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Impact of efflux mechanisms on susceptibility and resistance to beta-lactam antibiotics in *Pseudomonas aeruginosa*: beyond the usual concepts

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Dedicated to …

My beloved mother & father:
"Nahed Ibrahim El Hajj" & "Abdel Aziz Ali Chalhoub"

The three flowers of my family, my sisters:
Rima, Maha and Zainab

My wonderful brothers:
Ali, Mohammad and Mahdi

Cystic fibrosis patients... Your strength to suffer pain today will be the pen that writes a new destiny for the future of this disease. No matter what happens now, always remember that you are a survivor, not a victim. Get well soon!

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Nomenclature

AmpC	Ampicillin class C β-lactamase; molecular classe C cephalosporinase
CAZ/AVI	Ceftazidime and avibactam (AVYCAZ® or ZAVICEFTA®)
BSAC	British Society of Antimicrobial Chemotherapy
CDC	Centres for disease control and prevention (USA)
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CLSI	Clinical and Laboratory Standards Institute
ECDC	European centre for disease prevention and control
EDTA	Ethylenediaminetetraacetic acid
EMA	European Medicines Agency
BL	β-lactamase
BLI	β-lactamase inhibitor
ESBL	Extended-spectrum β-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
GES	Guiana extended-spectrum β-lactamase
IV	Intravenous
IMP	Imipenemase (molecular class B)
KPC	Klebsiella pneumoniae carbapenemase (molecular class A)
LPS	Lipopolysaccharide
MBL	Metallo β-lactamase (molecular class B)
MBC	Minimum bactericidal concentration
MDR	Multidrug resistance
MDR-PA	Multidrug-resistant *P. aeruginosa*
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
NDM	New-Delhi metallo-β-lactamase
OXA	Oxacillinase (molecular class D)
PA	*Pseudomonas aeruginosa*
PBP	Penicillin-binding proteins
PCR	Polymerase chain reaction
RND	Resistance-Nodulation-Division efflux pumps
TMO	Temocillin
QS	Quorum sensing
UTI	Urinary tract infection
VIM	Verona integron-encoded metallo-β-lactamase (molecular class B)

Summary

Pseudomonas aeruginosa (PA) is one of the major causes of respiratory tract infections in patients suffering from cystic fibrosis (CF). Because of the recurrent character of these infections, CF patients need frequent antibiotic treatments, leading to the selection of multiresistance. Our work was specifically directed towards the evaluation of active efflux as a resistance mechanism to β-lactam antibiotics in these patients. Using a collection of PA isolates collected from CF patients from 4 different European countries (France, UK, Belgium and Germany), we undertook two surveys: (1) the first one studied the current situation of antibiotic resistance in these isolates and determined the role of efflux in acquired resistance to specific β-lactam antibiotics, (2) the second one aimed at studying the role of active efflux in intrinsic resistance to temocillin, considered as inactive against PA.

Our findings support the increased prevalence of multidrug-resistant PA among CF patients in the different countries, limiting the utility of the current antibiotics. We could demonstrate that active efflux plays a major role in resistance to β-lactam antibiotics in this collection. First, we found that overexpression of efflux systems confers high-level resistance to carbapenems, particularly meropenem, when associated with reduced influx due to OprD porin alterations. Second, we showed that the new β-lactamase inhibitor avibactam, which restores the activity to ceftazidime against part of the collection, is also facing poor membrane permeability that impedes its activity in PA showing increased efflux and decreased influx. Third, we made the unanticipated observation that temocillin shows clinically-exploitable activity against a high proportion of isolates, which harbour natural mutations in the genes encoding MexAB-OprM efflux pump, impairing its efflux capabilities.

This work calls to revisit the generally-accepted concept that efflux mechanism is only involved in low-level resistance. Moreover, it allows to better position the interest of meropenem and ceftazidime-avibactam in CF patients and suggests a potential interest for temocillin in these patients.

1. Introduction

1.1 The nightmare of antibiotic-resistant bacteria: causes and impacts on public health

According to World Health Organization (WHO) [1], bacterial resistance represents a serious worldwide threat to public health. It's one of the biggest global health care problems of our time [2]. The world is headed for a post-antibiotic era that could bring to "an end to modern medicine as we know it" (Dr Margaret Chan, Director-General of the WHO). Discovered by Alexander Fleming in 1928, antibiotics have been used for the last 70 years (starting with penicillin in the 1946) to treat patients with bacterial infectious diseases. These drugs have greatly reduced illness and mortality rates from infectious diseases. However, they have been used so widely and for so long that the bacteria the antibiotics are designed to kill have adapted to them, making the drugs less effective [3-5].

❖ **How does antibiotic resistance occur?**

Different resistance mechanisms to antibiotics can be naturally present in bacteria or acquired over time, following several paths:

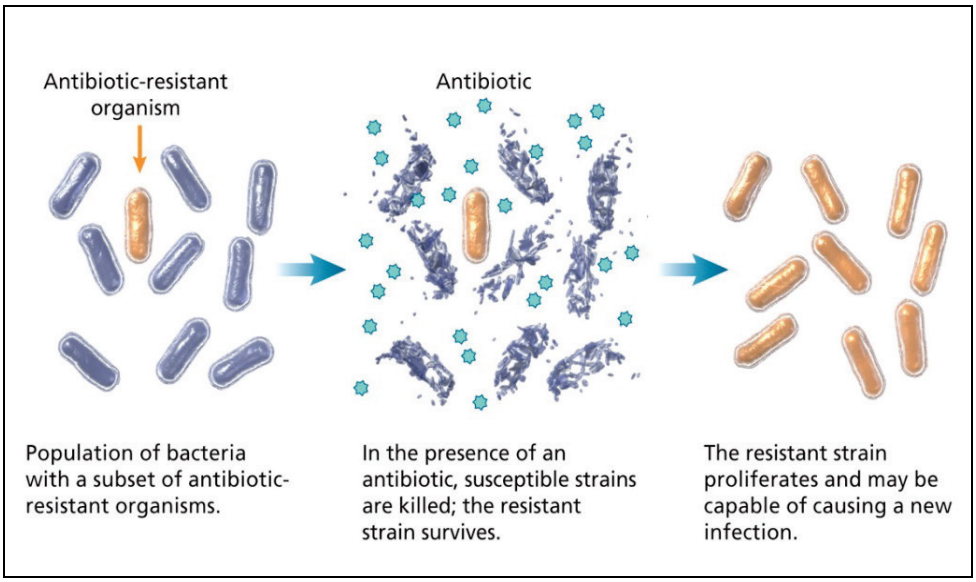
(1) Natural resistance in certain types of bacteria that produce antibiotics as a mean of defence against other bacteria, and need at the same time an appropriate mechanism to defend themselves against the produced antibiotics. Natural resistance can also result from a decreased drug permeability via loss of porin channels combined with active efflux in Gram-negative bacteria.

(2) Spontaneous mutations occurring during bacterial replication, which may help individual bacteria to survive exposure to an antibiotic when these mutations affect the antibiotic target.

(3) Exchange of resistance genes with other bacteria, then gaining the ability to resist to one or more antibiotics [5-7]. The spread of antibiotic resistance occurs through bacterial populations, vertically when new generations inherit antibiotic resistance genes, and horizontally by exchanging genetic material between bacteria [8]. Horizontal gene transfer can even occur between different bacterial species. Self-replicating plasmids [9-11], prophages [12], transposons [13], integrons [14] and resistance islands [15], all represent DNA elements that transfer resistance genes into sensitive bacteria. Environmentally, antibiotic resistance spreads as bacteria themselves move from place to place via people, who can transmit resistant bacteria by coughing or contact with unwashed hands, for example. The use of antibiotics in animals may also represent a risk to human health. Resistant bacteria can travel from livestock to humans via air, water and contact. They may affect all consumers, not only fish and meat eaters [16-18], via different ways of transmission: (1) contaminated wastewater flowing from a pipe into a lagoon, (2) insects (like house flies) that can also transmit pathogens to food, or (3) fomites (non-living objects) such as barbed wire, skin cells, hair, clothing, and bedding.

The overuse and misuse of antibiotics can worsen the problem by imposing a selective pressure that helps selecting microorganisms resistant to the used antibiotic or other ones if affected by the same resistance mechanism. The survivors will replicate, and their progeny will quickly become the dominant type throughout the bacterial population (figure 1.1).

Figure 1.1: Survival of resistant bacteria under the selective pressure of antibiotics, exerting a Darwinian selection. Picture from: http://mostlyscience.com/2014/06/antibiotic-resistance



❖ **Susceptible (S), intermediate (I) or resistant (R) bacteria?**

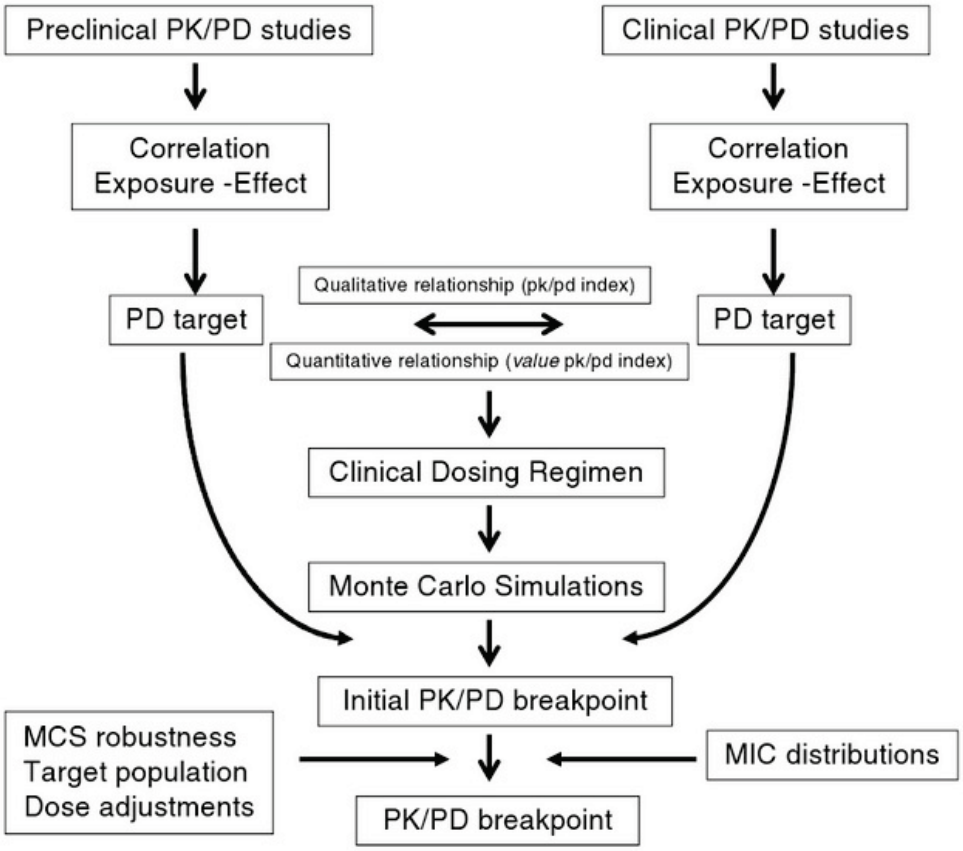
Antibacterial activity is measured *in vitro* by determination of the minimum inhibitory concentration (MIC), which are defined as the lowest concentration of an antibiotic that will inhibit the visible growth of a bacterium after overnight incubation. The MIC values do not account for fluctuations of drug concentrations within the body. Therefore, pharmacokinetic and pharmacodynamic (PK/PD) indices are required to predict the clinical efficacy of an antibiotic [19]. PK studies attempt to discover the fate of an antibiotic from the moment it is administered up to the point at which it is completely eliminated from the body. It describes what the body does to the antibiotic after its administration, through the mechanisms of absorption and distribution, as well as the metabolism and the routes of excretion of the metabolites. PK indices of an antibiotic may be affected by elements such as the mode of administration and the corresponding dose. PK studies are often

associated with PD studies that analyse the drug pharmacological effect on the body. This includes evaluation of antibiotic efficacy in experimental settings and clinical studies to derive pharmacodynamic targets such as the time where the antibiotic free concentration remains above the MIC (%*f*T > MIC) required for efficacy, and computational algorithms (Monte Carlo experiments) to estimate exposures of the antibiotic in the target patient population according to specific dosing regimens. Clinical breakpoints are then derived and evaluated with respect to the wild-type population of the target microorganisms (figure 1.2) [20;21]. These clinical breakpoints are used in clinical microbiology laboratories to categorize microorganisms as clinically susceptible (S), intermediate (I) or resistant (R) to the antibiotic. Clinical breakpoints are set, in Europe, by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), and in USA, formerly by the Clinical and Laboratory Standards Institute (CLSI) and now by the Food and Drug Administration (FDA), following a defined procedure [21].

According to EUCAST, a micro-organism is defined as susceptible (S, where the antimicrobial activity is associated with a high likelihood of therapeutic success), intermediate (I, where the antimicrobial activity is associated with an uncertain therapeutic effect) and resistant (R, where the antimicrobial activity is associated with a high likelihood of therapeutic failure). Thus, if the MIC is lower than or equal to the susceptibility breakpoint the bacterium is considered susceptible to the antibiotic, if the MIC is greater than this value, the bacterium is considered intermediate or resistant to the antibiotic.

The European Medicines Agency (EMA) and Food and Drug Administration (FDA) are always asked to ensure that antibiotic labels contain up-to-date breakpoints.

Figure 1.2: Summary of the process of setting PK/PD breakpoints by EUCAST [21]. Monte Carlo simulations (MCS) are a broad class of computational algorithms that model the probability of different outcomes in the target patient population, thereby taking into account interpatient variability in drug exposure, drug potency, as well as *in vivo* exposure targets predictive of positive therapeutic outcomes.



❖ Can bacteria lose their antibiotic resistance?

The outcome of a resistance mutation is determined by its fitness in the bacterial population. Mutations that have little fitness cost are more likely to persist in the absence of antibiotic treatment [22]. When the selective pressure that is applied by the presence of an antibiotic is removed, the bacterial population can occasionally revert back to susceptible population by losing its resistance mechanism [23-25]. Reversion from resistance to susceptibility will thus depend on the fitness cost of resistance. If this cost is high, susceptible bacteria will outcompete resistant bacteria upon removal of selective pressure. However, the process of reversibility tends to occur more slowly at the community level. A main reason for this poor reversibility is that several resistance mechanisms can be simultaneously present on a transmissible plasmid [26-29]. A co-selection of resistance can thus occur if replacing the original antibiotic by another one, due to a close genetic linkage among the respective antimicrobial resistance genes. This poor reversibility of resistance has been exemplified by a Swedish study showing that a drastic reduction in trimethoprim use was not accompanied by a commensurate decrease in resistance rates, due to a low fitness cost of trimethoprim resistance together with a strong co-selection of resistance to other antibiotics [30].

❖ Antibiotic prescribing: attitudes, behaviours and cost

Worldwide, it is estimated that half of antibiotics are inappropriately prescribed, dispensed or sold [1]. In some countries and over the Internet, antibiotics are purchased without a doctor's prescription or directly from the pharmacy, where patients most commonly seek care for viral illnesses, like the common cold or other acute respiratory infections that are caused by viruses. These unnecessary prescriptions could impact countries at all economic levels while bacterial resistance increases, causing more severe illnesses that require extended hospital stays, more toxic, or more expensive medications [31-34].

❖ **Required actions for antibiotic resistance**

The decline in the development of new antibiotics together with the rise in antibiotic resistance requires three main actions across all government sectors and society [35] :

a. Development of new drugs: this strategy implies a close collaboration between academy (universities laboratories, research centres) and pharmaceutical industries in order to find new molecules targeting still unexploited targets as potential antibiotics for the future [36;37].

b. Reviving of old antibiotics [38]: Many of the old-generation antibiotics were approved a long time ago, in an era when there were no clear drug development procedures. Reintroducing these old compounds and setting up their correct use to their full potential, could help to alleviate the pressure on more recent agents. To this effect, rational approaches for redevelopment are needed according to today's standards, integrating new knowledge into regulatory frameworks and communicating the knowledge from the research bench to the bedside. Without these systematic approaches, there is a significant risk of doing harm to patients and further increasing multidrug resistance. A number of these old antibiotics are re-emerging as valuable alternatives for the treatment of difficult-to-treat infections such as fosfomycin [39] and temocillin. Temocillin, which was developed in the 1980s in the UK, was recently reintroduced and proved efficacious in urinary tract infections against β-lactamases-producing bacteria [40].

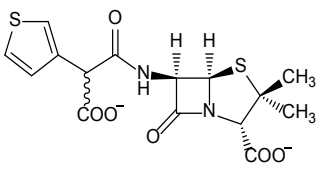
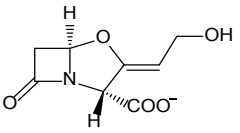
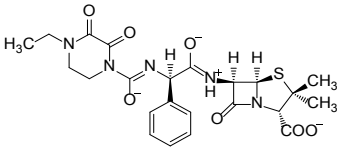
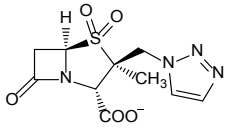
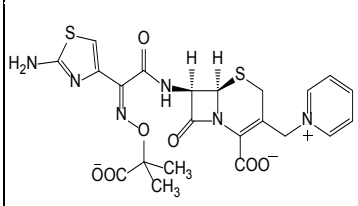
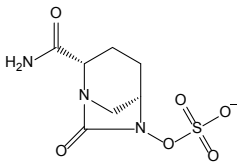
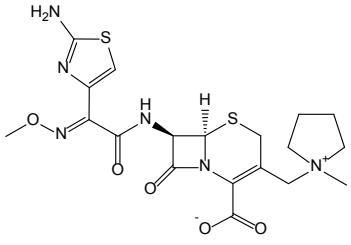
c. Antimicrobial stewardship: in order to preserve the antibiotics we have, our goal should be focused on minimizing the development of resistance at the individual patient level and at the community level. Government sectors in all countries should control and prevent the antimicrobial overuse, misuse, and abuse by working with health care practitioners. This will help each patient to receive the most appropriate antimicrobial agent with the correct dose and duration [41].

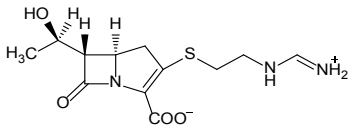
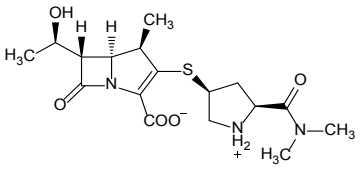
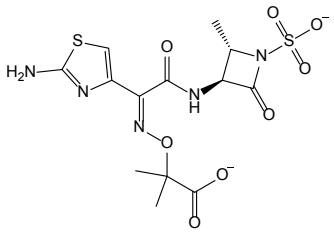
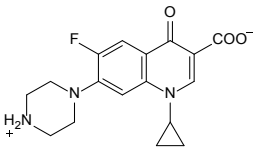
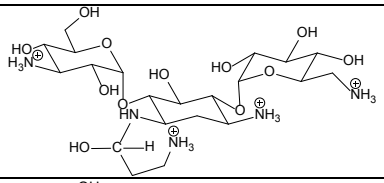
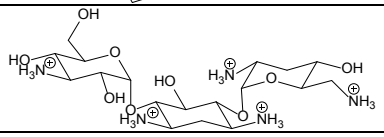
1.2 About *Pseudomonas aeruginosa:* an opportunistic pathogen

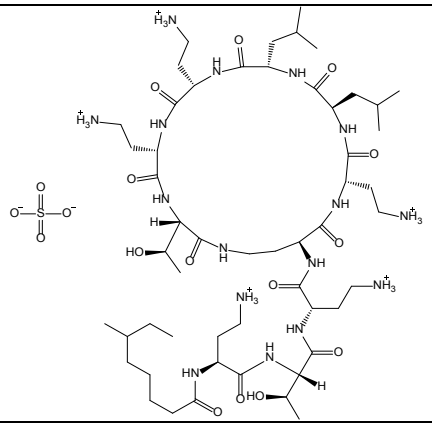
Pseudomonas aeruginosa (PA; Gram-negative bacterium) is an opportunistic human pathogen associated with hospital-acquired infections, especially in patients with weakened immune system (such HIV-infected, neutropenic, cancer or cystic fibrosis patients) or hospitalized in intensive care units (ICUs). PA causes acute and chronic infections in eyes, kidneys, blood, skin, heart valves and lungs (pneumonia). Moreover, it has been reported that PA can form biofilms, which play a major role in infections like pulmonary infections in cystic fibrosis patients, chronic pneumonia, chronic wound infections, medical device-related infections, etc. It forms biofilms with extreme tolerance to antibiotics, prompting the Centers for Disease Control and Prevention to classify PA under serious-threat level [42]. PA is one of the so-called ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, PA, and *Enterobacter species*), which currently cause the majority of hospital-acquired infections throughout the world and effectively "escape" the effects of antibacterial drugs [43]. Several studies have reported the hospital-acquired spread and transmission of clonal strains of PA [44-46]. Different antibiotic classes are used in the clinics to treat pseudomonal infections, they are called antipseudomonal drugs and include the classes of β-lactams, aminoglycosides, quinolones, and polymyxins (table 1.1). PA may resist to a wide-range of antibiotics. Such strains are referred to as multidrug-resistant PA (MDR-PA), extensively drug-resistant (XDR) or pandrug-resistant (PDR), which require double antibiotic coverage including β-lactams and other antibiotics like aminoglycosides, or in some cases colistin as last resort antibiotic [47]. Although there is no standard definition for the multiple resistance, a definition is proposed for MDR as acquired resistance to at least one agent in three or more antibiotic categories, XDR as resistance to at least one agent in all but one or two antibiotic categories and PDR as resistance to all agents in all antibiotic categories [48;49]. The high prevalence of MDR-PA is a serious challenge for antibacterial therapy

and becomes more challenging with each passing year. As this pathogen acquires more transferrable resistance mechanisms and continues to rapidly adapt to acquire additional resistance mechanisms during the course of antimicrobial therapy, we face the growing threat of pan-resistance.

Table 1.1. Common antipseudomonal antibiotics.

Anti-microbial category	Antimicrobial agent +/- β-lactamase inhibitors	Chemical structure at the physiological pH = 7.4
Penicillins +β-lactamase inhibitors	*Ticarcillin + clavulanic acid*	Ticarcillin  Clavulanic acid 
	Piperacillin + tazobactam	Piperacillin  Tazobactam 
Cephalosporins	*Ceftazidime +/- avibactam*	Ceftazidime  Avibactam 
	Cefepime #	

Carbapenems	*Imipenem*	
	Meropenem	
Monobactams	*Aztreonam* [#]	
Fluoro-quinolones	*Ciprofloxacin*	
Aminoglycosides	*Amikacin*	
	Tobramycin	

Polymyxins	*Colistin sulfate*	

[#] Susceptibility to cefepime and aztreonam were not tested in this work towards the whole collection of clinical isolates of PA. They were used only as resistance markers towards MexXY-OprM-, AmpC- or ESBL-overproducing control strains of PA.

1.3 *Pseudomonas* infections in cystic fibrosis

1.3.1 Cystic fibrosis

Cystic fibrosis (CF) is the most prevalent lethal hereditary disease in the Caucasian population, and much less common in Afro-Caribbean and Asian people. It is inherited in an autosomal recessive manner. The gene involved is located on chromosome 7 encoding cystic fibrosis transmembrane conductance regulator (CFTR). Different errors can occur in CFTR gene, they are associated with different severities of the disease. To date, more than 1800 mutations have been found in the CFTR gene. However, the deletion of the phe508-codon is the most prevalent mutation.

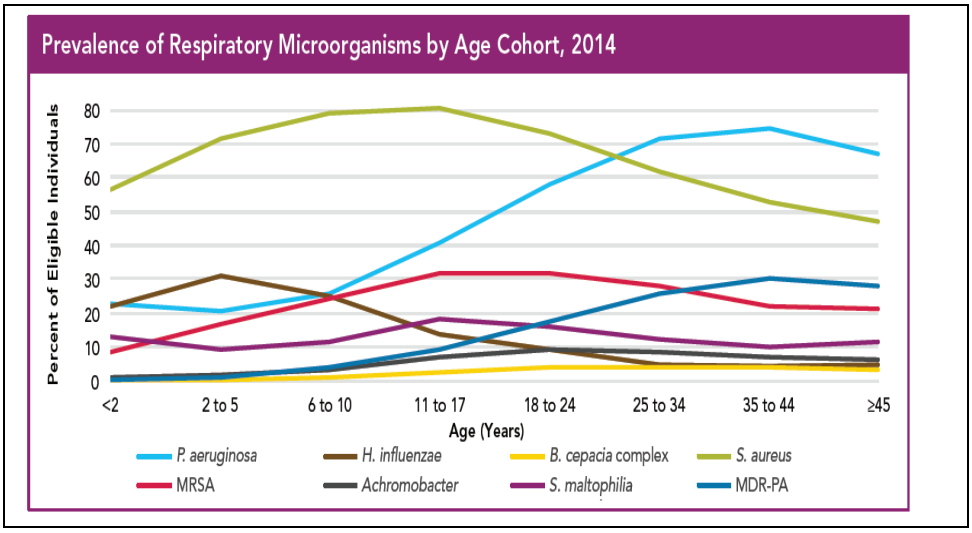
The widespread presence of CFTR throughout the body (lungs, salivary glands, pancreas, liver, kidneys, sweat ducts and reproductive tract) helps to explain why CF is a multisystem condition affecting many organs. The two major systems affected are the lungs and the gastrointestinal tract. As a transmembrane channel, CFTR protein helps to control anion transport (sodium and chloride ions), and mucociliary clearance in the airways. Functional failure of CFTR channels results in mucus retention in the lungs [50]. The accumulation of viscous and nutrient-rich mucus secretions in the lungs of CF patients creates an appropriate territory for bacteria [51], where they hide from the body's immune system and subsequently colonize lungs.

Moreover, mutations in the CFTR gene dysregulate a variety of components of the innate immune system in individuals suffering from CF. Macrophages are impaired and inflammatory signal transduction pathways exaggerated [52-54]. Consequently, CF patients suffer from recurrent and chronic bacterial airway infections that eventually lead to progressive pulmonary deterioration, and preterm death.

1.3.2 Pathogenesis of *Pseudomonas* infections

PA is one of the first pathogens that can be isolated from the airways of adult CF patients and in most patients, the same clone persists for many years, leading to chronic lung infection. Figure 1.3 shows the predominant respiratory bacteria in CF diseases at the childhood and adulthood. PA begins to invade these patients from 17 years to dominate their lungs in adulthood. In parallel, colonies of MDR-PA start to emerge as resistant to multiple classes of antibiotics, which are the most problematic phenotypes.

Figure 1.3. Invasion of PA and MDR-PA at the adulthood of CF patients [55].



PA infections represent a multi-stage process in the lungs of CF patients. PA exhibits complex interactions with the unique environment of the CF lung. The initial adhesion of PA to the epithelial lining of the respiratory tract may be transient and intermittent, with antimicrobial therapy initially eradicating the infection. CF patients acquire this bacterium from environmental sources, however patient-to-patient transmission could occur and a series of epidemic clones have been described [56;57]. In the majority of adult CF patients, a new pulmonary exacerbation is usually from their same chronic PA strain

isolated on prior cultures, and not caused by the acquisition of a new strain [58].
Once chronic colonization occurs, the phenotypes of PA switch to alginate producers, forming mucoid biofilms that enable bacteria to successfully establish chronic infections [59]. In CF lungs, the exopolysaccharide alginate is the major part of the PA biofilm matrix consisting of polysaccharides, protein and DNA. Biofilm growth in CF lungs is associated with an increased frequency of mutations, as well as adaptation of the bacteria to the conditions in the lungs (mucoid, non-mucoid, slow growth and small colony variants [60;61]), and to antibiotic therapy (resistance mechanisms such as chromosomal β-lactamase, upregulated efflux pumps) [62]. Colonization of the CF airways with mucoid biofilms, as well as high-level resistance to multiple antibiotics have been associated with an accelerated rate of decline in lung function [63].

Biofilm formation can be prevented by early aggressive antibiotic therapy [64;65]. Once the biofilm has been established, the clinical progression of CF proceeds in two characteristic directions: (1) an acute exacerbation caused by planktonic bacteria that have germinated from the biofilm, (2) a slow progression of disease that is induced by chronic immune response against alginate, as well as the formation of circulating immune complexes that are deposited on lung tissue [66]. As a consequence, chronic suppressive therapy becomes standard and imperative for CF patients [67].

1.3.3 Antibiotherapy: oral, intra-venous and inhaled

Medications used to treat CF patients may include antibiotics, pancreatic enzyme supplements, multivitamins [68], mucolytics [69], bronchodilators, anti-inflammatory agents [70;71], and CFTR modulators like ivacaftor and lumacaftor [72-74].
Here we focus on PA infections in CF, and treatment with antibiotics that may be given orally, intravenously or by inhalation. The choice of the antipseudomonal agent, its route of administration as well as the threshold for starting treatment vary at individual clinician level, both within the same

centre and between centres. The decision includes the following consideration(s): (1) susceptibility profile (antibiogram) of PA to various antibiotics, (2) past patient's response to specific oral and/or intravenous (IV) therapy, (3) allergy and tolerability to antibiotics. However, *In vitro* susceptibilities may not predict treatment outcome [75], which makes care for CF patients with pulmonary exacerbations all the more challenging and perplexing for prescribers.

❖ Oral

Initial therapy of PA infections usually involves a course of oral antibiotics. Oral fluoroquinolones (ciprofloxacin, levofloxacin) are the only oral antibiotics available for treatment of mild exacerbation caused by PA, or as part of antibiotic combination therapy for early treatment [76] or more severe exacerbations [77]. One course (2-week) of ciprofloxacin is commonly administered in outpatients with CF as first-line therapy. When chronic biofilm infection is established, its permanent eradication becomes extremely difficult or impossible [78]. Multiple courses of oral antibiotics have been shown to be less likely to prevent progression to IV therapy in patients who may have failed initial therapy [79]. Over time, PA isolates acquire resistance to fluoroquinolones and CF patients get more advanced disease. Failure to respond to the oral quinolones and pulmonary exacerbations requiring more than one course of oral antibiotics, are likely to select IV or/and inhaled therapy as the initial therapy. Using chronic suppressive antibiotic therapy [67;80], the bacterial load can be reduced and lung function of CF patients can be maintained, and the patients can survive for decades.

Other oral antibiotics used for PA-colonized CF patients include macrolides (azithromycin and clarithromycin), which are actually considered as gold standard for their anti-inflammatory and immunomodulatory properties [81;82]. It is less clear how macrolides ameliorate the lung disease associated with PA, which is considered to be intrinsically resistant to these drugs [83]. Clinical trials investigating the effectiveness and safety of macrolides in CF patients have demonstrated a benefit of chronic therapy with azithromycin or clarithromycin by improving lung function and reducing

pulmonary exacerbations [81;84;85]. *In vitro* studies have also shown that azithromycin and clarithromycin paired with tobramycin (IV) were synergistic and highly active against PA [86-88]. Interestingly, our laboratory has recently demonstrated that macrolides could be active against PA strains when tested in bronchoalveolar lavage fluid, serum or eukaryotic cell culture media, which may help explain the clinical efficacy of macrolides against pseudomonal infections [89]. Laboratories and pharmaceutical industries are studying the feasibility of macrolide inhalation as a novel approach for the treatment of pulmonary diseases due to their anti-inflammatory effects and antimicrobial spectrum at high concentrations [90].

❖ Intravenous (IV)

The duration of IV therapy varies and is often 10-21 days, but optimal duration is not clearly defined. IV antibiotics for acute respiratory exacerbations in CF are usually given in combinations because of concerns about development of antibiotic resistance in isolates of PA with single drug usage. The most frequently used combination therapy includes a β-lactam antibiotic (ticarcillin/clavulanate, piperacillin/tazobactam, ceftazidime, cefepime, imipenem/cilastin, meroperem, aztreonam) plus an aminoglycoside (amikacin, tobramycin). Since β-lactams and aminoglycosides have different bacterial targets, as well as different modes of entry into the bacterial cell, they are synergistic when used in combination. More specifically, by inhibiting peptidoglycan synthesis, β-lactam antibiotics increase the permeability of the bacterial cell wall resulting in greater aminoglycoside penetration and access to target ribosomes [91;92]. Although there are theoretical advantages to combination therapy shown by *in vitro* and animal studies, clinical data lead to conflicting conclusions [93]. Deciding on an optimal combination and specific dosing regimens (i.e. high dose aminoglycoside once daily and continuous infusion of a β-lactam antibiotic) depends on the patient's response to treatment and local hospital practices.

Given its superior *in vitro* activity, tobramycin is the most commonly used aminoglycoside, combined with ceftazidime or meropenem [94;95] or as monotherapy [96-98] for CF pulmonary exacerbations associated with PA.

IV meropenem monotherapy [99-101] also proved to be a valuable drug in the treatment of CF patients with acute or chronic pulmonary infection with MDR-PA [102;103].

IV colistin is associated with high toxicity and renal insufficiency [104], and is not indicated for routine use by this route. It is presently a second or third line agent, and used in case of allergy or resistance to other antimicrobial agents [105].

❖ Nebulized and dry powders forms for inhalation

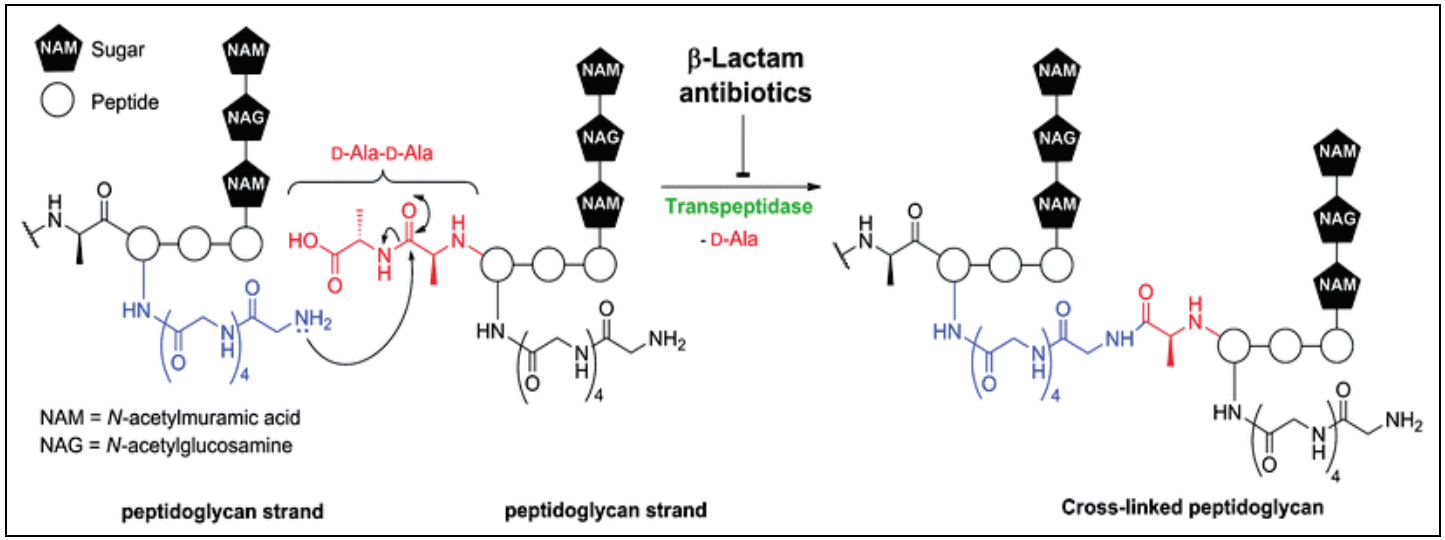
In addition to parenteral antibiotics, inhaled antibiotics act directly in the airway with minimal systemic absorption. There are many potential benefits of using the inhaled rather than the IV route: (1) providing direct deposition and access of inhaled particles to the infected lung parenchyma, (2) increasing the clinical and bactericidal efficacy by increasing lung tissue concentrations, (3) decreasing systemic toxicity, (4) preventing emergence of resistance, (5) promoting home therapy for CF patients and reducing hospital admissions. Antipseudomonal antibiotics currently marketed for inhalation [106] in CF patients include nebulized and dry powder forms of tobramycin [107] and colistin [108], and nebulized aztreonam [109]. These formulations have proven clinical efficacy in CF patients by improving lung function and reducing exacerbation rate, with the best evidence for inhaled tobramycin [110]. Additional inhaled antibiotics are in late-stage development, including ciprofloxacin, levofloxacin [111;112], and liposomal amikacin [113].

1.4 β-lactam antibiotics: Mode of action and resistance mechanisms in *P. aeruginosa*

β-Lactam antibiotics, which include penicillins, cephalosporins, carbapenems, and monobactams act by interfering with bacterial cell wall synthesis. They bind to and inhibit the enzymes involved in the terminal stages of peptidoglycan biosynthesis e.g. transpeptidase enzymes (also known as penicillin-binding proteins, PBPs) that catalyse the peptide cross-linking between two adjacent glycan chains (figure 1.4). As a result, there is inhibition of the peptidoglycan synthesis resulting in a weak cell wall and the bacterium lysis from osmotic pressure [114]. For that reason, β-lactam antibiotics require actively growing bacteria in order to exert their activity.

Regrettably, the clinical use of β-lactam antibiotics has exerted a Darwinian selection, killing susceptible bacteria and allowing the resistant ones to survive. The most common causes of resistance in Gram-positive cocci, like *Streptococcus pneumoniae* [115] and Methicillin-resistant *Staphylococcus aureus* (MRSA, [116]), are changes in the normal Penicillin-binding proteins (PBPs) or acquisition of additional β-lactam-insensitive PBPs, respectively. However, in the Gram-negative bacteria, resistance is mostly due to increased efflux, combined with porin alterations and/or β-lactamases expression [117].

Figure 1.4. Mechanism of action of β-lactam antibiotics [118]. In the absence of drug, transpeptidase enzymes (also known as Penicillin Binding Proteins; PBP) in the cell wall catalyse cross-links between adjacent glycan chains, which involves the removal of a terminal D-alanine residue (highlighted by the accolade) from one of the peptidoglycan precursors. β-lactam antibiotics, which bear a structural resemblance to the natural D-Ala-D-Ala substrate for the transpeptidase, exert their inhibitory effects on cell wall synthesis by tightly binding to the active site of the transpeptidase.



1.4.1 β-lactamases and their selective inhibitors

Resistance to β-lactam antibiotics occurs primarily through production of β-lactamases, i.e. enzymes that inactivate these antibiotics by splitting the amide bond of the β-lactam ring. The efficiency of the β-lactamase in hydrolysing a β-lactam antibiotic is mainly determined by five variables: (1) the amount of β-lactamase produced by the bacterial cell, (2) its affinity for the antibiotic, (3) its rate of hydrolysis, (4) the rate of diffusion of the antibiotic into the periplasmic space and (5) the susceptibility of the target protein (PBP) to the antibiotic. In Gram-positive bacteria, β-lactamases are excreted extracellularly while in Gram-negative bacteria, they remain trapped in the periplasmic space when no barrier exists to prevent antibiotic penetration. Resistance to extended spectrum β-lactams (cephalosporins) in PA may arise from over expression of the naturally occurring AmpC-type chromosomal cephalosporinase, or acquired β-lactamases such as extended-spectrum β-lactamases (ESBL), carbapenem-hydrolyzing β-lactamases (carbapenemases) and metallo-β-lactamases (MBL). The respective genes coding for acquired β-lactamases are often carried on transferable structures like plasmids, transposable genetic elements (transposons), DNA integration elements (integrons), and can therefore move among people and animals, from one country to another without notice, making therapeutic options scarce.

Nowadays, the problem of β-lactamases dissemination knows no boundaries, and poses a major therapeutic challenge in the treatment of hospitalized and community-based patients. Infections due to β-lactamase producing bacteria range from uncomplicated urinary tract infections to life-threatening sepsis.

❖ The Ambler molecular classification and the Bush-Jacoby-Medeiros functional classification

These classifications are the two most commonly used classification systems for β-lactamases. Ambler scheme divides β-lactamases into four major classes (A to D) based upon their protein homology (amino acid similarity).

In the Ambler classification scheme, β-lactamases of classes A, C and D are serine β-lactamases. In contrast, the class B enzymes are metallo-β-lactamases requiring a bivalent metal ion, usually Zn^{2+}, for activity. With the exception of OXA-type enzymes (class D enzymes), the ESBLs are of molecular class A. Alternatively, the Bush-Jacoby-Medeiros classification groups β-lactamases according to their substrate profile and susceptibility to β-lactamase inhibitors, such as clavulanic acid [119;120]. Inhibition by β-lactamase inhibitors such as clavulanic acid, and inability to hydrolyse cephamycins differentiates the ESBLs from the AmpC-type β-lactamases (group 1) that have third-generation cephalosporins as their substrates and are not inhibited by clavulanic acid. The metallo-β-lactamases (group 3) can hydrolyse third-generation cephalosporins (and carbapenems) but are inhibited by ethylenediaminetetraacetic acid (EDTA), a chelating agent but not clavulanic acid. Tables 1.2 and 1.3 resume the Ambler classification and the mechanism of action of different β-lactamases.

❖ AmpC-type chromosomal enzymes

These enzymes are chromosomally encoded and universally present in PA. AmpC enzymes are believed to have evolved from PBPs due to the presence of certain sequence homology, probably resulting from the selective pressure exerted by β-lactam-producing soil bacteria found in the environment [121;122]. AmpC-producing strains are typically resistant to 7-α-methoxy cephalosporins (cephamycins like cefoxitin or cefotetan), oxyimino-cephalosporins (cefotaxime, ceftazidime, ceftriaxone), as well as monobactams (aztreonam), and are susceptible to carbapenems. AmpC enzymes are not inhibited by β-lactamase inhibitors such as clavulanic acid, tazobactam. Selection of stably derepressed mutants, which hyperproduce the AmpC-type β-lactamases, has been associated with clinical failure when third-generation cephalosporins are used to treat serious infections with AmpC-producing bacteria. In general, fourth-generation cephalosporin “cefepime”, or carbapenems are clinically useful against AmpC-producing *Pseudomonas* but may be less useful in treating ESBL-producers as well as carbapenemase-producers. Avibactam, a novel β-lactamase

inhibitor with broad-spectrum activity, has received the most attention recently. It shows promising inhibition against AmpC and ESBL simultaneously [123].

❖ Acquired β-lactamases

They are often reported in PA as a result of the acquisition of ESBLs and/or carbapenemases. ESBLs are a group of complex and rapidly evolving β-lactamases enzymes, capable of hydrolysing penicillins like penicillinases, but also first-, second- and third-generation cephalosporins; and aztreonam (but not the cephamycins or carbapenems). Their genes are in general present on plasmids. Yet, novel chromosomally encoded ESBLs have also been described [124]. ESBLs are inhibited by β-lactamase inhibitors such as clavulanic acid, tazobactam or avibactam. Typical types of EBSL enzymes in PA are: PSE (pseudomonas specific enzyme), CARB (carbenicillinase), BEL (Belgium Extended-spectrum β-Lactamase), PER (Pseudomonas extended resistance), GES (Guyana extended-spectrum β-lactamase), VEB (Vietnam extended-spectrum β-lactamase), TEM (stands for Temoniera, a patient from whom first plasmid-mediated β-lactamase in Gram-negative bacteria TEM-1 was isolated in Greece), CTX-M (cefotaxime-M), OXA (oxacillin), SHV (sulfhydryl variable), and IBC (integron-born cephalosporinase)-type enzymes. Carbapenemases are enzymes with versatile hydrolytic capacities, able to hydrolyze penicillins, cephalosporins, monobactams, and carbapenems such as: metallo-β-lactamases (MBLs), including the Verona integron-encoded β-lactamase (VIM) and imipenem hydrolysing (IMP), New Delhi Metallo-β-lactamase (NDM), *Klebsiella pneumoniae* carbapenemase (KPC) enzymes and OXA-48-type enzymes. MBLs hydrolyze almost all clinically-available β-lactams except monobactams (aztreonam) [125;126].

❖ Avibactam: a novel β-lactamase inhibitor

While ceftazidime has been the mainstay of treatment for many years, its efficacy is limited by reduced susceptibility observed in cephalosporin-

resistant PA. Avibactam is a novel β-lactamase inhibitor, with a broader spectrum of activity than other currently available β-lactamase inhibitors, against multiple serine based β-lactamases including Ambler class A ESBLs, class A KPCs, class C (AmpC) enzymes and some class D (OXA) enzymes (table 1.2), but is not active against class B (the metallo-β-lactamases) [127]. It was recently approved by FDA and EMA in combination with ceftazidime, namely AVYCAZ® and ZAVICEFTA® respectively. Ceftazidime-avibactam is indicated for the treatment of complicated urinary tract infections (UTIs) and complicated intra-abdominal infections (cIAI), including those caused by expanded-spectrum cephalosporin-resistant Gram-negative organisms ([128;129]). Furthermore, ceftazidime-avibactam offers a potential advancement in the management of lung infections involving PA due to its excellent activity against prevalent β-lactamases [130], penetration into pulmonary secretions [131;132] and reduced potential for development of resistance [133]. The mechanism of action of avibactam as inhibitor of a serine β-lactamase is illustrated in table 1.3 in comparison with previous generations of β-lactamase inhibitors.

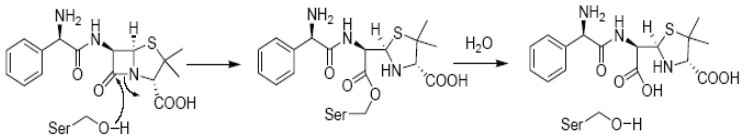
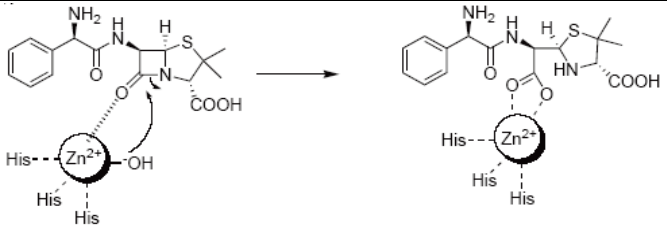
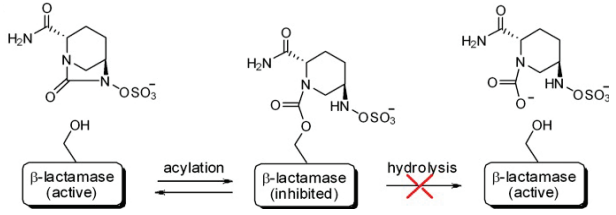
Table 1.2. Ambler classification of β-lactamases found in *P. aeruginosa* and their selective inhibitors.

Class	Active site	Enzyme Type	Substrates	Example types	Inhibitors or antibiotics used as indicators (diagnosis)
A	Serine	Penicillinases; extended-spectrum β-lactamase	❖ Benzylpenicillin, aminopenicillins, ureidopenicillins, narrow-spectrum cephalosporins, oxymino-β-lactams (cefotaxime, ceftazidime, ceftriaxone) and aztreonam ❖ Carboxypenicillins **(except temocillin)**	SHV; TEM; PSE; CARB; CTX-M; BEL; PER; VEB GES; IBC	Clavulanate#, tazobactam#, avibactam#, boronic acid$
		Carbapenemases	❖ Substrates of extended-spectrum plus cephamycins and carbapenems ❖ **Temocillin resists to KPC variants if other resistance mechanisms (e.g. efflux) are excluded** [134]	KPC; GES-2/IBC-2*	Avibactam#, boronic acid$
B	Metal ion (Zn^{2+})	Carbapenemases	❖ Substrates of extended-spectrum plus cephamycins and carbapenems ❖ **Temocillin resists to FIM-1 variant** [135]	VIM, IMP, NDM, GIM, SPM, SIM, FIM	Aztreonam$, EDTA$
C	Serine	Cephalosporinases	❖ Substrates of extended-spectrum **(except temocillin)** plus cephamycins	AmpC	Avibactam#, cloxacillin$, cefepime$, ceftolozane$, boronic acid$
D	Serine	Extended-spectrum	❖ Aminopenicillins, ureidopenicillin, carboxypenicillins **(except temocillin)**, cloxacillin, methicillin, oxacillin, and some narrow-spectrum cephalosporins, oxymino-β-lactams and monobactams	OXA-family in PA (OXA-18, OXA-20)	Clavulanate#, tazobactam#, avibactam#
		Carbapenemases	❖ Substrates of extended-spectrum plus cephamycins and carbapenems	OXA-family in *A. baumanii*	Avibactam#

* GES/IBC new variants emerged with carbapenemase activity [136].

used as inhibitors of β-lactamases in the clinics; $ used as indicator of the expression of the corresponding enzymes in diagnosis.

Table 1.3. Mechanism of action of β-lactamases (BL) and inhibition by avibactam [130].

Ambler class	BL active site	Substrate	Mechanism of action	Yield
A, C, D	Serine (hydrolysis)	β-lactam antibiotics and inhibitors with β-lactam core (clavulanate, tazobactam, sulbactam)		-Inactivated β-lactam core irreversibly, recycled enzyme
B	Metal ion (Zn^{2+})	β-lactam antibiotics and inhibitors with β-lactam core (clavulanate, tazobactam, sulbactam)		-Inactivated β-lactam core irreversibly, recycled enzyme
A,C and some of class D	Serine (hydrolysis)	Avibactam		-Inhibition of BL with reversible mechanism favouring recyclization of avibactam over hydrolysis

1.4.2 Decreased permeability of bacterial outer membrane

The main barrier impeding penetration of antibiotics into Gram-negative bacteria is the outer membrane. The outer membrane is an asymmetric bilayer of phospholipid and lipopolysaccharides (LPS). The core region of LPS is made up of tightly bound hydrocarbon molecules that plays a major role in providing a barrier to hydrophobic antibiotics. Strains that express full length LPS have an intrinsic resistance to these antibiotics [137]. In order to gain access to the cell interior, hydrophilic antibiotics preferentially use non-specific porins, channel-forming proteins that are built into lipid bilayer of the outer membrane. Porins are small β-barrel openings that can be found in the outer membrane of Gram-negative bacteria and are thought to be the entry point for most if not all antibiotics that are currently on the market [138]. Two major porin-based mechanisms for antibiotic resistance have been reported: alterations of the porin expression or altered function due to specific mutations reducing permeability.
Unlike *Escherichia coli*, which possesses a large number of general porins and a few specific ones, *Pseudomonas* spp. and other closely related soil organisms are unique in that the majority of small molecules are taken up by specific porins. As depicted in table 1.4, there are four types of porins in PA: general/nonspecific porins, substrate-specific porins, gated porins, and efflux porins.

The major general porin, OprF, forms an inefficient uptake pathway. The majority of the OprF channels are too “small” to allow sufficient passage of molecules.

The specific porins of PA characterized to date include the glucose-specific porin OprB (outer membrane protein B), the phosphate/polyphosphate-specific porins OprP and OprO, respectively, and the basic-amino-acid-specific porins of the OprD family. OprD (OccD1), which is the prototype of a large paralogous family of porins, takes up the structurally related antibiotics imipenem and meropenem. A common trend amongst the molecules that pass through the OprD family is that they contain a carboxylic acid or a

bioisostere thereof; so they are named Occ, for outer membrane carboxylate channels. There are 19 OprD homologues in PA whose primary amino acid sequences are 46 to 57% similar to that of OprD (OccD1). They can be divided into two distinct subfamilies (table 1.4): OccD subfamily (8 family members; OccD1 to 8) that has conserved cation selectivity and OccK subfamily (11 family members; OccK1 to 11) that has conserved anion selectivity. As a consequence, PA outer membrane (OM) is much less permeable to small polar molecules than in other bacterial species, making this bacterium intrinsically resistant towards many antibiotics [139-141]. Since β-lactams are polar hydrophilic compounds, alterations of OprD (OccD1) porin are known to contribute to carbapenem resistance and to affect imipenem (and meropenem to some extent) efficacy in PA in case of altered function or/and expression [142-144].

Thus, since we are facing OprD downregulation as main resistance mechanism against the permeation of antibiotics, researchers are currently trying to use the siderophore-dependent iron uptake systems (gated porins) as ”Trojan horse” strategies to get inside PA. Recent studies have indeed shown that the conjugation of an antibiotic to a siderophore could significantly increase the biological activity of the antibiotic, by enhancing its transport into the bacterium [145].

Table 1.4. Function of porins in uptake and efflux in *P. aeruginosa* [139].

TYPE	SUBSTRATES / FUNCTION
GENERAL PORINS	
OprF	Structural role
SPECIFIC PORINS	
OprP/O	Phosphate, pyrophosphate
OprB	Carbohydrate (e.g. glucose, glycerol) uptake system
OprD group (OccD subfamily) [140;143;146]	**Conserved cation selectivity**
OprD (OccD1)	Arginine, antibiotics (carbapenems), histidine, lysine
OpdC (OccD2)	Arginine, histidine
OpdP (OccD3)	Glycine-glutamate, antibiotics (carbapenems [147]), arginine
OpdT (OccD4)	Benzoate, tyrosine
OpdI (OccD5)	Arginine
OprQ (OccD6)	Arginine
OpdB (OccD7)	Proline
OpdJ (OccD8) [a]	(?)
OpdK group (OccK subfamily) [140;148]	**Conserved anion selectivity**
OpdK (OccK1)	Vanillate, caproate, benzoate, glucoronate, pyroglutamate, antibiotics (carbenicillin, cefoxitin and tetracycline)
OpdF (OccK2)	Glucuronate, benzoate, pyroglutamate, antibiotics (carbenicillin, cefoxitin and gentamicin)
OpdO (OccK3)	Pyroglutamate, benzoate, glucoronate, antibiotics (cefotaxime)
OpdL (OccK4)	Phenylacetic acid, benzoate, pyroglutamate, glucoronate
OpdH (OccK5)	Tricarboxylates, benzoate, pyroglutamate, glucoronate, antibiotics (cefotaxime)
OpdQ (OccK6)	Benzoate, pyroglutamate
OpdD (OccK7)	Benzoate, pyroglutamate, antibiotics (meropenem)
OprE (OccK8) [a]	(?), antibiotics (carbapenems) [149]
OpdG (OccK9) [a]	(?)
OpdN (OccK10) [a]	(?)
OpdR (OccK11) [a]	(?), phenylacetic acid [146]
GATED PORINS	
FpvA, FptA, OprC, OprH	Metals (e.g. iron, copper, magnesium) uptake system
EFFLUX PORINS	
OprM, OprJ, OprN, AprF	Efflux and protein secretion (type I, II, III) systems

[a] (?) Data available about substrates are poor or not available for these channels.

1.4.3 Active efflux

In order to protect the bacterium from β-lactam antibiotics, multiple antibiotic resistance in Gram-negative bacteria often starts with the overexpression of MDR efflux systems, which can export multiple unrelated antibiotics, in concert with the relatively limited outer membrane permeability to many antibiotic agents, and periplasmic β-lactamases. There are five families of chromosomally encoded efflux pumps that are associated with MDR: (1) the Resistance-Nodulation-Division (RND) family, (2) the major facilitator superfamily (MFS), (3) the small multidrug resistance (SMR) proteins family, (4) the multidrug and toxic compound extrusion (MATE) family, and (5) the ABC (ATP binding cassette) family [150]. In addition to their role in resistance to antibiotics, efflux systems have a physiological role and are responsible for the export of natural substances produced by the host, allowing survival in a harmful environment, such as bile and host-defence molecules [151;152].

Overexpression of efflux pumps of the RND family in PA, encoded by *mexAB-oprM, mexCD-oprJ, mexEF-oprN,* and *mexXY-oprM,* is often detected in clinical isolates and contributes to worrying MDR phenotypes. MexAB-OprM and MexXY-OprM are constitutively expressed efflux systems, which provide intrinsic multidrug resistance to a broad range of clinically significant antibiotics; while MexCD-OprJ and MexEF-OprN are inducible systems and promote acquired multidrug resistance as a result of mutational overexpression of their efflux genes. Not all antibiotic classes are exported to the same extent by RND-type efflux systems. The antibiotic affinity for these pumps varies not only between different classes of antibiotics but also between members of the same class illustrating the flexibility of these efflux systems; such as cefepime resistance (fourth-generation cephalosporin antibiotic) due to MexXY-OprM efflux pumps overexpression that do not affect the activity of ceftazidime (third generation cephalosporin) [153-155].

MexAB-OprM efflux pump is the most important RND transporter in PA. Being constitutively produced in wild-type strains, MexAB-OprM recognizes a wide variety of substrates, and contribute to natural resistance to a diverse

range of compounds such as β-lactams, β-lactamase inhibitors and carbapenems (not imipenem), aminoglycosides, fluoroquinolones, tetracyclines, tigecycline, macrolides, phenicols, novobiocin, sulfonamides, trimethoprim, some amphiphilic molecules, disinfectants, dyes, detergents, and several homoserine lactones involved in quorum sensing (QS) (see table 1.5). MexXY-OprM pumps, expressed constitutively at a basal level, are inducible in response to challenges by aminoglycosides, zwitterionic cephalosporins (cefepime, [156]), tetracyclines, macrolides, fluoroquinolones, and chloramphenicol [157-160]. Yet, the MexXY can utilize either OprA or OprM protein as an outer membrane channel. The *oprA* gene which is found in the multidrug resistant clinical isolate PA7 and relatives, is absent in most PA strains and often OprM protein encoded by the mexAB-oprM operon is associated with the MexXY constituent [161;162].

The substrates of MexCD-OprJ pump include chlorhexidine, benzalkonium, fluoroquinolones, zwitterionic cephalosporins, macrolides, chloramphenicol, trimethoprim, and tetracyclines. MexEF-OprN pumps recognize fluoroquinolones, chloramphenicol, trimethoprim, and tetracycline as substrates. Fluoroquinolones, like ciprofloxacin and levofloxacin, have been reported to be most efficiently extruded by MexEF-OprN and MexCD-OprJ pumps, with less efficiency by MexAB-OprM and MexXY-OprM [163;164].

Efflux mechanism may select for new resistant mutants, by reducing the intracellular concentration of the antibiotic, followed by a gradual decline to sub-inhibitory levels [165]. In addition, RND efflux pumps have been described to play a role in mediating colonization and invasiveness of PA to host tissues, by exporting QS signal molecules (MexEF-OprN pump) [166] and extruding virulence determinants (MexAB-OprM pump) [167;168].
Figure 1.5 shows the proposed structural model for the MexAB-OprM efflux pump, which is composed of three interconnected elements:

1. MexA, a periplasmic membrane fusion protein which stabilizes the complex. Its structure has been solved in a tridecameric structure (13-meric, a hexamer facing a heptamer in a head-to-head manner with large

internal tubular space and widely opened flared ends), during its assembly with OprM and MexB proteins [169].

2. MexB, which protrudes from the inner membrane into the periplasm, is a trimeric transporter that is assumed to carry drugs out of the cytoplasmic or periplasmic compartments using proton motive force according to a functionally rotating mechanism [170;171].
3. OprM protein, forming a β-barrel structure (3 monomers), is assumed to serve as the drug discharge duct across the outer membrane [172;173].

Table 1.5. Substrates of clinically relevant chromosomally encoded multidrug resistance efflux pumps in *P. aeruginosa.*

RND efflux system	Substrates	References
MexAB-OprM	Penicillins (e.g. ticarcillin, carbenicillin, temocillin), cephalosporins (cefotaxime), monobactams, carbapenems (not imipenem), β-lactamase inhibitors, aminoglycosides, fluoroquinolones, tetracyclines, tigecycline, macrolides, phenicols, novobiocin, sulfonamides, trimethoprim, some amphiphilic molecules, disinfectants, dyes, detergents, and homoserine lactones.	[157-160;174-179].
MexXY-OprM/OprA	Aminoglycosides, zwitterionic cephalosporins (cefepime), carbapenems (not imipenem), tetracyclines, macrolides, fluoroquinolones and chloramphenicol.	[156-159;175;177].
MexCD-OprJ	Fluoroquinolones (ciprofloxacin and levofloxacin), chlorhexidine, benzalkonium, zwitterionic cephalosporins (cefepime, cefpirome), carbapenems (not imipenem), macrolides, chloramphenicol, trimethoprim, and tetracyclines.	[163;174;175;177;180].
MexEF-OprN	Fluoroquinolones (ciprofloxacin and levofloxacin), chloramphenicol, trimethoprim, and tetracyclines.	[163;164;180;181].

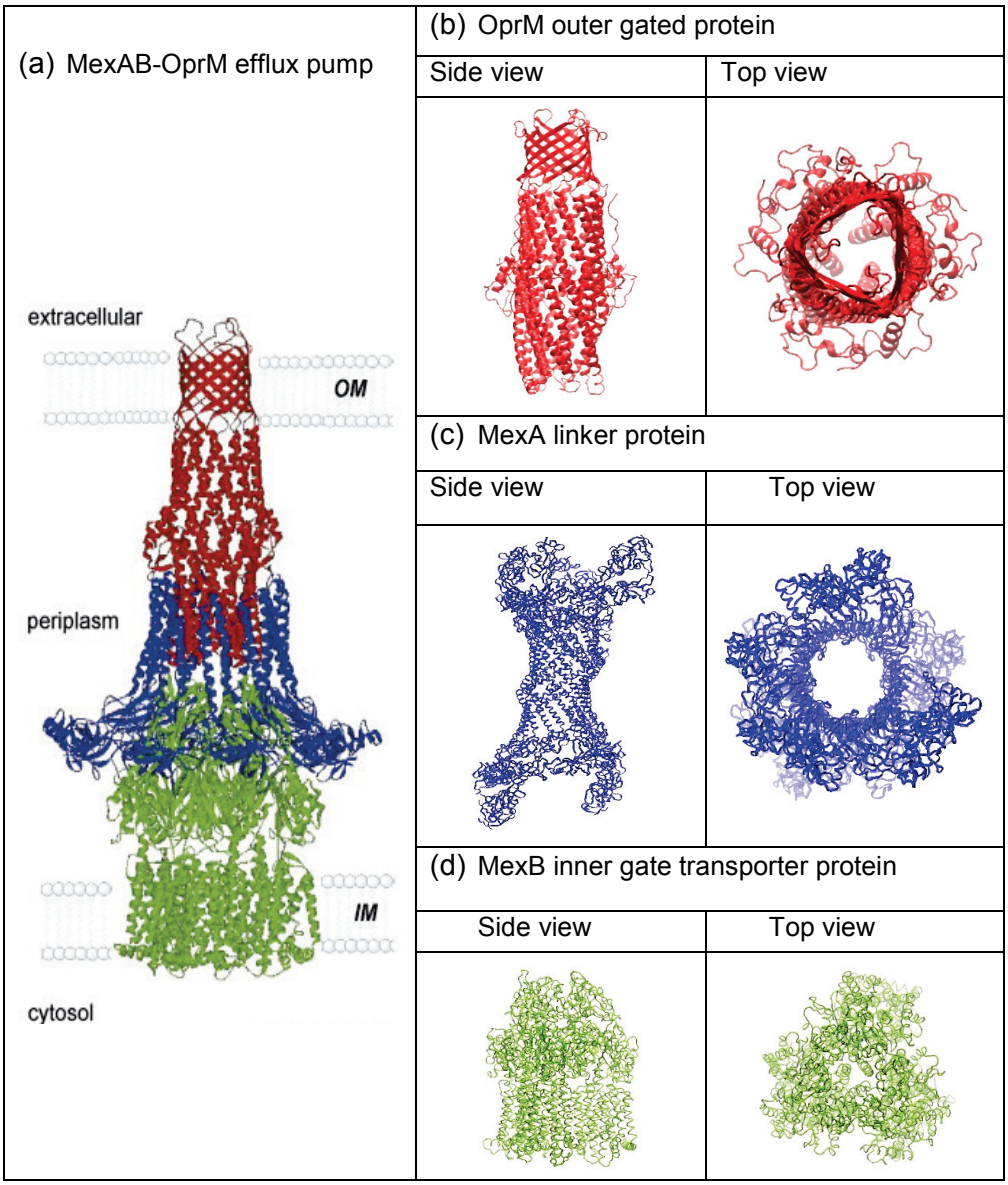


Figure 1.5. Proposed structural model for the MexAB-OprM tripartite multidrug efflux system in *P. aeruginosa* [182]. MexA, MexB and OprM proteins were rendered separately, using VMD program [183], based on Protein Data Bank (PDB) files 2V4D (MexA), 2V50 (MexB), and 3D5K (OprM).

1.5 Temocillin

1.5.1 Definition and mode of action

Temocillin (TMO; 6-α-methoxy-ticarcillin) is an injectable β-lactam antibiotic designed for parenteral use [IV or intramuscular injections]. It is a semi-synthetic 6-α-methoxy derivative of ticarcillin, bearing a methoxy side chain on the α-face of the β-lactam ring (figure 1.6). Along with ticarcillin and carbenicillin, temocillin belongs to the class of carboxypenicillins. Temocillin like all penicillins inhibits the synthesis of bacterial peptidoglycan, by binding irreversibly to the active site of specific transpeptidases and carboxypeptidases known as Penicillin Binding Proteins (PBPs). Temocillin is a narrow spectrum antibiotic active against multi-drug resistant Gram-negative bacteria and more particularly against the Enterobacteriaceae. After its introduction by Beecham Pharmaceuticals (London, UK) in the 1980s [184-186], temocillin was withdrawn from the UK market because of little commercial success due to the lack of activity against Gram-positive organisms, anaerobes and against *Pseudomonas* spp. Albeit, temocillin was recently reintroduced because of its activity against current ESBL-producing Enterobacteriaceae [40;187;188]. At the present time, it is commercialized by Eumedica Pharmaceuticals (Manage, Belgium) as Negaban® and registered in Belgium, Luxembourg, and the UK. It was also recently launched in France (June 2015), in response to the rise in ESBL-producing coliforms.

1.5.2 Spectrum of activity and resistance mechanisms

❖ **Microbiological properties of temocillin and clinical indications**

Temocillin has bactericidal activity; the MBCs are close to the MICs, or are only two to four times higher [189]. As for other β-lactam antibiotics, its activity depends on the duration that the free drug concentrations exceed the MIC for the microorganism.

Temocillin is indicated as empirical treatment for complicated urinary tract infections (UTI) due to ESBL producing coliforms or when there is history of ESBL [190-192]. It is also used as documented treatment for lower respiratory tract infections [193;194] wound infections [195] and bacteremia [196;197]. In 2004, temocillin gains the orphan drug designation in Europe (EMEA) and the United States (FDA) after proving efficacy for the treatment of *Burkholderia cepacia* infection in CF patients [198-202]. In mixed infections where gram-positive or anaerobic bacteria are also liable to be implicated, co-administration of temocillin with other appropriate antibacterial agents should be considered. Table 1.6 summarizes temocillin spectrum of activity against bacterial species, according to literature data and to its summary of product characteristics. Temocillin shows a good safety profile. It is not active on anaerobic flora and causes fewer gastrointestinal adverse events usually occurring in antibiotherapy [203], due to the disruption of the intestinal microflora and colonisation of *Clostridium difficile* [204].

The methoxy moiety of temocillin plays an important role in resistance to β-lactams degrading enzymes, by preventing the access of water to the active site of β-lactamases (figure 1.6) [205]. This characteristic confers high stability for temocillin against most β-lactamases including penicillinases, AmpC-type cephalosporinases, extended-spectrum ESBLs enzymes (including TEM, SHV, GES and CTX-M) [40;206;207]. *In vitro* and *in vivo* studies have also shown moderate activity against a high proportion of Enterobacteriaceae with KPC-type carbapenemases [187;197;208] although not against bacteria with OXA-48-like non-metalloenzymes or the metalloenzymes (including IMP, NDM and VIM types) [134;209]. Yet, more trials are warranted to evaluate whether temocillin offers, for example, an effective treatment for urinary tract infections caused by KPC producers. Interestingly, one recent study revealed that temocillin is indeed resilient against the activity of FIM-1 enzyme, a new purified metallo-β-lactamase from PA degrading penicillins and carbapenems [135].

Temocillin is thought to have limited efficacy in patients infected with PA and there is currently limited data regarding its use in CF patients. Because of its

stability to a wide range of β-lactamases, temocillin is often proposed as an alternative spare to carbapenems in severe hospital-acquired infections when *Pseudomonas* can be excluded [40]. This de-escalation from carbapenems helps to reserve these valuable agents, potentially prolonging their useful lives [210]. Temocillin has recently proven active *in vitro* against isolates of *Neisseria gonorrhoeae* that were penicillin and ciprofloxacin resistant [211].

The breakpoint for temocillin has not yet been established by the EUCAST. According to old and recent studies, the provisional breakpoint for temocillin in Enterobacteriaceae is 16 mg/L [40;194;212;213]. According to both BSAC [214;215] and the Belgian labelling [189], temocillin susceptibility breakpoint for Enterobacteriaceae varies between 8 and 32 mg/L if considering systemic and urinary tract infections, respectively. According to pharmacokinetic/pharmacodynamic indices, aided by Monte-Carlo simulations, a breakpoint of 8 mg/L is proposed for the registered maximum dosage of 4 g daily [188].

Figure 1.6. Top panels: Structure of temocillin (6-α-methoxy-ticarcillin) compared with ticarcillin. The arrow indicates the methoxy side chain that plays an important role in resistance to hydrolysis by β-lactamases. **Bottom panels**: Molecular docking of temocillin (left panel) and ticarcillin (right panel) in the active site of serine β-lactamase. The antibiotic structure is represented by bold lines. The hydroxy oxygen of the active Ser70 and the S-1 atoms of the antibiotics are shown as closed and open circles respectively. The α-methoxy moiety blocks the entry of a water molecule (represented here by W1) into the β-lactamase active site cavity, preventing activation of the serine and the chemical events leading to hydrolysis [205].

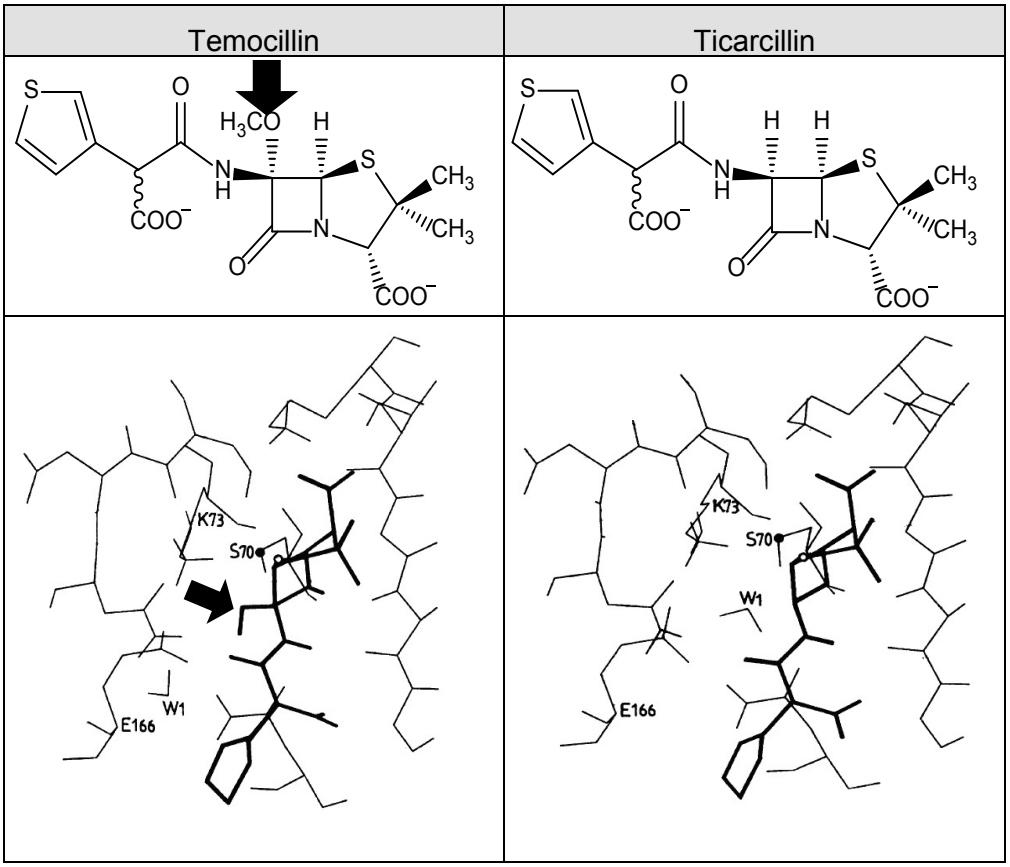


Table 1.6. Spectrum of activity of temocillin [216;217] and according to its summary of product [189].

<table>
<tr><td colspan="3">Susceptible organisms</td></tr>
<tr><td colspan="3">MIC in mg/L</td></tr>
<tr><td>MIC < 1mg/L</td><td>1 mg/L < MIC < 10 mg/L</td><td>10 mg/L < MIC < 100 mg/L</td></tr>
<tr><td>✓ Moraxella catarrhalis
✓ Haemophilus influenzae
✓ Legionella pneumophila
✓ Neisseria gonorrhoeae
✓ Neisseria meningitidis</td><td>✓ Brucella abortus
✓ Citrobacter spp.
✓ Escherichia coli
✓ Klebsiella pneumoniae
✓ Pasteurella multocida
✓ Proteus mirabilis
✓ Proteus spp.
✓ Providencia stuartii
✓ Salmonella Typhimurium
✓ Shigella sonnei
✓ Yersinia enterocolitica</td><td>✓ Serratia marcescens
✓ Enterobacter spp.</td></tr>
<tr><td colspan="3">Intrinsically resistant organisms</td></tr>
<tr><td colspan="3">• Gram(+) bacteria (probably related to reduced temocillin’s binding to many penicillin-binding proteins PBPs [40;218])
• Pseudomonas aeruginosa, Anaerobes (Bacteroides), Campylobacter spp., Acinetobacter spp.</td></tr>
</table>

❖ Pharmacokinetic properties of temocillin

Temocillin pharmacokinetic properties are similar to most other β-lactams. However, it shows a prolonged *in vivo* serum half-life (4.5 h after I.V. injection and 5.4 hours with I.M. injection) in comparison to other penicillins (ureidopenicillins, ticarcillin, carbenicillin and ampicillin, half-life of 1 h), and high area under the serum concentration curve (AUC). Regarding the temocillin distribution, the rate of binding to serum proteins is approximately 85% and depends on the plasma concentration as it is saturable. The metabolisation of temocillin is not significant. It is eliminated primarily by tubular excretion and about 80% of the administered dose is eliminated in 24 hours by the kidneys unchanged [184;219;220]. Protracted temocillin concentrations are obtained in the bile, prostatic tissue, interstitial fluid and lymph fluid [221-223]. It passes in very small amounts into the cerebrospinal fluid, except in cases of meningeal inflammation [224;225].

In subjects with normal renal function, intramuscular injection of temocillin (Negaban, 1g) results in urinary concentrations exceeding 1300 mg/L [189], highlighting its clinical efficacy in urinary tract infections [196;226]. Temocillin excretion may be delayed in cases of kidney failure so the dosage must be reduced, depending on the degree of kidney failure shown by the creatinine clearance values [227;228].

❖ Preliminary data on the activity of temocillin on *P. aeruginosa* in CF

Before the beginning of this thesis, our laboratory published a paper about the role of MexAB-OprM in intrinsic resistance of PA to temocillin and its impact on the susceptibility of strains isolated from patients suffering from CF [178]. They showed that MexAB-OprM-driven efflux strongly contributes to the intrinsic resistance of PA to temocillin, while the other Mex systems only play a minor role. Interestingly, they found 10 isolates obtained from CF patients showing hypersusceptibility to both ticarcillin and temocillin, with temocillin MICs ranging between 1 and 4 mg/L, due to natural mutations in *mexA* and *mexB* genes presumably inactivating the efflux capacities of the

MexAB-OprM system. When considering all isolates examined in this study, temocillin MICs were much higher than those of ticarcillin in isolates producing a functional or partially functional MexAB-OprM pump (with temocillin MICs being 3-5 $\log_2$ dilutions higher than those of ticarcillin). This suggests that temocillin is a preferential substrate for the MexAB-OprM transporter, pointing to a potential role of the 6-α-methoxy substituent in its recognition and efflux (figure 1.6). While intrinsic resistance of PA to temocillin makes this antibiotic unusable in most conventional clinical set-ups, the study showed that impairment of efflux lowers the MICs to values below the current clinical susceptibility breakpoint for Enterobacteriaceae (16 mg/L; UK and Belgium) or even the pharmacokinetic/pharmacodynamic breakpoint proposed for a 4 g daily dose (8 mg/L) [188].

While temocillin is thought to have limited efficacy in patients infected with PA, these data may have potential immediate application for CF patients. These patients can be co-infected by *Burkholderia cepacia* complex strains, against which temocillin has obtained an orphan drug designation [198-202].

These findings about the potential activity of temocillin in CF patients are also consistent with a previous cohort study from United Kingdom in 2008 [199;229], which found that temocillin administration was well tolerated in CF, associated with a significant improvement in white cell count and body mass index. This improvement was seen in CF patients with PA infection as well as those with proven *B. cepacia*. They concluded that further studies are needed to further evaluate the role of temocillin in CF patients [229].

Hypothesis and objectives

Active efflux is increasingly recognized as a common mechanism that leads to multidrug resistance in many clinically relevant pathogens like *Escherichia coli, Shigella* spp. and PA. Many of these transporter systems promote the active efflux of multiple classes of antibiotics, especially β-lactams. The Resistance-Nodulation-Cell Division (RND)-type multidrug efflux pumps are the most clinically relevant efflux systems detected in clinical isolates (e.g., MexAB-OprM, MexXY-OprM, MexCD-OprJ and MexEF-OprN).

Among β-lactam antibiotics, carbapenems show the broader spectrum and are considered as the most effective antipseudomonal antibiotics. Ceftazidime and piperacillin are inactivated by cephalosporinases or broad-spectrum β-lactamases (ESBL). The carboxypenicillin temocillin offers the advantage of remaining active against ESBL-producers, but it is considered as inactive against PA due to constitutive efflux by MexAB-OprM. Yet, before the beginning of this thesis, our laboratory demonstrated that natural mutations in the genes coding for this efflux pump restored temocillin activity; these mutations were observed in few isolates of PA collected from CF patients [178].

Based on these observations, our hypothesis was that efflux could play a more important role than usually suggested in defining the resistance profile of *P. aeruginosa* to β-lactam antibiotics. Therefore, the main objective of this thesis was to decipher the role of active efflux in intrinsic or acquired resistance to β-lactam antibiotics in PA isolated from patients suffering from CF in order to better position these drugs in the treatment of respiratory tracts infections, occurring frequently in this patients' population.

In this context, our work was constructed as follows:

i. A survey of the current situation of antibiotic resistance in PA coming from CF patients chronically exposed to different antipseudomonal agents. To this effect, we evaluated the susceptibility to antipseudomonal

agents in a collection of 153 PA isolated from 3 CF centres through Northern Europe (Belgium, Germany and UK) over the 2006-2012 period. Moreover, we aimed at determining prevalent resistance mechanisms in this collection and at evaluating novel therapeutic options. More specifically, we investigated:

a. The role of active efflux and/or other mechanisms in high-level resistance to carbapenems, and particularly meropenem.

b. The activity of the new antibiotic combination ceftazidime-avibactam against β-lactamase-producing PA isolated from CF patients and the role of active efflux in resistance to this combination.

ii. A survey of the prevalence of susceptibility/resistance to temocillin in a collection of PA from CF patients and the mechanisms associated with resistance/susceptibility to temocillin. More specifically, we investigated:

a. The impact of natural mutations occurring in *mexA* or *mexB* on the activity of MexAB-OprM efflux pump and temocillin.

b. The role of porins in the uptake of temocillin by PA.

2. Results

2.1 Collection of isolates and database

An international collection (n=333) of PA were isolated at random during routine visits of CF patients chronically exposed to different antipseudomonal agents (table 1.7). These isolates were kindly obtained from:

- The Queen's University of Belfast, United Kingdom: n=99
- Hôpital Erasme, Brussels, Belgium: n=88
- CHRU Besançon, Besançon, France: n=80
- University of Münster, Münster, Germany: n=66

Table 1.7. General description of the collection from 1996-2012

Country	Number of isolates	Number of patients	Age of patients	Year of sampling	Chronic antibiotic exposition	
					Oral	Inhaled (in alternance)
United Kingdom	99	46	18-48	2006-2009	Azithromycin	Tobramycin, colistin
Belgium	88	37	10-59	2010	Azithromycin	Tobramycin, colistin, aztreonam
France	80	36	1-33	1996-2012	Azithromycin	Tobramycin, colistin
Germany	66	36	1-54	2012	Azithromycin	Tobramycin, colistin, aztreonam
Total	**333**	**155**				

Isolates were frozen in CA-MHB sterile glycerol at -80°C. Hospitals were invited to send the clinical data; we received patient information including the sex, age, sample collection date, lung function testing (FEV), infection chronicity and antibiotic treatments. We noticed the chronic use of different antipseudomonal agents, according to the guidelines of the CF care centre as indicated in the table 1.7.

2.2 Characterization of resistance in the collection

i. Antimicrobial susceptibility of *P. aeruginosa* isolated from cystic fibrosis patients through Northern Europe

As a survey of the current status of bacterial resistance in CF patients, the following article compares the antimicrobial susceptibility of PA isolates from the United Kingdom, Belgium and Germany collected from CF patients during routine visits. Among the 333 PA isolated from 155 CF patients, only 153 isolates corresponding to 118 patients were selected in this study. The selection was operated as follows. Isolates from France were excluded for this epidemiological survey, because they were collected earlier than the other ones [63% isolated (50/80 isolates) before the year 2006], which may influence the resistance rates. Among the other collections, only isolates of the first sampling dates were considered. If more than one isolate was collected from a single patient, they were included only if phenotypic differences were evidenced among colonies. After this selection, the remaining collection consisted of 58 isolates from United Kingdom over the 2006-2009 period, 44 isolates from Belgium during the year 2010, and 51 isolates from Germany during the year 2012.

The combination of these three collections through Northern Europe provided valuable material, which allowed us to investigate the following parameters: (1) activity of common antipseudomonal antibiotics (β-lactams, fluoroquinolones, aminoglycosides, and colistin) used in CF patients; (2) prevalence of ESBL and/or carbapemenase producers; (3) multidrug-resistant clones (MDR) in routine isolates from three countries; (4) cross and co-resistance between different classes of antibiotics; (5) presence of single or multiple clones colonizing each CF patient; (6) correlation between the multidrug resistance and the patient's age; and (7) epidemic situation by examining the diversity of the clones and the dissemination of specific MDR clones in these countries.

The findings of this study present an interest to clinicians and microbiologists, particularly in Europe. Moreover, this type of survey has not been published in Belgium and our study is a first step in this direction. Only older studies were found in the UK ([230], collection in 2000) or in Germany ([231], collection in 2006).

Number of experimental replicates in this study. The determination of MICs by broth microdilution for each antibiotic against the whole collection of PA isolates was performed 1 to 4 times (for some isolates), and using as reference strain ATCC 27853. Moreover, control strains overexpressing AmpC cephalosporinases, ESBL, efflux systems (MexAB-OprM, MexXY-OprM, MexEF-OprN and MexCD-OprJ) were used as internal controls to ensure the reliability and validity of the technique.

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Antimicrobial Susceptibility of *Pseudomonas aeruginosa* Isolated from Cystic Fibrosis Patients in Northern Europe

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Pseudomonas aeruginosa **is a major cause of morbidity and mortality in cystic fibrosis patients. This study compared the antimicrobial susceptibilities of 153 *P. aeruginosa* isolates from the United Kingdom (UK) ($n = 58$), Belgium ($n = 44$), and Germany ($n = 51$) collected from 118 patients during routine visits over the period from 2006 to 2012. MICs were measured by broth microdilution. Genes encoding extended-spectrum β-lactamases (ESBL), metallo-β-lactamases, and carbapenemases were detected by PCR. Pulsed-field gel electrophoresis and multilocus sequence typing were performed on isolates resistant to ≥3 antibiotic classes among the penicillins/cephalosporins, carbapenems, fluoroquinolones, aminoglycosides, and polymyxins. Based on EUCAST/CLSI breakpoints, susceptibility rates were ≤30%/≤40% (penicillins, ceftazidime, amikacin, and ciprofloxacin), 44 to 48%/48 to 63% (carbapenems), 72%/72% (tobramycin), and 92%/78% (colistin) independent of patient age. Sixty percent of strains were multidrug resistant (MDR; European Centre for Disease Prevention and Control criteria). Genes encoding the most prevalent ESBL (BEL, PER, GES, VEB, CTX-M, TEM, SHV, and OXA), metallo-β-lactamases (VIM, IMP, and NDM), or carbapenemases (OXA-48 and KPC) were not detected. The Liverpool epidemic strain (LES) was prevalent in UK isolates only (75% of MDR isolates). Four MDR sequence type 958 (ST958) isolates were found to be spread over the three countries. The other MDR clones were evidenced in ≤3 isolates and localized in a single country. A new sequence type (ST2254) was discovered in one MDR isolate in Germany. Clonal and nonclonal isolates with different susceptibility profiles were found in 20 patients. Thus, resistance and MDR are highly prevalent in routine isolates from 3 countries, with meropenem, tobramycin, and colistin remaining the most active drugs.**

Pulmonary infection represents a major cause of morbidity and mortality among cystic fibrosis (CF) patients (1). These patients are therefore regularly exposed to antibiotics for the treatment of infectious exacerbations as well as for the prevention of chronic colonization. *Pseudomonas aeruginosa* is one of the most prevalent bacterial species, especially in the adult population (2). It is well known for its genetic plasticity and capacity to accumulate resistance mechanisms, including acquisition of foreign genetic material (3). The percentage of patients colonized by *P. aeruginosa* has decreased in recent years (2), but with improved life expectancy, the absolute number of colonized patients has increased. It has also been proposed that multidrug-resistant (MDR) strains are more frequent in older patients, primarily due to cumulative exposure to antibiotics (2). A further reason for the spread of antibiotic resistance in CF patients is the dissemination of MDR clones. The Liverpool epidemic strain (LES), first described in 1996 (4), has proven particularly successful at acquiring resistance mechanisms over the years (5, 6) and at spreading from the United Kingdom (UK) to other countries, such as Canada, Spain, and Australia (7).

In this study, we compared the antimicrobial susceptibility of *P. aeruginosa* isolated from CF patients in the UK, where the MDR LES clone is known to be highly prevalent (5), with those of an equivalent number of strains collected in Germany and Belgium, where no specific survey has been published in recent years. We determined the presence of coresistance to unrelated antibiotic classes and its possible association with MDR clones. We found that resistance was high in the three countries but was not related to the dissemination of a specific MDR clone in Germany or Belgium. Carbapenems, tobramycin, and colistin remain the drugs most active against *P. aeruginosa* respiratory isolates. Importantly, no carbapenemases were detected in these strains.

MATERIALS AND METHODS

Bacterial isolates. A total of 153 clinical *P. aeruginosa* isolates were selected at random among those collected between 2006 and 2012 in 3 CF centers in Belgium (Hôpital des enfants malades Reine Fabiola/Erasme Hospital; $n = 44$), Germany (University Hospital of Münster; $n = 51$), and the UK (Queen's University of Belfast; $n = 58$) during routine visits.

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TABLE 1 *P. aeruginosa* collection (2006 to 2012)

Country	No. of isolates	No. of patients	Period of sampling
Belgium	44	38	2010
Germany	51	34	2012
United Kingdom	58	46	2006–2009
Total	153	118	

The details on the collection are shown in Table 1. When successive strains were collected from a single patient, only those collected at the first occasion were considered. Nevertheless, more than one isolate were analyzed for some patients based on differences in their phenotypic appearance (see Fig. S1 in supplemental material).

Antibiotics. The following antibiotics were obtained as microbiological standards (with abbreviations and potencies shown in parentheses): amikacin disulfate (AMK; 74.80%), colistin sulfate (CST; 79.64%), piperacillin sodium (PIP; 94.20%), and ticarcillin disodium salt (TIC; 85.25%) from Sigma-Aldrich, St. Louis, MO; ciprofloxacin (CIP; 85.00%) from Bayer, Leverkusen, Germany; and tobramycin (TOB; 100%) from Teva, Wilrijk, Belgium. The remaining antibiotics were obtained as the corresponding branded product in Belgium for intravenous use and complied with the provisions of the European Pharmacopoeia with respect to content in active agent: ceftazidime as Glazidim (CAZ; 88.20%) from GlaxoSmithKline, Genval, Belgium; imipenem as Tienam (also containing cilastatin, which does not have any antibacterial activity) (IPM; 45.60%) from MSD, Brussels, Belgium; meropenem as Meronem (MEM; 74.00%) from AstraZeneca, Brussels, Belgium; and piperacillin-tazobactam as Tazocin (TZP; 97.00%) from Wyeth, Louvain-La-Neuve, Belgium (now part of Pfizer).

Susceptibility testing. MICs were determined by microdilution in cation-adjusted Mueller-Hinton broth by following Clinical and Laboratory Standards Institute (CLSI) recommendations, using *P. aeruginosa* ATCC 27853 as a quality control strain (8). Susceptibility was assessed according to the interpretive criteria of both the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (9) and the CLSI (8). Isolates were considered multidrug resistant (MDR) if they were resistant to at least three antibiotic classes among those tested (penicillins/cephalosporins, carbapenems, fluoroquinolones, aminoglycosides, and polymyxins), according to European Centre for Disease Prevention and Control (ECDC) criteria (10).

Screening for extended-spectrum β-lactamases (ESBL) and carbapenemases. For all isolates ($n = 51$) showing MICs of >8 mg/liter for ceftazidime and meropenem, the bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ (groups 1, 2, and 9), bla_{VIM}, bla_{IMP}, bla_{KPC}, and bla_{NDM} gene families were detected by real-time multiplex PCR, using group-specific primers (references 11 to 13 and references therein). Genes encoding OXA (OXA-1, -2, -9, -10, -18, -20, -23, -24, -30, -48, -58, and -198), BEL (BEL-1 to -3), PER (PER-1 to -5 and -7), GES (GES-1 to -18), and VEB (VEB-1 to -7) enzymes were also detected by multiplex PCR.

Molecular typing. All MDR isolates in the collection showing coresistance to penicillins and/or cephalosporins and two other classes ($n = 56$) were characterized by pulsed-field gel electrophoresis (PFGE) analysis (14). In addition, 40 pairs of isolates collected simultaneously and in the same sample from 20 patients (see Fig. S1 in the supplemental material) but differing in their profiles of susceptibility to at least one class of antibiotics were also genotyped by PFGE to determine their genetic relatedness. The pulsotype classification criteria designated a pulsotype by one or two letters, including patterns showing zero to six DNA fragment differences (14). An epidemic pulsotype was defined as a pulsotype recovered from ≥2 patients, while a sporadic pulsotype was recovered only once.

Multilocus sequence typing (MLST) was performed on a representative strain of epidemic pulsotypes detected in ≥3 strains, as previously described (15). The reference LES B58 strain (4) was used as a control. MLST data were uploaded to the *P. aeruginosa* MLST Database (http://pubmlst.org/paeruginosa) for allele type and sequence type (ST) assignments (16).

RESULTS

MIC distributions. Table 2 shows the MIC distribution for 9 antipseudomonal drugs against 153 isolates collected from 118 CF patients originating from three different countries over the period from 2006 to 2012, together with the percentages susceptible and resistant based on both EUCAST and CLSI interpretive criteria. The corresponding MIC cumulative distributions are illustrated in Fig. S2 in the supplemental material. Resistance was high in this collection. Using the EUCAST or the CLSI resistance breakpoints, respectively, the rates of full resistance for the isolates were ≥71% or ≥54% for penicillins (ticarcillin, piperacillin, and piperacillin-

TABLE 2 MIC distributions for antipseudomonal antibiotics and corresponding percent susceptibility according to EUCAST or CLSI breakpoints[a]

	MIC (mg/liter)				Susceptibility according to: EUCAST[b]			CLSI[c]		
Antibiotic	Min	Max	50%	90%	% S	% I	% R	% S	% I	% R
TIC	1	>512	128	>512	16	NA	84	16	23	61
PIP	0.5	>512	256	>512	24	NA	76	24	15	61
TZP	0.5	>512	128	512	29	NA	71	29	17	54
CAZ	1	>512	64	512	31	NA	69	31	10	59
IPM	0.25	128	4	32	48	19	33	48	19	33
MEM	0.032	256	2	16	44	36	20	63	17	20
AMK	1	>512	32	128	22	17	61	39	15	46
TOB	0.064	>512	2	16	72	NA	28	72	12	16
CIP	0.064	64	1	8	24	20	56	44	29	27
CST	0.25	>512	1	4	92	NA	8	78	14	8

[a] Min, minimum; max, maximum; S, susceptible; I, intermediate; R, resistant; NA, not applicable (no I category); TIC, ticarcillin; PIP, piperacillin; TZP, piperacillin-tazobactam; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; AMK, amikacin; TOB, tobramycin; CIP, ciprofloxacin; CST, colistin.

[b] EUCAST breakpoints (in milligrams per liter): for TIC, S ≤ 16 and R > 16; for PIP, S ≤ 16 and R > 16; for TZP, S ≤ 16 and R > 16; for CAZ, S ≤ 8 and R > 8; for IPM, S ≤ 4 and R > 8; for MEM, S ≤ 2 and R > 8; for AMK, S ≤ 8 and R > 16; for TOB, S ≤ 4 and R > 4; for CIP, S ≤ 0.5 and R > 1; and for CST, S ≤ 4 and R > 4.

[c] CLSI breakpoints (in milligrams per liter): for TIC, S ≤ 16, I = 32 to 64, and R ≥ 128; for PIP, S ≤ 16, I = 32 to 64, and R ≥ 128; for TZP, S ≤ 16, I = 32 to 64, and R ≥ 128; for CAZ, S ≤ 8, I = 16, and R ≥ 32; for IPM, S ≤ 4, I = 8, and R ≥ 16; for MEM, S ≤ 4, I = 8, and R ≥ 16; for CIP, S ≤ 1, I = 2, and R ≥ 4; for AMK, S ≤ 16, I = 32, and R ≥ 64; for TOB, S ≤ 4, I = 8, and R ≥ 16; and for CST, S ≤ 2, I = 4, and R ≥ 8.

TABLE 3 Percent cross-resistance or coresistance among pairs of antibiotics and multivariate correlation between MICs of each pair of antibiotics for individual strains[a]

r value (multivariate correlation) \ Percentage of cross- or coresistance										
	TIC	**68**	**71**	**69**	31	20	**54**	25	**48**	8
0.78	CAZ	**68**	**65**	29	20	**48**	24	**42**	7
0.72	**0.88**	PIP	**71**	31	20	**52**	24	**45**	7
0.73	**0.86**	**0.94**	TZP	30	20	**50**	24	**42**	7
0.53	0.47	0.47	0.45	IPM	16	24	12	24	4
0.66	0.55	0.48	0.54	**0.80**	MEM	14	7	18	3
0.37	0.46	0.40	0.36	0.34	0.26	AMK	28	**38**	8
0.26	0.40	0.31	0.28	0.29	0.17	**0.90**	TOB	22	5
0.26	0.30	0.27	0.28	0.39	0.43	0.31	0.31	CIP	6
0.18	0.16	0.14	0.11	0.13	0.04	0.32	0.34	0.01	CST

[a] Above the diagonal, figures correspond to the percentage of isolates categorized as resistant to the two antibiotics (row/column) using EUCAST breakpoints. Values in bold indicate combinations for which resistance is higher than 30%. The numbers below the diagonal correspond to the correlation coefficient between individual MICs for each pairs of antibiotics. Values higher than 0.75 are in bold. See Table 2, footnote *a*, for abbreviations of antibiotics and Fig. S4 in the supplemental material for the details of this analysis.

tazobactam), 69% or 59% for ceftazidime, 61% or 46% for amikacin, 56% or 27% for ciprofloxacin, ≥20% for carbapenems, and 28 or 16% for tobramycin. Full resistance to colistin was noted for only 8% of the isolates. Strains resistant to ceftazidime and meropenem were screened for the expression of frequent ESBLs, metallo-β-lactamases, and carbapenemases, which returned negative results.

Cross-resistance or coresistance. Cross-resistance or coresistance was examined among pairs of antibiotics. Cross-resistance is defined as the presence of a single resistance mechanism that confers resistance to antimicrobial molecules with a similar mechanism(s) of action. It thus describes resistance to an entire class of antibiotics, to different classes of agents with overlapping drug targets, or to different classes of antibiotics that are substrates for the same broad-spectrum efflux system. Coresistance refers to the presence of different mechanisms of resistance in the same bacterial isolate and thus necessarily confers resistance to unrelated antibiotic classes (17). Ninety-four strains were considered MDR according the ECDC (10). The upper right part of Table 3 shows the percentage of strains showing cross-resistance or coresistance to pairs of antibiotics according to EUCAST criteria. About two-thirds of the strains were resistant to both penicillins and ceftazidime and more than 40% were resistant to penicillins and ceftazidime together with amikacin or ciprofloxacin. The rates of coresistance between any studied drug and tobramycin, meropenem, and colistin were lower than 28%, 20%, and 8%, respectively. Of note, only 4 strains in the whole collection were coresistant to meropenem, tobramycin, and colistin (see Fig. S3 in the supplemental material).

The lower left part of Table 3 shows the correlation coefficient between the individual MIC for each pair of antibiotics, with the corresponding multivariate analysis presented in detail in Fig. S4 in the supplemental material. The highest degrees of correlation (>0.75) between individual MICs were observed for ticarcillin versus ceftazidime, piperacillin versus piperacillin-tazobactam, ceftazidime versus piperacillin-(tazobactam), imipenem versus meropenem, and amikacin versus tobramycin, suggesting common mechanisms of resistance between these pairs of antibiotics. Yet differences in the intrinsic potency were nevertheless observed between these pairs of drugs throughout the collection (illustrated in Fig. S4 and associated Table B in the supplemental material): tazobactam reduced the MIC of piperacillin by a factor of 1.5 dilution, while ceftazidime MICs were 0.5 and 1 dilution lower than those of ticarcillin and piperacillin, respectively, and similar to those of piperacillin-tazobactam. Meropenem MICs were 1 dilution lower than those of imipenem, and tobramycin MICs were 3 dilutions lower than those of amikacin.

Typing of MDR isolates. Among the 94 MDR isolates, most were resistant to penicillins and/or cephalosporins. Only those showing resistance to at least 2 other classes ($n = 56$) were characterized by PFGE analysis. A high genetic diversity was observed, with 19 sporadic pulsotypes and 9 epidemic pulsotypes (Table 4). With the exception of pulsotype YY recovered for 1 or 2 isolates in the three countries, each epidemic pulsotype remained localized in a single country. The CA epidemic pulsotype found in 3/4 of the UK isolates corresponded to the pulsotype of the LES clone. MLST analysis of epidemic pulsotypes CA, H, and YY showed ST146, ST2254 (new ST), and ST958, respectively (data not shown).

PFGE analysis was also performed on 40 isolates collected as pairs from 20 patients and displaying different susceptibility profiles (see Table S1 in the supplemental material). In 12 patients, the pair of *P. aeruginosa* isolates had the same pulsotype, while the 8 other patients had isolates with different pulsotypes.

Analysis per country or age group. Because of the genetic diversity observed between countries, we then examined the distribution of susceptible, intermediate (when applicable), and resistant isolates classified based on the country where they were collected (Fig. 1). Susceptibility rates differed among countries, with lower resistance in Belgium (significant for all antibiotics except ticarcillin and ciprofloxacin) and higher resistance in Germany and the UK (significant for piperacillin-tazobactam in Germany and for imipenem, ciprofloxacin, and colistin in the UK) than the mean value for the whole collection. There was no significant correlation between the patient's age when the isolate was

TABLE 4 Distribution of pulsotypes among the MDR *P. aeruginosa* clinical isolates

Country	No. of MDR strains	No. of pulsotypes		No. of strains in epidemic pulsotype								
		Sporadic	Epidemic	CA[a]	CK	CM	CD	H	WW	YI	CJ	YY
Belgium	10	3	4	0	0	2	2	0	2	0	0	1
Germany	22	11	5	0	2	0	0	3	0	2	2	2
United Kingdom	24	5	2	18	0	0	0	0	0	0	0	1

[a] CA pulsotype corresponds to the LES epidemic clone pulsotype.

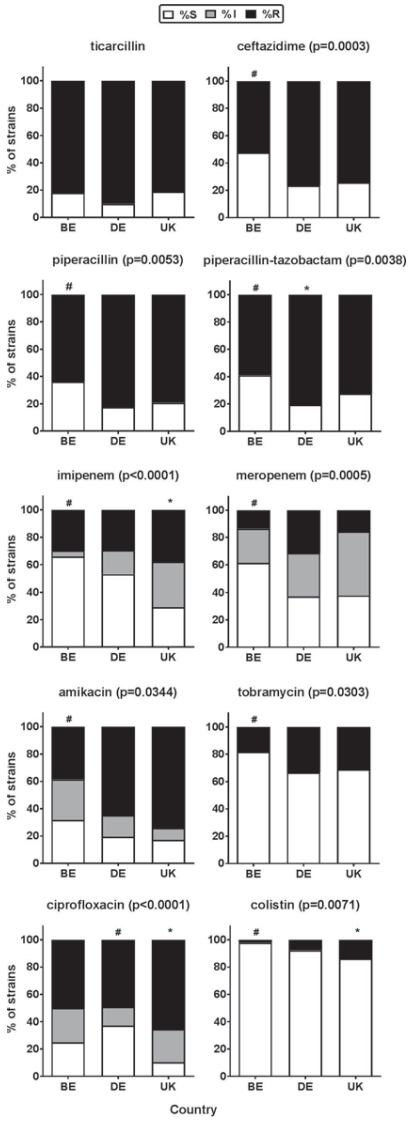

FIG 1 Comparison of the percentage of antibiotic resistance in the collection based on the country of origin of the strain (Belgium [BE]: $n = 44$; Germany [DE]: $n = 51$; United Kingdom [UK]: $n = 58$). Statistical analysis was done by chi square test (*P* values indicated are after the name of the antibiotic); Analysis of means of proportions was done with an α level of 0.05. *, the value is below the mean; #, the value is above the mean.

collected and the number of antibiotic classes to which the isolate was resistant (see Fig. S5 in the supplemental material).

DISCUSSION

In this study, we examined antibiotic susceptibility within a collection of *P. aeruginosa* isolates from CF patients in three northern European countries collected during routine examination, which provides a broader view than the majority of previous surveys, which have focused on a single country (18–20) or a single center (21–23). A key observation is that resistance rates were high in this population, confirming previous studies with CF patients (2), and notably much higher than that which has been reported for isolates collected in northern Europe from intensive care units (24–26). Resistance rates were also higher than those previously reported for strains from CF patients in a German survey from the University of Würzburg, except in the case of tobramycin (isolates collected in 2006 [27]), or in a multicentric study in the UK, except for meropenem and ciprofloxacin (isolates collected in 2000[28]). Moreover, a high degree of cross-resistance or coresistance among antibiotics was observed, which is important from both a pharmacological and a clinical perspective.

From a pharmacological perspective, we noticed, as expected, significant correlations between MICs for antibiotics belonging to the same or similar classes (penicillins and ceftazidime or other penicillins, imipenem and meropenem, and amikacin and tobramycin), but with systematic differences in the potency of each antibiotic within these pairs (see Fig. S4 and associated Table B in the supplemental material). Focusing on β-lactams, the impact of tazobactam on piperacillin activity was modest but of the same order of magnitude as that observed on MIC distribution for wild-type strains reported by EUCAST (29), probably denoting the inhibition by tazobactam of the low basal levels of AmpC produced by the wild-type strains (30, 31). Likewise, a higher potency of ceftazidime than for penicillins and of meropenem than for imipenem is reported in wild-type EUCAST distributions (29). Thus, differences in potency among these pairs of drugs in our collection are likely to reflect differences in intrinsic activity rather than in vulnerability to resistance mechanisms. Remarkably, no carbapenemase production was apparent in this collection. The same finding was reported in two recent investigations of *P. aeruginosa* isolates collected over the same period as those examined here. The first of these studies was performed in Australia and examined successively a collection of 662 carbapenem-resistant isolates assembled in 2007 to 2009 from diverse CF centers and of 517 isolates collected in a single CF center in 2011 (32). The second study was performed in Brazil and analyzed isolates from 75 patients collected from 2010 to 2011 (19). In contrast, carbapenemases have been detected in 63 out of 217 *P. aeruginosa* isolates collected from CF patients in China (22). The prevalence of carbapenemase genes could, however, be different in other bacteria infecting CF patients, but there is no large survey published so far for other Gram-negative species (33, 34).

Thus, carbapenem resistance in CF European isolates is probably primarily mediated by the combined effect of AmpC and of a reduced accumulation (porin mutations and/or increased efflux) (35, 53). Of note, however, carbapenem resistance has previously been described for the LES clone (5), but the underlying mechanism(s) has not been investigated to date. For aminoglycosides, the higher potency of tobramycin over amikacin in our collection also reflects what is observed in MIC distributions of wild-type

strains assembled by EUCAST (29). Tobramycin has been described as a poorer substrate than amikacin for the efflux pump MexXY-OprM, considered responsible for natural and adaptive resistance to aminoglycosides in *P. aeruginosa* (36, 37).

Considering our findings from a clinical perspective, a high degree of cross-resistance was observed between penicillins and ceftazidime, which was expected. However, a high degree of coresistance was also apparent between these antibiotics and both ciprofloxacin and amikacin, resulting in 60% of the isolates being categorized as multidrug resistant. In contrast, meropenem, colistin, and, to a lesser extent, tobramycin were active against a large fraction of the isolates, with few strains coresistant to these three antibiotics. Tobramycin and colistin by inhalation are often considered first line for the eradication of early *P. aeruginosa* infection, and tobramycin is also considered first line for chronic therapies (38–40). High concentrations delivered by this route of administration may help to overcome resistance (41, 42).

We also noticed an important genetic diversity among multiresistant isolates collected in Belgium and Germany, while those collected in the UK belong in majority to the Liverpool epidemic strain (LES) clone. Global studies of *P. aeruginosa* population structure concluded that CF isolates present a high genetic diversity but nevertheless belong to a "core lineage" ubiquitous in the natural environment (43), which is highly suggestive of a direct colonization of the patients from the environment. However, a series of epidemic clones have been described (7), among which are the LES clone (4), representing 18 of the 24 MDR isolates collected in the UK in our study, and ST17 (7), which differs by only 1 nucleotide from ST958, found in the three countries we investigated. ST2254, the new ST we describe, was distinct from ST146 (LES clone; 5 alleles different) and ST958 or ST17 (6 alleles different).

We observed that a single patient can be colonized by different strains and, conversely, that clonally related strains isolated at the same time from a single patient can harbor diverse susceptibility profiles. This could be a consequence of the previously described phenotypic variability among isolates with the same colony morphotype and being part of a single clonal lineage (44, 45), as well as of recombination occurring *in vivo* and generating phenotypic and genetic diversification (46, 47).

Although limited, differences in resistance rates between Belgium and the two other countries are raising questions about segmentation of clone distribution. For strains collected in the UK, higher resistance is clearly related to the high prevalence of the LES clone, which has been described as exhibiting a large proportion of MDR isolates (5). Of interest, we observed different resistance profiles within this clone, which is consistent with the previously described phenotypic variability among LES isolates (6). ST958, represented in the three countries, is also found among the MDR clonal complexes (7). In the German collection, higher resistance is essentially related to the presence of more sporadic MDR clones than in the two other countries. We cannot exclude differences in therapeutic management of patients among these three centers that may influence resistance selection (48), but this specific aspect was not within the scope of our study.

Resistance rates were not higher in the older population than in children and young adults. The interpretation of these data needs to be done with caution because (i) we did not follow the evolution of susceptibility over time in single patients and (ii) we do not know the age of first colonization for each patient. With this limitation in mind, the fact that MDR isolates could be found in young people and susceptible isolates in adults may suggest that resistance depends on the initial susceptibility of the infecting strain. A link between emergence of resistance and early antibiotic use in CF patients is still controversial, even though it was underlined in the last report of the Cystic Fibrosis Foundation (2). A recent study in Australia showed that multiresistance in children is correlated with duration of intravenous antibiotic treatment, which was not the case for adults (18). A correlation with antibiotic usage irrespective of patient age (49) or with time after colonization (6) has also been proposed. In contrast, other studies following the evolution of antibiotic susceptibility in successive isogenic isolates from a single patient suggest that resistance can occur either sporadically (50) or without correlation with the time of isolation (51). In these cases, the presence of mutator variants seems to predetermine the risk of developing resistance over time (6).

Our study has a number of limitations, primarily linked to the fact that samples collected during periodic routine examinations may not correspond to the first *P. aeruginosa* infections in these patients. Moreover, as we did not have the history of antibiotic use in these patients, we could not determine if there was a potential link between antibiotic usage and subsequent development of resistance. Nevertheless, this collection reflects the situation CF clinicians face daily, where they have to select antibiotics based on susceptibility testing performed on current isolates. In this context, our data may lead to three clinically meaningful conclusions. First, susceptibility testing is important to perform even for newly infected patients, because they can be colonized very early by MDR clones. Second, these tests should be performed on more than one colony (especially if different phenotypes are evidenced on culture plates), because of potential population heterogeneity with respect to susceptibility profiles (52). Third, prudent use of highly active drugs should be promoted in order to preserve their efficacy. This implies the use of optimized doses if administered by conventional routes or administration by inhalation to ensure high local concentrations that could minimize the risk of selection of resistance.

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Antimicrobial susceptibility of *Pseudomonas aeruginosa* isolated from Cystic Fibrosis patients through Northern Europe

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SUPPLEMENTARY MATERIAL

Table S1: MIC of isolates collected simultaneously in individual patients and classified according to their PFGE pattern

Patient ID	Country	PFGE	Age	Collection date	TIC	PIP	TZP	CAZ	IMI	MER	CIP	AMK	TOB	CST
pairs with identical PFGE patterns														
AD	UK	CA	18	13-09-06	**128**	**256**	2	**16**	4-8	2	**2**	8-16	1-2	2
AD	UK	CA	18	13-09-06	**32**	8	0.5	8	4-8	2	**2**	**64**	**8**	4
JP	UK	YY	19	10-05-06	16	8-16	4	1-2	1	0.125	0.5	**32-64**	4	1
JP	UK	YY	19	10-05-06	8	2	2	8	1-2	2-4	**8**	**256**	**128**	**16**
BAM75	BE	CV	35	26-09-10	**128**	**>512**	**512**	**>512**	2-4	1-2	**32**	16	2	2
BAM75	BE	CV	35	26-09-10	**32**	4-8	8	2	0.5	0.5	1	16	2	0.5
BCM75	BE	CP	35	02-09-10	**512**	**>512**	**128**	**>512**	**32**	**16**	**32**	**>512**	**>512**	4
BCM75	BE	CP	35	02-09-10	**512**	**>512**	**128**	**512**	**32**	**16**	**8**	4	0.5	1
JSF89	BE	CM	21	12-10-10	**>512**	**>512**	**512**	**256**	**32**	**16**	**2-4**	**64**	2	1
JSF89	BE	CM	21	12-10-10	**512**	**>512**	**256**	**512**	**32**	**16**	**8**	**128**	**16**	0.25
RCF62	BE	CB	48	09-09-10	**128**	**256**	**128**	**32**	**32**	8	**4**	16	1	1
RCF62	BE	CB	48	09-09-10	**64**	**128**	**32**	**8-16**	2	1	**2**	**32-64**	2	1
127	DE	YI	31	10-07-12	**>512**	16	16	8	**32-64**	8	1	**64**	**8-16**	1
127	DE	YI	31	10-07-12	**512**	**>512**	**512**	**256**	8	8	0.5	**64**	**8**	0.5
178	DE	CD	49	27-07-12	1	2	2	1	1	0.25	1	4	0.5	1
178	DE	CD	49	27-07-12	**>512**	**>512**	**>512**	**>512**	**128**	**256**	**8**	16	2	0.5
205	DE	CR	36	09-08-12	**>512**	**>512**	**>512**	**64**	2	**32**	**4**	16	1	1
205	DE	CR	36	09-08-12	**256**	**512**	**256**	**64**	2	**32**	**4**	**32**	2	**256**
158	DE	WY	23	18-07-12	2	4	4	4-8	1-2	0.5	**4**	**64**	**16**	4
158	DE	WY	23	18-07-12	**64**	**32-64**	**32**	4-8	1	0.25	**2**	**64**	**8**	2-4
191	DE	CK	36	03-08-12	**128**	**>512**	**512**	**>512**	1	0.25	**2**	**512**	**32**	2
191	DE	CK	36	03-08-12	**256**	**>512**	**256**	**>512**	0.5	0.5	0.25	**64**	4	2-4
208	DE	H	32	09-08-12	1	**256**	**256**	**256**	**16**	8	**32**	**256**	**64**	2-4
208	DE	H	32	09-08-12	**256**	**>512**	**512**	**>512**	1-2	0.25	**2**	**512**	**128**	2

pairs with different PFGE patterns														
AON	UK	WT	41	2007	**64**	**64**	**32**	4-8	**16**	4-8	**2**	4-8	0.25	1
AON	UK	CQ and CA	41	2007	**>512**	**512**	**256**	**128**	**16**	8	**2**	**64**	2	**128**
AW	UK	CA	48	2007	**>512**	**512**	**256**	**512**	**16-32**	**32**	**2**	**64**	4-8	**64**
AW	UK	WG	48	2007	**>512**	**>512**	**512**	**512**	**64**	**32**	**8**	**64**	4	1-2
CC	UK	CA	22	11-10-06	**>512**	**256-512**	**128**	**512**	8	2	**2**	**64**	4	2
CC	UK	CS	22	11-10-06	**>512**	**>512**	**>512**	**>512**	**32**	**16-32**	**8-16**	**>512**	**128**	1-2
CI	UK	WL	24	2007	**512**	**>512**	**256**	**256**	1	0.5	1	**64**	2	1-2
CI	UK	CA	24	2007	**>512**	**>512**	**512**	**512**	**16**	8	**2**	**256**	**32**	4
LS	UK	WN	20	2007	**64**	8	8	4	1	1	**2**	8	0.5	1
LS	UK	CA	20	2007	**>512**	**>512**	**512**	**512**	**16**	8	0.5-1	**128**	4-8	4
143	DE	WI	29	12-07-12	**64**	**512**	**256**	**128**	2	0.5	0.5	**128**	**16**	2
143	DE	CV	29	12-07-12	16	4	4	2-4	1	0.25	0.25-0.5	**32-64**	4	1
192	DE	WS	48	04-08-12	**32**	**32-64**	16	**16**	8	4	0.5	16	1-2	2
192	DE	CO	48	04-08-12	**>512**	**256**	**256**	**>512**	2	**32**	1	8	1	0.5
195	DE	WP and CJ	31	06-08-12	**128**	**256**	**256**	**16**	8	**16-32**	**64**	**64**	4	0.25
195	DE	CJ	31	06-08-12	**256**	4	4	8	2	**16**	**4**	**32**	1-2	0.5

values in bold: above the EUCAST R breakpoint

Difference in MIC conferring resistance to one of the two isolates (MIC > EUCAST breakpoint for one of the isolates in the pair)

Difference in MIC of at least 2 dilutions between the two isolates in the pair, but no change in S/R categorization

Figure S1: Distribution of replicates in the collection (total number of patients = 118)

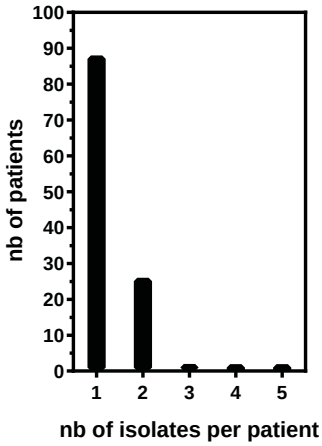


Figure S2: MIC distributions for the antibiotics under study (n=153)
CST: Colistin; CIP: Ciprofloxacin TOB: Tobramycin; AMK: Amikacin; MEM: Meropenem; IPM: Imipenem; CAZ: Ceftazidime; TZP: Piperacillin-tazobactam; PIP: Piperacillin; TIC: Ticarcillin

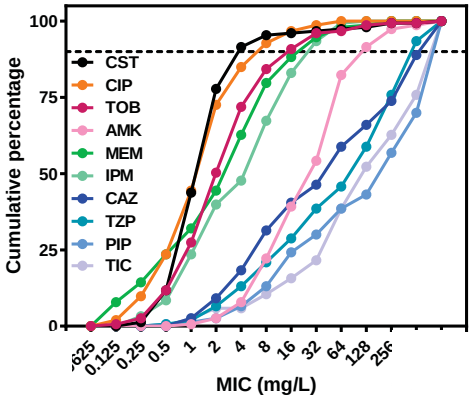


Figure S3

Tridimensional plot analysis of individual MICs of colistin (CST), meropenem (MEM) and tobramycin (TOB) among the whole collection.

Strains resistant (as per EUCAST criteria) to two or three of these antibiotics are highlighted in specific colors. MICs are expressed as $\log_2$ of their value.

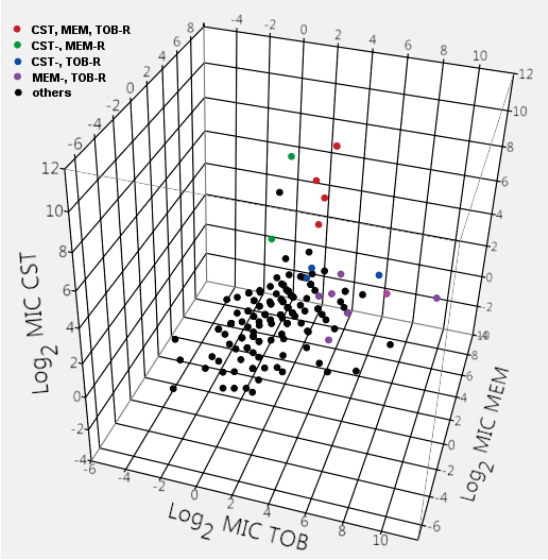
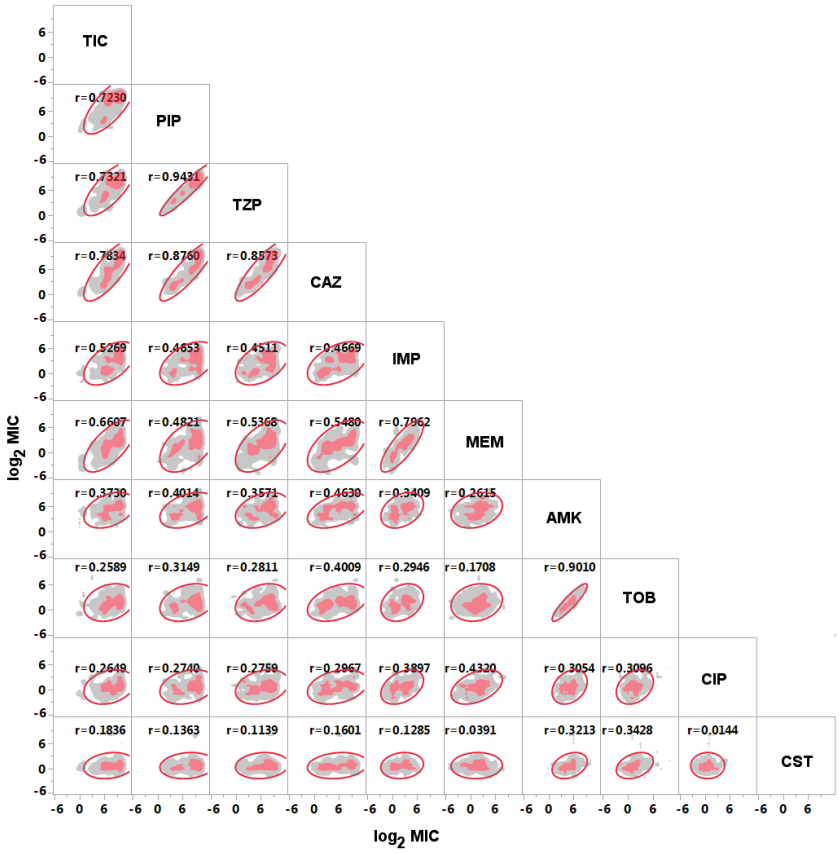


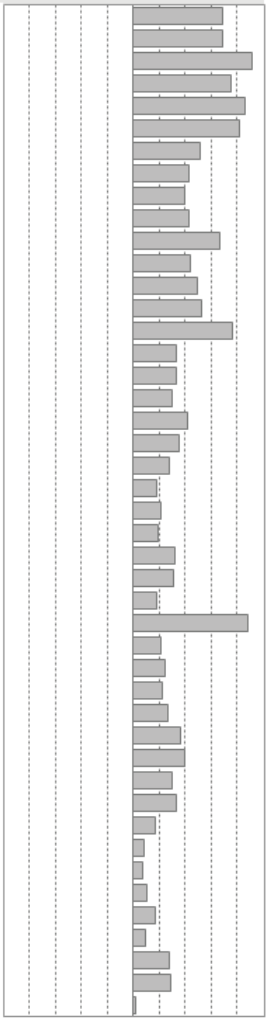
Figure S4: multivariate correlation analysis of MICs for pairs of antibiotics.
The graphs show the density plots and density ellipse (α: 95%) for individuals pairs of MICs (expressed in as log_2 of values) together with the correlation coefficient (r). The tables below the graph show respectively the details of statistical analyses for pairwise correlations and the equation of the linear correlation for antibiotic pairs with r values > 0.75.
TIC: Ticarcillin; PIP: Piperacillin; TZP: Piperacillin-tazobactam: CAZ: Ceftazidime; IPM: imipenem; MEM: Meropenem; AMK: Amikacin; TOB: Tobramycin; CIP: Ciprofloxacin; CST: Colistin



A. statistical analyses for pairwise correlations

Pairwise Correlations

Variable	by Variable	Correlation	Count	Lower 95%	Upper 95%	Signif Prob
PIP	TIC	0.6943	153	0.6019	0.7683	<.0001*
TZP	TIC	0.6888	153	0.5952	0.7640	<.0001*
TZP	PIP	0.9147	153	0.8844	0.9373	<.0001*
CAZ	TIC	0.7587	153	0.6822	0.8188	<.0001*
CAZ	PIP	0.8628	153	0.8159	0.8985	<.0001*
CAZ	TZP	0.8256	153	0.7675	0.8703	<.0001*
IPM	TIC	0.5199	153	0.3937	0.6268	<.0001*
IPM	PIP	0.4271	153	0.2879	0.5486	<.0001*
IPM	TZP	0.4016	153	0.2594	0.5267	<.0001*
IPM	CAZ	0.4370	153	0.2990	0.5570	<.0001*
MEM	TIC	0.6671	153	0.5686	0.7467	<.0001*
MEM	PIP	0.4476	153	0.3111	0.5661	<.0001*
MEM	TZP	0.4971	153	0.3674	0.6078	<.0001*
MEM	CAZ	0.5321	153	0.4078	0.6370	<.0001*
MEM	IPM	0.7680	153	0.6939	0.8260	<.0001*
AMK	TIC	0.3389	153	0.1904	0.4721	<.0001*
AMK	PIP	0.3306	153	0.1814	0.4648	<.0001*
AMK	TZP	0.3013	153	0.1498	0.4390	0.0002*
AMK	CAZ	0.4207	153	0.2807	0.5431	<.0001*
AMK	IPM	0.3591	153	0.2125	0.4898	<.0001*
AMK	MEM	0.2824	153	0.1296	0.4222	0.0004*
TOB	TIC	0.1878	153	0.0300	0.3365	0.0201*
TOB	PIP	0.2215	153	0.0651	0.3672	0.0059*
TOB	TZP	0.1903	153	0.0326	0.3388	0.0185*
TOB	CAZ	0.3287	153	0.1794	0.4632	<.0001*
TOB	IPM	0.3153	153	0.1649	0.4514	<.0001*
TOB	MEM	0.1869	153	0.0290	0.3356	0.0207*
TOB	AMK	0.8853	153	0.8454	0.9154	<.0001*
CIP	TIC	0.2171	153	0.0605	0.3633	0.0070*
CIP	PIP	0.2492	153	0.0943	0.3924	0.0019*
CIP	TZP	0.2247	153	0.0684	0.3701	0.0052*
CIP	CAZ	0.2650	153	0.1109	0.4065	0.0009*
CIP	IPM	0.3631	153	0.2169	0.4934	<.0001*
CIP	MEM	0.4021	153	0.2600	0.5271	<.0001*
CIP	AMK	0.3062	153	0.1551	0.4433	0.0001*
CIP	TOB	0.3330	153	0.1841	0.4670	<.0001*
CST	TIC	0.1782	153	0.0201	0.3276	0.0275*
CST	PIP	0.0873	153	-0.0723	0.2426	0.2831
CST	TZP	0.0760	153	-0.0837	0.2319	0.3506
CST	CAZ	0.1043	153	-0.0553	0.2587	0.1993
CST	IPM	0.1768	153	0.0186	0.3263	0.0288*
CST	MEM	0.1020	153	-0.0576	0.2565	0.2095
CST	AMK	0.2808	153	0.1279	0.4208	0.0004*
CST	TOB	0.2883	153	0.1359	0.4275	0.0003*
CST	CIP	0.0211	153	-0.1381	0.1791	0.7961

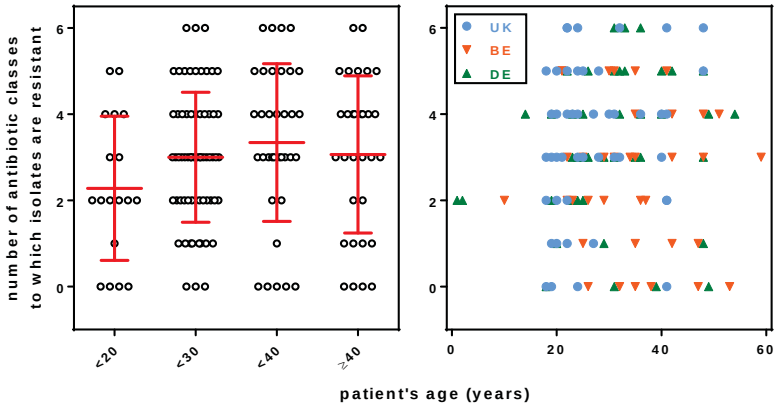


B. Equations of linear correlations for antibiotics pairs when r values are > 0.75

X variable	Y variable	slope	Y intercept (log_2)
TZP	PIP	0.94	1.4
TZP	CAZ	0.90	0.1
PIP	CAZ	0.92	-0.9
TIC	CAZ	0.87	-0.5
IPM	MEM	0.95	-0.7
AMK	TOB	0.99	-3.2

- All slopes are close to 1, denoting that MICs to both X and Y antibiotics increase in parallel.
- A Y intercept
    - close to 0 indicates that MICs of X and Y are globally similar;
    - close to +1, that MIC of Y are globally 1 doubling dilution higher than those of X;
    - close to -1, that MICs of Y are globally 1 doubling dilution lower than those of X.

Figure S5
Multiresistance as a function of patient's age.
The graph shows the number of antibiotic classes (among penicillins, cephalosporins, carbapenems, fluoroquinolones, aminoglycosides, polymyxins) to which each isolate is resistant (MICs > EUCAST "R" breakpoint) as a function of the patient's age pooled in categories (left) or individually but according to the country of origin (UK: United Kingdom; BE: Belgium; DE: Germany).
Statistical analysis: left panel: one-way ANOVA: p= 0.467; right panel: Pearson coefficient for the whole collection: 0.047



Additional discussion to this first article:

Figure S4 shows co-resistance between antimicrobials. When examining correlations between MICs of colistin and other antimicrobial agents, we observed that the correlation coefficient was lower with ciprofloxacin (0.01) than with β-lactams (0.04-0.18) or aminoglycosides (> 0.3).

A possible explanation is that mutations affecting lipopolysaccharide (LPS) and conferring resistance to colistin may also impair the activity of aminoglycosides and of β-lactams to a lower extent. For aminoglycosides, a previous study has indeed documented a reduced activity of aminoglycosides in strains harbouring mutations in the PhoP-PhoQ two-component regulatory system that confer resistance to polymyxins [232]. This co-resistance may be due to the fact that aminoglycosides share with polymyxins a polycationic character. They may thus suffer from a reduced outer membrane permeability in colistin-resistant mutants.

Likewise, previous studies have shown that modifications in the structure of LPS confer resistance to the carboxypenicillin ticarcillin and to carbenicillin in PA [233;234]. Similarly, permeability modifications, due to alterations in outer membrane proteins (porins) or to LPS, have been shown to play a role in resistance to imipenem [235].

ii. Resistance mechanisms to β-lactam antibiotics: the case of meropenem

As indicated previously (first article, section 2.2.i), meropenem was classified among the most active drugs along with colistin and tobramycin, but high-level meropenem resistance was worryingly observed in six isolates. In addition to that, a distinct phenotype was evidenced in these isolates, in which meropenem was less active than imipenem, with MICs ≥ 64 mg/L (EUCAST susceptibility breakpoint of 2 mg/L).

The aim of this second paper was therefore to determine the mechanisms responsible for high-level resistance to meropenem in these isolates.

First, a screening for the expression of carbapenemases was performed on the whole collection of *Pseudomonas* isolates (n=333) by adapting the "Carba NP test" developed to detect these enzymes in Enterobacteriaceae. This mechanism is indeed recognized at conferring the highest levels of resistance to carbapenems. Surprisingly, no carbapemenase expression was detected in the whole collection. The expression of frequently encountered ESBLs, metallo β-lactamases, and carbapenemases was also studied by PCR in all isolates that were resistant to ceftazidime and meropenem, but again negative results were obtained.

Focusing then on the 6 isolates highly-resistant to meropenem, we examined the role of other resistance mechanisms, namely (1) the level of expression of the genes encoding clinically relevant efflux systems i.e. MexAB-OprM, MexXY-OprM, MexCD-OprJ and MexEF-OprN; (2) OprD porin mutations and (3) overexpression of AmpC-type cephalosporinases. These isolates were compared with seven carbapenemase-positive isolates, taken as control, from patients suffering from hospital-acquired pneumonia (HAP). We show that overexpression of efflux combined with porin mutations and AmpC overexpression made these isolates as resistant as HAP isolates (which express carbapenemases). This combination of resistance mechanisms

also explains the unusual phenotype of higher resistance to meropenem than to imipenem, as meropenem is a better substrate for efflux than imipenem.

Thus, we conclude from this study that these two carbapenems should be included in susceptibility testing for CF isolates.

Number of experimental replicates in this study. The determination of MICs by broth microdilution for each antibiotic against the different isolates of PA (from CF or HAP patients) was performed at least 4 times and using as reference strain ATCC 27853. In the presence of different substrates, the MICs were determined at least 2 times. Moreover, control strains overexpressing AmpC cephalosporinases, ESBL and efflux systems (MexAB-OprM, MexXY-OprM, MexEF-OprN and MexCD-OprJ) were used as internal controls and as compared to the wild type strain PAO1. The real-time PCR experiments were performed over 3 independent replicates of RNA samples, with the standard deviation varying between 0.1 and 1.

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Short Communication

High-level resistance to meropenem in clinical isolates of *Pseudomonas aeruginosa* in the absence of carbapenemases: role of active efflux and porin alterations ☆

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ABSTRACT

High-level carbapenem resistance is worryingly increasing in clinical isolates and is often attributed to carbapenemase expression. This study aimed to determine the mechanisms leading to high-level meropenem resistance in six carbapenemase-negative *Pseudomonas aeruginosa* isolated from cystic fibrosis (CF) patients and seven carbapenemase-positive isolates from patients suffering from hospital-acquired pneumonia (HAP). MICs were determined in the absence or presence of L-arginine or glycine–glutamate as competitive substrates for OprD (OccD1) or OpdP (OccD3), respectively, or the efflux pump inhibitor Phe-Arg β-naphthylamide (PAβN). β-Lactamases were screened by phenotypic tests and/or PCR. The *oprD* gene and its promoter were sequenced; protein expression was evidenced by SDS-PAGE. *mexA*, *mexX*, *mexC* and *mexE* transcripts were evaluated by real-time and semiquantitative PCR. Meropenem/imipenem MICs were 64–128/16–32 mg/L and 128/128–256 mg/L in CF and HAP isolates, respectively; PAβN reduced meropenem MICs to 4–16 mg/L only and specifically in CF isolates; porin competitors had no effect on MICs. All isolates showed an increase in transcription levels of *mexA*, *mexX* and/or *mexC* and mutations in *oprD* leading to production of truncated proteins. AmpC-type cephalosporinases were overexpressed in CF isolates and VIM-2 was expressed in HAP isolates. Antibiotic exclusion from bacteria by concomitant efflux and reduced uptake is sufficient to confer high-level resistance to meropenem in isolates overexpressing AmpC-type cephalosporinases. As efflux is preponderant in these isolates, it confers a paradoxical phenotype where meropenem is less active than imipenem. Concomitant susceptibility testing of both carbapenems and rapid elucidation of the most probable resistance mechanisms is thus warranted.

1. Introduction

Meropenem is widely used in the treatment of pulmonary exacerbations in cystic fibrosis (CF) patients infected by multidrug-resistant (MDR) *Pseudomonas aeruginosa*. Its consumption has promoted the emergence of high-level resistance, often ascribed to expression of carbapenemases in hospital-acquired infections [1]. Upon screening of a collection of 333 *P. aeruginosa* isolates from CF patients, we observed meropenem minimum inhibitory concentrations (MICs) of ≥64 mg/L in six isolates that did not express carbapenemases. This study aimed to determine the mechanism(s) leading to this high-level meropenem resistance.

2. Materials and methods

2.1. Strains

A total of 13 carbapenem-resistant *P. aeruginosa* isolates were collected from two CF patients ($n = 6$ isolates) [2] and three patients ($n = 7$ isolates) suffering from hospital-acquired pneumonia (HAP) [3]. *Pseudomonas aeruginosa* PAO1 and derivatives thereof with overexpression

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or deletion of genes encoding efflux pumps or OprD were also studied (Table 1). Clonal relatedness was evaluated by pulsed-field gel electrophoresis (PFGE) following published recommendations [7].

2.2. Susceptibility testing

MICs were determined by broth microdilution following Clinical and Laboratory Standards Institute (CLSI) recommendations in cation-adjusted Mueller–Hinton broth (CA-MHB) (Becton Dickinson & Co., Franklin Lakes, NJ), in controlled conditions or in the presence of (i) 20 mg/L of the efflux pump inhibitor Phe–Arg β-naphthylamide (PAβN) combined with 1 mM $MgSO_4$ to strengthen the outer membrane, (ii) 10 mM L-arginine (L-Arg) (OprD [OccD1] substrate [8]) or (iii) 10 mM glycine–glutamate (Gly-Glu) (OpdP [OccD3] substrate [8,9]). PAβN and porin substrates were used at the highest non-toxic concentration (no effect on bacterial growth). The CA-MHB was adjusted to pH 7.4 after addition of porin substrates. Susceptibility was categorised according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) interpretive criteria (http://www.eucast.org/clinical_breakpoints/; accessed May 2016).

2.3. β-Lactamase screening and identification

Phenotypic screening for metallo-β-lactamases was performed by the double-disk synergy test (imipenem–0.5 M ethylene diamine tetra-acetic acid (EDTA) pH 8–meropenem) [10]. The carbapenemase Nordmann–Poirel (Carba NP) and extended-spectrum β-lactamase (ESBL) NDP (Nordmann/Dortet/Poirel) tests, primarily developed for Enterobacteriaceae, were validated for *P. aeruginosa* using appropriate control strains and were used at first as a phenotypic screening test for carbapenemase(s) or for overproduction of AmpC-type cephalosporinases (not inhibited by clavulanic acid), respectively [4,5].

Detection of bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$ (groups 1, 2 and 9), bla_{VIM}, bla_{IMP}, bla_{KPC} and bla_{NDM} gene families was performed by real-time multiplex PCR using group-specific primers. Other genes encoding OXA (1, 2, 9, 10, 18, 20, 23, 24, 30, 48, 58 and 198), BEL (1–3), PER (1–5 and 7), GES (1–18) and VEB (1–7) enzymes were also detected by multiplex PCR (see [6,11] and the references cited therein).

2.4. Efflux pump expression

Strains were grown to late log phase [optical density at 620 nm (OD_{620}) = 1] in Luria–Bertani broth (Becton Dickinson & Co.) at 37 °C under agitation (130 rpm) and were harvested by centrifugation. Total RNA was isolated using an RNeasy Mini Kit (Stratec Molecular GmbH, Berlin, Germany) with residual DNA removed by RNase-Free DNase (Thermo Fisher Scientific, Waltham, MA). The absence of contaminating DNA was checked by PCR reactions for *rpsL* using RNA samples as a template. cDNA was obtained by reverse transcription of purified RNA and was used to evaluate the expression

Table 1
Minimum inhibitory concentrations (MICs) and gene expression levels in reference strains and in cystic fibrosis (CF) and hospital-acquired pneumonia (HAP) clinical isolates of *Pseudomonas aeruginosa*.

Reference strains and clinical isolates (with patient's ID code and date of collection)	β-Lactamases[a]	MIC (mg/L)[b]								Relative expression of genes encoding efflux pumps[d]		
		Meropenem				Imipenem				*mexA*	*mexX*	*mexC*
		Control	+10 mM L-Arg	+10 mM Gly-Glu	+20 mg/L PAβN[c]	Control	+10 mM L-Arg	+10 mM Gly-Glu	+20 mg/L PAβN[c]			
PAO1 wild-type	–	1	4	2	0.5	2	4	4	2	1.0	1.0	1.0
PAO1 Δ*oprD*	–	4	4	4	1–2	8	8	8	8	1.7	0.9	0.4
PAO1 Δ*mexAB*::FRT	–	0.25	ND	ND	0.064	2	ND	ND	ND	1.0	0.5	0.6
PAO1 Δ*mexAB–oprM*, *mexXY*, *mexEF–oprN*, *mexCD–oprJ*, *mexJK*	–	0.12	ND	ND	Toxic	1	ND	ND	0.5	0.07	***2.1***	0.0
PAO1 Δ*mexXY*	–	1	ND	ND	0.5	2	ND	ND	ND	1.9	1.0	***2.0***
PAO1 MexAB–OprM overproducer	–	4	ND	ND	1–2	2	ND	ND	ND	***9.3***	1.3	0.7
PAO1 MexXY–OprM overproducer	–	1	ND	ND	0.5	2	ND	ND	ND	1.5	***7.7***	***2.0***
PAO1 MexEF–OprM overproducer	–	1	ND	ND	0.5	2	ND	ND	ND	1.0	0.2	0.8
PAO1 MexCD–OprM overproducer	–	1	ND	ND	0.5	2	ND	ND	ND	1.2	0.4	***7.5***
128-CF (DAF69, 09/09/2010)[e]	AmpC-type	128	128	128	16	32	32	32	32	***2.2***	0.8	0.0
180-CF (DAF69, 04/10/2010)[e]	AmpC-type	64	64	64	16	16	16	16	16	***3.7***	1.7	***2.3***
238-CF (DAF69, 19/10/2010)[e]	AmpC-type	64	64	64	4	16	16	16	16	***2.3***	1.2	0.2
254-CF (DAF69, 26/10/2010)[e]	AmpC-type	64	64	64	8	16	16	16	16	***14.3***	***3.1***	***2.7***
306-CF (DAF69, 09/11/2010)[e]	AmpC-type	64	64	64	16	16	16	16	16	***6.9***	***2.4***	1.7
132-3-CF (132, 08/07/2012)[f]	AmpC-type	64	64	64	16	32	16	16	32	0.3	***3.5***	0.7
228-HAP (DS, 26/12/2005)[e]	VIM-2	128	128	128	128	128	128	128	128	***2.3***	1.0	1.0
218-HAP (DS, 26/01/2006)[e]	VIM-2	128	128	128	128	128	128	128	128	***4.3***	***2.8***	1.4
222-HAP (DS, 13/02/2006)[e]	VIM-2	128	128	128	128	128	128	128	128	***6.5***	***4.3***	***13.6***
240-HAP (OG, 08/04/2006)[e]	VIM-2	128	128	128	128	256	256	256	256	***6.4***	***9.6***	***5.4***
241-HAP (OG, 02/05/2006)[e]	VIM-2	128	128	128	128	256	256	256	256	***2.8***	1.6	0.9
258-HAP (ND, 10/08/2006)[e]	VIM-2	128	128	128	128	256	256	256	256	***3.4***	***7.5***	1.2
252-HAP (ND, 11/09/2006)[e]	VIM-2	128	128	128	128	256	256	256	256	***3.7***	***4.8***	1.3

L-Arg, L-arginine; Gly-Glu, glycine–glutamate; PAβN, Phe–Arg β-naphthylamide; ND, not determined.

[a] AmpC-type overexpression (chromosomal and/or plasmid-mediated): phenotypic detection [4] using the extended-spectrum β-lactamase (ESBL) NDP (Nordmann/Dortet/Poirel) test compared with the wild-type strain PAO1 producing AmpC at very low basal levels; VIM-2: phenotypic detection [5] for carbapenemase(s) expression using the Carba NP test, with genotypic detection [6] for the $bla_{VIM\text{-}2}$ gene (multiplex PCR).

[b] European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (mg/L): meropenem, susceptible (S), ≤2, resistant (R), >8; imipenem, S, ≤4, R, >8.

[c] No toxicity detected at this concentration.

[d] Mean values of transcripts levels obtained in three independent experiments performed in duplicate were considered. Increased gene expression compared with PAO1 (expression level set at 1.0) of ≥2 was considered significant and is shown in italic bold characters. Student's *t*-test and analysis of variance (ANOVA): $P < 0.05$ (*mexA*); $P < 0.01$ (*mexX* and *mexC*). PAO1 mutants overexpressing efflux pumps were used as controls.

[e] From Erasme Hospital (Brussels, Belgium).

[f] From University Hospital Münster (Münster, Germany).

of *mexE* by semiquantitative PCR and that of *mexA*, *mexX* and *mexC* by real-time PCR, with *rpsL* as a housekeeping gene and using the primers and conditions described in Supplementary Appendix S1.

2.5. Porin expression and mutations

Outer membrane proteins were isolated and visualised by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) after Coomassie Brilliant Blue (Bio-Rad Laboratories, Hercules, CA) staining. Mutations in *oprD* and its promoter region were identified by PCR amplification and sequencing using the previously described primers and conditions, with the wild-type strain *P. aeruginosa* PAO1 used as a comparator [12].

3. Results

PFGE showed two different genotypes among isolates from CF patients (Supplementary Fig. S1). The same pulsotype was observed among all HAP isolates (three distinct patients over a 1-year period), corresponding to an epidemic clone in Belgian hospitals (see [13] and references cited therein). All isolates were multi-drug resistant (MDR; EUCAST criteria; Supplementary Table S1). They showed elevated MICs to meropenem (64–128 mg/L and 128 mg/L in CF and HAP isolates, respectively) and to imipenem, which were however less elevated in CF isolates (MICs of 16–32 mg/L) (Table 1).

Phenotypic and molecular screening tests for the production of carbapenem-hydrolysing enzymes in CF isolates showed neither carbapenemases nor ESBLs but overexpression of AmpC-type cephalosporinases (possibly associated with non-identified ESBLs, evidenced by the NDP test). In contrast, all HAP isolates were VIM-2 producers (known to increase MICs to 16 mg/L and >32 mg/L for meropenem and imipenem, respectively [12]). SDS-PAGE analysis showed no expression of the OprD porin (Supplementary Fig. S2). Sequencing showed no mutation in the promoter region of the gene, but several in the coding region (Table 2).

Ten isolates [the six CF isolates and four isolates (240, 241, 258 and 252) from two different HAP patients] showed nucleotide deletions and missense/nonsense mutations leading to a truncated (most probably inactive) porin of 117–295 amino acids (compared with 443 amino acids in the wild-type porin). Among all CF and HAP isolates, the different amino acid substitutions found in loops L1 (D43N, S57E and S59R), L2 (V127L), L3 (E185Q, P186G and V189T), L4 (E202Q, I210A and E230K), L5 (S240T, N262T and T276A), L6 (A281G, K296Q, Q301E, R310E, G312R and A315G), L7 (L347M) and L8 (S403A and Q424E) were previously described both in carbapenem-resistant and -susceptible *P. aeruginosa* strains [15]. More specifically, the long loops L2 and L3 are important for the binding and/or entrance of imipenem [16]. However, a specific impact of loop-affecting mutations on imipenem activity could not be evidenced here, presumably due to the absence of expression of the truncated porin, the presence of other mutations, the concomitant expression of carbapenemases (HAP isolates only) and/or other carbapenem resistance mechanisms. Mutations tended to accumulate over time in isogenic isolates collected successively from the DAF69 CF patient (namely isolates 128 and 180 vs. isolates 238, 254 and 306).

The three last isolates (228, 218 and 222, originating from the same HAP patient) had a set of 20 amino acid substitutions and a deletion of two amino acid residues in loop 7 (L7-short) leading to the formation of a slightly shortened protein (441 amino acids). Truncation of L7 has been previously reported to confer hypersusceptibility to meropenem without affecting imipenem activity [14] but, again, this effect could not be evidenced here due to the concomitant expression of VIM-2 in these isolates. One new mutation was detected in loop L8 (R412P) of these three HAP clonal isolates, the impact of which on carbapenem susceptibility remains to be established.

Table 1 shows the influence of porin expression on resistance to carbapenems (comparing the MICs of *P. aeruginosa* PAO1, PAO1 Δ*oprD* mutant and the clinical isolates). MICs were determined in control conditions and in the presence of L-Arg or Gly-Glu, considering that L-Arg is a competitor for OprD [8] and Gly-Glu is a competitor for OpdP (another member of the OccD porin family recently described as playing a minor role in carbapenem uptake by *P. aeruginosa* [9]). Deletion of OprD in PAO1 caused a 3 $\log_2$ dilution increase in the MIC of both carbapenems. In *P. aeruginosa* PAO1, the MICs of carbapenems increased 2–4-fold and 2-fold in the presence of L-Arg and Gly-Glu, respectively. However, no effect was observed in PAO1 Δ*oprD* mutant and in clinical isolates. Thus, the alterations in porin expression/permeability observed could not suffice to explain the high-level resistance in CF isolates.

We therefore investigated the expression of efflux pumps in clinical isolates and their impact on carbapenem activity (Table 1). All but one isolate (CF isolate 132-3) showed a significant increase in

Table 2
Molecular characterisation of OprD protein in cystic fibrosis (CF) and hospital-acquired pneumonia (HAP) clinical isolates of *Pseudomonas aeruginosa*.

Strain origin	Identifier (PFGE pattern)[a]	Carbapenem susceptibility/resistance		OprD porin			
		Meropenem	Imipenem	No. of amino acids	Amino acid changes in protein sequence[b]	Loops affected	Insertion/deletion in *oprD* gene
Reference	PAO1 wild-type	S	S	443	–	–	–
Reference	PAO1 Δ*oprD*	S	R	0	–	–	–
CF	128 (P1a), 180 (P1b)	R	R	117	D43N, S57E, S59R, D118STOP[c]	L1	Deletion of 2 nt (312–313)
CF	238 (P1b), 254 (P1a), 306 (P1a)	R	R	295	D43N, S57E, S59R, E202Q, I210A, E230K, S240T, N262T, A267S, A281G, K296STOP[c]	L1, L4, L5, L6	
CF	132-3 (P2)	R	R	228	D43N, S57E, S59R[c]	L1	Deletion of 1 nt (410)
HAP	228 (P3a), 218 (P3a), 222 (P3a)	R	R	441	V127L, E185Q, P186G, V189T, E202Q, I210A, E230K, S240T, N262T, T276A, A281G, K296Q, Q301E, R310E, G312R, A315G, L347M, S403A, R412P, Q424E	L2, L3, L4, L5, L6, L7, L8	Loop 7-short[d]
HAP	240 (P3b), 241 (P3b), 258 (P3b), 252 (P3b)	R	R	276	V127L, E185Q, P186G, V189T, E202Q, I210A, E230K, S240T, N262T, T276A, W277STOP[c]	L2, L3, L4, L5	

PFGE, pulsed-field gel electrophoresis; S, susceptible; R, resistant; nt, nucleotide(s).
[a] See Supplementary Fig. S1.
[b] Substitutions were determined by comparison with the sequence of PAO1 (GenBank accession no. **AE004091**) that encodes a protein of 443 amino acids.
[c] Premature stop codon.
[d] Shortening of putative loop L7 previously reported [14].

the transcripts of *mexA*, and eight of them showed an increase for *mexX* compared with *P. aeruginosa* PAO1. Two CF and two HAP isolates also showed an increase in *mexC* expression levels, but none of the isolates expressed the *mexE* gene (Supplementary Fig. S3).

To assess the involvement of specific pumps in carbapenem efflux, MICs for derivatives of *P. aeruginosa* PAO1 with overexpression or deletion of genes encoding each of the putative candidates were determined. Overexpression of MexAB–OprM caused a 2 $\log_2$ dilution increase in the meropenem MIC, whilst overexpression of each of the other pumps did not affect the MICs. Conversely, disruption of *mexAB* only or of the genes encoding five efflux pumps (*mexAB–oprM*, *mexXY*, *mexEF–oprN*, *mexCD–oprJ* and *mexJK*) reduced the meropenem MIC by 2 $\log_2$ or 3 $\log_2$ dilutions, respectively, designating MexAB–OprM as the main transporter for meropenem [12,17,18]. In contrast, the impact of efflux on imipenem activity was limited (only 1 $\log_2$ dilution reduction in MIC for the mutant deleted for five efflux pumps). Table 1 also shows that the efflux pump inhibitor PAβN reduced the meropenem MICs by 2–4 $\log_2$ dilutions in CF isolates but not in HAP isolates, emphasising a role for this mechanism of resistance in CF isolates. However, reversion of resistance was not complete upon addition of the efflux inhibitor, possibly due to incomplete inhibition of efflux at the concentration used and/or to the co-existence of other resistance mechanisms. PAβN did not modify the MIC of imipenem against clinical isolates.

4. Discussion

This study demonstrates that interplay between MexAB–OprM overexpression and OprD inactivation, combined with overexpression of AmpC-type cephalosporinases, is sufficient to confer high-level resistance to meropenem (MIC ≥ 64 mg/L) in *P. aeruginosa* from CF patients, in contrast to other studies showing an increase of meropenem MICs to ≤32 mg/L under similar conditions [17].

A key role of MexAB–OprM overexpression or chromosomal mutations (leading to both AmpC derepression and membrane impermeability) in high-level carbapenem resistance has already been described in CF isolates [18]. In line with previous work [19], the current data suggest that MexAB–OprM overexpression associated with OprD inactivation is probably sufficient to increase the meropenem MIC to ≥32 mg/L in CF isolates. Contrary to these authors, however, we found that imipenem was more active than meropenem against CF isolates, which is unusual [17] but has been described previously [15] for strains that do not express OprD. As meropenem MICs are brought back below those of imipenem in the presence of PAβN, we suggest that active efflux is the main reason for high-level resistance to meropenem in CF isolates. Building on earlier studies [19], the current work also identifies specific *oprD* mutations, including one that had not been previously described, and shows that these mutations can accumulate over the course of a chronic infection in isogenic isolates from the same patient, explaining an increase in resistance. This, moreover, underlines the change in expression of porins as a contributing factor for carbapenem resistance in Gram-negative bacteria [20].

These results confirm that high-level resistance to carbapenems in *P. aeruginosa* cannot be blindly ascribed to carbapenemase production, in particular among CF isolates in which active efflux appears to play an underestimated role. Concomitant susceptibility testing of both meropenem and imipenem is warranted and appropriate phenotypic tests may help in delineating the most probable mechanisms involved in carbapenem resistance.

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Competing interests: None declared.

Ethical approval: Not required.

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2016.09.012.

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Supplementary material

High-level resistance to meropenem in clinical isolates of *Pseudomonas aeruginosa* in the absence of carbapenemases: role of active efflux and porin alterations

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Appendix S1. Primers and conditions used for semi-quantitative and real-time PCR

a) Primers

1. Efflux systems
   - *mexE*-Fwd: 5'-CGACAACGCCAAGGGCGAGTTCACC-3'
   - *mexE*-Rev: 5'-CCAGGACCAGCACGAACTTCTTGC-3'
   - *mexA*-Fwd: 5'-CGACCAGGCCGTGAGCAAGCAGC-3'
   - *mexA*-Rev: 5'-GCGACAACGCGGCGAAGGTCTCC-3'
   - *mexX*-Fwd: 5'-TGAAGGCGGCCCTGGACATCAGC-3'
   - *mexX*-Rev: 5'-CGCTGACCCGCGTCGAGCAGATC-3'
   - *mexC*-Fwd: 5'-GTACCGGCGTCATGCAGGGTTC-3'
   - *mexC*-Rev: 5'-TTACTGTTGCGGCGCAGGTGACT-3'

2. Housekeeping genes
   - *rpsL*-Fwd: 5'-CGGCACTGCGTAAGGTATGC-3'
   - *rpsL*-Rev: 5'-AGCAGGGTCGTTCGAAGTACG-3'

b) PCR conditions

Three min of cDNA denaturation at 95 °C, followed by 40 cycles of 10 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. A melt curve was run at the end of the real-time PCR cycles, to check for the presence of a unique PCR reaction product.

Table S1: MIC of strains under study to other antibiotic classes

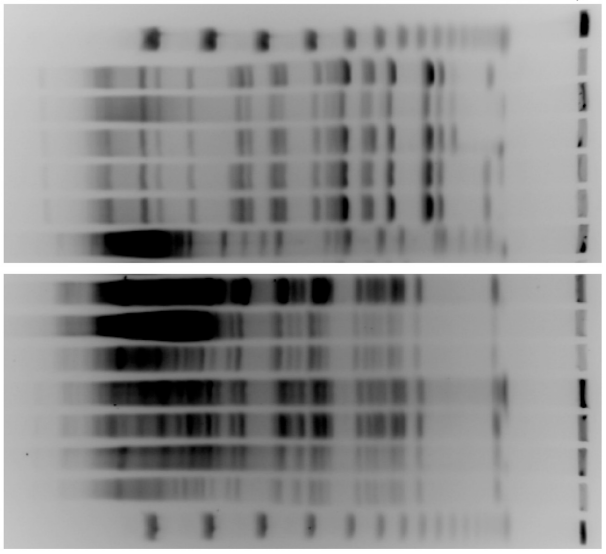
Reference strains and clinical isolates (with patient identification and date of collection)	MIC (mg/L) [a]							
	TIC	**PIP**	**TZP**	**CAZ**	**CIP**	**AMK**	**TOB**	**CST**
PAO1 Wild type	32	4	4	2	0.5	4	1	4
PAO1 Δ*oprD2*	32	4	4	2	0.25	4	1	2
PAO1 Δ*mexAB*::FRT	2	0.5	0.5	1	0.12	1	1	4
PAO1 Δ*mexAB-oprM*, *mexXY*, *mexEF-oprN*, *mexCD-oprJ*, *MexJK*	2	0.5	0.5	1	0.12	1	0.5	2
PAO1 Δ*mexXY*	8	2	2	1	1	2	1	2
PAO1 MexAB-OprM overproducer	128	8	16	4	0.5	4	0.5	2
PAO1 MexXY-OprM overproducer	32	2	2	2	1	16	2	2
PAO1 MexEF-OprM overproducer	32	0.5	2	1	2	2	0.5	1
PAO1 MexCD-OprM overproducer	32	2	4	4	0.5	4	1	2
128-CF (DAF69 - 09/09/10) [b]	1024	1024	1024	1024	8	64	4	1
180-CF (DAF69 – 04/10/10) [b]	1024	1024	1024	1024	4	64	4	0.5
238-CF (DAF69 – 19/10/10) [b]	1024	1024	1024	1024	16	256	16	2
254-CF (DAF69 – 26/10/10) [b]	512	1024	1024	256	16	256	16	2
306-CF (DAF69 – 09/11/10) [b]	1024	1024	1024	512	16	64	4	2
132-3-CF (132 - 08/07/12) [c]	128	64	32	16	2	64	4	2
198-3-CF (198 - 04/08/12) [c]	1024	256	256	1024	2	32	2	0.5
218-HAP (DS – 26/01/06) [b]	1024	256	256	128	32	128	32	1
222-HAP (DS – 13/02/06) [b]	1024	256	256	128	32	128	32	1
228-HAP (DS – 26/12/05) [b]	1024	64	64	64	32	128	32	1
240-HAP (OG - 08/04/06) [b]	1024	64	64	64	32	128	32	1
241-HAP (OG – 02/05/06) [b]	1024	256	256	128	32	128	32	1
252-HAP (ND – 11/09/06) [b]	1024	128	128	128	32	128	32	1
258-HAP (ND – 10/08/06) [b]	1024	128	128	128	32	128	32	1

[a] EUCAST breakpoints (NA: not applicable [no I category]): **TIC** (ticarcillin) S≤16 R>16; **PIP** (piperacillin) S≤16 R>16; **TZP** (piperacillin-tazobactam) S≤16 R>16; **CAZ** (ceftazidime) S≤8 R>8; **CIP** (ciprofloxacin) S≤ 0.5 R>1; **AMK** (amikacin) S≤ 8 R>16; **TOB** (tobramycin) S≤ 4 R>4; **CST** (colistin) S≤ 4 R>4.

[b] from Erasme hospital, Brussels, Belgium

[c] from University Hospital Münster, Münster, Germany

Figure S1: Pulsed-field gel electrophoresis (PFGE) of SpeI-digested genomic DNA from *P. aeruginosa* isolates. P1-P3 correspond to different PFGE patterns; P1a and P1b as well as P3a and P3b are closely related.



Isolates	PFGE Pattern
PFGE marker	
128-CF	P1a
180-CF	P1b
238-CF	P1b
254-CF	P1a
306-CF	P1a
132-3-CF	P2
228-HAP	P3a
218-HAP	P3a
222-HAP	P3a
240-HAP	P3b
241-HAP	P3b
258-HAP	P3b
252-HAP	P3b
PFGE marker	

Figure S2: Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using Coomassie Brilliant Blue staining of the outer membrane protein OprD extracted from *P. aeruginosa* CF and HAP isolates. The WT strain PAO1 and its OprD mutant were included as positive and negative control strains. Gel1: CF-isolates; loaded as follows: lane1: protein Ladder; lane 2: PAO1; lane 3: PAO1-ΔOprD; lane 4: 128-CF; lane 5: 180-CF; lane 6: 238-CF; lane 7: 254-CF; lane 8: 306-CF; lane 9: 132-3-CF. Gel 2: HAP-isolates; loaded as follows: lane1: protein Ladder; lane 2: PAO1; lane 3: PAO1-ΔOprD; lane 4: 228-HAP; lane 5: 218-HAP; lane 6: 222-HAP; lane 7: 240-HAP; lane 8: 241-HAP; lane 9: 258-HAP; lane 10: 252-HAP.

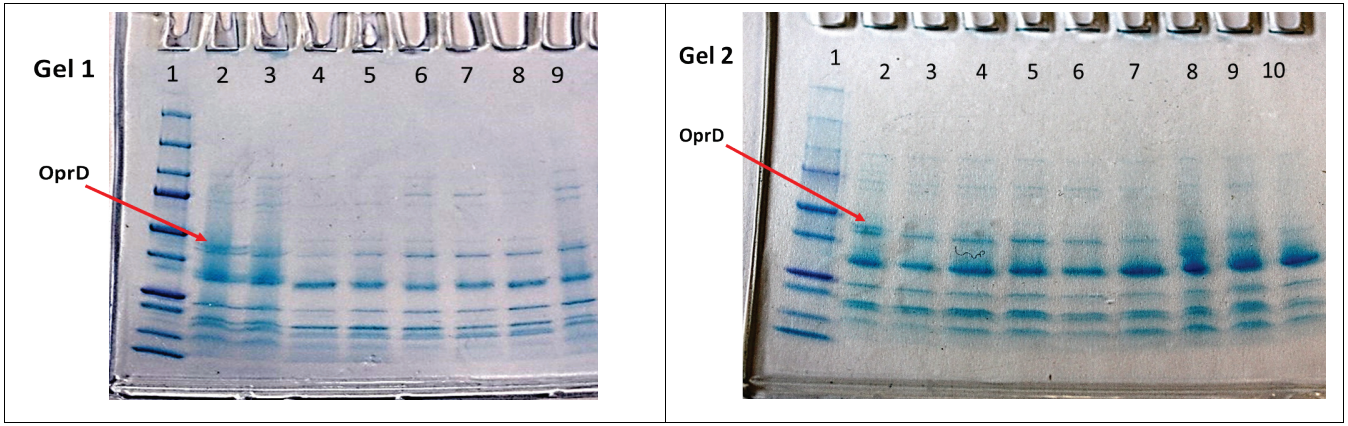
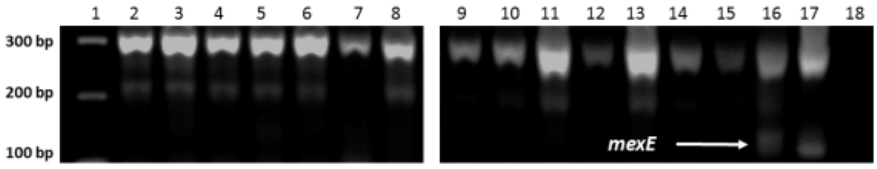


Figure S3: Detection of *mexE* expression by semi-quantitative RT-PCR. The figure shows the DNA-stained gels of RT-PCR products over CF and HAP isolates. The inducible target gene (*mexE*, 137 bp) is amplified together with the constitutive *mexA* gene (316 bp) used as internal positive control. Samples are loaded as follow: lane1: DNA ladder ; lane 2: PAO1 (negative control non-expressing *mexE* gene); lane 3: 128-CF; lane 4: 180-CF; lane 5: 238-CF; lane 6: 254-CF; lane 7: 306-CF; lane 8: 132-3-CF; lane 9: 228-HAP; lane 10: 218-HAP; lane 11: 222-HAP; lane 12: 240-HAP; lane 13: 241-HAP; lane 14: 258-HAP; lane 15: 252-HAP; lane 16: positive control with strain overexpressing *mexE* gene; lane 17: DNA template (positive control); lane 18: non template control.



iii. Resistance mechanisms to β-lactam antibiotics: the case of the newly approved combination ceftazidime-avibactam

As evidenced in the two first articles (section 2.2.i and 2.2.ii), a high proportion of the collection was resistant to ceftazidime. Ceftazidime-avibactam is a new approved drug that combines ceftazidime with avibactam, a broad spectrum non-β-lactam β‑lactamase inhibitor (BLI).

Before its approval by FDA in 2015 and by EMA in 2016, avibactam had not been studied in the context of PA lung infection in CF patients. In addition, the combination of resistance mechanisms like active efflux, OprD mutations and the high prevalence of AmpC derepressed isolates in our collection offers a specific context of clinical interest for testing the activity of avibactam as a new BLI.

In this paper, our aim was to compare the activity of ceftazidime alone or combined with avibactam against CF isolates and to determine the threshold level of ceftazidime resistance above which the addition of avibactam proved ineffective to restore ceftazidime activity.

This study suggests that combining avibactam with ceftazidime may offer a clear interest against 40% of the collection. Yet, part of the collection remains insensitive to this combination. In this context, the summary of product [236] and recent studies [237;238], suggest that ceftazidime-avibactam may not have activity against Gram-negative bacteria that overexpress efflux pumps or have porin mutations. We therefore started to investigate the impact of these mechanisms on resistance to avibactam in the collection.

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Avibactam confers susceptibility to a large proportion of ceftazidime-resistant *Pseudomonas aeruginosa* isolates recovered from cystic fibrosis patients

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Keywords: carbapenemases, extended-spectrum β-lactamases, ESBLs, β-lactamase inhibition, *P. aeruginosa*

Sir,

Pseudomonas aeruginosa is the predominant bacterial pathogen in cystic fibrosis (CF) patients and is associated with decline in pulmonary function.[1] Due to the chronic persistent nature of infections, CF patients receive frequent antibiotic courses for eradication of potential pathogens, treatment of acute infective exacerbations and as chronic suppressive therapy. Consequently, resistance to antipseudomonal β-lactams is common in the strains collected from CF patients,[2,3] narrowing therapeutic options. Clinicians are therefore forced to use aminoglycosides or polymyxins, increasing the risk of adverse effects.[4,5] Therefore, optimizing the activity of β-lactams may help to alleviate this burden. Ceftazidime is a well-established cephalosporin (on the WHO List of Essential Medicines) with an excellent safety profile and an antibacterial spectrum that includes *P. aeruginosa*. However, ceftazidime is degraded by many β-lactamases, including ESBLs (Ambler classes A and D), cephalosporinases (Ambler class C) and carbapenemases. Avibactam (formerly NXL-104) is a novel non-β-lactam, broad-spectrum β-lactamase inhibitor, with promising inhibitory activity against Ambler class A (including ESBLs and *Klebsiella pneumoniae* carbapenemases), C and D β-lactamases.[6] Combined with ceftazidime, it is currently in Phase III clinical trials for the treatment of complicated intra-abdominal infections, urinary tract infections and healthcare-associated pneumonia (http://clinicaltrials.gov identifiers NCT01499290, NCT01500239, NCT01726023, NCT01644643, NCT01595438 and NCT01808092). In *P. aeruginosa* from non-CF patients, avibactam has been shown to reverse ceftazidime resistance, bringing MICs to values lower than the EUCAST and CLSI breakpoints.[7,8] However, very little is known about the effect of avibactam on ceftazidime activity in *P. aeruginosa* isolated from CF patients.[9] We therefore assembled a collection of 334 non-duplicate *P. aeruginosa* isolates from 156 patients with a clinically confirmed diagnosis of CF equally distributed between four European countries with a predominance of recent isolates [Belgium (2010), France (1996–2012), Germany (2012) and the UK (2006–09)] and used them to assess the activity of ceftazidime alone or combined with avibactam. MICs were determined by microdilution in cation-adjusted Mueller–Hinton broth following the CLSI methodology for ceftazidime alone (procured as Glazidim®, the commercial product registered in Belgium for parenteral use; potency, 88.2%; GlaxoSmithKline; Genval, Belgium) and combined with 4 mg/L avibactam (NXL-104, potency 91.7%, batch number AFCH005151; AstraZeneca Pharmaceuticals, Waltham, MA, USA). *P. aeruginosa* ATCC 27853 (fully susceptible) and *K. pneumoniae* ATCC 700603 (resistant to ceftazidime by the production of SHV-18 β-lactamase) were used as quality controls. Correlations between MICs of ceftazidime and ceftazidime/avibactam for individual strains were assessed using quantile density contour analysis (JMP® version 10.0.2, SAS Institute Inc., Cary, NC, USA). Figure 1(a) shows that isolates in this collection had a high MIC_{90} of ceftazidime (512 mg/L), with only 36% being clinically susceptible (MIC ≤8 mg/L) according to EUCAST or CLSI interpretive criteria. When combined with avibactam, the proportion of susceptible strains increased to 76% and the MIC_{90} decreased to 64 mg/L. Figure 1(b) shows the fold reduction in MIC observed in the presence of avibactam for these isolates classified according to the MIC of ceftazidime. While the mean reduction in MIC observed for the whole collection was 2.6 dilutions, the amplitude of the effect was clearly dependent on the initial ceftazidime MIC. Thus, when combined with avibactam, the MIC of ceftazidime decreased by 0.6 dilutions for each doubling of ceftazidime MIC in the 1–128 mg/L range (0.6 is the slope value of a linear regression relating the $\log_2$ MIC of the combination to the $\log_2$ MIC of ceftazidime in that range; $R^2 = 0.965$), which would decrease the MIC to 8–16 mg/L, irrespective of the ceftazidime MIC in that range of concentrations. For more-resistant strains, the amplitude of the avibactam effect plateaued at a reduction of ∼4 dilutions in MIC for strains for which the ceftazidime MIC was ∼256 mg/L and decreased to a reduction of 3 dilutions for isolates for which the MICs were still higher. This shift in MIC is illustrated for individual strains in Figure 1(c), which shows the correlation between MICs of individual isolates for ceftazidime alone and ceftazidime combined with avibactam. Susceptibility to ceftazidime was restored in 40% of the strains, with avibactam proving more effective for strains for which the MIC was <256 mg/L. In accordance with the conclusion drawn from Figure 1(c), the ceftazidime MIC was now only 4–8 mg/L for most of the affected

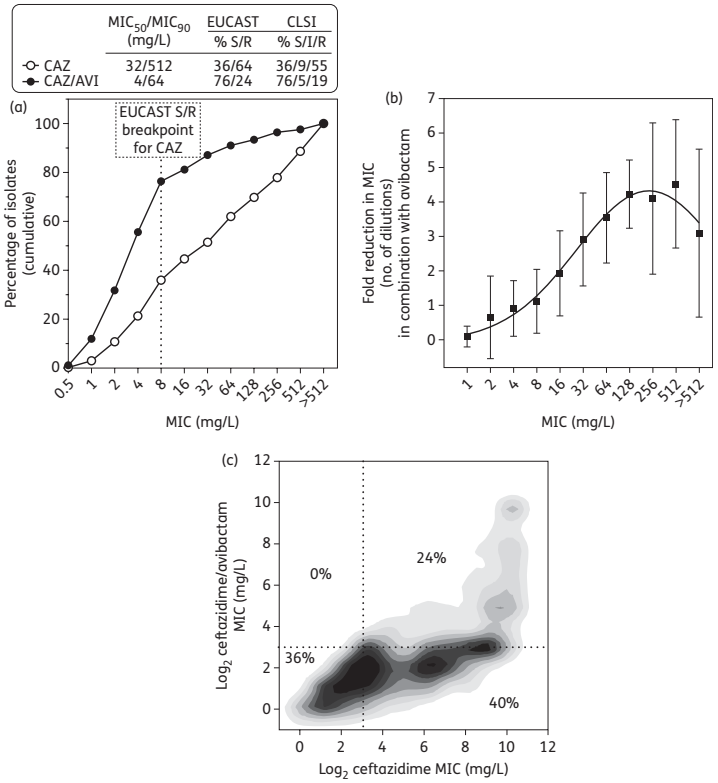

Figure 1. Effect of avibactam (4 mg/L) on the activity of ceftazidime against 334 isolates of *P. aeruginosa* collected from CF patients. (a) Cumulative MIC distribution with indication of MIC_{50}, MIC_{90} and percentage susceptibility according to the interpretive criteria of EUCAST (susceptible, ≤8 mg/L; resistant, >8 mg/L) and CLSI (susceptible, ≤8 mg/L; resistant, ≥32 mg/L). The broken line indicates the limit between susceptible and resistant strains according to EUCAST. (b) Reduction in the MIC (±SD) of ceftazidime (expressed as the number of dilutions) when combined with avibactam as a function of the ceftazidime MIC. The data were used to fit a log Gaussian equation (R^2=0.979) allowing us to calculate that the maximum amplitude of change (number of dilutions, 4.3±0.14) occurred at an MIC of 229±29 mg/L. (c) Correlation between MICs of ceftazidime alone (abcissa) and ceftazidime/avibactam (ordinate) for each individual strain in the collection using quantile density contour analysis. The intensity of each zone (from deep black to light grey) is indicative of the proportion of strains (from large to small) with MICs at the corresponding coordinates. The broken lines point to the MIC value above which the isolates are considered resistant strains according to EUCAST interpretive criteria for ceftazidime and the figures indicate the percentage of strains in each quadrant. AVI, avibactam; CAZ, ceftazidime; S, susceptible; I, intermediate; R, resistant.

strains, a value that is below the EUCAST and CLSI susceptibility breakpoints, extending to CF *P. aeruginosa* isolates the conclusions obtained for pseudomonal isolates of other origins[7] and for other Gram-negative bacteria.[8]

Taken together, these data highlight the potential utility of combining ceftazidime with avibactam for the treatment of *P. aeruginosa* infections, including in clinical situations where resistance rates are high. It also shows that a concentration of 4 mg/L is sufficient to bring into the susceptible range those *P. aeruginosa* strains with a ceftazidime MIC ≤256 mg/L.

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Transparency declarations

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❖ **Supplementary data related to ceftazidime/avibactam article**
(Prepared for submission as a correspondence to *the Journal of Antimicrobial Chemotherapy*)

Poor membrane permeability impedes avibactam activity in *Pseudomonas aeruginosa*

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Running tittle: Avibactam and resistance mechanisms in *P. aeruginosa*
Keywords (not in the title): RND efflux systems, OprD, cystic fibrosis

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Sir,

Avibactam is a broad-spectrum inhibitor of β-lactamases, recently registered in the USA in combination with ceftazidime for the treatment of complicated intra-abdominal or urinary infections caused by susceptible microorganisms [1]. Yet, it could be useful in other infections involving β-lactamase-producing Gram-negative pathogens. We previously showed that avibactam at 4 mg/L restored the activity of ceftazidime in 40 % of *Pseudomonas aeruginosa* isolates from cystic fibrosis (CF) patients [2], while 24 % of the collection still showed a MIC higher than the susceptibility breakpoint of ceftazidime (EUCAST: S ≤ 8 mg/L; R > 8 mg/L; CLSI: S ≤ 8 mg/L; R > 16 mg/L). These isolates were however negative for the production of carbapenemases or extended spectrum β-lactamases, but overexpressed AmpC-type cephalosporinases [3]. Poor drug accumulation due to porin alterations and/or active efflux (mainly mediated by MexAB-OprM) is another reason for β-lactam resistance in *P. aeruginosa*.

In this work, we investigated the possible mechanisms explaining resistance to ceftazidime-avibactam in these isolates. Twelve *P. aeuginosa* isolates recovered from 11 CF patients in UK, Belgium and Germany and showing ceftazidime MIC ≥ 128 mg/L were selected from the whole collection [2]. These isolates were previously characterized with respect to their genetic relatedness (multilocus sequence typing [MLST] and/or pulsed-field gel electrophoresis [PFGE]) [4], the rate of efflux of the fluorescent MexAB-OprM preferential substrate N-phenyl-1-naphthylamine (NPN) [5], and the sequence of *mexA* and *mexB* genes [5]. PAO1 and derivatives thereof overproducing or defective in the expression of efflux pumps or of the OprD porin were included as controls. MICs were determined by microdilution in CA-MHB according to CLSI criteria for ceftazidime alone (Glazidim®, potency, 88.2%; Glaxo-SmithKline; Genval, Belgium) or combined with avibactam (potency 91.7%; AstraZeneca Pharmaceuticals; Waltham, MA). Imipenem [0.25 mg/L] was added to the medium as an inducer of the expression of chromosomal AmpC cephalosporinases in control strains in order to evidence the effect of avibactam on MICs.

Examining first MICs for these induced control strains (table 1), avibactam was less effective to restore ceftazidime activity in PAO1 derivatives overexpressing MexAB-OprM or MexEF-OprN (to a slightly lower extend) or in the OprD-deficient mutant than in the parental strain, indicating a role of these mechanisms in resistance to avibactam. Interestingly, we previously showed that a large proportion of isolates collected from CF patients harbour natural mutations in the *mexA* or *mexB* genes, which impaired the efflux activity of the MexAB-OprM efflux system [5]. We therefore wondered whether these mutations could also affect avibactam activity. Twelve CF isolates with MIC ranging from 128 to 1024 mg/L for ceftazidime were studied. Among them, 6 isolates (CF53, 143-1, 135-1, CF15, BV1 and CF16) regained full susceptibility to ceftazidime (MIC ≤ 8 mg/L) in the presence of avibactam. They all present a low efflux capacity for NPN together with severe deletions in *mexA* or *mexB* presumably explaining the lack of activity of the efflux pump [5]. Three other isolates (CF19, 191-4, 207) showed an intermediate susceptibility to ceftazidime (MICs of 16 or 32 mg/L) in the presence of avibactam, but still no efflux activity attributed to severe deletions in *mexA*. Because these isolates harbour more elevated ceftazidime MICs in control conditions than the six previous ones, we assume that the partial restoration of activity observed in the presence of avibactam is due to a higher expression level of AmpC-type cephalosporinases. The last three isolates (208-2, 208-3, 128) remained resistant to ceftazidime in the presence of avibactam. They show less severe mutations (substitutions of up to 30 amino-acids) in MexB and higher NPN efflux rate than the other isolates. Interestingly, the effect of avibactam on MIC is inversely correlated with NPN efflux rate in these strains (viz. the fold-reduction in MICs vs. Vmax values). This strongly suggests that MexAB-OprM-mediated active efflux contribute to reduce the activity of avibactam in these isolates beyond the overexpression of AmpC-type cephalosporinase.

Taken together, our data show that the capacity of avibactam to restore ceftazidime activity depends not only of the level of expression of AmpC-type cephalosporinase, but also of the permeability of the OprD porin together with the activity of MexAB-OprM and MexEF-OprJ efflux pumps.* The

Summary of Product Characteristics of ceftazidime-avibactam mentions that '*AVYCAZ is not active against bacteria that produce metallo-β-lactamases and may not have activity against Gram-negative bacteria that overexpress efflux pumps or have porin mutations*' [1]. Our work adds to this statement (a) the identification of the main efflux transporters for avibactam in *P. aeruginosa*, and (b) the demonstration of the existence of some isolates from cystic fibrosis patients that remain susceptible to avibactam because of inactive efflux. These may represent as much as 30% of the isolates [5]. We also showed that meropenem resistance in these isolates is due to the same combination of resistance mechanisms (OprD mutations and MexAB-OprM mediated efflux in AmpC overexpressers) [3]. Avibactam-ceftazidime may therefore represent a carbapenem-sparing alternative for meropenem-susceptible strains in these patients.

* Ongoing studies are evaluating *ampC, mexA, and mexE* expression levels by real time PCR and OprD expression, by SDS page. These data will be added before submission of the paper.

Number of experimental replicates. The determination of MICs by broth microdilution for ceftazidime in the presence or absence of avibactam was performed 1 to 3 times.

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Table 1: description of bacterial isolates, MICs, and efflux characteristics

Control strains	description	MIC (mg/L)[a,b]			MexAB-OprM efflux system characteristics		
					mexA or *mexB* mutations		
		CAZ[c]	CAZ/AVI[c]	Fold reduction	Gene	Protein (AA[c] length)	Vmax (unit/s)[d]
PAO1	reference strain, ATCC	32	0.125	8	NA[e]	NA	**-0.48**
PAO1 mexAB [6]	PAO1 MexAB-OprM defective mutant	32	0.125	8	NA	NA	-0.12
PT629 [7]	PAO1 overproducing MexAB-OprM	32	**4**	3	NA	NA	-0.72
MutGr1 [6]	PAO1 overproducing MexXY-OprM	32	0.125	8	NA	NA	
PAO-7H [7]	PAO1 overproducing MexEF-OprN	32	**2**	4	NA	NA	
ERYR [8]	PAO1 overproducing MexCD-OprJ	32	0.125	8	NA	NA	
CC-03 [9]	PAOD1 OprD-defective mutant	32	**4**	3	NA	NA	

Isolates	Patient's code, origin or ref.	Clonality (PFGE / MLST)	MIC (mg/L)[b]			MexAB-OprM efflux system characteristics *mexA* or *mexB* mutations		
			CAZ	CAZ/AVI	Fold reduction	Gene	Protein (AA[c] length)	Vmax (unit/s)
CF53	DP, UK	CA / ST146 (MDR LES clone)[c]	256	4	6	*mexA*: C360G nonsense mutation	Truncated MexA (119)	-0.02
143-1	143, DE	WI	128	8	4	*mexA*: Δ 2 nt (837-838) nonstop mutation	MexA (0) [10]	-0.02
135-1	135, DE	H / ST2254 (new ST)	128	8	4	*mexB*: G1261_C1262insG + Δ 2 nt (1947-1948)	Truncated MexB (719)	-0.10
CF15	CT, UK	CA / ST146	256	8	5	*mexA*: C360G nonsense mutation	Truncated MexA (119)	-0.02

BV1	DC, UK	CA / ST146	512	8	6	*mexB*: Δ 8 nt (1555-1562) nonsense mutation	Truncated MexB (672)	-0.08
CF16	RC, UK	CA / ST146	512	8	6	*mexB*: Δ 154 nt (85-239) nonsense mutation	Truncated MexB (30)	-0.12
CF19	LS, UK	CA / ST146	512	**16**	5	*mexA*: C360G nonsense mutation	Truncated MexA (119)	-0.02
191-4	191, DE	CK	1024	**16**	6	*mexA*: G82T nonsense mutation	Truncated MexA (27)	-0.01
207	207, DE	CK	512	**32**	4	*mexA*: G82T nonsense mutation	Truncated MexA (27)	-0.01
208-2	208, DE	H / ST2254	256	**64**	2	*mexB*: Δ 1 nt (T1889) + Δ 2 nt (1947-1948)	19 AA substitutions in MexB (1045)	**-0.25**
208-3	208, DE	H / ST2254	1024	**128**	3	*mexB*: Δ 1 nt (T1854) + Δ 2 nt (1947-1948)	30 AA substitutions in MexB (1045)	**-0.32**
128	DAF69, BE	YY / ST958 (MDR clonal complex ST111)	1024	**512**	1	*mexB*: conservative missense mutation C1126G	1 AA substitution L376V in MexB (1046)	**-0.58**

[a] for control strains, MICs were measured in the presence of 0.25 mg/L imipenem (sub-MIC concentration) in order to induce *ampC* expression.
[b] Values in bold denote MICs that remain high (> that measured for PAO1 in control strains or > EUCAST susceptibility breakpoint [8 mg/L]for clinical isolates)
in the presence of 4 mg/L avibactam
[c] Abreviations: CAZ: ceftazidime; AVI: avibactam at 4 mg/L; AA, Amino Acids; LES, Liverpool Epidemic Strain; MDR, Multi-Drug Resistant
[d] kinetics of efflux of NPN (fluorescence arbitrary units)/s [5]
[e] not applicable

2.3 Susceptibility and resistance to temocillin in *P. aeruginosa* isolates from cystic fibrosis patients

In the previous chapters, we showed that resistance to broad spectrum β-lactams like carbapenems or even to the newly introduced avibactam-ceftazidime combination does exist in CF isolates. Alternative therapeutic options are thus warranted. Reviving old antibiotics is a clear opportunity in this context.

Based on the preliminary observation that temocillin may recover activity against part of the isolates collected in CF patients [178], we examined here its activity against a large collection of CF isolates and determined the mechanisms explaining acquired susceptibility.

We found that approximately 30% of the collection had clinically-exploitable MICs for temocillin. In this chapter, we describe the mutations in *mexAB-oprM* genes explaining this regain of activity, classify them according to the resulting MIC and correlate them with a measure of the efflux capacity of each isolate in real time. We show in parallel that temocillin is not a substrate of the same porin as carbapenems, yet it uses two redundant anion-specific porins. This may represent an advantage by avoiding cross-resistance with carbapenems and making more difficult the selection of resistance by porin mutations. Taken together, our data lead to the conclusion that temocillin may represent a reasonable alternative for the treatment of PA infections in CF patients and thus should be included in routine testing.

Number of experimental replicates. The determination of MICs by broth microdilution for temocillin (in the presence of absence of different substrates for efflux systems or porins) and other antimicrobials against the whole collection of PA strains or *E. coli* (overexpressing porins) was performed 2-4 times and 1-4 times, respectively. Moreover, control strains overexpressing AmpC

cephalosporinases, ESBL, efflux systems (MexAB-OprM, MexXY-OprM, MexEF-OprN and MexCD-OprJ) were used as internal controls to ensure the reliability and validity of different techniques (broth microdilution, efflux assay). For the sequencing part of mexA and mexB genes, some isolates were sequenced two times in the forward and reverse directions of each gene. The quantification of extracellular polymeric saccharides (EPS) was performed one time with 3 to 6 measurements for each isolate.

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Mechanisms of intrinsic resistance and acquired susceptibility of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients to temocillin, a revived antibiotic

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Abstract

The β-lactam antibiotic temocillin (6-α-methoxy-ticarcillin) shows stability to most extended spectrum β-lactamases, but is considered inactive against *Pseudomonas aeruginosa*. Mutations in the MexAB-OprM efflux system, naturally occurring in cystic fibrosis (CF) isolates, have been previously shown to reverse this intrinsic resistance. In the present study, we measured temocillin activity in a large collection (n=333) of *P. aeruginosa* CF isolates. 29% of the isolates had MICs ≤ 16 mg/L (proposed clinical breakpoint for temocillin). Mutations were observed in *mexA* or *mexB* in isolates for which temocillin MIC was ≤ 512 mg/L (nucleotide insertions or deletions, premature termination, tandem repeat, nonstop, and missense mutations). A correlation was observed between temocillin MICs and efflux rate of *N*-phenyl-1-naphthylamine (MexAB-OprM fluorescent substrate) and extracellular exopolysaccharide abundance (contributing to a mucoid phenotype). OpdK or OpdF anion-specific porins expression decreased temocillin MIC by ~1 two-fold dilution only. Contrarily to the common assumption that temocillin is inactive on *P. aeruginosa,* we show here clinically-exploitable MICs on a non-negligible proportion of CF isolates, explained by a wide diversity of mutations in *mexA* and/or *mexB*. In a broader context, this work contributes to increase our understanding of MexAB-OprM functionality and help delineating how antibiotics interact with MexA and MexB.

Introduction

Pseudomonas aeruginosa is the most prevalent pathogen isolated in the respiratory tract of adult cystic fibrosis (CF) patients and a major cause of morbidity and mortality in this population[1]. These patients are frequently exposed to antipseudomonal antibiotics and, as a result, become colonized by multidrug-resistant strains. Since therapeutic choices narrow, clinicians are increasingly forced to look after "forgotten" antibiotics against which resistance rates could be low because they were scarcely used[2].

Temocillin (6-α-methoxy-ticarcillin) is one of these old antibiotics recently revived, based on a renewed interest for its activity against many β-lactam-resistant *Enterobacteriaceae*[3]. Temocillin, indeed, shows remarkable stability to most β-lactamases, including AmpC-type cephalosporinases and extended-spectrum β-lactamases (ESBLs) such as TEM, SHV, and CTX-M enzymes[4]. Temocillin also obtained an orphan drug designation for the treatment of *Burkholderia cepacia* complex infection in CF patients[5]. However, as temocillin was long considered as intrinsically inactive against *P. aeruginosa,* it was not included in conventional susceptibility testing of this organism when isolated from CF patients. Previous work from our laboratory actually showed that the intrinsic resistance of *P. aeruginosa* to temocillin was due to active efflux by the constitutively-expressed MexAB-OprM efflux transporter, while the other major efflux transporters were not involved[6]. Moreover, some isolates from CF patients regained susceptibility to temocillin because of natural mutations in the proteins constituting the MexAB-OprM efflux pump[6]. MexAB-OprM belongs to the Resistance Nodulation Division superfamily of efflux transporters. It is energized by proton motive force and consists of three proteins, namely an inner membrane exporter (homotrimer of MexB), an outer membrane gated channel (homotrimer of OprM), and a periplasmic linker (6 to 13-mer of MexA), the concerted action of which allows for the extrusion of substrates from the inner membrane or the periplasmic space directly out of the bacteria ([7] for review).

The role of porins in the capacity of temocillin to cross the outer membrane is unknown so far. Among porins described in *P. aeruginosa,* the cation-selective channel OprD (OccD1) is involved in the entry of carbapenems (most notably, imipenem), while the anion-specific channels OpdK (OccK1) and OpdF (OccK2) are involved in the entry of carboxypenicillins like carbenicillin and of cefoxitin[8]. In this context, the objective of this study was to better document the mechanisms of intrinsic resistance and acquired susceptibility to temocillin in *P. aeruginosa* isolated from CF patients. We exploited a large collection of 333 isolates collected from 4 CF centres in Northern Europe, which has been partly characterized for its resistance to commonly used anti-pseudomonas agents[9]. We demonstrate that a non-negligible proportion of these isolates (~15-30%) showed low and clinically-exploitable MICs to temocillin, associated with a wide variety of mutations in *mexA* or *mexB* genes. No β-lactamase hydrolysing temocillin was found in the collection. A marginal role of anion-specific porins (OpdK and OpdF) in temocillin influx was also demonstrated. Besides its immediate interest for the management of infections in CF patients, this work also brings innovative pieces of information regarding mutations affecting the transport activity of the MexAB-OprM efflux system in *P. aeruginosa*, which can refine our understanding of the mechanism of substrate recognition and transport by this pump and may also help in reviving other antibiotics that are substrates of the same transporter.

Materials and methods

***P. aeruginosa* isolates.** A total of 333 isolates were randomly collected from 155 patients in four CF centres from different countries (*Hôpital des enfants malades Reine Fabiola/Erasme*; Belgium, n=88; *Hôpital Jean Minjoz*, Besançon, France; n=80; University Hospital of Münster, n=66, Germany; Queen's University of Belfast, UK, n=99) during routine periodic visits (Table S1). PAO1, mutants thereof overexpressing or deleted for MexAB-OprM (Table 1), and

PA14 were used as control strains. The molecular typing of part of this collection has been previously performed by pulse field gel electrophoresis, focusing on isolates collected simultaneously from the same patients but differing in their susceptibility profile[9]. It was completed here for selected sequenced isolates following the same procedure.

Antibiotics. The following antibiotics were obtained as microbiological standard from Sigma-Aldrich, St Louis, MO (sodium salts, potency in brackets): ticarcillin (TIC; 85.25%), piperacillin (PIP; 94.20%), carbenicillin (CAR; 89.16%), and cefoxitin (FOX; potency, 95.11%). The remaining antibiotics were obtained as powder for parenteral use from their corresponding manufacturers (potency in brackets) : temocillin (TMO, 78.12%) as Negaban® from Eumedica (Manage, Belgium), piperacillin-tazobactam (TZP; 97.00%) as Tazocin® from Wyeth (Louvain-La-Neuve, Belgium), ceftazidime (CAZ, 88.20%) as Glazidim® from Glaxo-SmithKline (Genval, Belgium), imipenem (IPM, 45.60% [due to the presence of cilastatin in the powder]) as Tienam® from MSD (Brussels, Belgium), and meropenem (MEM, 74.00%) as Meronem® from AstraZeneca (Brussels, Belgium).

Susceptibility testing. MICs were determined by broth microdilution following the guidelines from the Clinical and Laboratory Standards Institute and using *P. aeruginosa* ATCC27853 as a quality control. Susceptibility was assessed according to the interpretative criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) or using a cut-off value of 16 mg/L for temocillin (no EUCAST breakpoint) by analogy with the current susceptibility breakpoint of its parent compound ticarcillin. For specific isolates, MICs were also measured in the presence of 20 mg/L Phe-Arg-β-naphthylamide dihydrochloride (PAβN, broad-spectrum efflux pump inhibitor[10]; Sigma-Aldrich) and of 1 mM $MgSO_4$ (to prevent the outer membrane permeabilization caused by PAβN[33]) or in the presence of substrates of *P. aeruginosa* porins, namely vanillate (VNL, OpdK substrate[18]), or L-arginine (L-Arg, OprD substrate[19]), both obtained from Sigma-Aldrich.

β-lactamases screening and identification. Isolates showing MICs > 8 mg/L for both ceftazidime and meropenem (n=53) were screened for the presence of genes encoding acquired metallo-β-lactamases (VIM, IMP, NDM), carbapenemases (OXA-48, KPC), or widespread extended-spectrum β-lactamases (ESBLs) by PCR, as previously described[9]. The expression of carbapenemase(s) was also screened using a phenotypic method described as the Carbapenemase Nordmann-Poirel (Carba NP) test[34].

Sequencing of *mexA* and *mexB*. *mexA and mexB* genes were amplified by PCR using the following primers: *mexA-F* (5'-GCGAGGCTTTCGGACGTTTA-3'); *mexA-R* (5'-GGCAGACTGAGGATCGACA-3'), *mexB1-F* (5'-CAAGGGGATTCGTAATGTC-3'); mexB1-R (5'-GTGAACATCCAGATCATCC-3'), *mexB2-F* (5'-CGGATGTTCCTTTCCACCAC-3'); *mexB2-R* (5'-GACAGAACGACAGCGGCTA-3'). Annealing temperatures for each pair of primers and amplicon sizes were as follows: *mexA,* 60°C, 1375 bp; *mexB1,* 61°C, 1684 bp; *mexB2,* 67°C, 1638 bp. Sanger sequencing was performed in forward and reverse directions at GATC Biotech (Konstanz, Germany) using the same primers. PAO1 was used as a reference. Molecular graphics for MexA and MexB proteins were rendered using Visual Molecular Dynamics program (VMD[35]), based on Protein Data Bank (PDB) files 2V4D (MexA[24]), and 2V50 (MexB[25]).

Direct measurement of efflux activity. Efflux pump functionality was assayed by following the kinetics of efflux of *N*-phenyl-1-naphthylamine (NPN; Sigma-Aldrich), a MexAB-OprM preferential substrate[11], which is fluorescent when incorporated in bacterial membranes. As previously described[36], bacteria were treated with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 10 µM; Sigma-Aldrich) and then loaded with 20 µM NPN. Efflux was initiated by energizing bacteria with 50 mM D-glucose (Sigma-Aldrich). Decay in fluorescence signal was followed over time using a Spectramax® multiplate reader (Molecular devices, Sunnyvale, CA). Maximal velocity (V_{max}) was calculated by the

SoftMax® Pro *Microplate Data Acquisition and Analysis Software* (version 6.2). PAO1mexAB (Δ*mexAB*-mutant) was used as negative control.

Cloning and overexpression of porins. Genes encoding different porins *i.e.* OprD (OccD1), OpdK (OccK1), and OpdF (OccK2) were cloned in a heterologous system of *Escherichia coli*, which was lacking the main native porins OmpC and OmpF (*E.coli* K-12 W3110:Δ*ompF*Δ*ompC*)[37]. The genes *opdK* and *opdF* were PCR amplified from PAO1 (with HindIII and NsiI restriction site overhangs) and further cloned into pGOmpF (a vector with the signal sequence of *phoE* [phosphoporin protein E] attached to the *ompF* gene[38]) via HindIII/PstI, thereby replacing only *ompF*. This resulting vectors (pG-OpdK or pG-OpdF) had thus *opdK* and *opdF* attached to the *phoE* signal sequence and was further under the control of the *lac* promoter. The expression of *opdK or opdF* was inducible by 0.5 mM IPTG. The pB22-OprD construct was a kind gift from Prof. Bert van den Berg, Newcastle, UK. In this *E. coli* expression vector, the *oprD* gene with the signal sequence of the *E. coli* outer membrane protein YtfM is under the control of the P_{BAD} promoter inducible by 0.5% (w/v) arabinose. MICs were determined in the presence of inducer.

Single-porin *P. aeruginosa* mutants. Insertion mutants for the same 3 porins were obtained from the PA14 transposon insertion mutant library from the Harvard Medical School, Boston, MA[39]. This transposon library has been previously characterized and sequenced in order to identify insertion sites[40]; all transformants were resistant to gentamicin, confirming the expression of the gentamicin-resistance gene present on the insertion cassette.

Quantification of extracellular polymeric saccharides (EPS). EPS were quantified using calcofluor white (*BD*™ Diagnostics, Sparks Glencoe, MD), a fluorophore which binds to β-(*1,4)* and *β*-(*1,3)* polysaccharides[41], including alginate (co-polymer of O-acetylated β-(*1,4)* D-mannuronic acid and L-guluronic acid), a major constituent of slime produced by mucoid *P. aeruginosa*. Bacteria were cultured in 96-well plates as for MIC determinations (starting inoculum, 10^6 CFU/mL; 24 h incubation), after which bacterial density was evaluated by

measuring OD_{620nm}. The content of the wells was transferred to Eppendorf tubes and calcofluor white at a final concentration of 30 mg/L was added. The tubes were incubated for 30 minutes at room temperature and subsequently centrifuged at 20,800 *g*. Pellets were washed with phosphate buffered saline (PBS pH 7.4), resuspended in the same buffer, and transferred in black 96-well plates to measure fluorescence in a Spectramax® multiplate reader (λ_{exc} 370 nm; λ_{em} 440 nm). Fluorescence values were normalized *vs.* the *OD_{620nm} of the cultures, with the value measured for PAO1 set to 1 as a reference*.

Statistical analysis

Statistical analysis was performed using JMP® version 12.1.0, SAS Institute Inc., Cary, NC, USA or GraphPad Prism version 7.01 (GraphPad software Inc., San Diego, CA).

Ethics

The protocol for this study has been examined by the ethical committee of the *Université catholique de Louvain*, who determined that it did not fall under the scope of the law on human experimentation as (i) all isolates were collected during routine sampling and assembled retrospectively, and (ii) all patients' data were anonymized.

Results

Activity of temocillin against clinical isolates and comparison with other β-lactams.

Fig. 1a shows the MIC distribution of temocillin compared to ticarcillin against the whole collection, with MIC_{50} and MIC_{90} of conventional antipseudomonal β-lactams illustrated in the accompanying Table. For temocillin, 15% and 29% of isolates had an MIC ≤ 8 and 16 mg/L, respectively. These values are close to those observed with ticarcillin (13% and 18%, respectively). Of interest, a similar proportion of isolates were susceptible to first-line antipseudomonal

agents such as piperacillin, piperacillin/tazobactam, or ceftazidime. Carbapenems were active against a higher proportion of isolates.

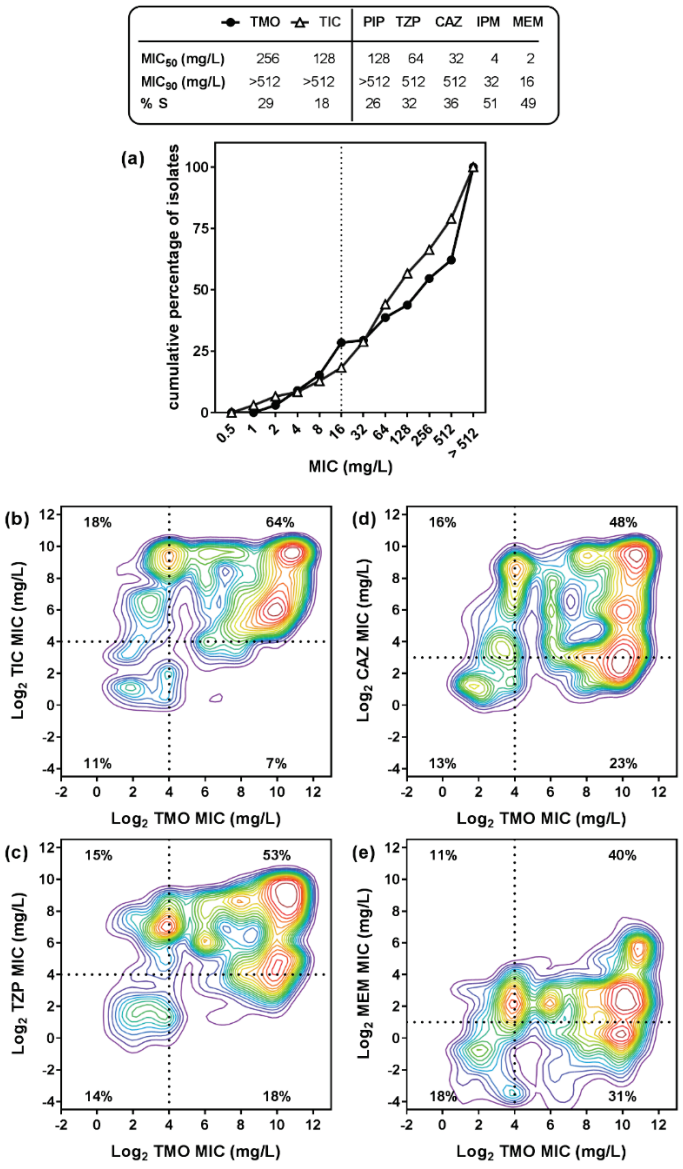

Fig. 1: Activity of temocillin and comparators against CF isolates of *P. aeruginosa*.

Panel (a): Cumulative MIC distribution for temocillin (TMO) compared to ticarcillin (TIC), with indication of MIC_{50}, MIC_{90} and percentage of susceptibility according to the interpretive criteria of EUCAST (S, susceptible; R, resistant) for ticarcillin (S≤16 mg/L; R>16 mg/L); piperacillin (PIP, S≤16 mg/L; R>16 mg/L); piperacillin-tazobactam (TZP, S≤16 mg/L; R>16 mg/L); ceftazidime (CAZ, S≤8 mg/L; R>8 mg/L); imipenem (IPM, S≤4 mg/L; R>8 mg/L); meropenem (MEM, S≤ 2 mg/L; R>8 mg/L). A value of 16 mg/L (dotted line in the graph; EUCAST susceptibility breakpoint of ticarcillin) has been considered as cut-off value for temocillin (TMO), for comparison purposes.
Panels (b-e): cross-resistance between TMO and other β-lactams**.** Correlation between MICs of TMO (abscissa) and TIC, CAZ, TZP or MEM (ordinate) for each individual isolate in the collection using quantile density contour analysis (JMP® version 12.1.0). The intensity of each zone (from warm to cold colours) is indicative of the proportion of isolates (from large to small) with MICs at the corresponding coordinates. The broken lines point to the MIC value above which the isolates are considered resistant for TIC, CAZ, TZP and intermediate for MEM according to EUCAST interpretive criteria. A value of 16 mg/L has been considered for TMO. The percentage of isolates is indicated in each quadrant of the figures. MICs values are expressed as the log_2 of their value.

Fig. 1b-e shows cross-resistance between temocillin and the other β-lactams for individual isolates. While a high proportion of isolates were cross-resistant to temocillin and ticarcillin (64%), piperacillin/tazobactam (53%), ceftazidime (48%), or meropenem (40%), a small but meaningful proportion of isolates that were resistant to the comparator (ranging from 11% for meropenem to 18% for ticarcillin) remained susceptible to temocillin.

β-lactamases screening and identification.

β-lactamase production is a main mechanism of β-lactam resistance in *P. aeruginosa.* We therefore screened the collection for extended spectrum β-lactamases (ESBLs) and carbapenemases. No carbapenemases were detected using both phenotypic and genotypic methods. Moreover, detection of genes encoding CTX-M, TEM, SHV, and BEL, PER, GES, VEB, or OXA β-lactamases

returned negative results in all isolates that were simultaneously resistant to CAZ and MEM (MIC > 8 mg/L).

Influence of active efflux on temocillin activity.

Previous studies suggested that active efflux by MexAB-OprM plays a major role in *P. aeruginosa* resistance to temocillin[6]. Using a representative subset of isolates (n=124) selected to cover the whole range of MICs (8-14 isolates for each MIC value), we therefore examined the influence of the broad spectrum efflux pump inhibitor Phe-Arg-β-naphthylamide (PAβN[10]) on temocillin activity. We checked that PAβN was not toxic by itself for these isolates in the conditions of the experiment. MICs were shifted towards lower values in the presence of the inhibitor (Fig. 2a), with its effect being particularly marked for isolates showing MICs ranging between 128 and 512 mg/L in the absence of PAβN (Fig. 2b). To confirm the role of MexAB-OprM-mediated efflux in resistance to temocillin, we followed in real-time the efflux of *N*-phenyl-1-naphthylamine (NPN), a preferential MexAB-OprM substrate[11], using temocillin as a competitor and comparing PAO1 to its MexAB-OprM deletion mutant (Fig. 2c). NPN efflux was much slower in the deletion mutant than in the parent strain. Temocillin was able to decrease the rate of efflux of NPN in PAO1 but had no effect in the MexAB-OprM deletion mutant even when using a temocillin concentration much higher than its MIC (2 mg/L). These data strongly suggest that temocillin is a preferential substrate of MexAB-OprM. The rate of efflux of NPN was therefore evaluated in 32 isolates (29 clinical isolates plus PAO1, PT629, PAO1*mexAB*) harbouring increasing temocillin MICs (Fig. 2d). NPN efflux was significantly slower for isolates with a temocillin MIC < 512 mg/L and increased in proportion to temocillin MIC.

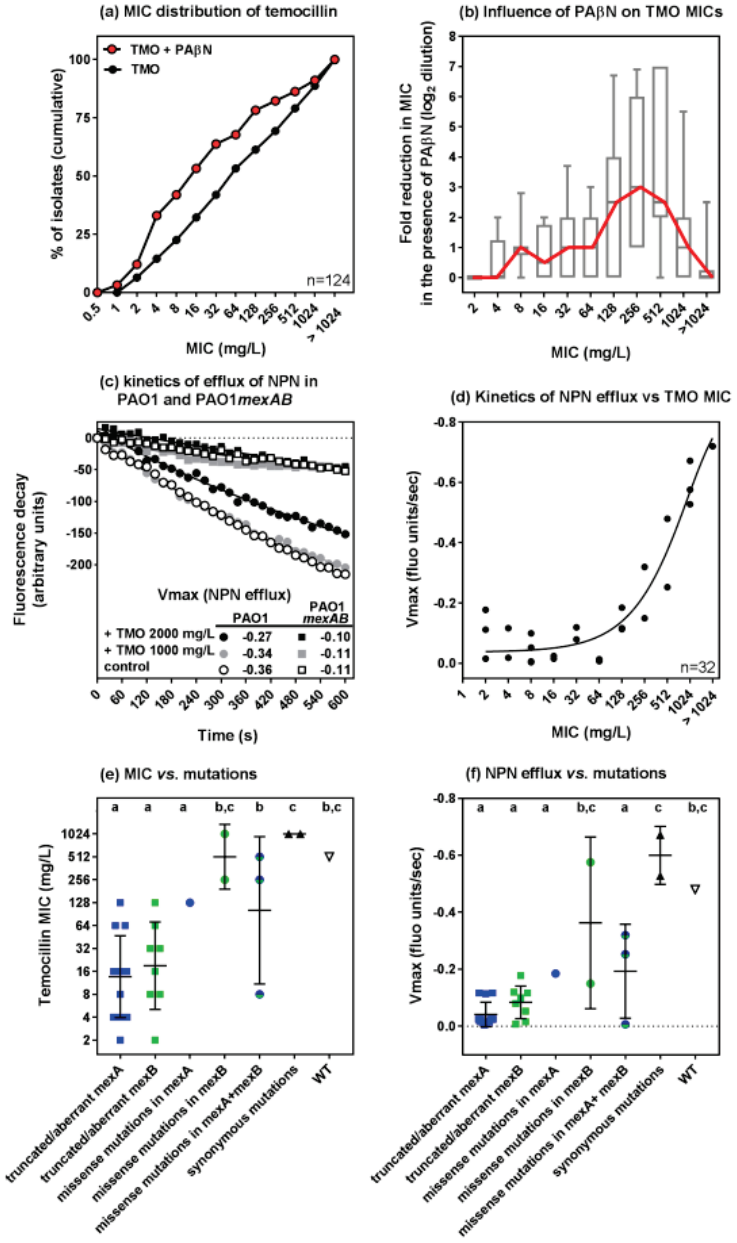
(a) MIC distribution of temocillin
TMO + PAβN
TMO
% of isolates (cumulative)
n=124
MIC (mg/L)
(b) Influence of PAβN on TMO MICs
Fold reduction in MIC in the presence of PAβN (log2 dilution)
MIC (mg/L)
(c) kinetics of efflux of NPN in PAO1 and PAO1mexAB
Fluorescence decay (arbitrary units)
Vmax (NPN efflux)
PAO1
PAO1 mexAB
+ TMO 2000 mg/L -0.27 -0.10
+ TMO 1000 mg/L -0.34 -0.11
control -0.36 -0.11
Time (s)
(d) Kinetics of NPN efflux vs TMO MIC
Vmax (fluo units/sec)
n=32
MIC (mg/L)
(e) MIC vs. mutations
Temocillin MIC (mg/L)
truncated/aberrant mexA
truncated/aberrant mexB
missense mutations in mexA
missense mutations in mexB
missense mutations in mexA+mexB
synonymous mutations
WT
(f) NPN efflux vs. mutations
Vmax (fluo units/sec)

Fig. 2: Influence of active efflux on temocillin activity.
(a) Cumulative MIC distribution of temocillin in a subset of the collection (n=124) selected to cover the whole range of MICs and influence of the efflux pump inhibitor PAβN (20 mg/L).
(b) Fold reduction (log_2 scale) in temocillin MIC in the presence of PAβN, according to temocillin MICs for the same isolates. The graph shows the box and whiskers plot with 10-90 percentiles, with the red line connecting the medians.
(c) Kinetics of NPN efflux from PAO1 or PAO1*mexAB* in the absence (control) or presence of temocillin (TMO) at the indicated concentrations. Vmax are expressed in reduction in the fluorescence signal per second.
(d) Kinetics of efflux of NPN as a function of temocillin MIC for the 32 isolates for which *mexA* and *mexB* were sequenced (Table 1). The ordinate is expressed as the V_{max} (arbitrary fluorescence units). R^2 for a one-phase association: 0.9043. Node splitting value for slower efflux: MIC < 512 mg/L (LogWorth statistic: 29.5157 [p<0.001]).
(e-f) MIC and rate of NPN efflux in sequences isolates classified according to the type of mutations observed in *mexA* and *mexB* (Table 1). 'Truncated/aberrant' refer to deletions of more than 3 consecutive nucleotides in the sequence, nonstop mutations, insertion of minisatellites or aberrant signal peptides. 'Missense mutations' refer to mutations leading to the replacement of at least on amino acid in the corresponding protein. The graphs shows individual values together with means and SD. Statistical analysis (1-way ANOVA; Tuckey post-hoc test): data series with different letters are different from one another ($p < 0.05$).

Mutations in *mexA* and *mexB* in relation with temocillin MIC and efflux pump activity.

MexA and MexB, but not OprM, determine the β-lactam specificity of the MexAB-OprM efflux system[12]. We therefore sequenced *mexA* and *mexB* genes in the 32 isolates for which NPN efflux was evaluated. Fig. 2e-f illustrates the relationship between the type of mutations evidenced and the MIC of temocillin or the rate of efflux of NPN. Isolates harboring deletions in *mexA* or *mexB* or aberrant sequences (deletion of ≥ 3 consecutive nucleotides, nonsense mutation, nonstop mutation[13], aberrant signal peptide, minisatellite repeat) had the slowest rate of efflux of NPN and temocillin MICs ranging between 2 and 128 mg/L. Isolates with missense mutations globally showed a slower efflux than PAO1 and temocillin MICs of 128 or 256 mg/L. Synonymous mutations did not impact NPN efflux or temocillin MICs. In addition, most of the isolates showed

a high frequency of synonymous mutations and codon degeneracy especially at the third position. Most of them were conserved between different isolates originating from different countries.

Table 1 summarizes these results and shows in parallel for each isolate the V_{max} for NPN efflux, together with (i) the MICs of temocillin in control conditions or in the presence of the efflux pump inhibitor PAβN or of the OpdK porin substrate vanillate, and (ii) the relative amount of exopolysaccharides. The constructed molecular graphics of the corresponding proteins are illustrated in Table S2 (see also Fig. S1 for a view of the wild-type proteins, with specification of their subdomains).

Table 1 (see next pages): Efflux characteristics, MIC and relative polysaccharide content for reference strains versus CF clinical isolates of *P. aeruginosa.* The table shows (a) the mutations detected in *mexA* and *mexB* and the changes in the corresponding proteins, the V_{max} for NPN efflux (see Fig. 2, panels c-d), (b) temocillin MICs in control conditions, or in the presence of 20 mg/L PAβN as efflux pump inhibitor and 2 mM vanillate (VNL) as OpdK substrate, and (c) the culture content in exopolysaccharides relative to PAO1 (see Fig. 3).
Abbreviations used: TM, transmembrane α-helix; MP, membrane-proximal domain; PN1/PN2 (periplasmic, *N-terminal)*, PC1/PC2 (periplasmic, *C-terminal)*, DN (*docking*, N-terminal) and DC (*docking*, C-terminal) are six periplasmic subdomains which build the pore and docking domains of MexB, respectively (see Fig. S1 for more details); nt, nucleotide; AA, amino acids; SCV, small colony variant; LES, Liverpool Epidemic Strain; EPS, extracellular polymeric saccharides. Molecular graphics for *mexA* and *mexB* mutants are represented in the supplementary data (Table S2a-e) together with the position of the different protein domains

Reference strains and clinical isolates (patient's identification code; country; date of collection)	Description (phenotype; length of MexA/B protein; AA deletions and affected MexA/B domains)	Efflux characteristics			MIC (mg/L)			
		mexA alterations	*mexB* alterations	Vmax (units/s)	TMO	TMO+PAβN	TMO+VNL	EPS (β-1,4 / β-1,3 glycosidic bonds)
PAO1	Wild type MexA (383 AA) MexB (1046 AA)	-	-	- 0.480	512	128	768	1
PT629[a]	PAO1 MexAB-OprM overproducer	-	-	-0.720	1024	256	1536	
PAO1*mexAB*[a]	PAO1 *ΔmexAB*	-	-	-0.112	2	2	2	

3724 (AL1; France; 14/12/2006)	-MexA (383 AA). -Truncated MexB (878 AA): **S879_Q1046del** deletion of 168 AA affecting 5 TMs (TM8-TM12).	-Uncommon[b] synonymous mutations: C546G -Prevalent[b] synonymous mutations: A345G G993A	-Uncommon synonymous mutations: C486T -Prevalent synonymous mutations: A495G C1308T C1692T T2280C T2730C G3117A **-Nonsense mutation: C2636A**	-0.178	2	2	4	1.2
144 (PCF79; Belgium; 20/09/2010)	-Truncated MexA (124 AA): **A125_G383del** deletion of 259 AA affecting the α-hairpin, second, third and MP domains. -MexB (1046 AA)	-Prevalent synonymous mutation: G993A **-Nonsense mutation, Δ 13 nt (367-379)**	Prevalent synonymous mutations: A495G T2280C T2730C G3117A	-0.016	2	2	4	1.1

W024 (DM; UK; 2009)	-Truncated MexA (69 AA): **V70_G383del** deletion of 314 AA in α-hairpin, second, third and MP domains. -MexB (1046 AA)	**Nonsense mutation, Δ 1 nt (164)**	No mutations	-0.117	4	2	6	1.4
W049 (DB; UK; 2009)	- Isogenic to W024 -Truncated MexA (69 AA): **V70_G383del** deletion of 314 AA in α-hairpin, second, third and MP domains. -MexB (1046 AA)	**Nonsense mutation: Δ 1 nt (164)**	No mutations	-0.117	4	2	6	2.2

143-1 (143; Germany; 12/07/2012)	-Nonstop mutation in *mexA* (**stalled ribosome,** proteolysis; recycled ribosome)[13]. -MexB (1046AA)	-Prevalent synonymous mutation: G993A **-Nonstop mutation: Δ 2 nt (837-838)**	-Uncommon synonymous mutations: C2412T -Prevalent synonymous mutations: A495G T2226C T2280C T2730C G3117A	-0.019	4	4	8	0.5
AG3 (JP; UK; 10/05/2006)	-Shortened MexA (372 AA): **G337_E347del** deletion of 11 AA in the MP domain. -MexB (1046AA)	-Prevalent synonymous mutations: A345G G993A **-Deletion: Δ 33 nt (1011-1043)**	Prevalent synonymous mutations: C474T C492T A495G T2280C T2730C G3117A	-0.004	8	4	12	1.4
129 (RCF62; Belgium; 09/09/2010)	-MexA (383 AA). -MexB (1045 AA): **N254del** deletion of asparagine 254 in the DN subdomain and missense mutation **(T557N)** in TM7.	-Uncommon synonymous mutations: C633T G702A C780T -Prevalent synonymous mutations: T333C A345G G447A A639G T655C T729C C732T A738G C789G G792A T1002C T1095C	-Uncommon synonymous mutations: C909T G1362A -Prevalent synonymous mutations: A495G T2730C G3117A **-Conservative missense mutation: C1670A** **-Deletion: Δ 3 nt (760-762)**	-0.053	8	2	12	1.1

135-1 (135; Germany; 10/07/2012)	-MexA (383 AA): **A206S** missense mutation (third domain). -**MexB truncated** (719 AA): **M720_Q1046del** deletion of 327 AA in DC, PN1, PC2 and TM8-TM12 and **300 AA substitutions (I186V; M421_719)** in TM5-7, DN, PC1, and PC2.	-Uncommon synonymous mutations: G438T G558C -Prevalent synonymous mutations: T333C A345G G447A A639G T655C T729C C732T A738G C789G G792A C951G T1002C T1095C **-Radical missense mutation: G616T**	-Uncommon synonymous mutations: G348A C1101T C1389A -Prevalent synonymous mutations: C315T A351G A495G C498G G591A C600G C537T A1938G T2067C A2079G T2280C T2730C **-Conservative missense mutation: A556G** **-Insertion and deletion leading to premature stop codon: G1261_C1262insG + Δ 2 nt (1947-1948)**	-0.100	8	8	12	3.8

126 (VIF68; Belgium; 09/09/2010)	-SCV isolate. -MexA (383 AA): aberrant signal peptide **(S19L)**. -MexB (1046 AA): missense mutations **(I186V, Q773P)** in DN and DC subdomains at the OprM-docking domain of MexB	-Uncommon synonymous mutations: C582T C912T -Prevalent synonymous mutations: T333C A345G G447A T655C T729C C789G G792A C951G T1002C T1095C **-Radical missense mutation: C56T**	-Uncommon synonymous mutations:C1314A G2004A C2014T -Prevalent synonymous mutations: C315T A351G A495G C498G C537T G591A C600G A804C G1587T T1749C A1938G T2067C A2079G T2226C T2280C T2730C T2826C A2892G C2955T **-Conservative missense mutation: A556G** **-Radical missense mutation: A2318C**	-0.007	8	2	12	3.3
279 (RRM59; Belgium; 28/10/2010)	-MexA (383 AA). -Aberrant MexB (1027 AA): **K151_T169del** deletion of 19 amino acids in the periplasmic pore domain (subdomain PN2)	No mutations	-Prevalent synonymous mutations: A351G C1308T T2280C T2730C G3117A **-Deletions: Δ 57 nt (455-511)**	-0.016	16	4	24	2

BM1 (AD; UK; 13/09/2006)	- LES isolate. -Truncated MexA (119 AA): **Y120_G383del** deletion of 264 AA in α-hairpin, second, third and MP domains. -MexB (1046 AA)	-Prevalent synonymous mutation: A345G **-Nonsense mutation: C360G**	-Uncommon synonymous mutation: T2015G -Prevalent synonymous mutations: A495G, C1692T, T2280C, T2730C, G3117A	-0.025	16	16	16	3.7

AJ3 (ML; UK; 22/05/2006)	- LES isolate. -Truncated MexA (119 AA): **Y120_G383del** deletion of 264 AA in α-hairpin, second, third and MP domains. -MexB (1046 AA)	-Prevalent synonymous mutation: A345G **-Nonsense mutation: C360G**	-Uncommon synonymous mutation: T2015G -Prevalent synonymous mutations: A495G, C1692T, T2280C, T2730C, G3117A	-0.025	16	16	32	1.4
CF15 (CT; UK; 2007)	-LES isolate. -Truncated MexA (119 AA): **Y120_G383del** deletion of 264 AA in α-hairpin, second, third and MP domains. -MexB (1046 AA)	-Prevalent synonymous mutation: A345G **-Nonsense mutation: C360G**	-Uncommon synonymous mutation: T2015G -Prevalent synonymous mutations: A495G, C1692T, T2280C, T2730C, G3117A	-0.025	16	16	24	3.2

CF53 (DP; UK; 2007)	-LES isolate. -Truncated MexA (119 AA): **Y120_G383del** deletion of 264 AA in α-hairpin, second, third and MP domains). -MexB (1046 AA)	-Prevalent synonymous mutation: A345G **-Nonsense mutation: C360G**	-Uncommon synonymous mutation: T2015G -Prevalent synonymous mutations: A495G, C1692T, T2280C, T2730C, G3117A	-0.025	16	16	32	1.6
CF19 (LS; UK; 2007)	-LES isolate. -Truncated MexA (119 AA): **Y120_G383del** deletion of 264 AA in α-hairpin, second, third and MP domains). -MexB (1046 AA)	-Prevalent synonymous mutation: A345G **-Nonsense mutation: C360G**	-Uncommon synonymous mutation: T2015G -Prevalent synonymous mutations: A495G, C1692T, T2280C, T2730C, G3117A	-0.025	16	16	24	1.9

CF16 (RC; UK; 2007)	-LES isolate. -MexA (383 AA). -Truncated MexB (30 AA): **P31_Q1046del** only TM1 is encoded.	Prevalent synonymous mutation: A345G	-Uncommon synonymous mutation: T2015G -Prevalent synonymous mutations: A495G, C1692T, T2280C, T2730C, G3117A **-Nonsense mutation: Δ 154 nt (85-239)**	-0.120	32	8	48	2.7
BV1 (DC; UK; 11/10/2006)	-LES isolate. -MexA (383 AA). -Truncated MexB (672 AA): **E673_Q1046del** deletion of 374 AA in DC, PN1, PC2 and TM8-TM12, and 154 AA substitutions (**F519_G672)** in TM6-7, PC1 subdomains.	Prevalent synonymous mutation: A345G	-Uncommon synonymous mutation: T2015G -Prevalent synonymous mutations: A495G, C1692T, T2280C, T2730C, G3117A **-Nonsense mutation: Δ 8 nt (1555-1562)**	-0.080	32	8	64	3.6

191-4 (191; Germany; 03/09/2012)	-Truncated MexA (27 AA): **E28_G383del** deletion of 356 AA. -MexB (1046 AA).	-Prevalent synonymous mutation: G993A **-Nonsense mutation: G82T**	Prevalent synonymous mutations: A495G T2280C T2730C G3117A	-0.014	64	32	128	4.1
207 (207; Germany; 2012)	-Truncated MexA (27 AA): **E28_G383del** deletion of 356 AA. -MexB (1046 AA).	-Prevalent synonymous mutation: G993A **-Nonsense mutation: G82T**	Prevalent synonymous mutations: A495G T2280C T2730C G3117A	-0.014	64	32	64	2.6
109 (ENM88; Belgium; 03/09/2010)	-MexA (383 AA). -15 base pairs (5 AA residues PAIAP [P36_P40]) **minisatellite repeat** in MexB (1051 AA), toxic or malfunctioning proteins.	-Uncommon synonymous mutation: G117A C468T -Prevalent synonymous mutations: T333C A345G G447A T729C T1002C	-Uncommon synonymous mutations: G1452A C1920T -Prevalent synonymous mutations: C315T A351G A495G T642C A804C C1308T T1749C A1938G T2067C A2079G T2226C T2280C **-15 nt minisatellite repeat: 118-132[c]**	-0.008	64	toxic	64	2.5

618 (FJ1; France; 01/01/1996)	-Truncated MexA (297 AA): **V298_G383del,** deletion of 86 AA in MP domain. -MexB (1046 AA)	-Prevalent synonymous mutation: A345G **-Nonsense mutation: Δ 1 nt (C869)**	Prevalent synonymous mutations: A495G C1308T C1851T T2067C T2226C T2280C T2730C T2826C A2892G C2955T G3117A	-0.114	128	toxic	128	2.9
3179 (MP1; France; 03/02/2004)	-SCV isolate. -MexA (383 AA). -Truncated MexB (719 AA): **M720_Q1046del** deletion of 327 AA in TM8-TM12, DC and PN1/PC2 subdomains.	Prevalent synonymous mutation: G993A	-Prevalent synonymous mutations: A495G C1308T C1851T T2067C T2226C T2280C T2730C T2826C A2892G C2955T G3117A **-Nonsense mutation: Δ 1 nt (G2147)**	-0.117	128	128	192	3.1
180-3 (180; Germany; 30/07/2012)	-MexA (383 AA): **W332R** missense mutation in MP domain; -MexB (1046 AA)	-Prevalent synonymous mutations: A345G T729C T1002C **-Radical missense mutation: T994C**	-Uncommon synonymous mutation: G1608A -Prevalent synonymous mutations: C492T A495G T2280C T2730C G3117A	-0.185	128	32	128	1.2

3319 (BV1; France; 03/08/2004)	-MexA (383 AA); -MexB (1046 AA): **Y182C, A707S, I963V, S1041E and V1042A,** missense mutations in PC2, DN, TM11, and cytoplasmic C-terminal domain.	-Uncommon synonymous mutations: G51T C147G C291T G399A C423T G426A C591T C651T C687T C705T C777A -Prevalent synonymous mutations: T333C A345G G447A T655C T729C C951G T1002C	-Uncommon synonymous mutations: C129T G165A T423G T519C G1080A C1152T T1332C C1383T C1476G T1563C C1581T C1614T A1713G G1748C T1758G G1836C C1980T G2001T C2070A C2134T A2139G C2148T C2223T G2319A T2457C G2469C C2604A T2907C T3033C -Prevalent synonymous mutations: A351G A495G C498G C537T T642C A804C G1587T T1749C T2067C T2226C T2280C T2730C A2892G C2955T **-Radical missense mutation: G2119T** **-Conservative missense mutations: A545G; A2887G; G3120T T3121G C3122A C3123G T3125C**	-0.150	256	32	384	1.8

208-3 (208; Germany; 09/08/12)	-Isogenic to 135-1. -MexA (383 AA): missense mutation **A206S** (third domain). -MexB (1045 AA): missense mutation **I186V** (DN subdomain), and **30 AA replaced by 29 new AA** (R620_R650 in PC1 subdomain).	-Uncommon synonymous mutations: G438T G558C -Prevalent synonymous mutations: T333C A345G G447A A639G T655C T729C C732T A738G C789G G792A C951G T1002C T1095C **-Radical missense mutation: G616T**	-Uncommon synonymous mutations: G348A C1101T -Prevalent synonymous mutations: C315T A351G A495G C498G C537T G591A C600G A1938G T2067C A2079G T2280C T2730C **-Conservative missense mutation: A556G** **-Deletions: Δ 1 nt (T1854) + Δ 2 nt (1947-1948)**	-0.320	256	256	256	4.4

208-2 (208; Germany; 09/08/12)	-Isogenic to 135-1. -MexA (383 AA): missense mutation **A206S** (third domain). -MexB (1045 AA): missense mutation **I186V** (DN subdomain), and **19 AA replaced by 18 new AA** (L631_R650 in PC1 subdomain)	-Uncommon synonymous mutations: G438T G558C -Prevalent synonymous mutations: T333C A345G G447A A639G T655C T729C C732T A738G C789G G792A C951G T1002C T1095C **-Radical missense mutation: G616T**	-Uncommon synonymous mutations: G348A C1101T -Prevalent synonymous mutations: C315T A351G A495G C498G C537T G591A C600G A1938G T2067C A2079G T2280C T2730C **-Conservative missense mutation: A556G** **-Deletions: Δ 1 nt (T1889) + Δ 2 nt (1947-1948)**	-0.253	512	256	512	4.6
128 (DAF69; Belgium; 09/10/2010)	-Isogenic to AG3. -MexA (383 AA). -MexB (1046 AA): **L376V,** missense mutation in TM3.	Prevalent synonymous mutations: A345G G993A	-Prevalent synonymous mutations: C474T C492T A495G T2280C T2730C G3117A **-Conservative missense mutation: C1126G**	-0.576	1024	1024	1024	4.9

129-6 (129; Germany; 11/07/2012)	-Isogenic to AG3. -Synonymous mutations in MexA (383 AA) and MexB (1046 AA)	Prevalent synonymous mutations: A345G G993A	Prevalent synonymous mutations: C474T C492T A495G T2280C T2730C G3117A	-0.672	1024	256	1536	2.1
4289 (JV1; France; 13/08/2007)	-Synonymous mutations in MexA (383 AA) and MexB (1046 AA)	Prevalent synonymous mutations: G993A	Prevalent synonymous mutations: A495G T2280C T2730C G3117A	-0.528	1024	512	1536	1.5

[a] see [6] for references

[b] uncommon synonymous mutations: rare mutations found in isolates belonging to only one clone or one single patient ; prevalent synonymous mutations: identical mutations found in different isolates belonging to different clones.

[C] The presence of minisatellites could be consecutive to bacterial exposure to genotoxic compounds[42,43]; the resulting proteins are malfunctioning.

Tables S2b-e specifically focus on sequenced isogenic isolates collected either from the same patient or from different patients originating either from the same or from different countries. Table S2b illustrates isolates collected in the UK and belonging to the Liverpool epidemic strain (LES)[9]. Isolates from five different patients with an MIC of 16 mg/L shared the same synonymous mutations and also the same nonsense mutation in *mexA* leading to the production of a 119 amino acid protein. Of note, two other LES isolates with an MIC of 32 mg/L shared the same synonymous mutations in *mexA* and *mexB* genes as those described for the first 5 LES isolates, but harboured different deletions in their *mexB* gene, leading to proteins of 30 and 672 amino acids, respectively. Tables S2c illustrates 2 pairs of isolates harbouring the same mutations although collected from different patients in the same country. Thus, in the isogenic isolates 191-4 and 207[9] with a temocillin MIC of 64 mg/L, MexA was truncated to 27 amino acids. Likewise, in isogenic isolates W024 and W049 with a MIC of 4 mg/L, MexA was truncated to 69 residues due to the loss of 1 nucleotide leading to a premature stop codon. Interestingly also, isogenic isolates can be found in different countries (Table S2d). These three isolates belonging to the same multidrug-resistant clonal complex[9] and originating from the UK (AG3), Belgium (128) and Germany (129-6) shared the same synonymous mutations. Only AG3 had a low MIC to temocillin (8 mg/L), associated with a MexA protein truncated in its MP domain (372 amino acids left). The two other isolates had MICs of 1024 mg/L, with one conservative missense mutation (L376V) found in the transmembrane domain TM3 of the MexB protein of isolate 128. Finally, Table S2e illustrates three other clonal isolates differing in their susceptibility to temocillin. They come from two different patients in Germany and share (i) a radical missense mutation (G616T) in the *mexA* part encoding the third domain of MexA interacting with the distal domain of MexB, (ii) a conservative missense mutation (A556G), and (iii) a deletion of nucleotides 1947 and 1948 in *mexB*. In addition, isolate 135-1, with a temocillin MIC of 8 mg/L, showed an insertion in *mexB* (G1261_C1262insG) leading to the production of an aberrant and truncated MexB protein. In contrast, isolates 208-3 and 208-2, with temocilllin MIC of

256 and 512 mg/L, differed only by the position of an additional 1 nucleotide deletion (in position 1854 or 1889, respectively) in the *mexB* gene part encoding the pore (PC1) subdomain, leading to the replacement of 30 by 29 amino acids, and of 19 by 18 amino acids, respectively. This difference could explain the 1 two-fold dilution difference in temocillin MIC between these two isolates.

Despite the evidenced truncations or mutations in MexA and MexB, most of the clinical isolates showed nevertheless higher temocillin MICs than the reference strain PAO1*mexAB* lacking MexA and MexB proteins (see Fig. 2e). This strongly suggests that other mechanisms of resistance independent of the functionality of MexAB-OprM are present.

Culture content in extracellular polymeric saccharides with β-1,4 and β-1,3 linkages.

P. aeruginosa isolated from CF patients often harbour a mucoid phenotype, related to the overproduction of extracellular polysaccharides, among which alginate contributes to the chronicity of infections. Alginate production has been shown to impede the activity of β-lactams, including ticarcillin[14,15].

We therefore compared exopolysaccharide abundance in cultures of 73 clinical isolates (included those which had been sequenced) *vs.* PAO1 by measuring calcofluor white binding to polysaccharides (Fig. 3). A high variability in fluorescence values was observed among isolates, but a global statistical analysis showed that the increase in fluorescence signal was significant for isolates with a temocillin MIC ≥ 4 mg/L. Notably, among sequenced isolates with truncated or aberrant MexA or MexB, the fluorescence signal was at least 2.6-fold higher than in PAO1 for those harbouring MICs of 32 to 128 mg/L.

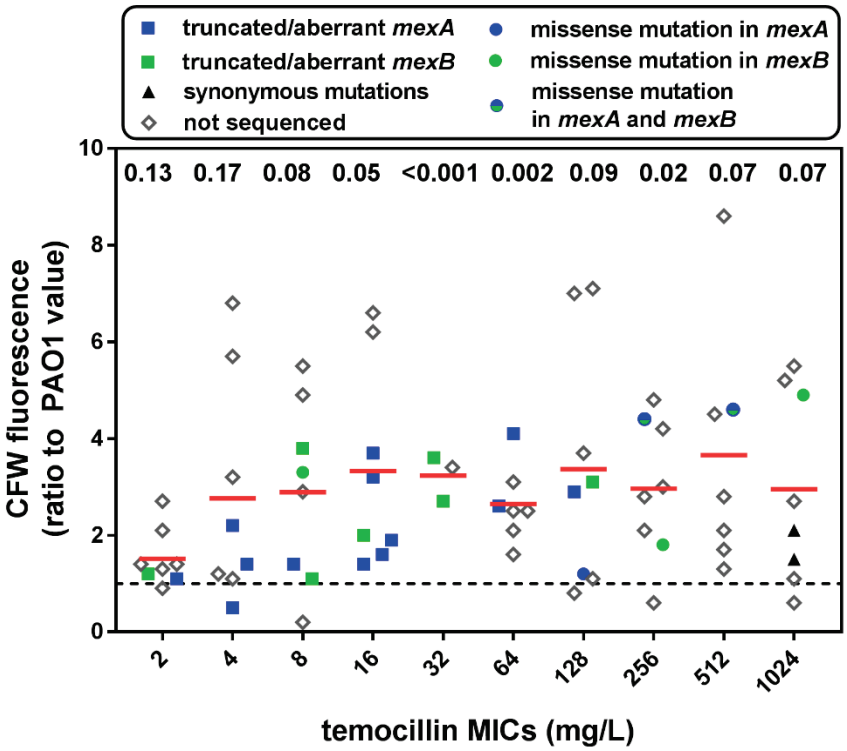

Fig. 3. Evaluation of exopolysaccharide abundance in cultures of clinical isolates as compared to the reference strain PAO1 as a function of temocillin MICs.

Calcofluor white (CFW) fluorescence was measured for 73 clinical isolates, including those for which *mexA* and *mexB* were sequenced (Table 1). Data expressed as a ratio to the value measured for the non-mucoid reference strain PAO1 (highlighted by the dotted line), the MIC of which is 512 mg/L). The red horizontal lines show the mean of values for 7-8 isolates for each MIC (3 only for 32 mg/L [no more isolates with this MIC value in the whole collection]). Statistical analysis: figures above each bar represents the p value (multiple t-test) versus PAO1. Node splitting value for significant increase in CFW fluorescence signal *vs*. PAO1: ≥ 4 mg/L; LogWorth statistic: 1.6343 [p 0.02].

Influence of porins on temocillin activity.

β-lactams are known to cross the outer membrane of Gram-negative bacteria via porins.

While OprD (OccD1) has been well characterized as facilitating the entry of carbapenems (mainly imipenem[16]) into *P. aeruginosa*, other members of the

OprD family like OpdK (OccK1, archetype of the OccK subfamily[17]) and OpdF (OccK2) are involved in the transport of the carboxypenicillin carbenicillin and of the anionic cephalosporin cefoxitin[8]. As temocillin is also a carboxypenicillin, we examined whether these anion-selective porins were involved in temocillin transport into *P. aeruginosa.* OprD was studied in parallel. Other members of the OprD family of porins were not investigated in the present work.

MICs of temocillin, cefoxitin, imipenem, and meropenem were measured in transformants of a porin-deficient *E. coli* strain expressing OpdK, OpdF, or OprD (Table 2; carbenicillin could not be tested due to the presence of a *β-lactamase*-encoding *gene* on the transforming plasmid). Consistent with previous studies[8], OprD expression increased the activity of carbapenems but not that of the other drugs. Conversely, the expression of OpdK, and to a slightly lesser extent, of OpdF, increased the activity of temocillin and cefoxitin; this effect was best seen when the antibiotics were combined with PAβN.

Table 2: Influence of porins on antibiotic activity in *E. coli* transformants expressing *oprD/Occd1, opdK/occK1*, or *opdF/occK2*

Strains	Antibiotic MIC (mg/L)[a]					
	TMO		FOX		IPM	MEM
	control	+ PAβN[b]	control	+ PAβN[b]		
W3110 Δ*ompF/C* + pBAD-Empty	40	18	80	20	1	1.5
W3110 Δ*ompF/C* + pBAD-*oprD*	40	18	80	20	**0.225**	**0.225**
W3110 Δ*ompF/C* + pG-Empty	40	18	80	20	1	1.5
W3110 Δ*ompF/C* + pG-*opdK* clone 1[c]	**20**	**9**	**56**	**16**	1	1.5
W3110 Δ*ompF/C* + pG-*opdK* clone 2[c]	**20**	**9**	**56**	**16**	1	1.5
W3110 Δ*ompF/C* + pG-*opdF* clone 1[c]	**22**	**14**	**60**	**18**	1	1.5
W3110 Δ*ompF/C* + pG-*opdF* clone 2[c]	**22**	**14**	**60**	**18**	1	1.5

[a] MICs (mg/L) of temocillin (TMO), cefoxitin (FOX), imipenem (IPM), and meropenem (MEM) were determined in the presence of IPTG 0.5 mM or arabinose 0.5% as gene inducers. Values in bold highlight increased activity *vs*. the corresponding control (no porin expressed)

[b] 20mg/L PAβN added as an inhibitor of *E.coli* AcrAB-TolC efflux pump

Subsequently, MICs were measured in *P. aeruginosa* mutants of each of these three porins (Table 3). MICs for carbapenems were markedly (3-4 $\log_2$ dilutions) increased by the loss of OprD while those of temocillin and carbenicillin were not affected. Conversely, deletion of OpdK or OpdF modestly (< 1 $\log_2$ dilution) increased the MIC of carboxypenicillins. This effect was amplified in the presence of vanillate (substrate for OpdK[17,18]) in the OpdF mutant for carboxypenicillins, suggesting that these two porins cooperate for the import of these antibiotics. Likewise, L-Arginine (substrate for OprD[19]) reduced carbapenem activity in the OpdK or OpdF mutants. The effect of vanillate on temocillin activity against clinical isolates is illustrated in Fig. 4a (MIC distribution) and in Table 1 (individual isolates). This effect was

modest, but best seen in isolates with temocillin MICs ≤ 64 mg/L, in which efflux was deficient (Fig. 4b).

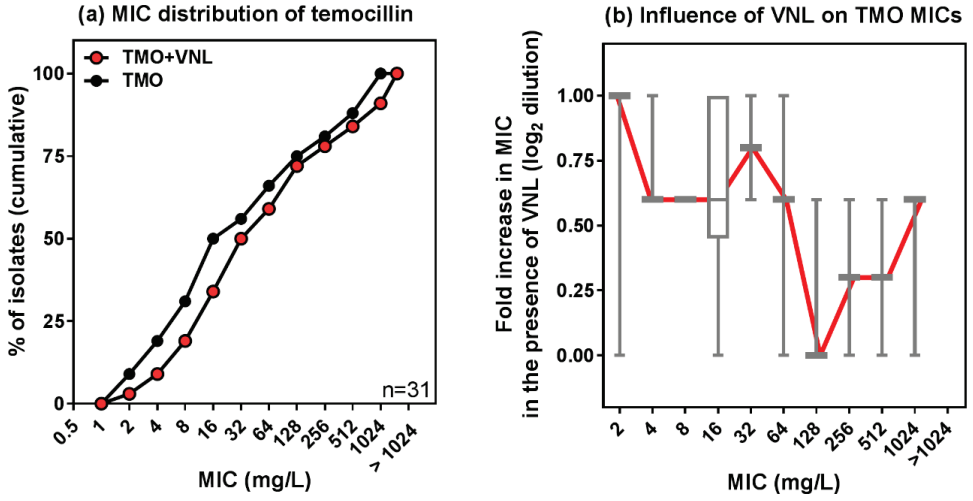

Fig. 4: Influence of porins on temocillin activity.
(a) Cumulative MIC distribution of temocillin in a subset of the collection (n=31) selected to cover the whole range of MICs and influence of the OpdK competitor vanillate (VNL 2 mM).
(b) Fold reduction (log_2 scale) in temocillin MIC in the presence of VNL, according to temocillin MICs for the same isolates. The graph shows the box and whiskers plot with 10-90 percentiles, with the red line connecting the medians.

Table 3: Influence of porins on antibiotic activity in *P. aeruginosa* PA14 and its **single disruptants for the *oprD*, *opdK*, or *opdF* genes.**

strain	Antibiotic MIC (mg/L)[a]											
	TMO			**CAR**			**MEM**			**IPM**		
	control	+ VNL	+ L-Arg	control	+ VNL	+ L-Arg	control	+ VNL	+ L-Arg	control	+ VNL	+ L-Arg
PA14	256	**384**	256	48	**64**	48	0.37	0.37	**1**	1	1	**2**
PA14::*oprD*	256	**384**	256	48	**64**	48	*3*	*3*	*3*	*4*	*4*	*4*
PA14::*opdK*[b]	*384*	384	*384*	*64*	64	*64*	0.37	0.37	**1**	1	1	**2**
PA14::*opdF*[b]	*384*	***786***	*384*	*64*	***128***	*64*	0.37	0.37	**1**	1	1	**2**

[a] MICs (mg/L) of temocillin (TMO), carbenicillin (CAR), imipenem (IPM), and meropenem (MEM) were determined in the presence of 2 mM vanillate (VNL) or 10 mM L-Arg as competitor substrates for OpdK and OprD, respectively. Values in bold highlight decreased activity *vs.* the corresponding control (no porin substrate added); values in italics highlight decreased activity vs the wild-type strain PA14.
[b] Two different clones transformed with pG-OpdK or pG-OpdF tested.

Discussion

This study is the first to describe the activity of temocillin in a large collection of *P. aeruginosa* collected from CF patients, and to determine the mechanisms that can lead to a phenotype of 'acquired susceptibility' to this antibiotic, which is uncommon in this pathogen[3]. Our results showed that a low but significant proportion of the isolates have MICs lower than the current temocillin BSAC (British Society for Antimicrobial Chemotherapy) susceptibility breakpoint for systemic infections, and to the current EUCAST breakpoint for the parent molecule ticarcillin, as well as to the breakpoint proposed for high dosing regimens of temocillin based on pharmacokinetic/pharmaocodynamic considerations[20]. Even if far from being the majority, these susceptible isolates cannot be ignored, as 30 to 60% of them are resistant to conventional antipseudomonal β-lactams, including carbapenems, making temocillin one of the last viable therapeutic option.

We confirm that altered active efflux mediated by MexAB-OprM is the main driver of restored susceptibility to temocillin. Non-functional efflux, as evidenced using NPN as a tracer, was observed in isolates with temocillin MICs < 512 mg/L, and these also harbour mutations in *mexA* and/or *mexB* leading to protein alterations. Among the sequenced isolates, we could evidence a whole panel of mutations that may lead to loss of functionality, many of which had not been reported in our earlier study of temocillin-susceptible CF isolates[6], neither in other studies investigating the effect of *mexA/B* mutations on the pump activity[21-23].

The most susceptible isolates show major deletions in *mexA* or *mexB* leading to the production of truncated or aberrant proteins. Over the 8-512 mg/L range of MICs, *mexA* mutations are found in the regions encoding the α-helical hairpin and/or of the second, third, and membrane-proximal (MP) domains, which interact with MexB and/or OprM proteins[24], possibly affecting the linkage with MexB or OprM, and therefore the correct assembly of the tripartite efflux system[22]. *mexB* mutations are located either in the regions building the pore domain (PC1, PC2, PN1, and PN2) implicated in drug recognition and extrusion[25], or in those encoding the periplasmic tip of the

docking subdomains (DN and DC) involved in the interaction with OprM[25]. Only one isolate (3319) showed an additional amino acid substitution outside these domains, namely in the cytoplasmic C-terminal domain of MexB[25]. Deletions in transmembrane helices TM8-12 were also identified in a few isolates. TM10 has been shown to mediate proton translocation (with the TM4) in the inner membrane domains[26]. Of interest, we noticed different mutations and hence, different susceptibility to temocillin, among clonal isolates, suggesting a high adaptability of each of them to its own environment.
Of note, most of the isolates also showed a high frequency of synonymous mutations and codon degeneracy especially at the third position. Most of these mutations were conserved among several isolates from different countries. Synonymous substitutions are often considered as silent mutations, but they can impact gene transcription and mRNA transport or translation, which could alter protein folding and function[27]. Codon degeneracy is described as highly frequent in *P. aeruginosa*[28].

Intriguingly, restoration of temocillin activity was only partial in some isolates that showed major deletions in *mexA* or *mexB.* Since we did not investigate the impact of each evidenced mutation on temocillin MIC in a PAO1 background, we cannot exclude that this is due to the expression of other, still undescribed mechanisms of resistance, or to an overexpression of other efflux systems that may compensate for the loss of MexAB-OprM activity, as previously described in CF isolates[29]. However, we previously showed that these systems only play a marginal role in temocillin extrusion[6]. Therefore, we suggest a possible phenotypic resistance related to an overproduction of extracellular polymeric substances, including alginate, in isolates with temocillin MICs ≥ 4 mg/L. Alginate has been shown to impair the diffusion, and therefore the activity, of ticarcillin, the parent compound of temocillin[14,15]. We show here that alginate production is systematically low in isolates with a temocillin MIC of 2 mg/L, *i.e*. a value corresponding to that measured for the *mexAB* deletion mutant of the non-mucoid reference PAO1, while it is high in clinical isolates with inactive efflux but temocillin MICs of 32-128 mg/L.

Of note, we were able to exclude or minimize the role of two known mechanisms of resistance to β-lactams, namely the expression of β-lactamase(s) and reduced expression of porins. Thus, no β-lactamase among the few ones capable of hydrolysing temocillin (VIM, NDM, IMP, OXA-48[30]) was genotypically detected in our isolates. For porins, while OprD alterations have been previously evidenced in this collection[31], we show here that, in contrast to carbapenems, temocillin does not require this porin to enter bacteria, but rather uses the anion-specific porins, OpdK and OpdF, the role of which seems redundant. Temocillin indeed shares a methoxy moiety with cefoxitine and vanillate, two well-established substrates of these porins. This moiety has been described for vanillate as interacting with the L3-L7 loops forming the internal constriction of OpdK[18]. Yet, the importance of these porins in temocillin uptake seems rather marginal since their inhibition increases temocillin MIC of 1 two-fold dilution only. For this reason, we did not specifically look for mutations or reduced expression of these porins in the collection. We cannot exclude that other porins expressed by *P. aeruginosa*[32] that were not investigated here also play a role in temocillin uptake. We acknowledge this is a limitation of this work.

Considering all data, and despite remaining uncertainty regarding the reason for variable susceptibility to temocillin in mutated isolates, our work brings three major pieces of scientific progress. Firstly, from a clinical perspective, we demonstrate a potential use for temocillin in the management of *P. aeruginosa* infections in CF patients. Although limited to a small proportion of isolates, this is important as temocillin susceptibility was observed for some isolates that were resistant to other β-lactams, either by production of β-lactamases or by alteration of the OprD porin, as well as in isolates belonging to clones described as multidrug-resistant. Thus our data call for introducing temocillin in routine susceptibility testing for *P. aeruginosa* collected from CF patients. Moreover, temocillin may offer the advantage of acting at the same time on *Burkholderia* spp[5] that can co-infect CF patients. Second, in a context of drug design, we observed a large diversity of mutations in *mexA* and *mexB,* opening perspectives for studying their role in

substrate recognition and/or transport activity. The mutants described here could also be used to study the efflux of other antibiotics and to help delineating how these interact with specific domains of MexA and MexB. Third, from an ecological perspective, our data reinforce the concept that a functional MexAB-OprM pump is not needed for *P. aeruginosa* survival in the CF lung[29] and illustrates the high adaptability of this bacteria to its environment.

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Author contributions

H.C., D.P., Y.B., and H.R.V. performed the experiments. H.C., P.M.T., and F.V.B. designed the studies. P.M.T., M.W., and F.V.B. supervised the work. D.P., Y.B., H.W., M.M.T., J.S.E., P.P., B.C.K., and O.D., collected or provided biological material and gave useful suggestions. H.C. and F.V.B. wrote the manuscript and all the authors revised the final text and agreed with its submission.

Competing financial interest

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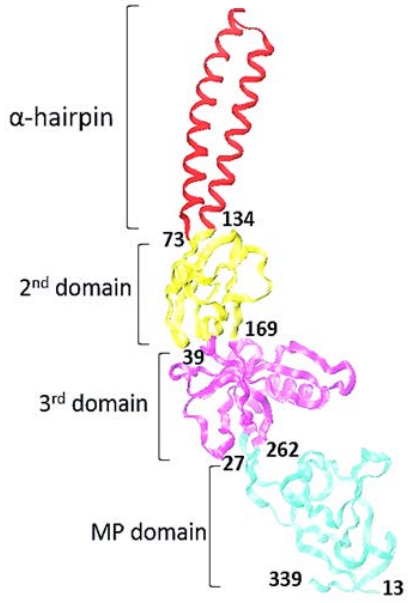
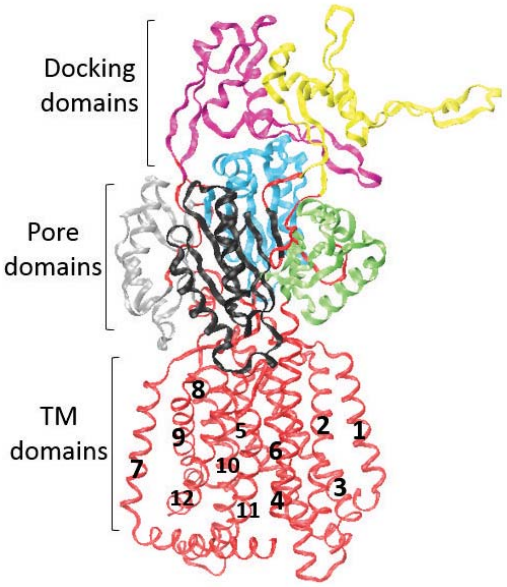
SUPPLEMENTARY MATERIAL

Fig. S1. Molecular representations of MexA and MexB monomers in PAO1, with indication of their respective domains (based on references[1-3]).

The MexA rod domain (constructed by the long α-helical hairpin of twisted coiled-coil) interacts with OprM. The second domain (adjacent to the rod α-helical domain; globular; cluster of 8 short β-sheets) interacts with OprM and MexB. The third domain (globular; 7 short β-sheets and one short α-helix) and membrane proximal domain (MP, β-roll) are located distal to the α-helical rod. They interact with the distal domain of MexB.

The multidrug transporter MexB has 12 transmembrane segments (TMs) inserted in the inner membrane. The periplasmic pore consists of 4 subdomains (PC1, PC2 [periplasmic, C-terminal], PN1, PN2 [periplasmic, N-terminal]), which are directly involved in the export of substrates. The docking domain (DC [docking, C-terminal] and DN [docking, N-terminal] subdomains) is at the periplasmic tip of MexB and interacts with the outer membrane protein (OprM). The DN subdomain forms a long protruding loop that inserts into the docking domain of the neighbouring MexB subunit.

Structure of MexA monomer (PAO1)	Structure of MexB monomer (PAO1)
The 4 domains are represented with AA residues numbered according to the *P. aeruginosa* MexA sequence: α-hairpin (red), second (yellow), third (violet), and membrane proximal MP domains (cyan).	*P. aeruginosa* MexB docking domains: DC (violet), DN (yellow); pore domains: PC1/ PC2 (Dark/clear grey), PN1/ PN2 (cyan, lime); transmembrane segments: TMs are numbered from 1 to 12.
	

References to Fig. S1:

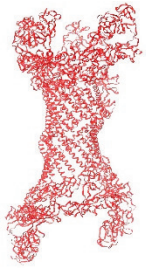
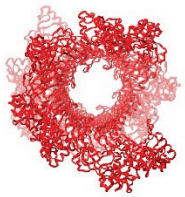
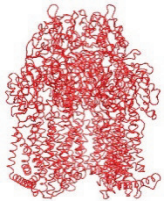
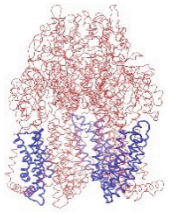
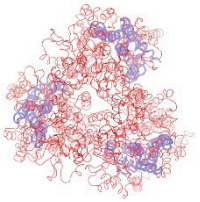
1. Akama H., *et al.* Crystal structure of the membrane fusion protein, MexA, of the multidrug transporter in Pseudomonas aeruginosa. *J Biol Chem.* **279,** 25939-25942 (2004).
2. Symmons M. F., Bokma E., Koronakis E., Hughes C., Koronakis V. The assembled structure of a complete tripartite bacterial multidrug efflux pump. *Proc. Natl. Acad. Sci. U S A* 106, 7173-7178 (2009).
3. Sennhauser G., Bukowska M. A., Briand C., Grutter M. G. Crystal structure of the multidrug exporter MexB from Pseudomonas aeruginosa. *J. Mol. Biol.* **389**, 134-145 (2009)

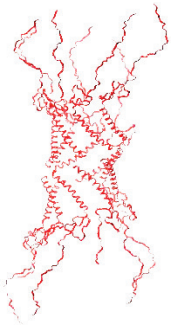
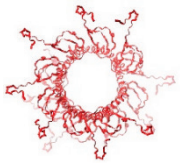
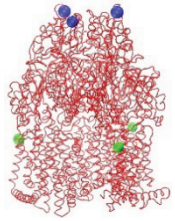
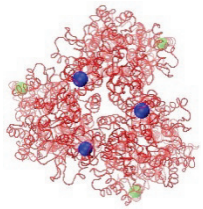
Table S1: General description of the collection from 1996-2012

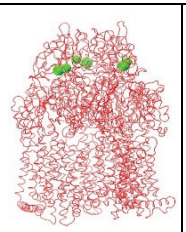
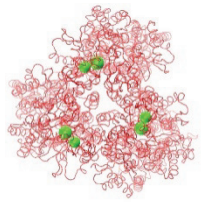
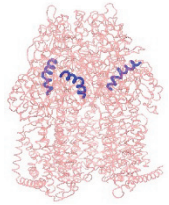
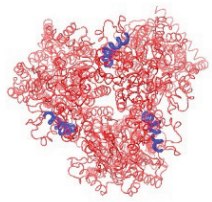
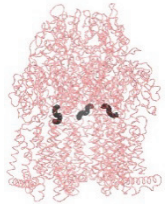
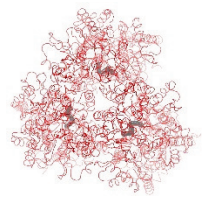
Country	Number of isolates	Number of patients	Year of sampling
United Kingdom	99	46	2006-2009
Belgium	88	37	2010
France	80	36	1996-2012
Germany	66	36	2012
Total	333	155	

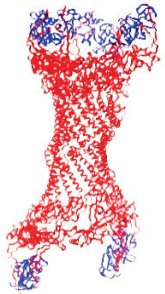
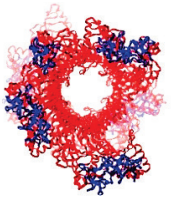
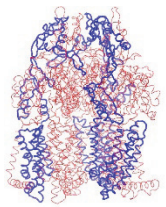
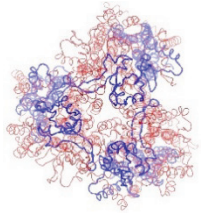
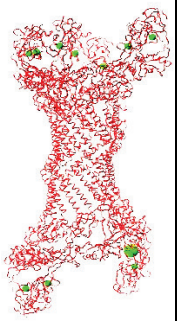
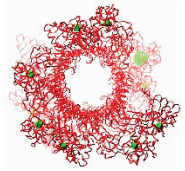
Table S2. Molecular representations for MexA and MexB proteins in clinical isolates of *P. aeruginosa* isolated from cystic fibrosis patients, as compared to the wild-type PAO1.
Color code for tables S2a-e: blue: deleted residues; green: nonsynonymous substitutions of amino acids; black: tandem repeated sequence of amino acids; red: encoded parts of MexA and MexB proteins.

Table S2a: non-clonal isolates

Isolates	TMO MIC (mg/L)	Protein length (AA)		Encoded MexA		Encoded MexB	
		MexA	MexB	Side view	Top view	Side view	Top view
PAO1 wild type	512	383 (13-mer)	1046 (trimer)				
3724	2	383	878	Synonymous mutations			

144	2	124	1046			Synonymous mutations
143-1	4	0	1046	Nonstop mutation in *mexA* (proteolysis; recycled ribosome)		Synonymous mutations
129	8	383	1045	Synonymous mutations		

126	8	383	1046	Radical missense mutation (S19L) in the signal sequence of MexA		
279	16	383	1027	No mutations		
109	64	383	1051	Synonymous mutations		

618	128	297	1046			Synonymous mutations
3179	128	383	719	Synonymous mutations		 
180-3	128	383	1046			Synonymous mutations

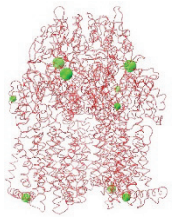
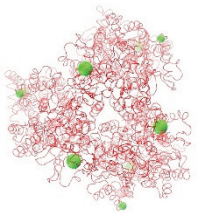
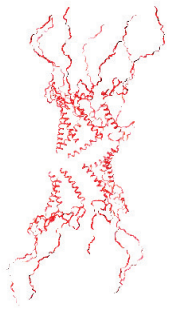
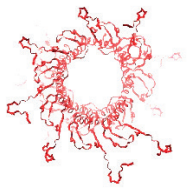
3319	256	383	1046	Synonymous mutations		
4289	1024	383	1046	Synonymous mutations	Synonymous mutations	

Table S2b: LES clonal isolates

Isolates	Patient ID, country and date of collection	TMO MIC (mg/L)	Protein length (AA)		Encoded MexA		Encoded MexB	
			MexA	MexB	Side view	Top view	Side view	Top view
BM1, AJ3, CF15, CF53, CF19	AD, UK (Sept. 2006), ML, UK (May 2006), CT, UK (2007) DP, UK (2007) LS, UK (2007)	16	119	1046			Synonymous mutations	
CF16	RC, UK (2007)	32	383	30	Synonymous mutations			

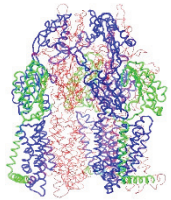
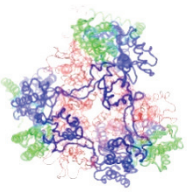
BV1	DC, UK (Oct. 2006)	32	383	672	Synonymous mutations		

Table S2c: isogenic isolates from 2 other pairs of patients in the same country, sharing the same mutations

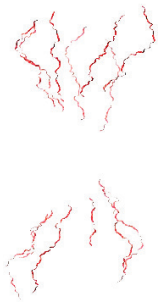
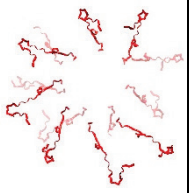
Isolates	Patient ID, country and date of collection	TMO MIC (mg/L)	Protein length (AA)		Encoded MexA		Encoded MexB	
			MexA	MexB	Side view	Top view	Side view	Top view
191-4, 207	191, Germany (2012), 207, Germany (2012)	64	27	1046	Nonsense mutation: G82T		Synonymous mutations	
W024, W049	DM, UK (2009), DB, UK (2009)	4	69	1046			No mutations	

Table S2d: isogenic isolates from three different patients originating from different coutries

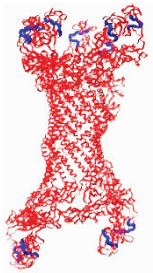
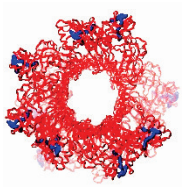
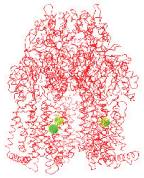
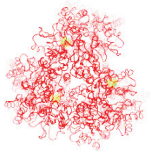
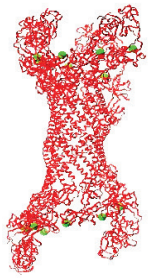
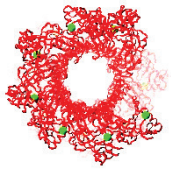
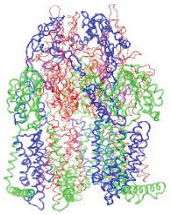
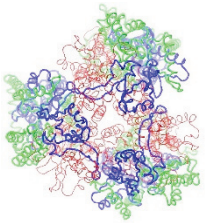
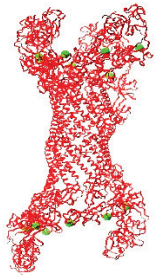
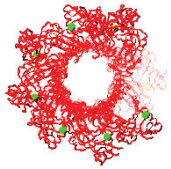
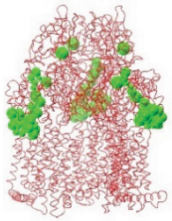
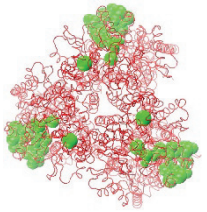
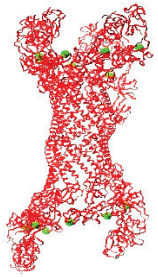
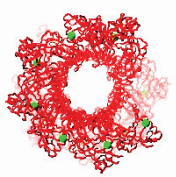
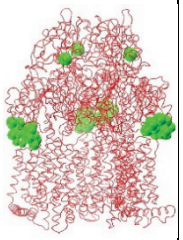
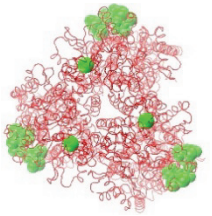
Isolates	Patient ID, country and date of collection	TMO MIC (mg/L)	Protein length (AA)		Encoded MexA		Encoded MexB	
			MexA	MexB	Side view	Top view	Side view	Top view
AG3	JP, UK (May 2006)	8	372	1046			Synonymous mutations	
128	DAF69, Belgium (Oct. 2010)	1024	383	1046	Synonymous mutations			
129-6	129, Germany (July 2012)	1024	383	1046	Synonymous mutations		Synonymous mutations	

Table S2e: isogenic isolates from 2 different patients originating from the same country

Isolates	Patient ID, country and date of collection	TMO MIC (mg/L)	Protein length (AA)		Encoded MexA		Encoded MexB	
			MexA	MexB	Side view	Top view	Side view	Top view
135-1	135, Germany (July 2012)	8	383	719				
208-3	208, Germany (Aug. 2012)	256	383	1045				

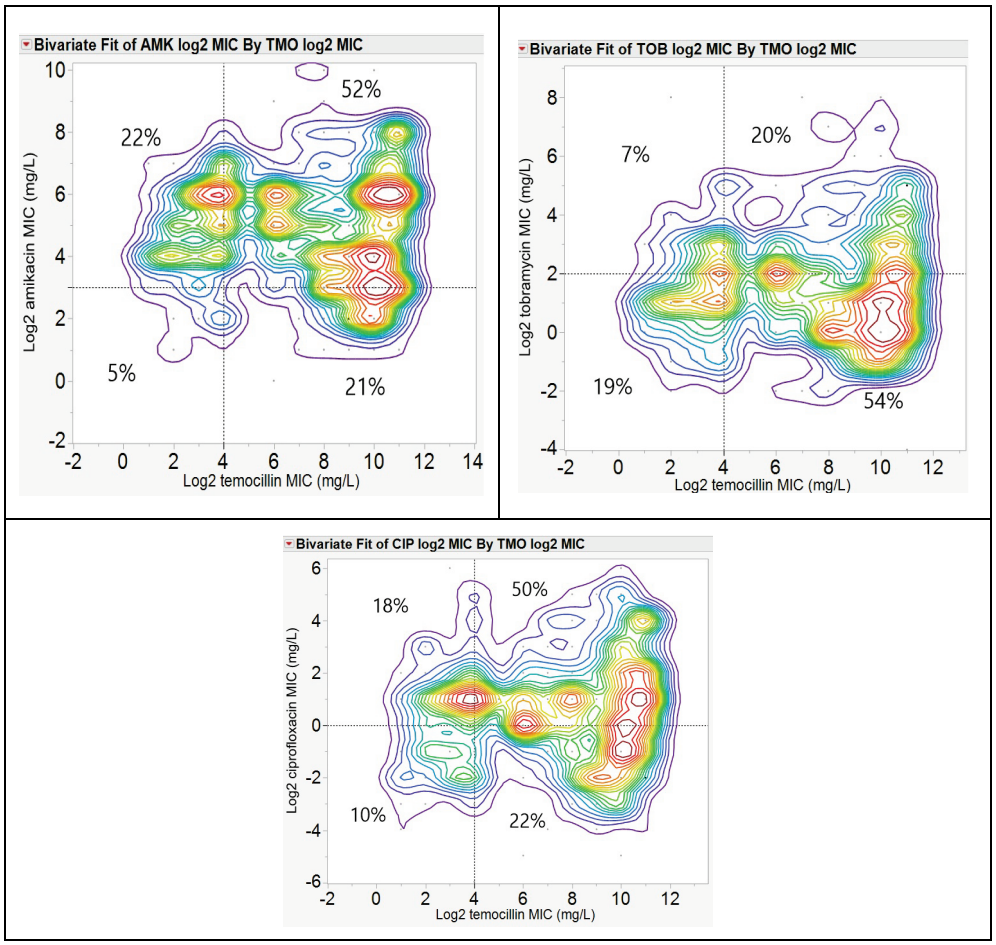
208-2	208, Germany (Aug. 2012)	512	383	1045				

❖ Supplementary data related to temocillin article

We conclude from our study that temocillin should be included in susceptibility testing of CF isolates. We therefore also examined whether it could offer an interest against isolates resistant to current therapeutic options. To this effect, we analysed correlations between MICs of temocillin and aminoglycosides (amikacin, tobramycin) or fluoroquinolones (ciprofloxacin) for each individual isolate in the whole collection (n=333), using quantile density contour analysis (JMP® pro version 12, SAS Institute Inc., Cary, NC, USA).

The data are presented in figure 1.7. About half of the collection was co-resistant to temocillin and amikacin or to temocillin and ciprofloxacin, while only 20% was co-resistant to temocillin and tobramycin. Interestingly enough, a non-negligible percentage of isolates was intermediate/resistant to amikacin (22%) or to ciprofloxacin (18%) but susceptible to temocillin, indicating the presence of specific resistance mechanisms affecting the activity of amikacin and ciprofloxacin. Yet, only 7% of isolates were found to be resistant tobramycin and susceptible to temocillin. Temocillin susceptible isolates harbour mutations inactivating efflux mediated by MexAB-OprM. Ciprofloxacin is a universal substrate for efflux in PA, including MexEF-OprN [239]. Amikacin, but not tobramycin, is specifically substrate for MexXY-OprM [162]. Based on the concept of resistance cost, we may suggest that overexpression of efflux pumps like MexEF-OprN and MexXY-OprM could be linked with the inactivation of MexAB-OprM as a sort of compensatory mechanism to allow the bacteria extruding toxic compounds at a sufficient rate and could be responsible for resistance to ciprofloxacin and amikacin in temocillin-susceptible isolates. This concept of efflux unbalance has been previously suggested [240-242], and considered to play a role in the capacity of PA to chronically colonize the lungs of CF patients [166;241]. This illustrates again the fitness costs for PA long-term adaptation to CF-lungs, and comforts the concept that MexAB-OprM pump is not needed for this adaptation. In order to verify this hypothesis, we are currently determining the expression level of the main efflux systems in the collection.

Figure 1.7: Co-resistance between temocillin (TMO; abscissa) MICs and aminoglycosides or fluoroquinolones MICs (ordinates) [amikacin (AMK), tobramycin (TOB) or ciprofloxacin (CIP)]. The intensity of each zone (from deep red to violet) is indicative of the proportion of isolates (from large to small) with MICs at the corresponding coordinates. The broken lines point to the MIC value above which the isolates are considered resistant according to EUCAST interpretive criteria for each antibiotic and the figures indicate the percentage of isolates in each quadrant.



3. Discussion and Perspectives

3.1 Main findings of this work

a) Resistance and clonality in CF isolates from Europe

This work allowed us to investigate the interplay between three key microbiological aspects of *Pseudomonas* infections in CF patients from Belgium, Germany and UK: the antimicrobial resistance, the occurrence of transmissible clonal isolates, and the individual heterogeneity of isolates in CF patients.

The antimicrobial resistance rates were high in the three European countries, with globally lower resistance in Belgium and higher resistance in Germany and UK. The multidrug-resistance of CF isolates reflects the selective pressure exerted by the intense use of different class of antibiotics. In parallel, we did not observe any significant correlation between the patient's age when the isolate was collected and the number of antibiotic classes to which the isolate was resistant. This absence of correlation could result from (1) differences in therapeutic management by CF caregivers among these three centres, which influence the emergence and selection of resistance in CF patients [243], (2) the presence of mutator variants evolving sporadically, as previously described [44].

Although limited, differences in resistance rates between the three countries are raising questions about segmentation of clone distribution. An important genetic diversity is observed among isolates collected in Belgium and Germany. In contrast, the presence of cross and co-resistance to different classes of antibiotics in UK is associated with the dissemination of a specific multidrug-resistant clone, namely the "Liverpool Epidemic strain LES B58". Of interest, we observed different resistance profiles within LES B58 clone, which underwent further interpatient spread. This spread and transmission of clonal strains of PA between and within hospitals has been previously

reported [44-46]. The higher resistance in German isolates is essentially related to the presence of more sporadic multidrug-resistant clones, among which a new clone with sequence type 'ST2254' was discovered. This new ST was distinct from the sequence types ST146 (LES epidemic clone; 5 alleles different) and ST958 or ST17 (6 alleles different), which belong to the largest group "ST111" of MDR/XDR epidemic clones of PA [57].

Global studies of PA populations concluded that CF isolates present a high genetic diversity but nevertheless belong to a 'core lineage' ubiquitous in the natural environment [244]. This concept was illustrated by the absence of acquired resistance genes coding for carbapenemases in the whole collection of CF isolates, which indicates that this *Pseudomonas* population is specific and different than that of the intensive-care units which may acquire carbapenemase genes by horizontal transfer [245].

Further, the individual population of PA could be heterogeneous for CF patients, and one patient may harbour in his/her lungs clonally-related and/or different isolates with diverse mutations affecting genes that encode efflux systems or OprD porin, for instance.

❖ **Global MDR/XDR clones and their derivatives: the case of ST958**

One clone, the ST958 was found in the three countries we investigated. This indicates the high spreading and colonisation abilities of this clone for different CF patients in Europe, or that the clone existed already in each country but evolved differently. ST958 shows different antibiotic resistance patterns among the different countries, suggesting different paths of resistance development. Indeed, recent concerning reports have provided evidence of the existence of MDR/XDR epidemic clones, disseminated in hospitals worldwide: ST235, ST111, and ST175 associated with hospital acquired infections [57]. Nowadays, "person-to-person" transmission [44;246] is an accepted route of PA acquisition among CF patients and certain clones are extensively recognized as epidemic and/or transmissible being world-wide distributed such as ST146 (LES), ST649 (AES-1,

Australian or Melbourne Epidemic Strains), ST775 (AES-2), ST242 (AES-3), ST17 (clone C, highly prone to infect CF patients in Canada, England, France and Germany [247]), ST217 (Manchester 1), ST148 (Midlands 1), ST274, ST782, DK1/DK2, ST406, ST497, ST192 (PES), Houston-1, Norway cluster 1, Ireland cluster. Although relationship between resistance mechanisms, virulence factors (production of cytotoxins e.g. ExoS, ExoT, ExoU, or ExoY) and colonisation/transmissibility of MDR/XDR epidemic clones is not yet well established to date, these strains show increased antibiotic resistance, long-term persistence within CF individuals, and are frequently associated with higher morbidity and mortality ([57;248] and references cited therein).

As for LES [249], AES isolates have shown increased antibiotic resistance, increased virulence gene expression and higher morbidity and mortality during chronic infection [250]. Concerning the clone C (ST17), characteristics such as biofilm formation and antibiotic resistance are not enhanced and it is not clear whether the prevalence of such strain types in CF is attributable to transmissibility, virulence or merely a reflection of their environmental abundance [247].

ST958 is closely related to ST17 clone (only one nucleotide difference). These two STs, together, belong to the international clonal complex ST111, which is the founder of subgroups from which an important number of MDR/XDR STs derive. Interestingly, the clone ST958 were among CF clones that showed high-level resistance to meropenem by active efflux in the absence of carbapenemases. Although still far from for ST235, ST111 epidemic clones have also been found to be associated with remarkable number of different acquired genetic structures, mostly integrons containing VIM, IMP, GES, or OXA enzymes. The integron structure found to be more geographically widespread among ST111 isolates is one containing *bla*$_{\text{VIM-2}}$ gene [251-253].

Our data was coherent with these studies and showed also that all our hospital-acquired pneumonia (HAP) isolates with high-level resistance to meropenem were VIM-2 producers and belong to the clone ST111. It also

indicates that the vast majority of the emerging XDR isolate (including ST958) belongs to wide-spread clone ST111, which can genetically diversify to generate variants with or without the acquisition of carbapenemases gene acquisition by using different resistance strategies like high-level efflux mechanism. Indeed, the high prevalence and diversity of exogenous genetic elements in ST111 clone has been suggested to be driven by the loss of the clustered regularly interspaced short palindromic repeats system (CRISPR-Cas), which acts as a bacterial defence system against foreign DNA, such as phage DNA and plasmids [253;254]. Thus, our clone ST958 showing no acquisition of carbapenemase genes may still have this bacterial defence system against foreign DNA and resist extensively by alternative strategies, highlighting the efflux mechanism as a special feature in this clone until its new diversification to a new variant. As an epidemic clone, ST111 is still evolving differently and dangerously. The early detection of ST111 and its derivative ST958, should have major epidemiological, infection control and public health relevance, by helping to avoid their spread in the hospital environment, as proposed for epidemic XDR clones.

MLST is likely the currently most widely accepted gold standard for the detection of MDR/XDR epidemic clones, but other faster typing techniques have been shown to provide satisfactory results such as: Multiple-Locus Variable Number Tandem Repeat Analysis (MLVA) [45], Double-Locus Sequence Typing (DLST) [255], matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) [256]. Whole genome sequencing approaches provide further valuable information in outbreak investigations, including the elucidation of the transmission dynamics and the time frame for the acquisition of resistance genes (e.g. VIM-2 acquisition in ST111 clone [254]). Therefore, the detection of MDR/XDR epidemic clones directly from clinical samples could be a future useful tool for the establishment of early appropriate targeted antimicrobial therapies. Otherwise, the development of specific control strategies, such as vaccines would be useful to fight back the world-wide dissemination of epidemic clones [257].

b) Role of efflux in high-level resistance to β-lactam antibiotics and β-lactamases inhibitors

We have shown that active efflux by the RND superfamily is a predominant resistance mechanism in this collection of PA isolates from CF patients. Active efflux appears as a key mechanism behind resistance to β-lactam antibiotics like meropenem and temocillin, or to the new β-lactamase inhibitor avibactam. Our findings create concern that even the development of new antimicrobial agents may encounter clinically important resistance in a context of multidrug-resistant bacteria. The design of antibiotic treatment strategies in the future must consider these factors.

❖ **Acquired resistance : meropenem and ceftazidime-avibactam**

Along with colistin and tobramycin, carbapenems (imipenem, meropenem) were the most active drugs against our collection of isolates from CF patients. However, a high-level of resistance to meropenem was observed in this population of CF isolates in the absence of carbapenemase activity.

Efflux pumps of the RND superfamily (mainly MexAB-OprM) are not only important players in the high-level resistance to meropenem, but are integral to conferring a paradoxical phenotype where meropenem shows less activity than imipenem. As active efflux can also confer cross-resistance to other antipseudomonal agents, i.e. other β-lactams or quinolones for example, determining the mechanism of resistance to meropenem is highly recommended in clinical settings in order to optimize the antibiotherapy.

Another example of resistance by high-level efflux was seen in the study of ceftazidime/avibactam combination. The study showed that poor membrane permeability due to overexpressed efflux and downregulated OprD porin systems impede strongly the activity of avibactam, coherently with the summary of product for ceftazidime/avibactam combination [236] and recent studies [237;238]. Moreover, our data ask the following questions: (1) is the concentration of avibactam sufficient in isolates overexpressing efflux,

downregulating porins or overexpressing specific β-lactamase(s)? (2) is there a production of avibactam-insensitive β-lactamase(s)?

❖ **Intrinsic resistance to temocillin**

Antibiotic resistance is often attributed to the horizontal acquisition of new genes by previously susceptible bacteria [8], or the occurrence of spontaneous mutations within chromosomally located genes that are subsequently transmitted vertically within a bacterial lineage [258]. In addition to the ability of bacteria to 'acquire' resistance, they are also intrinsically resistant to different classes of antibiotics; a trait that is universally found within the genome of bacterial species and not accredited to horizontal gene transfer. A conventional example of intrinsic antibiotic resistance is the multidrug resistant (MDR) phenotype of Gram-negative bacteria, which are insensitive to many classes of clinically effective Gram-positive antibiotics. The molecular basis of this phenomenon is the presence of the Gram-negative outer membrane, which is impermeable to many molecules, and expression of numerous MDR efflux pumps that effectively reduce the intracellular concentration of the given drug [259].

The intrinsic resistance of PA to various β-lactam antibiotics is due to the interplay of different factors [260;261]. The wild-type strains of PA extrude hydrophilic β-lactams using the RND family efflux pumps. Previous studies [174;262-264] have shown that PA is intrinsically resistant to carbenicillin that belongs to the class of "carboxypenicillins", along with temocillin. In the same context, our data shows that intrinsic resistance of PA to temocillin is due to active efflux by the constitutively-expressed transporter MexAB-OprM, explaining the lack of activity of temocillin against the wild-type strains of PA. Again, this confirms that the intrinsic resistance is independent of the selective pressure imposed by the clinical use of antibiotics.

c) Spontaneous mutations in efflux genes and modulation of intrinsic resistance

We showed that natural mutations in *mexA* and *mexB* may negatively modulate the efflux activity of the MexAB-OprM transporter toward temocillin. These mutations seem to occur specifically in CF isolates but not in other clinical isolates, like those collected from patients suffering from hospital-acquired pneumonia (HAP) [178]. Meanwhile, the development of high-level susceptibility to temocillin appears to occur efficiently in a PA mutator background such as the Liverpool Epidemic Strain (LES B58) among CF patients in the UK, where multiple mutations lead to downregulation and/or structural modifications of MexA and MexB proteins. In the same context, previous studies has shown that a relatively high prevalence of hypermutable PA in CF patients was associated with surprisingly low antibiotic resistance levels [265-267]. Consequently, resistance or susceptibility to a particular drug class may emerge even when the patient has never been treated with that drug class [268].

Noteworthy, the ST958 MDR clone that has been found in the three countries (first paper, section 2.2.i, table S1, patient JP) behaved differently in UK and was susceptible to temocillin and other antibiotics except ciprofloxacin, amikacin and/or tobramycin. This shows again that this clone may have adapted again by efflux unbalance, which appears as a key mechanism for the establishment of chronic infections in the lungs of people suffering from CF. Given this asymmetry in efflux balance, our study shows the importance of compensatory evolution in maintaining resistance. This concept should not be underestimated and highlights the need to understand how selection at other sites in the genome influences the dynamics of resistance alleles in clinical settings [269].

d) New therapeutic options in CF: revived temocillin or ceftazidime-avibactam

We have discussed the high prevalence of *mexAB* mutations in CF isolates, which make temocillin active against PA. Incidentally, these mutations were also beneficial for the activity of avibactam, which appeared to be also a substrate for MexAB-OprM. Therefore, ceftazidime-avibactam and temocillin may be used as sparing agents against meropenem-susceptible isolates in CF patients. Of note, while both ceftazidime-avibactam and meropenem loose activity in case of OprD porin alterations, which is not the case for temocillin. It may thus offer an advantage over the two other molecules in OprD mutants if also mutated in *mexAB*.

❖ Revived temocillin

Bacteria are primarily classified according to Gram staining. Antimicrobials are classified based on the mechanisms of action and the specific types of bacteria they target. Our findings on temocillin activity in CF define a new concept of classifying antibiotics according to their activity in specific populations of patients like the CF population. We show indeed that this drug could be specifically useful in this population, thanks to the presence of isolates with deficient MexAB-OprM efflux system. Tracking the behaviour and mutational changes of bacterial population infecting a specific patient population appears thus as an important indicator for choosing the correct antibiotic treatment.

Noteworthy, temocillin showed promising activity against LES and ST958 clones belonging to MDR clonal complexes, which are present in UK and among the three European countries, respectively. Even more, a fraction of the isolates that were susceptible to temocillin remained resistant to other antipseudomonals i.e. ticarcillin, piperacillin/tazobactam, ceftazidime and meropenem. These isolates were resistant either by production of β-lactamases (mainly AmpC type) or by alteration of the OprD porin, since temocillin is stable to most β-lactamases and does not use OprD to enter

inside PA. Temocillin may offer the possibility of using as single drug to act at the same time on *Burkholderia* spp that can co-infect CF patients [199], or in combination with aminoglycosides as demonstrated previously *in vitro* [186]. This study has shown that temocillin in combination with aminoglycoside antibiotics exerted a synergistic or partially synergistic effect against the majority of strains of PA tested. Unlike cefoxitin, temocillin exhibited no antagonism against isolates of Enterobacteriaceae producing inducible cephalosporinases, when combined with piperacillin and third generation cephalosporins (e.g. cefotaxime). For instance, temocillin may be combined with aminoglycosides and help CF caregivers to cope with difficult situations while sparing other agents like carbapenems, especially that temocillin have little apparent potential to select for *Clostridium difficile* infections. Therefore, testing for temocillin susceptibility of PA isolated from CF patients appears potentially useful.

❖ **Ceftazidime-avibactam**

Not much is known about the ability of avibactam to restore the activity of ceftazidime towards PA isolated from CF patients. Taking advantage of an existing large collection of clinical isolates from clinically-confirmed cases of CF in 4 European countries, our study [270] shows that the association of avibactam to ceftazidime significantly increases the susceptibility of MDR-PA.

Combined with ceftazidime, avibactam may therefore stand as a useful part of our chemotherapeutic armamentarium in situations where *Pseudomonas* resistance is due to the expression of avibactam-susceptible β-lactamases. Ceftazidime-avibactam could be recommended in clinical practice for CF patients. However, more clinical trials are needed in order to establish the suitable dosing regimen. We have seen indeed that the concentration of avibactam may be suboptimal in part of the isolates due to overexpression of AmpC-type cephalosporinases and/or poor permeability to the compound due to porin mutations and/or active efflux. The prudent use of ceftazidime-avibactam should be promoted in isolates showing susceptibility in order to preserve its efficacy and prevent the selection of resistant clones.

3.2 Carbapenems, ceftazidime/avibactam and temocillin: pros and cons

Based on the contributions of this thesis and literature data, we review here the pros and cons of these molecules in CF patients (table 1.8).

a) Carbapenems

PROS

Carbapenems have a broad spectrum of antibacterial activity against Gram-(+), Gram-(-) and anaerobe bacteria. They are important agents for the treatment of pulmonary exacerbations in CF patients with multidrug-resistant PA, *Burkholderia cepacia* or Enterobactericeace, and with hypersensitivity reactions or resistance to other β-lactam drugs. The unique molecular structure of carbapenems confers exceptional stability against most β-lactamases including AmpC-type cephalosporinases and the extended spectrum β-lactamases (ESBLs). Carbapenems proved effective in the treatment of acute or chronic lung infections with MDR-PA in CF, as a monotherapy on in combination with other class of antibiotics like aminoglycosides [94;95]. Studies have shown that meropenem is more active than imipenem against PA [271]. However, our study contradicts these data in the context of CF, but globally confirms the stability and efficacy of carbapenems against MDR-PA.

CONS

Carbapenem resistance mechanisms have emerged under the pressure of carbapenem use in clinical settings and may be classified as enzymatic, mediated by carbapenemases (emergence of carbapenemases like KPC-, IMP-, VIM-, NDM-, OXA-, and GES/IBC-types enzymes), and non-enzymatic [163]. Carbapenem resistance among PA develops frequently due to the concomitant presence of more than one mechanism: plasmid or integron-mediated carbapenemases, increased expression of RND efflux systems, reduced OprD porin expression and increased AmpC-type cephalosporinase

activity. However, the acquisition of carbapenemases genes does not seem to occur frequently in CF patients.

Carbapenems enter into the periplasmic space of PA through the OprD outer membrane porin. Diminished expression or loss of the OprD porin is rather frequent during carbapenems treatment and leads to reduced susceptibility, especially with imipenem that could also induce the expression of chromosomal cephalosporinases. While imipenem is not affected by the efflux activity of RND systems, high-level resistance to meropenem are observed in our CF isolates when active efflux coexists with reduced OprD expression and increased cephalosporinases activity.

The drug stability of carbapenems in solutions for continuous infusion is reduced at room temperature. Therefore, their home administration using portable pumps becomes difficult for patients with CF, especially in tropical countries with high ambient temperatures (> 30°C) [272]. Carbapenems pose similar risks as other β-lactam antibiotics, and adverse effects are rare but seizures could occur, especially with imipenem administration. They generally exhibit good activity against anaerobes and could be responsible for antibiotic associated diarrhoea (AAD).

b) Ceftazidime-avibactam

PROS

Ceftazidime/avibactam possesses *in vitro* efficacy against CF isolates of MDR-PA, as demonstrated in our study. In vitro, it showed activity against most MDR Gram-(-), including AmpC-type, most ESBL producing and carbapenem-resistant strains. Ceftazidime-avibactam could be used as a potential alternative to carbapenems for lung infections associated with CF patients against documented ceftazidime-resistant PA and Enterobacteriaceae. Ceftazidime has long-term stability (up to 24h) in continuous infusion [272] and could be combined with avibactam (stability ~12h in the infusion bags, [236]) for CF home therapy.

CONS

Ceftazidime is not usually affected by efflux, while recent studies showed that avibactam could be subjected to efflux and OprD-mediated resistance. As demonstrated in our studies on ceftazidime/avibactam combination, avibactam could not restore the activity of ceftazidime against CF isolates of PA overexpressing efflux pumps (MexAB-OprM / MexEF-OprN) or showing OprD porin alterations. In addition, no or few clinical data is available about the safety and efficacy of this drug combination against lung infections in CF, pneumonia, and bacteremia.

c) Temocillin

PROS

Temocillin is primarily used against lower respiratory tract infections with Enterobacteriaceae or *B. cepacia* in CF, and is stable against AmpC-type cephalosporinases, extended spectrum β-lactamases (ESBLs) and some carbapenemases (table 1.2). It is also active against PA isolates that shows deficiency in MexAB-OprM pumps or even OprD porin, such as the case of isolates from CF patients shown in our study. Since temocillin is not OprD-dependant, it could be useful as a potential alternative for carbapenems against CF lung infections with PA lacking OprD porin. In contrary to carbapenems, temocillin proved long-term stability in elastomeric pumps for outpatient antibiotic therapy in CF patients [273].

CONS

Temocillin shows no activity against Gram-positive and against PA isolates in which active efflux remains effective. It is inactivated by most metallo-β-lactamases (e.g. VIM-, IPM-, and NDM-type) or OXA-48-producing PA. Temocillin could not be used as empiric therapy for acute infections and lung exacerbations with Gram-positive infections. This is important to take into account in CF patients who are particularly vulnerable to *S. pneumoniae* infections, against which temocillin showed no activity. Furthermore, since the *in vitro* susceptibilities do not always predict treatment outcome [75], more clinical trials are warranted to establish the efficacy of temocillin against

documented infections by PA deficient in MexAB-OprM mediated efflux. Otherwise, the population of PA in CF patients could be individually heterogeneous and one patient could harbour several clones of PA with different pattern of resistance. Therefore, if a patient harbours two PA isolates with different susceptibility profiles, temocillin could be active only against the isolate with altered MexAB-OprM function, unless the use of efflux inhibitor in combination for the resistant phenotype.

In addition to active MexAB-OprM efflux system in resistant or intermediate isolates, we have also shown that OpdK/OpdF-porin loss may also play a marginal role in the resistance to temocillin.

Previous study [242], has shown that the hypersusceptibility to β-lactams could occur spontaneously in CF isolates, but they added that changes in expression of several intrinsic mechanisms in the hypersusceptible isolates did not correlate always with the observed phenotype, highlighting the complex interactions of resistance mechanisms in PA and their roles in drug susceptibility. As shown in temocillin paper, the overproduction of extracellular polymeric saccharides by PA isolates in CF lungs could impede the activity of temocillin even in MexA/MexB deficient isolates, by retarding the diffusion of temocillin to reach its target at the bacterial periplasmic space.

Furthermore, it was shown previously that temocillin concentrations in the sputum ranged from 1.5 to 2.8 mg/L [193]. Therefore, the use of temocillin could be inadequate for the treatment of respiratory *Pseudomonas* infection in CF if temocillin levels fall below a specific clinical breakpoint of 8 or 16 mg/L. In this context, temocillin dosage regimens for CF patients should be optimised when using the conventional routes, or switching to inhalation mode of administration to insure high local concentrations.

Table 1.8: Carbapenems, ceftazidime/avibactam versus temocillin: pros and cons in CF patients

Drugs	PROS	CONS
Carbapenems	✓ Active against Gram-(+), Gram-(-), anaerobes including most ESBL- and AmpC-type producers ✓ Efficacy and safety data for CF-related infections	✓ Reduced activity by reduced permeability of bacterial envelope ✓ OprD porin-dependant ✓ Meropenem is efflux-dependant ✓ Frequent dosing - three times daily dosing ✓ Short-term stability in portable devices for home-based therapy in CF patients ✓ Seizures could occur, especially with imipenem administration. ✓ Reported cases of antibiotic-associated diarrhoea (AAD)
CAZ/AVI	✓ Efficacy and safety (data for cUTI and cIAI) ✓ *In vitro* activity against most MDR Gram-negatives, including most ESBL producing and carbapenem-resistant strains ✓ *In vitro* efficacy against CF isolates of MDR-PA ✓ Long-term stability for continuous infusion and CF home therapy ✓ Rare antibiotic-associated diarrhoea as reported for systemic antibacterial drugs	✓ Limited activity against Gram-(+) bacteria, anaerobes ✓ Reduced activity by reduced permeability of bacterial envelope ✓ OprD porin-dependant ✓ Ceftazidime activity may be affected by alginate production in PA [274]; [275] ✓ No or few clinical data for other infections (e.g., CF, pneumonia, bacteremia) ✓ Limited anaerobic activity
Temocillin	✓ Active against Gram-(-) including most ESBL-, AmpC-type and some carbapenemases producers ✓ Extruded intrinsically by MexAB-OprM of PA but in CF isolates, cons turned to pros ✓ Long-term stability of temocillin in elastomeric pumps for outpatient antibiotic therapy in CF patients [273] ✓ OprD porin-independent (usage as carbapenem-sparing agent) ✓ Efficacy and safety (data for cUTI, pneumonia, bacteremia) ✓ Rare antibiotic-associated diarrhoea	✓ Limited activity against Gram-(+) bacteria, anaerobes and wild strains of PA ✓ Reduced activity by increased MexAB-OprM efflux ✓ Degraded by VIM, IMP, OXA-48, NDM carbapenemases ✓ Reduced activity associated with exopolysaccharides overexpression ✓ No clinical breakpoint against PA infections in CF patients ✓ Higher cost compared to standard therapy

3.3 Limitations of this work

This work provides more insights about old and new antibiotics agents that that could help in the management of *Pseudomona*s lung infections in CF patients. Yet, we have to acknowledge also some of its limitations.

a) Criticising our collection of CF isolates: can't we do better?

The sampling of PA isolates from the 155 CF patients was done during periodic routine examinations. Therefore, samples may not correspond to the first isolate of PA for each CF patient. Moreover, we do not know the age of first colonization for each patient and some replicates belonging to the same clone and showing the same resistance pattern were isolated from the same patient. Collecting first isolates from individual patients and then sequential isolates would be ideal in order to follow the accumulation of resistance overtime or the presence of mutator variants. Yet, assembling this type of collecting is highly challenging because it is difficult to make sure that patients has never been colonized by PA before the first sampling.

In addition, we do not have a complete view of the treatments received by the patients. This reflects however the clinical situations, because these patients receive so many antibiotics that they are not systematically indicated in the clinical file. Again, this type of data would be easier to collect if running a prospective study.

b) Tracking ESBL(s) and AmpC-type genes

While our results on screening the frequent ESBL(s) and carbapenems genes in PA turned negative, our screening for AmpC-type cephalosporinases overexpression (using the phenotypic ESBL NDP test) showed inhibition by clavulanate indicating an ESBL-activity for some isolates. We could not rule out the possible presence of unidentified-ESBL(s), like *Pseudomonas*-specific enzyme genes encoding for PSE/CARB-type carbenicillinase. In the same context, the overexpression of

ampC or variants (plasmidic or chromosomal) could have been checked by real-time PCR.

c) Investigating the impact of each evidenced mutation on temocillin activity

Different mutations affecting MexAB-OprM pump were evidenced in our sequencing data run on a subset of the collection. Cloning each mutation in a vector and transforming a wild-type background of PA would be useful to determine the impact of each mutation on the bacterial susceptibility to temocillin, out of other factors that could influence the activity of temocillin like porins (OpdK, OpdF) alterations, unidentified temocillinases (temocillin-degrading β-lactamase), or alginate overproduction, which are interfering in clinical isolates.

3.4 Perspectives

a) Short term perspectives:

1. <u>Studying the expression of genes encoding RND efflux systems</u>

Efflux unbalance was proposed previously [241] for MexAB-OprM and MexXY-OprM. This efflux unbalance confers resistance to aminoglycosides while β-lactam antibiotics remain active. Our preliminary data, however, show that a high proportion of temocillin susceptible isolates are resistant to both aminoglycosides and fluoroquinolones (figure 1.7). Therefore, efflux systems conferring resistance to fluoroquinolones (i.e. MexEF-OprN; MexCD-OprJ) could also be overexpressed in these temocillin susceptible isolates. To better document the concept of efflux unbalance in temocillin-susceptible CF isolates, it would be interesting to study the expression of the genes encoding MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM in a subset of CF isolates that are temocillin-susceptible but ciprofloxacin/amikacin-resistant versus other isolates showing other

susceptibility patterns. The expression of the genes encoding different RND efflux pumps could be measured by real-time quantitative PCR.

b) Long term perspectives:

1. Investigations related to efflux mechanisms towards temocillin and other antibacterial agents

In order to get more insights about the functionality of the MexAB-OprM pump and mutations that make temocillin active against PA, it would be of interest to:

- Check the prevalence of mutations in genes coding for MexAB-OprM pumps in PA isolates from a recent CF population (2016).
This will give an update about the prevalence of mutations and susceptibility to temocillin, as compared to the results obtained here in our temocillin study against PA isolated between 1996 and 2012. In addition, if temocillin had to be used against PA in CF patients in the future, it would be of interest to track the evolution of these mutations by comparing isolates from patients treated or not with temocillin.

- Sequence the *mexR* gene encoding the regulator of MexA-MexB-OprM operon expression. As a transcriptional regulator for MexAB-OprM, *mexR* mutations have been shown to affect the expression of MexAB-OprM and to occur in CF isolates, more specifically in Liverpool epidemic strain LES [276]. Therefore, we could study the occurrence of *mexR* mutations in our population of PA, especially for isolates from UK showing high prevalence of LES isolate.

- Investigate the impact of each evidenced mutation on temocillin MIC in a wild-type background of PA. As already underlined in the paragraph describing the limitations of the work, it would be useful to introduce the mutations highlighted here in a wild-type background of PA to investigate its impact on temocillin activity.

- Study the expression of MexA and MexB proteins by western blot and structural studies for efflux pumps using cryo-electron microscopy and tomography. Proteins sequence and structure in PA are processed thanks to specialized nanomachines of protein folding and secretion systems [277]. Depending on *mexA* and *mexB* mutations sites, their impact on the expression is purely empirical and different scenarios might happen (1) MexA and MexB proteins are not expressed, (2) expressed but misfolded, keeping them from reaching bacterial envelope, (3) expressed in insufficient quantities, (4) expressed and reaches cell surface, but does not function properly, or (5) highly expressed. In the temocillin paper (section 2.3), we were not able to check if the truncations observed could have affected the folding, the solubility and/or the expression of MexA/MexB proteins. Protein expression could therefore be checked by western blot, while protein architecture could be examined by cryo-electron microscopy and cryo-electron tomography as done in previous studies [278;279]. Moreover, computer simulations [280-282] would be helpful for integrating data from experiments on the molecular level, and help to interpret data on the functionality of efflux pumps.

- Determine whether *mexA/mexB* mutations are beneficial for the activity of temocillin only or could also affect other substrates. We have shown that avibactam may encounter active efflux and/or OprD alterations as resistance mechanisms. We have evaluated the impact of *mexAB* mutations on avibactam activity in a limited number of isolates. It would be interesting to extent this study to a larger number of isolates harbouring other types of mutations in order to determine if temocillin and avibactam share the same recognition determinants in the pump.

2. Investigations related to OpdK and OpdF porins of PA

Our study identified two entry gates “OpdK and OpdF” involved in temocillin uptake by PA. It would be of interest, therefore, to:

- Screen *opdK* and *opdF* mutations in CF population of PA. The isolates of this CF collection showed alterations in OprD leading to carbapenems resistance. In the same context, screening mutations for OpdK and OpdF porins will allow us to determine whether specific mutations also exist in these porins that can contribute to resistance to temocillin.

- Study the effect of *opdK* and *opdF* mutations on temocillin uptake. In case of presence of mutations in the collection of CF isolates, the effect of these mutations on temocillin activity could be studied by introducing the same mutations in a wild-type background of PA.

3. Overproduction of extracellular polymeric substances by PA

We showed that the production of extracellular polysaccharides by mucoid isolates can reduce temocillin activity (section 2.3). To better document this phenomenon, it would be of interest to:

-Measure the expression level of genes encoding the enzymes producing different exopolysaccharides in isolates showing different susceptibility to temocillin. Detecting specific genes involved in the synthesis of exopolysaccharides, mainly *algD* (GDP mannose deshydrogenase) and *algC* (phosphomannomutase) as key genes for the formation of alginate or other related genes encoding regulatory functions like *mucA*, *mucB*, *mucC*, *mucD* will confirm if alginate production is a key mechanism to reduced susceptibility to temocillin.

4. Investigations related to the activity of temocillin against persistent forms of infections by PA

It has been shown that PA can replicate intracellularly and survive within epithelial cells in CF disease [283]. Likewise, biofilm formation is widely described as a major cause of persistence of PA in the CF lung [78]. No studies have been performed so far regarding the activity of temocillin against these specific forms of infection. It would be of interest to:

- Study the activity of temocillin against intracellular infections of PA. A model of intracellular infection of THP-1 monocytes has been set up in our laboratory [284], which could be used to compare the intracellular fate of wild-type and MexAB-mutated strains as well as the activity of temocillin.

- Study temocillin activity on biofilms by PA with or without altered MexAB-OprM function. Efflux systems have been suggested to play a role in biofilm formation, notably by extruding quorum sensing molecules [285]. It would therefore be useful to compare biofilm formation by temocillin susceptible and resistant isolates and to investigate temocillin activity in these models. Since synergistic effect has been shown previously between aminoglycosides (tobramycin or amikacin) and temocillin *in vitro* against planktonic PA [186], testing these combinations against biofilms of PA from CF patients would be useful as well.

5. Investigations related to the affinity of penicillin-binding proteins for temocillin in PA

As previously reported [40], the stability of temocillin binding to many penicillin-binding proteins (PBPs) is still uncertain and needs to be more investigated. An alteration in this binding has indeed been shown to confer resistance against temocillin in *E.coli* [218]. In PA, it would be of interest to:

-Investigate the reduced or enhanced affinity of penicillin-binding proteins to temocillin. Low affinity PBP-4s were reported after imipenem treatment in PA, as well as after administration of high doses of piperacillin in patients suffering from CF [286]. Other studies reported a reduced susceptibility to β-lactams in PA strains with overproduction of PBP-3s [287]. The impact on such mutations of temocillin is unknown and should be studied, as it may constitute a non-enzymatic acquired resistance mechanism.

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