"Activity of antibiotics against in vitro mono-species biofilms of P. aeruginosa and S. aureus in the context of cystic fibrosis : the influence of the culture medium"

Diaz Iglesias, Yvan

ABSTRACT

Cystic fibrosis is a genetic disease characterized by the accumulation of viscous mucus in the patient's airways, which favors bacterial colonization by bacterial communities named biofilms. The most common pathogens are Staphylococcus aureus in children and Pseudomonas aeruginosa in adults. Our goal was to develop a relevant artificial sputum medium (ASM) that can be used to grow biofilms of each of these bacteria and study the activity of antibiotics administered to the patients. We showed that the rheological properties of this medium were similar to those of sputa from patients. Biofilms from both species were less responsive to antibiotics in ASM than in a conventional medium. In addition, some antibiotics selected small colony variants in ASM, another mode of bacterial growth involved in the pathology. These results show the importance of using a relevant culture medium to study the activity of antibiotics in the context of this pathology.

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Notice biographique

Yvan Diaz Iglesias est diplômé en sciences biomédicales de l'Université catholique de Louvain. Durant son master, il rejoint le laboratoire du Professeur F. Van Bambeke pour étudier les biofilms de *Streptococcus pneumoniae*. En 2015, il obtient une bourse du Fonds pour la Recherche dans l'Industrie et l'Agriculture (FRIA) pour effectuer des recherches sur l'activité des antibiotiques contre les biofilms de *Staphylococcus aureus* et *Pseudomonas aeruginosa* dans le contexte de la mucoviscidose.

Résumé: La mucoviscidose est une maladie génétique qui se caractérise par une accumulation de mucus visqueux dans les voies respiratoires, qui favorise la colonisation par des communautés bactériennes, les biofilms. Les espèces les plus fréquentes sont *Staphylococcus aureus* chez l'enfant et *Pseudomonas aeruginosa* chez l'adulte. Notre but était de développer un sputum artificiel (ASM) pertinent pour y faire croître des biofilms de chaque bactérie et étudier l'activité des antibiotiques administrés aux patients. Nous avons montré que les propriétés rhéologiques de ce milieu étaient similaires à celles de sputa de patients. Les biofilms répondent moins aux antibiotiques dans ce milieu que dans un milieu classique. De plus, certains antibiotiques y sélectionnent des variants petites colonies, un mode de croissance impliqué dans la pathologie. Ces résultats montrent l'importance d'utiliser un milieu de culture adéquat pour étudier l'activité des antibiotiques dans le contexte de cette pathologie.

Summary: Cystic fibrosis is a genetic disease characterized by the accumulation of viscous mucus in the patient's airways, which favors bacterial colonization by bacterial communities named biofilms. The most common pathogens are *Staphylococcus aureus* in children and *Pseudomonas aeruginosa* in adults. Our goal was to develop a relevant artificial sputum medium (ASM) that can be used to grow biofilms of each of these bacteria and study the activity of antibiotics administered to the patients. We showed that the rheological properties of this medium were similar to those of sputa from patients. Biofilms from both species were less responsive to antibiotics in ASM than in a conventional medium. In addition, some antibiotics selected small colony variants in ASM, another mode of bacterial growth involved in the pathology. These results show the importance of using a relevant culture medium to study the activity of antibiotics in the context of this pathology.

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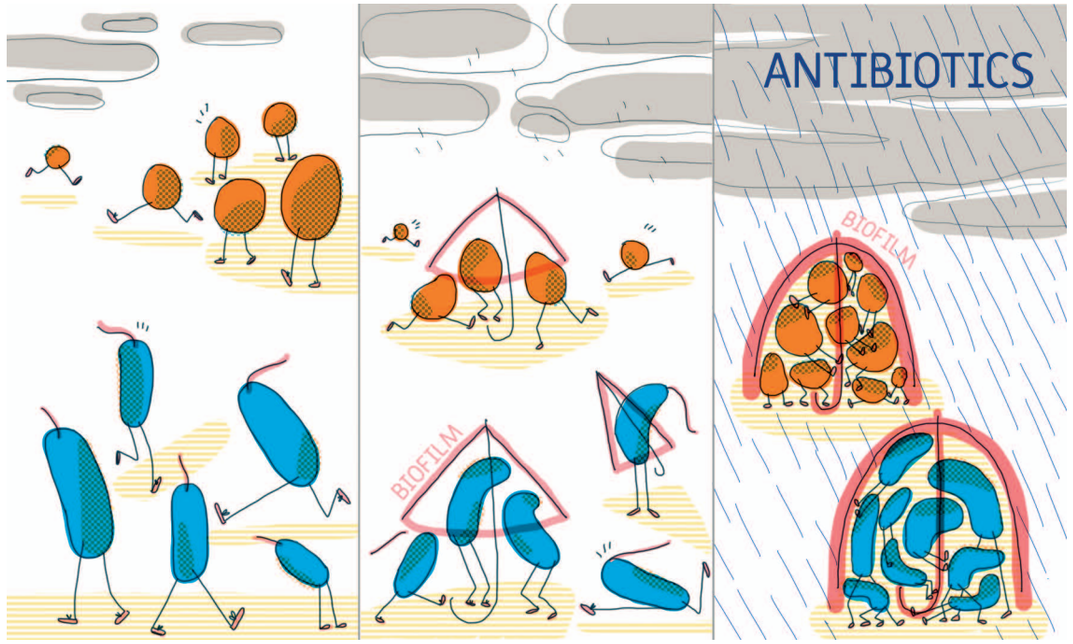

Thèses du secteur des Sciences de la santé

Activity of antibiotics against *in vitro* mono-species biofilms of *P. aeruginosa* and *S. aureus* in the context of cystic fibrosis: The influence of the culture medium

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Résumé. La mucoviscidose est une maladie génétique qui se caractérise par une accumulation de mucus visqueux dans les voies respiratoires, qui favorise la colonisation par des communautés bactériennes, les biofilms. Les espèces les plus fréquentes sont *Staphylococcus aureus* chez l'enfant et *Pseudomonas aeruginosa* chez l'adulte. Notre but était de développer un sputum artificiel (ASM) pertinent pour y faire croître des biofilms de chaque bactérie et étudier l'activité des antibiotiques administrés aux patients. Nous avons montré que les propriétés rhéologiques de ce milieu étaient similaires à celles de sputa de patients. Les biofilms répondent moins aux antibiotiques dans ce milieu que dans un milieu classique. De plus, certains antibiotiques y sélectionnent des variants petites colonies, un mode de croissance impliqué dans la pathologie. Ces résultats montrent l'importance d'utiliser un milieu de culture adéquat pour étudier l'activité des antibiotiques dans le contexte de cette pathologie.

Summary. Cystic fibrosis is a genetic disease characterized by the accumulation of viscous mucus in the patient's airways, which favors bacterial colonization by bacterial communities named biofilms. The most common pathogens are *Staphylococcus aureus* in children and *Pseudomonas aeruginosa* in adults. Our goal was to develop a relevant artificial sputum medium (ASM) that can be used to grow biofilms of each of these bacteria and study the activity of antibiotics administered to the patients. We showed that the rheological properties of this medium were similar to those of sputa from patients. Biofilms from both species were less responsive to antibiotics in ASM than in a conventional medium. In addition, some antibiotics selected small colony variants in ASM, another mode of bacterial growth involved in the pathology. These results show the importance of using a relevant culture medium to study the activity of antibiotics in the context of this pathology.

Table of contents

Abbreviations

ABC, adenosine triphosphate-binding cassette
ABPA, allergic bronchopulmonary aspergillosis
Agr, accessory gene regulator
AI-2, autoinducer 2
AIP, autoinducing peptide
AprA, alkaline protease
ASM, artificial sputum medium
Atl, major autolysin protein
ATP, adenosine triphosphate
BCFR, Belgian cystic fibrosis registry
BHL, *N*-butonoyl-L-homoserine lactone (BHL)
C, relative potency
cAMP, adenosine 3',5'-cyclic monophosphate
C-di-GMP, cyclic di-guanosine monophosphate
CF, cystic fibrosis
CFRD, cystic fibrosis-related diabetes
CFTR, cystic fibrosis transmembrane conductance regulator
CHIPS, chemotaxis-inhibiting proteins of *S. aureus*
ClfA, clumping factor A
CV, crystal violet
DNA, deoxyribonucleic acid
DOPC, dioleoylphosphatidylcholine
Eap, extracellular adherence protein
eDNA, extracellular DNA
E_{max}, maximal efficacy
ENaC, epithelial sodium channel
EPS, extracellular polymeric substances
ETA, exfoliative toxins A
ETB, exfoliative toxins B
ExoA/S/T/U/Y, exotoxin A/S/T/U/Y
FDA, fluorescein diacetate
GalNAc, *N*-acetylgalactosamine
GlcNAc, *N*-acetylglucosamine
HQNO, 2-heptyl-1-hydroxyquinolin-4-one
ICAM-1, intercellular adhesion molecule-1
IQS, 2-(2-hydroxylphenyl)-thiazole-4-carbaldehyde / integrated quorum sensing signal
LasA, elastase A
LasB, elastase B

LPS, lipopolysaccharides
LVE, linear viscoelastic
MBC, minimal bactericidal concentration
MBEC, minimum biofilm eradication concentration
MBIC, minimum biofilm inhibitory concentration
MDR-PA, multidrug-resistant *P. aeruginosa*
MIC, minimal inhibitory concentration
MRSA, methicillin-resistant *S. aureus* (MRSA)
MSCRAMM, microbial surface component recognizing adhesive matrix molecules
MSSA, methicillin-sensitive *S. aureus*
NBD, nucleotide-binding domain
NTM, nontuberculous mycobacteria
OdDHL, *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL)
PBP, penicillin binding protein
Pel, pellicle
PIA, polysaccharide intercellular adhesin
PIV, protease IV
PMN, polymorphonuclear cells
PNAG, poly-β-1,6-*N*-acetylglucosamine
PQS, 2-heptyl-3-hydroxy-4-quinolone / *Pseudomonas* quinolone signal
Psl, polysaccharide synthesis locus
PSM, phenol-soluble modulins
PVL, Panton-Valentine leukocidin
QS, quorum sensing
R, regulatory domain
ROS, reactive oxygen species
SarA, staphylococcal accessory regulator
SCC, staphylococcal cassette chromosomes
SCFM, synthetic cystic fibrosis sputum medium
SCV, small colony variants
SpA, staphylococcal protein A
TGN, tryptic soy broth supplemented by glucose and NaCl
TSST-1, toxic shock syndrome toxin-1

Chapter I. Introduction

1. Cystic fibrosis: a multi-systemic disease

1.1. Global overview

Cystic fibrosis (CF) is a recessive genetic disease affecting multiple organs, like the airways, pancreas, intestines, liver, reproductive tract, and sweat glands. The disease is caused by different mutations affecting a gene coding for a chloride channel, the cystic fibrosis transmembrane conductance regulator (CFTR).

The mutations in *CFTR* will result in an impaired, reduced or absent protein at the apical membrane of epithelial cells of multiple organs, which will lead to a defective ion transport through membranes, a change in ionic imbalance (with an increased absorption of sodium in the lungs) and an accumulation of a viscous, difficult-to-evacuate mucus in the affected tissues [1-3].

CF is the most prevalent lethal genetic recessive disease affecting Caucasian individuals with around 30 000 cases in the US, 10 000 in the UK, and 40 000 in the rest of Europe (with 1319 patients in Belgium for year 2017 [4]) for a total of 80 000 people in the world. In Europe, the prevalence of the disease varies between 1 in 1400 in Ireland to 1 in 7700 in Finland. Today, 75% of people with CF are diagnosed by the age of 2 years and more than half of the CF population is aged 18 or older. The therapeutic progress made during the last two decades has significantly increased the life expectancy of patients from over 70% risk of death at birth in the 1930s to over 95% survival today with a life expectancy estimated at 44 years [5-7].

The statistics from the Belgian CF registry (BCFR) show data similar to those from the other CF centers: 62.2% of the patients are adults (>18 years), the mean age at diagnosis is 5.4 months. The median life expectancy was 22.8 years in 2017 based on 6 deaths (age ranging from 11.8 to 70.4 years). With an estimated coverage of 90% of all people with CF living in Belgium, the data collection shows that population is aging as shown in Figure 1, with only 1.2% of people aged of 40 years or more in 2000 to 16.8% in 2017 [4].

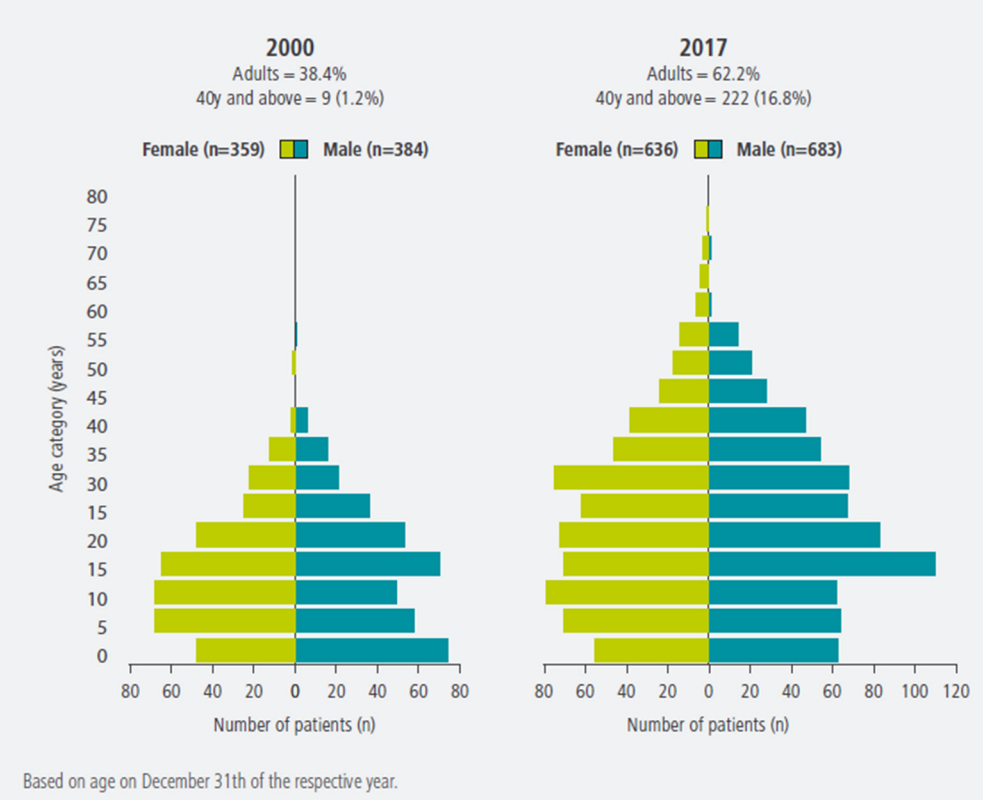


Figure 1: Age distribution by gender in 2000 (left) and 2017 (right) [4].

Despite the fact that CF is a multi-systemic disease, the pulmonary manifestations are the major cause of morbidity and mortality. The pathology is characterized by acute or chronic respiratory disorder associated to bacterial infections caused by the colonization of the stagnant mucus in the airways by different pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* [5;6;8].

1.2. Historical overview

Even if some symptoms (such as salty sweat) were known for centuries, the first clear description of this pathology was made in the 1930s by doctor Dorothy Andersen, a pathologist at the New York Babies Hospital, in her paper: "*Cystic fibrosis of the pancreas and its relation to celiac*

disease". In this work, she evidenced the presence of mucus plugging the glandular ducts of pancreas, damages to the tissues and the association with *meconium ileus* (congestion of the ileum due to the thickening of the first feces, described as the most precocious and serious syndrome of CF). Following this, links were made between pancreatic, pulmonary and intestinal symptoms as parts of the same pathology [1;9]. In the late 40s, Andersen and Hodges [10] formulated the hypothesis and proved that CF is a recessive genetic disease. In the early 60s, Gibson and Cooke [11] developed the pilocarpine iontophoresis sweat test to diagnose CF by measuring the concentration of chloride released in sweat. Pilocarpine is an agonist of muscarinic acetylcholine receptors present in sweat glands and induces sweat production, facilitating sampling for determination of its salt concentration. This test is still the gold standard for CF diagnosis. The 1980s were marked by an understanding on a biomolecular basis of the deregulation of chloride and sodium ion transport [12] as well as the cloning of the *CFTR* in 1989 [13], which led later to the characterization of CF as a result of a loss of function and/or expression of the CFTR.

1.3. Cystic Fibrosis Transmembrane conductance Regulator

1.3.1. *CFTR* gene and function of the CFTR protein

The *CFTR* gene is 180kbp long and is located in the long arm of chromosome 7 (7q31.2). It encodes a 1480 amino-acid chloride channel located in the apical membrane of epithelial cells of multiple organs such as lungs, pancreas, intestinal tract, liver, reproductive tract, and sweat glands [14;15]. Other functions have also been described for this protein, such as regulation of other membrane channels (eg. epithelial sodium channel (ENaC)). It is also known that CFTR can transport or regulate HCO_3^- transport through epithelial cell membranes [16].

The CFTR protein is a member of the adenosine triphosphate (ATP)-binding cassette (ABC) transporter protein family. This family regulates transmembrane transport of small molecules. The protein structure (Figure 2) consists of two transmembrane domains, two nucleotide-binding

domains (NBD1 and NBD2) interacting with ATP and one regulatory domain that can be phosphorylated by a protein kinase A dependent on adenosine 3',5'-cyclic monophosphate (cAMP). The phosphorylation of the R domain is followed by ATP binding to NBD and the opening of the channel. The channel is closed when ATP is hydrolyzed [1;14;16;17].

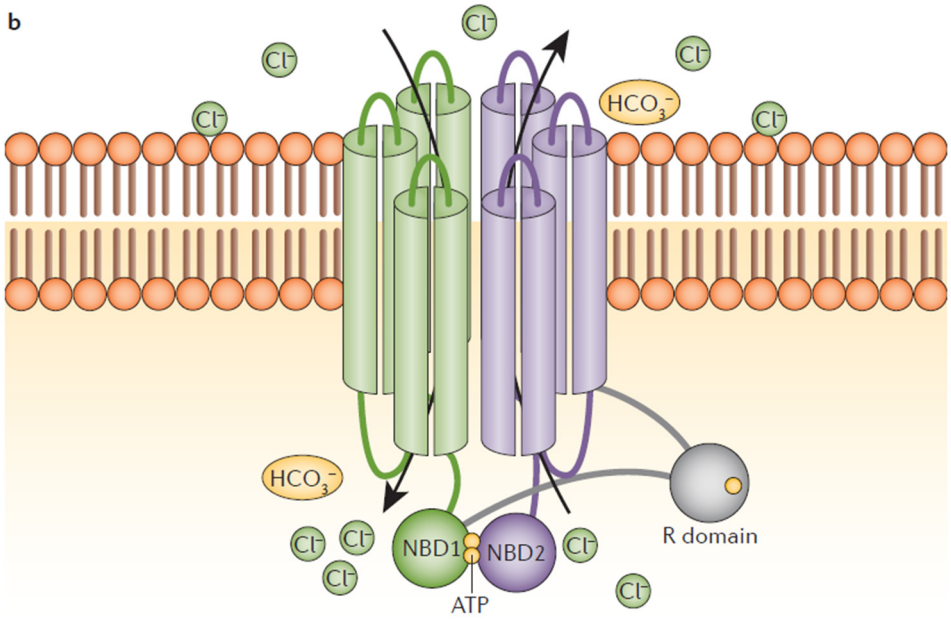


Figure 2: Structure of CFTR protein with its two transmembrane domains, two nucleotide-binding domains (NBD1 and NBD2) and one regulatory domain (R domain). The channel opens after the binding of ATP to NBD, this binding only happens when the R domain is phosphorylated by the protein kinase A (Image from: [16]).

1.3.2. *CFTR* gene mutations

The pathology is caused by mutations in *CFTR* gene. The mutations will result in an impairment of the function of the protein or in a reduction (or even absence) of its expression. Today, more than 2000 putative mutations are known for this pathology, with diverse ranges of disease severity. The most frequent and described mutation is the missense mutation **F508del** corresponding to the deletion of three base pairs encoding a phenylalanine in position 508 of the CFTR. This phenylalanine is

located in the NBD1 region and its deletion will result in an immature protein that will be predominantly degraded by the proteasome. This mutation accounts for 70% of all CF alleles but its repartition is not uniform, being found in 70-90% of the cases in northern European countries and North America but only 50% in Mediterranean countries [1;14;18;19].
In some Caucasian sub-populations, we can observe a higher occurrence of less frequent mutations (≤1%) than in the global Caucasian population. For example, the mutation W1282X (stop codon) has around 42% of occurrence in Ashkenazi Jewish populations while the mutation 621+1G>T (nucleotide insertion) is found in 23% of the French Canadian population [20;21].
CFTR mutations are classified in six classes depending on their consequences for the protein. These consequences and the severity of the pathology in the corresponding patients are listed in table 1.

Class	Approximate Worldwide Frequency	Mutation prototype	Consequence	Severity of the pathology
I	~10 %	W1282X, 621 +1G>T	The protein is not synthesized	Severe
II	70%	F508del	The protein is synthesized but in an immature form that will be degraded by the proteasome	Severe
III	2-3%	G551D	The protein is synthesized but its regulation is altered	Severe
IV	<2%	R334W	The protein is synthesized but transport of chloride ion is reduced	Milder
V	<1%	3849 + 10kb C>T	The protein synthesis is reduced	Milder
VI	<1%	1811 +1.6kb A>G	The protein is synthesized but the conformational stability is reduced	Severe

Table 1: The mutation affecting the *CFTR* are categorized in 6 classes depending on the consequences for the protein (adapted from [1;14;16;19]).

1.4. Clinical manifestations

Despite being a multi-systemic disease, the damages to the lungs of patients with CF are those that will most threaten their quality of life. They will determine the severity of the pathology and the life expectancy of the patients. Around 63% of CF deaths are due to pulmonary insufficiency, other

causes are related to unsuccessful transplantations (15%), liver disease (3%), suicide (1%) or other/unknown reasons (13%) [2;5;22;23]. Before developing the clinical manifestations related to the lung diseases in the following paragraph, the clinical manifestations affecting the other organs like the gastrointestinal tract, the endocrine system and the reproductive system will be briefly summarized in the next lines.

As the mucus in multiple organs is thicker, **gastrointestinal tract** is not spared by the disease. 15% of the newborns suffer from *meconium ileus*, an obstruction of the ileus. Between 85% and 90% of young patients suffer from exocrine pancreatic insufficiency, which is characterized by steatorrhea, flatulence, poor weight gain, low absorption of vitamins A, D, E and K (liposoluble vitamins) and malnutrition. Gallbladder function is also impaired because intrahepatic bile ducts can be obstructed which can lead to liver damages [23;24].

Different organs composing the **endocrine system** are also affected such as endocrine pancreas with pancreatic dysfunction caused by the obstruction of intrapancreatic ducts with thickened secretions. This plugging of ducts is associated with inflammation, formation of cysts and a diminution of functional Langerhans islets in all patients. This diminished number of functional islets will lead to a diminution of insulin secretion and the apparition of insulin insufficiency and the development of cystic fibrosis-related diabetes (CFRD). CFRD is different from type 1 and type 2 diabetes but CFRD shares some similarities with both diseases like insulin-dependence (diabetes type 1) and insulin-resistance (diabetes type 2). The frequency of CFRD is between 35% and 50% in adults [23;25].
Impaired growth hormone secretion has been reported in pediatric patients with CF and it is thought to be linked with short stature. Bones are also affected as vitamin D is not properly absorbed, this deficiency coupled with chronic systemic inflammation and intermittent cortico-steroid use will lead to osteoporosis and higher risks of bone fracture [23;25].

CFTR mutations can also affect **reproductive organs**. Men suffer from congenital bilateral absence of the *vas deferens*, the duct transporting spermatozoids from the epididymis to the prostate. This implies azoospermia and means that men with CF are infertile (but not sterile). Women are less affected as up to 50% of women are able to conceive children but they have thicker cervical mucus. This mucus can impair cervical penetration by sperm and it can reduce the chances of fecundation [23;26].

1.4.1. Lung pathophysiology

The *CFTR* mutations result in a deregulation of the homeostasis of ion transport in the respiratory tract. The ion imbalance is characterized by the reduction of chloride ions transport to the respiratory mucus. In normal conditions (Figure 3A), this transport creates an osmotic gradient with water flux from epithelial cells to mucus but in CF, as chloride ions are not transported, less water is transported to the lumen of the airways. At the same time, sodium ions are transported through the ENaC from the lumen to the cells, which creates an osmotic gradient with water flux from the mucus to the epithelial cells [16;27]. Those two mechanisms (reduction in chloride secretion and increase in sodium absorption) contribute to reduce the hydration of mucus, making it viscous. As the mucus is depleted in liquid, the mucociliary clearance is reduced (Figure 3B). In normal conditions, the mucociliary clearance works to clear pathogens and provides a protective barrier against toxic products. In the case of CF lungs, its action is reduced due to the stagnation of the thick mucus making possible the colonization of this stagnant mucus by bacteria [16;27].

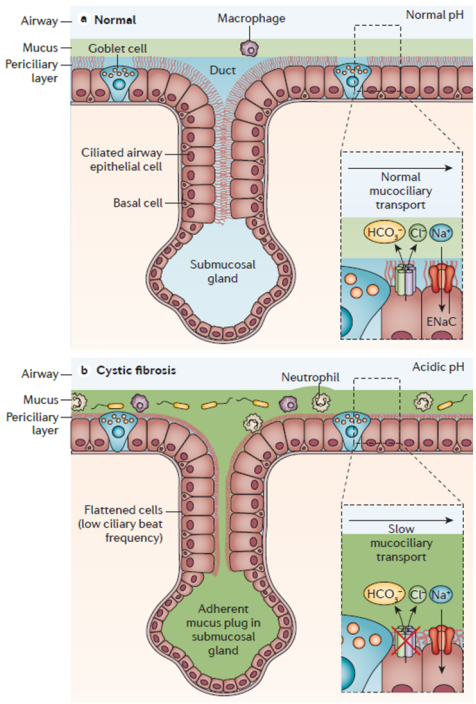


Figure 3: Impact of the *CFTR* mutation on mucociliary transport.
(A). In people with normal function of CFTR, the transport of ions through the membrane allows an adequate hydration of the mucus and a normal mucociliary transport. (B). In people with CF, the ion imbalance due to the *CFTR* dysfunction provokes a dehydration of the mucus in the lumen. Dehydrated mucus will plug into the glands, reduce the mucociliary transport and favor the colonization by pathogens.
(Image adapted from [16])

Shortly after birth, mucus is colonized by bacteria such as *Staphylococcus aureus* and *Haemophilus influenzae,* which are the major pathogens found in infants [5;8;28]. This early airway colonization is accompanied by a constant inflammation state demonstrated by the presence of polymorphonuclear cells (PMN), high levels of interleukin 8 and neutrophil elastase in brochoalveolar lavage fluid. PMN are recruited to combat bacterial and fungal pathogens through the release of proteases but this release can harm the lung tissue contributing to the chronic non-resolving inflammation [2;27]. Moreover, the death of PMN in the airways causes the release of their internal constituents such as DNA, which will further increase the viscosity of the mucus [2;23]. Recurrent infections and inflammation state will cause structural changes in the lungs due to the development of bronchiectasis, a permanent enlargement of parts of the airways [1;2;23].
The impossibility to clear bacterial colonization, the permanent inflammation state and the obstruction of the airways by the mucus will

result in pulmonary insufficiency and, in the worst cases, respiratory failure [5;23]. A vicious circle sets in between the obstruction caused by the mucus, the chronic infections and the chronic inflammation.

1.5. Microbiology of CF lung disease

Colonization of the airways occurs shortly after birth but the probability of finding one pathogen rather than another will depend on the age of the patient (Figure 4). A large variety of pathogens are found in CF populations and all of them are considered as opportunistic. Nevertheless, they are responsible for infections that, coupled with chronic inflammation and obstruction of the airways by the mucus, will result in pulmonary insufficiency which can lead to death [5;22;23].

1.5.1. Childhood and adolescence

One of the first bacteria to be cultured in infants is *S. aureus,* which is found in 60% of patients before the age of 2 years and around 80% in children aged of 10 years. Methicillin-Resistant *S. aureus* (MRSA) represents between 10 and 30% of the *S. aureus* isolated. [5;28;29].

The second most frequently cultivated species is *H. influenzae,* found in 30% of patients until the age of 10 years. Its prevalence tends to diminish under 10% at the end of adolescence. As the strains infecting patients with CF are nontypeable *H. influenzae*, the *H. influenzae* type b vaccines do not protect the children against the strains recovered in this population [5;28-30].

The median age of *Pseudomonas aeruginosa* detection is 5 years but its prevalence in children before 10 years remains around 20%, which makes it the third more frequent pathogen in this population. At the beginning of adolescence, its prevalence starts to grow up until 50% at 18 years. In parallel to the growth of *P. aeruginosa*, the first Multidrug-Resistant (MDR-PA) strains appear around age 11 years. At 18 years, they represent 10% of isolated *P. aeruginosa*. [5;28].

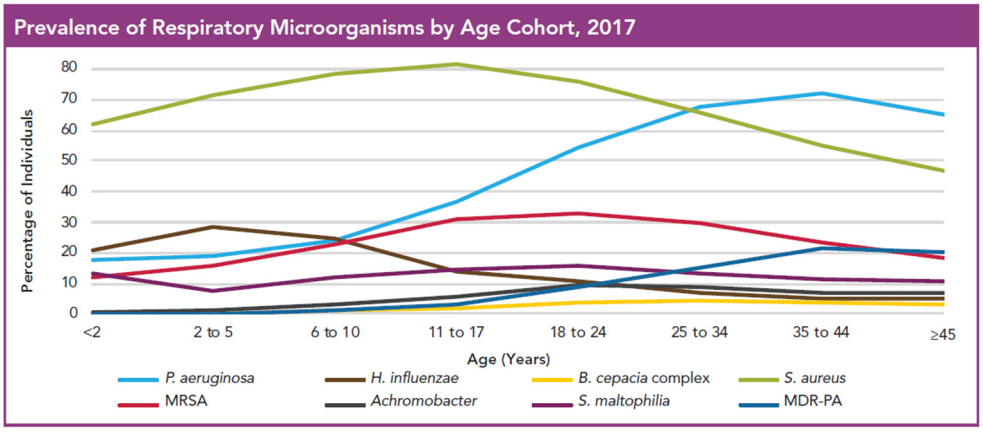


Figure 4: Prevalence of respiratory microorganisms found in lungs of patients with CF (From [5]).

Other pathogens that can be isolated in children with a frequency under 10% are *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans*, two emerging, intrinsically multi-drug resistant, gram-negative rods. The implication of both species in lung function decline is still not clear [5;28;31;32].

Interestingly, the major pathogens found in childhood and adulthood are the same but their prevalence is different. The main change concerns the fact that *P. aeruginosa* becomes the dominant pathogen instead of *S. aureus*. Different hypotheses have been formulated to explain those changes. First, *P. aeruginosa* will undergo phenotypic changes between early colonization (environmental phenotype) and chronic infection (mucoid phenotype, loss of motility and resistance to antibiotics) making it more adapted to CF environment [33]. Second, *P. aeruginosa* produces multiple extracellular virulence factors that target and kill *S. aureus* (*P. aeruginosa* virulence factors will be discussed later in the manuscript) [33]. Finally, the pulmonary microenvironment (low levels of oxygen and presence of nitrate [34]) will favor anaerobic respiration of *P. aeruginosa* that will express genes essential for denitrification and the use of nitrate as electron acceptor [33;35;36]. A last hypothesis that can explain the switch from *S. aureus* to *P. aeruginosa* is the use of staphylococcal prophylaxis in children that could

increase the pseudomonal infections rate, however the use of staphylococcal prophylaxis diverges between US and Europe [37-40].

All these changes in *P. aeruginosa* behavior (phenotypic change, production of virulence factors and anaerobic respiration) are probably part of the explanation of the change of dominant pathogen in CF at the end of adolescence where *S. aureus* prevalence diminishes to the advantage of *P. aeruginosa*.

1.5.2. Adulthood

The dominant pathogen in adults is *P. aeruginosa,* which can be found in 70% of the patients aged of 25 years or more. The MDR-PA strains represent until 20% of isolated strains of *P. aeruginosa* in patients older than 30 years. The acquisition of this microorganism is associated with clinical deterioration [5;6].

S. aureus remains as the second pathogen and it can be found in around 50% of patients. Besides, we can observe a diminution of the percentage of MRSA isolated [5;6]. It is interesting to note that *P. aeruginosa* and *S. aureus* are co-isolated in 20 to 30% of patients [41;42].

Other organisms cultured from adult sputum are *S. maltophilia*, *A. xylosoxidans*, *Burkholderia cepacia*, nontuberculous mycobacteria (NTM) and fungi such as *Aspergillus* spp.

Even if *S. maltophilia* and *A. xylosoxidans* are more frequent, *B. cepacia* complex is the most problematic pathogen. *B. cepacia* complex is a complex of closely related species and the two major species of the complex accounting for 85 to 95% are *Burkholderia cenocepacia* and *Burkholderia multivorans.* They are associated with the “cepacia syndrome” which starts with high fevers, bacteremia and rapid progression to severe necrotizing pneumonia and death. The prevalence of *B. cepacia* complex in people with CF is comprised between 3 and 5% [5;29;43].

Nontuberculous mycobacteria are isolated from respiratory secretion of patients with CF in 13% of the cases with *Mycobacterium avium* complex (72%) and *Mycobacterium abscessus* (16%) being the more

frequently isolated. The presence of NTM is associated with older patients and the presence of *S. aureus* rather than *P. aeruginosa* [5;6;29].

Fungi are frequently found late in the disease progression. The two most frequent fungi are *Candida* spp. and *Aspergillus* spp., with *Candida* spp. being the most prevalent as they can be found in up to 90% of adult patients having received multiple antibiotic treatments. The presence of *Aspergillus* spp. is detected in 65 and 75% of the patients [29;44;45].

Candida albicans is the most prevalent species from the *Candida* spp. and it may cause transient or permanent colonization of the respiratory tracts. The pathogenicity of *C. albicans* is still not clearly demonstrated as *C. albicans* is a commensal yeast but its presence has in some cases been associated with a decrease in lung function [29;44-49]. The second most prevalent species from the *Candida* spp. is *Candida glabrata* known to cause intermittent and chronic colonization. Such colonization has been associated with loss of lung function in adolescents [47].

On the contrary, the presence of *Aspergillus* spp. is clearly associated with lung function decline. *Aspergillus fumigatus* accounts for 70% of all *Aspergillus* spp. and it can be responsible for infections, bronchitis or allergic bronchopulmonary aspergillosis (ABPA). ABPA is an allergic syndrome caused by allergens from *A. fumigatus*. This syndrome is characterized by wheezing, pulmonary infiltrates and potential bronchiectasis and fibrosis. Its prevalence is approximately 25% [29;44;45;49-51].

1.6. Therapeutic strategies

As CF is a multi-systemic disorder, the therapeutic strategies will act to restore at best to the normal function. Figure 5 shows the different disorders and some strategies adopted by the physicians to restore the function.

In this section, therapeutic strategies to overcome **nutritional problems**, **mucociliary clearance**, **CFTR function** and **inflammation** will be shortly presented. **Anti-infective strategies** will be further discussed. If all the strategies failed, the last resort is the **lung transplantation**.

Nutritional problems (malabsorption and pancreatic insufficiency) are overcome with strict alimentary habits and supplementation with pancreatic enzymes and fat soluble vitamins (A/D/E/K) [3;23].

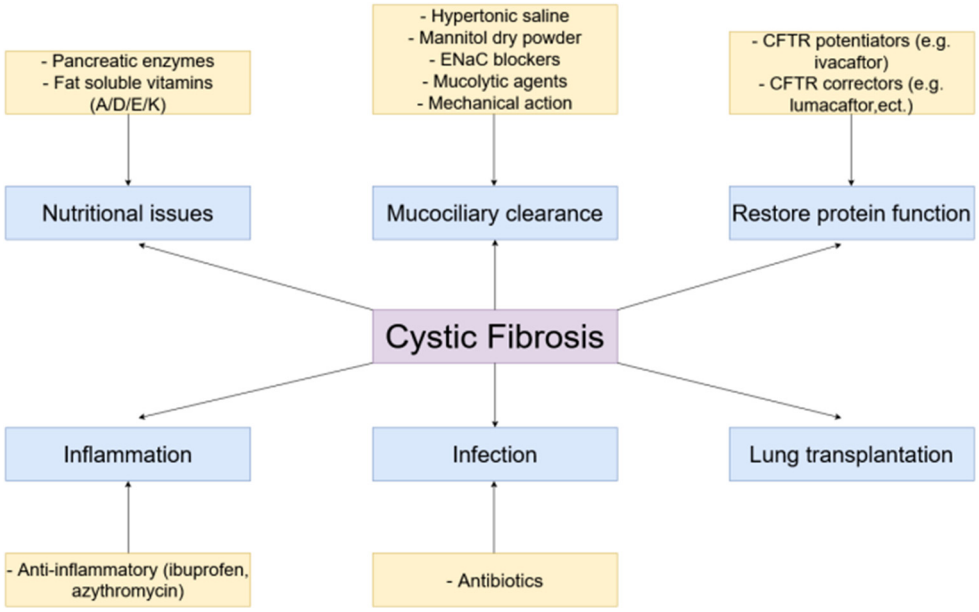


Figure 5: Therapeutic strategies available to treat patients with CF. For each disorder there is a strategy to restore at best to the normal function. This figure summarizes each disorder and the corresponding strategy (constructed based on [16]).

Multiple strategies acting on the **mucociliary clearance** can be implemented such as direct rehydration (hypertonic saline) [3;16;23;52], osmotic gradient (mannitol dry powder) [3;16;52], ionic channel blockers (ENaC blockers) [16;29;52-56], mucolytic agents (DNAse) [3;16] and mechanical actions (device assistance or breathing exercises) [16;57]. All these treatments will act on mucus and help to restore the mucociliary clearance.

Research to **restore the function** of the chloride channel has led to the development and the commercialization of different molecules acting as "CFTR potentiators" or "CFTR correctors".
The first one helps chloride flow through the CFTR. The main representative is ivacaftor [3;16;19;58]. The second class, represented by lumacaftor, helps

the misfolded protein to adopt its correct 3D configuration and improve the trafficking of CFTR mutants to increase the amount of protein expressed in the plasma membrane. However, as they have no potentiator effect, they are always given in combination with a CFTR potentiator [3;16;19;58]. In Belgium, lumacaftor is approved and commercialized in combination with ivacaftor under the name of Orkambi® [4].

To reduce **inflammation,** the American Cystic Fibrosis Foundation recommends the use of ibuprofen. It reduces the rate of decline in lung function, especially in children and adolescent patients [16;27;29]. The use of oral or inhaled corticosteroids has limited effects in patients and is often accompanied by side effects such as growth impairment; they are therefore not recommended [16;23;27;29].

1.6.1. Anti-infective therapies

Antibiotics can be used in four different manners to treat bacterial infections in patients with CF: prophylaxis, eradication of early infections, control of chronic infections and treatment of exacerbations.

- Prophylaxis

The UK (and EU) guidelines diverge from the US guidelines regarding prophylaxis recommendations. In Europe, guidelines recommend the prophylactic use of anti-staphylococcal antibiotics such as flucloxacillin in order to reduce the risk of infection by *S. aureus* [37;38;40]. However, US guidelines are against the use of prophylactic anti-staphylococcal antibiotics based on studies claiming for an increase of pseudomonal infections due to staphylococcal prophylaxis [39]. This controversial issue stimulated the UK "National Institute for Health Research" to launch in 2016 a randomized trial to assess the use of flucloxacillin as a prophylactic agent for children with CF. The study is still ongoing and all the information can be found on the website of the “National Institute for Health Research” :
https://www.journalslibrary.nihr.ac.uk/programmes/hta/142223#/

- Eradication of early infections

The objective is to promptly treat and eradicate an infection to reduce the risk of evolution to chronicity.

For *S. aureus*, when prophylaxis is failing, the guidelines recommend to continue flucloxacillin treatment and add a second anti-staphylococcal antibiotic for two to four weeks (fusidic acid or rifampicin). After this treatment, two possibilities are envisioned : either the culture is negative and the prophylactic flucloxacillin is continued while the second antibiotic is stopped, or the culture remains positive and intravenous antibiotics are administered, preferentially two antibiotics to which the pathogen is susceptible [40].

For *P. aeruginosa*, as there is no prophylactic therapy, and as it is the pathogen that impacts the most the quality of life and increases the risk of death by 2-3 folds over an 8-year period, it is important to treat this infection as quickly as possible to reduce the risk of chronic infection [40].

The strategies to treat early infection by *P. aeruginosa* may vary between countries but all comprise inhaled and systemic anti-pseudomonal antibiotics. In the US, inhaled tobramycin is the first line treatment whereas in the UK, the first line therapy is based on a regimen of nebulized colistin and oral ciprofloxacin [37;40].

- Infection control

At some point, the infection will become chronic and new strategies have to be implemented to control the infection. To act efficiently, the antibiotics concentration in sputum should be high enough. Sadly however, antibiotics given by oral or intravenous route cannot reach sufficiently high concentrations in the sputum. Yet, research to develop antibiotic formulation administered by inhalation has made great progress over the last years. The use of inhaled antibiotics has shown an interest to reduce the rate of decline of respiratory function in patients chronically infected with *P. aeruginosa* [37;40].

The UK recommendations propose the use of a cyclic approach based on the use of an inhaled antibiotic during one month followed by one month without antibiotic and then repeat. In the UK, the first antibiotic should be nebulized colistin because it achieves low systemic and high local concentration in the lung. The other approved inhaled antibiotics that can be used to treat chronic *P. aeruginosa* infection are tobramycin solution for inhalation, tobramycin dry powder for inhalation, or aztreonam. Oral azithromycin is often added to this treatment although not being active against *P. aeruginosa.* Its interest mainly resides in its anti-inflammatory activity (independent of its bacteriostatic activity), which impacts positively the quality of life [37;40;59].

In Europe, commercialized antibiotics for inhalation (nebulization or dry powder) include colistin, tobramycin, aztreonam, and levofloxacin for the management of chronic infections caused by *P. aeruginosa*. Moreover, vancomycin inhalation powder is currently in phase III according to the Cystic Fibrosis Foundation. [37;40].

- Treatment of exacerbations

Acute respiratory exacerbations are defined as the worsening of symptoms of a disease that lasts for several days. They are treated with two intravenous antibiotics that have different mechanisms of action to reduce potential emergence of resistance and to benefit from any potential antibiotic synergies. The treatment goes on for 14 days and the choice of the antibiotics will depend on the isolated pathogen [37;40].

- Which antibiotic for which pathogen?

In the previous section, we focused on *S. aureus* and *P. aeruginosa* infections but as indicated in section “Microbiology of Lung Disease” there are many other organisms that can be involved in lung infections in CF. Table 2 describes the antibiotics that can be administered to treat infections by the main pathogens encountered in patients with CF, according to the UK cystic fibrosis registry.

1.6.2. Lung transplantation

Lung transplantation is the very last therapeutic approach that can be considered if all the other ones fail to restore the pulmonary function. The recommendations state that lung transplantation is suitable for people (even children in Europe) with a 2-year predicted survival inferior to 50% or (for adults only) if the volume of air that can be blown in the first second (FEV1) is inferior to 30% of lung capacity. With a successful transplantation, patients can hope for a median survival of 10 years. However, the presence of some pathogens like *B. cepacia* complex can affect outcomes and their presence is systematically taken into account when assessing lung transplant candidates [16;23;60].

<table>
<tr><th colspan="4"><u>P. aeruginosa</u>:</th></tr>
<tr><td colspan="2">β-lactams</td><td colspan="2">ceftazidime, meropenem, ticarcillin-clavulanic acid, piperacillin-tazobactam, aztreonam</td></tr>
<tr><td colspan="2">Aminoglycosides</td><td colspan="2">tobramycin</td></tr>
<tr><td colspan="2">Polymyxins</td><td colspan="2">colistin</td></tr>
<tr><td colspan="2">Fluoroquinolones</td><td colspan="2">ciprofloxacin</td></tr>
<tr><td colspan="2">Others</td><td colspan="2">fosfomycin</td></tr>
<tr><th colspan="4"><u>S. aureus</u>:</th></tr>
<tr><td colspan="2">MSSA</td><td colspan="2">MRSA</td></tr>
<tr><td>β-lactams</td><td>flucloxacillin</td><td rowspan="2">Others</td><td rowspan="2">teicoplanin, vancomycin Linezolid</td></tr>
<tr><td>Others</td><td>rifampicin, fusidic acid</td></tr>
<tr><th colspan="4"><u>H. influenzae</u></th></tr>
<tr><td colspan="2">β-lactams</td><td colspan="2">Amoxicillin (sensitive strains), amoxicillin-clavulanic acid, doxycycline, cefaclor, cefixime</td></tr>
<tr><td colspan="2">Other</td><td colspan="2">Chloramphenicol</td></tr>
<tr><th colspan="2"><u>B. cepacia</u> <u>complex</u></th><th colspan="2"><u>S. maltophilia</u></th></tr>
<tr><td>β-lactams</td><td>meropenem, temocillin, ceftazidime</td><td>β-lactams</td><td>ticarcillin-clavulanic acid</td></tr>
<tr><td rowspan="2">Others</td><td rowspan="2">co-trimoxazole</td><td>Tetracyclines</td><td>minocycline, tigecycline</td></tr>
<tr><td>Others</td><td>co-trimoxazole</td></tr>
<tr><th colspan="4"><u>A. xylosoxidans</u></th></tr>
<tr><td colspan="2">β-lactams</td><td colspan="2">meropenem, imipenem, piperacillin-tazobactam</td></tr>
<tr><td colspan="2">Tetracyclines</td><td colspan="2">minocycline</td></tr>
<tr><th colspan="4"><u>NTM</u></th></tr>
<tr><th colspan="2"><u>M. avium</u> <u>complex</u></th><th colspan="2"><u>M. abscessus</u>:</th></tr>
<tr><td>Macrolides</td><td>clarithromycin, azithromycin</td><td>β-lactams</td><td>meropenem, cefoxitin</td></tr>
<tr><td rowspan="2">Others</td><td rowspan="2">rifampicin, ethambutol</td><td>Macrolides</td><td>clarithromycin</td></tr>
<tr><td>Aminoglycosides</td><td>amikacin</td></tr>
<tr><th colspan="4">Fungi</th></tr>
<tr><td colspan="2">Antifungals</td><td colspan="2">voriconazole, amphotericin-B, caspofungin</td></tr>
</table>

Table 2: antibiotics recommended by UK cystic fibrosis registry to treat different microorganisms when causing exacerbations in patients with CF [40].

2. *Staphylococcus aureus* & *Pseudomonas aeruginosa*: From opportunistic to life-threatening pathogens

2.1. *Staphylococcus aureus*

2.1.1. Definition and epidemiology

Staphylococcus aureus is a non-motile Gram-positive cocci-shaped bacterium measuring 0.5-1 µm diameter. *S. aureus* is a facultative anaerobic, able to lyse red blood cells, catalase-positive and coagulase-positive organism, which differentiates it from coagulase-negative staphylococci (like *Staphylococcus epidermidis*) [61].

S. aureus is carried by 30% of people as a skin and nasal commensal and is considered as an opportunistic pathogen associated with hospital-acquired infections in patients with weakened immune system. As a pathogen, *S. aureus* is involved in device-related infections (catheters, prosthetic heart valves, joint replacement, etc.), osteomyelitis, infective endocarditis, skin and soft tissues infections, pneumonia, toxic shock syndrome and it is an important cause of infections in young people with CF [61;62].
S. aureus is a member of the "E**S**KAPE pathogens" group. ESKAPE is the acronym for a group of bacteria, encompassing both Gram-positive and -negative species, made up of *Enterococcus faecium*, ***S**taphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species. They distinguish themselves from other species by developing high levels of antibiotic resistance that involves multiple mechanisms reflecting their ability to escape killing by antibiotics. ESKAPE pathogens are associated with a high risk of morbidity and mortality for patients [63;64].
This ability to easily develop resistance to antibiotics leads to the emergence in the 1960s of a sub-population of *S. aureus* resistant to methicillin (MRSA, see also part 2.1.4. Resistance to antibiotics) making difficult the treatment due to the impossibility to use β-lactam antibiotics (except ceftaroline, a fifth generation cephalosporin) [65].

2.1.2. Virulence factors

S. aureus uses a large number of virulence factors that allow it to adhere and colonize the host tissues, escape the immune system, and cause harmful toxic effects to the host [66].

Adhesins: Adhesins are needed to initiate the colonization process (Figure 6). The microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) are a major class of adhesins that are anchored in the cell peptidoglycan and that bind to different proteins found in connective tissue and blood (fibrinogen, fibronectin and collagens). The archetypal MSCRAMM of *S. aureus* is the clumping factor A (ClfA) that binds the fibrinogen [61;66;67]. Aside from "MSCRAMMs'", other proteins also have an adhesion role like the Extracellular adherence protein (Eap) that binds extracellular matrix molecules but also ICAM-1 (intercellular adhesion molecule-1) receptors present at the surface of endothelial cells [68;69]

Escape the host immunity: To escape immunity *S. aureus* uses different mechanisms (Figure 6). Most of the *S. aureus* possess a thin polysaccharide capsule that can confer resistance to phagocytosis and killing by PMN [61;68;70]. *S. aureus* can prevent opsonization through the production of staphylococcal Protein A that binds immunoglobulins [68].
S. aureus produces staphyloxanthin, a carotenoid pigment responsible for its gold color. This virulence factor acts as an antioxidant capable of neutralizing the reactive oxygen species (ROS) produced by the phagocytes [71].

S. aureus produces and secretes proteins that can form pores in the plasma membrane of cells, causing cell leakage and death. These include alpha-hemolysin (which inserts into the eukaryotic membrane and oligomerizes to form a pore causing cytolysis), Panton-Valentine leukocidin (PVL consists of two proteins that form porins on cell membrane of host cells leading to leak of cell contents and cell death) and chemotaxis-inhibiting proteins of *S. aureus* (CHIPS are secreted proteins that can bind to neutrophil receptors

for chemoattractants) [61;66;68]. *S. aureus* also produces different proteases that interact with the immune system, leading to the cleavage of surface proteins of neutrophils (staphopains A & B), of plasma proteins (V8 protease) and antimicrobial peptides (aureolysin) [69].

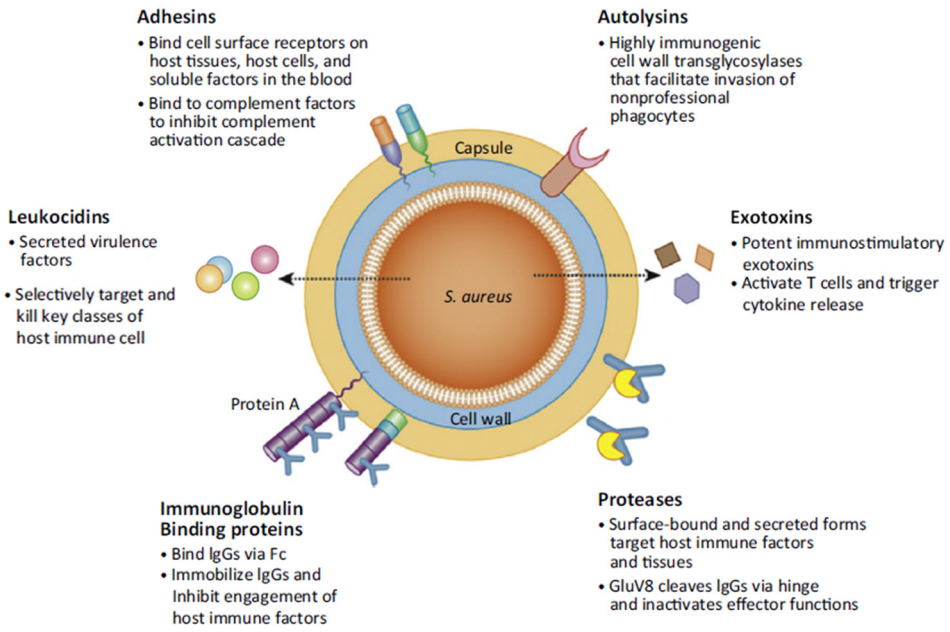


Figure 6: Virulence factors used by *S. aureus* to interact with its host. **Adhesins** promote host colonization. **To escape immunity**, *S. aureus* produces different proteins such as leukocidins, immunoglobulin binding proteins and proteases. **Exotoxins** are secreted toxins (Adapted from [72]).

Exotoxins: Another particularity of *S. aureus* is its capacity to secrete toxins (Figure 6) in its environment, inducing different pathologies like the toxic shock syndrome, the staphylococcal scalded skin syndrome or the staphylococcal food poisoning, which are respectively caused by the toxic shock syndrome toxin-1 (TSST-1), exfoliative toxins A & B (ETA & ETB), and the different staphylococcal enterotoxins [66;69].

Exopolysaccharide: *S. aureus* produces one main exopolysaccharide (Figure 7), the polysaccharide intercellular adhesin (PIA). It is a major component of the extracellular matrix in the development of the biofilm mode of growth (see section 3). PIA is composed of poly-β-1,6-*N*-acetylglucosamine (PNAG), a positively-charged polysaccharide that allows intercellular adhesion of bacterial cells and has important structural functions in the biofilm matrix architecture. It is also implicated in the evasion from host immune-response. Its synthesis is mediated by the proteins encoded by the *icaADBC* operon [73;74].

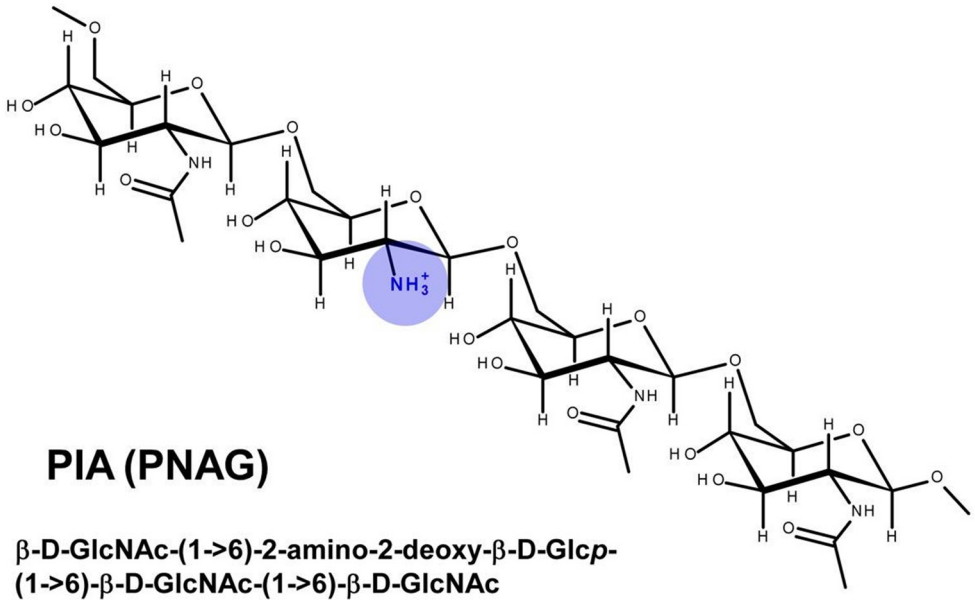


Figure 7: Major exopolysaccharide produced by *S. aureus* composed of poly-β-1,6-*N*-acetylglucosamine (PNAG) approximately one fourth is deacetylated creating a free amino group that is positively charged (in blue) at acidic pH (from [75]).

2.1.3. Quorum Sensing

Quorum sensing (QS) is an intercellular communication system used by bacteria to regulate the expression of a large diversity of genes involved in virulence, motility, and biofilm formation [76]. Quorum sensing is activated when a population size threshold is reached and it is based on self-generated

signal molecules called “autoinducers” [76;77]. The QS systems are binary systems composed of one enzyme, which synthesizes the autoinducer and a receptor, which binds the autoinducer and controls the expression/repression of genes. It is estimated that around 10% of the bacterial genome is under the influence of QS [77].

S. aureus possesses two QS systems, namely the agr and LuxS systems (Figure 8).

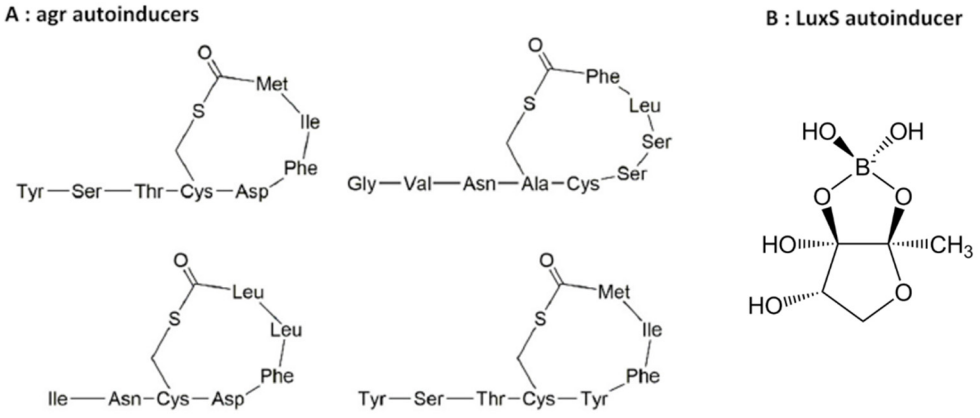


Figure 8: Chemical structures of the molecules composing the QS systems from *S. aureus*. (A) The 4 different forms of autoinducing peptides (AIP) from agr QS system. (B) Autoinducer 2 from LuxS QS system. (Adapted from [78]).

<u>The accessory gene regulator (Agr) system</u> is probably the most important QS systems of *S. aureus* (Figure 9). Agr system positively regulates the escape from host immunity (alpha-hemolysin, ETA, leukocidin, etc.) and production of toxins (TSST-1 and some enterotoxins) but it also negatively regulates the MSCRAMM (staphylococcal protein A, fibronectin binding protein) and biofilm formation.

The Agr system consists of 4 different peptides called “the autoinducing peptides” (AIPs), which can be 7 to 9 amino acids in length and contain a thiolactone ring (Figure 8).

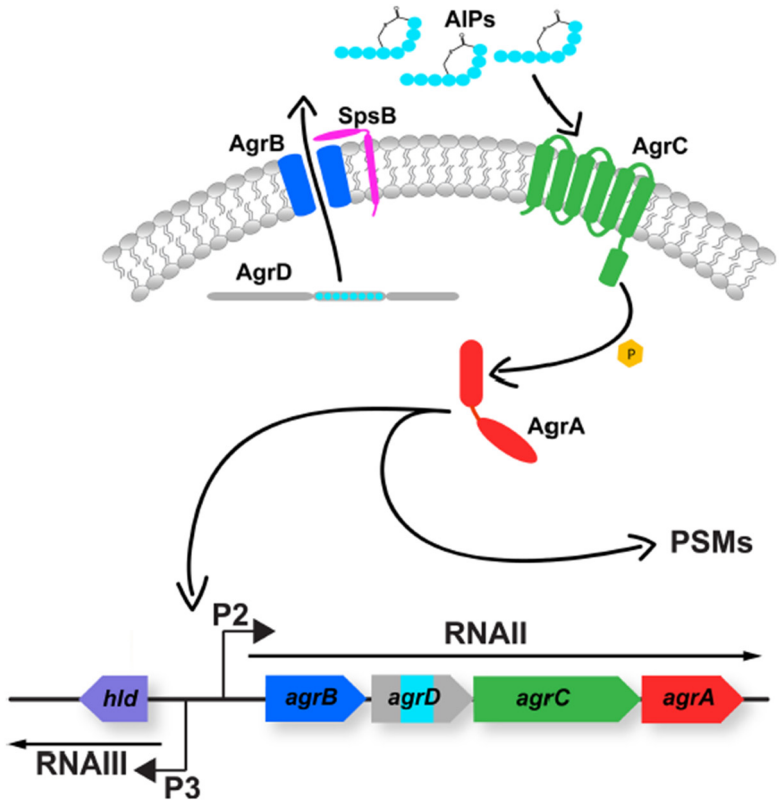


Figure 9: The *agr* operon is constructed around 2 transcriptional units, RNAII and RNAIII. RNAII encodes four genes, a*grB, agrD, agrC* and *agrA*. The gene *agrD* encodes the AgrD protein which is the precursor peptide of the autoinducer AIP, AgrD is maturated and exported by AgrB, encoded by *agrB* gene of the operon, outside of the cell. Once outside of the cell, AIP is linking AgrC protein, a surface receptor encoded by *agrC*, which phosphorylates AgrA, the response regulator. AgrA is encoded by *agrA* gene of the *agr* operon. AgrA activates RNAII and RNAIII (positive feedback loop) and the transcription of the *phenol-soluble modulins* (PSM: a family of staphylococcal peptide toxins). RNAIII acts as regulatory RNA and also encodes the delta-toxin. As a regulatory RNA, RNAIII regulates multiples genes like the MSCRAMMs [77;79-81]. (image from [79])

The second QS system is the LuxS system. It consists of the autoinducer 2 (AI-2), a furanosyl borate diester (Figure 8). AI-2 regulates the capsule synthesis, biofilm formation (through the production of IcaR), antibiotic susceptibility and virulence [78;80;82;83].

2.1.4. Resistance to antibiotics

S. aureus is part of the "ESKAPE pathogens" group due to its high level of antibiotic resistance. The resistance to antibiotics in *S. aureus* is mainly mediated by antibiotic inactivating-enzymes, modified protein targets and efflux pumps (Table 3). Most of these resistance mechanisms are acquired through horizontal gene transfer of mobile genetic elements (MGE). MGE regroup plasmids, transposons, bacteriophages and staphylococcal cassette chromosomes (SCC) [84]. The most known SCC is the staphylococcal cassette chromosome *mec* (SCC*mec*) coding for an alternative penicillin binding protein (PBP), the PBP2a. PBP2a has a lower affinity for β-lactams than other PBPs. This lower affinity confers resistance to the entire class of β-lactam antibiotics. *S. aureus* strains carrying this chromosomal cassette are regrouped under the name of methicillin resistant *Staphylococcus aureus* (MRSA). Today, the designation MRSA indicates resistance to all β-lactams antibiotics, including cephalosporins and carbapenems. However, new β-lactams with activity against MRSA have been discovered and brought on the market, among which the fifth generation cephalosporins ceftaroline (available in Belgium) and ceftobiprole (not available in Belgium) [64;65;85;86].

In CF adults infected by *S. aureus*, MRSA can represent up to 30% of all *S. aureus* isolates [5].

Class of antibiotic	Mechanism of the resistance	Consequence
β-lactams	β-lactamase enzymes	Hydrolysis of β-lactams
	Alternative penicillin binding protein 2a (PBP2a)	Lower affinity for β-lactams than normal PBP
Fluoroquinolones	Mutations in the subunit ParC of topoisomerase IV or in the subunit GyrA of DNA gyrase	Reduction of binding to the target
	Overexpression of efflux pumps	Reduction of accumulation within the bacteria
Aminoglycosides	Aminoglycoside modifying enzymes	Inhibition of binding to the target
Glycopeptides	Thickening of cell wall	Reduction of glycopeptides penetration in the cell wall
	Alternative synthesis of peptidoglycan precursor	Production of precursors with lower affinity for glycopeptides
Oxazolidinones	Methylation of ribosomal subunit 50S	Reduction of binding to the target
	Ribosomal 23S RNA mutation	Reduction of binding to the target
Macrolides	Methylation of ribosomal 23S RNA subunit	Inhibition of binding to the target
Ansamycins	RNA polymerase mutation	Inhibition of binding to the target

Table 3: Most common causes of resistance to classes of antibiotics given to patients suffering with CF for the treatment of *S. aureus* exacerbations [64;84;86;87].

2.2. *Pseudomonas aeruginosa*

2.2.1. Definition and epidemiology

Pseudomonas aeruginosa is a highly versatile, motile Gram-negative rod-shaped bacterium measuring 0.5-0.8 µm wide and 1.5-3.0 µm long. This bacterium mainly uses aerobic respiration but it is also capable of anaerobic respiration on nitrate [88]. *P. aeruginosa* is a ubiquitous microorganism that can be found in a large variety of places like soil, marine habitats, plants, fruits, vegetables and more rarely as commensal on human skin and gastrointestinal tract. The high versatility of *P. aeruginosa* allows it to grow on medical devices as surgical instruments and catheters [89].

P. aeruginosa is considered as an opportunistic pathogen associated with hospital-acquired infections in patients with weakened immune system. It commonly causes hospital-acquired pneumonia (including ventilator-associated pneumonia), bloodstream, skin and soft tissues, ear, eye and urinary tract infections. Beside hospital-acquired infections, *P. aeruginosa* also causes chronic infections in people with CF and is responsible for severe exacerbations [90]. As previously mentioned in paragraph "**1.5.2. Adulthood**", *P. aeruginosa* is the main pathogen found in adults where it can be isolated in around 70% of the patients.

As *S. aureus*, *P. aeruginosa* is a member of the ESKA**P**E pathogens because of its ability to resist to antibiotic treatments. *P. aeruginosa* deploys different strategies to resist to antibiotic therapies [63;64].

2.2.2. Virulence factors

P. aeruginosa produces numerous virulence factors (Figure 10) that will interact with the host immune system and modulate the host response. These interactions will modulate the severity of the infection by influencing the rate of bacterial clearance as well as by causing collateral damage to the host tissues [89]. The virulence factors can be divided into two categories depending on whether they are present at the surface of the bacteria or secreted by the bacteria.

2.2.2.1. Surface virulence factors

Flagella: *P. aeruginosa* possesses a polar flagellum which is critical for its motility through a screw-like motion in liquids (swimming) and in highly viscous environments or along surfaces (swarming) [91]. Flagellum presence is necessary for the initial infection in patients with CF and it is capable to cause an inflammatory response. However, in more chronic stages of the infection in CF, around 40% of *P. aeruginosa* isolates are found without flagellum showing that *P. aeruginosa* is capable of turning off the expression of flagellum and select aflagellar mutants to evade host response [89;92-94].

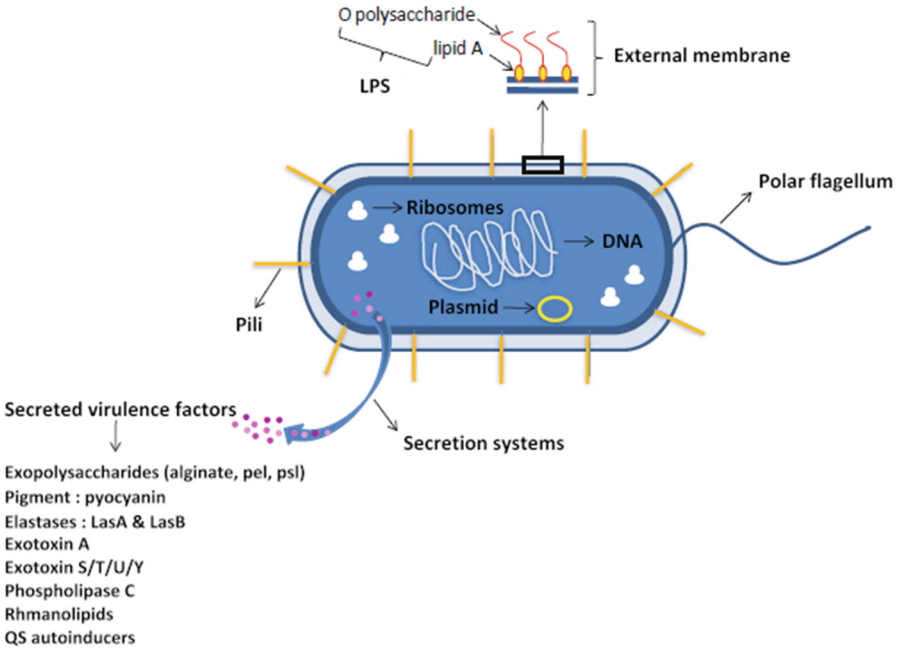


Figure 10: *Pseudomonas aeruginosa* with its virulence factors: polar flagellum, pilus, LPS, secreted factors (adapted from [95]).

Type IV pili: Pili are small filamentous appendages present at the surface of *P. aeruginosa*. Generally their main function is adherence but in the case of type IV pili, they can also provide a particular motility to *P. aeruginosa* called "twitching" due to their retractile properties. Twitching allows *P. aeruginosa* to spread along hydrated surfaces of moderate viscosity [92;96]. Type IV pili are involved in early colonization of the airways, they can initiate an

inflammatory response and they are also involved in the formation of microcolonies, one of the early stages of biofilm formation (see **part 3. Biofilms**) [89;92;96].

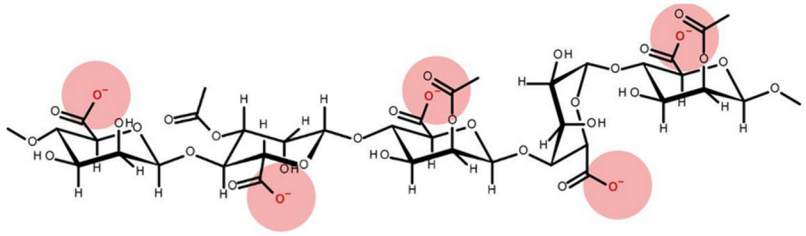
Lipopolysaccharides (LPS): LPS is a glycolipid located at the outer face of the outer membrane and it is part of the cell wall. It is considered as the major virulence factor of *P. aeruginosa*. LPS is composed by a hydrophobic domain (Lipid A) inserted in the phospholipid bilayer and a hydrophilic tail composed of the core polysaccharide and the O-antigen. The O-antigen is highly variable and immunogenic and can elicit a strong humoral response. The role of LPS is to give bacteria a protection against foreign molecules, especially those which are positively-charged (LPS being negatively-charged) or lipophilic [97]. During chronic infection, LPS undergoes important adaptive changes like specific Lipid A modifications, which give resistance to host antimicrobials, or loss of O-antigen, which promotes immune evasion [92;94;97].

2.2.2.2. Secreted virulence factors

Exopolysaccharides: *P. aeruginosa* is able to produce and secrete three different polysaccharides. They are all three involved as major components of the extracellular matrix in the development of the biofilm mode of growth that will be addressed later in this manuscript (See **part 3. Biofilms**) [75;98].

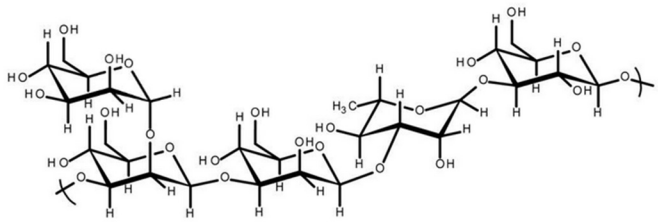
Alginate: Alginate is an anionic acetylated polymer composed of monomers of β-1,4-linked D-mannuronic acid and its epimer α-L-guluronic acid (Figure 11). Alginate has multiple functions. It can act as an adhesin and in CF lungs, it protects bacteria against the reactive oxygen species (ROS) produced by the recruited PMN and provides protection against phagocytic clearance. Alginate plays a role in the maturation of the biofilms in low water conditions, which is the case with poorly hydrated mucus found in CF lungs. Furthermore, most strains isolated in patients with CF are considered as mucoid due to an overproduction of alginate. This overproduction of

alginate increases the protective effects conferred to *P. aeruginosa* leading to higher resistance to antibiotics used to treat infections [92;94;98;99].



Alginate

β-D-ManUA-(1->4)-3-O-acetyl-β-D-ManUA-(1->4)-2-O-acetyl-β-D-ManUA-(1->4)-β-L-GulUA-(1->4)-2-O-acetyl-β-D-ManUA



Psl

[->3)-β-D-Man*p*-(1->3)-β-D-Man*p*-(1->3)-α-L-Rha*p*-(1->3)-β-D-Glc*p*-(1->]n-

2
↑
1
α-D-Man

Figure 11: 2 exopolysaccharides produced by *P. aeruginosa.* Alginate is composed of monomers of β-1,4-linked D-mannuronic acid and its epimer α-L-guluronic acid. It is negatively-charged (in red). Psl is a pentasaccharide composed of D-mannose, D-glucose and L-rhamnose (from [75]).

Polysaccharide synthesis locus (Psl): Psl is another exopolysaccharide produced by non-mucoid strains of *P. aeruginosa*. This exopolysaccharide is a neutral pentasaccharide repeat containing D-mannose, D-glucose and L-rhamnose (Figure 11). As alginate, Psl plays a role in the attachment of bacteria to surfaces and other cells, it also acts as a scaffold to help maintain biofilm structure and protects bacteria from antibiotics and phagocytic cells [75;94;98;100].

Pellicle (Pel): Pel is the last exopolysaccharide produced by non-mucoid strains of *P. aeruginosa*. This exopolysaccharide is probably composed of the amino sugars *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc) and their de-*N*-acetylated forms, galactosamine and glucosamine. The presence of deacetylated forms of GalNAc and GlcNAc will give to this exopolysaccharide a positive charge but the correct organization is still not known [101]. Pel is involved in the formation of biofilm at the air-liquid interface, it plays a role in the interactions between bacteria in the biofilm and gives protection against antibiotics [98;101].

Pyocyanin: Pyocyanin is a phenazine-derived pigment produced by *P. aeruginosa*. This pigment gives to *P. aeruginosa* colonies their characteristic blue-green color. Pyocyanin is a major virulence factor of *P. aeruginosa* not only against eukaryotic cells (pyocyanin acts as a proinflammatory molecule by stimulating the recruitment of PMN at the site of infection) but also against prokaryotic cells (pyocyanin can inhibit the growth of *Escherichia coli* by interacting with the respiratory chain). Among its functions, pyocyanin also works as a signaling molecule during the stationary growth phase of *P. aeruginosa* (including modulation of efflux, redox processes and iron acquisition genes) and it is also able to generate reactive oxygen species. [92;102;103].
In CF, pyocyanin acts against *S. aureus* inhibiting its respiratory chain and thus its growth [103;104].

Elastase B (LasB): LasB is the best characterized elastase from *P. aeruginosa*. This protease is able to cleave numerous proteins such as elastin, collagen type III and IV, tight junctions, components of immune defenses (like cytokines and immunoglobulins), components of the complement system and surfactant proteins like the “collectins” (Protein A and D) that are responsible for the recognition and the binding to oligosaccharides present on the surface of bacteria. Through its broad fields of action, LasB is involved

in inflammation and host immune escape by reducing phagocytosis. In brochoalveolar lavages from patients with CF, a reduction of proteins A and D is observed, which may indicate an infection by *P. aeruginosa* [95;105].

Elastase A (LasA): This elastase is also known as “staphylolysin” because one of its main function is to cleave the glycyl-glycine and glycyl-alanine links in the pentaglycine bonds in the peptidoglycan of *Staphylococcus aureus* and induces its lysis [106]. LasA is less potent to cleave elastin than LasB probably because this protease is only able to cleave selected sites within the elastin molecule but through this restricted specificity, LasA modifies the elastin molecule in order to make it more susceptible to other elastases [95;105-107].

Other important secreted virulence factors: *P. aeruginosa* is a highly versatile bacterium and it produces much more secreted virulence factors among which we can cite alkaline protease, protease IV, phospholipase C, exotoxin A and exotoxin S/T/U/Y.

Alkaline protease (AprA) and protease IV (PIV): These two proteases are important secreted virulence factors because AprA and PIV are able to degrade a large number of host proteins including interleukins (IL-6 for AprA and IL-22 for PIV), fibrin, antibodies and complement proteins. All these put together gives to *P. aeruginosa* the ability to escape phagocytosis [95;108].

Phospholipase C: This enzyme targets eukaryotic membrane phospholipids and has been shown to participate in inflammation of *P. aeruginosa* acute lung injury [92].

Exotoxin A (ExoA): ExoA is an ADP-ribosyl transferase inhibiting protein synthesis and leading to cell death and depressing host response to infection [92;109].

Exotoxin S/T/U/Y (ExoS, ExoT, ExoU, ExoY): This four toxins act, on different levels, by altering membrane integrity of human cells. They are directly injected inside the human cell through the type III secretion systems [93;110].

2.2.3. Quorum Sensing

Quorum sensing systems are widely studied in *P. aeruginosa* and four QS systems are known (Table 4), namely, Las system, Rhl system, Pqs system and Iqs system. Each QS system possesses its own autoinducer, respectively, OdDHL, BHL, *Pseudomonas* quinolone signal (PQS) and integrated quorum sensing signal (IQS) (Figure 12).

System name	Enzyme/Receptor	Autoinducer
Las System	LasI/LasR	*N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL)
Rhl System	RhlI/RhlR	*N*-butonoyl-L-homoserine lactone (BHL)
Pqs System	operon *pqsABCDE*/PqsR	2-heptyl-3-hydroxy-4-quinolone (PQS)
Iqs System	operon *ambBCDE*/IqsR	2-(2-hydroxylphenyl)-thiazole-4-carbaldehyde (IQS)

Table 4: The four quorum sensing systems of *P. aeruginosa*. The enzymes responsible for the production of autoinducer, the receptor that binds the autoinducer and the names of the four different autoinducers of *P. aeruginosa*.

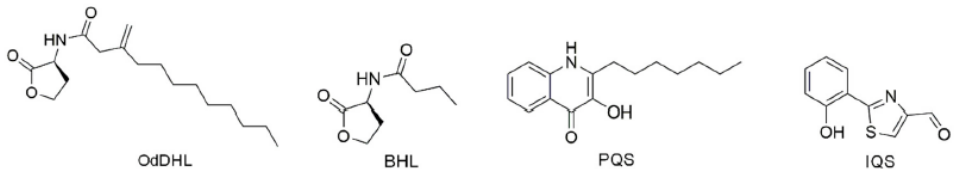


Figure 12: Structure of the four autoinducers from QS systems in *P. aeruginosa* (adapted from [78]).

The four systems are highly interconnected (Figure 13) but the LasI/LasR system oversees all of them [78]. The QS systems control the production of virulence factors (e.g. elastases, pyocyanin, proteases) needed to survive during host invasion in the early stage of infection but also to maintain the infection at later stages [78]. The four systems are implicated in different stages of the biofilm formation (this part will be discuss more in detail in **part 3.2. From planktonic lifestyle to biofilm**).

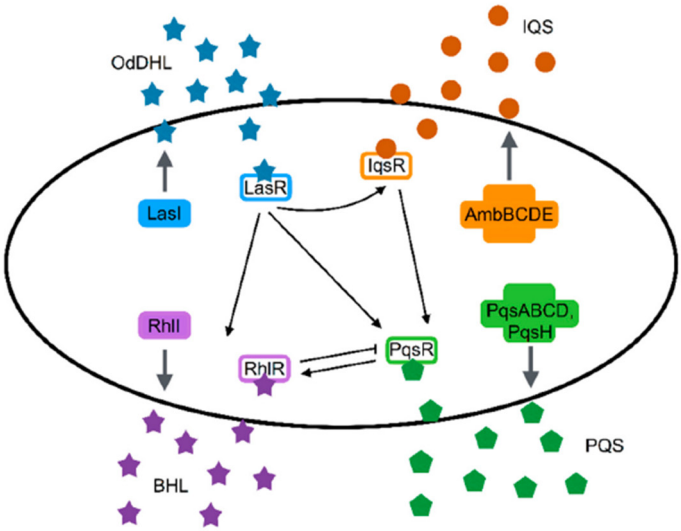


Figure 13: The interconnection of the four quorum sensing systems used by *P. aeruginosa*. All four QS systems positively regulate themselves through positive feedback loops. **Las system** is at the top of QS systems; it positively regulates Rhl, PQS and IQS QS systems and different virulence factors (elastases) [78;102]. **Rhl system** positively regulates virulence factors (pyocyanin, rhamnolipids) but represses PQS system [78;102;111;112]. **PQS system** regulates virulence factors (pyocyanin and HQNO, an intermediate molecule in the pathway of PQS that possesses antimicrobial properties and that can inhibit cytochromes) [78;102;113]. **Iqs system** is an alternative QS system that takes over the Las system under phosphate-depletion stress. In this particular condition, Iqs system positively controls the Pqs and Rhl systems and thus all the virulence factors they control [78;113;114] (image from [78]).

In patients with CF and during early infection, *P. aeruginosa* strains have a functional QS system but later, in chronically infected patients, it is frequent to isolate *LasR* mutants. The frequency of *LasR* mutants can represent up to 50% of isolated strains and paradoxically in these strains the Rhl system is still active and it stimulates the production of Rhl-dependent virulence factors [111;112;115]. It has been shown that in patients with CF, Iqs system can take over QS systems, in the absence of Las system, because of the low phosphate condition that can be found in CF lungs [112-114;116].

2.2.4. Resistance to antibiotics

The versatility of *P. aeruginosa* also comes from its intrinsic resistance to antibiotics through the **low permeability of the outer membrane**, the constitutive **expression of efflux pumps** and the **production of enzymes that inactivate the antibiotics** (Table 5). On top of that, *P. aeruginosa* can acquire resistance mechanisms to other classes of antibiotics (Figure 14). Because of all the previously mentioned mechanisms, it is not rare to isolate *P. aeruginosa* strains that possess resistance to one or more antibiotics classes. By definition, a strain is considered as multidrug-resistant (MDR) when it is non-susceptible to at least one agent in three or more antibiotic classes [117]. In CF adults infected by *P. aeruginosa*, MDR-PA can represent up to 20% of all *P. aeruginosa* isolates [5].

As described in section "1.6.5. Anti-infective therapies", *Pseudomonas aeruginosa* is mainly treated with 4 classes of antibiotics: β-lactams, fluoroquinolones, aminoglycosides and polymyxins [40;118].

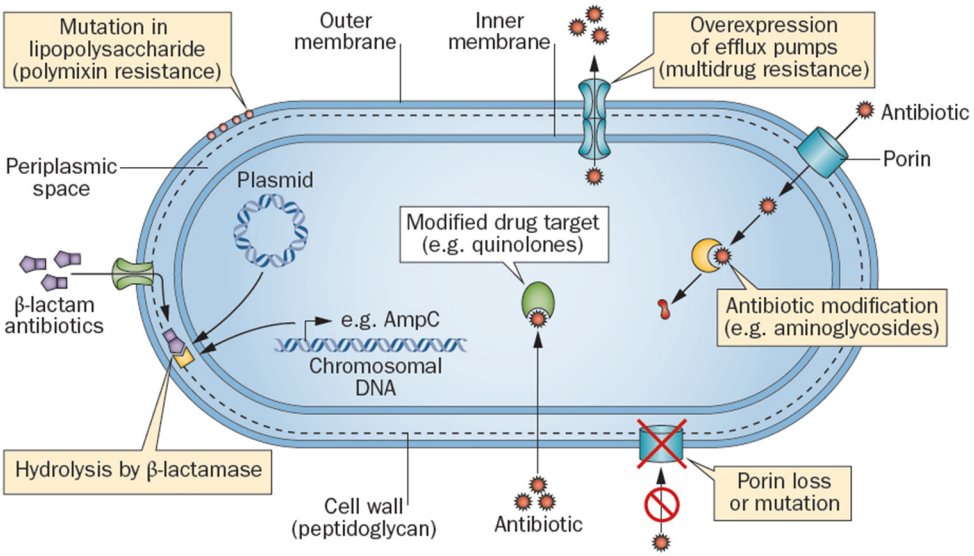


Figure 14: Antibiotic resistance mechanisms in *P. aeruginosa.* The main mechanisms of resistance are represented in this figure such as β-lactams hydrolysis, overexpression of efflux pumps, antibiotic-modifying enzymes, drug-target mutations and outer membrane modifications (mutations in LPS and porins) (adapted from [119]).

Class of antibiotic	Mechanisms of resistance	Consequence
β-lactams	Mutation in porins	Modification of membrane permeability
	Active efflux through pumps (MexAB-OprM & MexCD-OprJ)	Reduction of accumulation within the bacteria
	β-lactamases[1]	Hydrolysis of β-lactams
Fluoroquinolones	Underexpression of porins	Modification of membrane permeability
	Overexpression/mutations of efflux pumps (MexAB-OprM & MexEF-OprN)	Reduction of accumulation within the bacteria
	Mutation in the subunit ParC/ParE of topoisomerase IV or in the subunit GyrA/GyrB of DNA gyrase	Reduction of binding to the target
Aminoglycosides	Underexpression/mutations of porins	Modification of membrane permeability
	Overexpression of efflux pumps (MexXY-OprM & MexEF-OprN)	Reduction of accumulation within the bacteria
	Methylation of ribosomal 30S subunit	Reduction of binding to the target
	3 different aminoglycoside modifying enzymes	Modification of the structure of the antibiotic
Polymyxins	Modification of the lipid A moiety of the LPS	Decrease of the binding of polymyxins

Table 5: Most common causes of resistance to classes of antibiotics given to patients suffering with CF to treat *P. aeruginosa* exacerbations [64;118;120-123]

[1]: The production of β-lactamases is the most important mechanism of resistance and inhibitors (such as clavulanate, tazobactam and avibactam) have been developed in order to overcome this resistance. The combination of a β-lactamase inhibitor and a β-lactam (as for example: ticarcillin-clavulanate, piperacillin-tazobactam, ceftazidime-avibactam) has increased the efficacy of this class of antibiotics against *P. aeruginosa* [64;118;122].

2.2.5. Adaptation to cystic fibrosis lung

The previous section described the classical antibiotics resistance mechanisms that can be acquired by *P. aeruginosa* but in the context of CF, this pathogen will also display other adaptive mechanisms specific to the environment encountered in the lungs and that are observed in the context of chronic *P. aeruginosa* infections of patients with CF. These adaptive

mechanisms involve the appearance of specific phenotypes that can harbor reduced susceptibility to antibiotics.

The first phenotype is the mucoid phenotype. This phenotype is due to the overproduction of alginate [98]. Mucoid strains are mutated in the gene encoding the negative regulator MucA, producing MucA truncated proteins that lose their negative regulator function [99]. In normal conditions, the role of MucA is to repress the expression of *algU*, encoding the transcription factor that activates the alginate operon. When mutated, *mucA* cannot properly repress AlgU, which leads to the overproduction of alginate and to the mucoid phenotype [99]. The emergence of this phenotype is a key step in the establishment of the chronic lung infection [124]. This overproduction of alginate plays a major role in the biofilm formation by altering its architecture which results in more tolerant biofilms [125;126]. This higher tolerance is clearly observed with tobramycin, which is less active against mucoid strains than non-mucoid strains [125-128] and it is probably due to binding between positively-charged aminoglycosides and negatively-charged alginate [127].

Another specific phenotype of chronic lung infection is the hypermutator phenotype [124;129]. This phenotype is characteristic of strains that have a spontaneous mutation rate increased by 1000-fold compared to wild-type strains [129;130]. This increased rate of mutations is due to stable mutations affecting key genes in DNA repair systems like the mismatch repair system and the 7,8-dihydro-8-oxo-deoxyguanosine system [130-132]. The prevalence of hypermutators in CF isolates has been estimated as ranging between 30% and 60% depending on the geographical regions [129;130]. Interestingly, the prevalence of this phenotype is under 1% in acute infections making it a biomarker of chronic respiratory infections in CF [133;134]. This hypermutator phenotype allows *P. aeruginosa* to rapidly adapt to stressful environments and is thus associated to the development of resistance during antibiotic exposure through point mutations on genes involved in synthesis of antibiotic target proteins (see

table 5). The frequency of antibiotic resistance was found to be two times higher in hypermutable strains than in normal strains [124;130;132].

Other phenotypes are related to loss of functions due to the inactivation of the transcriptional regulator LasR (causing a reduction in the expression of acute virulence proteins), the loss of flagellum expression (to escape host immune response) and the production of a modified lipopolysaccharide. This last modification can be involved in polymyxin resistance [92-94;115;133;135].

3. Biofilms

3.1. Definition and global overview

Bacteria can adopt different types of survival mechanisms to adapt to their environment. In liquid conditions like fresh water, saliva or blood, bacteria have a planktonic mode of growth where they are able to move. On the other hand, when bacteria are attached to surfaces, they adopt a sessile growth mode where they are unable to move [136].

«*Biofilms are a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription»* [137].

The extracellular matrix is mainly composed of exopolysaccharides produced by the bacteria, proteins and extracellular DNA (eDNA), the origin of which can be autolysis of bacterial subpopulation or DNA from dead PMN fighting the infection. The distribution of these three main constituents varies among species [73;137-139]. For example, *P. aeruginosa* and *S. aureus* use different exopolysaccharides as components of the extracellular matrix. *P. aeruginosa* will use Alginate, Pel and Psl when most of the *S. aureus* strains will use PNAG [73;140].

Bacteria that adopt this mode of growth have an advantage over their planktonic counterparts (Figure 15). Biofilm growth mode confers to bacteria an increased tolerance to antimicrobials and human immune defenses.

The ability to escape human immune defenses and avoid phagocytosis is conferred by the thickness of the extracellular matrix [73;137].

Bacteria within the biofilm are 10 to 1000 fold less susceptible to antimicrobials than their planktonic counterparts [141]. Antibiotic tolerance is the ability to survive transient exposure to high concentrations of an

antibiotic. Biofilm tolerance is thought to be achieved through different mechanisms [142;143].

- Antibiotic penetration: It is generally accepted that the biofilm matrix does not inhibit antibiotics diffusion within the biofilm. However, a restricted penetration within the biofilm matrix is observed in some cases and can contribute to biofilms tolerance. The main mechanism proposed for reduce penetration is the binding of antibiotics to the components of the matrix [128;142;144].

- Altered microenvironment: The thickness of the extracellular matrix creates gradients in nutrients, oxygen and pH from top to bottom. The activity of many antibiotics rely on the metabolic activity (cell wall synthesis) and the growth of bacteria (replication). The presence of these gradients can be responsible for the tolerance observed between outer (higher rate of nutrients) and inner (lower rate of nutrients) parts of the biofilm [128;142;144].

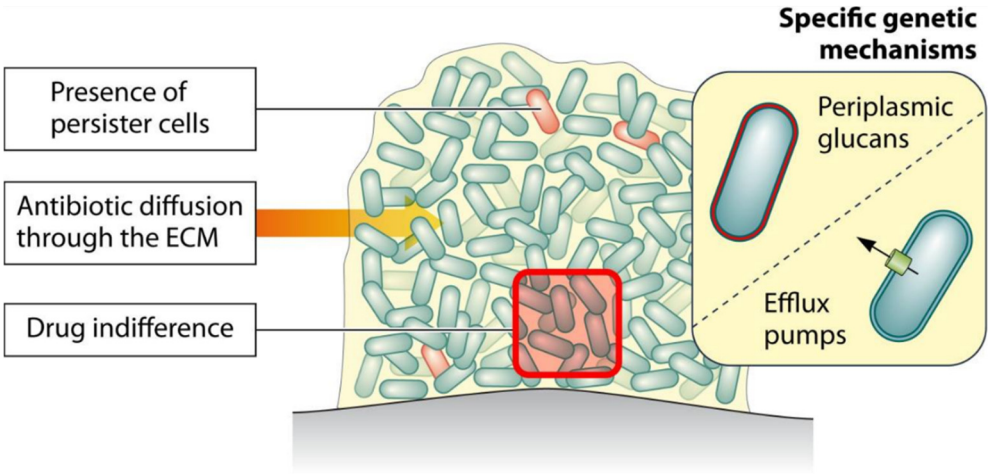


Figure 15: Schematic representation of the protections given by the biofilm growth mode to bacteria composing it: Possible alteration of antibiotic penetration. Drug indifference through gradients of oxygen, nutrients and pH. Genetic mechanisms induced by antibiotic stress and persister cells (from [144]).

- Genetic mechanisms: Antibiotic stress can induce transcriptional adaptation of bacteria. Clinical isolates of *P. aeruginosa* from patients with CF show a high level of production of β-lactamases that can accumulate in the biofilm matrix degrading the β-lactams and reducing its diffusion in the matrix [144;145]. Another genetic mechanism is the production of glucans that are secreted in matrix and can sequester aminoglycosides [144;146]. Efflux pumps could be involved, since *P. aeruginosa* recovered in sputa from patients with CF show higher level of mutation in *mexZ*, the repressor controlling the expression of MexXY-OprM (mainly involved in aminoglycoside extrusion) [118;144].

- A last mechanism of tolerance is the persistence through persister cells that are a subpopulation of bacteria tolerant to antibiotics. They represent less than 1% of the total population composing a biofilm, they do not multiply in the presence of antibiotics, their tolerance is not related to genetic mutations and once they resume growth, they show a non-persister antibiotic tolerance profile [128;143;144;147].

The last benefit of the growth mode is the potential for cellular detachment. Bacteria can detach from the biofilm and spread in the organism to uninfected region of the host, attach and promote new biofilm formation [139].

For all these reasons, biofilms are a major health concern and today it is considered that biofilms account for 80% of the microbial infections in the body [138;141]. Biofilms are found on tissues in valve endocarditis, otitis media, CF and periodontitis but also on medical devices like prosthetic heart valves, central venous catheters, urinary catheters, contact lenses and prosthetic implants (knee joint, hip replacement, etc.) [137].

3.2. From planktonic lifestyle to biofilm: The development stages

Biofilm development is a complex dynamic process that follows 5 steps (i) reversible attachment, (ii) irreversible attachment, (iii) microcolonies formation, (iv) biofilm maturation, (v) dispersion (Figure 16) [73;77;148]. This last stage completes the circle, as dispersed bacteria will spread in the organism to uninfected regions of the host, attach and promote new biofilm formation [139].

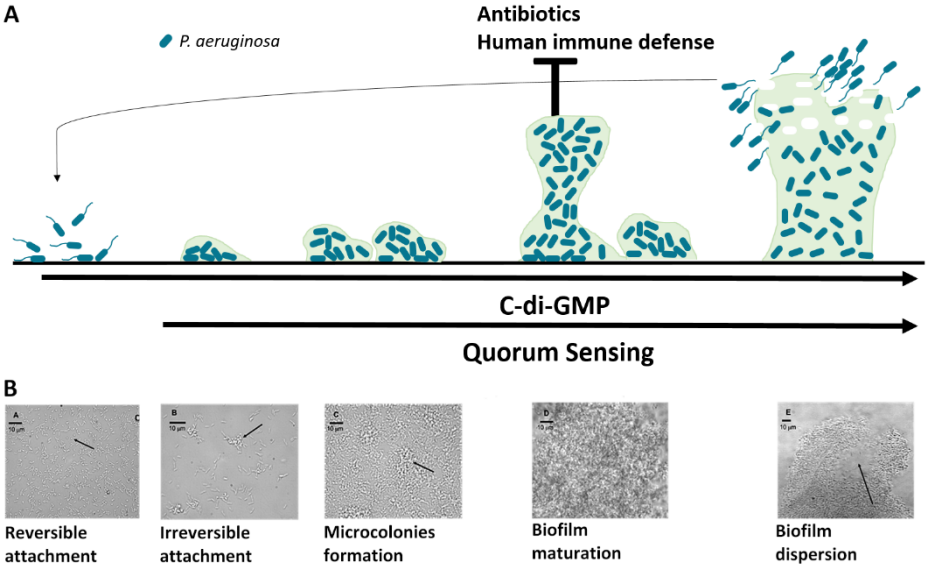


Figure 16: The five stages of *in vitro* biofilm development. (A) Schematic representation of the five stages, they are regulated by the production of c-di-GMP and quorum sensing molecules. (B) The five stages of *P. aeruginosa* biofilm development (adapted from [148]).

The first stage of biofilm development, (i) reversible attachment, is characterized by the switch from planktonic lifestyle to sessile biofilm lifestyle. This switch happens when bacteria arrive next to a surface and undergo a number of physiological, metabolic and phenotypic changes [149]. During this early stage of attachment, quorum sensing molecules are not involved but other molecules are synthetized by the bacteria.

In *P. aeruginosa*, the switch from planktonic to sessile lifestyle is associated to levels of cyclic di-guanosine monophosphate (c-di-GMP), a second messenger recognized as the coordinator of the "lifestyle transition" (Figure 17). Planktonic lifestyle is associated with low level of c-di-GMP while high level of c-di-GMP promotes surface attachment and biofilm formation. C-di-GMP down-regulates bacterial motility, mainly via the flagellar motor (its assembly and its function) [150;151] but the flagella also have an adhesin function that allows *P. aeruginosa* to adhere to surfaces [148]. At the same time, c-di-GMP up-regulates type IV pili and adhesins to facilitate the (ii) irreversible attachment. The c-di-GMP is also involved in the up-regulation of the genes required for the production of the three exopolysaccharides (Pel, Psl and Alginate) that compose the extracellular matrix of the biofilm [150;152].

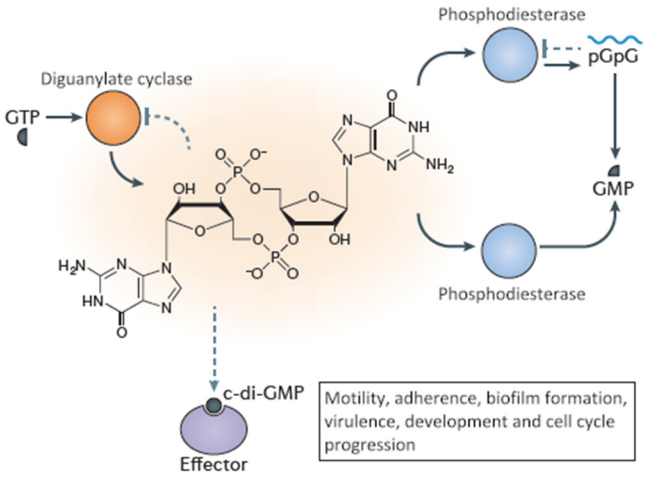


Figure 17: cyclic di-guanosine monophosphate (c-di-GMP) is synthetized by diguanylate cyclase from 2 GTP. C-di-GMP is degraded by two different classes of phosphodiesterases. One class degrades c-di-GMP in 5'-phophoguanylyl- (3'-5 ') - guanosine (pGpG), then pGpG is hydrolyzed into 2 GMP molecules by the oligoribonuclease Orn. The second class is thought to catalyze both reactions (adapted from [151]).

S. aureus does not metabolize c-di-GMP but can respond to it if it is added in the medium. Adding c-di-GMP to the growth medium of *S. aureus* causes a reduction of biofilm formation [153]. However, as a non-motile bacterium,

S. aureus gains contact with a surface passively. Its adhesion to a surface is mainly mediated by the MSCRAMMs proteins that are covalently linked to the peptidoglycan or by the Eap protein that binds extracellular matrix molecules but also ICAM-1 (intercellular adhesion molecule-1) receptors present at the surface of endothelial cells [139].

Thus, at the end of stage (i) reversible attachment, bacteria are attached to surfaces through adhesins and start to secrete polymeric molecule to form the extracellular matrix.

The second stage of development, (ii) irreversible attachment, starts when bacteria are regrouped as clusters and any motility has ceased [139;148].
During this stage, in *P. aeruginosa,* QS starts to be active with the Las systems controlling the expression of virulence factors [148]. The synthesis of exopolysaccharide is still under the control of c-di-GMP [77].

The production of exopolysaccharides for *S. aureus* biofilm is under the control of the staphylococcal accessory regulator (SarA) [154]. This regulator stimulates the *ica* operon responsible for the synthesis of PNAG. Moreover, SarA is known to be up-regulated in biofilms (when compared to planktonic cultures) and inhibits the expression of nucleases and proteases [73;74;77;139;154] . Yet, all *S. aureus* strains do not possess the *ica* operon and they still produce a biofilm considered as “PNAG-independent” (in opposition to biofilm PNAG-dependent formed by strains having the *ica* operon) (Figure 18). This exopolysaccharide-independent biofilm is mainly formed by adhesion proteins like staphylococcal protein A, fibronectin-binding proteins and biofilm-associated proteins. All these proteins will promote cell-to-cell aggregation. Another major component of this biofilm is eDNA produced by the lysis of subpopulation of *S. aureus*. The lysis is mediated by the major autolysin protein (Atl) [73;74;85;139;155].

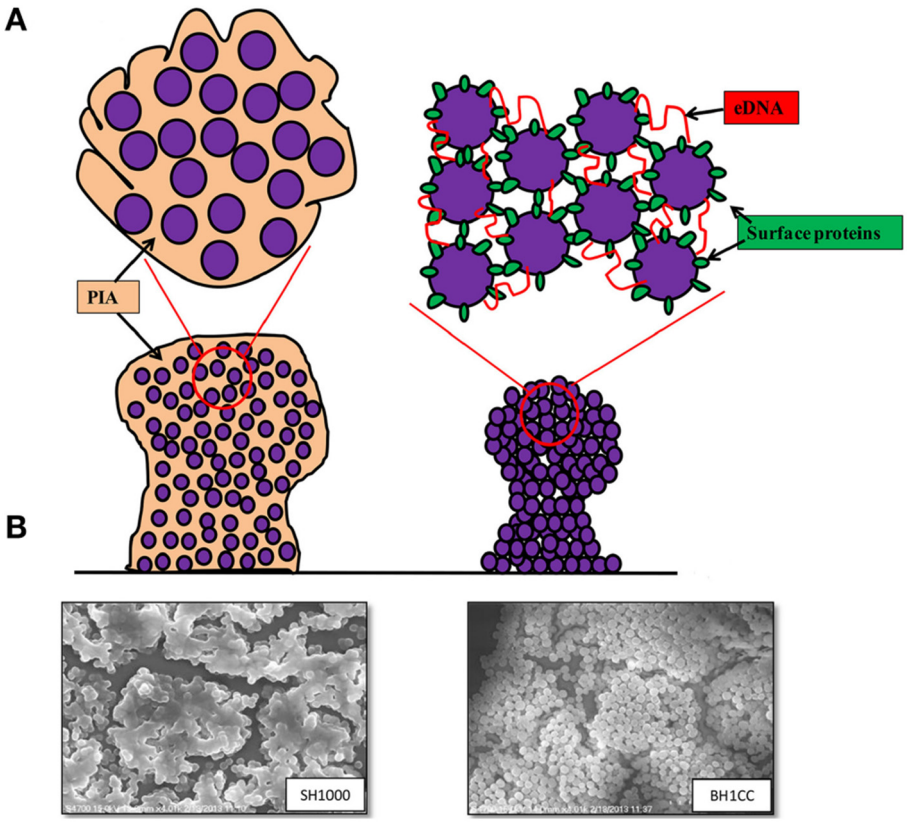


Figure 18: Graphic representation of the two types of biofilms produced by *S. aureus* strains. (A-left) PNAG-dependent biofilm due to the presence of the operon *ica*. (A-right) PNAG-independent biofilm in the absence of *ica* operon. The major components of the biofilm are proteins and eDNA. (B) Examples of PNAG-dependent biofilm (left) and PNAG-independent biofilm (right). Scanning electron micrographs (adapted from [85]).

The third stage of development, (iii) microcolonies formation, is observed when clustered bacteria become progressively layered.

For *P. aeruginosa*, this stage is accompanied by the activation of Rhl system controlling the expression of virulence factors [148]. The synthesis of exopolysaccharides is still under the control of c-di-GMP and the clusters now form microcolonies [77]. Interestingly, during this stage of formation, proteins linked to anaerobic processes seem to be upregulated. This implies

that, during this phase of development, some parts of the biofilm become oxygen-limited (most likely near the attachment surface) [148].

For *S. aureus* (iii) microcolonies formation phase, the determining factor is cell-cell adhesion mediated by production of PNAG and surface proteins [80;139].

The fourth stage is characterized by the maturity of the biofilm (iv); this stage is reached when cell clusters attain their maximum thickness. It is also the stage where sessile bacteria are the most different from planktonic bacteria with respect to the gene expression.
In the case of *P. aeruginosa*, it has been shown that more than 50% of all proteins undergo changes in expression (upregulation) when compared to planktonic bacteria. The majority of these proteins are involved in anaerobiotic processes [148]. During this stage, it is thought that rhamnolipids play an important role by contributing to the formation of internal cavities within the mature biofilm (allowing water and nutrient to reach deeper zones of the biofilm) (Figure 19). Rhamnolipids also play an important role of protection against PMN in patients with CF. They are able to kill PMNs that will release their DNA in the extracellular space. *P. aeruginosa* can utilize the released DNA to enhance its biofilm [111;140].

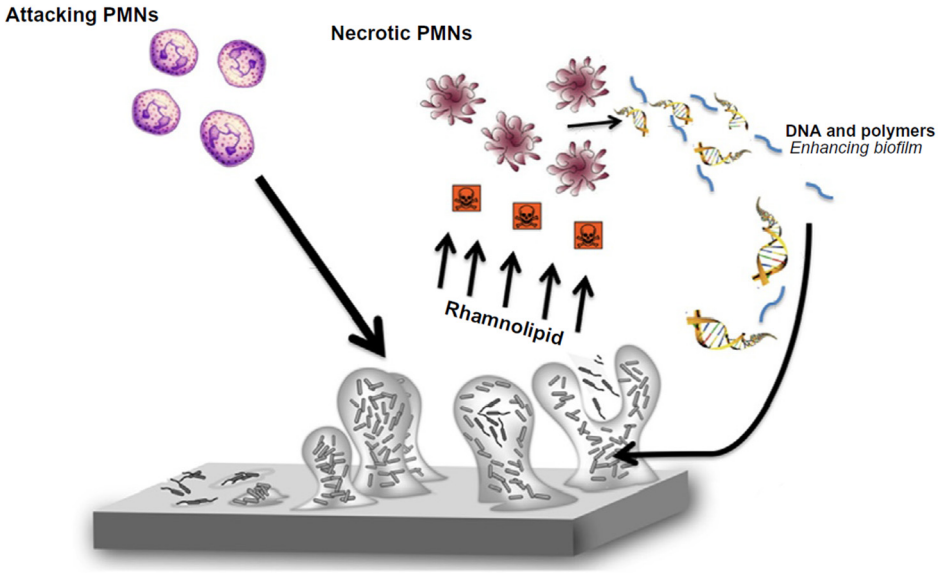


Figure 19: Rhamnolipids protect the biofilm against PMNs, provoking their death. Once they are dead, they will release their DNA in the extracellular space. This eDNA will be absorbed by the biofilm to enhance its thickness (adapted from [111]).

For *S. aureus*, the maturation stage is an equilibrium of adhesive mechanisms and disruptive forces. The adhesive forces are mainly cell-cell adhesion (PNAG, proteins, eDNA). The disruptive forces are mediated by enzymes that are able to degrade the biofilm polymers. Phenol-soluble modulins (PSM) are known to disrupt non-covalent interactions. PSM are amphipathic proteins that have surfactant-like properties and are under the control of the agr system [139;156]. PSM also have cytolysis activity and are able to lyse PMNs and erythrocytes [156].

The last stage is the dispersion (v) of the biofilm. Bacteria leave the biofilm and are able to spread in the organism, adhere and form a new biofilm in an unaffected zone. The dispersion is probably the consequence of multiple factors like degradation of the extracellular matrix, changes in gene expression or changes in environmental conditions.

Dispersed *P. aeruginosa* shows a phenotypic pattern closer to planktonic counterparts (motile bacteria with the expression of the flagellum) than to bacteria from mature biofilm stages (non-motile bacteria) [148]. *P. aeruginosa* biofilm dispersion is probably due to rhamnolipids overproduction [157;158] but it is also linked to Las system which positively regulates the expression of the tyrosine phosphatase TpbA. TpbA is known to negatively act on levels of c-di-GMP which will cause a decrease expression of the *pel* operon and adhesin genes [158;159]. All these modifications will lead to the dispersion of the biofilm by an enhancement of the bacterial motility [158].

The loss of equilibrium between adhesive and disruptive forces is one of the factors that provokes the dispersion of *S. aureus* biofilm. PSM, proteases and nucleases are under the regulation of agr QS system and when they are overproduced they play a role in the disruption of the biofilm [139;158;160]. LuxS QS system also contributes to the dispersion of *S. aureus* biofilm by stimulation of the *ica* repressor IcaR which will negatively regulate the production of PNAG [83;158]

3.3. Biofilms in cystic fibrosis

Despite aggressive antibiotic treatments, CF lungs are permanently infected by different bacteria. Bacterial damages to lungs are reduced by the treatments but the chronic infection is never eradicated. As previously mentioned in section **1.4.1. Lung pathophysiology**, the impossibility to clear bacterial colonization, the permanent inflammation state and the obstruction of the airways by the mucus will result in pulmonary insufficiency and lung damages [5;23].

The first *in vivo* visualizations of biofilm in the context of CF was made by Lam and colleagues in 1980 using electron microscope [161]. They show *P. aeruginosa* surrounded by a “thick fibrous mass of bacterial

exopolysaccharide" (Figure 20) in the alveoli of an explanted lung from a CF person [161].

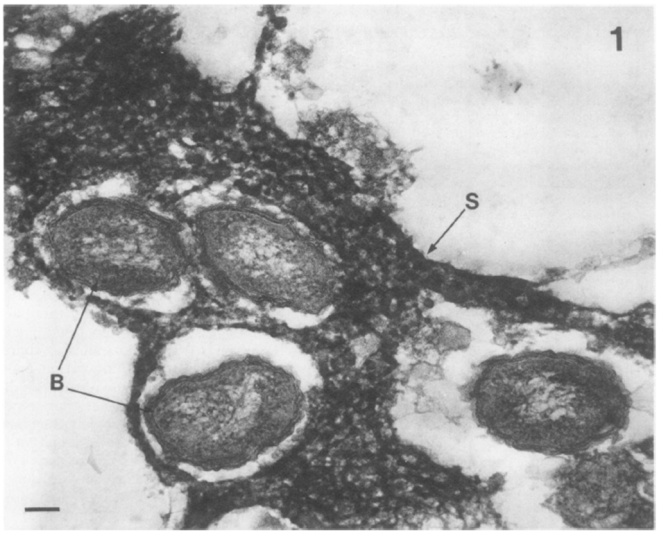


Figure 20: Electron micrograph of a thin section of alveoli from a patient with CF. Alveolar space colonized by *P. aeruginosa* (B) embedded in a "thick fibrous mass of bacterial exopolysaccharide" (S) (from [161]).

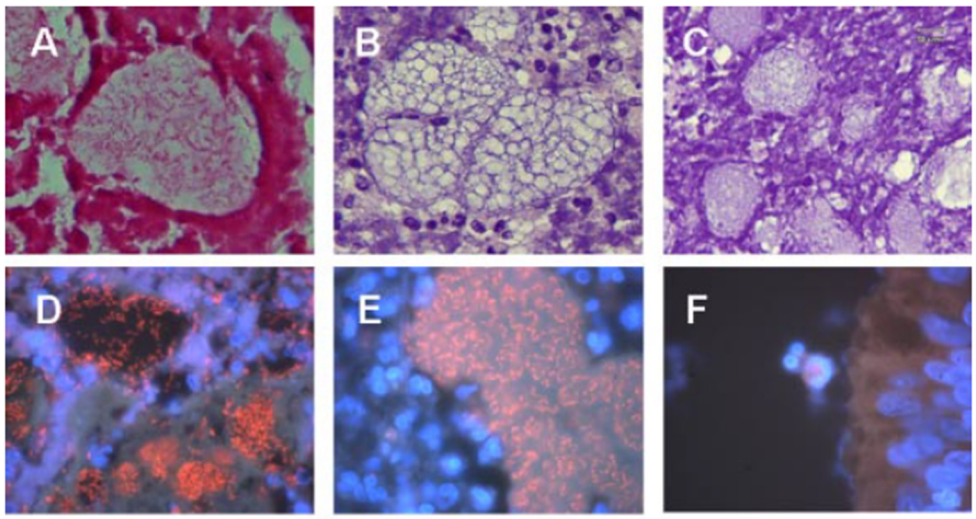


Figure 21: Visualization of *P. aeruginosa* biofilms in the CF lung. (**A**) Bacteria in biofilm within a bronchus visualized using Gram stain. (**B** and **C**) Hematoxylin and eosin staining of bacteria-filled bronchiole. (**D** and **E**) Intraluminal *P. aeruginosa* biofilms surrounded by polymorphonuclear leucocytes visualized using peptide nucleic acid-fluorescence *in situ* hybridization and DAPI. (**F**) Intact bronchi wall (from [162]).

Bjarnsholt and colleagues [162] evaluated the distribution of *P. aeruginosa* in the conductive and respiratory zone of explanted lungs from patients with CF (Figure 21). They made three important discoveries. First they showed that bacteria were more frequently found in the conductive zone (upper part of the lungs: large bronchi) than in the respiratory zone (lower part of the lungs: alveoli) if the patient was highly medicated (if not, they detected bacteria in the respiratory zone: alveolar ducts and sacs). Second, they also showed the presence of PMNs at infection site surrounding the bacterial aggregates in both highly and non-highly medicated explanted lungs. And last but not least, bacteria composing the aggregates were not attached to the epithelial wall, indicating that they grow within the mucus rather than attach to the epithelial cells [162].

In vivo visualization of *S. aureus* biofilm in the context of CF was made in late 90s by Ulrich and colleagues [163] using lung biopsy from three young patients with CF and infected by *S. aureus* for at least 6 years (Figure 22). In their study, they showed that *S. aureus* was detectable in obstructed bronchioli and moreover that *S. aureus* was embedded in the sputum and did not adhere to the respiratory cells [163].

Other species previously described as colonizing the lungs of patients with CF have also been found growing as non-adherent biofilms in their airways like *A. xylosoxidans*, *B. multivorans*, *S. maltophilia* or *M. abscessus*. Non-adherent biofilms of these species have been found in the sputum of patients with CF with chronic lung infection (Figure 23) [164].

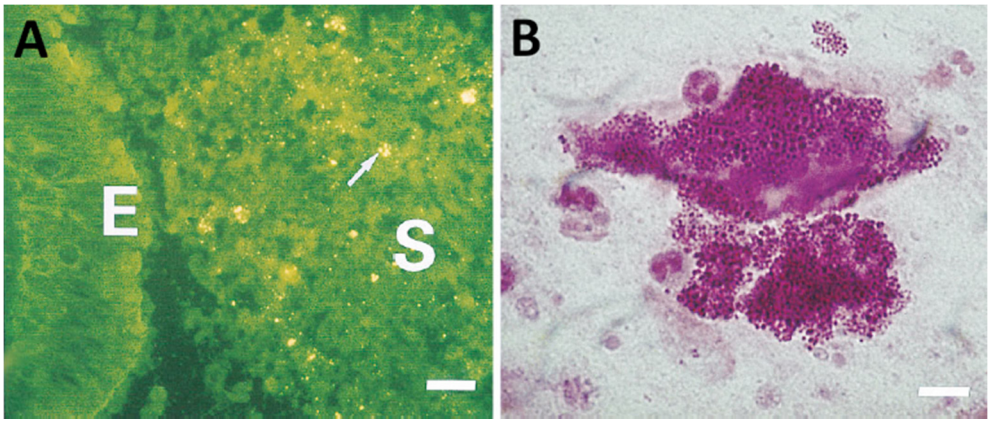


Figure 22: *In vivo* visualization of *S. aureus* biofilms. **(A)** Intraluminal *S. aureus* biofilms embedded in sputum (S), note that there is no adhesion of *S. aureus* to the epithelium (E).Staining by indirect immunofluorescence against teichoic acid. Magnifications: (A) x400; *bar:* 25 µm. **(B)** Gram stain of sputum isolated from the bronchus of a patient with CF showing *S. aureus* clusters. Magnification: x1,000; *bar:* 10 mm (adapted from [163]).

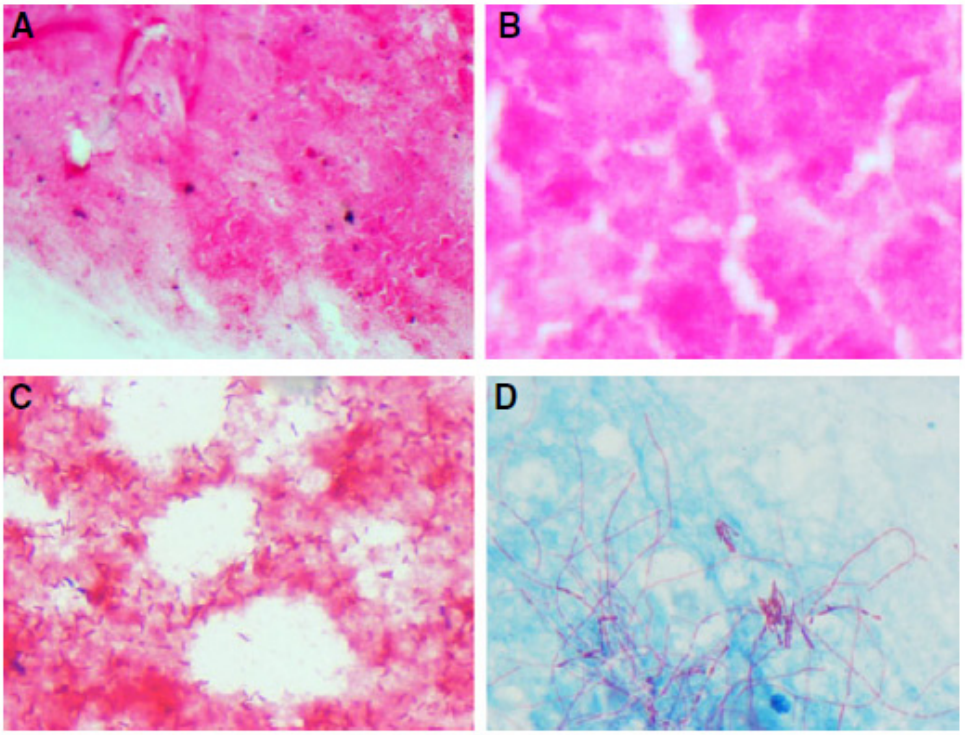


Figure 23: Gram staining of biofilms of *A. xylosoxidans* (**A**), *B. multivorans* (**B**), *S. maltophilia* (**C**) and *M. abscessus* (**D**) in sputum from a patient with CF [164].

4. Methodology

This last part of the introduction will present specific aspects of the methodology used during the experimental work that serves as a basis for the two papers published during this thesis.

4.1. Artificial Sputum Medium

In CF, biofilms grow in the stagnant mucus present in the airways. We therefore developed a medium that mimics the properties of the mucus found in these airways. The composition of our "Artificial Sputum Medium" (ASM) is based on three different papers [165-167] (Table 6). We compared the amount of each ingredient in the media presented in these three papers with the literature describing the content of sputum in mucin (concentration ranging from 10 to 47 g/L; mean 23.7g/L) [168], DNA (from 0.8 to 6.6; mean 3.7g/L) [168;169], amino acids (5.7g/L) [170], or salts (Cl^- 5.5g/L, N^+ 1.9g/L, K^+ 1.15g/L) [169;171;172] as well as it pH value (from 6.3 to 7.7, mean 7) [172;173]. Based on this analysis, we decided to keep all the concentrations as described in the three papers except for mucin. Based on a recent study [168], we increased its concentration from 5g/L to 10g/L.

	Ghani *et al.* [165]	Sriramulu *et al.* [166]	Tavernier *et al.* [167]	Medium developed in the context of this thesis [174]
Name / Compounds	**Artificial sputum medium**	**Artificial sputum medium**	**BHI supplemented**	**Artificial sputum medium**
Mucin	5g/L[a]	5g/L[a]	5g/L[a]	10g/L[a]
DNA	4g/L	4g/L	No	4g/L
L-amino acids	No	5g/L	No	5g/L
DTPA	5.9 mg/L	5.9mg/L	No	5.9mg/L
Salts	NaCl (5g/L), KCl (2.2 g/L)	NaCl (5g/L), KCl (2.2 g/L)	no	NaCl (5g/L), KCl (2.2 g/L)
Tris base	1.81g/L	No	No	1.81g/L
Other	/	/	5% (w/v) bovine serum albumin, 3g/L agar	3g/L agar
Egg yolk emulsion	5mL	5mL	No	5mL
pH	6.9	7	/	7
Sterilization	autoclave	autoclave	/	autoclave

Table 6: Comparative table of the composition media that were used to develop our ASM.
[a]: Origin of mucin: Porcine stomach

Other formulations for artificial sputum do exist (Table 7), like the artificial sputum medium from Kirchner *et al*. [175] and Rozenbaum *et al*. [176] but both are based on Sriramulu *et al.* [166] and do not add anything new to the composition. Two other media that exist are the synthetic CF sputum medium (SCFM) from Palmer *et al*. [177] and a more complete version called SCFM2 from Turner *et al*. [178]. However, none of those groups evaluated the rheological properties of their medium.

	Kirchner *et al.* [175]	Rozenbaum *et al.* [176]	Palmer *et al.* [177]	Turner *et al.* [178]	Diaz Iglesias *et al.* [174]
Name / Compounds	**Artificial sputum medium**	**Artificial sputum medium**	**Synthetic CF sputum medium**	**Synthetic CF sputum medium 2**	**Artificial sputum medium**
Mucin	5g/L	5g/L	No	5g/L	10g/L
DNA	4g/L	4g/L	No	0.6g/L	4g/L
L-amino acids	5g/L	5g/L	2.4g/L	2.4g/L	5g/L
DTPA	5.9mg/L	5.9mg/L	No	No	5.9mg/L
Salts	NaCl (5g/L), KCl (2.2 g/L)	NaCl (5g/L), KCl (2.2 g/L)	NaH_2PO_4 (0.156g/L), Na_2HPO_4 (0.178g/L), KNO_3 (0.035g/L), NH_4Cl (0.122g/L), KCl (1.114g/L), NaCl (3.03g/L) $CaCl_2$ (0.195g/L), $MgCl_2$, (0.06g/L), $FeSO_4$ (0.001g/L)		NaCl (5g/L), KCl (2.2 g/L)
Tris base	No	No	No	No	1.81g/L
Other	/	/	Glucose (0.54g/L), Lactate (0.84g/L)	Glucose (0.54g/L), Lactate (0.84g/L) GlcNAc (0.066g/L), DOPC (0.1g/L)	3g/L agar
Egg yolk emulsion	5mL	5mL	No	No	5mL
pH	6.9	7	6.8	6.8	7
Sterilization	Filtration	Filtration	Filtration	Filtration	autoclave

Table 7: Comparative table of the composition of artificial sputum media.
DOPC: dioleoylphosphatidylcholine

4.2. Rheology study

Rheology is the study of the deformation and flow behavior of materials. An interesting parameter to study is the viscoelastic behavior of artificial media as compared to that of patient's sputum because it possesses specific rheological properties in terms of viscosity (resistance to flow) and elasticity (representing the tendency to recover the original shape after a stress-induced deformation). The physical behavior of sputum is complex as

it can be considered as a non-Newtonian fluid (the viscosity can change to either more liquid or more solid when the fluid undergoes a force), with properties that are between those of a viscous liquid and an elastic solid [179]. Those properties directly impact on the main functions of sputum which are to act as a lubricant, selective barrier, and defense against infection. This explains why a thicker sputum, with higher viscoelastic properties, will be one of the reasons for inadequate mucus clearance, hence facilitating the colonization by opportunistic pathogens [179].

To study the viscoelastic behavior of our culture medium we used the **shear strain amplitude sweep test,** which aims at describing the deformation behavior of samples in the non-destructive deformation range and to describe the upper limit of this range (Figure 24).

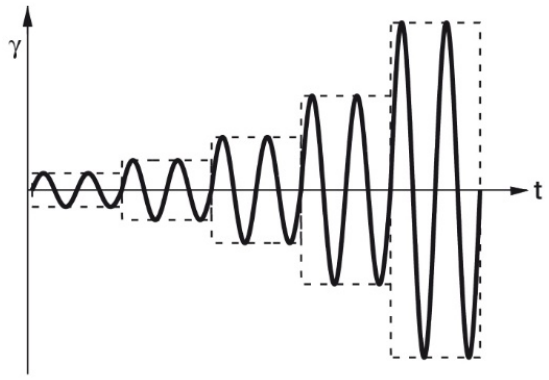


Figure 24: Preset of the shear strain amplitude sweep test: Y axis represents the controlled shear strain (γ) applied to the sample and the increase in the amplitude in five steps through time (X axis: time [t]). Frequency is kept constant at all five measuring points (from: https://wiki.anton-paar.com/en/amplitude-sweeps/).

The results of this test are usually presented as graphics with shear strain plotted on the X axis and elastic modulus (also called storage modulus: G') or viscous modulus (also called loss modulus: G'') plotted on the Y axis (Figure 25). Generally, the values are represented on both axes on a logarithmic scale. The main parameter determined through this kind of graphic (Figure 25) is the limit of the linear viscoelastic region (LVE). The LVE region indicates the range in which the test can be carried out without

destroying the structure of the samples. This region corresponds to the plateau on the left side of the curves on the graphics.

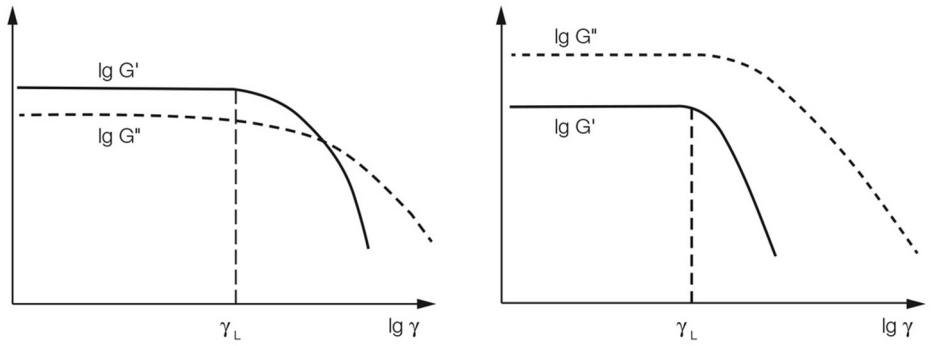


Figure 25: Illustration of two shear strain amplitude sweep tests. The functions of elastic modulus (G', solid curve) and viscous modulus (G'', dotted curve) show constant plateau values within the LVE region (plateau on the left part of the curves). Left panel: elastic modulus G' > viscous modulus G'' in the LVE region for a sample with a gel-like or solid structure. Right panel: viscous modulus G'' > elastic modulus G' in the LVE region for a fluid sample. Axes are in logarithmic scale (Y axis for the modulus G' and G''; X axis for shear strain γ). γ_L corresponds to the end of the LVE of elastic modulus G' (from: https://wiki.anton-paar.com/en/amplitude-sweeps/).

The results obtained when evaluating the viscoelastic behavior of ASM show that our medium (Figure 26) behaves like a gel-like structure (elastic modulus G' > viscous modulus G'' in the LVE region).

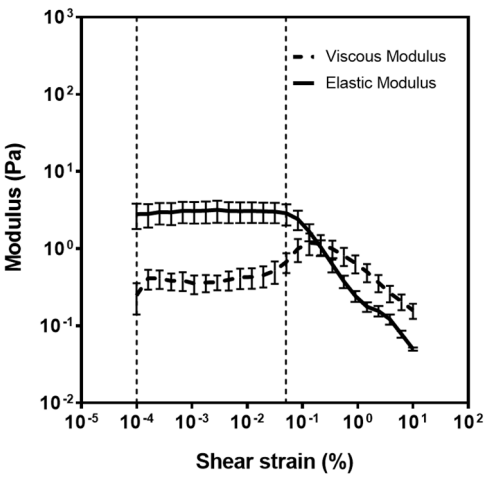


Figure 26: shear strain amplitude sweep test of ASM. These results show elastic modulus (G') and viscous modulus (G'') plotted on Y axis and shear strain on X axis. For ASM, elastic modulus G' > viscous modulus G'' in the LVE region (vertical dotted line), thus the sample has a gel-like structure. This graphic presents the same results (for elastic modulus and viscous modulus) as in figure 1 of paper 1 from this thesis but plotted on the same graphic instead of two distinct graphics.

Chapter II. Objectives

Today, around 80 000 people suffer from CF. Since 1938 and the first paper ever written about this condition, medicine has moved forward in order to better understand the underlying pathophysiology and develop strategies to improve the quality of life of these patients. Nevertheless, this population remains confronted during all its life with bacterial infections and repeated antibiotic treatments. Those bacterial infections appear as biofilms that develop directly in the thick mucus present in the airways. In our studies, we have chosen to study, separately, the most representative pathogens respectively found in children (*Staphylococcus aureus)* and in adults (*Pseudomonas aeruginosa)* and some of the most representative antibiotics frequently used in patients with CF. An important consideration to enhance the relevance of our work was to try to develop an *in-vitro* model that could mimic as well as possible the environment in which these pathogens grow in the lungs of the patients.

In this context, the objectives of this thesis were to study the activity of antibiotics in pertinent *in vitro* models of biofilms by these two pathogens. This implied as a preliminary step the development of an appropriate culture medium to achieve this goal.

Our work was constructed as followed:

The first step (Chapter III, paper 1) was to develop an artificial medium that can be representative of the conditions prevailing *in vivo* in the lungs of the patients. The composition of the Artificial Sputum Medium was set up to be as close as possible of patients' mucus, as described in the previous section. We pushed further its characterization by studying its rheological properties in terms of viscosity and elasticity.

With this tool in hands, our first goal consisted in developing a mono species biofilm model in 96-well plates for *S. aureus* in order to study the activity of

antibiotics commonly administered to patients suffering from *S. aureus* infections (Chapter III, paper 1). To achieve this goal, we started from a model in 96-well plates developed by our team to study *S. aureus* biofilms [180] and adapted it to the use of ASM. As antibiotics, we selected representative molecules among antistaphylococcal agents (vancomycin, linezolid, rifampicin) but also among those that are frequently used in patients with CF, based on their broad spectrum of activity (carbapenems, fluoroquinolones, aminoglycosides) or on their anti-inflammatory effect (azithromycin, which also shows antibiotic activity against *S. aureus*). The activity of antibiotics was studied on pharmacodynamic basis to evaluate parameters like the maximal efficacy (E_{max}), i.e. the effect extrapolated for an infinitely large concentration of antibiotics, and the relative potency (C), i.e. the concentration needed to achieve a specified effect.

Lastly, the second goal (Chapter III, paper 2) consisted in transposing the model we developed for *S. aureus* to use it with *P. aeruginosa* in order to study once again the activity of anti-pseudomonal antibiotics administered to patients. We selected 5 antibiotics frequently used on these patients, namely, the fluoroquinolone ciprofloxacin, the β-lactams ceftazidime and meropenem, the aminoglycoside tobramycin and the polymyxin colistin. The same pharmacodynamic parameters were studied to evaluate the activity of the antibiotics used against *P. aeruginosa* biofilms.

Chapter III. Results

Adapted from paper 1: Activity of antibiotics against *Staphylococcus aureus* in an *in vitro* model of biofilms in the context of cystic fibrosis: influence of the culture medium

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[A specific reference list is provided at the end of this paper]

Abstract

Staphylococcus aureus is a highly prevalent pathogen in the respiratory tract of young patients with cystic fibrosis (CF) and causes biofilm-related infections. Here, we set up an in-vitro model of biofilm grown in tryptic soy broth supplemented by glucose and NaCl (TGN) or in artificial sputum medium (ASM) and used it to evaluate on a pharmacodynamic basis the activity of antibiotics used in CF patients and active on staphylococci (meropenem, vancomycin, azithromycin, linezolid, rifampicin, ciprofloxacin, tobramycin). Rheological studies showed that ASM was more elastic than viscous, as also observed for sputa from CF patients, with elastic and viscous moduli, respectively, similar and slightly lower than those of CF sputa. Biofilms formed by methicillin-sensitive *S. aureus* ATCC 25923 and methicillin-resistant *S. aureus* ATCC 33591 reached maturity after 24 h, with biomass (measured by crystal violet staining) and metabolic activity (assessed by following resazurin metabolization) being lower in ASM than in TGN, and viability (bacterial counts) being similar in both media. Full concentration-response curves of antibiotics obtained after 24h of incubation of biofilms showed that all antibiotics were drastically less potent and less efficient in ASM than in TGN towards viability, metabolic activity, and biomass. Tobramycin selected for small colony variants, specifically in biofilms grown in ASM; the auxotrophism of these variants could not be established. These data highlight the major influence exerted by the culture medium on *S. aureus* responsiveness to antibiotics in biofilms. Use of ASM may help to determine effective drug concentrations or to evaluate new therapeutic options against biofilms in CF patients.

Introduction

Cystic fibrosis (CF) is a recessive genetic disease affecting mainly Caucasian individuals, with around 70,000 cases around the world. The disease results from a malfunction of a chloride channel (cystic fibrosis transmembrane conductance regulator) affecting multiples organs like airways, pancreas, intestines, liver, reproductive tract, and sweat glands and causing in these tissues the accumulation of a viscous, difficult-to-evacuate, mucus. This stagnation is a propitious field for bacterial colonization and biofilm-related infections [1;2]. Biofilms are defined as microbial communities embedded in a self-produced matrix of extracellular polymeric substances (EPS), mainly extracellular DNA, polysaccharides, and proteins [3;4]. This lifestyle protects bacteria against host defense and antibiotics not only through the barrier effect exerted by the biofilm matrix, but also because of metabolic changes affecting bacterial response to antibiotics [4;5].

Staphylococcus aureus is the most prevalent microorganism detected in infants and children suffering from CF, being detected in about 80% of patients aged 10 to 17 years [6]. In chronic infections, *S. aureus* can adopt specific phenotypes, like small colony variants (SCVs), characterized by a slow growth and, therefore, the generation of small colonies on agar plates. Among them, the best characterized are electron transport-deficient SCVs (auxotroph for hemin, menadione, or thiamine) that can be induced by aminoglycoside-treatment, and thymidine-dependent SCVs, mainly recovered after long-term treatment by trimethoprim–sulfamethoxazole in cystic fibrosis patients [7]. Fatty acid-dependent and CO_2-dependent SCVs have also been described [8;9].

S. aureus is well known to form biofilms that contribute to its capacity to cause chronic infections, including in CF patients [4;10-12]. Until now, many studies have mainly concentrated on the activity of antibiotics against *Pseudomonas aeruginosa* biofilms, because they are the most prevalent in the adult CF population [6]. Data are thus critically lacking regarding staphylococcal biofilms.

The aim of the present study was to set up an *in vitro* model of biofilm by *S. aureus* using a medium mimicking the viscoelastic properties of the sputum found in CF patients, and referred to as Artificial Sputum Medium (ASM) [13]. This model was then used to study the activity of antibiotics on a pharmacodynamic basis in order to evaluate their relative potency and maximal efficacy [14]. As antibiotics, we selected representative molecules among antistaphylococcal agents (vancomycin, linezolid, rifampicin), but also among those that are frequently used in CF patients based on their broad spectrum of activity (carbapenems, fluoroquinolones, aminoglycosides) or on their anti-inflammatory effect (azithromycin) [5;15;16].

Results

Rheological properties of ASM

As a preliminary step to this work, we compared the viscoelasticity of Artificial Sputum Medium (ASM) with that of sputa collected from CF patients in order to evaluate the capacity of this medium to mimic, in this respect, the environment faced by microbes in the lungs of CF patients. To this effect, 6 sputum specimens which were previously categorized as purulent (uniformly green or yellow) or mucopurulent (mixture of purulent and mucoid parts) by visual inspection [17]. Their deformation and flow under an applied stress were studied by rheology (Figure 1). Upon application of an increasing shear strain to the samples, we observed that the linear viscoelastic region of CF samples remained steady for both the elastic and viscous moduli between 0.0001% and 0.1% of shear strain. For ASM, stability was observed at shear strain between 0.0001% and 0.1% for the elastic modulus but only at shear strains between 0.0001% and 0.05% for the viscous modulus. In this range of non-destructive shear strains, purulent samples showed higher elastic and viscous moduli than mucopurulent samples, while ASM had an elastic modulus very similar to the values measured for mucopurulent and a viscous modulus about 3 times lower than the values measured for mucopurulent samples. Both CF sputa and ASM were more elastic than viscous.

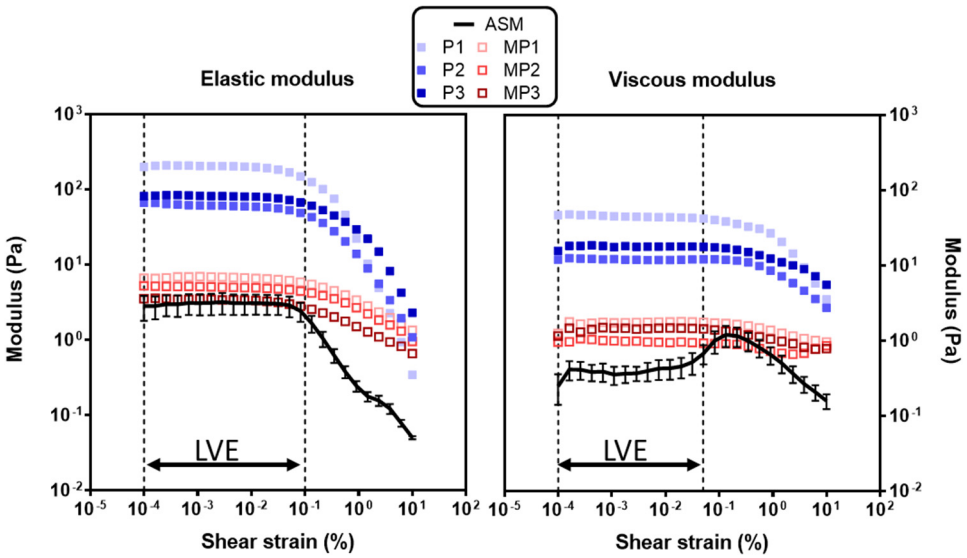


Figure 1. Viscoelasticity of artificial sputum medium (ASM; black curve) as compared to six sputum specimens that were collected from CF patients and showing a purulent (samples P1, P2, P3 [blue closed squares]) or mucopurulent (samples MP1, MP2, MP3 [red open squares]) appearance, shown as individual data. The graphs show the elastic modulus (left) and the viscous modulus (right), both expressed in Pascal (Pa; $kg.m^{-1}.s^{-2}$). A linear viscoelastic (LVE) region is observed between 0.0001% and 0.1% of shear strain.

Kinetics of biofilm development in different media

The kinetics of biofilm development were then studied for two *S. aureus* strains, namely the methicillin-sensitive (MSSA) strain ATCC 25923 and the methicillin-resistant (MRSA) strain ATCC 33591. Two different media were compared, namely, tryptic soy broth supplemented with glucose and NaCl (TGN), previously used for growing staphylococcal biofilms [14;18], and ASM. Figure 2 shows the evolution over time of viability (assessed by CFU counts), metabolic activity (metabolization of resazurin in fluorescent resorufin), and biomass (absorbance of crystal violet) in these biofilms. In both media, both strains grew quickly during the first 12 h to reach a plateau at approximately 10^7 CFU/mL that remained stable over three days (Figure 2, left). Metabolic activity (Figure 2, middle) also increased rapidly over the first 12 h and reached a plateau after approximately 24 h in TGN, with fluorescence signals being higher for ATCC 33591 than ATCC 25923.

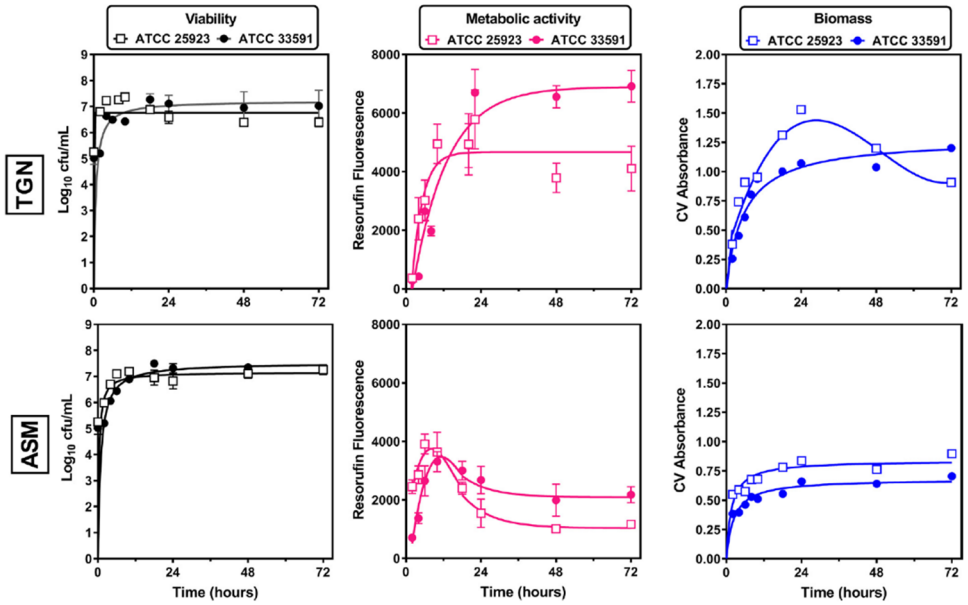


Figure 2. Characterization of the biofilm model in two different media. Evolution over time in Trypticase Soy Broth supplemented with 1% glucose and 2% NaCl (TGN; top) and in artificial sputum medium (ASM; bottom) of viability (assessed by CFU counts in black), metabolic activity (metabolization of resazurin in fluorescent resorufin in pink), and biomass (absorbance of crystal violet in bleu).Medium was changed every 24h. Data are means ± standard deviations (SD) of at least 3 independent experiments. When the error bars are not visible, the SD were smaller than the size of symbols.

In ASM, metabolic activity was lower than that in TGN and similar for both bacterial strains, reaching a maximum after 12 h and decreasing thereafter to reach a plateau at fluorescence values 1/3 of those observed in TGN at 12 h. Biomass also grew quickly over the first 24 h in both media and for both strains (Figure 2, right), with no major change being seen thereafter, except for ATCC 25923 in TGN, for which biomass decreased from day 1 to day 3. Again, absorbance value were higher in TGN than in ASM. Based on these results, we opted to use 24 h biofilms for further experiments in which antibiotics were added for 24 additional hours.

Antibiotic activity against planktonic cultures

Table 1 shows the MICs of the selected antibiotics against the two bacterial strains in the above-described media used for biofilms studies compared to those in cation-adjusted Mueller Hinton Broth (MHB-ca). ATCC 25923 was fully susceptible to all antibiotics tested, while ATCC 33591 was resistant to meropenem, azithromycin, and tobramycin.

In TGN, MICs were similar (± 1 dilution), slightly higher than (2 dilutions for vancomycin against ATCC 25923 and azithromycin against ATCC 33591) or higher than (3 or more dilutions for vancomycin and azithromycin against ATCC 33591 and tobramycin against ATCC 25923) those in MHB-ca. In ASM, MICs were similar (± 1 dilution), slightly higher than (2 dilutions for vancomycin against ATCC 25923 or azithromycin and tobramycin against ATCC 33591) or lower than (for linezolid against ATCC 25923) those in MHB-ca.

Antibiotics	MIC (mg/L)						EUCAST[c] Resistance breakpoint (mg/L)	Human C_{max} (mg/L)[d]
	ATCC 25923			ATCC 33591				
	MHB-ca[a]	TGN[a]	ASM[a,b]	MHB-ca[a]	TGN[a]	ASM[a,b]		
Meropenem	0.06	0.125	0.125	8	16	8	4	50
Vancomycin	1	4	4	1	8	2	2	60
Linezolid	2	2	0.5	1	1	0.5	4	15
Azithromycin	1	1	1	128	512	512	2	0.5
Rifampicin	0.015-0.03	0.0039	0.031	0.03-0.06	0.001	0.015	0.5	18
Ciprofloxacin	0.25	0.25	0.5	0.25	0.25	0.5	1	3.7
Tobramycin	0.25	4	0.5	64	1024	256	1	15

Table 1: Antibiotic susceptibility of bacteria strains in different media

[a] MHB-ca: Muller-Hinton broth cation-adjusted; TGN: Trypticase Soy Broth supplemented with 1% glucose and 2% NaCl; ASM: Artificial Sputum Medium

[b] The MIC determined by CFU counting after plating due to the turbidity of the medium

[c] EUCAST: European Committee on Antimicrobial Susceptibility Testing

[d] based on the Belgian summary of product characteristics for each drug

Antibiotic activity against biofilms

We then evaluated the activity of the same antibiotics against bacterial viability, metabolic activity and biofilm biomass after 24h of incubation in TGN or in ASM.

Results are presented as concentration-response curves in Figure 3A for drugs known to be slowly bactericidal (meropenem, vancomycin), or bacteriostatic (linezolid, azithromycin), and in Figure 3B for drugs that are rapidly bactericidal (rifampicin, ciprofloxacin and tobramycin). Pharmacodynamic parameters, calculated based on the sigmoidal regression of the concentration-response curves, are summarized in Figure 4 for maximal efficacy (E_{max}; corresponding to the maximal decrease in metabolic activity or number of CFU extrapolated for an infinitively large concentration) and in Figure 5 for relative potency (estimated by the concentration needed to reduce the number of CFU by 1 $\log_{10}$ [C_{-1log}]) and metabolic activity or biomass, of 33 % [C_{33}]).

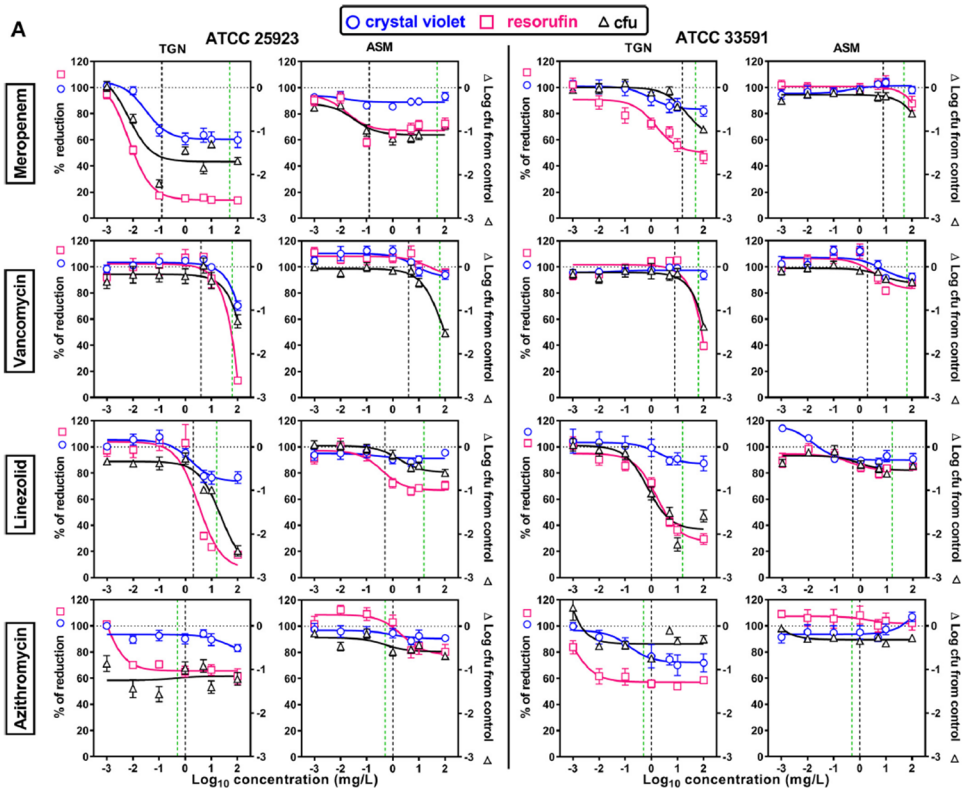
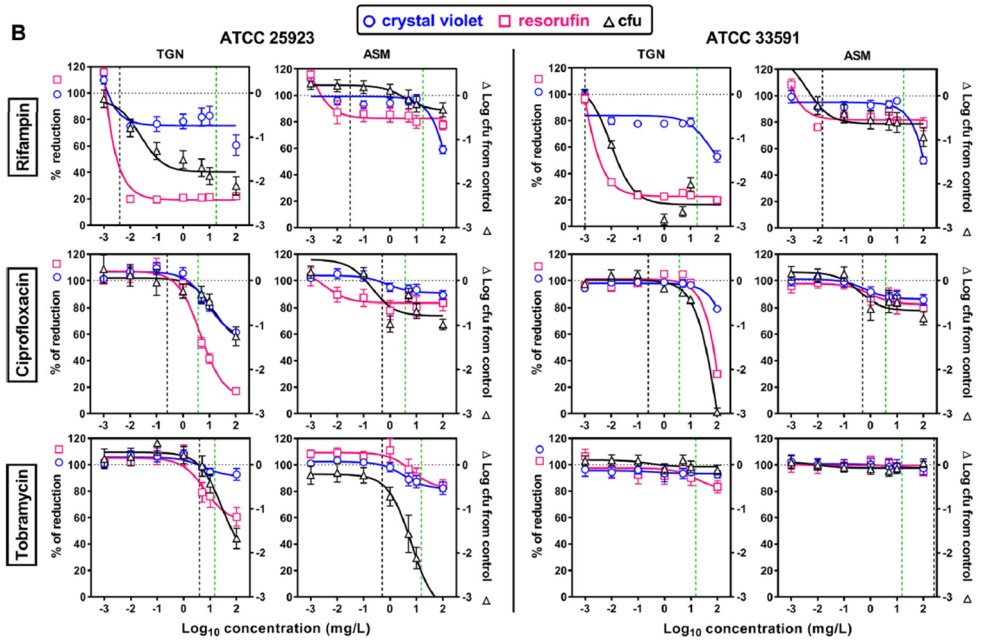


Figure 3. Activities of antibiotics against biofilms. Concentration-responses activity of antibiotics against 24h old-biofilms of strains ATCC 25923 (left) and ATCC 33591 (right) grown in Trypticase Soy Broth supplemented with 1% glucose and 2% NaCl (TGN) or in Artificial Sputum Medium (ASM). Biofilms were incubated with increasing concentration of antibiotics during 24 h (**Panel A**: meropenem, vancomycin, linezolid, azithromycin; **Panel B**: rifampicin, ciprofloxacin, tobramycin). The left ordinate shows the decrease in metabolic activity (resazurin assay, pink open squares) or biofilm mass (crystal violet assay [CV], blue open circles) as a percentage of the control value (no antibiotic present). The right ordinate shows the change in viability (CFU counts, black open triangles) as the reduction (in log scale) from the control value (no antibiotic present). The vertical black dotted line are the MIC of the antibiotic in the corresponding medium and the green dotted line are the human C_{max} after conventional dosing (see Table 1). Note that the MIC of tobramycin against ATCC 33591 is higher than 1000mg/L and, therefore, not visible on the graph. All values are means ± SEM of 3 to 7 independent experiments performed in triplicates (when not visible the error bars are smaller than the size of symbols).



Considering first the results as a whole, all antibiotics showed concentration-dependent effects on both bacterial viability within the biofilm and biomass, with metabolic activity decreasing in parallel with the number of CFU in most of the cases (a 33% reduction in metabolic activity corresponding to approximately 1 $\log_{10}$ CFU decrease). The largest discrepancy between concentration-response curves for viability and metabolic activity was observed with tobramycin in ASM: the decrease in CFU counts was indeed much more marked than the reduction in metabolic activity at high tobramycin concentrations.

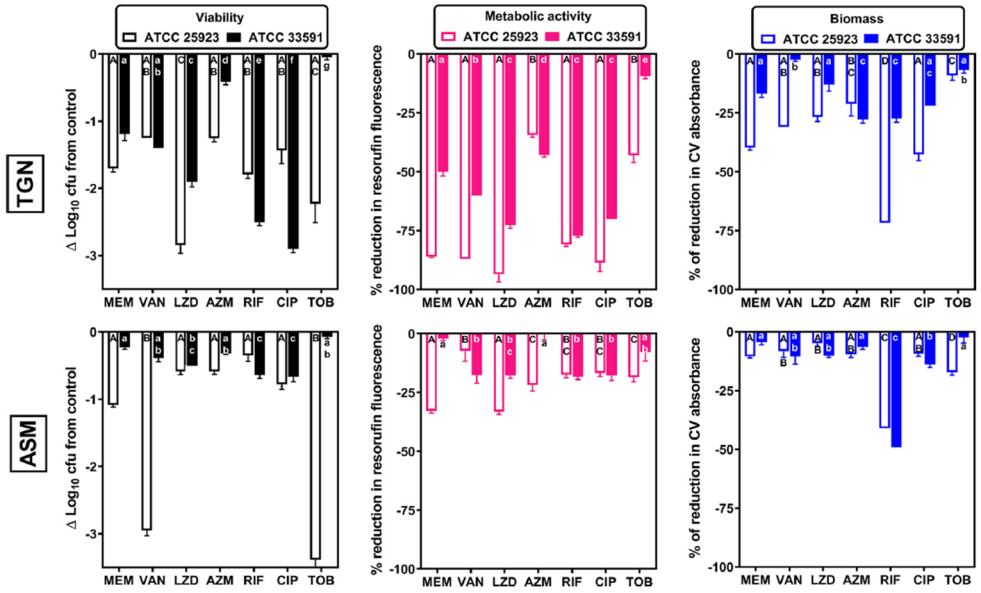


Figure 4. Comparison of antibiotics maximal efficacies (E_{max}) expressed as reduction in CFU from that for the control (left), or as a percentage of the reduction in metabolic activity (resorufin fluorescence; middle) or biofilm mass (crystal violet [CV] absorbance, right) compared to that for an untreated biofilm for strains ATCC 25923 (open bars) or ATCC 33591 (closed bars) grown in Trypticase Soy Broth supplemented with 1% glucose and 2% NaCl (TGN) or in Artificial Sputum Medium (ASM). MEM, meropenem; VAN, vancomycin; LZD, linezolid; AZM, azithromycin; RIF, rifampicin; CIP, ciprofloxacin; TOB, tobramycin. Values are means ± SEM. Statistical analyses: by one-way analysis of variance (ANOVA) with Tukey's post-test for multiple comparisons; values with different letters are significantly different from each other ($p<0.05$). Capital letters: comparison between antibiotics for strain ATCC 25923; lowercase letters: comparison between antibiotics for strain ATCC 33591.

Looking, then, to the data in more details, four main observations can be made. First, the activity of antibiotics was globally lower in ASM than in TGN with respect to both their maximal efficacy and their relative potency. Second, as expected, the efficacy of antibiotics to which ATCC 33591 is resistant was low for viability, metabolic activity and biomass. Third, when comparing efficacy among antibiotics, the bacteriostatic drug azithromycin was globally less effective (it has a less negative E_{max}). The other bacteriostatic drug, linezolid, was as effective as rapidly bactericidal drugs

(rifampicin, ciprofloxacin, tobramycin) towards viability and as effective as slowly bactericidal drugs (meropenem, vancomycin), rifampicin, and ciprofloxacin towards metabolic activity causing a higher reduction in crystal violet staining than other antibiotics against both bacterial strains in both media. Rifampicin was the most effective antibiotic towards biomass, causing a more marked reduction in crystal violet staining than other antibiotics against both bacterial strains in both media. Fourth, the potency of drugs was low in biofilms, since C_{-1log} (viability) and C_{33} (metabolic activity and biomass) were higher than their MIC (except for azithromycin [towards viability and metabolic activity] and meropenem [against ATCC 25923] in TGN). If we now consider these results in a clinical perspective, a 1 log_{10} reduction in the number of CFU or a 33% reduction in metabolic activity was obtained at concentrations that can be reached in human serum (see Table 1 for values) in TGN for all drugs except (i) ciprofloxacin and tobramycin against both strains and (ii) meropenem and azithromycin against ATCC 33591 (ATCC 33591 is resistant to both meropenem and azithromycin). In contrast, only modest effects were observed in ASM at concentrations found in human serum (see green vertical dotted lines in Figures 3 or green horizontal lines in Figure 5 corresponding to the human C_{max} after administration of a conventional dose).

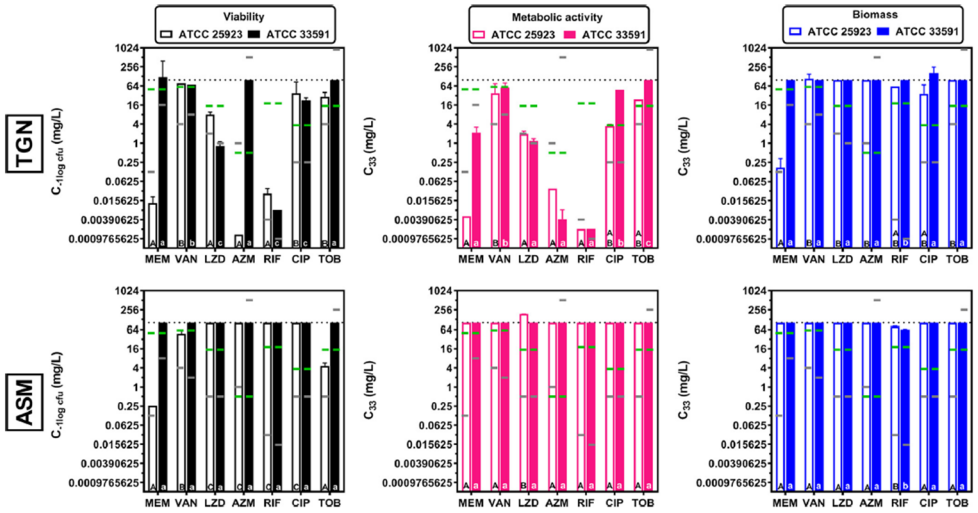


Figure 5. Comparison of antibiotics relative potencies (C_{-1log} or C_{33}) expressed in mg/L for viability (left), metabolic activity (resorufin fluorescence; middle) or biofilm mass (crystal violet [CV] absorbance, right) for strains ATCC 25923 (open bars) or ATCC 33591 (closed bars) grown in Trypticase Soy Broth supplemented with 1% glucose and 2% NaCl (TGN) or in Artificial Sputum Medium (ASM). Green horizontal lines correspond to the human C_{max} after administration of a conventional dose. Gray horizontal lines correspond to MIC shown in table 1. MEM, meropenem; VAN, vancomycin; LZD, linezolid; AZM, azithromycin; RIF, rifampicin; CIP, ciprofloxacin; TOB, tobramycin. Values are means ± SEM. Statistical analyses: one-way analysis of variance (ANOVA) with Tukey's post-test for multiple comparisons; values with different letters are significantly different from each other ($p<0.05$). Capital letters compare the results for the antibiotics for strain ATCC 25923; lowercase letters compare the results for the antibiotics for strain ATCC 33591. The gray horizontal lines are the MIC of the antibiotic in the corresponding medium and the green horizontal line, the human C_{max} after conventional dosing (see Table 1).

SCV analysis and resazurin metabolization

The large difference in the activity of tobramycin when assessed by CFU count *vs*. the resorufin fluorescence against ATCC 25923 biofilms grown in ASM, suggests the presence of high metabolizer phenotypes in the remaining bacterial population. We observed indeed that metabolic activity in ASM biofilm is high when measured with resorufin fluorescence while the reduction in CFU counts is high. When carefully examining the morphology of colonies grown on Columbia Blood agar, we observed, specifically for these samples exposed to tobramycin, the presence of large hemolytic colonies and small non-hemolytic colonies (Figure 6). Aminoglycosides are known to induce the formation of hemin- or menadione-dependent Small Colony Variants (SCVs) [7]. This implies the presence of two phenotypes in our biofilm, a normal phenotype and a SCV phenotype. We therefore also plated samples collected from tobramycin-treated biofilms on TSA and TSA supplemented with hemin (TSAH), menadione (TSAM), or both hemin and menadione (TSAHM). Large and small colonies were observed on plates exposed to tobramycin (tested at 5, 10 and 100 mg/L), with no significant differences in the total number of colonies counted on the various types of agar plates.

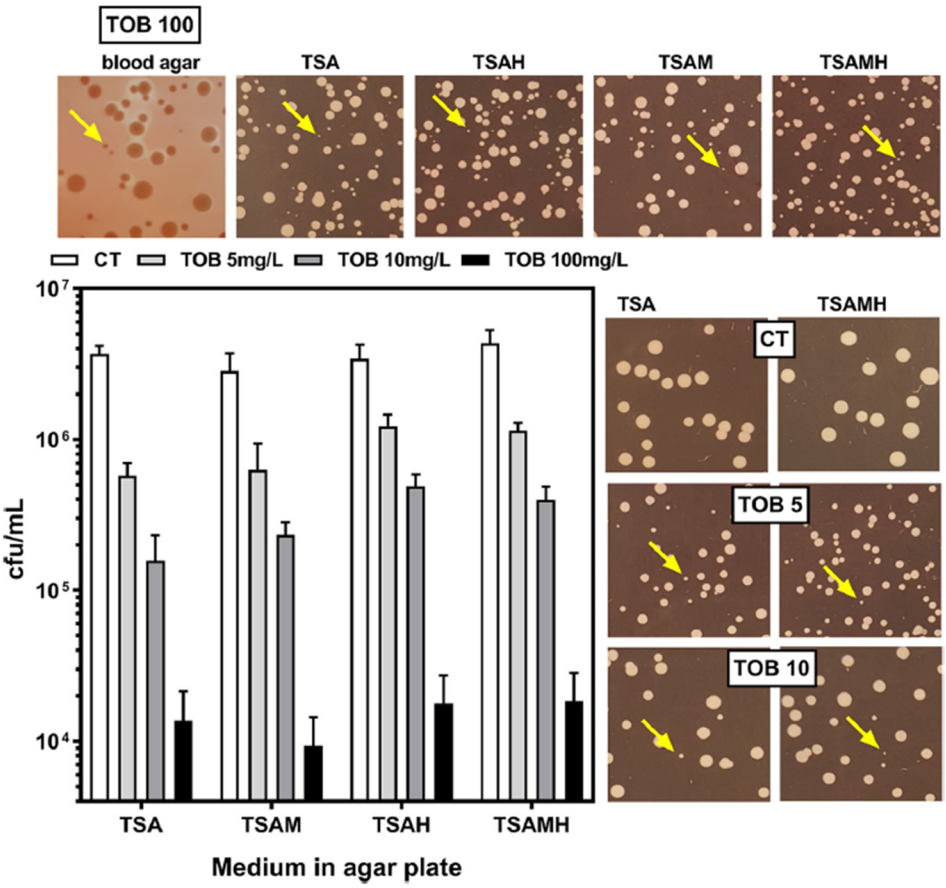


Figure 6. Morphology and counts of colonies from biofilms of ATCC 25923 cultivated in artificial sputum medium (ASM) and exposed during 24 h to tobramycin (TOB) at 5 (TOB 5), 10 (TOB 10), or 100 (TOB 100) mg/L or in control conditions (no antibiotic added; CT). Samples were plated on Columbia blood agar, TSA, or TSA supplemented with either 1 mg/L hemin (TSAH), menadione (TSAM) or both (TSAMH). Yellow arrows indicate a typical small colony.

To further explore the reasons for discrepancies between fluorescence signal and CFU counts in biofilms exposed to high concentrations of tobramycin, we compared the capacity to metabolize resazurin of planktonic and biofilm cultures of isogenic strains, namely COL (K7) and its two stable SCVs variants COL-*menD* (K8; menadione-dependent) and COL-*hemB* (K9; hemin-dependent). Planktonic cultures at increasing OD_{620nm} were incubated for 30 minutes with resazurin (Figure 7, left). We observed for all strains that resazurin metabolization increased linearly with

the optical density of the bacterial suspension, and was not lower for SCVs than for the normal phenotype parental strain. In biofilms grown in TGN, the fluorescence signal was not lower for both SCVs than for COL (K7). In ASM, the metabolic capacity of all strains was markedly reduced, but to a lower extent for COL (K7) than for SCVs (Figure 7B; right). This may contribute to explain the disproportion between the decrease in metabolic activity and in CFU counts inducted by exposure to tobramycin.

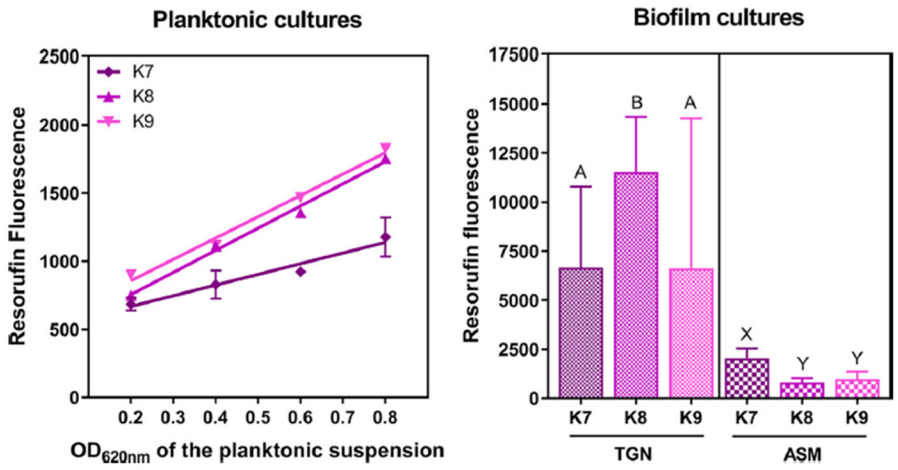


Figure 7. Metabolic activity in planktonic cultures grown in MHB-ca or biofilm cultures grown in Trypticase Soy Broth supplemented with 1% glucose and 2% NaCl (TGN) or in Artificial Sputum Medium (ASM). (Left) Resorufin fluorescence signal recorded after 30 minutes of incubation of planktonic bacteria at increasing inocula with 10 mg/L resazurin. (Right) Resorufin fluorescence signal recorded after 30 minutes of incubation of 24h-old biofilm with 10 mg/L resazurin. Data are means ± SD of triplicates in a single experiment or means ± SEM of at least 3 independent experiments performed in triplicate. Statistical analyses comparing strains in each individual medium: one-way analysis of variance (ANOVA) with Tukey's post-test for multiple comparisons; values with different letters are significantly different from each other ($p<0.05$).

Discussion

Many studies investigating the activity of antibiotics against biofilms are considering only parameters like the minimum biofilm inhibitory concentration (MBIC) and the minimum biofilm eradication concentration (MBEC) to establish their potential interest [19-21]. Yet, these methods lack standardization [19] and do not allow examination of antibiotic pharmacodynamics in detail.

In this work, we set up an *in vitro* model to study the pharmacodynamics of antibiotics against staphylococcal biofilms in the context of cystic fibrosis. This model allows for a quick quantification of biofilm biomass and bacterial metabolic activity and viability. Biofilms were grown in microtiter plates, a support that is widely used [14;18;22-24] because they combine a series of advantages for high-throughput experiments, like the possibility to test many conditions in parallel and the need for small amounts of material and drug [21].

Compared to our previous work [14], we introduce here two major improvements to our *in vitro* model to study biofilm formation by *S. aureus* and activity of antibiotics.

The first one consisted in comparing TGN, a broth medium optimized for *S. aureus* biofilm growth, with ASM, a medium designed to mimic the viscoelastic properties of the mucus produced by patients with cystic fibrosis. To the best of our knowledge the present work is the first to experimentally study the viscoelastic properties of the ASM. This has indeed not been examined in previous papers using ASM media of similar composition [13;25-27]. The general shape of the viscous modulus measured with the agar-containing ASM used here differs from that of sputum but is reminiscent of that described for solid agar preparations subjected to the same amplitude sweep oscillatory test [28]. More importantly, we show that agar-containing ASM shares with CF sputa a higher elasticity than viscosity [17;29;30] and that the values of these parameters in ASM are close from those measured in mucopurulent sputa and approximately 10-fold lower than those of purulent sputa. Although,

only a small number of sputum samples could be tested here, we know from larger series that there is a broad dispersion in mucus viscoelastic properties for infected sputa [30]. ASM may thus reasonably represent mucus characteristics from part of the CF population.

By comparing the influence of culture conditions on biofilm growth, we demonstrate that a stable biofilm can be obtained after 24 h of culture in both media, with however, lower metabolic activity for equivalent bacterial counts and a slightly lower biomass in ASM than in TGN. This lower metabolic activity in ASM than in TGN seems to be specific for the biofilm mode of life, as it is observed (i) for both bacterial strains as well as for K7 or its SCVs in biofilms, but not in planktonic cultures and (ii) for incubation times longer than 12 h (when both the number of CFU and the biomass have reached a plateau value). When examining antibiotic activity in these media, all data from concentration-effect curves could be fitted to sigmoidal regressions, the equation of which allowed us to compare two pharmacodynamic parameters, namely the relative potency and maximal efficacy [14].

Regarding the relative potency, we noticed that a reduction of 33 % of metabolic activity or of 1 $\log_{10}$ CFU was rarely reached in ASM, and at drug concentrations that were higher than those in TGN or than the MIC measured for planktonic cultures. Although these effects do not strictly correspond to bacteriostasis, our results confirm that higher concentrations of antibiotics are needed to act against biofilms than against planktonic cultures [14;18;22-24;31-35].

The low bioavailability of antibiotics in the biofilm matrix is probably a main reason for this loss in potency [3;4;18] and may become even more critical in ASM. Supporting this assumption, it has been shown that the diffusion of antimycobacterial antibiotics was low in mucus from cystic fibrosis patients or in a synthetic CF mucus (composed of DNA, bovine serum albumin and purified pig gastric mucus) [36]. These authors correlated this decrease in diffusion with the length of mucin glycoprotein backbone as well as the presence of branching and of DNA molecules linked to mucin strands.

According to this study, higher viscoelastic properties of CF mucus or synthetic CF mucus are related to the length of mucin strands and its molecular weight. In addition to viscoelastic properties from the environment, biofilms possess their own viscoelastic properties that critically depend on their structure and on the composition of the extracellular matrix. The global viscoelastic properties (from both mucus components and extracellular matrix of biofilm) play a pivotal role in their protective effect against mechanical and chemical challenges [37].

Regarding the maximal efficacy, we noticed that a bactericidal effect (3 $\log_{10}$ CFU reduction) was rarely obtained, with, however, the limitation that a plateau value was not reached in many cases. Maximal efficacy is also markedly lower in ASM than in TGN for all drugs. This might be ascribed to a reduced metabolic rate in ASM (documented here by measuring resazurin reduction), which may render bacteria more tolerant to antibiotics relying on bacterial multiplication to exert their effect [4]. We cannot totally exclude the possibility, however, that slow metabolism also indirectly affects relative potency, by impairing the uptake inside bacteria of antibiotics requiring active transport like aminoglycosides. Intriguingly, linezolid is more effective than other bacteriostatic or slowly bactericidal drugs in TGN, in accordance with our previous data with this molecule [14;38]. A direct comparison with other literature data is difficult because most of the papers report MBEC values only [39;40].

The second improvement brought to the model consists of following bacterial viability by measuring in parallel metabolic activity and bacterial counts. The detection of fluorescent metabolites as a surrogate for viability is essentially justified by ease and rapidity of their use in high throughput assays [41]. We complemented it here with CFU counting because we were suspecting that the metabolization of molecules used as probes could be altered in dormant but viable bacteria that are present in biofilms [42;43].

While, in most of the cases, CFU counts and metabolic activity were affected to a similar extent by antibiotics, a noticeable exception concerns

tobramycin: it caused a marked reduction in the number of CFU for ATCC 25923 in biofilms grown in ASM, but only a marginal reduction in metabolic activity. We suggest that the metabolization of resazurin is essentially operated by the normal phenotype and only marginally influenced by the presence of SCV, while decrease in CFUs counts concern both phenotypes. Aminoglycosides are known to induce the formation of small colony variants of *S. aureus* both *in vivo* and *in vitro* [7;44-47]. We demonstrate here the presence of small colonies, which strongly suggests that tobramycin selects for SCVs specifically in biofilms cultivated in ASM. At this stage, we could not determine a single metabolic defect associated with this phenotypic switch, since small colonies were still observed on media enriched in hemin and/or in menadione, *i.e.* the two factors that usually revert SCV phenotype when induced by aminoglycosides. This result is coherent with a recent study describing the existence of SCVs for which no known auxotrophism could be evidenced [48]. SCVs are thought to play a pathological role in patients with cystic fibrosis [45]. Our data suggest that viscous sputum may contribute to facilitate this selection upon antibiotic treatment.

From a clinical point of view our study suffers from three major limitations. First we only used reference strains and not clinical isolates collected from patients with cystic fibrosis. This study should therefore be considered as a first step to establish a strong pharmacodynamic model that can be now applied to other strains or other drugs. Second, we did not consider the possible role of the microaerophilic environment that may exist in the sputum of patients with cystic fibrosis and may affect antibiotic activity [27]. Third, bacteria were exposed to constant concentrations of antibiotics for a fixed period of time. These conditions do not mimic the pharmacokinetic profile of the drugs in the lungs. Our high throughput system may be helpful to select the most effective drugs to test them thereafter in a dynamic model reproducing pharmacokinetic fluctuations overtime [38]. In the meantime, however, the broad range of concentrations we tested covers human peak serum levels (C_{max}). Our data therefore leads to the conclusion that serum concentrations are probably too low to be active on biofilms developing in

the respiratory tract. Delivery of drugs directly to the lungs via inhalation may help to overcome this issue.

Materials and Methods

Bacterial strains and antibiotics

S. aureus ATCC 25923 (methicillin-sensitive strain) and ATCC 33591 (methicillin-resistant strain) were used for all experiments. COL (K7; wild-type, hospital-acquired methicillin-resistant *Staphylococcus aureus*), and its *menD* and *hemB* SCV stable mutants (K8 and K9, constructed by allelic replacement with an *ermC* cassette-inactivated *menD* gene and an *ermB* cassette-inactivated *hemB* gene, respectively [49;50] were used for experiments focusing on the small colony variant (SCV) phenotype. All strains, including SCVs, were routinely grown on TSA. SCVs remained stable in these conditions (no revertants observed).

Antibiotics were obtained as microbiological standards as follows: azithromycin (potency 100%), from Teva (Petach Tikva, Israel); ciprofloxacin HCl (potency 93.9%), from Bayer (Leverkusen, Germany); tobramycin (potency 100%), from Galephar (Marche-en-Famenne, Belgium); linezolid (potency 100%), from Rib-X Pharmaceuticals (presently Melinta Therapeutics, New Haven, CT); rifampicin (potency 97%), from Sigma-Aldrich. Meropenem (potency 74%) and vancomycin (potency 97.5%) were procured as generic drug branded products for human parenteral use distributed for clinical use in Belgium by Sandoz (Holzkirchen, Germany) and Mylan (Cannonsburg, PA), respectively.

Culture media for biofilm cultures

Two media were used in parallel, namely Trypticase soy broth (VWR; Radnor, PA) supplemented with 1% glucose and 2% NaCl (TGN [14;18]) or Artificial Sputum Medium (ASM, adapted from [13;25;26]). This medium aims at mimicking the composition and viscoelastic properties of the mucus found in the respiratory tract of patients with cystic fibrosis. It contains per liter: 10 g mucin (Sigma-Aldrich; St. Louis, MO), 4 g DNA (Sigma-Aldrich), 5.9 mg DTPA (diethylenetriaminepentaacetic acid) (Sigma-Aldrich), 5 g NaCl (VWR), 2.2 g KCl (Sigma-Aldrich), 3 g agar (Becton Dickinson ; Franklin Lakes, NJ), 5 g amino acids (Becton Dickinson), Tris 1.81g (Calbiochem ; San Diego, CA), and 5 mL egg yolk emulsion (Sigma-Aldrich). All compounds were autoclaved

except egg yolk emulsion which was added aseptically to the autoclaved medium, after which the pH was adjusted to 7 with NaOH.

Rheology

Rheology was used to evaluate the viscoelastic properties of artificial sputum medium in comparison with CF sputa. These sputa were previously collected from six CF patients by expectoration during physiotherapy and immediately frozen at -80°C [17]. The experimental protocol was approved by the Ethics Committee of the *Université catholique de Louvain* (UCL; registration number: B403201422928). Among the six sputa, two were characterized at the time of collection for the presence of microorganisms, and found to be contaminated by *S. aureus* (sample P3) and by *Pseudomonas aeruginosa* and *Aspergillus fumigatus* (sample MP3), respectively. Upon application of an increasing shear strain to the samples, we followed, during an amplitude sweep oscillatory test, their storage/elastic modulus, representing the tendency to recover the original shape after a stress-induced deformation, and their loss/viscous modulus, corresponding to the resistance to flow [29]. We also determined the linear viscoelastic region (LVER) of each sample, i.e. the region of small deformations in which the viscoelastic parameters remain constant [17]. As previously described [17], 600 mg of sample were loaded on a MCR102 rheometer (Anton Paar, Graz, Austria) set up at 37°C. Elastic and viscous moduli were recorded during application of strains between 0.0001% and 10% at a constant frequency of 10 Hz, using the RheoCompass™ software.

Development of the biofilm model

Biofilms were grown in 96 well plates (European catalog number 734-2327; VWR) as previously described [18] using TGN or ASM. In brief, a bacterial suspension was prepared in cation-adjusted MHB starting from overnight cultures on Trypticase soy agar. When using TGN for biofilm growth, 96-well plates were inoculated (200 µL/well) at approximately 10^7 CFU/mL ($OD_{620\ nm}$ adjusted to 0.005) and then incubated at 37°C for 24h so as to obtain a mature biofilm. When ASM was used for biofilm growth, 20 µL of a suspension at 10^8 CFU/mL in cation-adjusted MHB were inoculated in the

96-well plates and incubated during 2h at 37°C to favor attachment, after which 180 µL of ASM were added to also reach an inoculum of 10^7 CFU/mL. Plates were then incubated at 37°C for 24h.

Susceptibility testing

MICs were determined by microdilution following the guidelines of the Clinical and Laboratory Standards Institute using cation-adjusted Mueller-Hinton Broth (MHB-ca) [51], and compared to those measured in TGN and ASM following the same protocol. Yet, direct visual reading of MICs was not possible in ASM due to the natural turbidity of this medium. Aliquots were therefore spread on TSA and incubated overnight; MICs were defined as the lowest concentration for which there was no change in CFU as compared to initial values.

Activity of antibiotics against biofilms

After 24h of incubation, the culture medium of biofilms was removed and replaced with fresh medium (control) or medium supplemented with antibiotics at concentrations ranging from 10^{-3} to 10^3 mg/L in order to obtain full concentration-response curves. Biofilms were reincubated for 24h at 37°C. At the end of the incubation period, the medium was removed and the biofilm was washed once with 200 µL of phosphate buffered saline (PBS). Biofilm biomass was quantified using crystal violet, a cationic dye that non-specifically stains negatively-charged constituents in biofilms [18]. Washed biofilms were fixed by heat at 60°C for about 1 hour and incubated during 10 minutes at room temperature with 200 µL of crystal violet (VWR) (final concentration 0.2 g/L). After removing the excess of crystal violet, plates were washed under running water and dried. The dye fixed to the biofilm was resolubilized in 200 µL of 66% acetic acid and incubated 1h at room temperature. Absorbance was measured at 570 nm using a SPECTRAmax Gemini XS microplate spectrophotometer (Molecular Devices LLC, Sunnyvale, CA). Metabolic activity in biofilms was quantified using the resazurin assay, which is based on the reduction by living bacteria of the weakly fluorescent blue-colored dye resazurin in the pink-colored highly fluorescent resorufin [18]. Washed biofilms were incubated with 10 mg/L

resazurin (Sigma-Aldrich) for 30 min at room temperature in the dark. Resorufin fluorescence was measured at a wavelength of 590 nm with an excitation wavelength of 550 nm using a SPECTRAmax Gemini XS microplate spectrofluorometer. Bacterial counts were also determined in washed biofilms resuspended in 1mL of sterile PBS in microcentrifugation tubes. The tubes were vortexed and placed during 5 minutes in a sonication bath (Bransonic® Ultrasonic cleaner 3510E-MT, frequency 40 kHz; Danbury, CT) to disrupt the biofilm, and vortexed again, after which aliquots were taken and diluted before spreading on TSA plates. Unless stated otherwise, colonies were counted after 24h of incubation at 37°C.

Identification and metabolic activity of small colony variants

When appropriate, the presence of SCV was determined by spreading bacteria recovered from biofilms on 5 types of agar plates, namely TSA, TSA supplemented with 1 mg/L hemin (TSAH) or menadione (TSAM), TSA supplemented with both 1 mg/L hemin and menadione (TSAMH) and Columbia blood agar plates (CBA) [44;52]. Colonies were counted after 24h of incubation at 37°C. In parallel, we compared the capability of stable SCV and of normal phenotype strains to metabolize resazurin into resorufin. Bacterial suspensions at different OD_{620nm} were prepared in PBS starting from overnight cultures on TSA. 96-well plates were inoculated with 200 µL of bacterial suspension mixed with resazurin (final concentration: 10 mg/L) and then incubated for 30 min at room temperature in the dark, after which resorufin fluorescence was measured as described above.

Curve fitting and statistical analyses

Curve fitting analyses were made using Graph-Pad Prism version 8.02 (GraphPad Software, San Diego, CA). Data were used to fit a sigmoid function, which allowed us to calculate maximal efficacy (E_{max}; maximal reduction in viability or biomass for an infinitely large concentration of antibiotic) and relative potencies (C_{33}; antibiotic concentration needed to reach 33% of viability or biomass reduction within the biofilm). Statistical analyses were performed with Graph Pad Instat version 3.06 (GraphPad Software) or Graph-Pad Prism version 8.02.

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References to paper #1

[1] Ciofu O, Tolker-Nielsen T, Jensen PO, Wang H, & Hoiby N (2015) Antimicrobial resistance, respiratory tract infections and role of biofilms in lung infections in cystic fibrosis patients. *Adv. Drug Deliv. Rev.*, **85,** 7-23.

[2] Cohen TS & Prince A (2012) Cystic fibrosis: a mucosal immunodeficiency syndrome. *Nat. Med.*, **18,** 509-519.

[3] Donlan RM & Costerton JW (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.*, **15,** 167-193.

[4] Otto M (2018) Staphylococcal Biofilms. *Microbiol. Spectr.*, **6**.

[5] Bhattacharya M, Wozniak DJ, Stoodley P, & Hall-Stoodley L (2015) Prevention and treatment of Staphylococcus aureus biofilms. *Expert. Rev. Anti. Infect. Ther.*, **13,** 1499-1516.

[6] Cystic Fibrosis Foundation Patient Registry. Annual Data Report 2017. Cystic Fibrosis Foundation . 2018. 16-11-2018.

[7] Garcia LG, Lemaire S, Kahl BC, Becker K, Proctor RA, Denis O, Tulkens PM, & Van Bambeke F (2013) Antibiotic activity against small-colony variants of Staphylococcus aureus: review of in vitro, animal and clinical data. *J. Antimicrob. Chemother.*, **68,** 1455-1464.

[8] Gomez-Gonzalez C, Acosta J, Villa J, Barrado L, Sanz F, Orellana MA, Otero JR, & Chaves F (2010) Clinical and molecular characteristics of infections with CO2-dependent small-colony variants of Staphylococcus aureus. *J. Clin. Microbiol.*, **48,** 2878-2884.

[9] Kaplan ML & Dye W (1976) Growth requirements of some small-colony-forming variants of Staphylococcus aureus. *J. Clin. Microbiol.*, **4,** 343-348.

[10] Tan X, Coureuil M, Ramond E, Euphrasie D, Dupuis M, Tros F, Meyer J, Nemazanyy I, Chhuon C, Guerrera IC, Ferroni A, Sermet-Gaudelus I, Nassif X, Charbit A, & Jamet A (2019) Chronic Staphylococcus aureus lung infection correlates with

proteogenomic and metabolic adaptations leading to an increased intracellular persistence. *Clin. Infect. Dis.*.

[11] Gabryszewski SJ, Wong Fok Lung T, Annavajhala MK, Tomlinson KL, Riquelme SA, Khan IN, Noguera LP, Wickersham M, Zhao A, Mulenos AM, Peaper D, Koff JL, Uhlemann AC, & Prince A (2019) Metabolic Adaptation Supports Persistent Methicillin-Resistant Staphylococcus aureus Pulmonary Infection. *Am. J. Respir. Cell Mol. Biol.*.

[12] Hirschhausen N, Block D, Bianconi I, Bragonzi A, Birtel J, Lee JC, Dubbers A, Kuster P, Kahl J, Peters G, & Kahl BC (2013) Extended Staphylococcus aureus persistence in cystic fibrosis is associated with bacterial adaptation. *Int J. Med. Microbiol.*, **303,** 685-692.

[13] Sriramulu DD, Lunsdorf H, Lam JS, & Romling U (2005) Microcolony formation: a novel biofilm model of Pseudomonas aeruginosa for the cystic fibrosis lung. *J. Med. Microbiol.*, **54,** 667-676.

[14] Bauer J, Siala W, Tulkens PM, & Van Bambeke F (2013) A combined pharmacodynamic quantitative and qualitative model reveals the potent activity of daptomycin and delafloxacin against Staphylococcus aureus biofilms. *Antimicrob. Agents Chemother.*, **57,** 2726-2737.

[15] Kiedrowski MR & Horswill AR (2011) New approaches for treating staphylococcal biofilm infections. *Ann. N. Y. Acad. Sci.*, **1241,** 104-121.

[16] Goss CH & Muhlebach MS (2011) Review: Staphylococcus aureus and MRSA in cystic fibrosis. *J. Cyst. Fibros.*, **10,** 298-306.

[17] Guichard MJ, Kinoo D, Aubriot AS, Bauwens N, Gougue J, Vermeulen F, Lebecque P, Leal T, & Vanbever R (2018) Impact of PEGylation on the mucolytic activity of recombinant human deoxyribonuclease I in cystic fibrosis sputum. *Clin. Sci. (Lond)*, **132,** 1439-1452.

[18] Siala W, Mingeot-Leclercq MP, Tulkens PM, Hallin M, Denis O, & Van Bambeke F (2014) Comparison of the antibiotic activities of Daptomycin, Vancomycin, and the investigational Fluoroquinolone Delafloxacin against biofilms from Staphylococcus aureus clinical isolates. *Antimicrob. Agents Chemother.*, **58,** 6385-6397.

[19] Coenye T, Goeres D, Van Bambeke F, & Bjarnsholt T (2018) Should standardized susceptibility testing for microbial biofilms be introduced in clinical practice? *Clin. Microbiol. Infect.*, **24,** 570-572.

[20] Gomes IB, Meireles A, Goncalves AL, Goeres DM, Sjollema J, Simoes LC, & Simoes M (2018) Standardized reactors for the study of medical biofilms: a review of the principles and latest modifications. *Crit Rev. Biotechnol.*, **38,** 657-670.

[21] Azeredo J, Azevedo NF, Briandet R, Cerca N, Coenye T, Costa AR, Desvaux M, Di Bonaventura G, Hebraud M, Jaglic Z, Kacaniova M, Knochel S, Lourenco A,

Mergulhao F, Meyer RL, Nychas G, Simoes M, Tresse O, & Sternberg C (2017) Critical review on biofilm methods. *Crit Rev. Microbiol.*, **43,** 313-351.

[22] Waryah CB, Wells K, Ulluwishewa D, Chen-Tan N, Gogoi-Tiwari J, Ravensdale J, Costantino P, Gokcen A, Vilcinskas A, Wiesner J, & Mukkur T (2017) In Vitro Antimicrobial Efficacy of Tobramycin Against Staphylococcus aureus Biofilms in Combination With or Without DNase I and/or Dispersin B: A Preliminary Investigation. *Microb. Drug Resist.*, **23,** 384-390.

[23] Smith K, Perez A, Ramage G, Gemmell CG, & Lang S (2009) Comparison of biofilm-associated cell survival following in vitro exposure of meticillin-resistant Staphylococcus aureus biofilms to the antibiotics clindamycin, daptomycin, linezolid, tigecycline and vancomycin. *Int. J. Antimicrob. Agents*, **33,** 374-378.

[24] Salem AH, Elkhatib WF, & Noreddin AM (2011) Pharmacodynamic assessment of vancomycin-rifampicin combination against methicillin resistant Staphylococcus aureus biofilm: a parametric response surface analysis. *J. Pharm. Pharmacol.*, **63,** 73-79.

[25] Tavernier S & Coenye T (2015) Quantification of Pseudomonas aeruginosa in multispecies biofilms using PMA-qPCR. *PeerJ.*, **3,** e787.

[26] Ghani M & Soothill JS (1997) Ceftazidime, gentamicin, and rifampicin, in combination, kill biofilms of mucoid Pseudomonas aeruginosa. *Can. J. Microbiol.*, **43,** 999-1004.

[27] Kirchner S, Fothergill JL, Wright EA, James CE, Mowat E, & Winstanley C (2012) Use of artificial sputum medium to test antibiotic efficacy against Pseudomonas aeruginosa in conditions more relevant to the cystic fibrosis lung. *J. Vis. Exp.*, e3857.

[28] Manickam K, Machireddy RR, & Seshadri S (2014) Characterization of biomechanical properties of agar based tissue mimicking phantoms for ultrasound stiffness imaging techniques. *J. Mech. Behav. Biomed. Mater.*, **35,** 132-143.

[29] Lai SK, Wang YY, Wirtz D, & Hanes J (2009) Micro- and macrorheology of mucus. *Adv. Drug Deliv. Rev.*, **61,** 86-100.

[30] Serisier DJ, Carroll MP, Shute JK, & Young SA (2009) Macrorheology of cystic fibrosis, chronic obstructive pulmonary disease & normal sputum. *Respir. Res.*, **10,** 63.

[31] Olson ME, Slater SR, Rupp ME, & Fey PD (2010) Rifampicin enhances activity of daptomycin and vancomycin against both a polysaccharide intercellular adhesin (PIA)-dependent and -independent Staphylococcus epidermidis biofilm. *J. Antimicrob. Chemother.*, **65,** 2164-2171.

[32] Vergidis P, Rouse MS, Euba G, Karau MJ, Schmidt SM, Mandrekar JN, Steckelberg JM, & Patel R (2011) Treatment with linezolid or vancomycin in combination with

rifampin is effective in an animal model of methicillin-resistant Staphylococcus aureus foreign body osteomyelitis. *Antimicrob. Agents Chemother.*, **55,** 1182-1186.

[33] Howlin RP, Brayford MJ, Webb JS, Cooper JJ, Aiken SS, & Stoodley P (2015) Antibiotic-loaded synthetic calcium sulfate beads for prevention of bacterial colonization and biofilm formation in periprosthetic infections. *Antimicrob. Agents Chemother.*, **59,** 111-120.

[34] Salem AH, Elkhatib WF, Ahmed GF, & Noreddin AM (2010) Pharmacodynamics of moxifloxacin versus vancomycin against biofilms of methicillin-resistant Staphylococcus aureus and epidermidis in an in vitro model. *J. Chemother.*, **22,** 238-242.

[35] Molina-Manso D, del Prado G, Ortiz-Perez A, Manrubia-Cobo M, Gomez-Barrena E, Cordero-Ampuero J, & Esteban J (2013) In vitro susceptibility to antibiotics of staphylococci in biofilms isolated from orthopaedic infections. *Int. J. Antimicrob. Agents*, **41,** 521-523.

[36] Bhat PG, Flanagan DR, & Donovan MD (1996) Drug diffusion through cystic fibrotic mucus: steady-state permeation, rheologic properties, and glycoprotein morphology. *J. Pharm. Sci.*, **85,** 624-630.

[37] Peterson BW, He Y, Ren Y, Zerdoum A, Libera MR, Sharma PK, van Winkelhoff AJ, Neut D, Stoodley P, van der Mei HC, & Busscher HJ (2015) Viscoelasticity of biofilms and their recalcitrance to mechanical and chemical challenges. *FEMS Microbiol. Rev.*, **39,** 234-245.

[38] Siala W, Rodriguez-Villalobos H, Fernandes P, Tulkens PM, & Van Bambeke F (2018) Activities of Combinations of Antistaphylococcal Antibiotics with Fusidic Acid against Staphylococcal Biofilms in In Vitro Static and Dynamic Models. *Antimicrob. Agents Chemother.*, **62,** e00598-18.

[39] Abad L, Tafani V, Tasse J, Josse J, Chidiac C, Lustig S, Ferry T, Diot A, Laurent F, & Valour F (2018) Evaluation of the ability of linezolid and tedizolid to eradicate intraosteoblastic and biofilm-embedded Staphylococcus aureus in the bone and joint infection setting. *J. Antimicrob. Chemother.*, 10.

[40] Van den Driessche F, Brackman G, Swimberghe R, Rigole P, & Coenye T (2017) Screening a repurposing library for potentiators of antibiotics against Staphylococcus aureus biofilms. *Int J. Antimicrob. Agents*, **49,** 315-320.

[41] Herten M, Bisdas T, Knaack D, Becker K, Osada N, Torsello GB, & Idelevich EA (2017) Rapid in Vitro Quantification of S. aureus Biofilms on Vascular Graft Surfaces. *Front Microbiol.*, **8,** 2333.

[42] Hoiby N, Bjarnsholt T, Givskov M, Molin S, & Ciofu O (2010) Antibiotic resistance of bacterial biofilms. *Int J. Antimicrob. Agents*, **35,** 322-332.

[43] Sandberg ME, Schellmann D, Brunhofer G, Erker T, Busygin I, Leino R, Vuorela PM, & Fallarero A (2009) Pros and cons of using resazurin staining for quantification of

viable Staphylococcus aureus biofilms in a screening assay. *J. Microbiol. Methods*, **78,** 104-106.

[44] Vestergaard M, Paulander W, Leng B, Nielsen JB, Westh HT, & Ingmer H (2016) Novel Pathways for Ameliorating the Fitness Cost of Gentamicin Resistant Small Colony Variants. *Front Microbiol.*, **7,** 1866.

[45] Kahl BC (2014) Small colony variants (SCVs) of Staphylococcus aureus--a bacterial survival strategy. *Infect. Genet. Evol.*, **21,** 515-522.

[46] Tuchscherr L, Kreis CA, Hoerr V, Flint L, Hachmeister M, Geraci J, Bremer-Streck S, Kiehntopf M, Medina E, Kribus M, Raschke M, Pletz M, Peters G, & Loffler B (2016) Staphylococcus aureus develops increased resistance to antibiotics by forming dynamic small colony variants during chronic osteomyelitis. *J. Antimicrob. Chemother.*, **71,** 438-448.

[47] Pelletier LLJ, Richardson M, & Feist M (1979) Virulent gentamicin-induced small colony variants of Staphylococcus aureus. *J. Lab Clin. Med.*, **94,** 324-334.

[48] Idelevich EA, Kriegeskorte A, Schleimer N, Peters G, von Eiff C, & Becker K (2018) In Vitro Susceptibility of Clinical Staphylococcus aureus Small-Colony Variants to beta-Lactam and Non-beta-Lactam Antibiotics. *Antimicrob. Agents Chemother.*, **62**.

[49] Bates DM, von Eiff C, McNamara PJ, Peters G, Yeaman MR, Bayer AS, & Proctor RA (2003) Staphylococcus aureus menD and hemB mutants are as infective as the parent strains, but the menadione biosynthetic mutant persists within the kidney. *J. Infect. Dis.*, **187,** 1654-1661.

[50] von Eiff C, Heilmann C, Proctor RA, Woltz C, Peters G, & Gotz F (1997) A site-directed Staphylococcus aureus hemB mutant is a small-colony variant which persists intracellularly. *J. Bacteriol.*, **179,** 4706-4712.

[51] Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; 28th Informational Supplement. CLSI document M100-S28. Wayne, PA. 2018.

[52] Kastbjerg VG, Hein-Kristensen L, & Gram L (2014) Triclosan-induced aminoglycoside-tolerant Listeria monocytogenes isolates can appear as small-colony variants. *Antimicrob. Agents Chemother.*, **58,** 3124-3132.

Adapted from paper 2: Activity of antibiotics against *Pseudomonas aeruginosa* in an *in vitro* model of biofilms in the context of cystic fibrosis: influence of the culture medium.

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Abstract

Pseudomonas aeruginosa is a major cause of respiratory biofilm-related infections in patients with cystic fibrosis. We developed an in-vitro pharmacodynamic model to study the activity of antipseudomonal antibiotics against PAO1 biofilms grown in artificial sputum medium with agar (ASM(+)) versus that against biofilms grown in trypticase soy broth supplemented with glucose and NaCl (TGN). We measured bacterial counts, metabolic activity (fluorescein diacetate [FDA] hydrolysis) and biomass (crystal violet absorbance). Biofilms grew slower in ASM(+) than in TGN but reached the same CFU counts and metabolic activity in both media and a slightly higher biomass after 48h in ASM(+) than in TGN. The concentration-response curves of antibiotics after 24h of incubation with mature biofilms showed maximal effects ranging from a 3 (ciprofloxacin) to a 1.5 (ceftazidime, meropenem) $\log_{10}$ CFU decrease, with tobramycin and colistin showing intermediate values. These maximal reductions in the numbers of CFU were similar in both media for ciprofloxacin and β-lactams but lower in ASM(+) than in TGN for tobramycin and colistin; they were reached at concentrations lower than human maximum concentration in plasma for ciprofloxacin and β-lactams only. The reduction in metabolic activity and in biomass were low in both media. Small colony variants were selected by tobramycin in ASM(+) and by ciprofloxacin in both media. The model was then successfully applied to 4 isolates from patients with cystic fibrosis. These biofilms showed CFU counts similar to those of PAO1 biofilms in ASM(+) but a higher biomass than PAO1 in ASM(+) and moderate differences in their susceptibility to antibiotics from that of PAO1 biofilms grown in this medium. This model proved useful to establish the pharmacodynamic profile of drugs against *P. aeruginosa* biofilms in the context of cystic fibrosis.

Introduction

Pseudomonas aeruginosa is the most prevalent microorganism in the respiratory tract of adult patients suffering from cystic fibrosis (CF), being detected in 70% of adults aged 25 years or older [1]. It is a major cause of mortality in this population thanks to its capacity to colonize the sputum where it forms microcolonies embedded in an alginate matrix, strongly suggestive of biofilms [2]. Biofilms are indeed defined as microbial communities living in a self-produced matrix essentially composed of polysaccharides, extracellular DNA, and proteins [3-5]. These structures offer a protection against host defenses and antibiotics due to the barrier effect of the extracellular matrix as well as to metabolic changes in bacterial populations, leading to dormant phenotypes less responsive to antibiotics [3;6].

One of the determining factors in the capacity of *P. aeruginosa* to colonize the lungs and form biofilms therein is the accumulation of a thickened mucus as a consequence of the dysfunction of the cystic fibrosis transmembrane conductance regulator channel, and the subsequent impaired mucociliary clearance of inhaled microbes [7;8]. In addition, *P. aeruginosa* may display a large variety of phenotypes like mucoid or small colony variants (SCV). The latter can be selected by antibiotics, which inhibit their growth, adding another risk factor for both the persistence of the infection [9;10] and the biofilm formation, as SCVs overproduce matrix polysaccharides [5;9;10].

In spite of the clinical importance of these infections, only a few studies have examined in details the activity of antibiotics in a medium that mimics the sputum of patients with CF [11-13]. In the present study we used a previously described medium (artificial sputum medium [ASM] with agar [ASM(+)]) that mimics much better the composition and rheological properties of the sputum of CF patients [14] than the so-called synthetic CF sputum medium (SCFM) [15]. An improved version of SCFM containing major constituents of sputum like DNA, *N*-acetyl-D-glucosamine, mucin, and dioleoylphosphatidylcholine could also have been used [16], but its

rheological properties have not been established so far. We study the activity of antibiotics *in vitro* against biofilms made by *P. aeruginosa* in this medium and on a pharmacodynamic basis, in order to establish their relative potency and maximal efficacy [17]. We systematically compared ASM(+) with Trypticase soy broth (TSB) supplemented by glucose and NaCl (TGN), a medium optimized to favor the growth of biofilms of *S. aureus* and other bacterial species ([17] and unpublished data from our team). We selected 5 antibiotics frequently used in these patients, namely the fluoroquinolone ciprofloxacin, the β-lactams ceftazidime and meropenem, the aminoglycoside tobramycin, and the polymyxin colistin [6].

Results

Establishing experimental conditions for fluorescein diacetate (FDA) assay with PAO1.

As a preliminary experiment, we determined the optimal conditions for assaying bacterial metabolic activity using the fluorescein diacetate (FDA) assay, in which FDA is cleaved into fluorescein by bacterial enzymes produced by metabolically active bacteria. To this end, we examined the influence of the time of incubation with FDA on fluorescein fluorescence for planktonic cultures of PAO1 at different inocula in cation-adjusted Mueller Hinton broth (MHB-ca). The numbers of CFU were measured in parallel. Figure 1A shows the evolution of the fluorescence signal as a function of the optical density at 620nm (OD_{620nm}) at 3 different incubation times. After 15 minutes of incubation, the fluorescence signal increased almost linearly (R^2 of the linear regression: 0.986) for OD_{620nm} values ≤ 0.5 (corresponding to approximately 4 10^8 CFU/mL), after which a trend to a saturation of the signal was observed. When the time of incubation was prolonged to 30 or 60 minutes, the range over which the relationship between OD_{620nm} and fluorescence was linear was restricted to OD_{620nm} values < 0.17 (corresponding to approximately 10^8 CFU/mL). Figure 1B shows the relationship between the fluorescence signal and bacterial counts after 15 minutes of incubation. This relationship was linear over counts ranging from $10^{6.5}$ to $10^{8.5}$ CFU/ml (R^2 of the linear regression: 0.9985), covering the numbers recovered from biofilms (see below). This time of incubation was therefore adopted for all experiments.

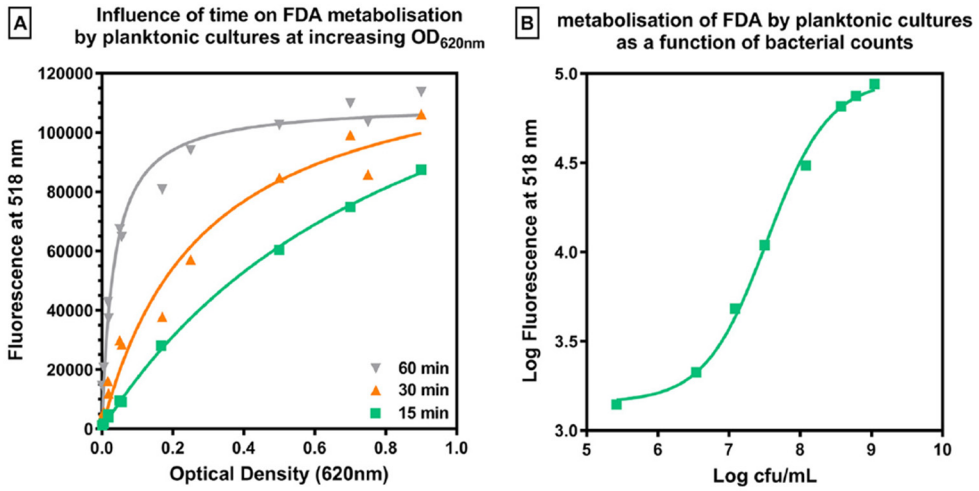


Figure 1. Establishing experimental conditions for fluorescein assay. A. Fluorescein fluorescence signal measured for planktonic cultures of PAO1 at increasing OD_{620nm} after 15, 30, and 60 minutes of incubation with 100mg/L fluorescein diacetate (FDA). B. Correlation between fluorescence signal measured after 15 minutes of incubation and CFU counts. Data are means ± SD of 3 independent determinations.

Setting up the biofilm model with PAO1.

The kinetics of growth of PAO1 in biofilm was followed over 72 h in TGN and ASM with agar (ASM(+)). Preliminary experiments showed that PAO1 was unable to grow and form a biofilm if directly inoculated in ASM(+), which we attributed to the high viscosity of this medium. We therefore incubated the bacteria first in ASM without agar (ASM(-)) for 24h to allow for adhesion, after which the medium was replaced by ASM(+). Figure 2 shows the evolution of the numbers of CFU (left), metabolic activity, as assessed by the FDA assay (middle), or biomass, as evaluated by crystal violet staining (right) over time. In TGN, bacterial counts increased from 10^7 CFU/mL to 7 10^8 CFU/mL over the first 24h, after which a plateau was reached. Fluorescein fluorescence and crystal violet absorbance increased in parallel. In ASM, bacterial growth was minimal during the first day of incubation in ASM(-), after which it rapidly recapitulated to reach a plateau at 7 10^8 CFU/mL in 8h. The fluorescence signal followed a similar pattern, with, however, a slow increase in the fluorescence signal being seen in the first hours following the

change of medium from ASM(-) to ASM(+). The biomass also increased over time at a rate similar to that observed in TGN but with slightly higher values all over the time of incubation. On this basis, an incubation time of 24h in TGN and of 24h in ASM(-) followed by 24h in ASM(+) was considered to generate a stable biofilm (with respect to bacterial counts, metabolic activity, and biomass) that could be exposed to antibiotics.

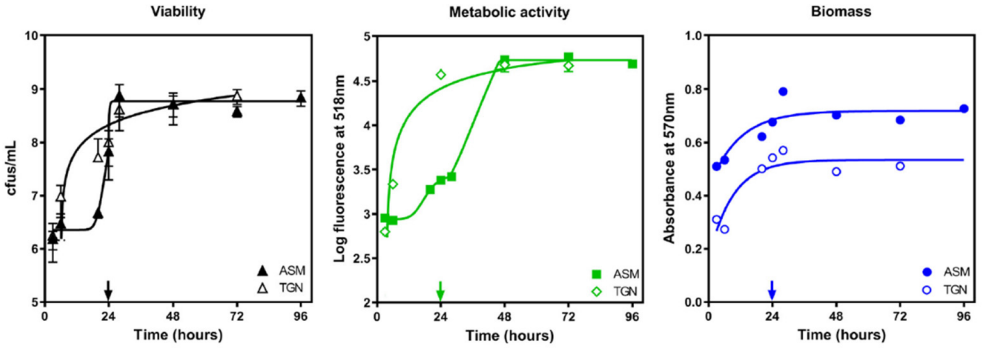


Figure 2. Kinetics of growth of biofilm in TGN and ASM. Initial inocula were $3x10^7$ CFU/mL in TGN and $5.5x10^7$ in ASM(-). In this case, attachment was allowed during 24 h in ASM(-), after which the medium was replaced by ASM(+) (highlighted by an arrow on the X axis). The graphs show the evolution over time of CFUs, fluorescein fluorescence generated by hydrolysis of fluorescein diacetate (FDA) by metabolically active bacteria (metabolic activity or vitality), or crystal violet absorbance (biomass). Data are means ± SD of at least 3 independent experiments performed in 3 replicates. When not visible, the SD are smaller than the size of the symbols.

Antibiotic activity against planktonic cultures of PAO1.

Table 1 shows the MICs of the selected antibiotics against PAO1 in TGN and ASM(+) media compared to cation-adjusted Mueller-Hinton broth (MHB-ca). PAO1 was fully susceptible to all antibiotics tested. In TGN, MICs were similar (± 1 doubling dilution) to those in MHB-ca for all antibiotics except tobramycin, the MIC of which was 8-fold (3 doubling dilutions) higher in TGN. The MICs were 2-fold (1 doubling dilution) lower in ASM(+) than in MHB-ca for ciprofloxacin and tobramycin and 4-fold (2 doubling dilutions) lower for β-lactams, but 4-fold (2 doubling dilutions) higher for colistin.

Antibiotics	MIC (mg/L)			EUCAST Resistance breakpoint (mg/L) [b]	Human C_{max} (mg/L) [c]
	MHB-ca	TGN	ASM(+) [a]		
Ciprofloxacin	0.125	0.125	0.06	0.5	3.7
Ceftazidime	1	0.5	0.25	8	87-170
Meropenem	0.5	0.5	0.125	8	50-115
Tobramycin	0.25	2	0.125	4	16-30
Colistin	0.5	0.5	2	2	2.7

Table 1 : Antibiotic susceptibility of PAO1 in different media

[a] MIC determined by CFU counting after plating due to the turbidity of the medium

[b] based on the Belgian summary of product characteristics (prescribing information) for each drug at conventional and high (as recommended for *P. aeruginosa* infections) registered dose or on reference [48] for colistin

Antibiotic activity against biofilms of PAO1.

The activities of these five antibiotics against bacterial viability, vitality (metabolic activity), and biofilm biomass were then evaluated after 24h of incubation of stable biofilms in TGN, in ASM(+) or ASM(-) as a comparator. Stable biofilms were defined as biofilm precultivated during 24h in TGN, 24h in ASM(-) followed by 24h in ASM(+) and 4 days of incubation with medium renewal every 24h for ASM(-), respectively. The corresponding concentration-response curves are presented in Figure 3 for TGN and ASM(+) and Unpublished Figure 1 for ASM(-). The pertinent calculated pharmacodynamic parameters (the maximal effect [E_{max}] extrapolated for an infinitely large concentration or the concentration needed to achieve a reduction in the number of CFU of 1 log_{10} [viability] [$C_{-1\ log}$], which corresponds to the concentration needed of a 0.1-log_{10} reduction in fluorescein fluorescence [metabolic activity] [$C_{-0.1}$] or a 0.3-log_{10} decrease in the crystal violet OD_{570nm} [biomass] [$C_{-0.3}$]; see reference [14] for more explanations of these parameters) are presented in Figure 4.

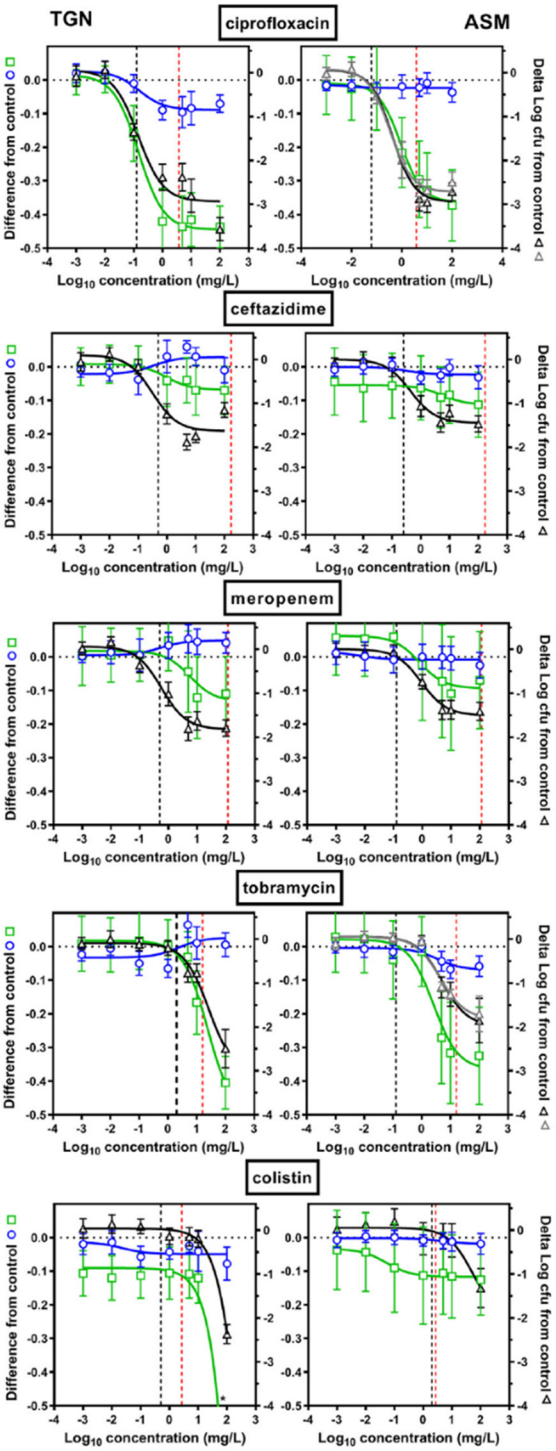
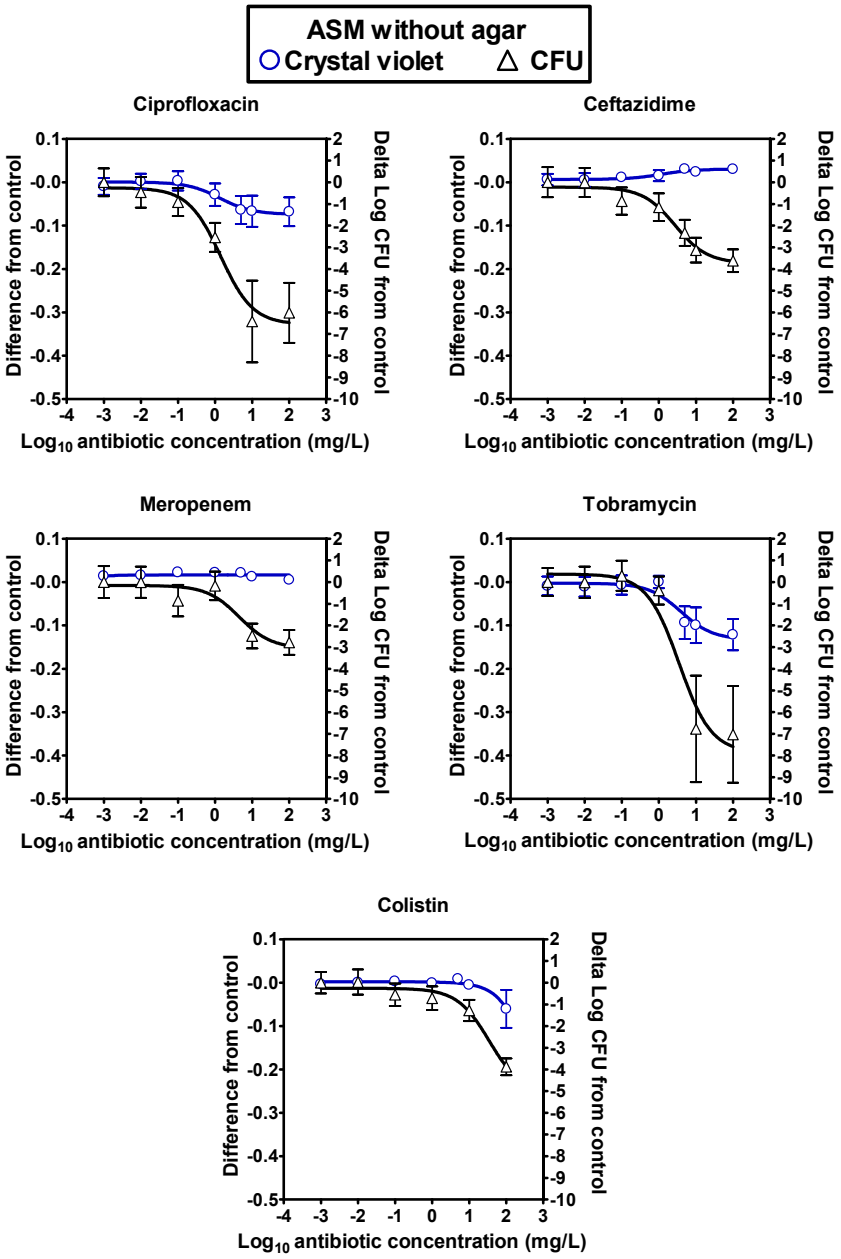

TGN
ciprofloxacin
ASM
ceftazidime
meropenem
tobramycin
colistin
Difference from control
Delta Log cfu from control
Log10 concentration (mg/L)

Figure 3. Activities of antibiotics against biofilms in TGN (left) or in ASM(+) (right). Concentration-responses curves of antibiotics against 24 h-biofilms of PAO1. Biofilms were incubated with increasing concentration of antibiotics during 24 h (ciprofloxacin, ceftazidime, meropenem, tobramycin and colistin). The left ordinate shows the decrease in metabolic activity (fluorescein assay, green open square) or biofilm mass (crystal violet assay, blue open circle) expressed as decreased from the control value (no antibiotic present; Δlog fluorescence units for metabolic assay; Δlog OD_{570nm} for crystal violet staining). The right ordinate shows the change in viability (CFU counting, CFUs were allowed to grow during 24 h [black open triangles] or 48 h [gray open triangles] on PIA plates), expressed as the reduction in log scale from the control value (no antibiotic present). The vertical black dotted line is the MIC of the antibiotic in the corresponding medium and the red dotted line, the human C_{max} after intra-venous administration of a high dose (see Table 1). All values are means ± SEM of 3 to 4 independent experiments performed in triplicates (when not visible the error bars are smaller than the size of symbols). Note that the last data point is at a value lower than -0.5 for metabolic activity with colistin in TGN (-0.9; highlighted by *) but the scale was maintained to allow comparison with all the other graphs.



Unpublished figure 1. Activities of antibiotics against biofilms in ASM(-). Concentration-responses curves of antibiotics against 96 h-biofilms of PAO1. Biofilms were incubated with increasing concentration of antibiotics during 24 h (ciprofloxacin, ceftazidime, meropenem, tobramycin and colistin). The left ordinate shows the decrease in biofilm mass (crystal violet

assay, blue open circle) expressed as decreased from the control value (no antibiotic present; Δlog OD_{570nm} for crystal violet staining). The right ordinate shows the change in viability (CFU counting, CFUs were allowed to grow during 24 h [black open triangles] on TSA plates), expressed as the reduction in log scale from the control value (no antibiotic present). All values are means ± SEM of 3 independent experiments performed in triplicates (when not visible the error bars are smaller than the size of symbols).

As a comparison, we also determined concentration-response curves in ASM(-), to compare these data with those obtained with ASM(+) and evidence a possible influence of medium viscoelastic properties on the activity of antibiotics (Unpublished Figure 1). We observed that the activity of antibiotics was globally higher as compared to that measured in ASM(+): In this model, all 5 antibiotics achieve at least 3 log of reduction in CFU counts with the highest concentration used (100mg/L). The effect on biomass is also slightly higher for ciprofloxacin and tobramycin but not for the three other antibiotics.

Considering antibiotic effects on CFU first, we observed a concentration-dependent reduction in all cases, which could be fitted to sigmoidal regressions. Minor differences between the two media were observed for all antibiotics except tobramycin and colistin, which were more active in TGN than in ASM(+) (Figure 4A, left). The maximal effect ranged from 3 log_{10} CFU decrease for ciprofloxacin, which was the most effective, to a 1.5 to 1.8 log_{10} CFU decrease for β-lactams, which were the least effective. For tobramycin and colistin, the maximal effect ranged from a 2 and 3 log_{10} CFU decrease (note that a plateau value was not reached at the highest concentration tested in these cases, so that we rather report the effect observed at the highest concentration tested [100 mg/L]). This E_{max} was obtained at clinically-achievable concentrations (lower than the maximum concentration in plasma [C_{max}] in humans) for ciprofloxacin and β-lactams but not for tobramycin and colistin (see the dashed red lines in Figure 3 which point to C_{max} values). The concentrations needed to reduce the inoculum by 1 log_{10} (Figure 4B, left) were close to the MIC in TGN for

ciprofloxacin and β-lactams but higher than the MIC for tobramycin and colistin in TGN and for all drugs in ASM(+). They were lower than the human C_{max} in both media for ciprofloxacin and β-lactams, close to this value for tobramycin, but higher for colistin. We noticed the presence of small colonies on plates from biofilms incubated with ciprofloxacin in both media or with tobramycin in ASM(+), the proportion of which increased with the antibiotic concentration (Figure 5). We therefore compared the CFU counts obtained under these conditions after 24h and 48h of incubation of *Pseudomonas* isolation agar (PIA) plates (black and gray curves, respectively, on the corresponding graphs in Figure 3), but did not find any difference in the global reduction in bacterial counts, suggesting we did not underestimate the CFU counts after 24h despite the small size of some of them.

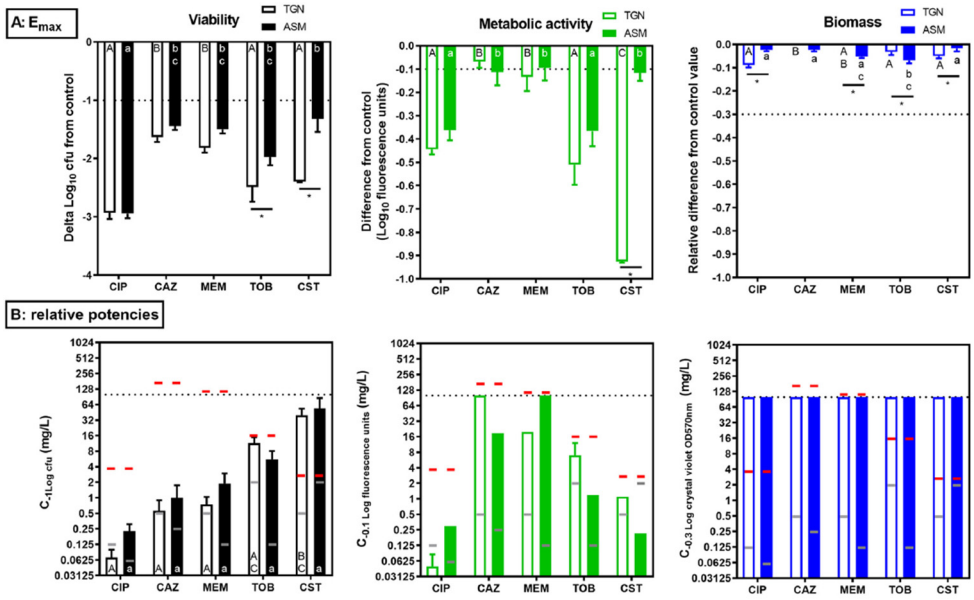


Figure 4. Antibiotic maximal efficacies (panel A; E_{max}) and antibiotic relative potencies (panel B; C_{-1log}, $C_{-0.1}$ or $C_{-0.3}$) compared to untreated biofilm for PAO1 in TGN (open bars) and in ASM(+) (closed bars). Panel A: The graphs show the maximal reduction in CFU (viability, left panel), in log fluorescein fluorescence (middle panel; metabolic activity) or in log crystal violet OD_{570nm} (right panel; biofilm mass) as compared to untreated controls, and as extrapolated from the sigmoid regression of the concentration-response curve for an infinitively large concentration (Figure 3). For tobramycin and colistin in TGN, and for colistin

in ASM(+), the E_{max} could not be calculated based on the equation of the sigmoidal regression (plateau not reached); the graph therefore shows the effect observed at the highest concentration tested. In the other cases, the E_{max} value is very similar to the effect observed at a concentration of 100 mg/L, as the plateau was already reached at this concentration. Panel B: The graphs show the antibiotic concentration (in mg/L) causing a reduction of 1 log_{10} in CFU (viability, left panel [C_{-1log}]), of 0.1 log in fluorescein fluorescence (middle panel; metabolic activity) [$C_{-0.1}$] or of 0.3 log in crystal violet OD_{570nm} (right panel; biofilm mass) [$C_{-0.3}$] as compared to untreated controls, and as intrapolated from the sigmoid regression of the concentration-response curve (Figure 3). These effects are highlighted by the horizontal dotted line in the upper panels of this figure. Red horizontal lines correspond to the human C_{max} after intravenous administration of a high dose; grey horizontal lines, to MICs in the corresponding medium (Table 1). CIP, ciprofloxacin; CAZ, ceftazidime, MEM, meropenem; TOB, tobramycin; CST, colistin. Values are means ± SEM of 3 to 4 independent experiments performed in triplicates. Statistical analyses: two-way analysis of variance (ANOVA) with Tukey's post-test for multiple comparisons; values with different letters are significantly different from each other ($p<0.05$). Capital letters: comparison between antibiotics in TGN; lowercase letters: comparison between antibiotics in ASM(+) (statistics could not be performed on $C_{-0.1}$ values [metabolic activity] because the confidence interval on values were large due to the shape of the curves around this value). * significant difference between media for a given antibiotic.

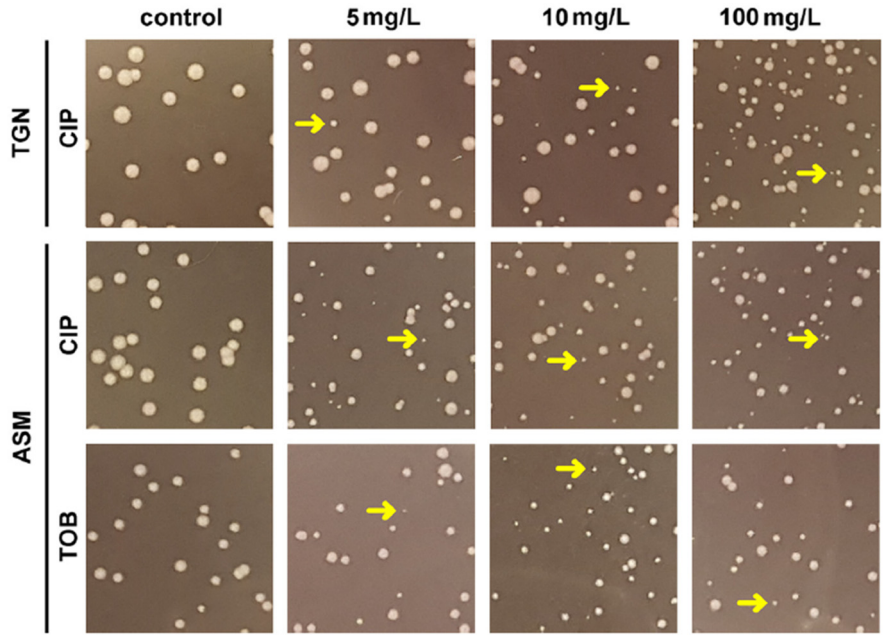


Figure 5. Morphology of colonies of bacteria recovered from biofilms of PAO1 cultivated in TGN medium or ASM(+) and exposed during 24 h to ciprofloxacin (CIP) or tobramycin (TOB)

at 5, 10, 100 mg/L or in control conditions and plated on PIA. The arrows point to typical small colony variants colonies.

Considering, then, the effects of antibiotics on metabolic activity, we observed globally low reductions in the fluorescence signal (compared to those observed in planktonic cultures for equivalent reductions in CFU counts [a 0.1 log10 difference versus a 0.8 log10 difference in the fluorescence signal over a 1 log 10 change in the numbers of CFU in biofilms and planktonic cultures, respectively]; see Figure S1), but the range of concentrations over which a reduction in the fluorescence signal was observed matched that over which antibiotics reduced the CFU counts.

Regarding biomass, all antibiotics failed to cause a significant decrease in crystal violet absorbance in both media.

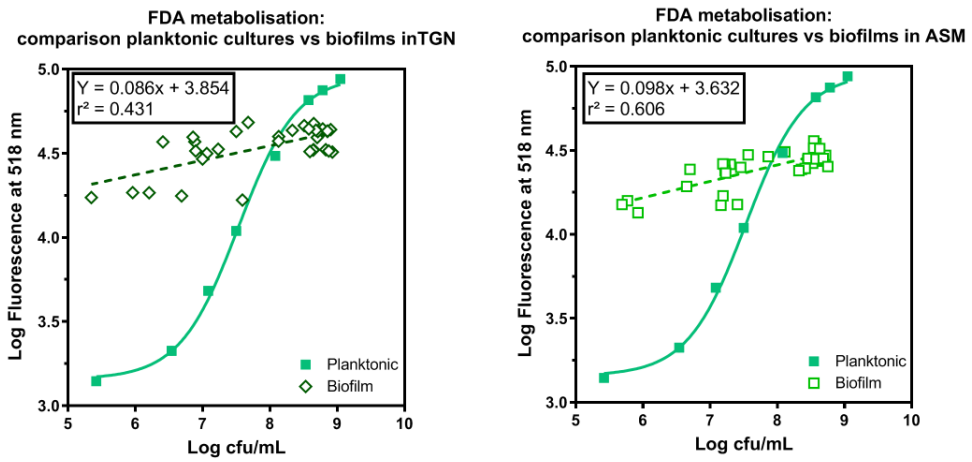


Figure S1. Fluorescein fluorescence signal measured for PAO1 at increasing inocula after 15 minutes of incubation with 100mg/L fluorescein diacetate (FDA). The graphs compare data from planktonic cultures in MHB-ca (same curve on both graphs; this curve is also shown in Figure 1) and from biofilms grown in TGN (left) or in ASM (right). In biofilms, the bacterial counts were those recovered from biofilms incubated with antibiotics at different concentrations and FDA fluorescence values were those measured in independent wells exposed to the same treatments. The linear regression of the data obtained in biofilms are shown on each graph. The slope is approx. 0.1 in both media, meaning that increasing of 1 $\log_{10}$ CFUs generates a 0.1 $\log_{10}$ increase in fluorescein fluorescence signal.

Adaptability of the model to CF clinical isolates.

Clinical isolates collected from patients with CF markedly differ from PAO1, and are usually considered as higher biofilm producers, partially due to their greater twitching motility [18]. We therefore examined whether the model we set up with PAO1 could also be used for CF clinical isolates. To this effect, we selected 4 clinical isolates fully susceptible to the antibiotics that we were testing here (Table 2) and compared them to PAO1 for their twitching motility, their capacity to form biofilms, and their susceptibility to antibiotics in planktonic cultures and biofilms grown in ASM(+). As illustrated in Figure 6, these isolates showed higher twitching motility than PAO1 (except for isolate 193-2) and formed a well visible biofilm below the surface of the culture medium. We then quantified the CFU and biomass for biofilms grown in ASM(+). We did not study the metabolisation of fluorescein diacetate for these isolates, because we showed it was poorly sensitive to the detection of changes in viability after antibiotic exposure for PAO1. Figure 7 shows that, while the CFU counts were similar in clinical isolates and PAO1, biofilm biomass was significantly higher for these isolates than for the reference strain.

Antibiotics	MIC (mg/L)							
	616 (PA921) [a]		3807 (PA970) [a]		154-1 (PA1024) [b]		193-2 (PA1060) [b]	
	MHB-ca	ASM(+)[c]	MHB-ca	ASM(+)	MHB-ca	ASM(+)	MHB-ca	ASM(+)
Ciprofloxacin	0.5	0.5	0.032	0.064	0.5	0.5	2	2
Ceftazidime	2	0.5	1	1	2	0.5	2	1
Meropenem	0.125	0.064	0.125	0.064	0.5	1	2	4
Tobramycin	4	4	2	1	4	8	1	2
Colistin	2	2	2	4	2	4	1	8

Table 2: Antibiotic susceptibility of clinical isolates in MHB-ca and ASM(+)

[a] Laboratoire de Bactériologie, Hôpital Jean Minjoz, Besançon, France

[b] University Hospital Münster, Münster, Germany

[c] MIC determined by CFU counting after plating due to the turbidity of the medium

In a next step, we examined the activity of the five antibiotics tested previously against these 4 isolates. MICs in ASM(+) were similar (in most of

the cases, ± 1 or 2 doubling dilution, as for PAO1) to those in MHB-ca (Table 2). We then studied the antibiotic activity against biofilms grown in ASM(+), focusing on concentrations corresponding to their respective human C_{max} and on the maximal concentration tested against PAO1 (100 mg/L). Considering the effects of the antibiotic on viability within the biofilms, ciprofloxacin was the more active and colistin was the less active against all strains, as already reported for PAO1 (Figure 8, left). Some variations in the reduction of the number of CFU were noticed among isolates for each individual antibiotic. They were the most important for ciprofloxacin at 100 mg/L, which was significantly more active against 2 out of the 4 isolates than against PAO1 (see Figure S2 for a picture of the corresponding biofilms). As observed for PAO1, drug effects on biomass were minimal (Figure 8, right). SCVs were observed in the same conditions as for PAO1 (not shown).

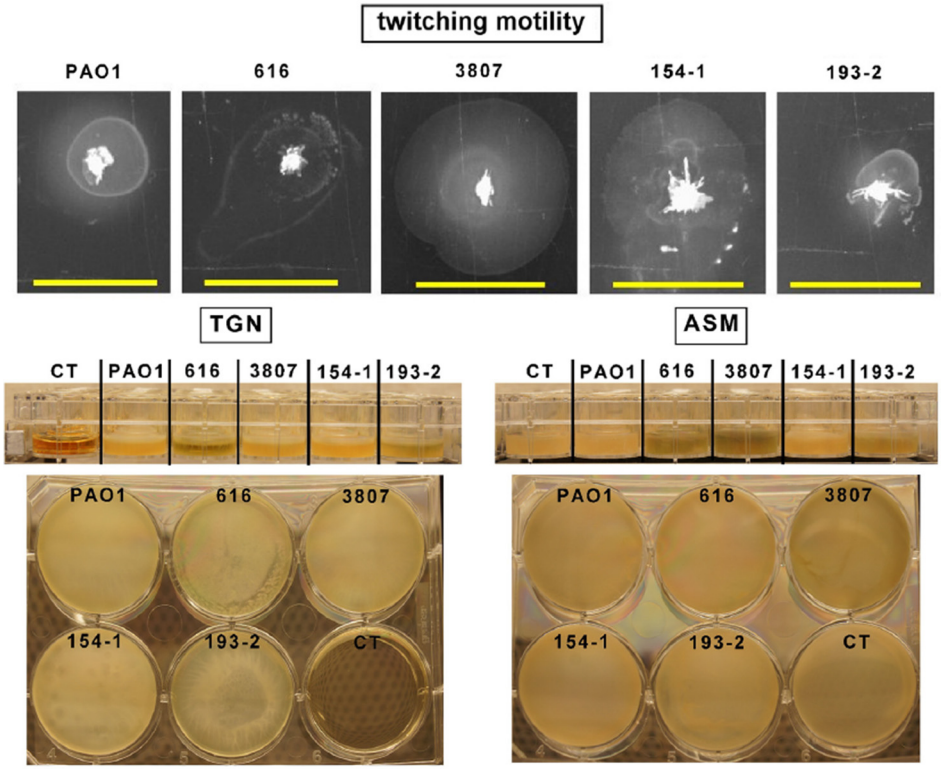


Figure 6. Upper panel: twitching motility of PAO1 and of the 4 CF isolates under study. Scale: the yellow bar in each subpanel corresponds to 1 cm. Lower panel: Lateral and bottom view (using a mirror) of biofilms made by PAO1 and the 4 CF isolates under study in TGN (left) or

ASM(+). Biofilms were cultured in 24 (lateral view) or 6 (bottom view) well plates during 24 h (TGN) or 24 h in ASM(-) followed by 24 h in ASM(+) (ASM) and pictures taken in control conditions. The control (CT) is a well containing the culture medium only.

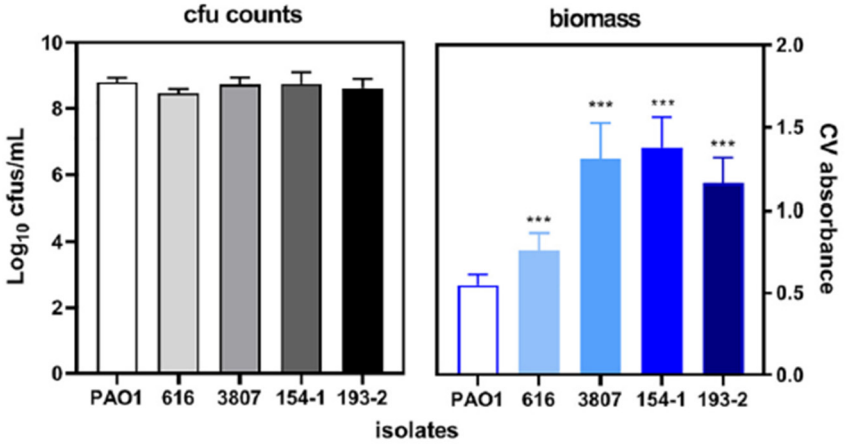


Figure 7. quantification of CFUs (left) and biomass (right; evaluated by crystal violet absorbance) in biofilms made by 4 CF clinical isolates in comparison with PAO1 after 24 h growth in ASM(-) followed by 24 h incubation in ASM(+). Data are means ± SEM of 2 independent experiments in 3 replicates for CFUs and of 2 experiments in 2 replicates for biomass. Statistical analyses: 1-way ANOVA with Dunnett's post hoc text comparing each isolate with PAO1: ***: $p < 0.001$

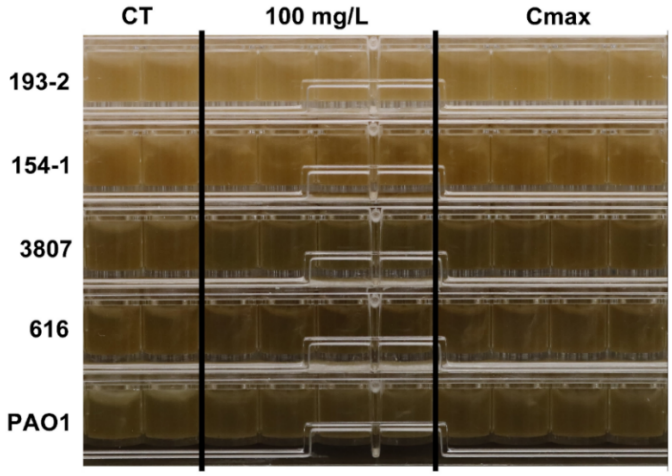


Figure S2 : lateral view of biofilms of PAO1 and 4 CF clinical isolates grown during 24 h in ASM(-), then 24 h in ASM(+), and incubated during 24 h additional hours in control conditions (no antibiotic added) or in the presence of ciprofloxacin at 100 mg/L or at its human C_{max} (3.7 mg/L).

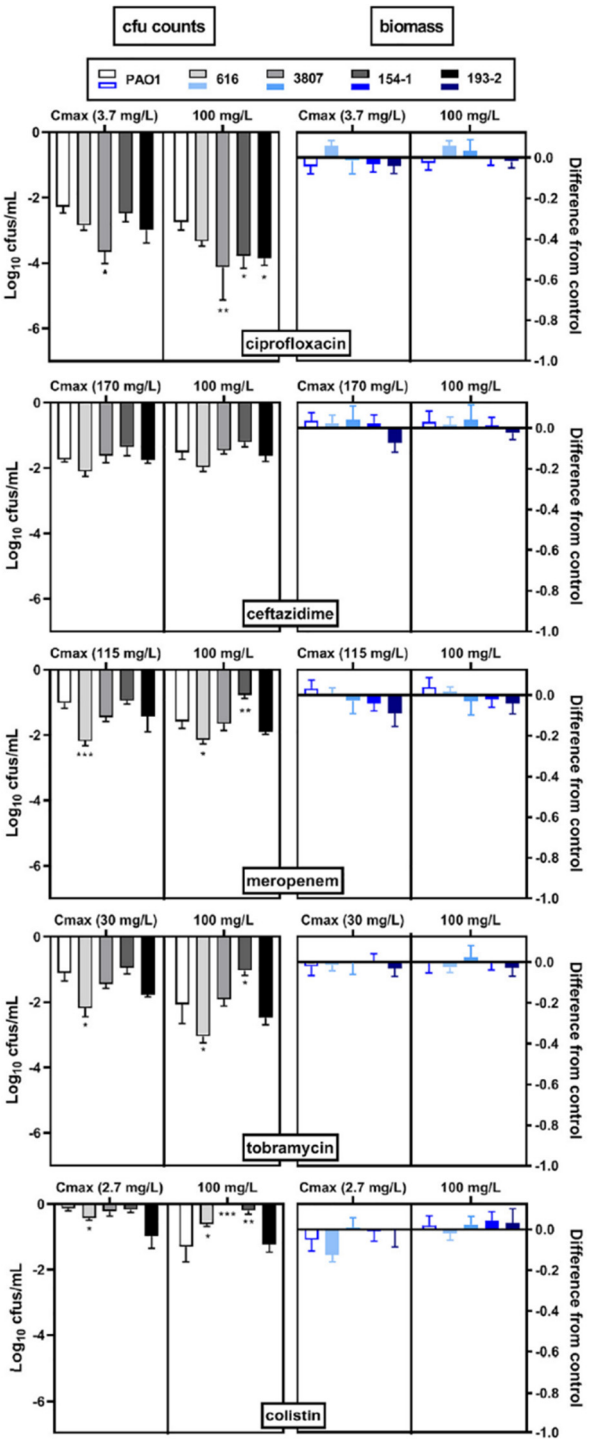


Figure 8. Activity of antibiotics against biofilms grown in ASM(+) for 4 CF clinical isolates in comparison with PAO1 and thereafter incubated with antibiotics (ciprofloxacin, ceftazidime, meropenem, tobramycin and colistin) at their human C_{max} (Table 1) or at 100 mg/L (maximal concentration tested in concentration-response curves for PAO1 in Figure 3) during 24 h. The graphs show the reduction in $\log_{10}$ CFUs number (left) or in the $\log_{10}$ OD_{570nm} of crystal violet as compared to the untreated control. Data are means ± SEM of 2 independent experiments in 3 replicates for CFUs and of 2 experiments in 2 replicates for biomass. Statistical analyses: 1-way ANOVA with Dunnett's post hoc text comparing each isolate with PAO1: ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$.

Discussion

This study is, to the best of our knowledge, the first one to present a comparative view of the pharmacodynamics of antibiotics against *P. aeruginosa* biofilms in the context of cystic fibrosis. Of interest also, we used here *in vitro* models comparable to those previously published for *S. aureus* [14], allowing for drawing a point-by-point parallel between these two pathogens that play an important role in lung infections of patients with cystic fibrosis.

A first point of interest is thus to compare the kinetics of biofilm development for both bacterial species. While a plateau value (corresponding to a stable biofilm) was obtained after 24h of incubation for *S. aureus* or for *P. aeruginosa* in TGN, we noticed a delay in CFU growth and in increase in metabolic activity for *P. aeruginosa* biofilms cultivated in ASM(+), since the plateau was reached after 48h only. We attribute this delay to the need of a 24h adhesion phase in medium without agar to insure *P. aeruginosa* further growth. In addition, although the number of CFU was similar for *S. aureus* and *P. aeruginosa* in both media for mature biofilms, biomass, evaluated by crystal violet staining was lower for *P. aeruginosa* than *S. aureus,* as well as in TGN versus ASM(+). As previously suggested [19], this could possibly be explained by an inadequate fixation of crystal violet to biofilms, especially in TGN, because of the larger amounts of water present in the rather slimy matrix built up by *P. aeruginosa*.

Another salient observation concerns the major difference in the rate of metabolisation of FDA by *P. aeruginosa* in biofilms versus planktonic cultures. Like Peeters *et al*. [19], we found a linear relationship between the fluorescence signal and CFU counts over a range of approximately 10^5 to 10^8 CFU for both planktonic and biofilm-embedded bacteria, but with much higher signals being seen in biofilms at low bacterial counts, suggesting higher metabolic activity in these conditions than in planktonic cultures. Although this observation is counterintuitive if taking into account the sessile character of bacteria in biofilms, it has already been reported and interpreted as denoting higher levels of hydrolytic enzymes in biofilms [20].

Corroborating this hypothesis, secreted esterases are known to be overexpressed during biofilm formation by *P. aeruginosa* [21]. Yet, this high rate of conversion of FDA in biofilms considerably reduces the sensitivity and suitability of the method to evaluate antibiotic activity in biofilms, pleading for the systematic use of CFU counting.

Considering these antibiotic effects on CFU counts, we observed maximal efficacies ranging from 1.5 to 3 $\log_{10}$ decrease in the number of CFU. These values are similar to those we previously described for *S. aureus* in TGN [14] but lower (less negative) than those we observed in ASM(-), where maximal efficacies ranged from 3 to 7.8 $\log_{10}$ decrease in the number of CFUs. Very few *in vitro* studies have looked into the details at the pharmacodynamics of antibiotic activity against *P. aeruginosa* in the context of cystic fibrosis, generally concentrating on the determination of Minimal Biofilm Eradication Concentration (MBEC) values against clinical isolates. These MBEC have been determined in TSB supplemented by 1% glucose and found to be 300-600-fold higher than MICs for ciprofloxacin, tobramycin and colistin and more than 2500-fold higher for β-lactams [22], which is coherent with our finding than the maximal efficacy of β-lactams is lower than that of the other drugs.

In contrast with our previous observations with *S. aureus* [14], the only drugs for which we observed a reduction in maximal efficacy in ASM(+) versus TGN wre tobramycin and colistin; i.e. the two drugs which are hydrophilic polycations. This result is coherent with the resultas of previous studies. First, tobramycin and, to a lesser extend colistin, were found more active against biofilms grown in Luria-Bertani broth than in ASM(-), but at concentrations higher than those tested here [23]. These authors attribute this difference to an enrichment of the biofilm matrix in polysaccharides in ASM(-). This explanation may, however, not apply to our study because TGN is supplemented by glucose, which upregulates the expression of the extracellular polysaccharide-related gene *pslA* required for biofilm formation [24]. Second, the addition of frozen mucus from patients with CF to preformed biofilms was shown to decrease the activity of tobramycin,

which was related to a reduced transport of this polycationic drug in the biofilm [25] due to its strong ionic interactions with components of the biofilm matrix and/or mucus [26]. Likewise, colistin strongly binds to mucin *in vitro*, which considerably decreases its activity [27].

In our previous work with *S. aureus,* we evidenced SCVs specifically in biofilms cultivated in ASM(+) and exposed to tobramycin [14]. The same observation was made here, but extended to ciprofloxacin in both culture media. While the selection of SCVs by aminoglycosides has been described a long time ago in different species including *S. aureus* [28], *P. aeruginosa* [29], or enterobacteriaceae [30], less is known about those selected by fluoroquinolones. They were described in *Salmonella enterica* as a transient response to stress [31], but also in *P. aeruginosa* biofilms grown in TSB [32] or in ASM(-) [33]. No specific auxotrophism could be determined for these SCVs [33]. *P. aeruginosa* SCVs are generally considered as high biofilms producers [34], less motile [33] and hyperpiliated [35], to be adapted to oxidative stress [35], and producing less siderophores [36]. They are frequently isolated from the sputum of patients with CF but revert quickly to normal growing phenotype [9;37]. Similarly to SCVs selected *in vitro*, those isolated from patients do not show any auxotrophism [37]. Thus, these SCVs are considered as participating to the physiopathology of biofilm-related infections in the lungs of patients with CF [38].

In contrast to many studies which use extremely high antibiotic concentrations in order to measure MBEC, our concentration-response curves cover the range of concentrations that can be achieved *in vivo* after intravenous administration. This allows us to conclude that, in both media, a 1 $\log_{10}$ decrease in CFU counts could be achieved at concentrations below the human C_{max} for all drugs except colistin. For β-lactams and ciprofloxacin, decrease of approximately 1.5 $\log_{10}$ and 3 $\log_{10}$, respectively, were obtained at this C_{max}, which would argue in favor of the use of these drugs. Yet tobramycin and colistin are also administered by inhalation, generating higher local concentrations, but it should be mentioned that SCVs were more

frequently recovered in the sputum from patients receiving aerosolized antibiotics than in patients receiving intravenous antibiotics [37].

Importantly, the biofilm model we set up here is applicable to isolates from patients suffering from CF. Of interest, these biofilms all form below the surface of the medium, as previously described, and show the presence of bacterial aggregates similar to those found in the sputum of patients with CF [13;39]. We confirm that these isolates globally show a higher twitching motility, recognized as necessary for exploration and attachment to surfaces and well as for the formation and development of biofilm architecture [18]. They also produce more biofilm biomass than the reference strain. In spite of this higher matrix amount, however, they do not show a lower susceptibility to antibiotics than PAO1, and they even sometimes show a higher susceptibility. Further studies are thus needed to establish the respective role of ASM(+) components and matrix constituents in limiting antibiotic access to bacteria embedded in biofilms.

Our study suffers from some limitations. We only included a few clinical isolates in our work, which may not represent the diversity encountered in clinical isolates, especially because we selected on purpose only fully-susceptible ones. We did not modulate the pH or the oxygenation of the medium, which may differ from the physiological conditions in the CF lungs, possibly affecting bacterial growth and antibiotic activity [40;41]. We exposed our biofilms to fixed antibiotic concentrations and for a specific incubation time, which do not mimic the pharmacokinetic variations observed in patients but keep the advantage of facilitating a direct comparison of pharmacodynamic profiles among drugs.

Taking these drawbacks into account, our data nevertheless contribute to rationalize therapeutic failures in the treatment of pulmonary infections by *P. aeruginosa* in cystic fibrosis, while our model paves the way to further studies aiming at examining in more details clinical isolates, including those harboring multiresistance, other drugs or drug combinations, as well as innovative therapeutic options.

Materials and Methods

Bacterial strain and antibiotics

P. aeruginosa PAO1 was used as a reference strain. Four isolates from cystic fibrosis previously characterized as fully susceptible to the antibiotics used in this study were included in specific experiments [42]. Isolates 616 and 3807 were collected in the *Hôpital Jean Minjoz* (Besançon, France from patients aged 13 and 3 years, respectively. They are characterized respectively as overproducing pyocyanin and harboring a mucoid phenotype. Isolates 154-1 and 193-2 were collected at the University Hospital Münster (Münster, Germany) from patients aged 31 and 20 years, respectively, both being chronically infected for at least two years. Strain 154-1 is also showing a mucoid phenotype.

These strains were routinely grown on Trypticase soy agar (TSA; VWR; Radnor, PA) for overnight culture and on Pseudomonas Isolation Agar (PIA; Sigma-Aldrich, St. Louis, MO) for determination of colony forming units (colonies were easier to count [round shape] and contaminations could be excluded on this medium).

Antibiotics were obtained as microbiological standards as follows: ciprofloxacin HCl (potency 93.9%), from Bayer (Leverkusen, Germany); tobramycin (potency 100%), from Galephar (Marche-en-Famenne, Belgium); ceftazidime pentahydrated (potency 72.5%), from Panpharma (Luitré-Dompierre, France) and colistin sulfate salt (potency 79.6%), from Sigma-Aldrich. Meropenem (potency 74%) was procured as generic drug branded product for human parenteral use distributed for clinical use in Belgium by Sandoz (Holzkirchen, Germany).

Culture media for biofilm cultures

Three media were used in parallel, namely Trypticase soy broth (VWR) supplemented with 1% glucose and 2% NaCl (TGN) [[17;43]], and two types of Artificial Sputum Media (adapted from [11;44;45]), differing by the fact one is supplemented in agar (ASM(+)) and the other one is not (ASM(-)). ASM(-) contains per liter: 10 g mucin (Sigma-Aldrich), 4 g DNA (Sigma-Aldrich), 5.9 mg DTPA (diethylenetriaminepentaacetic acid) (Sigma-Aldrich),

5 g NaCl (VWR), 2.2 g KCl (Sigma-Aldrich), 5 g amino acids (Becton Dickinson; Franklin Lakes, NJ), Tris 1.81g (Calbiochem ; San Diego, CA), and 5 mL egg yolk emulsion (Sigma-Aldrich). ASM(+) contains in addition 3 g/L agar (Becton Dickinson). All constituents except egg yolk emulsion were autoclaved (egg yolk emulsion was added aseptically to the autoclaved medium), after which the pH was adjusted to 7 with NaOH. We previously showed that ASM(+) better mimics the composition and viscoelastic properties of the mucus found in the respiratory tract of patients with cystic fibrosis [14].

Macroscopic Twitching Assay

Twitching motility was determined as previously described [46]. Briefly, bacteria were plated onto 1.5% lysogeny broth (LB) agar plates (prepared using Lysogeny Broth [Alfa Aesar, Ward Hill, MA] and 1.5% agar) and incubated overnight at 37°C, after which a small portion of the bacterial streak was collected with a toothpick and stabbed in the center of a 1% LB agar plate, which was then incubated at 37°C for 24h. To better visualize the twitching motility, the plates were flooded with a small volume of cold Twitching Motility (TM) developer solution (400mL deionized water, 100mL glacial acetic acid (Merck-Millipore, Burlington, MA), 500mL methanol (Merck-Millipore), stored at 4°C) for 30 minutes, after which the top colonies were gently scrapped with a plastic disposable loop. The TM developer solution and scrapped colonies were carefully decanted into a beaker and images of plates were taken using a Bio-Rad Molecular Imager (Gel Doc XR+ system; Bio-Rad, Hercules, CA).

Development of the biofilm model

Except when stated otherwise, biofilms were grown in 96 well plates (European catalog number; 655 180 VWR) as previously described [14] when using TGN but with some modifications in ASM(+). In brief, a bacterial suspension was prepared in cation-adjusted MHB (MHB-ca; Sigma-Aldrich) starting from overnight cultures on TSA. When using TGN for biofilm growth, the 96-well plates were inoculated (200 µL/well) at approximately $3x10^7$ CFU/mL ($OD_{620\ nm}$ adjusted to 0.05) and then incubated at 37°C for 24 h

(unless stated otherwise) to obtain a mature biofilm. When ASM(+) was used, 200 µL of a suspension at $5.5x10^7$ CFU/mL in ASM(-) was inoculated in the 96-well plates and the plates incubated for 24h at 37°C to allow for bacterial attachment, after which the medium was removed and replaced by 200 µL of ASM(+) and plates were reincubated for 24h at 37°C (unless stated otherwise). In all cases, plates were covered by Breathe-Easy® sealing membranes permeable to gas but limiting evaporation (Sigma-Aldrich). All changes of media or washing steps were carefully performed by slow pipetting from the bottom and close to the edge of the well to avoid removing biofilms growing below the surface of the medium.

Susceptibility testing

Minimal Inhibitory Concentrations (MICs) were determined by microdilution following the guidelines of the Clinical and Laboratory Standards Institute using cation-adjusted Mueller-Hinton Broth (MHB-ca) [47], and compared to those measured in TGN and ASM(+) following the same protocol. As previously described [14], direct visual reading of MICs was not possible in ASM(+) due to the intrinsic turbidity of this medium. Aliquots were therefore spread on PIA and incubated overnight, with MICs defined as the lower concentration for which there was no change in the number of CFU as compared to initial values. As bacteria tended to form aggregates in ASM(+), the content of each well was carefully homogenized by back-and-forth pipetting before taking aliquots, diluting them, and spreading them on agar plates.

Activity of antibiotics against biofilms

After reaching maturity (24h for TGN, 48h for ASM [24h in ASM(-) followed by 24h in ASM(+)]), the culture medium of biofilms was removed and replaced with fresh medium (control) or medium supplemented with antibiotics at concentrations ranging from 10^{-3} to 10^2 mg/L in order to obtain full concentration-response curves. The biofilms were reincubated for 24h at 37°C. At the end of the incubation period, the medium was removed and the biofilm was washed once with 200 µL of 3-morpholinopropane-1-sulfonic acid (MOPS) buffer (20.9 g/L of MOPS [Sigma-Aldrich] and 5.6 g/L

NaCl; pH adjusted to 7 with NaOH) [19]. Biofilm biomass was quantified using crystal violet, a cationic dye that non-specifically stains negatively-charged constituents in biofilms. The washed biofilms were fixed by heat at 60°C for about 24h and incubated during 10 minutes at room temperature with 200 µL of crystal violet (final concentration 0.2 g/L; VWR). After removing the excess of crystal violet, plates were washed under running water and dried. The dye fixed to the biofilm was resolubilized in 200 µL of 66% acetic acid and incubated 1h at room temperature. The absorbance at 570 nm was measured using a SPECTRAmax Gemini XS microplate spectrophotometer (Molecular Devices LLC, Sunnyvale, CA) [14]. The metabolic activity (vitality) in biofilms was quantified using the fluorescein diacetate assay (FDA). It is based on the hydrolysis by living bacteria of the non-fluorescent white-colored dye fluorescein diacetate in the yellow-colored highly fluorescent fluorescein [19]. This dye is preferred to resazurin (that we used in *S. aureus* biofilms) to evaluate metabolic activity of *P. aeruginosa*, generating much high fluorescence signals [19]. The washed biofilms were incubated with 100 mg/L fluorescein diacetate (Sigma-Aldrich) for 15 min (unless stated otherwise) at 37°C in the dark. Fluorescein fluorescence was measured at a wavelength of 518nm with an excitation wavelength of 494nm using a SPECTRAmax Gemini XS microplate spectrofluorometer. Bacterial counts were determined in washed biofilms resuspended in 1mL of sterile PBS in microcentrifugation tubes. The tubes were vortexed and placed during 5 minutes in a sonication bath (Bransonic® Ultrasonic cleaner 3510E-MT, frequency 40kHz; Danbury, CT) to disrupt the biofilm, and vortexed again, after which aliquots were taken and diluted before spreading on PIA plates. Unless stated otherwise, colonies were counted after 24 h of incubation at 37°C.

Curve fitting and statistical analyses

Curve fitting analyses were made using GraphPad Prism (version 8.02) software (GraphPad Software, San Diego, CA). Data were used to fit a sigmoid function, which allowed us to calculate maximal efficacy (E_{max}; the maximal reduction in viability, vitality or biomass for an infinitely large

concentration of antibiotic) and relative potencies (the antibiotic concentration needed for a reduction in the number of CFU in the biofilms of 1 log_{10} [C_{-1log}; vitality], which corresponds to the concentration needed for a 0.1 log_{10} reduction in fluorescein fluorescence [vitality] [$C_{-0.1}$] or a 0.3 log_{10} decrease in the crystal violet OD_{570nm} [biomass] [$C_{-0.3}$]). Statistical analyses were performed with GraphPad Instat (version 3.06) software (GraphPad Software) or Graph-Pad Prism (version 8.02) software.

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References to paper #2

[1] Cystic Fibrosis Foundation Patient Registry. Annual Data Report 2017. Cystic Fibrosis Foundation . 2018. 16-11-2018.

[2] Bjarnsholt T, Jensen PO, Fiandaca MJ, Pedersen J, Hansen CR, Andersen CB, Pressler T, Givskov M, & Hoiby N (2009) Pseudomonas aeruginosa biofilms in the respiratory tract of cystic fibrosis patients. *Pediatr. Pulmonol.*, **44,** 547-558.

[3] Ciofu O & Tolker-Nielsen T (2019) Tolerance and Resistance of Pseudomonas aeruginosa Biofilms to Antimicrobial Agents-How P. aeruginosa Can Escape Antibiotics. *Front Microbiol.*, **10,** 913.

[4] Donlan RM & Costerton JW (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.*, **15,** 167-193.

[5] Mann EE & Wozniak DJ (2012) Pseudomonas biofilm matrix composition and niche biology. *FEMS Microbiol. Rev.*, **36,** 893-916.

[6] Ciofu O, Rojo-Molinero E, Macia MD, & Oliver A (2017) Antibiotic treatment of biofilm infections. *APMIS*, **125,** 304-319.

[7] Ciofu O, Tolker-Nielsen T, Jensen PO, Wang H, & Hoiby N (2015) Antimicrobial resistance, respiratory tract infections and role of biofilms in lung infections in cystic fibrosis patients. *Adv. Drug Deliv. Rev.*, **85,** 7-23.

[8] Cohen TS & Prince A (2012) Cystic fibrosis: a mucosal immunodeficiency syndrome. *Nat. Med.*, **18,** 509-519.

[9] Evans TJ (2015) Small colony variants of Pseudomonas aeruginosa in chronic bacterial infection of the lung in cystic fibrosis. *Future. Microbiol.*, **10,** 231-239.

[10] Malone JG (2015) Role of small colony variants in persistence of Pseudomonas aeruginosa infections in cystic fibrosis lungs. *Infect. Drug Resist.*, **8,** 237-247.

[11] Sriramulu DD, Lunsdorf H, Lam JS, & Romling U (2005) Microcolony formation: a novel biofilm model of Pseudomonas aeruginosa for the cystic fibrosis lung. *J. Med. Microbiol.*, **54,** 667-676.

[12] Hoiby N, Bjarnsholt T, Moser C, Jensen PO, Kolpen M, Qvist T, Aanaes K, Pressler T, Skov M, & Ciofu O (2017) Diagnosis of biofilm infections in cystic fibrosis patients. *APMIS*, **125,** 339-343.

[13] Kirchner S, Fothergill JL, Wright EA, James CE, Mowat E, & Winstanley C (2012) Use of artificial sputum medium to test antibiotic efficacy against Pseudomonas aeruginosa in conditions more relevant to the cystic fibrosis lung. *J. Vis. Exp.*, e3857.

[14] Diaz Iglesias Y, Wilms T, Vanbever R, & Van Bambeke F (2019) Activity of Antibiotics against Staphylococcus aureus in an In Vitro Model of Biofilms in the Context of Cystic Fibrosis: Influence of the Culture Medium. *Antimicrob. Agents Chemother.*, **63,** e00602-19.

[15] Palmer KL, Aye LM, & Whiteley M (2007) Nutritional cues control Pseudomonas aeruginosa multicellular behavior in cystic fibrosis sputum. *J. Bacteriol.*, **189,** 8079-8087.

[16] Turner KH, Wessel AK, Palmer GC, Murray JL, & Whiteley M (2015) Essential genome of Pseudomonas aeruginosa in cystic fibrosis sputum. *Proc. Natl. Acad. Sci. U. S. A*, **112,** 4110-4115.

[17] Bauer J, Siala W, Tulkens PM, & Van Bambeke F (2013) A combined pharmacodynamic quantitative and qualitative model reveals the potent activity of daptomycin and delafloxacin against Staphylococcus aureus biofilms. *Antimicrob. Agents Chemother.*, **57,** 2726-2737.

[18] Burrows LL (2012) Pseudomonas aeruginosa twitching motility: type IV pili in action. *Annu. Rev. Microbiol.*, **66,** 493-520.

[19] Peeters E, Nelis HJ, & Coenye T (2008) Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. *J. Microbiol. Methods*, **72,** 157-165.

[20] Tribedi P, Gupta AD, & Sil AK (2015) Adaptation of Pseudomonas sp. AKS2 in biofilm on low-density polyethylene surface: an effective strategy for efficient survival and polymer degradation. *Bioresour. Bioprocess.*, **2,** 14.

[21] Tielen P, Rosenau F, Wilhelm S, Jaeger KE, Flemming HC, & Wingender J (2010) Extracellular enzymes affect biofilm formation of mucoid Pseudomonas aeruginosa. *Microbiology*, **156,** 2239-2252.

[22] Dosler S & Karaaslan E (2014) Inhibition and destruction of Pseudomonas aeruginosa biofilms by antibiotics and antimicrobial peptides. *Peptides*, **62,** 32-37.

[23] Rozenbaum RT, van der Mei HC, Woudstra W, de Jong ED, Busscher HJ, & Sharma PK (2019) Role of Viscoelasticity in Bacterial Killing by Antimicrobials in Differently Grown Pseudomonas aeruginosa Biofilms. *Antimicrob. Agents Chemother.*, **63**.

[24] She P, Wang Y, Liu Y, Tan F, Chen L, Luo Z, & Wu Y (2019) Effects of exogenous glucose on Pseudomonas aeruginosa biofilm formation and antibiotic resistance. *Microbiologyopen.*, e933.

[25] Muller L, Murgia X, Siebenburger L, Borger C, Schwarzkopf K, Sewald K, Haussler S, Braun A, Lehr CM, Hittinger M, & Wronski S (2018) Human airway mucus alters susceptibility of Pseudomonas aeruginosa biofilms to tobramycin, but not colistin. *J. Antimicrob. Chemother.*, **73,** 2762-2769.

[26] Tseng BS, Zhang W, Harrison JJ, Quach TP, Song JL, Penterman J, Singh PK, Chopp DL, Packman AI, & Parsek MR (2013) The extracellular matrix protects Pseudomonas aeruginosa biofilms by limiting the penetration of tobramycin. *Environ. Microbiol.*, **15,** 2865-2878.

[27] Huang JX, Blaskovich MAT, Pelingon R, Ramu S, Kavanagh A, Elliott AG, Butler MS, Montgomery AB, & Cooper MA (2015) Mucin Binding Reduces Colistin Antimicrobial Activity. *Antimicrob. Agents Chemother.*, **59,** 5925-5931.

[28] Schaaff F, Bierbaum G, Baumert N, Bartmann P, & Sahl HG (2003) Mutations are involved in emergence of aminoglycoside-induced small colony variants of Staphylococcus aureus. *Int J. Med. Microbiol.*, **293,** 427-435.

[29] Gerber AU & Craig WA (1982) Aminoglycoside-selected subpopulations of Pseudomonas aeruginosa: characterization and virulence in normal and leukopenic mice. *J. Lab Clin. Med.*, **100,** 671-681.

[30] Musher DM, Baughn RE, & Merrell GL (1979) Selection of small-colony variants of Enterobacteriaceae by in vitro exposure to aminoglycosides: pathogenicity for experimental animals. *J. Infect. Dis.*, **140,** 209-214.

[31] Drescher SPM, Gallo SW, Ferreira PMA, Ferreira CAS, & Oliveira SDd (2019) Salmonella enterica persister cells form unstable small colony variants after in vitro exposure to ciprofloxacin. *Sci. Rep.*, **9,** 7232.

[32] Belfield K, Bayston R, Hajduk N, Levell G, Birchall JP, & Daniel M (2017) Evaluation of combinations of putative anti-biofilm agents and antibiotics to eradicate biofilms of Staphylococcus aureus and Pseudomonas aeruginosa. *J. Antimicrob. Chemother.*, **72,** 2531-2538.

[33] Sousa AM, Monteiro R, & Pereira MO (2018) Unveiling the early events of Pseudomonas aeruginosa adaptation in cystic fibrosis airway environment using a long-term in vitro maintenance. *Int J. Med. Microbiol.*, **308,** 1053-1064.

[34] Kirisits MJ, Prost L, Starkey M, & Parsek MR (2005) Characterization of colony morphology variants isolated from Pseudomonas aeruginosa biofilms. *Appl. Environ. Microbiol.*, **71,** 4809-4821.

[35] von Gotz F, Haussler S, Jordan D, Saravanamuthu SS, Wehmhoner D, Strussmann A, Lauber J, Attree I, Buer J, Tummler B, & Steinmetz I (2004) Expression analysis of a highly adherent and cytotoxic small colony variant of Pseudomonas aeruginosa isolated from a lung of a patient with cystic fibrosis. *J. Bacteriol.*, **186,** 3837-3847.

[36] Sabra W, Haddad AM, & Zeng AP (2014) Comparative physiological study of the wild type and the small colony variant of Pseudomonas aeruginosa 20265 under controlled growth conditions. *World J. Microbiol. Biotechnol.*, **30,** 1027-1036.

[37] Haussler S, Tummler B, Weissbrodt H, Rohde M, & Steinmetz I (1999) Small-colony variants of Pseudomonas aeruginosa in cystic fibrosis. *Clin. Infect. Dis.*, **29,** 621-625.

[38] Drenkard E & Ausubel FM (2002) Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature*, **416,** 740-743.

[39] Haley CL, Colmer-Hamood JA, & Hamood AN (2012) Characterization of biofilm-like structures formed by Pseudomonas aeruginosa in a synthetic mucus medium. *BMC. Microbiol.*, **12,** 181.

[40] Pompilio A, Crocetta V, Pomponio S, Fiscarelli E, & Di Bonaventura G (2015) In vitro activity of colistin against biofilm by Pseudomonas aeruginosa is significantly improved under "cystic fibrosis-like" physicochemical conditions. *Diagn. Microbiol. Infect. Dis.*, **82,** 318-325.

[41] Quinn RA, Comstock W, Zhang T, Morton JT, da Silva R, Tran A, Aksenov A, Nothias LF, Wangpraseurt D, Melnik AV, Ackermann G, Conrad D, Klapper I, Knight R, & Dorrestein PC (2018) Niche partitioning of a pathogenic microbiome driven by chemical gradients. *Sci. Adv.*, **4,** eaau1908.

[42] Mustafa MH, Chalhoub H, Denis O, Deplano A, Vergison A, Rodriguez-Villalobos H, Tunney MM, Elborn JS, Kahl BC, Traore H, Vanderbist F, Tulkens PM, & Van Bambeke F (2016) Antimicrobial Susceptibility of Pseudomonas aeruginosa Isolated from Cystic Fibrosis Patients in Northern Europe. *Antimicrob. Agents Chemother.*, **60,** 6735-6741.

[43] Siala W, Mingeot-Leclercq MP, Tulkens PM, Hallin M, Denis O, & Van Bambeke F (2014) Comparison of the antibiotic activities of Daptomycin, Vancomycin, and the investigational Fluoroquinolone Delafloxacin against biofilms from Staphylococcus aureus clinical isolates. *Antimicrob. Agents Chemother.*, **58,** 6385-6397.

[44] Ghani M & Soothill JS (1997) Ceftazidime, gentamicin, and rifampicin, in combination, kill biofilms of mucoid Pseudomonas aeruginosa. *Can. J. Microbiol.*, **43,** 999-1004.

[45] Tavernier S & Coenye T (2015) Quantification of Pseudomonas aeruginosa in multispecies biofilms using PMA-qPCR. *PeerJ.*, **3,** e787.

[46] Turnbull L & Whitchurch CB (2014) Motility assay: twitching motility. *Methods Mol. Biol.*, **1149,** 73-86.

[47] Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; 28th Informational Supplement. CLSI document M100-S28. Wayne, PA. 2018.

[48] Karaiskos I, Friberg LE, Pontikis K, Ioannidis K, Tsagkari V, Galani L, Kostakou E, Baziaka F, Paskalis C, Koutsoukou A, & Giamarellou H (2015) Colistin Population Pharmacokinetics after Application of a Loading Dose of 9 MU Colistin Methanesulfonate in Critically Ill Patients. *Antimicrob. Agents Chemother.*, **59,** 7240-7248.

Chapter IV. Discussion & Perspectives

1. Main achievements of this work

1.1. Development of Artificial Sputum Medium

Biofilms are a major health concern, accounting for up to 80% of bacterial infections. Those infections are hard to eradicate because of the biofilm properties (tolerance to antibiotics and escape from immune system). Therefore it is important to use *in vitro* models relevant to the environment where these biofilms develop. This relevance also concerns the culture medium. A first achievement of this work was therefore to develop an artificial sputum medium as a tool mimicking the properties of the mucus from CF. It is also to note that before the elaboration of this version of artificial sputum medium with agar, we started our experiments on *Pseudomonas aeruginosa* with the exact same medium without agar and generated results for the five antibiotics of interest in our study (see **Paper 2 Results** and unpublished figure 1). However, the rheological studies performed on this medium did not correspond to results observed on patients' mucus and thus we discarded the medium without agar due to its lack of relevance.

Mucus can be classified in two groups: purulent or mucopurulent (based on macroscopic characteristics [169;181]). Purulent and mucopurulent mucus show different viscoelastic properties but in both groups, elastic moduli was higher than viscous moduli for the same range of shear strains [169;181-183]. Similarly, the artificial sputum medium developed in this thesis has a higher elasticity than viscosity and our rheology results for ASM are close to those observed for mucopurulent sputa. We have to acknowledge, however, that we compared our ASM to a small number of sputum samples (we used 6 samples, including 3 mucopurulent and 3 purulent) although we know from literature that sputum rheological properties can fluctuate from patient to patient [181;182] and even in one patient over time [183]. Taking these limitations into account, it remains that the artificial sputum medium developed during this thesis possesses rheological properties that are close to those of the mucus from at least part of the CF population.

1.2. *In vitro* models for mono species biofilms

Our laboratory previously published an *in vitro* model in 96-well plates to investigate the activity of antibiotics against *S. aureus* [180]. The use of microtiter plates for *in vitro* models of biofilm is widespread [166;180;184-187] because it has some advantages: there is no need for advanced equipment, it allows to test many conditions in parallel and it is quite inexpensive because it only requires small amounts of material and drugs [188]. However, the model used in our laboratory until now uses as medium trypticase soy broth supplemented with glucose and NaCl (TGN), a medium optimized for *S. aureus* biofilms growth but which is not a relevant model for CF. The use of TGN allows quick growth of biofilm and quick evaluation of the activity of antibiotics and we used it here for comparison purposes with ASM. Both media can be used to study the kinetics of biofilm development and the pharmacodynamic parameters of antibiotics administered to patients.

To define the optimal experimental conditions to test antibiotics against the biofilms of *S. aureus* and *P. aeruginosa* we studied the kinetics of biofilm development and the time needed to reach a stable biofilm (maturity) in terms of bacterial counts and metabolic activity. We optimized the use of TGN for *P. aeruginosa* in order to obtain a stable biofilm after 24h of incubation as we observed for *S. aureus*. In ASM, the maturity of the biofilm was reached after 24h of incubation for *S. aureus*. However, we observed a delay in the increase of bacterial counts and metabolic activity for *P. aeruginosa,* the maturity being reached only after 48h of incubation. This illustrates that the kinetics of development is heavily dependent on the medium and the species used. For example, Pompilio *et al*. [189] worked on *P. aeruginosa* biofilms using cation-adjusted MHB (MHB-ca) as medium and observed that 5 days of culture were needed to obtain a stable biofilm and use it to test antibiotics. In our hands (unpublished data), we demonstrated for strain PAO1 (not used by Pompilio *et al*.) that maturity was reached after

4 days of incubation in MHB-ca (Figure 27) based on metabolic activity and biomass measures.

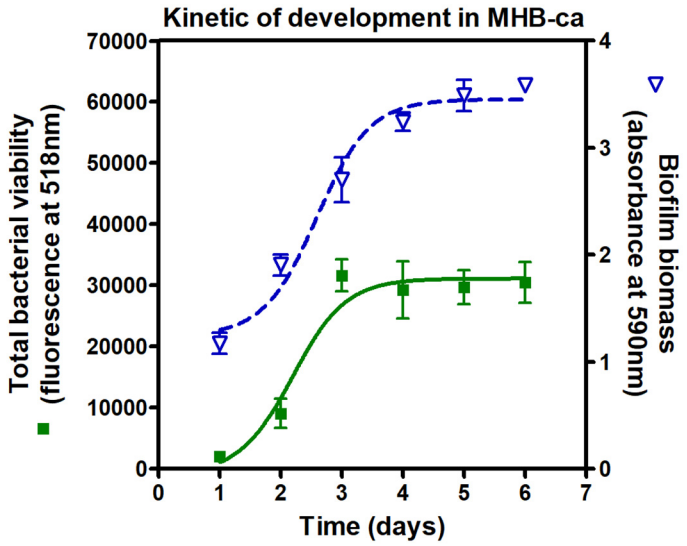

Figure 27: Kinetics of biofilm of *P. aeruginosa* using cation-adjusted Mueller-Hinton broth. The graph shows the evolution over time of fluorescein fluorescence generated by hydrolysis of fluorescein diacetate (FDA) by metabolically active bacteria (metabolic activity or vitality; left axis) and crystal violet absorbance (biomass; right axis).

In the context of our study, we decided to expose biofilms to antibiotics once they have reached maturity because, with the exception of *S. aureus* prophylaxis [37;38;40], treatments are given to patients when they present an established infectious exacerbation [4-6], which implies that biofilms are already present in the airways of those patients.

Different parameters exist to evaluate the activity of antibiotics on biofilms like the minimum biofilm inhibitory concentration (MBIC) and the minimum biofilm eradication concentration (MBEC) [128;189-197]. MBIC and MBEC are sessile counterparts of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) used for planktonic bacteria. Values obtained for MBIC and MBEC are much higher than the MIC and MBC [129;188;198;199]. For example, Dosler and Karaaslan [191] determined for *P. aeruginosa* that the MBEC of ciprofloxacin, tobramycin and colistin were

300 to 600 fold higher than the MIC in TSB supplemented with 1% glucose and that the MBEC for β-lactams was more than 2500 fold higher than the MIC. In another study on 12 strains of *P. aeruginosa,* Pompilio et al. [189] found that the $MBEC_{90}$ for colistin was 256 fold higher than the MIC_{90} when using MHB-ca but 512 fold higher when the experiment was performed in "CF-like conditions" (anaerobic atmosphere and acidic pH). Abad et al. [192] found that MBEC of linezolid against *S. aureus* was 1400 fold higher than the MIC.
These high MBIC and MBEC concentrations are difficult to reach in vivo, as antibiotic dosing is limited by the risk of adverse events [128].

Very few studies have looked into the details of the pharmacodynamics of antibiotic activity, which implies the analysis of concentration-response curves or of time-response effects. MBIC/MBEC are fixed values and they do not give any detailed information about the time- or concentration-dependence of the effect of antibiotics. Together with the lack of standardization of the models, a proper comparison between the results found in the literature is thus difficult [199].

In our studies, we focus on pharmacodynamic parameters like the maximal efficacy (E_{max}) and the relative potency (C) as measured after a fixed time of exposure to antibiotics. These parameters are based on concentration-response curves obtained by exposing mature biofilms to increasing antibiotic concentrations, covering the range that can be achieved *in vivo*. This analysis allowed us to conclude that antibiotics are less effective (E_{max} less negative) against biomass than viability (CFU counts) or metabolic activity for both *S. aureus* and *P. aeruginosa*. Moreover, we also showed that efficacy is influenced by the culture medium. Antibiotics are generally more effective on biofilms growing in TGN, since $\mathbf{E_{max}}$ determined for CFU counts are more negative or similar to those measured in ASM. There are only two exceptions, namely vancomycin and tobramycin, for which E_{max} were more negative in ASM than in TGN against *S. aureus* strain ATCC 25923.

To evaluate relative potency, we calculated concentrations allowing us to reach small but reproducibly measurable effects, like a 1 $\log_{10}$ decrease in CFU or a 20% reduction in biomass. This contrasts with many studies, which use extremely high antibiotic concentrations in order to measure MBEC and was made possible by the design of our experiments that explored full concentration-response curves, covering also the range of concentrations that can be achieved *in vivo* after intravenous administration. When we compare these values of relative potencies for both strains to the concentration achievable in human blood (C_{max}), we clearly see that a decrease of 1 $\log_{10}$ in CFU counts (**C_{-1log}** decrease in CFU counts) is rarely reached in ASM for *S. aureus* but is easily achieved for *P. aeruginosa* in both media.

In our work, three antibiotics where used against both species: ciprofloxacin, meropenem and tobramycin. The results show that tobramycin was the antibiotic with the most consistent efficacy against both *P. aeruginosa* and *S. aureus* biofilms in the two different media used here, with maximal efficacies that are higher than a 2 $\log_{10}$ CFU decrease, indicating a high reduction of CFU counts. All the other antibiotics were only used against one bacterial species and they did not show more activity than tobramycin, ciprofloxacin or meropenem. However, antibiotics like ceftazidime or colistin for *P. aeruginosa* and vancomycin for *S. aureus*, showed an effect similar to that of tobramycin, meropenem and ciprofloxacin. Other antibiotics (linezolid, azithromycin and rifampicin) lost efficacy in ASM (measured by CFU counts) against *S. aureus*.

As we already discussed in **Chapter I, part 3.1. Definition and global overview**, decreased antibiotic penetration does not totally explain increased tolerance of biofilms [144;200] but nutritional limitation (nutrient, oxygen, pH) could probably partially explain the loss of metabolic activity in ASM we observed against *S. aureus* and *P. aeruginosa*. However, we did not modulate nor measure such parameters in our studies [174;201]. This could be the starting point of further investigations because it has been shown

that *P. aeruginosa* can sustain metabolic activity under anaerobic conditions through arginine (which enters in the composition of our ASM) fermentation. Thus arginine can improve the activity of antibiotics like tobramycin or ciprofloxacin by increasing metabolic activity in the less oxygenated areas of the biofilms [35].

In *P. aeruginosa* biofilm, the activity of tobramycin was higher in TGN than in ASM. A similar observation was made by Rozenbaum *et al.* [176] who demonstrated higher activity of tobramycin in LB medium than in their version of artificial sputum medium (without agar). However, they used much higher concentrations (from 10 to 50 fold higher than our highest tested concentration of 100mg/L) than those we tested here. These authors attributed this difference between the two media to an enrichment of the biofilm matrix in polysaccharides in their version of artificial sputum medium. This explanation may, however, not apply to our study because TGN is supplemented by glucose, which upregulates the expression of the extracellular polysaccharide-related gene *pslA*, required for biofilm formation [196]. In another study performed on *P. aeruginosa* biofilms, the addition of frozen mucus from patients with CF to preformed biofilms was shown to decrease the activity of tobramycin when compared to biofilm grown in PBS. The decreased activity of tobramycin in mucus was related to a reduced transport of this polycationic drug in the biofilm [202] due to its strong ionic interactions with components of the biofilm matrix and/or mucus [197].

Concerning *S. aureus* biofilm, the activity of tobramycin was also higher in TGN than in ASM. In a study made by Waryah *et al*. [187] using a unique tobramycin concentration of 0.75mg/L to treat a biofilm grown in nutrient broth supplemented with 1% glucose, they showed a reduction of 1 $\log_{10}$ CFU count after 2.5h of incubation in an orbital shaker at 60rpm while we did not observe any effect at this concentrations. This discrepancy could possibly be ascribed to major differences in the experimental conditions. More specifically, our own biofilm were not exposed to orbital movements

which create shear stress influencing biofilm formation and response to antibiotics [203;204].

The effect of ciprofloxacin against the biofilm of *P. aeruginosa* and *S. aureus* is variable among these two species. In fact, ciprofloxacin is highly active in both media against PAO1 biofilm with a high reduction in the CFU counts (around 3 $\log_{10}$ of reduction in CFU counts, which is close to the definition of the MBEC) but its effect on staphylococci is higher in TGN than in ASM and did not reach 3 $\log_{10}$ of reduction.

Dosler and Karaaslan [191] determined for *P. aeruginosa* that ciprofloxacin MBECs were 300 to 600 fold higher than the MIC depending on the strain. Another group [205] using the same laboratory strain found that MBEC for ciprofloxacin were 150-fold higher than MIC. In our experiments using ciprofloxacin, we measured with the highest concentration (100mg/L corresponding to 1600-fold the MIC in ASM) a reduction of around 3 $\log_{10}$ in CFU counts. Considering that a reduction of 3 $\log_{10}$ corresponds to a 99.9% reduction in viability, this effect is thus close to the definition of the MBEC, which is the lowest concentration of an antibiotic that prevents visible growth in the recovery medium used to collect biofilm cells [129]. Thus we can estimate that the MBEC is close to 100 mg/L in both media; i.e. 800-fold higher than the MIC in TGN and 1600-fold higher than the MIC in ASM, which is slightly higher than the value described in the literature.

Concerning *S. aureus*, we only observe a 3 $\log_{10}$ of reduction in CFUs counts for the MRSA strain in TGN. This reduction could correspond to an MBEC that is 400-fold higher than the MIC measured in the corresponding medium. Those values are lower than those observed by Masadeh *et al*. [205] who found that MBEC of ciprofloxacin against a MRSA was 1800-fold higher than the MIC. The discrepancy in these results could probably be explained by the differences in strains and/or medium used in the work of Masadeh *et al*. and our study.

Meropenem showed a moderate effect against the biofilm of PAO1 but its effect against ATCC 25923 biofilm in ASM is two times lower than in TGN and its effect against ATCC 33591 is low because the strain is resistant to β-lactams.

Dosler *et al*. [191] show, for *P. aeruginosa*, that the MBEC for β-lactams (piperacillin and doripenem) were more than 1250-fold higher than the MIC. Ceftazidime and meropenem, the two β-lactams we used at 100mg/L, almost reached 2 $\log_{10}$ reductions in CFU counts, which correspond to a 99% reduction in viability and correspond respectively to 400 and 800 fold the MIC values for TGN. However, we did not reach 3 $\log_{10}$ reduction in CFU counts in the range of concentration used, therefore we cannot compare our results with these published MBEC values.

Those studies thus highlight the importance of having a standardized model to easily compare the results obtained for different drugs, bacteria, or media.

1.3. Small-Colony variants

An interesting observation we made during our studies was the presence of small colony variants for both *S. aureus* and *P. aeruginosa* when biofilms were grown in ASM after a treatment with tobramycin. In general, tobramycin and other aminoglycosides are known to induce the formation of small colony variants of both *S. aureus* and *P. aeruginosa, in vitro* and *in vivo* [206-213]. We also showed that ciprofloxacin induced the formation of *P. aeruginosa* SCVs in both media which was less anticipated. Much less is known about SCVs selected by fluoroquinolones.

The presence of *S. aureus* and *P. aeruginosa* SCVs in the CF population is well described, as they can emerge after long-term exposure to antibiotics [208;209;214;215]. Moreover, *P. aeruginosa* SCVs are considered as participating to the physiopathology of biofilm-related infections in the lungs of patients with CF [216]. *S. aureus* SCVs can represent

14% to 24% of the total *S. aureus* isolations in the CF population and are associated with worse CF respiratory outcomes [207;217].

Importantly, SCVs of *S. aureus* can emerge as a survival strategy in the presence of *P. aeruginosa* [42;218]. *P. aeruginosa* produces molecules like HQNO and pyocyanin that are known to block the oxidative respiration which inhibit *S. aureus* growth, and select for the SCV phenotype that promotes protection to killing by *P. aeruginosa* [42;104;218;219].

1.4. Clinical Isolates

Lastly, we demonstrated that our model of *P. aeruginosa* biofilm was applicable to clinical isolates recovered from patients with CF. *P. aeruginosa* clinical isolates are known to produce a variable amount of biomass [189], and biomass is known to influence antibiotic activity [73;128]. For our study, we selected four strains that were fully susceptible to the antibiotics we tested. Although this is not representative of the situation faced by many patients since our aim was to run a pharmacological comparison with PAO1 and thus evaluate the possible interest of the tested drugs against isolates susceptible to them.

The four clinical strains produced, as expected, different levels of biomass which were in all cases more elevated than for the reference strain. It is interesting that all the strains tested form biofilms below the surface of the medium, as previously described, and show the presence of bacterial aggregates similar to those found in the sputum of patients with CF [175;220]. Even though these strains produce more biomass with similar CFU counts, we did not observe a lower susceptibility to antibiotic activity when comparing clinical strains to reference strain, which was rather unexpected. Biomass is known to play a role in the antibiotic penetration and a plausible explanation can come from the composition of the matrix as none of our clinical strains is characterized in terms of exopolysaccharide production (two were mucoid thus probably producing alginate while we

know that PAO1 mainly produces Psl polysaccharide [200]). Exopolysaccharide are known to play a role in biofilm tolerance but there are many other parameters that could play a role like antibiotic penetration, gradients of nutrients, etc. [144;200].

Unfortunately, we did not have the opportunity to use clinical isolates of *S. aureus* which would have been a clear perspective of this work. As for *P. aeruginosa*, *S. aureus* clinical isolates show variable levels of metabolic activity and biomass which may affect antibiotic effects [185].

2. Limitations of this work

The models we developed have some merits, as described above, but still do not optimally take into account a series of parameters, among which the most important ones are discussed in this chapter.

2.1. Rheological properties of ASM

One of our objectives was to develop a relevant medium to study the activity of antibiotics in the context of CF. We showed in this thesis that we achieved to develop a medium that shares the same rheological properties as mucopurulent sputa collected from part of the CF population. However, our artificial sputum medium does not mimic the rheological properties of the purulent mucus produced by the other part of the CF population. The viscosity in our medium was brought by the addition of agar to the medium [167]. Further increasing the concentration of agar in the medium to obtain viscoelastic properties closer to those of purulent mucus may bring difficulties to keep the medium liquid enough to pipet it or to work with it in 96-well plates. Moreover, changing the medium to add the antibiotics without detaching the biofilm will become extremely difficult to do.

2.2. The growth environment

The main objective of this thesis was to develop models of mono-species biofilm for high throughput screening of antibiotics using a relevant

medium. To reach our goals, we set up an easy model that can be quickly adapted to different species and to clinical strains. However, pH and oxygenation levels in the CF lungs may affect the bacterial growth and the antibiotic activity [189;221].

For example, the pH of the mucus can vary between 6.3 and 7.7 [165;222] and it has been shown that the activity of some antibiotics like aminoglycosides could be impaired by a reduction of the pH [223;224].

Gradients of oxygen are observed in CF lungs, with some zones characterized as microaerobic or anaerobic [225]. It has been demonstrated, in planktonic cultures, that anaerobic conditions can affect the activity of antibiotics like tobramycin (amikacin and aztreonam) with elevation of the MICs compared to those measured in aerobic conditions [226]. Anaerobic conditions also impact the activity of antibiotic against biofilms, ceftazidime (cefepime and imipenem) show less activity [227] which is also the case for ciprofloxacin and tobramycin [175;228].

Both *S. aureus* and *P. aeruginosa* are facultative aerobes which makes their growth in low oxygen environment possible [61;88]. Moreover, in this particular condition of lack of oxygen, *S. aureus* and *P. aeruginosa* can adapt by producing exopolysaccharides, respectively, PIA [229] and alginate [225], which favor the biofilm growth and survival.

2.3. The pharmacokinetic profile of antibiotics

Our experiments were performed by exposing biofilms to a broad range of concentrations of antibiotics but which remained constant during the whole period of incubation of 24h. Obviously, our static model does not mimic the pharmacokinetic profile of the drugs in the lungs. Dynamic models do exist and have previously been used in our laboratory [230]. They allow to reproduce pharmacokinetic fluctuations over time. For example, in the context of the CF, we can simulate the intravenous administration of an

antibiotic or the inhaled/nebulized administration. However, the development of a relevant pharmacokinetic model is not straightforward. Indeed, if we model the pharmacokinetics by intravenous route, this will not take into consideration that (i) the antibiotics will first need to access the lungs, where their pharmacokinetics could be different than in plasma, and (ii) biofilms grow in mucus which cannot be used to mimic plasmatic pharmacokinetic profile. For inhaled or nebulized administration, there is no counter-argument to try mimicking antibiotic pharmacokinetics in this compartment because antibiotics directly reach lungs and the mucus. Specific devices also exist to evaluate drug deposition in the lungs by this route but these are not compatible with models of infection.

2.4. Concentration of antibiotics in the sputum of CF

In our studies, we compared the concentration of antibiotics needed to reduce the viability (through CFU counts) in our ASM model to the maximal concentration of antibiotic present in human blood. We conclude in the *S. aureus* paper [174] that blood concentrations were globally too low to reduce by 1 log the CFU counts, the only exceptions being meropenem, vancomycin and tobramycin against the MSSA strain ATCC 25923. In the *P. aeruginosa* paper [201], we conclude that blood concentrations were high enough to reduce by 1 log the CFU counts in our ASM model, the only exception being colistin that did not achieve this effect at concentrations reached in blood.

Yet, blood concentrations do not systematically correspond to those that effectively reach the lungs and the biofilms that grow in the sputum. Thus, the concentration of antibiotic in the sputum is probably a better indicator for the efficacy of antibiotics against biofilms in the sputum. However, the concentrations that can be measured in the sputum may differ depending of the route on administration of the drug (intravenous, oral or inhaled). Inhaled antibiotics reach higher local concentrations that can compensate for poor penetration of antibiotics within the biofilm or drug adsorption to sputum. Such high concentrations also prevent the

development of resistance by hypermutators strains or act upon persister phenotypes [124;197;231]. Unfortunately, only few antibiotics are available for inhalation: only 3 antibiotics out of 9 used in our studies are clinically available for inhalation (ciprofloxacin, tobramycin and colistin) and all directed towards *Pseudomonas.* Another one is still in phase III of development against *S. aureus* (vancomycin).

Table 8 shows the concentrations measured in sputum in function of the administration route for all the antibiotics used in our studies. Globally, we observed that inhaled antibiotics can reach higher concentrations in sputum but the results show large variations (ciprofloxacin 35 to 376 mg/L [232]; tobramycin 35 to 7414 mg/L [233-236]; vancomycin 10 to 300 mg/L [237]; colistin 6 to 178 mg/L [234;238-240]). The concentrations reachable in sputum with inhaled antibiotics are thus much higher than the concentrations needed to reduce by 1 log the CFU counts in our model. This indicates that this route of administration is probably better to achieve an effective reduction of the bacterial populations forming biofilms in the sputum. The two other administration routes (oral and intravenous) achieve lower concentrations in the sputum that do not always cover the concentrations needed to reduce by 1 log the CFU counts in our model.

Antibiotics	Route	C_{max} (mg/L) in sputum	References	$C_{(-1log\ CFU)}$ (mg/L)		
				ATCC		
				25923	33591	PAO1
Meropenem	Intravenous	2.2	[241]	0.26	100	1.88
Ceftazidime	Intravenous	9.5	[236]	-	-	0.99
Linezolid	Oral	3.6 - 17.4	[242]	100	100	-
Azithromycin	Oral	9.5 - 53	[243-245]	100	100	-
Ciprofloxacin	Inhalation	35 - 376	[232]	100	100	0.23
	Intravenous	0.39 - 1.02	[246]			
Tobramycin	Inhalation	35 - 7414	[233-235]	4.59	100	5.57
	Intravenous	1.8 - 5.7	[234;236]			
Vancomycin	Inhalation	10 - 300	[237]	47.59	100	-
Colistin	Inhalation	6 - 178	[234;238-240]	-	-	54.05
	Intravenous	0.12 - 0.72	[238]			
Rifampicin	Oral	1 – 3[a]	[247]	100	100	-
	Oral	12[b]	[248]			

Table 8: Concentration of antibiotics in the sputum following oral, intravenous or inhaled administration.

[a]: values measured in mucopurulent sputum from chronic bronchitis

[b]: values measured in sputum from tuberculous patients

In conclusion, sputum concentrations reached with inhaled antibiotics (ciprofloxacin, tobramycin and vancomycin) will achieve better efficacy against *S. aureus* strains when compared to the results we measured in our model to reduce by 1 log the CFU count. The same observation is made for inhaled colistin, the measured sputum concentrations are higher than the value we calculated to reduce by 1 log the CFU counts.

These comparisons with sputum concentrations need however to be considered with caution, because pharmacokinetic studies in sputum are usually performed on a limited number of patients and show huge variability (2 to 30 patients per study, mean 11 patients) [231-234;236-238;241-248]. The concentrations measured in sputum are also highly variable depending on the route of administration.

2.5. Clinical isolates

The principal objective of this thesis was to set up a model of mono species biofilms to be used for high throughput screening of antibiotic pharmacodynamics profile. To achieve our goal, we set up our models using laboratory strains of *S. aureus* and *P. aeruginosa*. Those strains are well characterized in terms of antibiotic susceptibility and their whole genome is sequenced. Yet, they do not represent the diversity that can be encountered with clinical isolates because these present different characteristics for biofilm growth, susceptibility to antibiotics, biomass production, etc. [185;189]. In our work with *P. aeruginosa*, we included four clinical strains, however, those strains were selected on purpose to be fully susceptible to the antibiotics used in the study. Thus they do not represent the diversity encountered in clinical isolates and the future experiments should consider the use of clinical isolates from *S. aureus* and *P. aeruginosa*.

3. Perspectives

3.1. Modulation of the experimental conditions for growing biofilms or testing antibiotics

3.1.1. pH and oxygenation

As previously described in the paragraph on **Chapter IV. Part 2. Limitations of this work**, two parameters, pH and oxygenation, can affect the bacterial growth and the antibiotic activity. We did not modulate them during this thesis but this could be easily done in the future. The pH of ASM can be adjusted to more acidic values in order to study the effect on biofilm growth and antibiotic activity and microaerophilic conditions can be obtained using CampyGen gas generation packs in large anaerobic jars, those packs will generate a microaerophilic environment composed of 5% O_2, 10% CO_2 and 85% N_2 [175].

3.1.2. Dynamic *in vitro* biofilm model

Our static *in vitro* biofilm model only allows pharmacodynamic evaluation. To evaluate the pharmacokinetic profiles, the best option is the use of a dynamic *in vitro* model. Our laboratory used the CDC biofilm reactor

(Figure 28) [230], in which biofilms grow under shear stress and fluid flow. To test antibiotic activity, this fluid flow can be adjusted to simulate different pharmacodynamic profiles. For example in the context of the CF, we can simulate the intravenous administration of an antibiotic or the inhaled/nebulized administration, with, however, the caveats expressed in the paragraph on the limitations of the study.

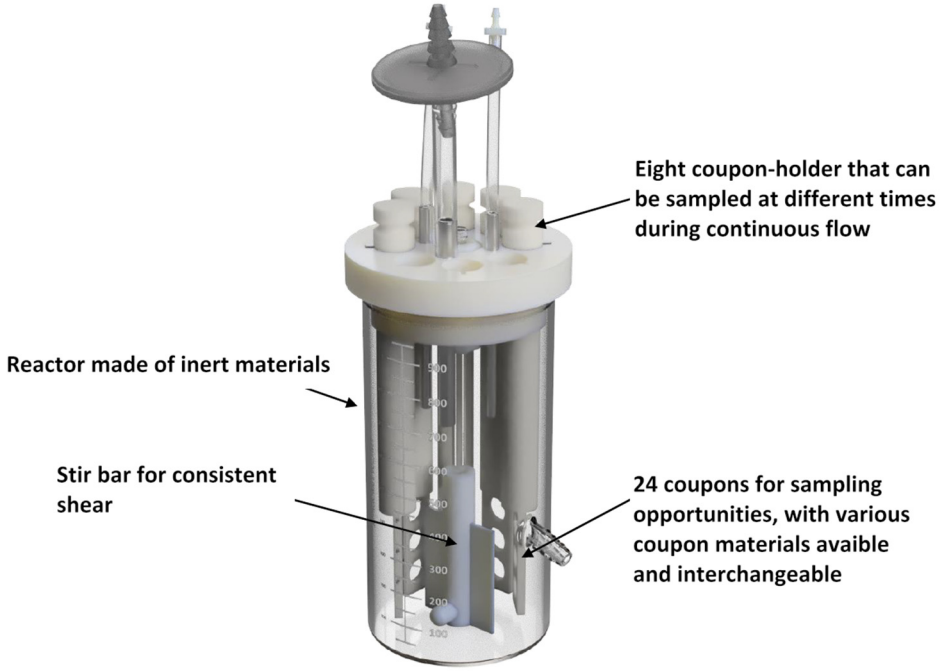

Figure 28: CDC biofilm reactor is composed of a 1L jar with an effluent spout to evacuate used medium and a lid that supports eight independent coupon-holder, a stir bar and a tube for fresh medium entry. Each coupon-holder can handle three removable coupons. Coupons will allow biofilm growth and they can be made from different materials (polycarbonate, stainless steel, titanium, PVC, etc.). The lid also holds a stir bar that is magnetically driven and due to this rotation, the coupons will experience high shear. The CDC biofilm reactor operates as a continuous flow stirred tank reactor, meaning nutrients are continuously pumped into and flow out of the reactor at the same rate. The addition of a peristaltic pump upstream of the jar allow to modulate the flow speed (image adapted from [249]).

3.2. Clinical isolates

Future works should integrate clinical isolates for both *S. aureus* and *P. aeruginosa* species and they should be selected on the basis of their profile of resistance. As we discussed in **Chapter I. part 1.5. Microbiology of CF lung disease**, the rate of resistant strains in both species is high with almost 30% of MRSA strains and 20% of MDR-PA recovered from patients. Such strains could be included, for example, to investigate other therapeutic strategies.

3.3. Other therapeutic strategies (combination therapy)

In this work, we only evaluated the activity of antibiotics in monotherapy against biofilms. A common strategy to cope with difficult-to-treat infections consists of combining antibiotics or combining antibiotics with adjuvants that do not kill bacteria but increase their activity by inhibiting resistance mechanisms or acting on the biofilm itself.

Focusing first on antibiotic combinations, there are multiple rationales for using them. First, they permit to cover a broad spectrum of pathogens during polymicrobial infections or in non-documented infections. Second, the combination of two antibiotics has the potential to decrease the rate of resistance [250]. And third, antibiotic combinations might result in synergistic effects [251;252].

The use of combinations in *in vitro* or *in vivo* models is already well established, different studies having shown that combining two antibiotics increases the effect when compared to antibiotics alone.

Rifampicin is often use in combination against *S. aureus* biofilms, first to avoid the quick emergence of resistant mutants [86] and second because of demonstrated synergy, as illustrated for vancomycin [253] or linezolid [254]. Another antibiotic used in combination is fusidic acid. When combined to daptomycin, linezolid or vancomycin, fusidic acid also shows increased effects against *S. aureus* biofilms [230].

Against *P. aeruginosa* biofilms, a combination of tobramycin with ceftazidime [255] or clarithromycin [251] showed increased effects.

P. aeruginosa is resistant to β-lactams because of the production of β-lactamases which are secreted in the matrix of the biofilms. The secreted β-lactamases protect producing and non-producing bacteria from the activity of the β-lactams and thus they play a role in biofilm resistance [145]. To counter the action of β-lactamases, anti-β-lactamases molecules were developed and used in combination with β-lactams (ticarcillin & clavulanic acid, piperacillin & tazobactam) and are commonly given to the patients [40].

Combination therapy has the potential to decrease the rate of resistance [250;256] because the occurrence of multiple resistance mutations in a single bacterium is very unlikely yet not impossible. Yet, some resistance mechanism can affect simultaneously unrelated antibiotic classes. Vestergaard *et al*. [257] showed that the exposition of planktonic *P. aeruginosa* to a combination of ceftazidime and ciprofloxacin leads to the emergence of resistance to both drugs through the overexpression of the MexAB-OprM efflux pump. This efflux pump is known to facilitate the efflux of the two antibiotics and thus confers broad-spectrum antibiotic resistance to *P. aeruginosa*.

In a poster for the Annual Symposium of the Belgian Society of Microbiology [258] (See annexes for the poster), we had the opportunity to develop and test a protocol for *S. epidermidis* biofilms in the context of endocarditis. In this work, we used the combination of daptomycin and one β–lactam (nafcillin or ceftaroline) because this combination has already showed synergetic effects against *S. aureus* bacteremia [259]. We have shown in this study that the effect of the combination of daptomycin and ceftaroline was only slightly improved compared to the effect of each of these drugs given alone, while the combination of daptomycin with nafcillin was markedly more active than the corresponding monotherapies. This illustrates the importance to look for combination between antibiotics.

Another kind of strategy that can be evaluated consists of the combination of an antibiotic and an adjuvant molecule. Mucolytic agents could be of prime interest in the context of CF. Their role is to interact with the stagnant mucus in the airways and to disrupt or alter its structure in order to facilitate its evacuation. Among the mucolytic agents, we can cite the dornase α (a deoxyribonuclease which is recommended in the CFF treatment guidelines under the name of Pulmozyme®, see **Chapter I. part 1.6. Therapeutic strategy**) and N-acetylcysteine (the effect of which has been demonstrated in CF population) [260;261]. Both molecules are excellent candidates to be investigated in our model for their action against matrix biofilm [262;263]. Indeed, dornase α could hydrolyze the eDNA present in the biofilm matrix and N-acetylcysteine has been suggested to impair biofilm formation [263-265]. Such molecules can easily be combined to an antibiotic to search possible synergistic effects against bacteria within the biofilms.

In this general context, another interesting strategy could consist of using nanocarriers to prevent infection (inhibition of the bacterial adhesion and biofilm formation through nanoparticles) or to fight the infection (targeting the biofilm and killing the bacteria through liposomes). Nanocarriers' properties are partly attributed to their dimensions (size between 1-100nm for nanoparticles and 25-1000nm for liposomes) [266;267]. Nanocarriers can have an antimicrobial effect *per se* (inorganic materials like silver) or they can be used as drug delivery systems to achieve high concentrations of drug at the site of infection [266;267]. Liposomes are the most widely used nanocarriers, they are vesicles composed of phospholipid bilayers. They are known to be able to penetrate within the biofilm and can protect the antibiotics from interactions with the matrix or from enzymatic inactivation. Their lipid structure can fuse with the outer membrane of Gram-negative bacteria and release the drug directly into the cell [266]. In the context of the CF, some studies already examined the potential of liposomal amikacin [268] or ciprofloxacin [269] for aerosol delivery.

Finally, an innovating option is the use of bacteriophages, i.e. viruses that infect and kill bacteria and are not affected by resistance mechanisms to antibiotics. However, resistance against bacteriophages is also observed and persistent bacteria are less susceptible to them [270]. The combination of bacteriophages and antibiotics has been tested *in vitro* against mono-species *P. aeruginosa* [271] and *S. aureus* [272] biofilms and the results have shown an increase in killing of bacteria within the biofilm [273]. However, the models of biofilms used to test the combination phage/antibiotic used conventional growth medium and reference strains. Such experiments can be extended to our artificial sputum medium to study the influence of the medium on the activity of the phages alone or in combination against biofilms.

3.4. Multi-species biofilms (*S. aureus* / *P. aeruginosa*)

In this work, we only focused on the development of models of mono-species biofilms. However, respiratory infections are not always caused by a single pathogen but are rather co-infections caused by different pathogens [42;274]. In patients with CF, *S. aureus* and *P. aeruginosa* are co-cultured from respiratory specimens in 20 to 25% of the cases [42] and their co-culture seems to contribute independently and additively to the disease severity (increased lung inflammation and thus increased lung damages) [104;274].

As described earlier in this dissertation, *S. aureus* and *P. aeruginosa* are well adapted to survive and invade human tissues. Interestingly, both species may have an impact on the metabolism of the other.

The presence of *S. aureus*, more specifically the production of the quorum sensing molecule AI-2 and the exopolysaccharide PNAG (both detected in the sputum of patients with CF), can upregulate the production of virulence factors like LasB, rhamnolipids, exotoxins, HQNO and pyocyanin by *P. aeruginosa* [102;275;276].

Conversely, *P. aeruginosa* produces many molecules that can outcompete other microorganisms in space and nutrients [274]. *S. aureus* is

not an exception to the rule and *P. aeruginosa* strongly outcompetes (Figure 29) it during co-culture *in vitro* [277]. Molecules like HQNO and pyocyanin are known to block the oxidative respiration, to inhibit *S. aureus* growth and to select the SCV phenotype that promotes protection to killing by *P. aeruginosa* [42;104;218;219]. Other secreted molecules like LasA (staphylolysin) can lyse *S. aureus* cells as described in **Chapter I. part 2.2.2.2 Secreted virulence Factors** [95;105-107]. This lysis will lead to the liberation of iron that will be scavenged by *P. aeruginosa* [278]. Other molecules like cis-2-decenoic acid and rhamnolipids can induce *S. aureus* (and even *P. aeruginosa*) biofilm dispersal [111;279]. Quorum sensing molecules like OdDHL (that regulate the production of pyocyanin and rhamnolipids) can inhibit the growth and virulence of *S. aureus* [219;274;280].

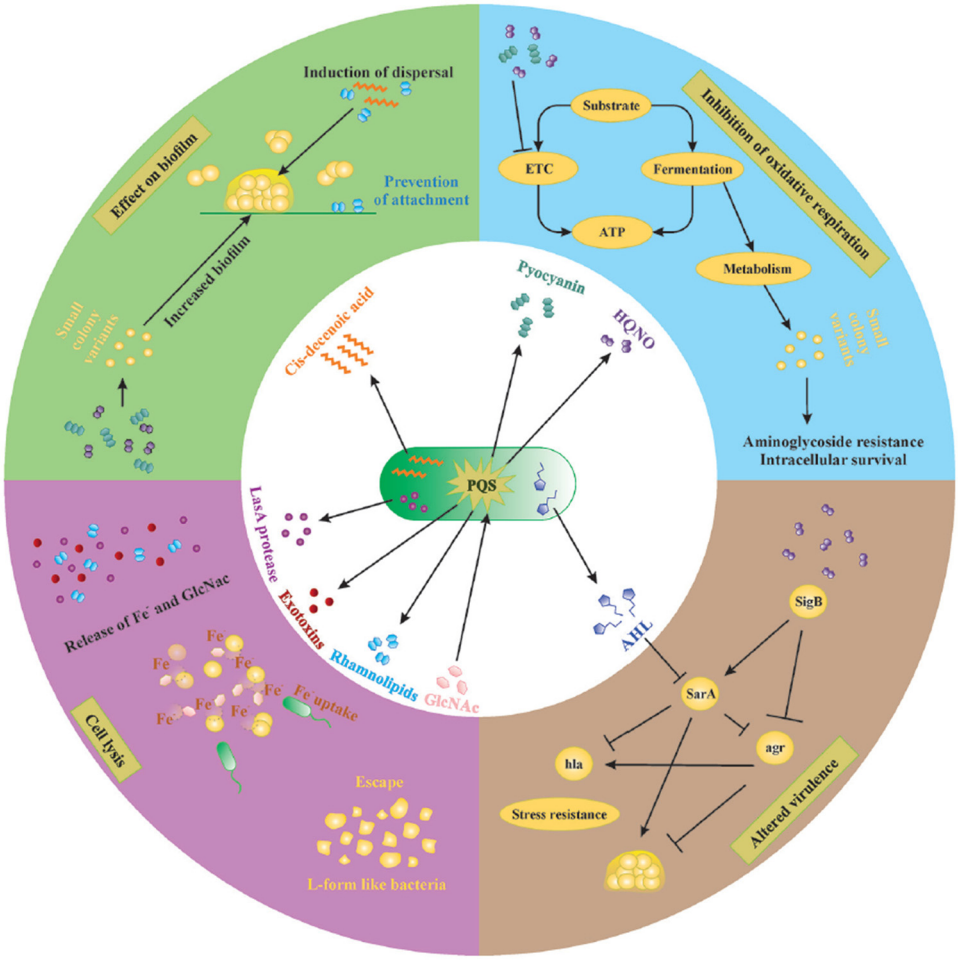

Figure 29: *P. aeruginosa* secretes extracellular factors that affect biofilm formation, oxidative respiration, cell lysis, and virulence of *S. aureus*. [274]

Concerning the interactions between *S. aureus* and *P. aeruginosa* in dual species biofilms, it seems that the exopolysaccharides produced by *P. aeruginosa* will influence the behavior of *S. aureus* and its ability to infiltrate into the biofilm and form multi-species biofilms [274]. Another *P. aeruginosa* factor that seems important to multi-species biofilms is the presence of type IV pili (essential to *P. aeruginosa* biofilm formation) [274;281] because it has been demonstrated that staphylococcal protein A (SpA) can bind to this structure and inhibit adhesion to surfaces (and thus

biofilm formation) and this binding confers to *P. aeruginosa* a protection to phagocytosis [281].

All these data emphasize the interest of developing a relevant model of dual species biofilm by *S. aureus* and *P. aeruginosa* in the context of CF to study (i) the behavior (positive and negative interactions) of both species when put together in a relevant medium, (ii) the evolution of both species within the dual species biofilms over time and (iii) the activity of antibiotics in monotherapy or in combination.

3.5. Adaption of the model to other species found in CF lungs

As already presented in "**Chapter I part 3.3**. **Biofilms in cystic fibrosis"**, other species like *Achromobacter xylosoxidans*, *Burkholderia multivorans*, *Stenotrophomonas maltophilia* and *Mycobacterium abscessus* can be found growing as biofilms in the airways of patients with CF [164]. Different papers had been published over the last years to study the biofilms of *A. xylosoxidans* [282], *B. multivorans* [283], or *S. maltophilia* [284] in the context of CF but they only used "classical" medium like lysogeny broth, cation-adjusted Mueller-Hinton Broth or brain heart infusion to grow their biofilm. Only Hunt-Serracin *et al*. [285] used an "artificial cystic fibrosis medium" based on the work from Sriramulu *et al*. [166] but they did not make rheological studies. Thus we think that our model of artificial sputum medium can be extended to such species to study the ability to form biofilm and to study the activity of antibiotic used to treat their exacerbation.

Chapter V: Annexes

Poster for "Microbes without Frontiers", Symposium of the Belgian Society for Microbiology, October 18 2019

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Daptomycin enhances nafcillin and ceftaroline activity against *S. epidermidis* biofilms

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Introduction & Purpose

- *Staphylococcus epidermidis* is a commensal bacterium of the skin flora. It is an opportunistic pathogen causing nosocomial infections.
- Here, we report about a methicillin-resistant *S. epidermidis* (MRSE) strain that caused a bacteremia related to endocarditis. The infection was poorly receptive to daptomycin (DAP) alone but when DAP was combined with nafcillin (NAF), the bacteraemia was cleared in 24h.
- This combination was already tested and shown synergistic against *S. aureus* bacteremia[1].
- The aim of this study is to evaluate the activity of the combination between daptomycin and nafcillin or ceftaroline (CPT, a β-lactam active against methicillin-resistant strains) against this *S. epidermidis* in an *in vitro* model of biofilm.

Methods

- The clinical isolate was provided by G. Sakoulas (University of California San Diego School of Medicine, La Jolla, CA).
- Biofilms were grown in TGN (Tryptic soy broth + 1% Glucose + 2% NaCl) using 96 well cell culture microplates (clear, TC surface treatment) for cell culture during 24h to reach maturity.
- In a first step, these biofilms were exposed during 24h to daptomycin, nafcillin or ceftaroline alone at concentrations ranging from 0.001 to 1000 mg/L in order to obtain full concentration-response curves.
- In a second step, there were exposed to one of these 2 β-lactams in the same conditions, but combined with 2 fixed concentrations of daptomycin (1 and 10 mg/L, corresponding approximatively to free C_{min} and C_{max} in human serum).
- At the end of the incubation, plates were harvested and used to evaluate metabolic activity within biofilm by the resazurin assay (RF)[2].
- Two pharmacodynamic parameters were calculated based on the equation of the sigmoidal regression fitted of the data:
  - E_{max} (maximal efficacy): reduction in viability for an infinitely large concentration
  - C_{50} (relative potency): concentration causing a reduction of 50% in viability

Conclusions

- Daptomycin alone is more effective than β-lactams alone
- Using C_{min} and C_{max} as fixed concentration for daptomycin in combination with nafcillin allows to improve the maximal efficacy and the relative potency
- Ceftaroline is not sensitive to that combination

References

1. Sakoulas G *et al.* Nafcillin enhances innate immune-mediated killing of MRSA. Journal of Molecular Medicine (2014) 92:139-49 PMID: 24297496
2. Peeters E. *et al.* Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. Journal of microbiological methods (2008) 72:157-65 PMID : 18155789

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Results

Concentration-response curves

- When used alone, drugs showed concentration-dependent activity
  - DAP : almost complete reduction in metabolic activity almost
  - β-lactams : maximal reduction close to 50%
- When combined with DAP at 1 mg/L, a slight increase in maximal effect was observed for both β-lactams.
- When NAF was combined with DAP at 10 mg/L, the activity of the combination was higher than that of each drug alone over the whole range of NAF concentration. With CPT, the activity of the combination was similar to that of DAP alone.

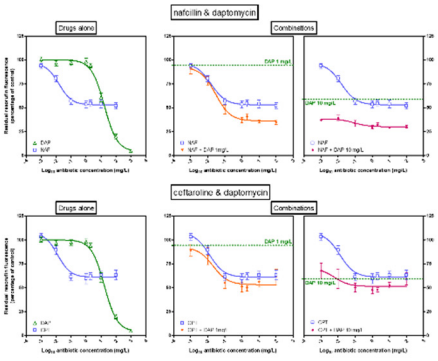

Fig. 1: 24h biofilms treated with antibiotics. Left panels show biofilms treated with antibiotics alone (daptomycin, DAP ; nafcillin, NAF ; ceftaroline, CPT). Center and right panels show biofilms treated with combination of antibiotics (upper: NAF+DAP. Lower: CPT+DAP).

Pharmacodynamic parameters:

- NAF maximal efficacy and relative potency were markedly improved by DAP at both 1 and 10 mg/L, indicating synergistic effects
- CPT maximal efficacy was slightly improved by DAP at 1mg/L ; its relative potency was slightly increased (lower C_{50}) by DAP 10 mg/L.

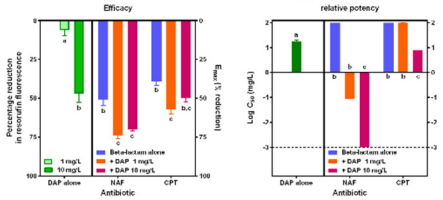

Fig. 2: pharmacodynamic parameters of the activity of antibiotics alone or in combination against biofilms. Left: antibiotic efficacy as the reduction in resorufin fluorescence as compared to control biofilms. Right: antibiotic relative potency as the concentration of antibiotic needed to reduce by 50% the fluorescence signal of resorufin (as compared to control biofilms)..

Chapter VI: Bibliography

[1] Lubamba B, Dhooghe B, Noel S, & Leal T (2012) Cystic fibrosis: insight into CFTR pathophysiology and pharmacotherapy. *Clin. Biochem.*, **45,** 1132-1144.

[2] Cohen TS & Prince A (2012) Cystic fibrosis: a mucosal immunodeficiency syndrome. *Nat. Med.*, **18,** 509-519.

[3] Rafeeq MM & Murad HAS (2017) Cystic fibrosis: current therapeutic targets and future approaches. *J. Transl. Med.*, **15,** 84.

[4] Belgian Cystic Fibrosis Registry. Annual Data Report 2017 BCFR. Belgian Cystic Fibrosis Registry . 2020.

[5] Cystic Fibrosis Foundation Patient Registry. Annual Data Report 2017. Cystic Fibrosis Foundation . 2018. 16-11-2018.

[6] UK Cystic Fibrosis Registry. Annual Data Report 2018. UK Cystic Fibrosis Registry . 2019.

[7] ECFS Patient Registry. ECFS Patient Registry : Annual Data Report 2017. ECFS Patient Registry . 2017.

[8] Harrison F (2007) Microbial ecology of the cystic fibrosis lung. *Microbiology*, **153,** 917-923.

[9] Andersen DH (1938) Cystic Fibrosis of the pancreas and its relation to caeliac disease: a clinical and pathologic study. *American Journal of Diseases of Children*, **56,** 344-399.

[10] Andersen DH & Hodges RG (1946) Celiac syndrome; genetics of cystic fibrosis of the pancreas, with a consideration of etiology. *Am. J. Dis. Child*, **72,** 62-80.

[11] Gibson LE & Cooke RE (1959) A Test for concentration of electrolytes in sweat in cystic fibrosis of the pancrease utilizing pilocarpine by iontophoresis. *Pediatrics*, **23,** 545.

[12] Knowles M, Gatzy J, & Boucher R (1981) Increased Bioelectric Potential Difference across Respiratory Epithelia in Cystic Fibrosis. *N Engl J Med*, **305,** 1489-1495.

[13] Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, & Chou JL (1989) Identification of the cystic

fibrosis gene: cloning and characterization of complementary DNA. *Science*, **245,** 1066-1073.

[14] Rogan MP, Stoltz DA, & Hornick DB (2011) Cystic fibrosis transmembrane conductance regulator intracellular processing, trafficking, and opportunities for mutation-specific treatment. *Chest*, **139,** 1480-1490.

[15] Wainwright BJ, Scambler PJ, Schmidtke J, Watson EA, Law HY, Farrall M, Cooke HJ, Eiberg H, & Williamson R (1985) Localization of cystic fibrosis locus to human chromosome 7cen-q22. *Nature*, **318,** 384-385.

[16] Ratjen F, Bell SC, Rowe SM, Goss CH, Quittner AL, & Bush A (2015) Cystic fibrosis. *Nat. Rev. Dis. Primers.*, **1,** 15010.

[17] Liu F, Zhang Z, Csanady L, Gadsby DC, & Chen J (2017) Molecular Structure of the Human CFTR Ion Channel. *Cell*, **169,** 85-95.

[18] Morral N, Bertranpetit J, Estivill X, Nunes V, Casals T, Gimenez J, Reis A, Varon-Mateeva R, Macek MJ, & Kalaydjieva L (1994) The origin of the major cystic fibrosis mutation (delta F508) in European populations. *Nat. Genet.*, **7,** 169-175.

[19] Veit G, Avramescu RG, Chiang AN, Houck SA, Cai Z, Peters KW, Hong JS, Pollard HB, Guggino WB, Balch WE, Skach WR, Cutting GR, Frizzell RA, Sheppard DN, Cyr DM, Sorscher EJ, Brodsky JL, & Lukacs GL (2016) From CFTR biology toward combinatorial pharmacotherapy: expanded classification of cystic fibrosis mutations. *Mol. Biol. Cell*, **27,** 424-433.

[20] Rozen R, De Braekeleer M, Daigneault J, Ferreira-Rajabi L, Gerdes M, Lamoureux L, Aubin G, Simard F, Fujiwara TM, & Morgan K (1992) Cystic fibrosis mutations in French Canadians: three CFTR mutations are relatively frequent in a Quebec population with an elevated incidence of cystic fibrosis. *Am. J Med Genet.*, **42,** 360-364.

[21] Shoshani T, Augarten A, Gazit E, Bashan N, Yahav Y, Rivlin Y, Tal A, Seret H, Yaar L, & Kerem E (1992) Association of a nonsense mutation (W1282X), the most common mutation in the Ashkenazi Jewish cystic fibrosis patients in Israel, with presentation of severe disease. *Am. J Hum. Genet.*, **50,** 222-228.

[22] Martin C, Hamard C, Kanaan R, Boussaud V, Grenet D, Abély M, Hubert D, Munck A, Lemonnier L, & Burgel PR (2016) Causes of death in French cystic fibrosis patients: The need for improvement in transplantation referral strategies! *Journal of Cystic Fibrosis*, **15,** 204-212.

[23] O'Sullivan BP & Freedman SD (2009) Cystic fibrosis. *Lancet*, **373,** 1891-1904.

[24] Wilschanski M & Novak I (2013) The cystic fibrosis of exocrine pancreas. *Cold Spring Harb. Perspect. Med*, **3,** a009746.

[25] Blackman SM & Tangpricha V (2016) Endocrine Disorders in Cystic Fibrosis. *Pediatr. Clin. North Am.*, **63,** 699-708.

[26] Ahmad A, Ahmed A, & Patrizio P (2013) Cystic fibrosis and fertility. *Curr. Opin. Obstet. Gynecol.*, **25,** 167-172.

[27] Cantin AM, Hartl D, Konstan MW, & Chmiel JF (2015) Inflammation in cystic fibrosis lung disease: Pathogenesis and therapy. *J Cyst. Fibros.*, **14,** 419-430.

[28] Saiman L (2004) Microbiology of early CF lung disease. *Paediatr. Respir. Rev.*, **5 Suppl A,** S367-S369.

[29] Gibson RL, Burns JL, & Ramsey BW (2003) Pathophysiology and management of pulmonary infections in cystic fibrosis. *Am. J Respir. Crit Care Med*, **168,** 918-951.

[30] Cardines R, Giufre M, Pompilio A, Fiscarelli E, Ricciotti G, Di Bonaventura G, & Cerquetti M (2012) Haemophilus influenzae in children with cystic fibrosis: antimicrobial susceptibility, molecular epidemiology, distribution of adhesins and biofilm formation. *Int. J Med Microbiol.*, **302,** 45-52.

[31] Firmida MC, Pereira RHV, Silva EASR, Marques EA, & Lopes AJ (2016) Clinical impact of Achromobacter xylosoxidans colonization/infection in patients with cystic fibrosis. *Braz. J Med Biol. Res.*, **49,** e5097.

[32] Barsky EE, Williams KA, Priebe GP, & Sawicki GS (2017) Incident Stenotrophomonas maltophilia infection and lung function decline in cystic fibrosis. *Pediatr. Pulmonol.*, **52,** 1276-1282.

[33] Pallett R, Leslie LJ, Lambert PA, Milic I, Devitt A, & Marshall LJ (2019) Anaerobiosis influences virulence properties of Pseudomonas aeruginosa cystic fibrosis isolates and the interaction with Staphylococcus aureus. *Sci Rep.*, **9,** 6748.

[34] Jones KL, Hegab AH, Hillman BC, Simpson KL, Jinkins PA, Grisham MB, Owens MW, Sato E, & Robbins RA (2000) Elevation of nitrotyrosine and nitrate concentrations in cystic fibrosis sputum. *Pediatr. Pulmonol.*, **30,** 79-85.

[35] Borriello G, Richards L, Ehrlich GD, & Stewart PS (2006) Arginine or nitrate enhances antibiotic susceptibility of Pseudomonas aeruginosa in biofilms. *Antimicrob Agents Chemother*, **50,** 382-384.

[36] Yoon SS, Hennigan RF, Hilliard GM, Ochsner UA, Parvatiyar K, Kamani MC, Allen HL, DeKievit TR, Gardner PR, Schwab U, Rowe JJ, Iglewski BH, McDermott TR, Mason RP, Wozniak DJ, Hancock REW, Parsek MR, Noah TL, Boucher RC, & Hassett DJ (2002) Pseudomonas aeruginosa anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. *Dev. Cell*, **3,** 593-603.

[37] Edmondson C & Davies JC (2016) Current and future treatment options for cystic fibrosis lung disease: latest evidence and clinical implications. *Ther Adv Chronic Dis*, **2016/05/01,** 170-183.

[38] Hurley MN, Fogarty A, McKeever TM, Goss CH, Rosenfeld M, & Smyth AR (2018) Early Respiratory Bacterial Detection and Antistaphylococcal Antibiotic Prophylaxis in Young Children with Cystic Fibrosis. *Ann. Am. Thorac. Soc.*, **15,** 42-48.

[39] Stutman HR, Lieberman JM, Nussbaum E, & Marks MI (2002) Antibiotic prophylaxis in infants and young children with cystic fibrosis: a randomized controlled trial. *J Pediatr.*, **140,** 299-305.

[40] UK Cystic Fibrosis Registry. Antibiotic treatment for cystic fibrosis - 3rd edition. UK Cystic Fibrosis Registry . 2009.

[41] Ahlgren HG, Benedetti A, Landry JS, Bernier J, Matouk E, Radzioch D, Lands LC, Rousseau S, & Nguyen D (2015) Clinical outcomes associated with Staphylococcus aureus and Pseudomonas aeruginosa airway infections in adult cystic fibrosis patients. *BMC Pulm. Med*, **15,** 67.

[42] Hoffman LR, Deziel E, D'Argenio DA, Lepine F, Emerson J, McNamara S, Gibson RL, Ramsey BW, & Miller SI (2006) Selection for Staphylococcus aureus small-colony variants due to growth in the presence of Pseudomonas aeruginosa. *Proc. Natl. Acad. Sci. U. S. A*, **103,** 19890-19895.

[43] Frost F, Shaw M, & Nazareth D (2019) Antibiotic therapy for chronic infection with Burkholderia cepacia complex in people with cystic fibrosis. *Cochrane. Database. Syst. Rev.*, **6,** CD013079.

[44] Middleton PG, Chen SCA, & Meyer W (2013) Fungal infections and treatment in cystic fibrosis. *Curr. Opin. Pulm. Med*, **19,** 670-675.

[45] Delfino E, Del Puente F, Briano F, Sepulcri C, & Giacobbe DR (2019) Respiratory Fungal Diseases in Adult Patients With Cystic Fibrosis. *Clin. Med Insights. Circ. Respir. Pulm. Med*, **13,** 1179548419849939.

[46] Chotirmall SH, O'Donoghue E, Bennett K, Gunaratnam C, O'Neill SJ, & McElvaney NG (2010) Sputum Candida albicans presages FEV(1) decline

and hospital-treated exacerbations in cystic fibrosis. *Chest*, **138,** 1186-1195.

[47] Hector A, Kirn T, Ralhan A, Graepler-Mainka U, Berenbrinker S, Riethmueller J, Hogardt M, Wagner M, Pfleger A, Autenrieth I, Kappler M, Griese M, Eber E, Martus P, & Hartl D (2016) Microbial colonization and lung function in adolescents with cystic fibrosis. *J Cyst. Fibros.*, **15,** 340-349.

[48] Gileles-Hillel A, Shoseyov D, Polacheck I, Korem M, Kerem E, & Cohen-Cymberknoh M (2015) Association of chronic Candida albicans respiratory infection with a more severe lung disease in patients with cystic fibrosis. *Pediatr. Pulmonol.*, **50,** 1082-1089.

[49] Tracy MC & Moss RB (2018) The myriad challenges of respiratory fungal infection in cystic fibrosis. *Pediatr. Pulmonol.*, **53,** S75-S85.

[50] King J, Brunel SF, & Warris A (2016) Aspergillus infections in cystic fibrosis. *J Infect.*, **72 Suppl,** S50-S55.

[51] Warris A, Bercusson A, & Armstrong-James D (2019) Aspergillus colonization and antifungal immunity in cystic fibrosis patients. *Med Mycol.*, **57,** S118-S126.

[52] Ratjen FA (2009) Cystic fibrosis: pathogenesis and future treatment strategies. *Respir. Care*, **54,** 595-605.

[53] Moody M, Pennington C, Schultz C, Caldwell R, Dinkel C, Rossi MW, McNamara S, Widdicombe J, Gabriel S, & Traynor-Kaplan AE (2005) Inositol polyphosphate derivative inhibits Na+ transport and improves fluid dynamics in cystic fibrosis airway epithelia. *Am. J Physiol Cell Physiol*, **289,** C512-C520.

[54] Traynor-Kaplan AE, Moody M, Nur M, Gabriel S, Majerus PW, Drumm ML, & Langton-Webster B (2010) INO-4995 therapeutic efficacy is enhanced with repeat dosing in cystic fibrosis knockout mice and human epithelia. *Am. J Respir. Cell Mol. Biol.*, **42,** 105-112.

[55] Scott DW, Walker MP, Sesma J, Wu B, Stuhlmiller TJ, Sabater JR, Abraham WM, Crowder TM, Christensen DJ, & Tarran R (2017) SPX-101 Is a Novel Epithelial Sodium Channel-targeted Therapeutic for Cystic Fibrosis That Restores Mucus Transport. *Am. J Respir. Crit Care Med*, **196,** 734-744.

[56] Gardiner P, Malmgren A, Ersdal E., Goldwater R., & Patel N. Enac Inhibitor Azd5634 First In Human Trial Reveals Promising Clinical Profile For The Treatment Of Cystic Fibrosis. ATS Conferences 2017 . 2017.

[57] McIlwaine M, Button B, & Nevitt SJ (2019) Positive expiratory pressure physiotherapy for airway clearance in people with cystic fibrosis. *Cochrane Database of Systematic Reviews*, CD003147.

[58] Joshi D, Ehrhardt A, Hong JS, & Sorscher EJ (2019) Cystic fibrosis precision therapeutics: Emerging considerations. *Pediatr. Pulmonol.*, **54 Suppl 3,** S13-S17.

[59] Wolter J, Seeney S, Bell S, Bowler S, Masel P, & McCormack J (2002) Effect of long term treatment with azithromycin on disease parameters in cystic fibrosis: a randomised trial. *Thorax*, **57,** 212-216.

[60] Weill D (2018) Lung transplantation: indications and contraindications. *J Thorac Dis*, **10,** 4574-4587.

[61] Gnanamani A, Periasamy H, & Paul Satyaseela M (2017) Staphylococcus aureus: Overview of Bacteriology, Clinical Diseases, Epidemiology, Antibiotic Resistance and Therapeutic Approach.

[62] Tong SYC, Davis JS, Eichenberger E, Holland TL, & Fowler VGJ (2015) Staphylococcus aureus infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clin. Microbiol. Rev.*, **28,** 603-661.

[63] Ma YX, Wang CY, Li YY, Li J, Wan QQ, Chen JH, Tay FR, & Niu LN (2020) Considerations and Caveats in Combating ESKAPE Pathogens against Nosocomial Infections. *Adv. Sci. (Weinh.)*, **7,** 1901872.

[64] Santajit S & Indrawattana N (2016) Mechanisms of Antimicrobial Resistance in ESKAPE Pathogens. *Biomed. Res. Int.*, **2016,** 2475067.

[65] Turner NA, Sharma-Kuinkel BK, Maskarinec SA, Eichenberger EM, Shah PP, Carugati M, Holland TL, & Fowler VGJ (2019) Methicillin-resistant Staphylococcus aureus: an overview of basic and clinical research. *Nat. Rev. Microbiol.*, **17,** 203-218.

[66] Bien J, Sokolova O, & Bozko P (2011) Characterization of Virulence Factors of Staphylococcus aureus: Novel Function of Known Virulence Factors That Are Implicated in Activation of Airway Epithelial Proinflammatory Response. *J. Pathog.*, **2011,** 601905.

[67] Foster TJ, Geoghegan JA, Ganesh VK, & Hook M (2014) Adhesion, invasion and evasion: the many functions of the surface proteins of Staphylococcus aureus. *Nat. Rev. Microbiol.*, **12,** 49-62.

[68] Foster TJ (2005) Immune evasion by staphylococci. *Nat. Rev. Microbiol.*, **3,** 948-958.

[69] Pietrocola G, Nobile G, Rindi S, & Speziale P (2017) Staphylococcus aureus Manipulates Innate Immunity through Own and Host-Expressed Proteases. *Front Cell Infect. Microbiol.*, **7,** 166.

[70] O'Riordan K & Lee JC (2004) Staphylococcus aureus capsular polysaccharides. *Clin. Microbiol. Rev.*, **17,** 218-234.

[71] Xue L, Chen YY, Yan Z, Lu W, Wan D, & Zhu H (2019) Staphyloxanthin: a potential target for antivirulence therapy. *Infect. Drug Resist.*, **12,** 2151-2160.

[72] Sause WE, Buckley PT, Strohl WR, Lynch AS, & Torres VJ (2016) Antibody-Based Biologics and Their Promise to Combat Staphylococcus aureus Infections. *Trends Pharmacol. Sci.*, **37,** 231-241.

[73] Archer NK, Mazaitis MJ, Costerton JW, Leid JG, Powers ME, & Shirtliff ME (2011) Staphylococcus aureus biofilms: properties, regulation, and roles in human disease. *Virulence.*, **2,** 445-459.

[74] Arciola CR, Campoccia D, Ravaioli S, & Montanaro L (2015) Polysaccharide intercellular adhesin in biofilm: structural and regulatory aspects. *Front Cell Infect. Microbiol.*, **5,** 7.

[75] Joo HS & Otto M (2012) Molecular basis of in vivo biofilm formation by bacterial pathogens. *Chem. Biol.*, **19,** 1503-1513.

[76] Castillo-Juarez I, Maeda T, Mandujano-Tinoco EA, Tomas M, Perez-Eretza B, Garcia-Contreras SJ, Wood TK, & Garcia-Contreras R (2015) Role of quorum sensing in bacterial infections. *World J. Clin. Cases.*, **3,** 575-598.

[77] Wolska KI, Grudniak AM, Rudnicka Z, & Markowska K (2016) Genetic control of bacterial biofilms. *J. Appl. Genet.*, **57,** 225-238.

[78] Scoffone VC, Trespidi G, Chiarelli LR, Barbieri G, & Buroni S (2019) Quorum Sensing as Antivirulence Target in Cystic Fibrosis Pathogens. *Int J Mol Sci*, **20,** 1838.

[79] Jenul C & Horswill AR (2018) Regulation of Staphylococcus aureus Virulence. *Microbiol Spectr*, **6,** 10.

[80] Kong KF, Vuong C, & Otto M (2006) Staphylococcus quorum sensing in biofilm formation and infection. *Int. J. Med. Microbiol.*, **296,** 133-139.

[81] Tan L, Li SR, Jiang B, Hu XM, & Li S (2018) Therapeutic Targeting of the Staphylococcus aureus Accessory Gene Regulator (agr) System. *Front Microbiol.*, **9,** 55.

[82] De Keersmaecker SCJ, Sonck K, & Vanderleyden J (2006) Let LuxS speak up in AI-2 signaling. *Trends Microbiol.*, **14,** 114-119.

[83] Yu D, Zhao L, Xue T, & Sun B (2012) Staphylococcus aureus autoinducer-2 quorum sensing decreases biofilm formation in an icaR-dependent manner. *BMC. Microbiol.*, **12,** 288.

[84] McGuinness WA, Malachowa N, & DeLeo FR (2017) Vancomycin Resistance in Staphylococcus aureus. *Yale J. Biol. Med.*, **90,** 269-281.

[85] McCarthy H, Rudkin JK, Black NS, Gallagher L, O'Neill E, & O'Gara JP (2015) Methicillin resistance and the biofilm phenotype in Staphylococcus aureus. *Front Cell Infect. Microbiol.*, **5,** 1.

[86] Foster TJ (2017) Antibiotic resistance in Staphylococcus aureus. Current status and future prospects. *FEMS Microbiol. Rev.*, **41,** 430-449.

[87] Sieradzki K & Tomasz A (1997) Inhibition of cell wall turnover and autolysis by vancomycin in a highly vancomycin-resistant mutant of Staphylococcus aureus. *J. Bacteriol.*, **179,** 2557-2566.

[88] Arai H (2011) Regulation and Function of Versatile Aerobic and Anaerobic Respiratory Metabolism in Pseudomonas aeruginosa. *Front Microbiol.*, **2,** 103.

[89] Sadikot RT, Blackwell TS, Christman JW, & Prince AS (2005) Pathogen-host interactions in Pseudomonas aeruginosa pneumonia. *Am. J. Respir. Crit Care Med.*, **171,** 1209-1223.

[90] Crousilles A, Maunders E, Bartlett S, Fan C, Ukor EF, Abdelhamid Y, Baker Y, Floto A, Spring DR, & Welch M (2015) Which microbial factors really are important in Pseudomonas aeruginosa infections? *Future. Microbiol.*, **10,** 1825-1836.

[91] Chaban B, Hughes HV, & Beeby M (2015) The flagellum in bacterial pathogens: For motility and a whole lot more. *Semin. Cell Dev. Biol.*, **46,** 91-103.

[92] Kipnis E, Sawa T, & Wiener-Kronish J (2006) Targeting mechanisms of Pseudomonas aeruginosa pathogenesis. *Med. Mal Infect.*, **36,** 78-91.

[93] Veesenmeyer JL, Hauser AR, Lisboa T, & Rello J (2009) Pseudomonas aeruginosa virulence and therapy: evolving translational strategies. *Crit Care Med.*, **37,** 1777-1786.

[94] Faure E, Kwong K, & Nguyen D (2018) Pseudomonas aeruginosa in Chronic Lung Infections: How to Adapt Within the Host? *Front Immunol.*, **9,** 2416.

[95] Galdino AC, Branquinha MH, Santos ALS, & Viganor L (2017) Pseudomonas aeruginosa and Its Arsenal of Proteases: Weapons to Battle the Host. In *Pathophysiological Aspects of Proteases* (Chakraborti S & Dhalla NS, eds), pp. 381-397. Springer Singapore, Singapore.

[96] Burrows LL (2012) Pseudomonas aeruginosa twitching motility: type IV pili in action. *Annu. Rev. Microbiol.*, **66,** 493-520.

[97] Huszczynski SM, Lam JS, & Khursigara CM (2019) The Role of Pseudomonas aeruginosa Lipopolysaccharide in Bacterial Pathogenesis and Physiology. *Pathogens.*, **9,** pii: E6.

[98] Ryder C, Byrd M, & Wozniak DJ (2007) Role of polysaccharides in Pseudomonas aeruginosa biofilm development. *Curr. Opin. Microbiol.*, **10,** 644-648.

[99] Hay ID, Wang Y, Moradali MF, Rehman ZU, & Rehm BHA (2014) Genetics and regulation of bacterial alginate production. *Environ. Microbiol.*, **16,** 2997-3011.

[100] Wu H, Wang D, Tang M, & Ma LZ (2019) The advance of assembly of exopolysaccharide Psl biosynthesis machinery in Pseudomonas aeruginosa. *Microbiologyopen.*, **8,** e857.

[101] Jennings LK, Storek KM, Ledvina HE, Coulon C, Marmont LS, Sadovskaya I, Secor PR, Tseng BS, Scian M, Filloux A, Wozniak DJ, Howell PL, & Parsek MR (2015) Pel is a cationic exopolysaccharide that cross-links extracellular DNA in the Pseudomonas aeruginosa biofilm matrix. *Proc. Natl. Acad. Sci. U. S. A*, **112,** 11353-11358.

[102] Jimenez PN, Koch G, Thompson JA, Xavier KB, Cool RH, & Quax WJ (2012) The multiple signaling systems regulating virulence in Pseudomonas aeruginosa. *Microbiol. Mol. Biol. Rev.*, **76,** 46-65.

[103] Jayaseelan S, Ramaswamy D, & Dharmaraj S (2014) Pyocyanin: production, applications, challenges and new insights. *World J. Microbiol. Biotechnol.*, **30,** 1159-1168.

[104] Filkins LM, Graber JA, Olson DG, Dolben EL, Lynd LR, Bhuju S, & O'Toole GA (2015) Coculture of Staphylococcus aureus with Pseudomonas aeruginosa Drives S. aureus towards Fermentative Metabolism and Reduced Viability in a Cystic Fibrosis Model. *J. Bacteriol.*, **197,** 2252-2264.

[105] Toder DS, Ferrell SJ, Nezezon JL, Rust L, & Iglewski BH (1994) lasA and lasB genes of Pseudomonas aeruginosa: analysis of transcription and gene product activity. *Infect. Immun.*, **62,** 1320-1327.

[106] Nair N, Biswas R, Gotz F, & Biswas L (2014) Impact of Staphylococcus aureus on pathogenesis in polymicrobial infections. *Infect. Immun.*, **82,** 2162-2169.

[107] Kessler E, Safrin M, Olson JC, & Ohman DE (1993) Secreted LasA of Pseudomonas aeruginosa is a staphylolytic protease. *J. Biol. Chem.*, **268,** 7503-7508.

[108] Bradshaw JL, Caballero AR, Bierdeman MA, Adams KV, Pipkins HR, Tang A, O'Callaghan RJ, & McDaniel LS (2018) Pseudomonas aeruginosa Protease IV Exacerbates Pneumococcal Pneumonia and Systemic Disease. *mSphere*, **3,** e00212-e00218.

[109] Javanmardi F, Emami A, Pirbonyeh N, Keshavarzi A, & Rajaee M (2019) A systematic review and meta-analysis on Exo-toxins prevalence in hospital acquired Pseudomonas aeruginosa isolates. *Infection, Genetics and Evolution*, **75,** 104037.

[110] Anantharajah A, Faure E, Buyck JM, Sundin C, Lindmark T, Mecsas J, Yahr TL, Tulkens PM, Mingeot-Leclercq MP, Guery B, & Van Bambeke F (2016) Inhibition of the Injectisome and Flagellar Type III Secretion Systems by INP1855 Impairs Pseudomonas aeruginosa Pathogenicity and Inflammasome Activation. *J. Infect. Dis.*, **214,** 1105-1116.

[111] Alhede M, Bjarnsholt T, Givskov M, & Alhede M (2014) Pseudomonas aeruginosa biofilms: mechanisms of immune evasion. *Adv. Appl. Microbiol.*, **86,** 1-40.

[112] Bjarnsholt T, Jensen PO, Jakobsen TH, Phipps R, Nielsen AK, Rybtke MT, Tolker-Nielsen T, Givskov M, Hoiby N, & Ciofu O (2010) Quorum sensing and virulence of Pseudomonas aeruginosa during lung infection of cystic fibrosis patients. *PLoS. One.*, **5,** e10115.

[113] Ahator SD & Zhang L (2019) Small Is Mighty-Chemical Communication Systems in Pseudomonas aeruginosa. *Annu. Rev. Microbiol.*, **73,** 559-578.

[114] Lee J & Zhang L (2015) The hierarchy quorum sensing network in Pseudomonas aeruginosa. *Protein Cell*, **6,** 26-41.

[115] Feltner JB, Wolter DJ, Pope CE, Groleau MC, Smalley NE, Greenberg EP, Mayer-Hamblett N, Burns J, Déziel E, Hoffman LR, & Dandekar AA (2016) LasR Variant Cystic Fibrosis Isolates Reveal an Adaptable Quorum-Sensing Hierarchy in Pseudomonas aeruginosa. *mBio*, **7,** e01513-e01516.

[116] Lee J, Wu J, Deng Y, Wang J, Wang C, Wang J, Chang C, Dong Y, Williams P, & Zhang LH (2013) A cell-cell communication signal integrates quorum sensing and stress response. *Nature Chemical Biology*, **9,** 339-343.

[117] Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL, Rice LB, Stelling J, Struelens MJ, Vatopoulos A, Weber JT, & Monnet DL (2012) Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.*, **18,** 268-281.

[118] Pang Z, Raudonis R, Glick BR, Lin TJ, & Cheng Z (2019) Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and alternative therapeutic strategies. *Biotechnol. Adv.*, **37,** 177-192.

[119] Zowawi HM, Harris PNA, Roberts MJ, Tambyah PA, Schembri MA, Pezzani MD, Williamson DA, & Paterson DL (2015) The emerging threat of multidrug-resistant Gram-negative bacteria in urology. *Nat. Rev. Urol.*, **12,** 570-584.

[120] Aldred KJ, Kerns RJ, & Osheroff N (2014) Mechanism of quinolone action and resistance. *Biochemistry*, **53,** 1565-1574.

[121] Chalhoub H, Saenz Y, Rodriguez-Villalobos H, Denis O, Kahl BC, Tulkens PM, & Van Bambeke F (2016) High-level resistance to meropenem in clinical isolates of Pseudomonas aeruginosa in the absence of carbapenemases: role of active efflux and porin alterations. *Int. J. Antimicrob. Agents*, **48,** 740-743.

[122] Chalhoub H, Saenz Y, Nichols WW, Tulkens PM, & Van Bambeke F (2018) Loss of activity of ceftazidime-avibactam due to MexAB-OprM efflux and overproduction of AmpC cephalosporinase in Pseudomonas aeruginosa isolated from patients suffering from cystic fibrosis. *Int. J. Antimicrob. Agents*, **52,** 697-701.

[123] Garneau-Tsodikova S & Labby KJ (2016) Mechanisms of Resistance to Aminoglycoside Antibiotics: Overview and Perspectives. *Medchemcomm.*, **7,** 11-27.

[124] Oliver A, Canton R, Campo P, Baquero F, & Blazquez J (2000) High frequency of hypermutable Pseudomonas aeruginosa in cystic fibrosis lung infection. *Science*, **288,** 1251-1254.

[125] Hentzer M, Teitzel GM, Balzer GJ, Heydorn A, Molin S, Givskov M, & Parsek MR (2001) Alginate overproduction affects Pseudomonas aeruginosa biofilm structure and function. *J Bacteriol.*, **183,** 5395-5401.

[126] Waine DJ, Honeybourne D, Smith EG, Whitehouse JL, & Dowson CG (2008) Association between hypermutator phenotype, clinical variables, mucoid

phenotype, and antimicrobial resistance in Pseudomonas aeruginosa. *J Clin Microbiol*, **46,** 3491-3493.

[127] Cao B, Christophersen L, Kolpen M, Jensen PO, Sneppen K, Hoiby N, Moser C, & Sams T (2016) Diffusion Retardation by Binding of Tobramycin in an Alginate Biofilm Model. *PLoS. One.*, **11,** e0153616.

[128] Ciofu O, Tolker-Nielsen T, Jensen PO, Wang H, & Hoiby N (2015) Antimicrobial resistance, respiratory tract infections and role of biofilms in lung infections in cystic fibrosis patients. *Adv. Drug Deliv. Rev.*, **85,** 7-23.

[129] Ciofu O, Rojo-Molinero E, Macia MD, & Oliver A (2017) Antibiotic treatment of biofilm infections. *APMIS*, **125,** 304-319.

[130] Rees VE, Deveson Lucas DS, Lopez-Causape C, Huang Y, Kotsimbos T, Bulitta JB, Rees MC, Barugahare A, Peleg AY, Nation RL, Oliver A, Boyce JD, & Landersdorfer CB (2019) Characterization of Hypermutator Pseudomonas aeruginosa Isolates from Patients with Cystic Fibrosis in Australia. *Antimicrob Agents Chemother*, **63,** pii: e02538-18.

[131] Oliver A, Sanchez JM, & Blazquez J (2002) Characterization of the GO system of Pseudomonas aeruginosa. *FEMS Microbiol Lett.*, **217,** 31-35.

[132] Ciofu O, Riis B, Pressler T, Poulsen HE, & Hoiby N (2005) Occurrence of hypermutable Pseudomonas aeruginosa in cystic fibrosis patients is associated with the oxidative stress caused by chronic lung inflammation. *Antimicrob Agents Chemother*, **49,** 2276-2282.

[133] Mena A, Smith EE, Burns JL, Speert DP, Moskowitz SM, Perez JL, & Oliver A (2008) Genetic adaptation of Pseudomonas aeruginosa to the airways of cystic fibrosis patients is catalyzed by hypermutation. *J Bacteriol.*, **190,** 7910-7917.

[134] Macia MD, Blanquer D, Togores B, Sauleda J, Perez JL, & Oliver A (2005) Hypermutation is a key factor in development of multiple-antimicrobial resistance in Pseudomonas aeruginosa strains causing chronic lung infections. *Antimicrob Agents Chemother*, **49,** 3382-3386.

[135] D'Argenio DA, Wu M, Hoffman LR, Kulasekara HD, Deziel E, Smith EE, Nguyen H, Ernst RK, Larson Freeman TJ, Spencer DH, Brittnacher M, Hayden HS, Selgrade S, Klausen M, Goodlett DR, Burns JL, Ramsey BW, & Miller SI (2007) Growth phenotypes of Pseudomonas aeruginosa lasR mutants adapted to the airways of cystic fibrosis patients. *Mol Microbiol*, **64,** 512-533.

[136] Kevin C (2013) Planktonic Versus Sessile Life of Prokaryotes. In *The Prokaryotes: Prokaryotic Communities and Ecophysiology* (Rosenberg E, DeLong EF, Lory S, Stackebrandt E, & Thompson F, eds), pp. 191-201. Springer Berlin Heidelberg, Berlin, Heidelberg.

[137] Donlan RM & Costerton JW (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.*, **15,** 167-193.

[138] Kumar A, Alam A, Rani M, Ehtesham NZ, & Hasnain SE (2017) Biofilms: Survival and defense strategy for pathogens. *Int. J. Med. Microbiol.*, **307,** 481-489.

[139] Otto M (2018) Staphylococcal Biofilms. *Microbiol Spectr*, **6,** 10.

[140] Moradali MF, Ghods S, & Rehm BHA (2017) Pseudomonas aeruginosa Lifestyle: A Paradigm for Adaptation, Survival, and Persistence. *Front Cell Infect. Microbiol.*, **7,** 39.

[141] Davies D (2003) Understanding biofilm resistance to antibacterial agents. *Nat. Rev. Drug Discov.*, **2,** 114-122.

[142] Yan J & Bassler BL (2019) Surviving as a Community: Antibiotic Tolerance and Persistence in Bacterial Biofilms. *Cell Host. Microbe*, **26,** 15-21.

[143] Balaban NQ, Helaine S, Lewis K, Ackermann M, Aldridge B, Andersson DI, Brynildsen MP, Bumann D, Camilli A, Collins JJ, Dehio C, Fortune S, Ghigo JM, Hardt WD, Harms A, Heinemann M, Hung DT, Jenal U, Levin BR, Michiels J, Storz G, Tan MW, Tenson T, Van Melderen L, & Zinkernagel A (2019) Definitions and guidelines for research on antibiotic persistence. *Nat. Rev. Microbiol.*, **17,** 441-448.

[144] Lebeaux D, Ghigo JM, & Beloin C (2014) Biofilm-related infections: bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. *Microbiol Mol Biol. Rev*, **78,** 510-543.

[145] Rojo-Molinero E, Macia MD, & Oliver A (2019) Social Behavior of Antibiotic Resistant Mutants Within Pseudomonas aeruginosa Biofilm Communities. *Front Microbiol*, **10,** 570.

[146] Hall CW, Hinz AJ, Gagnon LBP, Zhang L, Nadeau JP, Copeland S, Saha B, & Mah TF (2018) Pseudomonas aeruginosa Biofilm Antibiotic Resistance Gene ndvB Expression Requires the RpoS Stationary-Phase Sigma Factor. *Appl Environ Microbiol*, **84,** e02762-17.

[147] Lewis K (2010) Persister cells. *Annu. Rev. Microbiol.*, **64,** 357-372.

[148] Sauer K, Camper AK, Ehrlich GD, Costerton JW, & Davies DG (2002) Pseudomonas aeruginosa displays multiple phenotypes during development as a biofilm. *J. Bacteriol.*, **184,** 1140-1154.

[149] Tolker-Nielsen T (2015) Biofilm Development. *Microbiol. Spectr.*, **3,** MB-0001-2014.

[150] Valentini M & Filloux A (2016) Biofilms and Cyclic di-GMP (c-di-GMP) Signaling: Lessons from Pseudomonas aeruginosa and Other Bacteria. *J. Biol. Chem.*, **291,** 12547-12555.

[151] Jenal U, Reinders A, & Lori C (2017) Cyclic di-GMP: second messenger extraordinaire. *Nat. Rev. Microbiol.*, **15,** 271-284.

[152] Yi L, Li J, Liu B, & Wang Y (2019) Advances in research on signal molecules regulating biofilms. *World J. Microbiol. Biotechnol.*, **35,** 130.

[153] Purcell EB & Tamayo R (2016) Cyclic diguanylate signaling in Gram-positive bacteria. *FEMS Microbiol. Rev.*, **40,** 753-773.

[154] Valle J, Toledo-Arana A, Berasain C, Ghigo JM, Amorena B, Penades JR, & Lasa I (2003) SarA and not sigmaB is essential for biofilm development by Staphylococcus aureus. *Mol. Microbiol.*, **48,** 1075-1087.

[155] Bose JL, Lehman MK, Fey PD, & Bayles KW (2012) Contribution of the Staphylococcus aureus Atl AM and GL murein hydrolase activities in cell division, autolysis, and biofilm formation. *PLoS. One.*, **7,** e42244.

[156] Otto M (2014) Phenol-soluble modulins. *Int. J. Med. Microbiol.*, **304,** 164-169.

[157] Boles BR, Thoendel M, & Singh PK (2005) Rhamnolipids mediate detachment of Pseudomonas aeruginosa from biofilms. *Mol. Microbiol.*, **57,** 1210-1223.

[158] Solano C, Echeverz M, & Lasa I (2014) Biofilm dispersion and quorum sensing. *Curr. Opin. Microbiol.*, **18,** 96-104.

[159] Xu K, Li S, Yang W, Li K, Bai Y, Xu Y, Jin J, Wang Y, & Bartlam M (2015) Structural and Biochemical Analysis of Tyrosine Phosphatase Related to Biofilm Formation A (TpbA) from the Opportunistic Pathogen Pseudomonas aeruginosa PAO1. *PLoS. One.*, **10,** e0124330.

[160] Boles BR & Horswill AR (2011) Staphylococcal biofilm disassembly. *Trends Microbiol.*, **19,** 449-455.

[161] Lam J, Chan R, Lam K, & Costerton JW (1980) Production of mucoid microcolonies by Pseudomonas aeruginosa within infected lungs in cystic fibrosis. *Infect. Immun.*, **28,** 546-556.

[162] Bjarnsholt T, Jensen PO, Fiandaca MJ, Pedersen J, Hansen CR, Andersen CB, Pressler T, Givskov M, & Hoiby N (2009) Pseudomonas aeruginosa biofilms in the respiratory tract of cystic fibrosis patients. *Pediatr. Pulmonol.*, **44,** 547-558.

[163] Ulrich M, Herbert S, Berger J, Bellon G, Louis D, Munker G, & Doring G (1998) Localization of Staphylococcus aureus in infected airways of patients with cystic fibrosis and in a cell culture model of S. aureus adherence. *Am. J. Respir. Cell Mol. Biol.*, **19,** 83-91.

[164] Hoiby N, Bjarnsholt T, Moser C, Jensen PO, Kolpen M, Qvist T, Aanaes K, Pressler T, Skov M, & Ciofu O (2017) Diagnosis of biofilm infections in cystic fibrosis patients. *APMIS*, **125,** 339-343.

[165] Ghani M & Soothill JS (1997) Ceftazidime, gentamicin, and rifampicin, in combination, kill biofilms of mucoid Pseudomonas aeruginosa. *Can. J. Microbiol.*, **43,** 999-1004.

[166] Sriramulu DD, Lunsdorf H, Lam JS, & Romling U (2005) Microcolony formation: a novel biofilm model of Pseudomonas aeruginosa for the cystic fibrosis lung. *J. Med. Microbiol.*, **54,** 667-676.

[167] Tavernier S & Coenye T (2015) Quantification of Pseudomonas aeruginosa in multispecies biofilms using PMA-qPCR. *PeerJ.*, **3,** e787.

[168] Sanders NN, De Smedt SC, Van Rompaey E, Simoens P, De Baets F, & Demeester J (2000) Cystic fibrosis sputum: a barrier to the transport of nanospheres. *Am. J Respir Crit Care Med*, **162,** 1905-1911.

[169] Guichard MJ, Kinoo D, Aubriot AS, Bauwens N, Gougue J, Vermeulen F, Lebecque P, Leal T, & Vanbever R (2018) Impact of PEGylation on the mucolytic activity of recombinant human deoxyribonuclease I in cystic fibrosis sputum. *Clin. Sci. (Lond)*, **132,** 1439-1452.

[170] Barth AL & Pitt TL (1996) The high amino-acid content of sputum from cystic fibrosis patients promotes growth of auxotrophic Pseudomonas aeruginosa. *J Med Microbiol*, **45,** 110-119.

[171] Knowles MR, Robinson JM, Wood RE, Pue CA, Mentz WM, Wager GC, Gatzy JT, & Boucher RC (1997) Ion composition of airway surface liquid of patients with cystic fibrosis as compared with normal and disease-control subjects. *J Clin Invest*, **100,** 2588-2595.

[172] Grandjean Lapierre S, Phelippeau M, Hakimi C, Didier Q, Reynaud-Gaubert M, Dubus JC, & Drancourt M (2017) Cystic fibrosis respiratory tract salt concentration: An Exploratory Cohort Study. *Medicine (Baltimore)*, **96,** e8423.

[173] McShane D, Davies JC, Davies MG, Bush A, Geddes DM, & Alton EWFW (2003) Airway surface pH in subjects with cystic fibrosis. *Eur Respir J*, **21,** 37-42.

[174] Diaz Iglesias Y, Wilms T, Vanbever R, & Van Bambeke F (2019) Activity of Antibiotics against Staphylococcus aureus in an In Vitro Model of Biofilms in the Context of Cystic Fibrosis: Influence of the Culture Medium. *Antimicrob. Agents Chemother.*, **63,** e00602-19.

[175] Kirchner S, Fothergill JL, Wright EA, James CE, Mowat E, & Winstanley C (2012) Use of artificial sputum medium to test antibiotic efficacy against Pseudomonas aeruginosa in conditions more relevant to the cystic fibrosis lung. *J. Vis. Exp.*, e3857.

[176] Rozenbaum RT, van der Mei HC, Woudstra W, de Jong ED, Busscher HJ, & Sharma PK (2019) Role of Viscoelasticity in Bacterial Killing by Antimicrobials in Differently Grown Pseudomonas aeruginosa Biofilms. *Antimicrob Agents Chemother*, **63,** e01972-18.

[177] Palmer KL, Aye LM, & Whiteley M (2007) Nutritional cues control Pseudomonas aeruginosa multicellular behavior in cystic fibrosis sputum. *J. Bacteriol.*, **189,** 8079-8087.

[178] Turner KH, Wessel AK, Palmer GC, Murray JL, & Whiteley M (2015) Essential genome of Pseudomonas aeruginosa in cystic fibrosis sputum. *Proc Natl Acad Sci U S A*, **2015/03/16,** 4110-4115.

[179] Lai SK, Wang YY, Wirtz D, & Hanes J (2009) Micro- and macrorheology of mucus. *Adv. Drug Deliv. Rev.*, **61,** 86-100.

[180] Bauer J, Siala W, Tulkens PM, & Van Bambeke F (2013) A combined pharmacodynamic quantitative and qualitative model reveals the potent activity of daptomycin and delafloxacin against Staphylococcus aureus biofilms. *Antimicrob. Agents Chemother.*, **57,** 2726-2737.

[181] Serisier DJ, Carroll MP, Shute JK, & Young SA (2009) Macrorheology of cystic fibrosis, chronic obstructive pulmonary disease & normal sputum. *Respir. Res.*, **10,** 63.

[182] Alcaraz-Serrano V, Fernandez-Barat L, Scioscia G, Llorens-Llacuna J, Gimeno-Santos E, Herrero-Cortina B, Vazquez N, Puig de la Bellacasa J,

Gabarrus A, Amaro-Rodriguez R, Menendez R, & Torres A (2019) Mucoid Pseudomonas aeruginosa alters sputum viscoelasticity in patients with non-cystic fibrosis bronchiectasis. *Respir. Med.*, **154,** 40-46.

[183] Radtke T, Boni L, Bohnacker P, Fischer P, Benden C, & Dressel H (2018) The many ways sputum flows - Dealing with high within-subject variability in cystic fibrosis sputum rheology. *Respir. Physiol Neurobiol.*, **254,** 36-39.

[184] Salem AH, Elkhatib WF, & Noreddin AM (2011) Pharmacodynamic assessment of vancomycin-rifampicin combination against methicillin resistant Staphylococcus aureus biofilm: a parametric response surface analysis. *J. Pharm. Pharmacol.*, **63,** 73-79.

[185] Siala W, Mingeot-Leclercq MP, Tulkens PM, Hallin M, Denis O, & Van Bambeke F (2014) Comparison of the antibiotic activities of Daptomycin, Vancomycin, and the investigational Fluoroquinolone Delafloxacin against biofilms from Staphylococcus aureus clinical isolates. *Antimicrob. Agents Chemother.*, **58,** 6385-6397.

[186] Smith K, Perez A, Ramage G, Gemmell CG, & Lang S (2009) Comparison of biofilm-associated cell survival following in vitro exposure of meticillin-resistant Staphylococcus aureus biofilms to the antibiotics clindamycin, daptomycin, linezolid, tigecycline and vancomycin. *Int. J. Antimicrob. Agents*, **33,** 374-378.

[187] Waryah CB, Wells K, Ulluwishewa D, Chen-Tan N, Gogoi-Tiwari J, Ravensdale J, Costantino P, Gokcen A, Vilcinskas A, Wiesner J, & Mukkur T (2017) In Vitro Antimicrobial Efficacy of Tobramycin Against Staphylococcus aureus Biofilms in Combination With or Without DNase I and/or Dispersin B: A Preliminary Investigation. *Microb. Drug Resist.*, **23,** 384-390.

[188] Azeredo J, Azevedo NF, Briandet R, Cerca N, Coenye T, Costa AR, Desvaux M, Di Bonaventura G, Hebraud M, Jaglic Z, Kacaniova M, Knochel S, Lourenco A, Mergulhao F, Meyer RL, Nychas G, Simoes M, Tresse O, & Sternberg C (2017) Critical review on biofilm methods. *Crit Rev. Microbiol.*, **43,** 313-351.

[189] Pompilio A, Crocetta V, Pomponio S, Fiscarelli E, & Di Bonaventura G (2015) In vitro activity of colistin against biofilm by Pseudomonas aeruginosa is significantly improved under "cystic fibrosis-like" physicochemical conditions. *Diagn. Microbiol. Infect. Dis.*, **82,** 318-325.

[190] Van den Driessche F, Brackman G, Swimberghe R, Rigole P, & Coenye T (2017) Screening a repurposing library for potentiators of antibiotics

against Staphylococcus aureus biofilms. *International Journal of Antimicrobial Agents*, **49,** 315-320.

[191] Dosler S & Karaaslan E (2014) Inhibition and destruction of Pseudomonas aeruginosa biofilms by antibiotics and antimicrobial peptides. *Peptides*, **62,** 32-37.

[192] Abad L, Tafani V, Tasse J, Josse J, Chidiac C, Lustig S, Ferry T, Diot A, Laurent F, & Valour F (2019) Evaluation of the ability of linezolid and tedizolid to eradicate intraosteoblastic and biofilm-embedded Staphylococcus aureus in the bone and joint infection setting. *J. Antimicrob. Chemother.*, **74,** 625-632.

[193] Marumo K, Komukai D, Hirose M, Nakamura H, Tanaka H, Ugajin K, Nagashima G, & Yoshimura A (2013) Evaluation in vitro of the efficacy of colistin methanesulfonate against biofilm-forming multidrug-resistant Pseudomonas aeruginosa (MDRP). *J. Infect. Chemother.*, **19,** 348-351.

[194] Molina-Manso D, del Prado G, Ortiz-Perez A, Manrubia-Cobo M, Gomez-Barrena E, Cordero-Ampuero J, & Esteban J (2013) In vitro susceptibility to antibiotics of staphylococci in biofilms isolated from orthopaedic infections. *Int. J. Antimicrob. Agents*, **41,** 521-523.

[195] Sabaeifard P, Abdi-Ali A, Soudi MR, & Dinarvand R (2014) Optimization of tetrazolium salt assay for Pseudomonas aeruginosa biofilm using microtiter plate method. *J Microbiol Methods*, **105,** 134-140.

[196] She P, Wang Y, Liu Y, Tan F, Chen L, Luo Z, & Wu Y (2019) Effects of exogenous glucose on Pseudomonas aeruginosa biofilm formation and antibiotic resistance. *Microbiologyopen.*, e933.

[197] Tseng BS, Zhang W, Harrison JJ, Quach TP, Song JL, Penterman J, Singh PK, Chopp DL, Packman AI, & Parsek MR (2013) The extracellular matrix protects Pseudomonas aeruginosa biofilms by limiting the penetration of tobramycin. *Environ. Microbiol.*, **15,** 2865-2878.

[198] Macia MD, Rojo-Molinero E, & Oliver A (2014) Antimicrobial susceptibility testing in biofilm-growing bacteria. *Clin Microbiol Infect.*, **20,** 981-990.

[199] Coenye T, Goeres D, Van Bambeke F, & Bjarnsholt T (2018) Should standardized susceptibility testing for microbial biofilms be introduced in clinical practice? *Clin. Microbiol. Infect.*, **24,** 570-572.

[200] Hall CW & Mah TF (2017) Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. *FEMS Microbiol Rev*, **41,** 276-301.

[201] Diaz Iglesias Y & Van Bambeke F (2020) Activity of Antibiotics against Pseudomonas aeruginosa in an In Vitro Model of Biofilms in the Context of Cystic Fibrosis: Influence of the Culture Medium. *Antimicrobial agents and chemotherapy*, **64,** e02204-e02219.

[202] Muller L, Murgia X, Siebenburger L, Borger C, Schwarzkopf K, Sewald K, Haussler S, Braun A, Lehr CM, Hittinger M, & Wronski S (2018) Human airway mucus alters susceptibility of Pseudomonas aeruginosa biofilms to tobramycin, but not colistin. *J. Antimicrob. Chemother.*, **73,** 2762-2769.

[203] Kostenko V, Salek MM, Sattari P, & Martinuzzi RJ (2010) Staphylococcus aureus biofilm formation and tolerance to antibiotics in response to oscillatory shear stresses of physiological levels. *FEMS Immunol. Med Microbiol*, **59,** 421-431.

[204] Moreira JMR, Gomes LC, Araujo JDP, Miranda JM, Simoes M, Melo LF, & Mergulhao FJ (2013) The effect of glucose concentration and shaking conditions on Escherichia coli biofilm formation in microtiter plates. *Chemical Engineering Science*, **94,** 192-199.

[205] Masadeh MM, Alzoubi KH, Ahmed WS, & Magaji AS (2019) In Vitro Comparison of Antibacterial and Antibiofilm Activities of Selected Fluoroquinolones against Pseudomonas aeruginosa and Methicillin-Resistant Staphylococcus aureus. *Pathogens*, **8,** 12.

[206] Idelevich EA, Kriegeskorte A, Schleimer N, Peters G, von Eiff C, & Becker K (2018) In Vitro Susceptibility of Clinical Staphylococcus aureus Small-Colony Variants to β-Lactam and Non-β-Lactam Antibiotics. *Antimicrob Agents Chemother*, **62,** e02532-17.

[207] Suwantarat N, Rubin M, Bryan L, Tekle T, Boyle MP, Carroll KC, & Jennings MT (2018) Frequency of small-colony variants and antimicrobial susceptibility of methicillin-resistant Staphylococcus aureus in cystic fibrosis patients. *Diagn. Microbiol Infect. Dis.*, **90,** 296-299.

[208] Garcia LG, Lemaire S, Kahl BC, Becker K, Proctor RA, Denis O, Tulkens PM, & Van Bambeke F (2013) Antibiotic activity against small-colony variants of Staphylococcus aureus: review of in vitro, animal and clinical data. *J. Antimicrob. Chemother.*, **68,** 1455-1464.

[209] Kahl BC (2014) Small colony variants (SCVs) of Staphylococcus aureus--a bacterial survival strategy. *Infect. Genet. Evol.*, **21,** 515-522.

[210] Pelletier LLJ, Richardson M, & Feist M (1979) Virulent gentamicin-induced small colony variants of Staphylococcus aureus. *J Lab Clin Med.*, **94,** 324-334.

[211] Tuchscherr L, Kreis CA, Hoerr V, Flint L, Hachmeister M, Geraci J, Bremer-Streck S, Kiehntopf M, Medina E, Kribus M, Raschke M, Pletz M, Peters G, & Loffler B (2016) Staphylococcus aureus develops increased resistance to antibiotics by forming dynamic small colony variants during chronic osteomyelitis. *J. Antimicrob. Chemother.*, **71,** 438-448.

[212] Vestergaard M, Paulander W, Leng B, Nielsen JB, Westh HT, & Ingmer H (2016) Novel Pathways for Ameliorating the Fitness Cost of Gentamicin Resistant Small Colony Variants. *Front Microbiol.*, **7,** 1866.

[213] Gerber AU & Craig WA (1982) Aminoglycoside-selected subpopulations of Pseudomonas aeruginosa: characterization and virulence in normal and leukopenic mice. *J. Lab Clin. Med.*, **100,** 671-681.

[214] Evans TJ (2015) Small colony variants of Pseudomonas aeruginosa in chronic bacterial infection of the lung in cystic fibrosis. *Future. Microbiol.*, **10,** 231-239.

[215] Malone JG (2015) Role of small colony variants in persistence of Pseudomonas aeruginosa infections in cystic fibrosis lungs. *Infect. Drug Resist.*, **8,** 237-247.

[216] Drenkard E & Ausubel FM (2002) Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature*, **416,** 740-743.

[217] Wolter DJ, Emerson JC, McNamara S, Buccat AM, Qin X, Cochrane E, Houston LS, Rogers GB, Marsh P, Prehar K, Pope CE, Blackledge M, Deziel E, Bruce KD, Ramsey BW, Gibson RL, Burns JL, & Hoffman LR (2013) Staphylococcus aureus small-colony variants are independently associated with worse lung disease in children with cystic fibrosis. *Clin Infect. Dis.*, **57,** 384-391.

[218] Biswas L, Biswas R, Schlag M, Bertram R, & Gotz F (2009) Small-colony variant selection as a survival strategy for Staphylococcus aureus in the presence of Pseudomonas aeruginosa. *Appl. Environ. Microbiol*, **75,** 6910-6912.

[219] Fugere A, Lalonde Seguin D, Mitchell G, Deziel E, Dekimpe V, Cantin AM, Frost E, & Malouin F (2014) Interspecific small molecule interactions between clinical isolates of Pseudomonas aeruginosa and Staphylococcus aureus from adult cystic fibrosis patients. *PLoS. One.*, **9,** e86705.

[220] Haley CL, Colmer-Hamood JA, & Hamood AN (2012) Characterization of biofilm-like structures formed by Pseudomonas aeruginosa in a synthetic mucus medium. *BMC Microbiol*, **12,** 181.

[221] Quinn RA, Comstock W, Zhang T, Morton JT, da Silva R, Tran A, Aksenov A, Nothias LF, Wangpraseurt D, Melnik AV, Ackermann G, Conrad D, Klapper I, Knight R, & Dorrestein PC (2018) Niche partitioning of a pathogenic microbiome driven by chemical gradients. *Sci. Adv.*, **4,** eaau1908.

[222] Hill DB, Long RF, Kissner WJ, Atieh E, Garbarine IC, Markovetz MR, Fontana NC, Christy M, Habibpour M, Tarran R, Forest MG, Boucher RC, & Button B (2018) Pathological mucus and impaired mucus clearance in cystic fibrosis patients result from increased concentration, not altered pH. *Eur Respir J*, **52,** 1801297.

[223] Moriarty TF, Elborn JS, & Tunney MM (2007) Effect of pH on the antimicrobial susceptibility of planktonic and biofilm-grown clinical Pseudomonas aeruginosa isolates. *Br. J Biomed. Sci*, **64,** 101-104.

[224] Baudoux P, Bles N, Lemaire S, Mingeot-Leclercq MP, Tulkens PM, & Van Bambeke F (2007) Combined effect of pH and concentration on the activities of gentamicin and oxacillin against Staphylococcus aureus in pharmacodynamic models of extracellular and intracellular infections. *J Antimicrob Chemother*, **59,** 246-253.

[225] Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC, Birrer P, Bellon G, Berger J, Weiss T, Botzenhart K, Yankaskas JR, Randell S, Boucher RC, & Doring G (2002) Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients. *J Clin Invest*, **109,** 317-325.

[226] King P, Citron DM, Griffith DC, Lomovskaya O, & Dudley MN (2010) Effect of oxygen limitation on the in vitro activity of levofloxacin and other antibiotics administered by the aerosol route against Pseudomonas aeruginosa from cystic fibrosis patients. *Diagn. Microbiol Infect. Dis.*, **66,** 181-186.

[227] Gottaslo R & Salahi B (2013) Effects of Oxygen on In-vitro Biofilm Formation and Antimicrobial Resistance of Pseudomonas aeruginosae. *Pharm Sci*, **19,** 96-99.

[228] Borriello G, Werner E, Roe F, Kim AM, Ehrlich GD, & Stewart PS (2004) Oxygen limitation contributes to antibiotic tolerance of Pseudomonas aeruginosa in biofilms. *Antimicrob Agents Chemother*, **48,** 2659-2664.

[229] Cramton SE, Ulrich M, Gotz F, & Doring G (2001) Anaerobic conditions induce expression of polysaccharide intercellular adhesin in Staphylococcus aureus and Staphylococcus epidermidis. *Infect. Immun.*, **69,** 4079-4085.

[230] Siala W, Rodriguez-Villalobos H, Fernandes P, Tulkens PM, & Van Bambeke F (2018) Activities of Combinations of Antistaphylococcal Antibiotics with Fusidic Acid against Staphylococcal Biofilms in In Vitro Static and Dynamic Models. *Antimicrob Agents Chemother*, **62,** e00598-18.

[231] Dalhoff A (2014) Pharmacokinetics and pharmacodynamics of aerosolized antibacterial agents in chronically infected cystic fibrosis patients. *Clin Microbiol Rev*, **27,** 753-782.

[232] Stass H, Delesen H, Nagelschmitz J, & Staab D (2015) Safety and pharmacokinetics of ciprofloxacin dry powder for inhalation in cystic fibrosis: a phase I, randomized, single-dose, dose-escalation study. *J Aerosol Med Pulm. Drug Deliv.*, **28,** 106-115.

[233] Ruddy J, Emerson J, Moss R, Genatossio A, McNamara S, Burns JL, Anderson G, & Rosenfeld M (2013) Sputum tobramycin concentrations in cystic fibrosis patients with repeated administration of inhaled tobramycin. *J Aerosol Med Pulm. Drug Deliv.*, **26,** 69-75.

[234] Chmiel JF, Aksamit TR, Chotirmall SH, Dasenbrook EC, Elborn JS, LiPuma JJ, Ranganathan SC, Waters VJ, & Ratjen FA (2014) Antibiotic management of lung infections in cystic fibrosis. I. The microbiome, methicillin-resistant Staphylococcus aureus, gram-negative bacteria, and multiple infections. *Ann. Am. Thorac. Soc.*, **11,** 1120-1129.

[235] Electronic Medicines Compendium. UK Summary of Product Characteristics - tobramycin nebuliser solution. https://www.medicines.org.uk/emc/product/2683/smpc . 2017.

[236] Moriarty TF, McElnay JC, Elborn JS, & Tunney MM (2007) Sputum antibiotic concentrations: implications for treatment of cystic fibrosis lung infection. *Pediatr. Pulmonol.*, **42,** 1008-1017.

[237] Waterer G, Lord J, Hofmann T, & Jouhikainen T (2020) Phase I, Dose-Escalating Study of the Safety and Pharmacokinetics of Inhaled Dry-Powder Vancomycin (AeroVanc) in Volunteers and Patients with Cystic Fibrosis: a New Approach to Therapy for Methicillin-Resistant Staphylococcus aureus. *Antimicrob Agents Chemother*, **64**.

[238] Yapa S, Li J, Patel K, Wilson JW, Dooley MJ, George J, Clark D, Poole S, Williams E, Porter CJH, Nation RL, & McIntosh MP (2014) Pulmonary and systemic pharmacokinetics of inhaled and intravenous colistin methanesulfonate in cystic fibrosis patients: targeting advantage of inhalational administration. *Antimicrob Agents Chemother*, **58,** 2570-2579.

[239] Electronic Medicines Compendium. UK Summary of Product Characteristics - colistin inhalation powder. https://www.medicines.org.uk/emc/product/3063/smpc . 2018.

[240] Ratjen F, Rietschel E, Kasel D, Schwiertz R, Starke K, Beier H, van Koningsbruggen S, & Grasemann H (2006) Pharmacokinetics of inhaled colistin in patients with cystic fibrosis. *J Antimicrob Chemother*, **57,** 306-311.

[241] Fish DN & Singletary TJ (1997) Meropenem, a new carbapenem antibiotic. *Pharmacotherapy*, **17,** 644-669.

[242] Saralaya D, Peckham DG, Hulme B, Tobin CM, Denton M, Conway S, & Etherington C (2004) Serum and sputum concentrations following the oral administration of linezolid in adult patients with cystic fibrosis. *J Antimicrob Chemother*, **53,** 325-328.

[243] Baumann U, King M, App EM, Tai S, Konig A, Fischer JJ, Zimmermann T, Sextro W, & von der Hardt H (2004) Long term azithromycin therapy in cystic fibrosis patients: a study on drug levels and sputum properties. *Can. Respir J*, **11,** 151-155.

[244] Wilms EB, Touw DJ, & Heijerman HGM (2008) Pharmacokinetics and sputum penetration of azithromycin during once weekly dosing in cystic fibrosis patients. *J Cyst. Fibros.*, **7,** 79-84.

[245] Wilms EB, Touw DJ, & Heijerman HGM (2006) Pharmacokinetics of azithromycin in plasma, blood, polymorphonuclear neutrophils and sputum during long-term therapy in patients with cystic fibrosis. *Ther. Drug Monit.*, **28,** 219-225.

[246] Davis RL, Koup JR, Williams-Warren J, Weber A, Heggen L, Stempel D, & Smith AL (1987) Pharmacokinetics of ciprofloxacin in cystic fibrosis. *Antimicrob Agents Chemother*, **31,** 915-919.

[247] Citron KM & May JR (1969) Rifamycin antibiotics in chronic purulent bronchitis. *Lancet*, **2,** 982-983.

[248] Acocella G (1983) Pharmacokinetics and metabolism of rifampin in humans. *Rev Infect. Dis.*, **5 Suppl 3,** S428-S432.

[249] BioSurface Technologies Corporation. CDC Biofilm Reactor - Operator's manual. https://biofilms.biz/biosurfacetechnologies.com/wp-content/uploads/2020/01/CBR-Operators-Manual-Dec2019.pdf . 2019.

[250] Hill JA & Cowen LE (2015) Using combination therapy to thwart drug resistance. *Future. Microbiol*, **10,** 1719-1726.

[251] Tre-Hardy M, Nagant C, El Manssouri N, Vanderbist F, Traore H, Vaneechoutte M, & Dehaye JP (2010) Efficacy of the combination of tobramycin and a macrolide in an in vitro Pseudomonas aeruginosa mature biofilm model. *Antimicrob Agents Chemother*, **54,** 4409-4415.

[252] Foucquier J & Guedj M (2015) Analysis of drug combinations: current methodological landscape. *Pharmacol. Res. Perspect.*, **3,** e00149.

[253] Jorgensen NP, Skovdal SM, Meyer RL, Dagnaes-Hansen F, Fuursted K, & Petersen E (2016) Rifampicin-containing combinations are superior to combinations of vancomycin, linezolid and daptomycin against Staphylococcus aureus biofilm infection in vivo and in vitro. *Pathog. Dis.*, **74,** ftw019.

[254] El Haj C, Murillo O, Ribera A, Lloberas N, Gomez-Junyent J, Tubau F, Fontova P, Cabellos C, & Ariza J (2018) Evaluation of linezolid or trimethoprim/sulfamethoxazole in combination with rifampicin as alternative oral treatments based on an in vitro pharmacodynamic model of staphylococcal biofilm. *Int. J Antimicrob Agents*, **51,** 854-861.

[255] Kapoor P & Murphy P (2018) Combination antibiotics against Pseudomonas aeruginosa, representing common and rare cystic fibrosis strains from different Irish clinics. *Heliyon.*, **4,** e00562.

[256] Raymond B (2019) Five rules for resistance management in the antibiotic apocalypse, a road map for integrated microbial management. *Evol. Appl.*, **12,** 1079-1091.

[257] Vestergaard M, Paulander W, Marvig RL, Clasen J, Jochumsen N, Molin S, Jelsbak L, Ingmer H, & Folkesson A (2016) Antibiotic combination therapy can select for broad-spectrum multidrug resistance in Pseudomonas aeruginosa. *Int. J Antimicrob Agents*, **47,** 48-55.

[258] Diaz Iglesias Y & Van Bambeke F (2019) Daptomycin enhances nafcillin and ceftaroline activity against *S. epidermidis* biofilms. *Annual symposium of the Belgian Society of Microbiology*, poster presentation.

[259] Sakoulas G, Okumura CY, Thienphrapa W, Olson J, Nonejuie P, Dam Q, Dhand A, Pogliano J, Yeaman MR, Hensler ME, Bayer AS, & Nizet V (2014) Nafcillin enhances innate immune-mediated killing of methicillin-resistant Staphylococcus aureus. *J Mol Med (Berl)*, **92,** 139-149.

[260] Sun F, Tai S, Lim T, Baumann U, & King M (2002) Additive effect of dornase alfa and Nacystelyn on transportability and viscoelasticity of cystic fibrosis sputum. *Can. Respir. J*, **9,** 401-406.

[261] Conrad C, Lymp J, Thompson V, Dunn C, Davies Z, Chatfield B, Nichols D, Clancy J, Vender R, Egan ME, Quittell L, Michelson P, Antony V, Spahr J, Rubenstein RC, Moss RB, Herzenberg LA, Goss CH, & Tirouvanziam R (2015) Long-term treatment with oral N-acetylcysteine: affects lung function but not sputum inflammation in cystic fibrosis subjects. A phase II randomized placebo-controlled trial. *J Cyst. Fibros.*, **14,** 219-227.

[262] Belfield K, Bayston R, Hajduk N, Levell G, Birchall JP, & Daniel M (2017) Evaluation of combinations of putative anti-biofilm agents and antibiotics to eradicate biofilms of Staphylococcus aureus and Pseudomonas aeruginosa. *J. Antimicrob. Chemother.*, **72,** 2531-2538.

[263] Blasi F, Page C, Rossolini GM, Pallecchi L, Matera MG, Rogliani P, & Cazzola M (2016) The effect of N-acetylcysteine on biofilms: Implications for the treatment of respiratory tract infections. *Respir. Med*, **117,** 190-197.

[264] Thornton RB, Wiertsema SP, Kirkham LA, Rigby PJ, Vijayasekaran S, Coates HL, & Richmond PC (2013) Neutrophil extracellular traps and bacterial biofilms in middle ear effusion of children with recurrent acute otitis media--a potential treatment target. *PLoS. One.*, **8,** e53837.

[265] Dinicola S, De Grazia S, Carlomagno G, & Pintucci JP (2014) N-acetylcysteine as powerful molecule to destroy bacterial biofilms. A systematic review. *Eur. Rev Med Pharmacol. Sci*, **18,** 2942-2948.

[266] Koo H, Allan RN, Howlin RP, Stoodley P, & Hall-Stoodley L (2017) Targeting microbial biofilms: current and prospective therapeutic strategies. *Nat. Rev Microbiol*, **15,** 740-755.

[267] Vallet-Regi M, Gonzalez B, & Izquierdo-Barba I (2019) Nanomaterials as Promising Alternative in the Infection Treatment. *Int J Mol Sci*, **20,** pii: E3806.

[268] Clancy JP, Dupont L, Konstan MW, Billings J, Fustik S, Goss CH, Lymp J, Minic P, Quittner AL, Rubenstein RC, Young KR, Saiman L, Burns JL, Govan JRW, Ramsey B, & Gupta R (2013) Phase II studies of nebulised Arikace in CF patients with Pseudomonas aeruginosa infection. *Thorax*, **68,** 818-825.

[269] Cipolla D, Blanchard J, & Gonda I (2016) Development of Liposomal Ciprofloxacin to Treat Lung Infections. *Pharmaceutics.*, **8,** pii: E6.

[270] Pires DP, Melo L, Vilas Boas D, Sillankorva S, & Azeredo J (2017) Phage therapy as an alternative or complementary strategy to prevent and control biofilm-related infections. *Curr. Opin. Microbiol*, **39,** 48-56.

[271] Chaudhry WN, Concepcion-Acevedo J, Park T, Andleeb S, Bull JJ, & Levin BR (2017) Synergy and Order Effects of Antibiotics and Phages in Killing Pseudomonas aeruginosa Biofilms. *PLoS. One.*, **12,** e0168615.

[272] Kumaran D, Taha M, Yi Q, Ramirez-Arcos S, Diallo JS, Carli A, & Abdelbary H (2018) Does Treatment Order Matter? Investigating the Ability of Bacteriophage to Augment Antibiotic Activity against Staphylococcus aureus Biofilms. *Front Microbiol*, **9,** 127.

[273] Lusiak-Szelachowska M, Weber-Dàbrowska B, & Gorski A (2020) Bacteriophages and Lysins in Biofilm Control. *Virologica Sinica*, **35,** 125-133.

[274] Hotterbeekx A, Kumar-Singh S, Goossens H, & Malhotra-Kumar S (2017) In vivo and In vitro Interactions between Pseudomonas aeruginosa and Staphylococcus spp. *Front Cell Infect. Microbiol.*, **7,** 106.

[275] Duan K, Dammel C, Stein J, Rabin H, & Surette MG (2003) Modulation of Pseudomonas aeruginosa gene expression by host microflora through interspecies communication. *Mol. Microbiol*, **50,** 1477-1491.

[276] Li H, Li X, Wang Z, Fu Y, Ai Q, Dong Y, & Yu J (2015) Autoinducer-2 regulates Pseudomonas aeruginosa PAO1 biofilm formation and virulence production in a dose-dependent manner. *BMC Microbiol*, **15,** 192.

[277] Baldan R, Cigana C, Testa F, Bianconi I, De Simone M, Pellin D, Di Serio C, Bragonzi A, & Cirillo DM (2014) Adaptation of Pseudomonas aeruginosa in Cystic Fibrosis airways influences virulence of Staphylococcus aureus in vitro and murine models of co-infection. *PLoS. One.*, **9,** e89614.

[278] Mashburn LM, Jett AM, Akins DR, & Whiteley M (2005) Staphylococcus aureus serves as an iron source for Pseudomonas aeruginosa during in vivo coculture. *J Bacteriol.*, **187,** 554-566.

[279] Petrova OE & Sauer K (2016) Escaping the biofilm in more than one way: desorption, detachment or dispersion. *Curr. Opin. Microbiol*, **30,** 67-78.

[280] Qazi S, Middleton B, Muharram SH, Cockayne A, Hill P, O'Shea P, Chhabra SR, Camara M, & Williams P (2006) N-acylhomoserine lactones antagonize virulence gene expression and quorum sensing in Staphylococcus aureus. *Infect. Immun.*, **74,** 910-919.

[281] Armbruster CR, Wolter DJ, Mishra M, Hayden HS, Radey MC, Merrihew G, MacCoss MJ, Burns J, Wozniak DJ, Parsek MR, & Hoffman LR (2016) Staphylococcus aureus Protein A Mediates Interspecies Interactions at the Cell Surface of Pseudomonas aeruginosa. *mBio*, **7,** e00538-16.

[282] Nielsen SM, Penstoft LN, & Norskov-Lauritsen N (2019) Motility, Biofilm Formation and Antimicrobial Efflux of Sessile and Planktonic Cells of Achromobacter xylosoxidans. *Pathogens.*, **8,** 14.

[283] Kennedy S, Beaudoin T, Yau YCW, Caraher E, Zlosnik JEA, Speert DP, LiPuma JJ, Tullis E, & Waters V (2016) Activity of Tobramycin against Cystic Fibrosis Isolates of Burkholderia cepacia Complex Grown as Biofilms. *Antimicrob Agents Chemother*, **60,** 348-355.

[284] Ciacci N, Boncompagni S, Valzano F, Cariani L, Aliberti S, Blasi F, Pollini S, Rossolini GM, & Pallecchi L (2019) In Vitro Synergism of Colistin and N-acetylcysteine against Stenotrophomonas maltophilia. *Antibiotics (Basel)*, **8,** 101.

[285] Hunt-Serracin AC, Parks BJ, Boll J, & Boutte CC (2019) Mycobacterium abscessus Cells Have Altered Antibiotic Tolerance and Surface Glycolipids in Artificial Cystic Fibrosis Sputum Medium. *Antimicrob Agents Chemother*, **63,** e02488-18.