64	64	64	4	8	4	4	1	32	32	8	32	<u>2</u>
<i>B. cenocepacia</i> LMG 16656	32	32	32	32	32	32	16	16	16	32	>512	>512	>512	>512	>512	256	256	256	256	256	512	512	512	512	512	>512	>512	>512	>512	>512
<i>B. cepacia</i> LMG 1222	32	32	32	32	32	8	8	8	8	8	>512	>512	>512	>512	>512	256	256	256	256	256	256	128	64	256	64	>512	256	256	512	512
<i>B. lata</i> LMG 6992	1	0.50	0.25	1	0.50	0.50	0.50	0.25	0.25	0.25	>512	>512	>512	>512	>512	32	16	8	16	32	16	16	8	<u>4</u>	<u>1</u>	32	16	8	16	<u>2</u>
<i>B. multivorans</i> LMG 18822	2	2	0.50	2	1	4	4	2	2	4	>512	>512	>512	>512	>512	256	128	256	128	256	2	2	1	1	1	8	4	<u>2</u>	8	<u>2</u>
<i>B. multivorans</i> LMG 18825	8	8	<u>2</u>	8	8	32	32	32	32	32	>512	>512	>512	>512	>512	512	512	256	512	512	32	16	<u>2</u>	<u>2</u>	<u>8</u>	32	16	16	16	16
<i>B. vietnamiensis</i> LMG 10929	16	16	16	32	16	0.50	0.50	0.25	0.25	0.50	16	32	<u>2</u>	16	<u>1</u>	16	16	16	16	16	2	2	2	1	1	>512	>512	>512	>512	>512
<i>E. coli</i> ATCC 25922	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	8	8	<u>2</u>	4	<u>2</u>	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1
<i>P. aeruginosa</i> ATCC 27853	2	2	2	2	2	0.50	0.25	0.50	0.50	0.25	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	2	2	2	2	2	4	4	4	4	4

Bold values indicate combinations leading to at least 4-fold MIC-reduction upon addition of the β -lactamase inhibitor. Underlined values indicate a shift from resistant to susceptible upon addition of the β -lactamase inhibitor, according to PK/PD (non-species related) EUCAST breakpoints.[235] “-” represents treatment with β -lactam antibiotic alone. CAZ, ceftazidime; MEM, meropenem; AMOX, amoxicillin; CFX, cefoxitin; CFP, cefepime; AZT, aztreonam. Cla, clavulanic acid; Sul, sulbactam; Taz, tazobactam; Avi, avibactam.

Table 18. MICs of six antibiotics (in mg/L), alone or in combination with a β -lactamase inhibitor.

	CAZ			MEM			AMOX			CFX		CFP				AZT		
	-	+ Sul	+ Avi	-	+ Sul	+ Avi	-	+ Sul	+ Avi	-	+ Sul	-	+ Sul	+ Taz	+ Avi	-	+ Sul	+ Avi
<i>B. ambifaria</i> LMG 19467	2	1	2	4	4	4	>512	>512	>512	4	4	8	4	<u>1</u>	8	8	4	8
<i>B. anthina</i> LMG 20980	4	0.25	4	4	4	4	>512	>512	>512	128	128	4	1	1	2	16	<u>1</u>	16
<i>B. anthina</i> LMG 20983	2	0.25	2	4	4	4	>512	>512	>512	64	64	2	1	1	1	16	8	8
<i>B. arboris</i> LMG 24066	32	16	32	16	16	16	>512	>512	>512	128	128	256	64	128	128	>512	>512	>512
<i>B. arboris</i> R-132	>128	<u>16</u>	<u>16</u>	32	32	32	>512	>512	>512	256	256	128	64	64	128	512	512	512
<i>B. cenocepacia</i> LMG 18828	128	128	128	32	32	32	>512	>512	>512	512	512	>512	>512	>512	>512	>512	>512	>512
<i>B. cenocepacia</i> LMG 18829	16	8	8	4	4	4	>512	>512	>512	64	64	64	64	64	64	512	512	512
<i>B. cenocepacia</i> LMG 18830	16	16	16	16	16	16	>512	>512	>512	128	128	128	128	64	64	>512	512	512
<i>B. cepacia</i> LMG 18821	64	64	64	32	32	32	>512	>512	>512	512	512	256	256	256	256	>512	>512	>512
<i>B. contaminans</i> LMG 16227	64	64	64	16	16	16	>512	>512	>512	128	128	256	256	256	256	>512	>512	>512
<i>B. contaminans</i> R-12710	16	16	16	8	8	8	>512	>512	>512	128	128	64	32	16	64	>512	512	>512
<i>B. diffusa</i> LMG 24065	16	32	16	8	8	8	>512	>512	>512	256	256	128	128	128	128	>512	>512	>512
<i>B. diffusa</i> LMG 24266	32	32	32	4	8	8	>512	>512	>512	128	128	128	128	128	128	512	512	512
<i>B. dolosa</i> LMG 18941	64	32	64	64	32	32	>512	>512	>512	128	128	>512	512	512	>512	>512	>512	>512
<i>B. dolosa</i> LMG 18943	>128	>128	>128	64	64	64	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512
<i>B. lata</i> R-9940	1	0.25	1	1	1	1	>512	>512	>512	32	32	4	2	1	2	2	2	2
<i>B. latens</i> LMG 24064	4	4	4	4	4	4	>512	>512	>512	64	64	1	1	1	2	256	256	128
<i>B. latens</i> R-11768	8	8	8	32	32	32	>512	>512	>512	256	256	32	32	32	32	32	32	32

<i>B. metallica</i> LMG 24068	32	32	32	32	32	32	>512	>512	>512	256	256	128	128	128	128	512	512	512
<i>B. metallica</i> R-2712	8	8	8	16	16	16	>512	>512	>512	256	256	64	16	16	16	128	64	128
<i>B. multivorans</i> LMG 13010	8	<u>2</u>	4	8	8	8	>512	>512	>512	256	256	2	1	2	2	32	<u>4</u>	16
<i>B. multivorans</i> LMG 17588	16	<u>2</u>	8	16	16	16	>512	>512	>512	256	256	16	16	<u>4</u>	8	16	<u>4</u>	16
<i>B. pyrrocinia</i> LMG 21824	32	16	32	8	8	8	>512	>512	>512	32	32	512	128	128	512	>512	>512	512
<i>B. seminalis</i> LMG 24067	32	16	16	8	8	8	>512	>512	>512	256	256	128	128	128	128	512	512	512
<i>B. seminalis</i> LMG 24272	8	2	4	4	4	4	>512	>512	>512	128	128	8	<u>2</u>	4	8	32	32	32
<i>B. stabilis</i> LMG 14086	8	8	8	8	8	8	>512	>512	>512	128	128	32	32	32	8	64	64	64
<i>B. stabilis</i> LMG 14294	>128	>128	>128	16	16	16	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512
<i>B. ubonensis</i> LMG 20358	2	2	2	16	16	16	>512	>512	>512	256	128	16	16	8	16	128	128	128
<i>B. ubonensis</i> LMG 24263	8	4	4	32	32	32	>512	>512	>512	128	128	128	128	128	128	256	256	128
<i>B. vietnamiensis</i> LMG 18835	4	0.25	4	2	2	2	512	512	512	128	128	16	<u>1</u>	16	16	8	<u>1</u>	8

Bold values indicate combinations leading to at least 4-fold MIC-reduction upon addition of the β -lactamase inhibitor. Underlined values indicate a shift from resistant to susceptible upon addition of the β -lactamase inhibitor, according to PK/PD (non-species related) EUCAST breakpoints.[235] “-” represents treatment with β -lactam antibiotic alone. CAZ, ceftazidime; MEM, meropenem; AMOX, amoxicillin; CFX, cefoxitin; CFP, cefepime; AZT, aztreonam. Cla, clavulanic acid; Sul, sulbactam; Taz, tazobactam; Avi, avibactam

3.4.2. β -lactamase activity assay

For the Bcc strains for which active β -lactam antibiotic/ β -lactamase inhibitor combinations were identified, we investigated whether there is a relation between differences in β -lactamase activity and the altered susceptibility observed upon addition of a β -lactamase inhibitor. Therefore, a β -lactamase activity assay was used to measure β -lactamase activity. Only the combinations which showed at least a 4-fold MIC decrease upon addition of a β -lactamase inhibitor were included (i.e. CAZ + SUL, CAZ + AVI, AMOX + SUL, AMOX + AVI, CFX + SUL, CFP + SUL, CFP + TAZ, CFP + AVI, AZT + SUL and AZT + AVI).

Differences in β -lactamase activity were explored between untreated samples, samples treated with a β -lactam antibiotic alone and samples treated with β -lactam/ β -lactamase inhibitor combination treatments mentioned above (Figure 28). Results of the β -lactamase screening are expressed as relative β -lactamase activities compared to untreated bacterial samples. For *B. multivorans* LMG 18825 (Fig. 27a) the MIC for CFP decreased from 32 mg/L in the absence of an inhibitor to 8 mg/L in the presence of AVI, or to 2 mg/L in the presence of SUL or TAZ. As expected, treatment with 0.5 mg/L CFP significantly ($p < 0.05$, $n = 5$) increased relative β -lactamase activity to 5.44 and addition of 4 mg/L SUL significantly ($p < 0.05$, $n = 5$) decreased β -lactamase activity to 1.43. However, addition of 4 mg/L AVI had no significant ($p > 0.05$, $n = 5$) influence on β -lactamase activity (5.99) and addition of TAZ to CFP-treatment caused a significant ($p < 0.05$, $n = 5$) increase in relative β -lactamase activity to 13.64. These data demonstrate that there is no correlation between measured β -lactamase activity and MIC, suggesting that non- β -lactamase-mediated resistance mechanisms play an important role in CFP resistance in *B. multivorans* LMG 18825. For *B. arboris* R-132 (Fig. 27b), the situation is different. We observed high β -lactamase activity (7.29) when cells were exposed to CAZ, and a high MIC for CAZ (128 mg/L). Combination treatment with SUL or AVI led to a statistically significant ($p < 0.05$, $n = 4$) decrease in β -lactamase activity (1.10 and 3.30, respectively), associated with an 8-fold MIC decrease for CAZ. These data suggest that for this Bcc strain β -lactamase is the major cause of resistance to CAZ. For *B. cenocepacia* LMG 16656 (Fig. 27c), no differences in MIC-values for CAZ (MIC = 512 mg/L) or CFP (MIC = 32 mg/L) were observed upon addition of a β -lactamase inhibitor (SUL, AVI or TAZ). β -lactamase activity assay results showed that treatment with CAZ or CFP induces β -lactamase activity in this strain, and that addition of a β -lactamase inhibitor to CAZ- or CFP-treatment has no significant ($p > 0.05$, $n = 3$) influence on β -lactamase activity. A likely explanation for this is that 15 of the 21 β -lactamases currently identified in the genome of *B. cenocepacia* LMG 16656 belong to the metallo- β -lactamase protein family.[245] As mentioned, this class

of β -lactamases is not inhibited by any of the currently available β -lactamase inhibitors. The MIC- and β -lactamase activity results for *B. cenocepacia* LMG 16656 confirm that SUL, AVI or TAZ are not able to effectively inhibit metallo- β -lactamases and thus have no effect on the resistance of this strain against β -lactam antibiotics. Susceptibility of *B. vietnamiensis* LMG 18835 (Fig. 27d) for CAZ, CFP and AZT is altered upon addition of SUL; addition of SUL decreased the MIC for CAZ from 4 mg/L to 0.25 mg/L, for CFP from 16 mg/L to 1 mg/L and for AZT from 8 mg/L to 1 mg/L. However, relative β -lactamase activity for all three β -lactams is significantly increased upon addition of SUL. We currently have no explanation for these at first sight contradictory results, but it seems likely that both β -lactamase dependent and independent mechanisms are involved in β -lactam resistance in this strain. Results for the other Bcc strains investigated (see Fig. S1-S5) confirm that for most Bcc strains investigated the influence of a β -lactamase inhibitor on β -lactamase activity is both strain- and/or antibiotic-dependent.

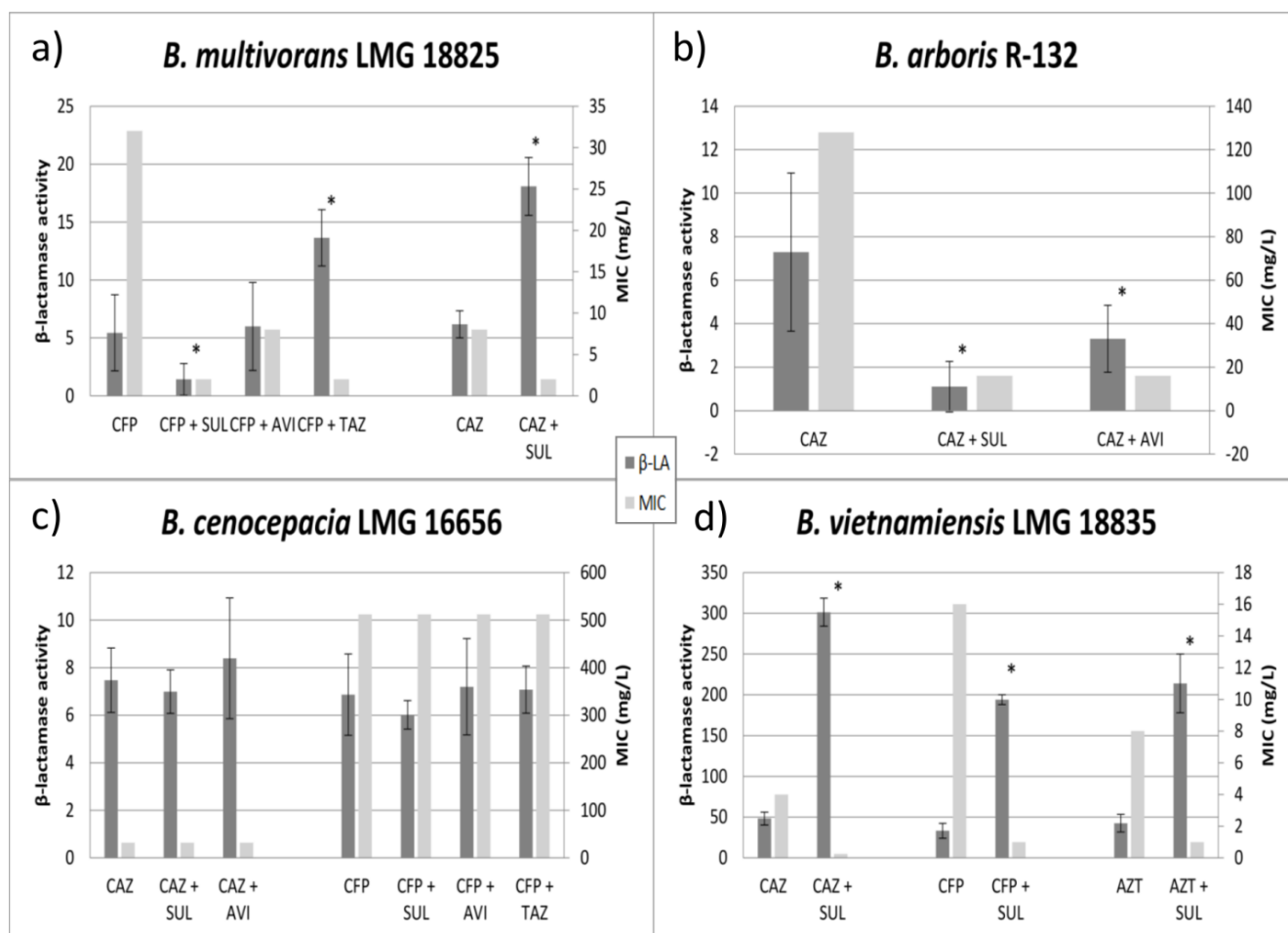


Figure 28. β -lactamase activity and corresponding MIC values for a selection of Bcc strains tested. Dark grey bars: β -lactamase activity relative to untreated controls, light grey bars: MIC values (mg/L) for a) *B. multivorans* LMG 18825, b) *B. arboris* R-132, c) *B. cenocepacia* LMG 16656 and d) *B. vietnamiensis* LMG 18835. * represents statistically significant differences compared to treatment with the β -lactam antibiotic alone ($p < 0.05$, $n \geq 3$)

3.5. Conclusions

There are considerable differences in susceptibility of Bcc strains to different β -lactam antibiotics. In the present study we investigated the effect of β -lactamase inhibitors on the susceptibility of Bcc species against β -lactam antibiotics. CLA had no effect on this susceptibility for any of the strains tested, but addition of SUL, TAZ or AVI to CAZ, AMOX, CFX, CFP or AZT leads to increased susceptibility (at least 4-fold decrease in MIC) in several Bcc strains. An important fact to take into account is that up to date, there is no clear evidence that *in vitro* susceptibility of Bcc species against a specific antimicrobial compound is related to clinical outcome.[246]

Investigation of the relation between altered susceptibility upon addition of SUL, TAZ or AVI and differences in β -lactamase activity was performed with a β -lactamase activity assay kit. Pronounced differences in β -lactamase activity after exposure to different β -lactam antibiotics and β -lactamase inhibitors, as well as between the Bcc strains tested were observed. Some of the results observed are in line with our expectations, confirming that resistance against β -lactam antibiotics is mediated by expression of β -lactamase enzymes which can be successfully inhibited *in vitro* by β -lactamase inhibitors. However, results obtained with other strains suggest that non- β -lactamase-mediated resistance mechanisms to β -lactam antibiotics (likely including reduced membrane permeability, altered PBPs and presence of efflux pumps) are also important.

Production of β -lactamases is clearly not the only mechanism Bcc strains use to survive treatment with β -lactam antibiotics, and it is therefore questionable that adding β -lactamase inhibitors to β -lactam therapy will be a valuable approach to combat Bcc infections.

3.6. Declarations

Ethics approval and consent to participate. Not applicable.

Consent for publication. Not applicable.

Availability of data and materials. All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests. None of the authors have any competing interests in the manuscript.

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Authors' contributions. AE performed all microbiological and analytical aspects of the work described above and was a major contributor in writing the manuscript. TC was responsible for the set-up of this project and a large contribution in writing and improving the manuscript. All authors read and approved the final manuscript.

Acknowledgements. Not applicable.

SUPPLEMENTARY DATA

Table S1. Biological and geographic origin of all *Burkholderia cepacia* complex strains used in this study.[247, 248]

Species	Strain	Biological origin	Geographic origin
<i>B. cepacia</i>	LMG 1222 ^T	Allium cepa	USA
	LMG 18821	CF patient	Australia
<i>B. multivorans</i>	LMG 18822	CF patient	Canada
	LMG 18825	CF patient	UK
	LMG 13010 ^T	CF patient	Belgium
	LMG 17588	Soil	Berkeley, USA
<i>B. cenocepacia</i>	LMG 16656 ^T	CF patient	Edinburgh, UK
	LMG 18828	CF patient	Canada
	LMG 18829	CF patient	USA
	LMG 18830	CF patient	Australia
<i>B. vietnamiensis</i>	LMG 10929 ^T	Oryza sativa, rhizosphere soil	Vietnam
	LMG 18835	CF patient	USA
<i>B. ambifaria</i>	LMG 19182 ^T	Pea rhizosphere	Wisconsin, USA
	LMG 19467	CF patient	Australia
<i>B. lata</i>	LMG 6992	Soil	Trinidad & Tobago
	R-9940	CF patient	Canada
<i>B. stabilis</i>	LMG 14294 ^T	CF patient	Belgium
	LMG 14086	Respirator	UK
<i>B. dolosa</i>	LMG 18943 ^T	CF patient	USA
	LMG 18941	CF patient	USA
<i>B. anthina</i>	LMG 20980 ^T	Soil rhizosphere	Nashville, USA
	LMG 20983	CF patient, sputum	Blackpool, UK
<i>B. pyrrocinia</i>	LMG 21824	CF patient	USA
<i>B. ubonensis</i>	LMG 20358 ^T	Surface soil	Thailand
	LMG 24263	Nosocomial infection	Thailand
<i>B. latens</i>	LMG 24064 ^T	CF patient	Italy
<i>B. arboris</i>	LMG 24066	Soil	USA
	R-132	CF patient	USA
<i>B. seminalis</i>	LMG 24067 ^T	CF patient	USA

	LMG 24272	Nosocomial infection	Thailand
<i>B. metallica</i>	LMG 24068 ^T	CF patient	USA
	R-2712	CF patient	Canada
<i>B. contaminans</i>	LMG 16227	CF patient, respiratory tract	Sweden
	R-12710	Sheep with mastitis, milk	Spain

^T = Type strain

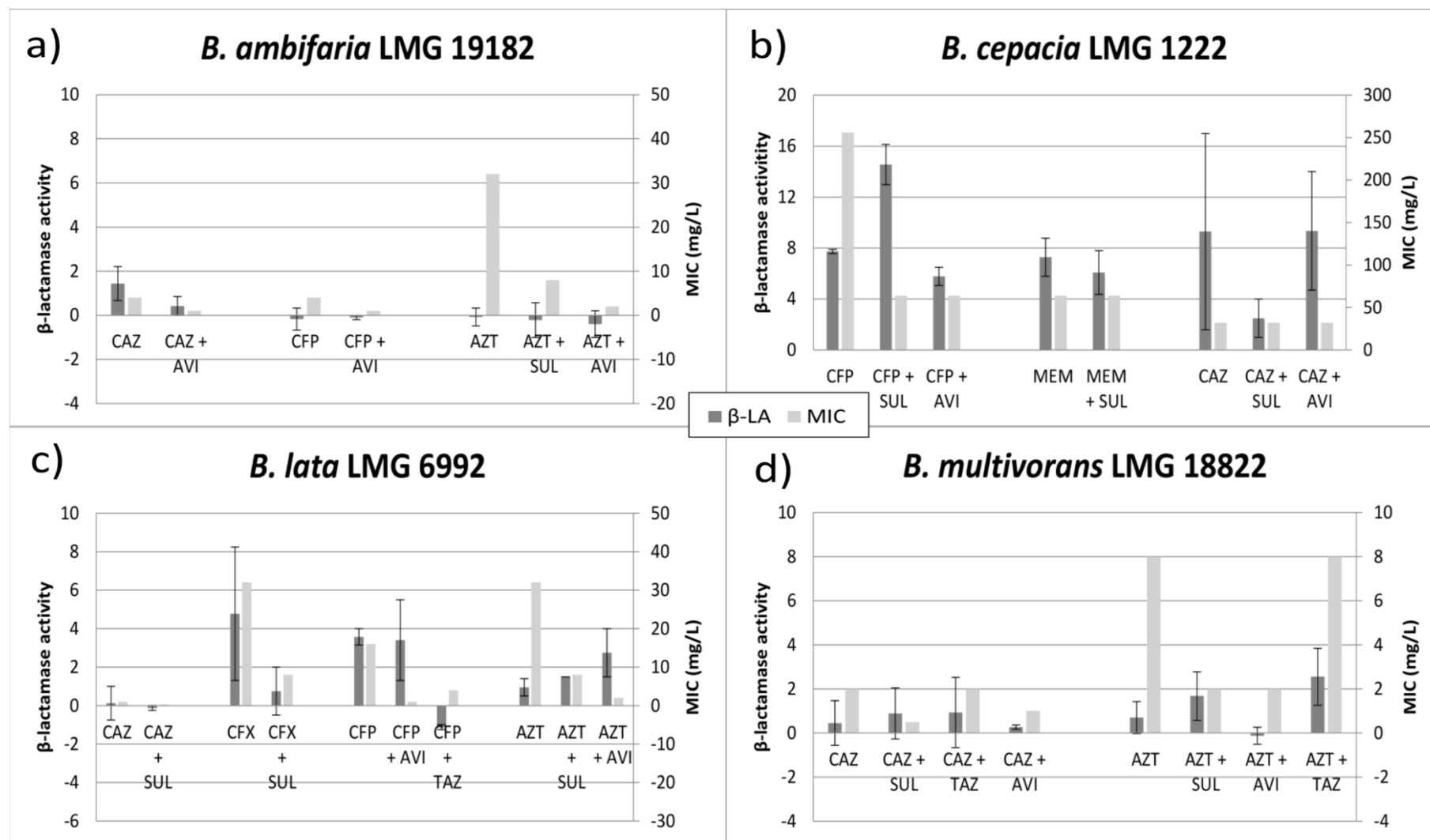


Figure S1. β -lactamase activity (dark grey bars, relative to untreated controls) and corresponding MIC values (light grey bars, mg/L) for a) *B. ambifaria* LMG 19182, b) *B. cepacia* LMG 1222, c) *B. lata* LMG 6992 and d) *B. multivorans* LMG 18822. Error bars represent min-max (n=2).

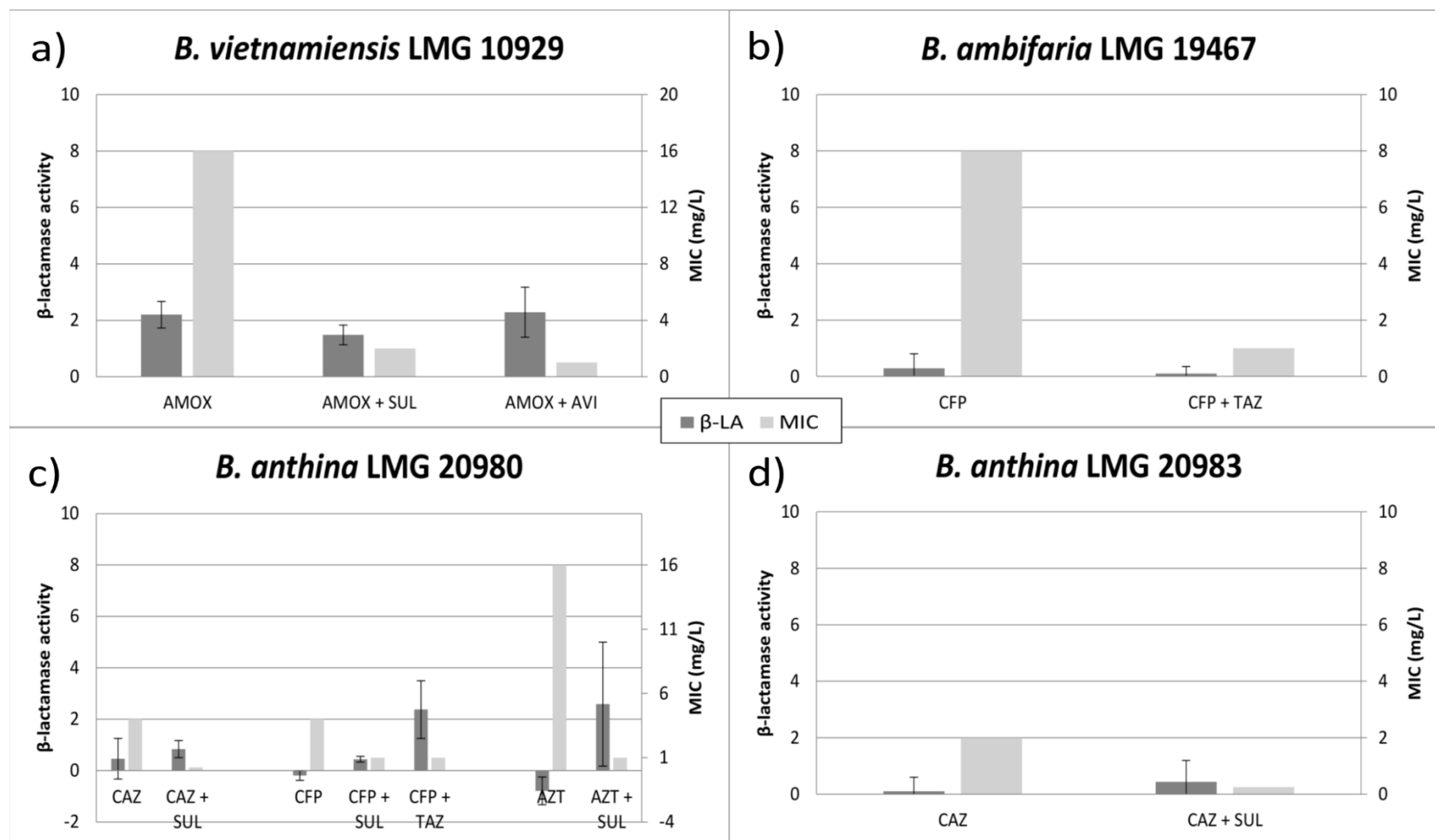


Figure S2. β -lactamase activity (dark grey bars, relative to untreated controls) and corresponding MIC values (light grey bars, mg/L) for a) *B. vietnamiensis* LMG 10929, b) *B. ambifaria* LMG 19467, c) *B. anthina* LMG 20980 and d) *B. anthina* LMG 20983. Error bars represent min-max ($n=2$).

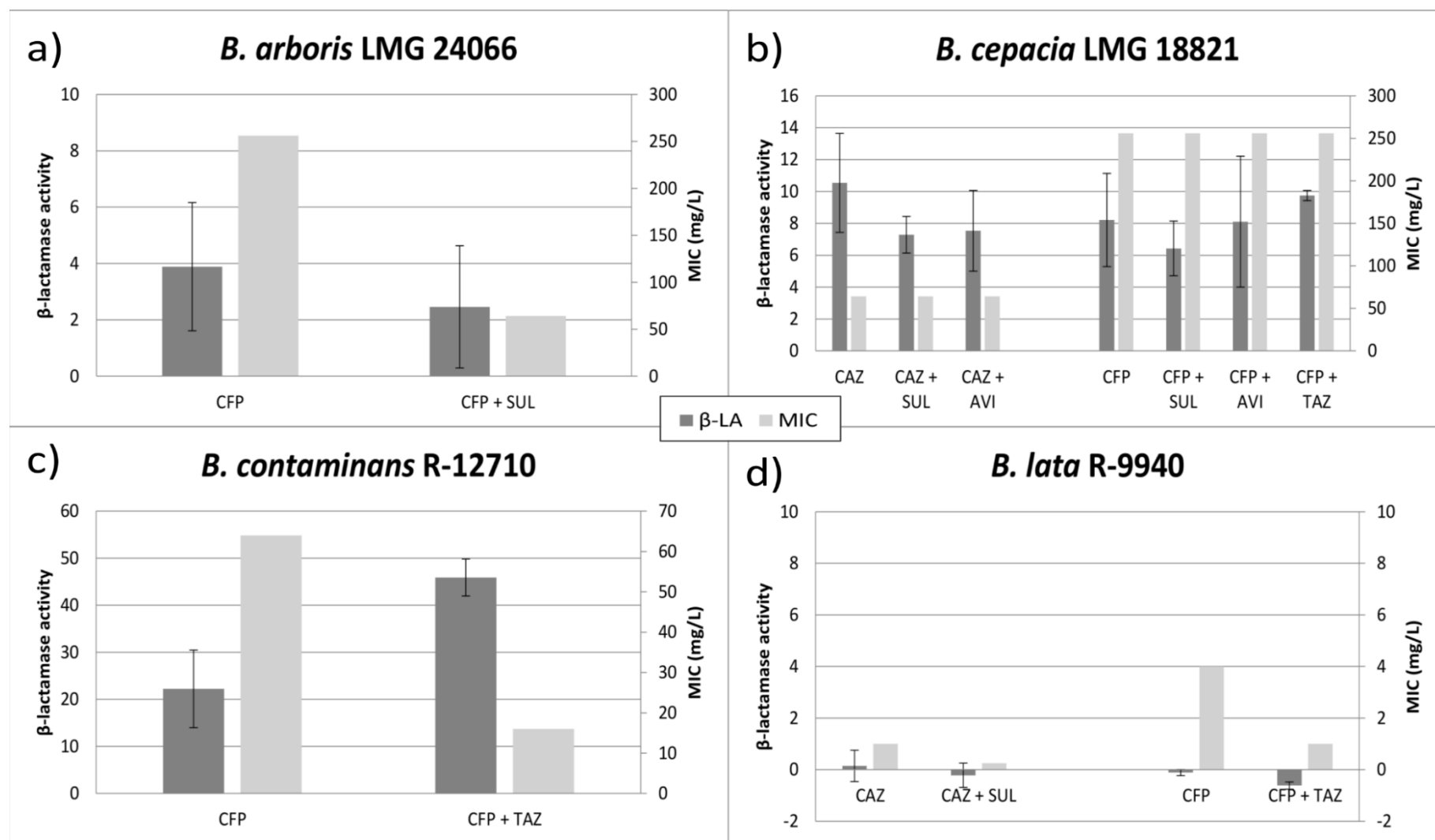


Figure S3. β -lactamase activity (dark grey bars, relative to untreated controls) and corresponding MIC values (light grey bars, mg/L) for a) *B. arboris* LMG 24066, b) *B. cepacia* LMG 18821, c) *B. contaminans* R-12710 and d) *B. lata* R-9940. Error bars represent min-max ($n=2$).

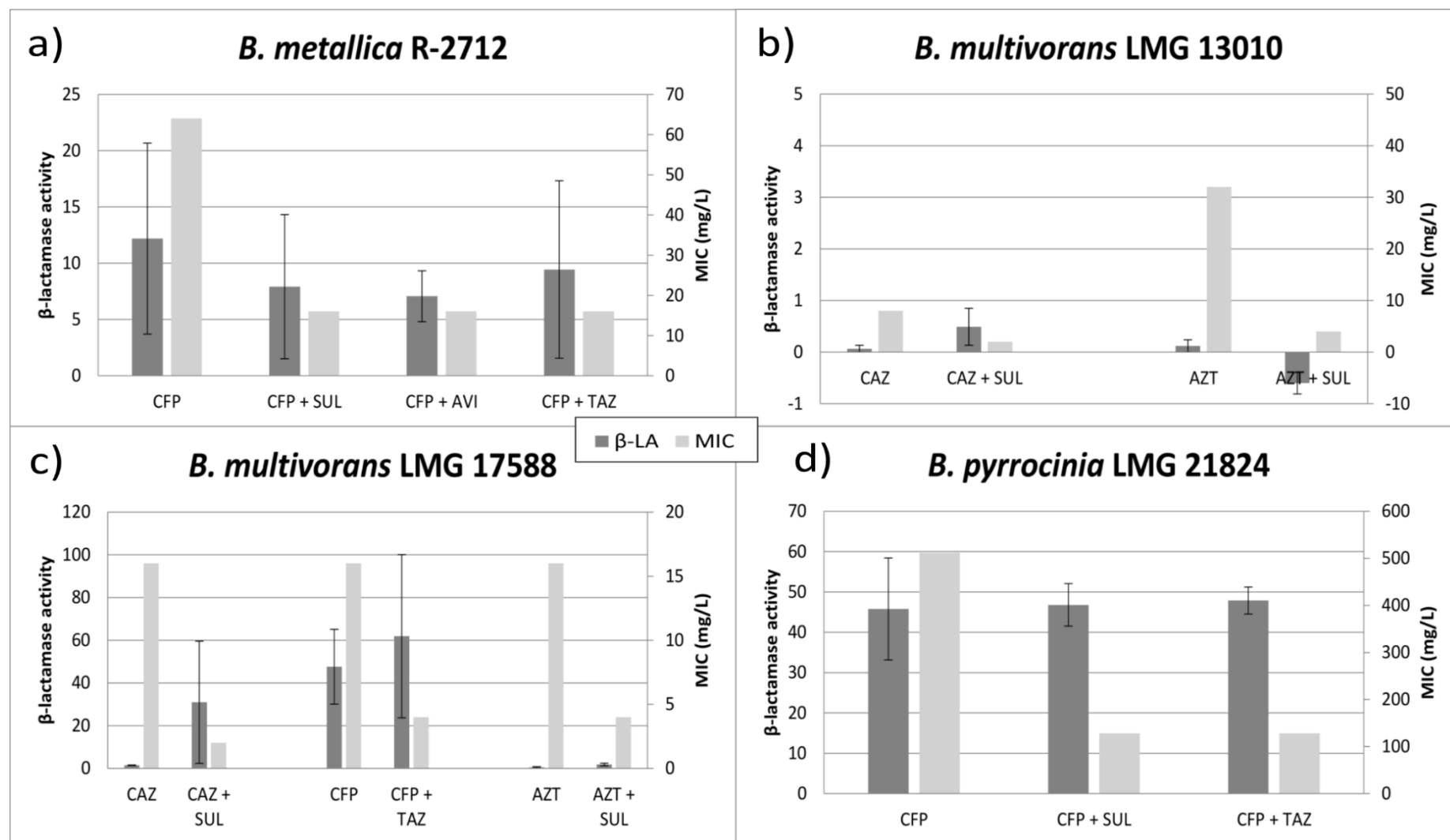


Figure S4. β -lactamase activity (dark grey bars, relative to untreated controls) and corresponding MIC values (light grey bars, mg/L) for a) *B. metallica* R-2712, b) *B. multivorans* LMG 13010, c) *B. multivorans* LMG 17588 and d) *B. pyrrocinia* LMG 21824. Error bars represent min-max ($n=2$).

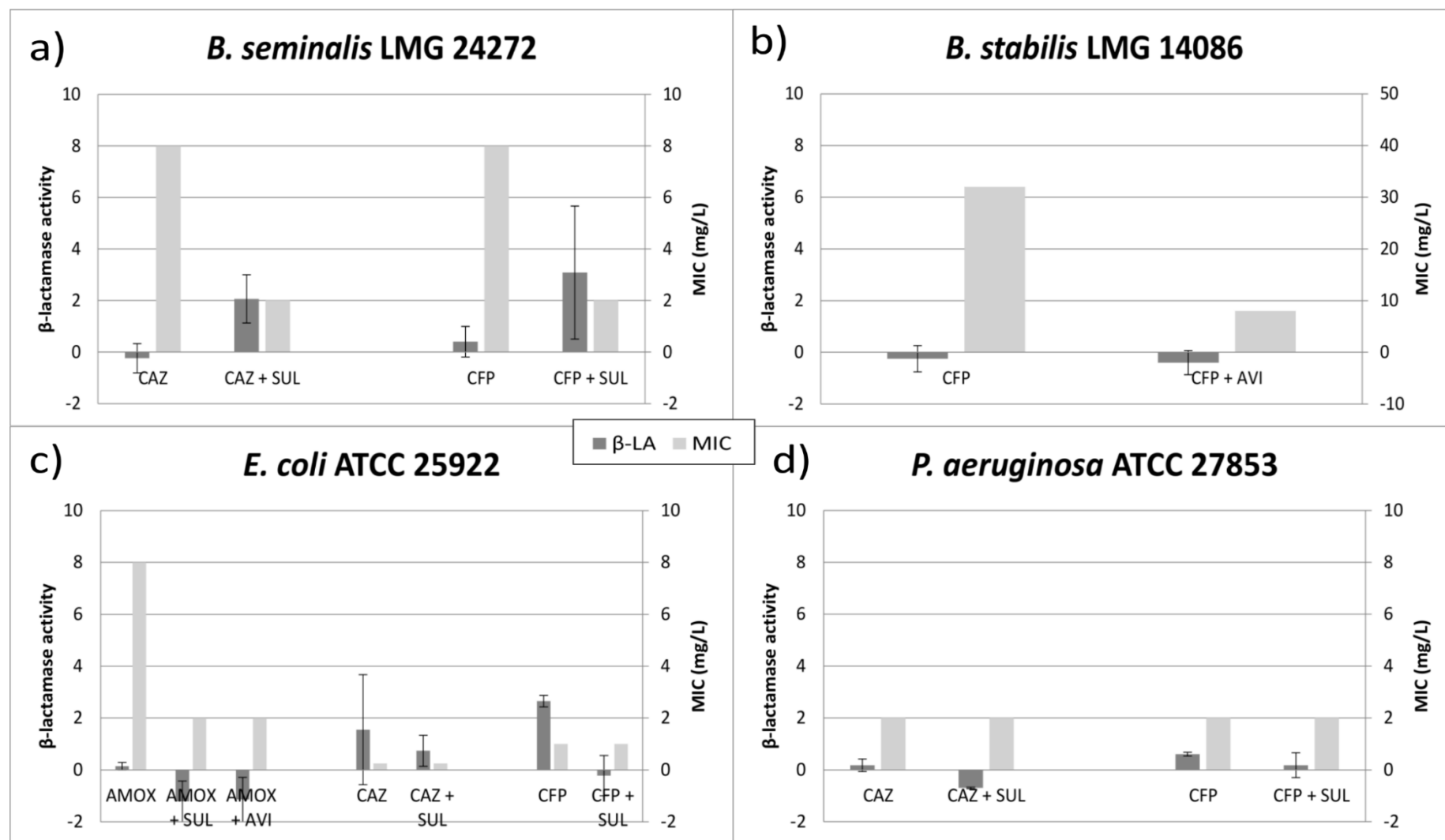


Figure S5. β -lactamase activity (dark grey bars, relative to untreated controls) and corresponding MIC values (light grey bars, mg/L) for a) *B. seminalis* LMG 24272, b) *B. stabilis* LMG 14086 and two control strains: c) *E. coli* ATCC 25922 and d) *P. aeruginosa* ATCC 27853. Error bars represent min-max (n=2)

CHAPTER IV.BROADER INTERNATIONAL CONTEXT, RELEVANCE, AND FUTURE PERSPECTIVES

1. Drugs versus bugs: an everlasting battle?

The discovery of penicillin by Alexander Fleming in 1928 was the beginning of a golden era for antibacterial drug discovery.[249] From the 1930s to the 1960s almost every currently available antibiotic was discovered and introduced to the market (Figure 29).[250] Newer antibiotic compounds and classes have been introduced to the market since the golden era, but the rate has slowed down radically. Lipopeptides were discovered in 1987, representing the latest novel antibiotic class that was successfully commercialized for the market.[87] Antibiotics with novel mechanisms of action have not been discovered since; the period from 1987 until today is therefore referred to as the 'discovery void'.[175]

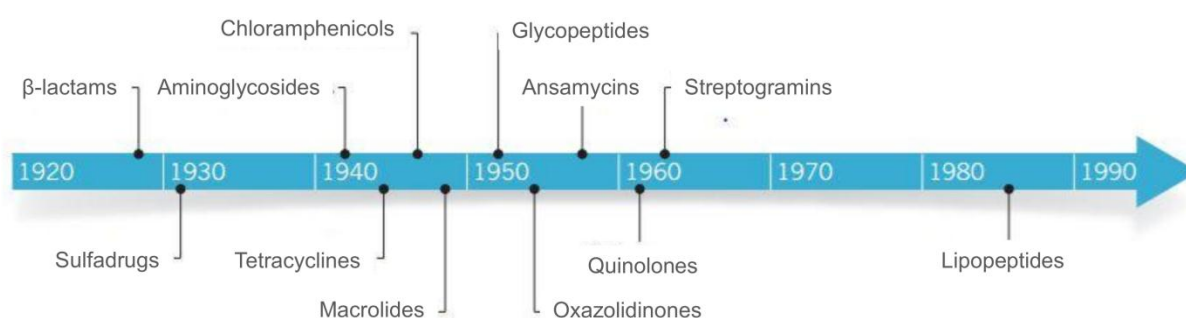


Figure 29. Timeline where the discovery of the first antibiotic of each class is pinpointed.[250]

Infections remain one of the major sources of disease and death worldwide with respiratory infections, malaria, tuberculosis and HIV/AIDS being the most important diseases causing mortality in third-world countries.[173] Respiratory infections also belong to the top 10 causes of death in middle- and high-income countries.[174] The introduction of antimicrobials caused a transformation of human and animal health systems by providing a weapon against infectious diseases. Unfortunately, the euphoria was overshadowed almost as soon as antibiotics were deployed, since bacteria began to respond by developing resistance against them.[87, 176] During the golden age alternative therapies could be offered by the introduction of new antibiotic classes, but the lack of innovation in the last two decades is causing major problems.[177] As the usage of antibiotics increased over the years, so did the complexity of resistance mechanisms exhibited by bacterial pathogens.[251] Furthermore, because of cumulative acquisition of resistance traits against multiple antibiotics, more bacterial pathogens with multiple-drug resistance are being reported worldwide. Major human and animal pathogens such as *Mycobacterium*, *Staphylococcus*, *Pseudomonas* and *Salmonella* species have consequently become resistant to antibiotics that were previously quite efficacious.[87] For every major group of bacterial pathogens, multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) species have been described. MDR pathogens are

resistant to at least one compound in three or more antibiotic classes, XDR species to at least one antibiotic in all but one or two classes and PDR pathogens are resistant to every currently available antibiotic.[176, 252] The increasing prevalence of drug-resistant bacterial pathogens and the lack of novel antibiotics has led to a *de facto* post-antibiotic era where antimicrobial chemotherapy often is no longer effective.[87, 177, 253, 254]

Infections with drug-resistant pathogens pose a global threat for public health due to higher morbidity and mortality rates, increased treatment costs and extended hospital stays, and the emergence of bacterial pathogens that cannot be adequately treated anymore.[237, 238, 255] Frequently encountered MDR organisms include vancomycin-resistant enterococci (VRE), methicillin-resistant and MDR *S. aureus* (MRSA), penicillin-resistant pneumococci, MDR *Streptococcus pneumoniae*, XDR *M. tuberculosis* and MDR Gram-negative species such as *P. aeruginosa*, *Acinetobacter baumannii*, *Enterobacter* spp., ESBL-producing *E. coli* and *K. pneumoniae*, carbapenemase-producing *Enterobacteriaceae* (CPE) and the recently emerging colistin-resistant CPE.[62, 237, 256-259]

To be able to win the battle against antimicrobial resistance we first need to reduce the selective pressure for the development of resistance, by limiting the use of antibacterial drugs, by using the appropriate dosage and by respecting the treatment duration.[176] A vital aspect to avoid inappropriate and ineffective therapies is knowledge of the intrinsic antibacterial resistance of a pathogen. Examples of intrinsic resistance traits and their resistance mechanisms are shown in Table 19. Some bacterial pathogens, including *M. tuberculosis* and *P. aeruginosa*, which are intrinsically resistant to a large variety of antibiotics pose a particular concern due to limited treatment options. Consequently, frequent use of the remaining possible treatments further increase the risk of acquiring new resistance mechanisms.[87, 260, 261] Secondly, strict attention to infection control guidelines (especially in healthcare facilities) should reduce the spread of resistant microbial organisms. The third and probably most important aspect is the development of antibacterial agents with new mechanisms of action.[176, 177] With sufficient efforts from researchers, healthcare facilities and patients we should be able to remain at least one step ahead of the next resistant outbreak and prevent the post-antibiotic era.

Table 19. Examples of intrinsic resistance and their respective mechanisms.[260, 261]

ORGANISMS	NATURAL RESISTANCE AGAINST:	MECHANISM
Anaerobic bacteria	Aminoglycosides	Lack of oxidative metabolism to drive uptake of aminoglycosides
Aerobic bacteria	Metronidazole	Inability to anaerobically reduce drug to its active form
Gram-positive bacteria	Aztreonam (β -lactam)	Lack of penicillin binding proteins (PBPs) that bind and are inhibited by this beta lactam antibiotic
Gram-negative bacteria	Vancomycin	Lack of uptake resulting from inability of vancomycin to penetrate outer membrane
<i>Klebsiella</i> spp.	Ampicillin (β -lactam)	Production of enzymes (beta-lactamases) that destroy ampicillin before the drug can reach the PBP targets
<i>S. maltophilia</i>	Imipenem (β -lactam)	Production of enzymes (beta lactamases) that destroy imipenem before the drug can reach the PBP targets.
Lactobacilli and <i>Leuconostoc</i>	Vancomycin	Lack of appropriate cell wall precursor target to allow vancomycin to bind and inhibit cell wall synthesis
<i>P. aeruginosa</i>	Sulfonamides, trimethoprim, tetracycline, or chloramphenicol	Lack of uptake resulting from inability of antibiotics to achieve effective intracellular concentrations
Enterococci	Aminoglycosides	Lack of sufficient oxidative metabolism to drive uptake of aminoglycosides
	All cephalosporins	Lack of PBPs that effectively bind and are inhibited by these beta lactam antibiotics

2. The *Burkholderia cepacia* complex in cystic fibrosis patients: a silent killer

Cystic fibrosis is the most common hereditary disease among the European population, with an incidence of approximately 1 in 2500 newborns in Belgium.[262] The median life expectancy of patients suffering from CF in the EU currently stands at 41 years old, according to the clinical CF registry of the CF Trust.[263] The major contributor to morbidity and mortality in CF patients is the occurrence of chronic lung infections. In children, these infections are mainly caused by *H. influenzae*, *S. aureus* or *P. aeruginosa* with the latter being the major pathogen in adults.[264, 265] The prevalence of major respiratory CF pathogens in 2015 is shown in Figure 30 ([265]). Members of

the Bcc are less frequently recovered from CF lungs, but the clinical outcome of Bcc infections is highly unpredictable and these microorganisms often cause a rapid decline in lung function.[7] Bcc species produce many different virulence factors, are capable of person-to-person transmission and Bcc infections are difficult to eradicate since these pathogens are intrinsically resistant to a wide range of available antibiotics.[7, 73] Therefore, research on the multifarious Bcc should focus on understanding their intrinsic resistance, virulence factors and treatment and eradication possibilities to enable CF patients to survive Bcc infections.

The research for this dissertation mainly focused on two aspects to combat Bcc infections; the ability of β -lactam- β -lactamase inhibitor combinations to eradicate different members of the Bcc on the one hand and the potential of the nonmevalonate pathway as a novel antibacterial target on the other hand.

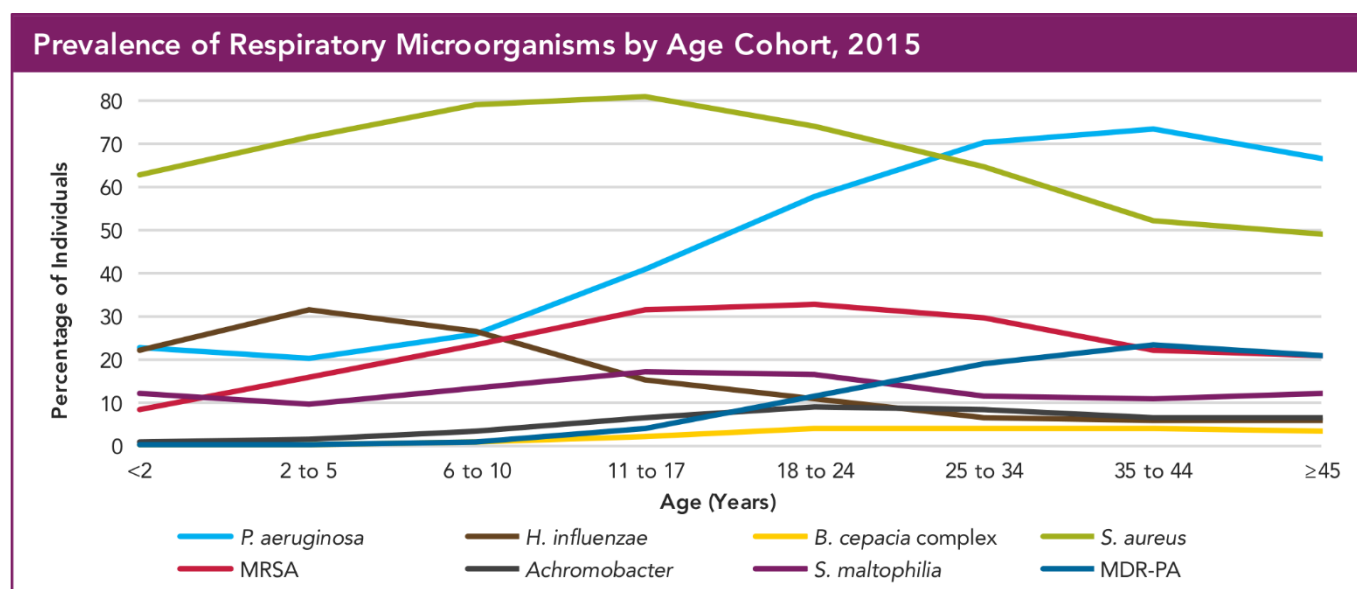


Figure 30. Prevalence of major respiratory microorganisms isolated from CF lungs in 2015, by age cohort.[265]

3. Revival of the β -lactams?

β -lactam antibiotics are among the most frequently prescribed group of antibiotics for both Gram-positive and Gram-negative bacterial infections.[66, 147] This group includes penicillins, cephalosporins, carbapenems and monobactams and they work by inhibiting the bacterial cell wall biosynthesis.[148] The use of this life-saving antibiotic class has become compromised due to the production of β -lactamases which are able to inhibit the antibacterial activity of β -lactams through hydrolysis of their functional group, the β -lactam ring.[66] Two different strategies can be applied to

circumvent the clinical threat of β -lactamase-mediated resistance: (i) the design of β -lactams that can evade enzymatic inactivation by β -lactamases (e.g. derivatives of carbapenems) and (ii) inhibition of β -lactamases by β -lactamase inhibitors.[65, 266]

Since the 1980s the development of β -lactamase inhibitors provided a new approach to combat this clinical challenge. All clinically available inhibitors share the β -lactam backbone, but do not possess antimicrobial activities.[65] Clavulanic acid (CLA) is a natural compound that was isolated from strains of *Streptomyces clavuligerus* in 1977 and was first introduced to the market in 1985.[267, 268] Sulbactam (SUL) and tazobactam (TAZ) are synthetic compounds developed by the pharmaceutical industry in 1978 and 1980, respectively, and share structural resemblance with penicillin.[269, 270] These three β -lactamase inhibitors are effective against most class A β -lactamases (including TEM, ESBL, CTX-M and SHV) but have limited activity against class B, C and D enzymes.[271-274] Hence, there is still a considerable need for inhibitors with activity against class B metallo- β -lactamase, class C cephalosporinases and class D oxacillinases.[152] Avibactam (AVI), formerly known as NXL104 or AVE1330A, is a newer β -lactamase inhibitor based on a bridged diazabicyclooctane (DBO) structure. AVI was recently approved for the market in combination with ceftazidime for the treatment of Gram-negative complicated intra-abdominal infections (cIAs), complicated urinary tract infections (cUTIs) and aerobic Gram-negative infections with limited treatment options.[152, 161, 275, 276] AVI has increased potency and an expanded spectrum compared to older inhibitors, including inhibitory activity against class A β -lactamases, class C enzymes (e.g. AmpC cephalosporinases) and against some class D enzymes. This compound is the first novel β -lactamase inhibitor introduced into clinical use in the past 20 years and offers new hope in the battle against multidrug-resistant Gram-negative bacterial infections.[152, 277-279]

Extensive research has been conducted on the antibacterial potential of β -lactam- β -lactamase inhibitor combinations, and eight different combinations are currently approved for clinical use including amoxicillin-CLA (co-amoxiclav), ticarcillin-CLA (co-ticarclav), ampicillin-SUL, cefoperazone-SUL, piperacillin-TAZ, ceftolozane-TAZ, ceftazidime-AVI and ceftolozane-AVI.[65, 275, 276, 280, 281] Some studies also suggest the combination of CLA with cefepime or cefpirome to be more effective against ESBL-producing organisms, but clinical trials have not yet been conducted.[282, 283] New combinations of AVI with ceftaroline or aztreonam are also currently under development.[284, 285]

Several studies claim that ceftazidime-avibactam is a promising addition to our current therapeutic armamentarium in the biological chess match between antibiotics and multidrug-resistant Gram-negative organisms (including Enterobacteriaceae (e.g. *K. pneumoniae*) and *P. aeruginosa*).[46, 161, 240, 241, 286] However, the effect of β -lactam- β -lactamase inhibitor combinations on members of

the Bcc is still poorly investigated. Mushtaq et al. evaluated the activity of ceftazidime-AVI and piperacillin-TAZ against various Bcc isolates by MIC determination of ceftazidime or piperacillin plus 4 mg/L AVI or TAZ, respectively. They included 54 clinical CF isolates, comprising 37 *B. multivorans*, 8 *B. cepacia*, 6 *B. cenocepacia* and 3 *B. vietnamiensis* strains. The majority of the Bcc isolates exhibited resistance against ceftazidime, piperacillin and piperacillin-TAZ. However, their results showed a MIC-reduction of ceftazidime by at least four- to tenfold in the presence of AVI, for most Bcc isolates screened.[242] Several research groups have studied the susceptibility of a variety of clinical Bcc isolates to ticarcillin-CLA. In these studies, all isolates demonstrated high resistance rates.[287-290] One Czech Republican and one Swiss study evaluated the antibacterial activity of piperacillin and piperacillin-TAZ against *B. multivorans* and *B. cenocepacia* strains isolated from both non-CF and CF-patients. The authors demonstrated a 4-fold MIC-decrease of piperacillin in the presence of TAZ, for 92.9% of the *B. multivorans* isolates and 60.7% of the *B. cenocepacia* isolates tested.[289, 291] The data from previous studies ([242, 287-291]) suggest that β -lactam resistance of Bcc isolates is not only related to β -lactamase production but also to the action of efflux pumps and/or altered PBPs. However, this has not been investigated in detail, nor for a larger collection of Bcc strains.

In chapter 3.III we wanted to fill this gap by testing the susceptibility of a larger Bcc panel against combination therapy of β -lactam antibiotics of several classes with the β -lactam inhibitors CLA, SUL, TAZ and AVI. We included the β -lactams amoxicillin, ceftazidime, cefepime, meropenem and aztreonam to cover every available class of β -lactam antibiotics. Not only did we investigate the susceptibility of our test panel (containing representatives of 16 Bcc species) towards different combinations, we also investigated the differences in β -lactamase activity upon addition of a β -lactamase inhibitor. Our results indicated that some combinations, including ceftazidime-SUL, ceftazidime-AVI, amoxicillin-SUL, amoxicillin-AVI, ceftazidime-SUL, cefepime-SUL, cefepime-TAZ, cefepime-AVI, aztreonam-SUL and aztreonam-AVI caused at least a 4-fold MIC decrease upon addition of the β -lactamase inhibitor. However, investigation of β -lactamase activity induced by these antibiotic combinations did not lead to unambiguous conclusions. We observed pronounced differences in β -lactamase activity after treatment with different β -lactams and β -lactamase inhibitors, as well as between the Bcc strains tested, confirming the results from previous studies. Expression of β -lactamases is clearly not the only mechanism that Bcc species use to circumvent the action of β -lactam antibiotics. The approach of β -lactam- β -lactamase inhibitor combinations can offer an addition for Bcc-therapy, but it is important to evaluate every clinical situation individually.

4. The future of β -lactam- β -lactamase inhibitor combinations

The future prospects of β -lactam antibiotics are highly dependent on the therapeutic design of effective β -lactamase inhibitors. The success of avibactam illustrates several important features for the development of new inhibitors. First, avibactam does not contain a β -lactam ring, and is therefore referred to as “non- β -lactam-inhibitor”, but it shows structural similarity to β -lactams at the carbonyl group.[65, 152] This structural distinction leads to rapid recognition and formation of a stable complex with β -lactamases.[292, 293] Secondly, analyses revealed that a covalent bond with class A, C and D β -lactamases is formed by a strong carbamoyl link between avibactam and the serine-residue of the β -lactamase.[293, 294] Thirdly, the enzymatic inhibition by avibactam is thought to be reversible and the active inhibitor can be regenerated through deacylation and recyclization of the 5-membered ring.[293] Avibactam’s characteristics serve as a lesson for the design of new β -lactamase inhibitors and two novel “non- β -lactam inhibitors” with DBO structure are currently being evaluated in clinical trials. Relebactam (MK-7655) has a similar activity spectrum as AVI and is currently in Phase III clinical trials for treatment of cUTIs and cIAls in combination with imipenem or cilastatin.[152, 295] RG6080 (or OP0565), currently in Phase I clinical trials, has a similar spectrum of activity compared to other DBOs but additionally exhibits antibacterial activity against MBL-producing *Enterobacteriaceae* when combined with aztreonam, cefepime or piperacillin.[296]

Two additional groups of inhibitors have recently received attention: boronic acid β -lactamase inhibitors that recently completed Phase I clinical trials, whereas cyclobutanone and penam sulfone inhibitors have the potential to inhibit class A and class C β -lactamases.[152, 281] The boronate RPX7009 represents a novel class of synthetic non- β -lactam inhibitors and is currently in Phase III clinical trials in the USA in combination with meropenem.[281, 297, 298] Although boronates have been described as effective serine β -lactamase inhibitors since the late 1970s, new modifications at the carboxamide group have rendered them clinically more relevant.[152, 299] RPX7009 serves as a reversible, competitive inhibitor showing very potent growth inhibition of microorganisms expressing class A and C β -lactamases, when combined with biapenem (a carbapenem antibiotic). In 2013, this combination showed strong potentiation of biapenem activity against class A carbapenemase-producing *Enterobacteriaceae*. [300] Despite this promising result, RPX7009 is currently only developed in combination with meropenem to treat pathogens expressing serine carbapenemases.[281, 297] The second group of inhibitors comprises cyclobutanone-based compounds which demonstrate reversible activity against all four groups of β -lactamases.[301]

To conclude, we can state that there are several interesting β -lactam- β -lactamase inhibitor combinations in the pipeline, but a close eye has to be kept on the evolution of two particular β -

lactamase classes. Class B (MBL) β -lactamases pose a particular challenge since none of the available inhibitors have significant activity against members of this enzyme class. The increasing emergence of NDM-1 β -lactamase illustrates the rapid spread of MBL enzymes from one country to the whole world. Only two combinations show inhibitory activity against class B enzymes; meropenem-cyclobutanone and aztreonam-AVI.[163, 301] Also class D (OXA) enzymes pose an increasing risk for multidrug-resistant traits by deactivation of both narrow- and broad-spectrum antibiotics, recently including carbapenems.[302, 303] Available β -lactamase inhibitors cannot effectively inhibit OXA-enzymes and there is a scarcity of research addressing this challenge.[304-306]

5. Evaluation of the nonmevalonate pathway as novel drug target

Since the discovery of the mevalonate (MVA) pathway it has been generally accepted that this was the unique pathway for the formation of IPP and DMAPP, the fundamental building blocks in isoprenoid biosynthesis.[109, 307, 308] In the 1990s however, Rohmer and colleagues discovered that several bacteria, green algae and chloroplasts of higher plants utilize an alternative pathway; the nonmevalonate, DOXP or MEP pathway.[114, 131, 309-311] The distribution of the MVA and DOXP pathway is listed in Table 20, including some examples of each group of organisms.

Isoprenoids or terpenoids are the largest class of naturally occurring organic compounds, comprising over 30 000 compounds, and play an essential role in several cellular processes in all living organisms.[119] They are vital for growth and survival by influencing cell wall and membrane biosynthesis (sterols in eukaryotes, bactoprenols and hopanoids in bacteria), regulation of transcription, electron transport (quinones), photosynthesis in plants (carotenoids) and many other processes.[128, 132, 311-313]

Extensive research has focused on inhibitors of the MVA pathway and downstream isoprenoid pathways for prevention and therapy of cardiovascular diseases through reduction of the blood cholesterol.[114, 314] This research led to the development of statins which interrupt cholesterol synthesis in the liver by blocking the conversion of HMG-CoA to mevalonic acid,[315-317] and bisphosphonates which are effective drugs to treat metabolic bone disease through inhibition of osteoclast-mediated bone resorption by isoprenoid lipids.[307, 318]

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Table 20. Distribution of the mevalonate (MVA) and the nonmevalonate (DOXP) pathway in different groups of organisms.[115]

GENUS/SPECIES	MVA	DOXP	GENUS/SPECIES	MVA	DOXP
Animals	+	-	Bacteria		
<i>Gorilla gorilla</i> (gorilla)	+	-	<i>Enterococcus</i>	+	(+)
<i>Homo sapiens</i> (human)	+	-	<i>Staphylococcus</i>	+	-
<i>Canis familiaris</i> (dog)	+	-	<i>Streptococcus</i>	+	-
<i>Mus musculus</i> (mouse)	+	-	<i>Borrelia</i>	+	-
<i>Rattus norvegicus</i> (rat)	+	-	<i>Acinetobacter</i>	-	+
<i>Sus scrofa</i> (pig)	+	-	<i>Bacillus</i>	-	+
Other animals	+	-	<i>Bacteroides</i>	-	+
Plants	+	+	<i>Burkholderia</i>	-	+
<i>Glycine max</i> (Soy bean)	+	+	<i>Campylobacter</i>	-	+
<i>Vitis vinifera</i> (Wine grape)	+	+	<i>Chlamydia</i>	-	+
<i>Zea mays</i> (Maize)	+	+	<i>Clostridium</i>	-	+
Fungi	+	-	<i>Enterobacter</i>	-	+
<i>Aspergillus</i>	+	-	<i>Streptomyces</i>	-	+
<i>Candida</i>	+	-	<i>Escherichia</i>	-	+
<i>Penicillium</i>	+	-	<i>Haemophilus</i>	-	+
<i>Saccharomyces cerevisiae</i>	+	-	<i>Helicobacter</i>	-	+
Parasites			<i>Klebsiella</i>	-	+
<i>Plasmodium</i>	-	+	<i>Mycobacterium</i>	-	+
<i>Toxoplasma</i>	-	+	<i>Propionibacterium</i>	-	+
<i>Trichomonas</i>	+	-	<i>Pseudomonas</i>	-	+
Nematodes			<i>Salmonella</i>	-	+
<i>C. elegans</i>	+	-	<i>Vibrio</i>	-	+

MVA; mevalonate pathway, DOXP; nonmevalonate pathway. ‘+’ indicates the presence and ‘-’ indicates the absence of the pathway listed on top of the column. (+) indicates the presence of an incomplete pathway.

We focused on the nonmevalonate pathway as a target for antibacterial chemotherapy, since this pathway is present in a variety of bacterial species and absent in human cells. In 1998, Kuzuyama and colleagues discovered fosmidomycin (previously disclosed as FR-31564) to be an inhibitor of purified DXR, the second enzyme in the nonmevalonate pathway, of *E. coli* K-12. Besides this activity towards the purified enzyme, it was shown that bacterial growth was also completely inhibited by 3.13 µg/mL fosmidomycin.[116, 117] Subsequently, several research groups described inhibitory activity of fosmidomycin and FR900098, fosmidomycin’s acetyl derivative, against important pathogens including *P. falciparum*, *P. aeruginosa* and *K. pneumoniae*. [118, 119, 137, 210] Fosmidomycin also demonstrates potent inhibition of purified DXR from *M. tuberculosis*, but is inactive against whole

cells of *M. tuberculosis* due to the absence of a GlpT-transporter for fosmidomycin uptake.[118, 119] Fosmidomycin is currently clinically used for treatment of malaria caused by *P. falciparum* and combination treatment with clindamycin showed favorable results.[319-321] However, problems with toxicity, short half-life and low bioavailability limit its potential as antimalarial drug.[122-124]

Efforts to overcome low bioavailability and cellular uptake have been described extensively, by designing derivatives with altered physicochemical properties or prodrugs of fosmidomycin and FR900098.[133-135, 137, 145, 146, 322] The antimicrobial activity of fosmidomycin analogues was mainly evaluated in *P. falciparum*, *M. tuberculosis* and *E. coli* but the nonmevalonate pathway can also be an interesting target for antibacterial therapy against a variety of other bacterial pathogens. Therefore, we focused in this work on four groups of bacteria which use the nonmevalonate pathway as sole route for isoprenoid biosynthesis; *Propionibacterium acnes*, *M. smegmatis* as a model for *M. tuberculosis*, *P. aeruginosa* and especially members of the Bcc.[115, 125-127]

In chapter 3.I., we evaluated the *in vitro* susceptibility of our test panel towards different β -aryl-, β -hydroxamate substituted derivatives and lipophilic phosphoramidate prodrugs of fosmidomycin, but also a variety of DXS-inhibitors and IspF-inhibitors were included. Our results showed limited *in vitro* activity of most compounds tested, with MIC values > 250 μ M. Additional experiments to identify the resistance mechanisms against inhibitors of the DOXP pathway suggest that resistance is not mediated by the activity of efflux pumps. Literature upon resistance mechanisms describe that the major problem is low membrane permeability leading to limited cellular uptake. However, the results obtained in this study did not prove this hypothesis.

The negative results in chapter 3.I. upon *in vitro* activity of promising inhibitors led to the question of the essentiality of the DOXP pathway in the studied bacteria. Also, insufficient research in the past has focused on the nonmevalonate pathway in Bcc species and therefore the goal in chapter 3.II. was to obtain a better insight in this pathway in *B. cenocepacia*. We constructed a rhamnose-inducible conditional mutant of *dxr* in *B. cenocepacia* K56-2 to determine the essentiality of this pathway. The conditional DXR mutant shows significant differences in planktonic growth compared to the wild-type strain, suggesting that DXR is important for growth of *B. cenocepacia* K56-2, but not essential. The mutant strain also shows a significant reduction in biofilm formation when grown in LB without rhamnose. In pSC200 synthetic medium, disruption of *dxr* causes a significant decrease in CFU/biofilm, but no significant differences in biofilm formation are observed when quantification happened with resazurin staining. Currently, we do not have an explanation for this discrepancy between quantification methods or between media, although we know that it cannot be attributed to differences in CFU/biofilm between LB and pSC200. Experiments to evaluate differences in

virulence in *C. elegans* between the mutant and wild-type showed that virulence of *B. cenocepacia* K56-2 is considerably affected when *dxr* is not expressed. The fact that DXR affects *in vivo* virulence of *B. cenocepacia* in a *C. elegans* infection model can be of substantial value. However, it is important to evaluate differences in virulence in more complex *in vivo* models appropriate to study Bcc infections. Brackman et al. evaluated survival of *Galleria mellonella* larvae and of BALB/c mice in a lung infection model after infection with different Bcc strains.[323] Other *in vivo* models used earlier to study virulence profiles of bacteria belonging to the Bcc include zebrafish embryos [324-326], rat lung infection model [327], murine infection model [328-331] and a 3D human lung epithelial cell culture model.[332] Finally, we also evaluated differences in metabolism of 190 different carbon sources between wild-type and mutant K56-2. Eight compounds can be used as carbon source by K56-2 but not by the conditional DXR mutant, with D-galactonate being of most interest. D-galactonate is required for the formation of G3P which is, together with pyruvate, the start product for isoprenoid biosynthesis through the nonmevalonate pathway.[213, 236] These results suggest that disruption of DXR in *B. cenocepacia* does inactivate the nonmevalonate pathway but also influences upstream metabolic pathways like the De Ley-Doudoroff (DD) pathway for biosynthesis of G3P. To our knowledge, no previous studies have described a relation between DXR and metabolic pathways upstream of the nonmevalonate pathway, although Ramos et al. described that the DD pathway greatly enhances isoprenoid production from D-galactose through the nonmevalonate pathway.[333]

6. The future of the nonmevalonate pathway as an antibacterial target

The enzymes of the nonmevalonate pathway are attractive targets for the development of novel antimicrobials targeting serious infectious diseases including malaria, tuberculosis and MDR bacterial infections.[113, 334-337] Hasan et al. described the enzymes of the nonmevalonate pathway as 'chokepoints', meaning that the functionality of these enzymes and the specific products cannot be compensated by other enzymes.[338] Fosmidomycin and FR900098 effectively inhibit DXR, the first dedicated step in the DOXP pathway, in multiple organisms.[120, 126, 211] However, due to their highly charged structures, both compounds show suboptimal pharmacokinetic properties and low cellular uptake by microorganisms.[120, 126] Despite these challenges, fosmidomycin is currently in Phase II clinical trials for combination treatment against malaria.[339-342]

Substantial research has already been performed on developing analogues of fosmidomycin and FR900098 with improved physicochemical properties. Most of the previous work focused on targeting DXR from *P. falciparum* ([120, 133, 135, 139, 145, 322, 343-348]) and from *M.*

tuberculosis. [118, 119, 137, 139, 349-353] Recently, more research has focused on the nonmevalonate pathway as a target for new antimicrobial agents to combat Gram-negative bacterial infections (including *P. aeruginosa*, *Salmonella enterica* and species of the Bcc). [127, 218, 354, 355] Improvement of cellular uptake of fosmidomycin and its derivatives remains one of the most important hurdles to overcome. However, if future research could lead to the development of DOXP inhibitors that are active against their target enzymes and efficiently get into bacterial cells, these compounds could become new weapons to fight infections by MDR organisms. Moreover, this pathway is probably important for viability and virulence in many more microorganisms and therefore, future research should also focus on investigating the importance of this pathway in other important pathogenic bacteria.

This work demonstrates that the nonmevalonate pathway certainly poses an interesting target for development of novel antibiotics against *B. cenocepacia* infections, if the issue of permeability can be addressed. Future research on this pathway should focus on determining essentiality of other DOXP genes and especially on the synthesis of active compounds able to inhibit this pathway and efficiently get into bacterial cells of *B. cenocepacia*.

Notably, two other factors about the nonmevalonate pathway as antibacterial target have to be taken into account. First, inhibitors of the DOXP pathway are broad-spectrum antibiotics since they can affect many Gram-negative bacteria, some Gram-positives and some parasites. [117] The advantages of broad-spectrum compounds include a broader spectrum of activity in comparison with narrow-spectrum antibiotics and less need to identify the pathogen with certainty before treatment. On the other hand, treatment with broad-spectrum antibiotics pose a higher risk to develop resistance and children who receive broad-spectrum therapy have a higher risk to develop asthma. Also, broad-spectrum antibiotics can affect commensal microorganisms in the human body influencing the function of our immune system, our capacity to process food and our capability to resist secondary infections. [234, 356] Narrow-spectrum antibiotics such as azithromycin or erythromycin are less likely to cause resistance because only specific bacteria will be killed and they have less ability to cause superinfection on normal microorganisms present in the human body. However, narrow-spectrum compounds can only be applied if the specific pathogenic organism is identified and a very careful selection of the antibiotic is required. [87, 357] Secondly, new resistance mechanisms against fosmidomycin or related agents might develop upon clinical use. Up to date, the most commonly described mechanism is mutation or duplication of the target enzyme, DXR. [206, 209, 231] A single point mutation in DXR of *E. coli* has been described to confer resistance against fosmidomycin due to alterations in the fosmidomycin-binding site of DXR. Because this resistance

does not include apparent fitness disadvantages, the genetic barrier to resistance may be quite low and careful monitoring during treatment will be necessary.[231] Fosmidomycin resistance in *P. falciparum* was described due to a *dxr* gene duplication, leading to a bypass for isoprenoid biosynthesis during fosmidomycin treatment.[209] Other resistance mechanisms include expression of a fosmidomycin resistance gene (e.g. in *B. cenocepacia* [127]) and mutations or the absence of a specific GlpT transporter ([126, 207, 208]).

7. Methods to identify essential bacterial genes

Essential genes encode proteins that are absolutely required to maintain survival of any living organism. Essential genes are usually involved in key cellular functions including metabolism, replication of DNA, translation of genes into proteins, maintenance of cellular structure and transport processes in and outside the cell.[227, 358] Recently, newly-identified genes encoding essential cellular functions are increasingly being investigated as potential targets for the development of new antimicrobial compounds.

Genome-wide essential gene studies have been carried out using two major strategies: directed gene deletion and random transposon mutagenesis. The first approach systematically deletes complete individual genes or open-reading frames (ORFs) from the genome. Baba et al. used this approach to construct a collection of 3985 single-gene knockout mutants of *E. coli*. The Keio collection provides a useful resource for systematic genotypic analyses and also for genome-wide screening of similar bacterial strains.[359] With the second method, transposons are randomly inserted in as many genome positions as possible, with inactivation of the target essential gene as objective.[358, 360] Several studies prove the usefulness of transposon mutagenesis in the reliable identification of essential genes as drug targets.[361] Mobegi and colleagues report a proof of concept for the combination of genome-scale high-density transposon mutagenesis, high-throughput sequencing and integrative genomics to investigate potential essential targets in human respiratory pathogens.[362] A recent study of Wong describes a similar genome-wide screening tool for drug targets in *B. cenocepacia*; TraDIS or Transposon Directed Insertion-site Sequencing.[224] TraDIS uses shearing of genomic DNA followed by specific PCR amplification of DNA fragments containing transposons and Illumina sequencing to map unique transposon insertion sites.[363-365] Using this approach, Wong et al. predicted 383 genes to be essential for *B. cenocepacia* growth and viability in rich, undefined medium. Furthermore, an additional repertoire of 439 genes was revealed to be important for growth in nutrient-depleted minimal medium, providing an indication on how *B. cenocepacia* can adapt to nutritional limitation in the host environment.[224] Most genome-wide

studies also use computational analysis approaches such as comparative genomics between experimental data and data predicted using bioinformatics [366-370], online essential gene prediction servers [371] and determination of a universal minimal set of essential genes (minimal genomes) [227, 372]. The minimal genome concept currently plays a key role in the identification of essential genes that are conserved across species because these genes are candidate targets for broad-spectrum drug development.[358, 373]

However, for this PhD work we were interested in genes of a particular pathway in a specific bacterial organism and only to a lesser extent in genome-wide essential gene studies. The approaches to experimentally study essential genes are rather limited since, by definition, inactivation of an essential gene is lethal to the organism. By simply deleting or mutating essential genes it is impossible to characterize genotypic and phenotypic features and therefore it is more appropriate to construct inducible conditional mutants to study essential genes. Kofoed et al. constructed temperature-sensitive mutants of essential genes for which the products lose function at high temperatures and phenotypic differences are thus only observed at increased temperatures.[374] Several studies describe the construction of conditional unmarked deletion mutants. Flannagan et al. developed plasmids pDAI-SceI and pGPI-SceI to construct targeted, non-polar unmarked deletion mutants of *B. cenocepacia* and Bloodworth et al. applied this approach, with some modifications, to construct rhamnose-inducible mutants of an electron transfer flavoprotein in *B. cenocepacia*. [221, 223] Conditional antisense mutagenesis is another approach that was applied earlier to investigate candidate essential genes.[220, 375] Wang et al. repressed the expression of certain candidate essential genes in *P. aeruginosa* by using antisense phosphorothioate oligomers and Greenberg et al. were able to inhibit growth of Bcc strains by the administration of antisense phosphorodiamidate-morpholino oligomers (PMOs) directed against the gene coding for the essential acyl-carrier protein AcpP.[220, 375] Because Greenberg et al. reported the proof of concept in *B. multivorans* we wanted to apply this approach in our study for *B. cenocepacia*. Rhamnose-inducible antisense overexpression mutants of *dxr* and *ispD* were constructed in *B. cenocepacia* LMG 16656 but unfortunately, every attempt failed to construct the desired antisense mutants. We currently do not have an explanation for this. However, there are alternative (and potentially more suitable) methods to investigate essential genes in the Bcc. Cardona et al. describe a transposon mutagenesis system in *B. cenocepacia* consisting of two vectors that deliver a rhamnose-inducible promoter (P_{rhaB}) at the transposon insertion site and in an enhanced green fluorescent protein gene (*e-gfp*) downstream from P_{rhaB} . This system enabled the research group to systematically identify essential genes and operons in *B. cenocepacia*. [225] Later on, plasmid pSC200 was constructed by Ortega and colleagues which enables the delivery of P_{rhaB} into the chromosome

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of *B. cenocepacia* K56-2 to drive the expression of a targeted gene.[226] Juhas et al. also used plasmid pSC200 for mutagenesis of *B. cenocepacia* H111 and both studies succeeded to construct conditional mutants in which expression of the target gene is dependent on the rhamnose concentration in the medium.[226, 228] In Table 21, an overview is shown of previous studies that describe the identification of novel essential genes in *B. cenocepacia* and other Bcc species. In the present study, we applied plasmid pSC200 to study the essentiality of nonmevalonate genes in *B. cenocepacia* and succeeded to construct a rhamnose-inducible conditional mutant of DXR in *B. cenocepacia* K56-2.

Table 21. Overview of previous studies on the identification of essential genes in Bcc species.

Study	Outcomes	Reference
Juhas <i>et al.</i> , 2012	84 essential genes (species-specific for <i>Burkholderiales</i>)	[228]
Bloodworth <i>et al.</i> , 2013	117 essential genes * 66 with essential orthologs in <i>E. coli</i> or <i>P. aeruginosa</i> * 51 species-specific for <i>Burkholderiales</i> (whereof 38 present in <i>B. cenocepacia</i>)	[222]
Wong <i>et al.</i> , 2016	383 genes essential in <i>B. cenocepacia</i> (90% located on chromosome 1) * including five genes of nonmevalonate pathway	[224]

CHAPTER V. SUMMARY – SAMENVATTING

Bacterial infections remain one of the main causes of disease and death, with respiratory infections belonging to the top 10 causes of death worldwide. The *Burkholderia cepacia* complex (Bcc) is a complex of 20 closely related opportunistic Gram-negative bacterial species. Bcc species are important human pathogens in patients with cystic fibrosis (CF) with *Burkholderia cenocepacia* and *B. multivorans* currently being the most frequently isolated Bcc species from CF lungs. Chronic Bcc infections can cause a rapid decrease in lung function, ultimately leading to mortality and treatment options are limited due to their intrinsic resistance to a variety of available antibiotics. The main objective of my work was to explore two novel strategies to tackle Bcc infections, including the nonmevalonate pathway for isoprenoid biosynthesis and combination therapy of β -lactam antibiotics with β -lactamase inhibitors.

The nonmevalonate pathway is a potential target for the development of new antibiotics against multidrug-resistant bacteria. Fosmidomycin and FR900098 are active inhibitors of DXR, the second nonmevalonate enzyme, from several bacteria but have limited effect on whole bacterial cells. Therefore, several attempts have previously been made to improve cellular uptake, by synthesizing analogues with modified physicochemical properties.

The first goal of this work was to determine the *in vitro* antibacterial activity of fosmidomycin, FR900098 and new analogues against various bacterial pathogens, and to identify different resistance mechanism against these nonmevalonate inhibitors. Susceptibility screening showed that *in vitro* antibacterial activity of most inhibitors tested was low, with MIC values $> 250 \mu\text{M}$. Only ritanserin, an inhibitor of the fifth enzyme IspF, does partially inhibit planktonic and biofilm growth of two *P. acnes* strains, three *Mycobacterium* strains and some Bcc strains. Moreover, our data suggest that the role of efflux pumps in resistance towards these compounds is limited. Previous publications describe that the main issue is low permeability leading to limited cellular uptake, however, the results obtained in this study did not prove this hypothesis.

The second goal was to determine the essentiality of *dxr* and *ispD*, the second and third gene of the nonmevalonate pathway, in *B. cenocepacia* by constructing conditional knock-down mutants. Therefore, we used plasmid pSC200 which enables the delivery of a rhamnose-inducible promotor into the chromosome to drive the expression of a target gene. We succeeded to construct a rhamnose-inducible *dxr* mutant of *B. cenocepacia* K56-2, but all attempts failed to insert a correct *ispD* construct in K56-2. Growth experiments showed that sufficiently high expression of *dxr* is essential for planktonic and

biofilm growth of *B. cenocepacia* K56-2. *In vitro* susceptibility for ceftazidime and aztreonam increased 16- and 8-fold, respectively, upon disruption of *dxr* in *B. cenocepacia* K56-2. Moreover, data obtained in a *C. elegans in vivo* infection model showed that expression of *dxr* is required for full virulence of *B. cenocepacia* K56-2. Also, *dxr* appears to be important in *B. cenocepacia* to efficiently metabolize several carbon sources including D-galactonate which is required for biosynthesis of D-glyceraldehyde-3-P, a start product of the nonmevalonate pathway. In summary, DXR seems to play an important role in different physiological processes of *B. cenocepacia* and is therefore an interesting target for antibacterial chemotherapy against *B. cenocepacia*.

Intrinsic resistance of Bcc species to β -lactam antibiotics is frequently observed and most commonly mediated by efflux pumps, alterations in penicillin-binding proteins or the expression of β -lactamases. The third goal of this work was to investigate the ability of β -lactamase inhibitors (clavulanic acid, sulbactam, tazobactam and avibactam) to restore the *in vitro* antibacterial activity of β -lactam antibiotics against members of the Bcc. Clavulanic acid did not cause an increased susceptibility, but addition of sulbactam, tazobactam or avibactam to ceftazidime, amoxicillin, cefoxitin, cefepime or aztreonam led to at least 4-fold MIC decrease in some Bcc strains. Moreover, we observed that the effect of β -lactamase inhibitors on β -lactamase activity is both strain and/or antibiotic dependent, suggesting that β -lactam resistance in Bcc species is mediated by both β -lactamase production and non- β -lactamase related resistance mechanisms.

To conclude, our work demonstrates that the nonmevalonate certainly is a potential target for the development of antibiotic chemotherapy against multidrug-resistant *B. cenocepacia* infections, if the issue of permeability can be addressed. Moreover, β -lactam- β -lactamase inhibitor combinations can offer an additional approach for Bcc-therapy, but it is important to evaluate every clinical situation individually.

Bacteriële infecties zijn één van de grootste oorzaken van ziekte en sterfte, en respiratoire infecties behoren momenteel tot de top 10 van belangrijkste doodsoorzaken wereldwijd. Het *Burkholderia cepacia* complex of Bcc is een complex van 20 nauw gerelateerde opportunistische Gram-negatieve bacteriën. Bcc species zijn belangrijke humane pathogenen in patiënten met mucoviscidose (CF), waarvan *Burkholderia cenocepacia* en *Burkholderia multivorans* de Bcc species zijn die meest frequent geïsoleerd worden uit CF longen. Chronische Bcc infecties kunnen een snelle achteruitgang in longfunctie veroorzaken, eventueel met sterfte als gevolg, en behandelingsopties zijn beperkt omwille van hun intrinsieke resistentie tegen verschillende beschikbare antibiotica. Het voornaamste doel van mijn werk was het evalueren van twee nieuwe strategieën om Bcc infecties aan te pakken, waaronder de ‘nonmevalonaat pathway’ voor biosynthese van isoprenoïden en combinatiebehandeling met β -lactam antibiotica en β -lactamase inhibitoren.

De ‘nonmevalonaat pathway’ is een potentieel target voor de ontwikkeling van nieuwe antibiotica tegen multidrug-resistente bacteriën. Fosmidomycine en FR900098 zijn actieve inhibitoren van DXR, het tweede enzym in de ‘nonmevalonaat pathway’, van verschillende bacteriën maar hebben slechts beperkte activiteit op volledige bacteriële cellen. Bijgevolg hebben reeds verschillende onderzoekers pogingen ondernomen om opname in de cel te verhogen via de aanmaak van fosmidomycine analogen met gemodificeerde fysicochemische eigenschappen.

Het eerste doel was de bepaling van *in vitro* antibacteriële activiteit van fosmidomycine, FR900098 en nieuwe analogen tegen bepaalde bacteriële pathogenen, en de identificatie van resistentie mechanismen tegen deze inhibitoren. MIC experimenten toonden aan dat de *in vitro* antibacteriële activiteit van de meeste inhibitoren laag is, met MIC-waarden $> 250 \mu\text{M}$. Enkel ritanserine, een inhibitor van het vijfde enzym IspF, was in staat om planktonische en sessiele groei te inhiberen van twee *P. acnes* stammen, drie *Mycobacterium* stammen en verschillende Bcc stammen. Bovendien suggereren onze data dat resistentie slechts in beperkte mate wordt veroorzaakt door de activiteit van efflux pompen. Literatuur beschrijft dat de voornaamste oorzaak beperkte cellulaire opname is door een lage membraanpermeabiliteit, doch konden wij deze hypothese niet aantonen in deze studie.

Ten tweede trachtten we om de essentialiteit te bepalen van *dxr* en *ispD*, het tweede en derde enzym van de ‘nonmevalonaat pathway’, in *B. cenocepacia* via het aanmaken van conditionele ‘knock-down’ mutanten. Derhalve gebruikten we plasmide pSC200 dat een rhamnose-induceerbare promotor

integreert in het chromosomaal DNA en zo de expressie van een bepaald gen beheerst. We slaagden erin om een rhamnose-induceerbare conditionele *dxr* mutant van *B. cenocepacia* K56-2 aan te maken, maar bovenstaande techniek bleek niet geschikt voor de aanmaak van een *ispD* mutant. Onze experimenten toonden aan dat expressie van *dxr* essentieel is voor planktonische en biofilm groei van *B. cenocepacia* K56-2. Bovendien nam *in vitro* antibacteriële activiteit van ceftazidime en aztreonam toe met een factor 16 en 8, respectievelijk, wanneer *dxr* was uitgeschakeld. Overigens toonden onze data van een *C. elegans in vivo* infectiemodel aan dat voldoende hoge expressieniveaus van *dxr* nodig zijn voor optimale virulentie van *B. cenocepacia* K56-2. Tenslotte lijkt *dxr* ook belangrijk te zijn voor de metabolisatie van bepaalde koolstofbronnen in *B. cenocepacia* K56-2, waaronder D-galactonaat dat noodzakelijk is voor de biosynthese van D-glyceraldehyde-3-fosfaat (startproduct van de 'nonmevalonaat pathway'). Samenvattend kunnen we stellen dat DXR een belangrijke rol speelt in verschillende fysiologische processen in *B. cenocepacia* waardoor dit een interessant target is voor de ontwikkeling van antibacteriële therapie tegen *B. cenocepacia*.

Bcc species zijn intrinsiek resistent aan allerhande β -lactam antibiotica en deze resistentie wordt vooral veroorzaakt door efflux pompen, wijzigingen in pencilline-bindende eiwitten of de productie van β -lactamase enzymen. In het derde hoofdstuk wouden we daarom onderzoeken of β -lactamase inhibitoren (clavulaanzuur, sulbactam, tazobactam en avibactam) in staat zijn om de *in vitro* antibacteriële activiteit van β -lactam antibiotica tegen Bcc species te herstellen. Clavulaanzuur bleek geen effect te hebben op de *in vitro* activiteit, maar toevoeging van sulbactam, tazobactam of avibactam aan ceftazidime, amoxicilline, cefoxitine, cefepime of aztreonam gaf aanleiding tot een minstens viervoudige daling in MIC-waarden tegen sommige Bcc stammen. Vervolgens observeerden we ook dat het effect van β -lactamase inhibitoren op β -lactamase activiteit zowel stam- als antibioticum afhankelijk is, wat suggereert dat resistentie tegen β -lactam antibiotica veroorzaakt wordt door zowel β -lactamases als door niet- β -lactamase-gerelateerde resistentiemechanismen.

Dit werk toont dus aan dat de 'nonmevalonaat pathway' een mogelijk target is voor ontwikkeling van nieuwe antibiotica tegen infecties met *B. cenocepacia*, als de permeabiliteitsproblemen van mogelijke inhibitoren kunnen worden aangepakt. Daarenboven kan ook de combinatie van β -lactam antibiotica met β -lactamase inhibitoren een nieuwe methode bieden voor therapie tegen Bcc bacteriën, waarbij het belangrijk is om elke klinische situatie individueel te beoordelen.

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PERSONAL INFORMATION

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EDUCATION

- 2013 - 2016: PhD in Pharmaceutical Sciences, Ghent University
Laboratory of Pharmaceutical Microbiology, Ghent University
- 2010 – 2012: Master in Pharmaceutical Sciences, Ghent University (graduated with distinction)
- 2006 – 2010: Bachelor in Pharmaceutical Sciences, Ghent University
- 2000 – 2006: Secondary school (Science and Mathematics), Sint-Pietersinsituut, Ghent

PUBLICATIONS

Everaert A., Sass A., Van Acker H., Van den Driessche F., Coenye T. 2016. Is the nonmevalonate pathway an essential biochemical pathway in *Burkholderia cenocepacia*? *Manuscript in preparation*.

Everaert A., Coenye T. 2016. Effect of β -lactamase inhibitors on *in vitro* activity of β -lactam antibiotics against *Burkholderia cepacia* complex species. *Antimicrob Resist Infect Control*. 5:44

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CONFERENCES

2016	Knowledge for Growth, Ghent, Belgium	Poster presentation
2015	Knowledge for Growth, Ghent, Belgium	Poster presentation
2015	IBCWG Meeting, Vancouver, Canada	Oral presentation
2015	Eurobiofilms, Brno, Czech Republic	Poster presentation
2014	Knowledge for Growth, Ghent, Belgium	Poster presentation
2014	IBCWG Meeting, Nimes, France	Oral presentation
2013	Eurobiofilms Conference, Ghent, Belgium	Poster presentation

TRAININGS

Doctoral schools UGent	Advanced Academic English, writing skills Grow your personal leadership – Grow your future career Effective graphical displays Communication skills Project management
KULeuven	Summerschool on bacterial biofilms

SUPERVISION OF GRADUATE STUDENTS

- **Kim Bigler**, 2014 – 2015
Bepaling van de antimicrobiële activiteit van potentiële inhibitoren van de DOXP pathway bij een diverse collectie bacteriën.
 - **Lisa Maes**, 2013 – 2014
Nonmevalonaat pathway voor isoprenoid biosynthese als potentieel doelwit voor antibacteriële therapie.
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