Study of the activity of murepavadin on Pseudomonas aeruginosa cystic fibrosis clinical strains and identification of resistance mechanisms

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Doctorat en Biologie Moléculaire et Microbiologie

Par

Aya GHASSANI

Etude de l'activité de la murepavadine sur des souches cliniques de *Pseudomonas aeruginosa* dans le contexte de la mucoviscidose et identification des mécanismes de résistance

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Abbreviations

ABC: ATP binding cassette

ACP: acyl carrier protein

AMP: antimicrobial peptides

AMR: antimicrobial resistance

ATP: adenosine tri-phosphate

BAM: β-barrel assembly machinery

BET: ethidium bromide

CAMP: cationic antimicrobial peptide

cDNA: complementary DNA

CF: cystic fibrosis

CFTR: CF transmembrane conductance regulator

Cm: carbamoyl

cMHB: cation adjusted mueller-hinton broth

CPA: common polysaccharide antigen

CPS: capsule polysaccharide

DMSO: dimethyl sulfoxide

Etn: ethanolamine

EUCAST: european committee on antimicrobial susceptibility testing

GalN: 2-amino-2-deoxy-D-galactose

Glc: D-glucose

GPAT: glycerol 3-phosphate acyltransferase

HIFI: high fidelity

HK: histidine kinase

ICU: intensive care unit

IM: inner membrane

IPTG: isopropyl- β-D-thiogalactopyranoside

kDa: kilodalton

Kdo: 3-deoxy-D-manno-oct-2-ulosonic acid

L-Ara4N: 4-amino-4-deoxy-L-arabinose

LC-ESI-MS: liquid chromatography-electrospray ionization-mass spectrometry

LP: lipoprotein

LPS: lipopolysaccharide

LPT: lipopolysaccharide transport

LptD: lipopolysaccharide transport protein D

MHA: mueller-hinton agar

MDR: multidrug resistant

MIC: minimum inhibitory concentration

mRNA: messenger RNA

NBD: nucleotide-binding domains

NEB: new england biolabs

NPN: 1-N-phenylnapthylamine

NRT: non reverse transcriptase

OD: optical density

OM: outer membrane

OMP: outer membrane protein

OMPTA: outer-membrane protein targeting antibiotic

OSA: O-specific antigen

PCR: polymerase chain reaction

PDC: *Pseudomonas d*erived AmpC

PEM: protein epitope mimetic

PIA: *Pseudomonas* isolation agar

POTRA: polypeptide transport associated

PG-1: protegrin-1

Rha: L-rhamnose

RND: resistance-nodulation-cell division

Rpm: round per minute

RR: response regulator

RT: reverse transcription

SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis

Skp: seventeen kilodalton protein

SurA: survival protein A

TAE: tris-acetate EDTA

TCS: two component system

TGS: tris, Glycine, SDS

TMD: transmembrane domain

Und-PP: pyrophosphate undecaprenyl

UV: ultraviolet

VAP: ventilator-associated pneumonia

WHO: World Health Organisation

WGS: whole genome sequencing

XDR: extensively drug resistant

I. Introduction

Pseudomonas aeruginosa is a Gram-negative, aerobic, rod-shaped bacterium that is widely distributed in nature. It is distributed widely across diverse habitats, including soil, marshes, coastal marine environments, and can even colonize both plant and animal tissues (Sitaraman, 2015).

In healthcare facilities, it can be found in ventilators, taps, sinks, etc, (Lister et al., 2009). Their presence in hospitals' equipments makes patients admitted in intensive care unit (ICU), having immune deficiences or suffering from respiratory diseases at high risk of pneumonia and colonization by this pathogen. Indeed *Pseudomonas aeruginosa* accounted for 16.2% of patients infections in ICU based on an international observational point-prevalence study (Reynolds and Kollef, 2021). In addition to that, it is responsible for 10% -20% of isolates related to ventilator-associated pneumonia (VAP) (Reynolds and Kollef, 2021).

In cystic fibrosis (CF), chronic infections with *P. aeruginosa* often lead to respiratory airways inflammation and decline in lung functioning (Taccetti et al., 2021). In fact, it is estimated that over 50% of adult CF patients will have at least one *P. aeruginosa* infection in their lifetime (Registre francais de la mucoviscidose, 2021). Unfortunately, infections caused by *P. aeruginosa* has become a predominant cause morbidity and mortality in CF patients (Bhagirath et al., 2016; Reynolds and Kollef, 2021).

The treatment of patients infected with *P. aeruginosa* poses significant challenges and complexities as the bacterium has the ability to develop resistance to multiple classes of antibiotics, even during the course of treatment (Lister et al., 2009; Taccetti et al., 2021). It possesses intrinsic resistance mechanisms to several anti-Gram-negative antibiotic classes, and may acquire additional ones, notably under antibiotic selective pressure (Poole, 2004). Intrinsic resistance is mainly due to the collaboration between the efficient drug efflux mechanisms that export some antibiotics outside the cell, the low permeability of the outer membrane, and the production of antibiotic-hydrolyzing enzymes such as β-lactamases (*Pseudomonas* Derived AmpC, PDC) (Pang et al., 2019). In addition, *P. aeruginosa* can acquire foreign DNA encoding resistance determinants carried by plasmids or transposons through horizontal gene transfer from different *Pseudomonas* species (Pang et al., 2019). But the most frequent way of becoming resistant to antimicrobial agents remains the acquisition of genetic mutations particularly in cystic fibrosis isolates (Dale et al., 2018; Hancock and Speert, 2000; Lambert, 2002). Several mutations in various genetic determinants have been identified, but some are more prevalent in cystic fibrosis isolates (Lambert, 2002). Thus, the overproduction of the RND efflux pump MexXY/OprM subsequent to the *mexZ* inactivation mutation is frequent among CF isolates

resistant to aminoglycosides ((Muller et al., 2011). This gene codes for the local repressor (TetR family of transcriptional regulators) of the efflux pump operon *mexXY*. The overproduction of MexXY/OprM is associated with a reduced (2- to 4-fold) susceptibility to aminoglycosides, fluoroquinolones, cephalospirins zwitterionic as cefepime (Aires et al., 1999). Another important determinant of antimicrobial resistance is driven by the overproduction of the PDC enzyme. This Ambler class C enzyme is the most frequent resistance mechanism to ceftazidime, and piperacillin/tazobactam in *P. aeruginosa* isolates from acute and chronic-infections (Berrazeg et al., 2015). Mutations in several genes participating in the regulatory pathways of gene *ampC* (*dacB, ampR, ampD,*…) are associated with the upregulation of this one (Moya and Oliver, 2009). Moreover, the PDC activity spectrum can be extended to other β-lactams (cefepime, cefiderocol, and combinations of β-lactams/inhibitor of β-lactamases as ceftazidime/avibactam, and ceftolozane/tazobactam) through deletions and/or aminoacid substitutions in the sequence of PDC enzyme, particularly in the omega loop region (Berrazeg et al., 2015).

P. aeruginosa is well-known for its ability to accumulate antimicrobial resistance mechanisms, leading to multidrug resistant isolates. To better categorize these isolates, a classification has been proposed by Magiorakos and collaborators in 2012 (Magiorakos et al., 2012). This latter included three categories, the first (i) corresponding to isolates that are resistant to at least one agent in three or more antimicrobial categories is designed MultiDrug Resistant (MDR), the second (ii) corresponding to Extensively Drug-Resistant category (XDR) included isolates that are susceptible to only one or two antimicrobial categories, and the third (iii) PanDrug Resistant (PDR) isolates, that are resistant to all agents in all antimicrobial categories (Magiorakos et al., 2012) (**table 1**). Approximately 30% of *P. aeruginosa* isolates identified in respiratory samples of patients with healthcare associated pneumonia are considered MDR (Martin-Loeches et al., 2018a). These isolates pose significant challenges for the treatment of associated infections, requiring last-resort treatment options, such as polymyxins (colistin, and polymyxin B).

Antimicrobial resistance (AMR) has become a growing global health concern. It is estimated by the World Health Organization (WHO) that by 2050, 10 million lives per year will be at risk of death due to the rise of drug resistance against antibiotics (O'NEILL, 2016). Therefore, huge efforts have been implemented around the world in order to discover new antibiotics and to slow down the spread of drug resistance (Rima et al., 2021).

Table 1: Antimicrobial categories and agents as defined by (Magiorakos et al., 2012).

Antimicrobial categories	Antimicrobial agents
Aminoglycosides	gentamicin, tobramycin, amikacin, netilmicin
Antipseudomonal carbapenems	imipenem, meropenem
Antipseudomonal cephalosporins	ceftazidime, cefepime
Antipseudomonal fluoroquinolones	ciprofloxacin, levofloxacin
Antipseudomonal penicillins + β-lactamase inhibitors	ticarcillin-clavulanic acid, piperacillin-tazobactam
Monobactams	aztreonam
Phosphonic acids	fosfomycin
Polymyxins	colistin, polymyxin B

The main objective of my PhD project was to characterize the antimicrobial activity of a newly developed antimicrobial agent called murepavadin on a large collection of CF clinical isolates. Murepavadin is a peptidomimetic with specific activity against *Pseudomonas* spp, currently in-phase I clinical trials, that is the first of a new class of outer membrane protein targeting antibiotics (OMPTA). This antibiotic displays excellent *in vitro* activity against drug resistant *P. aeruginosa* clinical strains (Martin-Loeches et al., 2018a). A special attention was payed on isolates that were less susceptible to murepavadin and efforts were put to identify the associated resistance mechanisms. A second part of the project was focused on understanding the molecular response of *P. aeruginosa* following the exposure to murepavadin. Finally, a third part, done in collaboration with Xavier Vuillemin (PhD student), focuses on identifying the role of the multidrug efflux system MexXY/OprM in resistance to polymyxins and murepavadin.

II. Bibliographical synthesis

1. Cystic Fibrosis

Cystic fibrosis (CF) is a life-shortening genetic disorder commonly found in Caucasian population, with approximately an incidence of 1 out of 3000 births in both Europe and North America (Malhotra et al., 2019). This disease is due to mutations in *CFTR* gene (CF transmembrane conductance regulator), situated in the long arm of chromosome 7, and codes for the ABC transporter CFTR protein. CFTR is a chloride channel found on apical surfaces of secretory epithelial cells that regulate Cl^- and HCO_3^- secretions for water secretion (Bhagirath et al., 2016). To date, more than 2,000 mutations have been identified, with some being more common than others (Registre francais de la mucoviscidose, 2021). These mutations could be classified into different classes based on the dysfunction of CFTR protein (**Figure 1**).

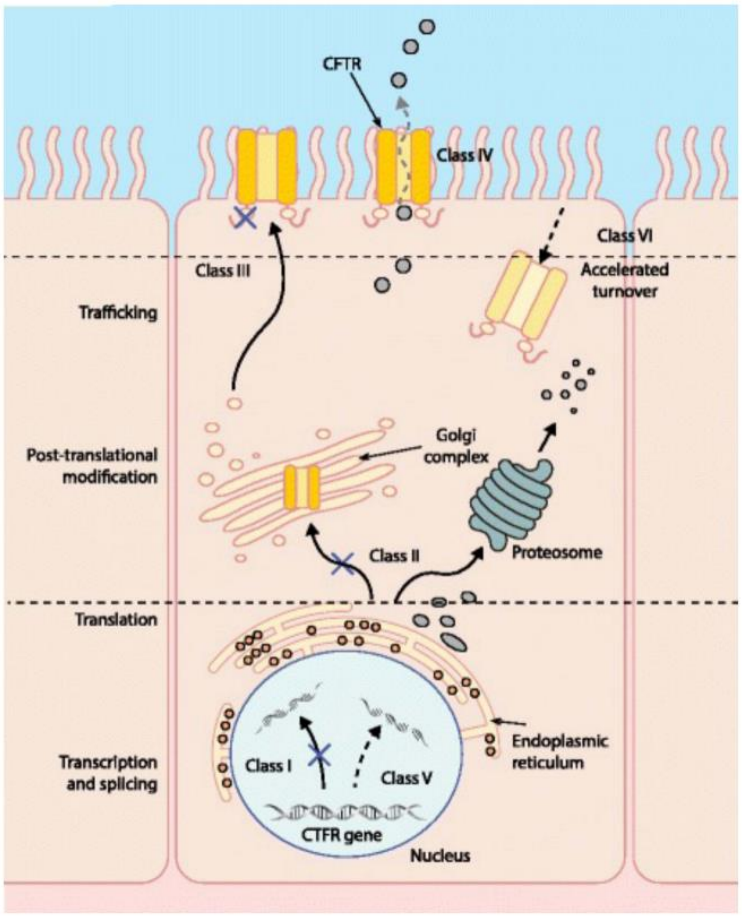

Figure 1: Different classes of CFTR mutations. Class I mutations cause defects in protein synthesis. Class II mutations impacts protein maturation. Class III mutations cause defects in protein regulation and functioning. Class IV mutations cause dysfunctions in ion conductance. Class V mutations reduce protein expression. Class VI mutations reduce protein stability in the membrane; (Haq et al., 2022).

Mutations of class I, result in no-protein synthesis, class II mutations, the most common and accounting for 80% of CF cases in France, cause defects in folding and maturation of CFTR.

Class III affects protein regulation (defects in ATP binding and hydrolysis), Class IV mutations cause defective chloride conductance, class V mutations lead to reduced expression of CFTR, and finally class VI mutations reduce protein stability at the surface of epithelial cells (Haq et al., 2022).

Despite the presence of CFTRs in different body organs, their malfunctioning in lung epithelial cells account for highest morbidity rates (Davies, 2002). A malfunctioning CFTR in lung epithelial cells prevents Cl^- and HCO_3^- ions secretion, causing their accumulation in the intracellular space and increase in the intracellular osmotic pressure. These events drive the unstoppable water reabsorption, leading to mucus dehydration and its transformation into a sticky and thick consistency (**Figure 2**) (Li and Schneider-Futschik, 2023; Malhotra et al., 2019).

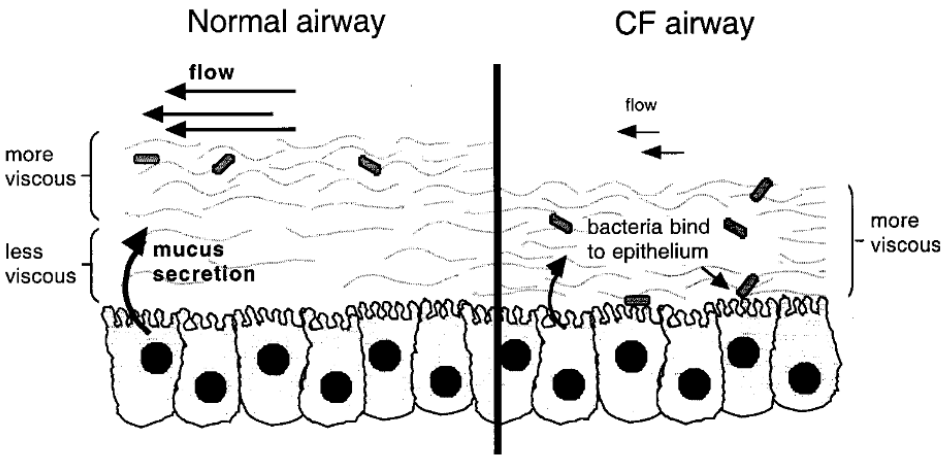

Figure 2: Comparison of the mucociliary clearance in a normal airway and in a CF airway. In the normal airway, two layers of mucus cover the epithelium. The inner layer is less viscous in which cilia beat, and the outer viscous layer traps particles and microorganisms. Epithelial cells beating causes unidirectional flow of the mucus toward the esophagus. In CF aiway, one visous layer covers the epithelial cells and traps microorganisms. This consistency of mucus limits the movement of cilia and the elimination of microorganisms towards the esophagus; (Lyczak et al., 2002).

As a consequence of this accumulation, mucociliary clearance in respiratory airways is reduced and major antimicrobial proteins involved in defence mechanisms cannot reach their target. This issue favors the colonization of pathogens like *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae* with *P. aeruginosa* being the most severe, accounting for 80% of chronic lung infections in CF (Cogen et al., 2022).

Since 2002, systematic neonatal screening for CF has been extended throughout France. This screening enables the identifications of 95% of newborns with CF (Registre francais de la mucoviscidose, 2021). Today, with better management of CF infections and thanks to the early

diagnosis, the life expectancy has already improved to more than 47 years old in the United States, United Kingdom, Europe (Malhotra et al., 2019; McKone et al., 2021). As a result, the number of deaths has decreased. Also, the number of adult CF patients have increased since 1992 while that of enfant patients have decreased; 38.3 % of CF patients in France were adults and 61.7 % were enfants in 2021, (Registre francais de la mucoviscidose, 2021).

Despite the improvement in managing infections caused by *P. aeruginosa*, they are still challenging. Its ability to resist naturally to antibiotics and to adapt to high antibiotic concentrations and prolonged treatments, favors the occurance of genetic mutations that ensure stable resistance, thus multi-drug resistance (Rossi et al., 2021). The CF environment not only supports the survival of the same initial pathogenic strains, but also new sub-populations, also refered as hypermutator strains often emerge and are very commonly isolated from CF patients (Bhagirath et al., 2016).

In CF lung, the host immune system is hyperinflammed and is uncapable of clearing the infection effectively. Even though immune cells accumulate and release powerful antimicrobial compounds and reactive oxygen species for the aim of bacterial infections, their presence in addition to the pathogen's virulence factors enhance progressive damage, and eventually leading to respiratory failure (Malhotra et al., 2019).

For more efficient treatments for CF patients, inhaled antibiotics are currently used and are advantageous over systemic treatments. This is due to more efficient delively of antibiotic directly into the airway with high concentrations and with less toxic effects (Li and Schneider-Futschik, 2023). Inhaled tobramycin, inhaled aztreonam lysine and colistin methane sulfonate are commonly prescribed as anti-*Pseudomonas* inhaled therapy (Cogen et al., 2022). Unfortunatlely, theiy might fail in performing their function and thus the development alternatives is desirable.

Indeed, Gram-negative bacteria are of particular threat when it comes to eradicating them from airway of CF patients. A principal cause for the failure of antibiotics to target them is their outer membrane (OM) that serves as a major player in intrinsic resistance (Hancock, 1997). The OM is considered a major obstacle due to its high impermeability that adds more difficulties for drug discovery (Lehman and Grabowicz, 2019). However, by taking adventage of its significant role, it is considered an attractive target for the development of new antibiotics due to its richness in potential targets.

2. The cell envelope of Gram-negative bacteria

The bacterial envelope of Gram-negative bacteria is an essential structure which participates in the first steps of interaction between the antibiotic and the bacteria. It is a key factor in antimicrobial susceptibility because it modulates the penetration of many antibiotics according to the hydrophobicity of the molecules. Thus, understanding the mechanisms contributing to its permeability is essential in developing effective antimicrobial strategies (Tang et al., 2021). Currently, several groups are attempting to bypass the outer membrane using various strategies such as combining antibiotics with compounds like siderophores which are recognized by the bacteria and internalized. The typical example is the cefiderocol, a new antibiotic active against *P. aeruginosa* isolates where the cephalosporin is conjugated with a chlorocathecol group (Candel et al., 2022). The antibiotic enters into the periplasmic space through the siderophores transporters. Thus, classical resistance mechanisms to cephalosporins such the overproduction of efflux pumps, and the alteration of porins are avoided. Understanding the structure, function, and regulation of the outer membrane components is essential for understanding resistance of Gram-negative bacteria. (MacNair et al., 2020). By identifying vulnerabilities in the outer membrane structure or disrupting essential functions, researchers aim to develop novel drugs that can overcome the challenges posed by the impermeability and resistance mechanisms of Gram-negative bacteria. Therefore, exploring the outer membrane and its associated functions can provide valuable insights for antibiotic discovery.

2.1 Composition

The cell envelope of Gram-negative bacteria consists of several layers that provide protection and structural integrity to the bacterial cell. It is composed of three main components: the outer membrane (OM), the peptidoglycan layer, and the inner membrane (IM) (**Figure 3**). The outer membrane is the outermost layer of the cell envelope in Gram-negative bacteria. It is the most distinguishing feature from Gram-positive bacteria (Silhavy et al., 2010). Since the outer membrane of Gram-negative bacteria is an essential part in this project, it will be discussed more in details in the later parts.

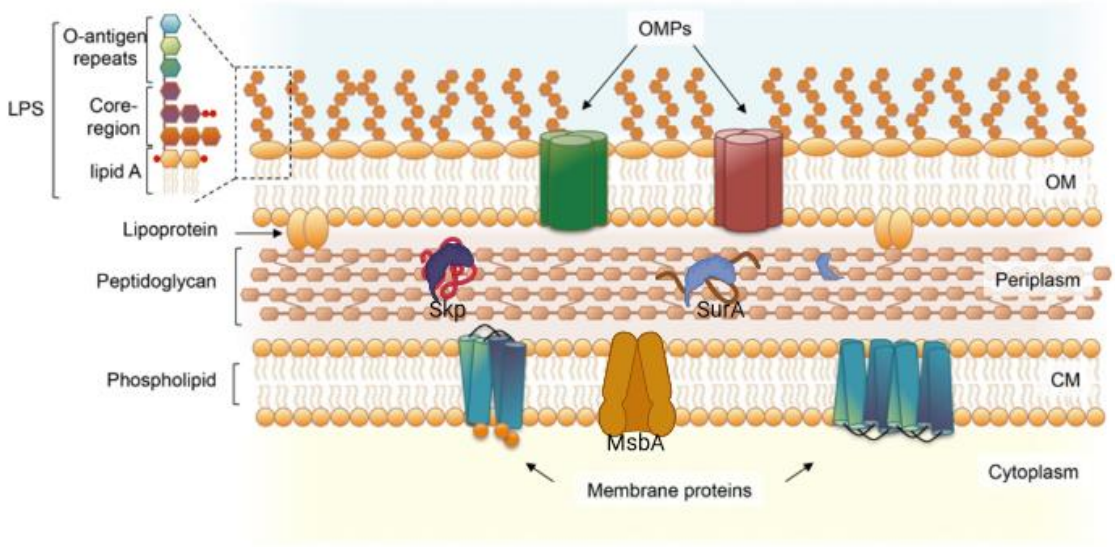

Figure 3: Structure of the cell envelope of Gram-negative bacteria. The cell envelope is composed mainly of two phospholipid bilayers: an outer membrane (OM) mainly composed of the LPS and outer membrane proteins (OMPs) in addition to lipoproteins that staples the OM to the peptidoglycan layer, an inner membrane or cytoplasmic membrane (CM) that contains membrane proteins, separated by the peptoglycan layer situated in the periplasm. Chaperones like Skp and SurA are present in the periplasm to assist in folding of OMPs; adapted from (Maldonado et al., 2016).

Beneath the OM lies the rigid peptidoglycan layer. It provides structural support and protects the cell against osmotic stress. The peptidoglycan is a polymer of repeating units of disaccharide N-acetyl glucosamine-N-acetyl muramic acid linked by glycosidic bonds forming glycan strands. They are cross-linked into a mesh-like structure that is rigid enough to determine the cell's shape (Silhavy et al., 2010). While the peptidoglycan layer in Gram-negative bacteria is few nanometers thick, that of Gram-positive bacteria ranges between 30-100 nm thick (Silhavy et al., 2010; Sperandeo et al., 2017a).

Finally, the semi-permeable IM, also called the cytoplasmic membrane or plasma membrane, is the innermost layer of the cell envelope. It is a symmetrical phospholipid bilayer composed of phospholipids, mainly phosphatidyl ethalonamine (70-80%), phosphatydilglycerol and cardiolipin and harbors α-helical membrane proteins (Koebnik et al., 2000). Other proteins for the transportation of lipopolysaccharides are present in the IM like MsbA, responsible for flipping lipid A-core complex from the cytoplasm towards the periplasm during LPS biosynthesis. Also located in the inner membrane are subunits of the lipopolysaccharide transport (LPT) complex for transporting the LPS across the membrane, and histidine kinases (HK) of two-component systems (Gao and Stock, 2009; Maldonado et al., 2016). The IM plays a crucial role in nutrient uptake, energy production, and the transport of molecules in and out of the cell (Koebnik et al., 2000).

Above the inner membrane is the periplasmic space, that holds several periplasmic-binding proteins that serve crucial functions such as protein folding or aminoacid transport. Those include chaperon survival protein A (SurA) and seventeen kilodalton protein (Skp) (Silhavy et al., 2010). Those are essential chaperons for the transportation of nascent outer membrane proteins in order to get properly folded and inserted in the outer membrane (Xu et al., 2023).

2.2 Outer membrane of Gram-negative bacteria

The OM of Gram-negative bacteria acts as a barrier against various environmental factors, including antibiotics and detergents (Whitfield and Trent, 2014). It consists of an assymetric lipid bilayer of lipopolysaccharides (LPS) situated on the outer leaflet, and phospoholipids in the inner leaflet (Andolina et al., 2018a). In addition, several OMPs with diverse functions decorate the OM, and account for approximately 50% of its mass (Silhavy et al., 2010). They also play a role in maintaining the integrity of the outer membrane (Rollauer et al., 2015). They can be divided into lipoproteins, anchored into the inner leaflet, and β-barrel proteins inserted into the outer leaflet (Andolina et al., 2018a; Silhavy et al., 2010). Porins constitute an important element of OMP that facilitate nutrient transport and uptake of small molecules (Koebnik et al., 2000).

The LPS is the major component of the outer membrane; it is composed of 3 distinct parts: the lipid A, the core-oligosaccharide and the O-antigen (King et al., 2009). The LPS part will be discussed below in more details. This unique structure of the OM maintains the selective permeability barrier and integrity of bacterial cells, and prevents the entry of cytotoxic molecules (Lin et al., 2002; Tang et al., 2021).

2.3 Biogenesis of the outer membrane

The biogenesis of the outer membrane usually occurs at three different levels (Brown, 2016; Silhavy et al., 2010):

- Folding and translocation of the β-barrel OMPs,
- Lipoproteins biosynthesis,
- Lipopolysaccharide biosynthesis and translocation,

2.3.1 Folding and translocation of the β-barrel OMPs

Outer membrane proteins (OMPs) that are integrated into the outer leaflet of the OM adopt β-barrel-shaped structures with a number of antiparallel β-sheets ranging from 8 to 26 strands in length (Rollauer et al., 2015). This specific structure maintains their stability towards harsh environmental conditions. The central space of the β-barrel OMPs structure is hydrophilic, while the outer surface is hydrophobic (Rollauer et al., 2015). The assembly of the OMPs is carried through the Sec/Bam pathway, which ensures their proper folding and insertion into the outer leaflet of the outer membrane (**Figure 4**) (Brown, 2016; Steenhuis et al., 2021). Initially, a nascent OMP is synthesized by ribosomes with a signal peptide at the N-terminal end. This nascent OMP can be delivered to SecYEG translocon by the SecA and SecB chaperones after being translated or during synthesis on the ribosome (Rollauer et al., 2015). The SecYEG translocon transports the precursor through the inner membrane to the periplasmic space (Brown, 2016). Transportation across the periplasm is facilitated by the SurA and Skp chaperones, which interact with the Bam complex and donates the nascent OMPs (Xu et al., 2023; Zerbe et al., 2017). The Bam complex will finalise the folding and insertion of the OMPs into the OM. OMPs that get aggregated or misfolded are degraded by DegP chaperone, that functions as a protease (Rollauer et al., 2015).

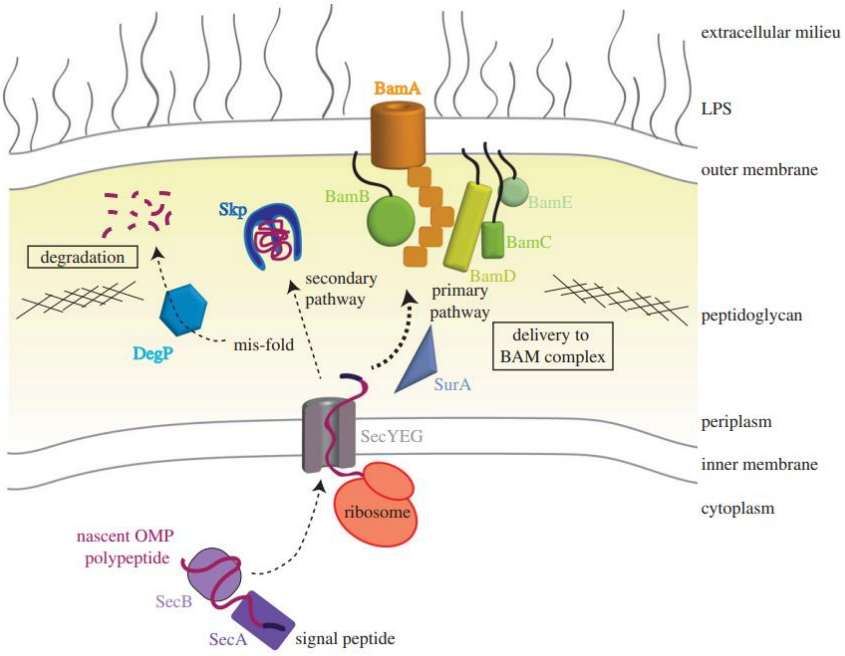

Figure 4: Pathway for OMPs translocation and folding in the outermembrane of Gram-negative bacteria. A nascent OMP is delivered to the SecYEG translocon either by the two cytoplasmic chaperone SecA and SecB, or by the ribosome during synthesis. The Sec translocase transports the precursor OMP to the periplasm. It is then delivered to the Bam complex by Skp and SurA chaperones where it will be integrated into the outer membrane. In case of aggregations or misfolding, the nascent OMP is degraded by DegP chaperone (Rollauer et al., 2015).

2.3.1.1 The Bam complex

The Bam complex is a conserved protein complex in Gram-negative bacteria that plays a critical role in the assembly and insertion of β-barrel OMPs. In *P. aeruginosa*, this complex consists of five core subunits, a large β-barrel BamA integral protein, and four lipoproteins BamBCDE (Plummer and Fleming, 2016).

BamA, which is also an OMP itself, is the central component of the complex and is an essential protein for cell viability. This transmembrane protein interacts with newly synthesized OMPs and facilitates their folding and assembly. In *E. coli*, the absence of BamA results in misfolding of OMPs and cell death (Xu et al., 2023). BamA is constituted of 5 POlypeptide TRansport Associated domains (POTRA) that are extended in the periplasm, with the β-barrel part at the C-terminal domain (Steenhuis et al., 2021). BamB, BamC, BamD, and BamE are accessory proteins that fulfill functions in reception and transfer of the nascent OMPs to the BamA, in addition to the complex stability (Steenhuis et al., 2021). BamB and BamD are lipoproteins that are anchored to the inner leaflet while BamC and BamE are periplasmic proteins. The

mechanism by which BamA inserts the folds OMP into the outer leaflet is not completely elucided. Two models have been proposed, (i) the « assisted » model and (ii) the « budding » model (**Figure 5**) (Steenhuis et al., 2021; Xu et al., 2023). In the first one, BamA facilitates the localisation and installation of the folded/partially folded OMP by permeabilizing locally the outer membrane (Steenhuis et al., 2021; Xu et al., 2023). In the budding model, an opening is formed in BamA called the « lateral gate », due to the weekness of hydrogen bond between the first β-strand and the last β-strand of the barrel. Once the OMP precursor reaches the interior of BamA, the β-strands gets templated and folded, and buds off laterally from BamA into the OM (Konovalova et al., 2017; Xu et al., 2023).

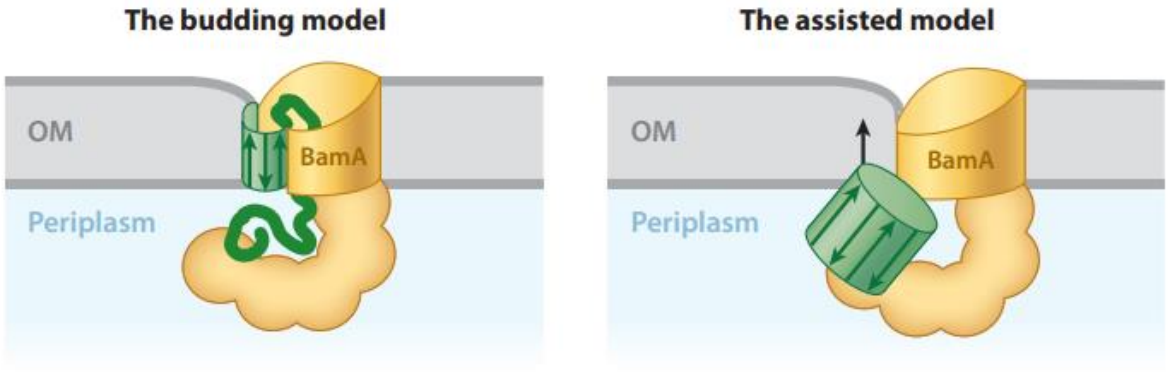

Figure 5: Models proposed for folding and insertion of OMPs by BamA. In the budding model, the β-strands of the β-fold barrel at the lateral gate serve as a template for folding the OMP. In the assisted model, folding occurs at the periplasmic side. When folding is completed, BamA forces the insertion of the OMP into the OM by causing defects. OM= outer membrane; (Konovalova et al., 2017; Xu et al., 2023).

The Bam complex is a potential target for new inhibitors against Gram-negative bacteria due to its essential role in the OM biogenesis. In addition, BamA is highly conserved among Gram-negative bacteria (Xu et al., 2023). Currently, two inhibitor peptides with potent antimicrobial activity are under development called JB-95 and Darobactin A, target the Bam complex (Overly Cottom et al., 2023). The β-hairpin peptidomimetic JB-95 show mostly an activity against *E. coli* (*E. coli* ATCC25922) with low MIC (0.25 µg/mL) including multi-drug resistant clinical strains, and less activity on *Acinetobacter bumanii* ATCC17978 (MIC = 1 µg/ml), *S. aureus* ATCC29213 (MIC= 2 µg/ml) and *P. aeruginosa* ATCC27853 (MIC = 4 µg/ml) (Overly Cottom et al., 2023; Urfer et al., 2016). Darobactin A has also demonstrated a good antimicrobial activity on *E. coli* (*E. coli* ATCC25922) and PAO1 reference strain with MIC = 2 µg/mL (Böhringer et al., 2021; Overly Cottom et al., 2023).

Mutations in the Bam complex were observed in *E. coli*, *P. aeruginosa* and *P. fluorescens* resistant to Bam inhibitors (Steenhuis et al., 2021) or in *in vitro* selected *P. aeruginosa* mutants resistant to murepavadin (**table 2**) (Díez-Aguilar et al., 2021b). However, due to the importance of Bam complex, it is expected that low selection pressure might exist to develop resistance against Bam inhibitors. Therefore, targeting the Bam complex can potentially overcome the protective barrier of the outer membrane, allowing antibiotics to reach their targets more effectively thus can provide a novel approach to combat antibiotic-resistant bacteria.

Table 2: Substitutions in BamA identified in bacterial species and impact on resistance to antimicrobial peptides.

Substitutions	Species	Impact on MIC to antimicrobials	Reference
$S_{509}G$	*P. aeruginosa*	Colistin resistance (2-fold)	(Jochumsen et al., 2016)
$G_{429}R$, $G_{429}V$, $T_{434}A$, $Q_{445}P$, $A_{705}T$, $E_{435}K$, $F_{394}V$, $G_{807}V$, $Q_{445}P$, $G_{443}D$, $L_{501}Q$	*E. coli*	Darobactin resistance	(Imai et al., 2019; Kaur et al., 2021; Miller et al., 2022)
$E_{470}K$	*E. coli*	MRL-494 resistance	(Hart et al., 2019; Steenhuis et al., 2021)
$T_{663}P$, $G_{540}D$,	*E. coli*	LlpA resistance	(Steenhuis et al., 2021)
$G_{667}V$, $T_{671}A$, $R_{666}C$	*P. fluorescens*	LlpA resistance	(Ghequire et al., 2018)
$D_{703}Y$	*E. coli*	Peptide 8 resistance	(Steenhuis et al., 2021)
$L_{501}Q$, $G_{429}V$, $G_{807}V$	*E. coli*	Dynobactin A resistance	(Miller et al., 2022)
$G_{504}D$	*P. aeruginosa*	Murepavadin resistance	(Díez-Aguilar et al., 2021b)

2.3.2 Lipoproteins biosynthesis

This pathway involves the localization and transport of lipoproteins destined to be integrated into the inner leaflet of the OM. Unfolded lipoproteins are transported to the the periplasmic space by the Sec translocation system, where they undergo post-translational modifications

(Brown, 2016). A cap of the aminoacid glycerylcysteine is added to the precursor of mature lipoproteins, to which three fatty acid chains are attached (Brown, 2016). Due to the presence of both fatty acid chains at the N-terminal side and a peptide region at the C-terminal side, the lipoprotein can anchor both phospholipids on the inner leaflet of the OM and the peptidoglycan layer with its peptide part (**Figure 6**) (Brown, 2016). Lipoproteins are thought to contribute to the structural integrity, rigidification, and stability of the outer membrane (Brown, 2016).

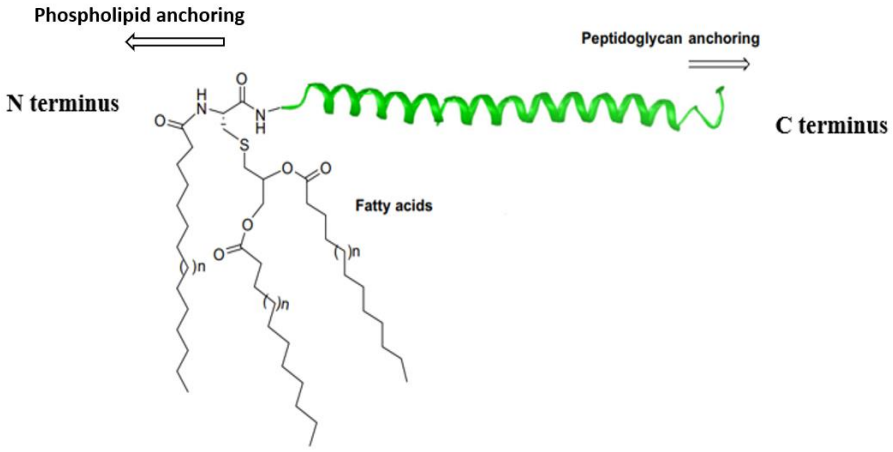

Figure 6: Structural representation of lipoproteins showing three fatty acid tails and a cap region represented in green; adapted from (Brown, 2016).

2.3.3 Lipopolysaccharide biosynthesis and translocation

2.3.3.1 LPS role and structure

LPS, a complex and essential component of the OM in Gram-negative bacteria, plays multifaceted roles that contribute to bacterial survival, immune interactions, and pathogenicity. The unique structure of LPS forms a protective barrier against the external environment, guarding bacteria from various harmful agents, including detergents and antibiotics, thus enabling their resilience in challenging conditions (Sperandeo et al., 2017b). Beyond its structural function, LPS is a highly conserved structure within all Gram-negative bacteria, making it a pattern to be recognized by the immune system, and serves as a potent virulence factor which can subsequently initiate bacterial infection clearance (Steimle et al., 2016). This is primarily achieved through the lipid A or endotoxin region, an immunostimulatory molecule

that is recognized by the host's immune cells, leading to an inflammatory response (Alexander and Rietschel, 2001).

The LPS comprises three distinct components: the lipid A region, the core oligosaccharide, and the O antigen or O-chain (King et al., 2009) (**Figure 7**).

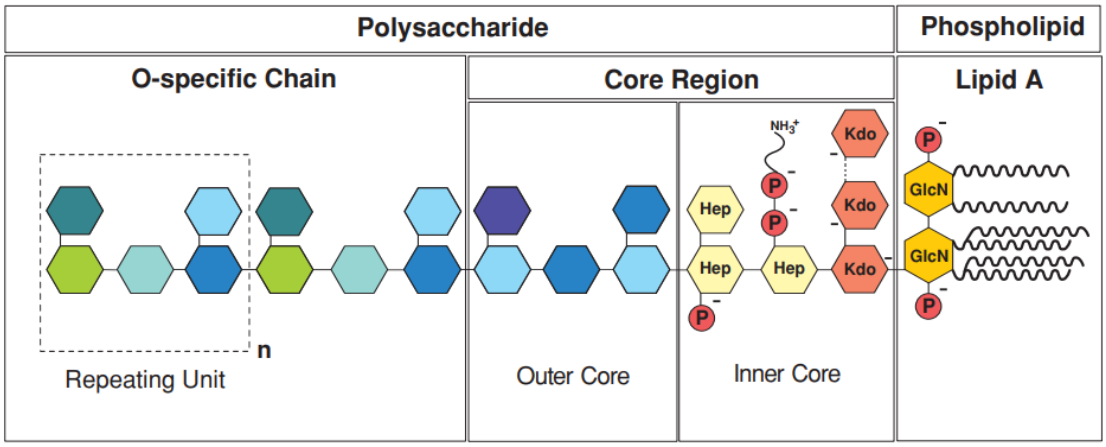

Figure 7: A representation of the general LPS structure of Gram-negative bacteria. It consists of the phospholipid lipid A structure linked to the polysaccharide made of the core region and the O-specific antigen. The lipid A is a double phosphorylated diglucosamine (GlcN), with a number of sugar residues that vary slightly with different species. The inner core is composed of three sugar residues Kdo and three Hep residues that are phosphorylated while the outer core is composed of three different sugar types. The O-specific chain is a repetitive unit of carbohydrate units. Kdo: 2-keto-3- deoxyoctulosonic acid; Hep: D-glycero-D-manno-heptose, P: phosphate; (Alexander and Rietschel, 2001).

The lipid A region, located at the innermost layer and highly conserved among bacterial species, consists of a disaccharide backbone to which fatty acid chains that anchor the LPS to the OM are attached. Positioned in the middle part, the core oligosaccharide, composed of 9 to 11 oligosaccharide units, adds complexity to LPS structure. Meanwhile, the O-antigen or O-chain, occupying the outermost and variable section, features repetitive carbohydrate units that not only confer variability among strains but also aid bacteria in evading phagocytosis and resisting the lytic action of the complement system (King et al., 2009; Sperandeo et al., 2017a). The complex forms of O-antigen and core oligosaccharides are not essential for bacterial growth, however they are needed for protective and virulence functions, and thus they are present in clinical and environmental isolates (Raetz et al., 2007). While the majority of Gram-negative bacteria possess the O-antigen, there are strains that lack this component, highlighting the diverse adaptations within this bacterial group.

2.3.3.2 LPS biosynthesis

The process of LPS biosynthesis starts at the cytoplasmic side of the inner membrane and is gradually completed with the maturation of the LPS and its incorporation into the outer membrane (**Figure 8**).

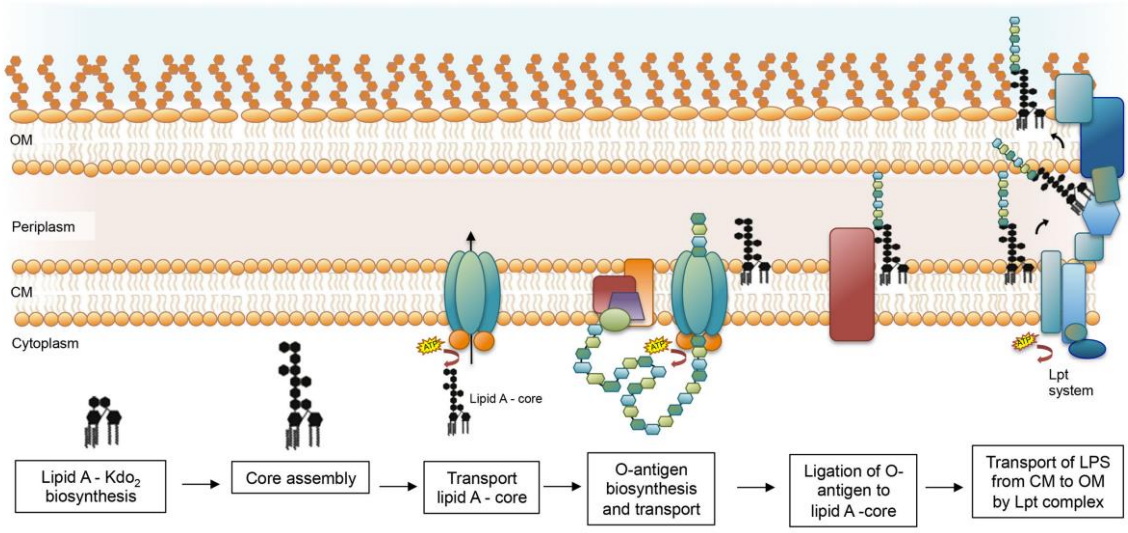

Figure 8: A simplified overview of LPS biosynthesis mechanism. After the completion of the biosynthesis of the mature lipid A and its attachement with the core oligosaccharide, the latter intermediate is flipped through the inner membrane by the action of MsbA complex. The O-antigen after being synthesized, gets attached to lipid A-core. The mature LPS is transported through the LPT complex situated in the membrane and is incorporated into the outer side of the outer membrane. CM: cytoplasmic membrane, OM: outer membrane, Lpt: LPS transport; (Maldonado et al., 2016).

The different parts are synthesized separately before being assembled and integrated into a cohesive structure that gets positioned on the outer leaflet that faces the external environment (King et al., 2009). The biosynthesis of each part of the LPS will be discussed in details in the next parts. Briefly, the biosynthesis of lipid A is initiated with a coordinated action of several enzymes at the cytosolic face of the inner membrane that results in the formation of the lipid A. A flippase lipid-activated ATP-ase MsbA flips lipid A-core towards the periplasmic site (Maldonado et al., 2016). Finally the mature LPS is translocated through the outer membrane with the help of the LPT complex (Brown, 2016). The process of LPS biosynthesis is very well coordinated, otherwise it can lead to defects in LPS structure and function (Maldonado et al., 2016). The end result of this process is the formation of a densely packed LPS layer interacting with numerous divalent cations like Mg^{2+} and Ca^{2+} present in the surrounding environment, which interact with the negatively charged lipid A and oligosaccharide core. Consequently, this rigid and tightly packed layer enhances the outer membrane's impermeability to hydrophobic

molecules and large hydrophilic compounds, which are restricted from passage through the narrow porin channels (Sperandeo et al., 2017a), thus mediating resistance from the external stress factors.

2.3.3.2.1 Lipid A biosynthesis

The lipid A is composed of double phosphorylated diglucosamine backbone to which penta-acyl or hexa-acyl chains are attached (**Figure 9**).

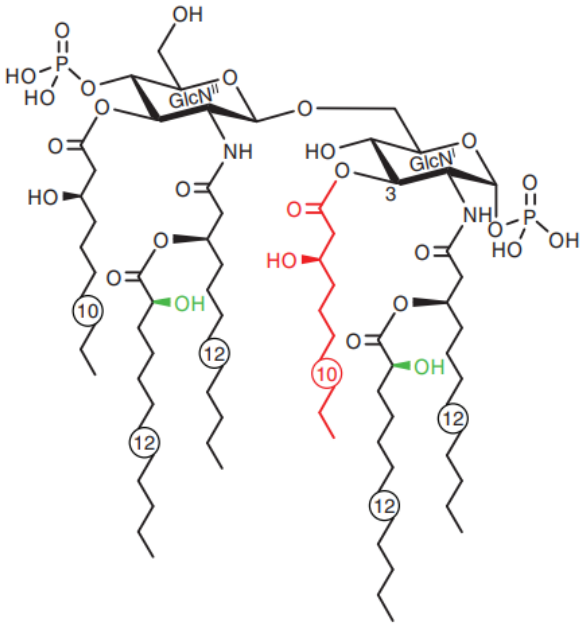

Figure 9: Representation of a hexa-acyl lipid A structure of *Pseudomonas aeruginosa*. Lipid A is composed of two phosphorylated diglucosamine residues ($GlcN^{I}$ and $GlcN^{II}$). Two 12-carbon acyl chains are added at position 2 and 2', to which 12-carbon secondary acyl chains, hydroxylated are added. O-linked 10 carbon hydroxydecanoyl substituents are added at position 3 of $GlcN^{I}$ and $GlcN^{II}$. The latter at position $GlcN^{I}$(red) is not present in 75% of lipid A of laboratory strains. (King et al., 2009).

Lipid A is the LPS part that is reponsible for inducing inflammatory response (Alexander and Rietschel, 2001). The number or carbons as well as acylchains vary between clinical strains or laboratory strains of PA, as well as between different Gram-negative bacteria. About 75% of laboratory *P. aeruginosa* strains have the penta-acyl form, wherease *E. coli* lipid A is hexa-acylated. Usually, hyperacylated / hexa-acylated clinical strains induce high inflammatory reponse against the immune system (King et al., 2009).

The enzymes involved in the lipid A pathway are constitutively expressed and are highly conserved. They can be found in the cytoplasm or in the inner surface of the inner membrane (Raetz et al., 2008). The first step of lipid A synthesis begins with the 3-O-acylation of the lipid

A precursor UDP-N-acetylglucosamine (UDP-GlcNAc), catalysed by LpxA enzyme in the cytoplasm (**Figure 10**).

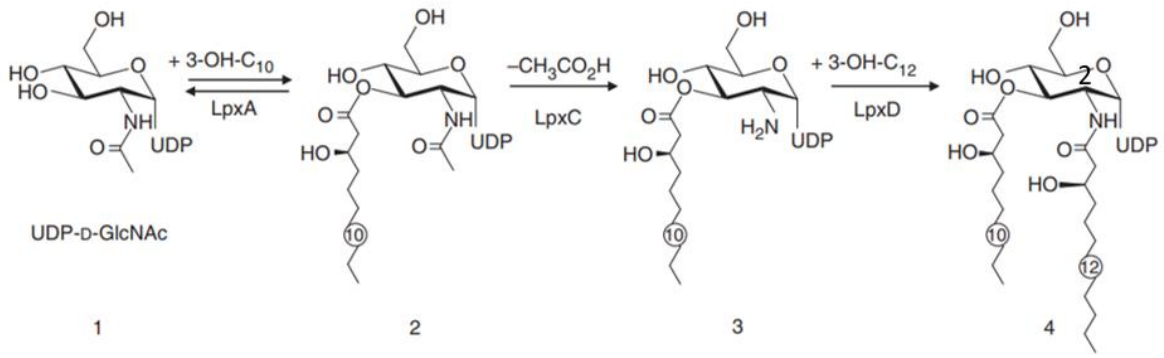

Figure 10: The first steps of lipid A biosynthesis. The starting compound is UDP-N-acetylglucosamine (1) that receives a R-3-hydroxydecanoic fatty by LpxA. A second deacetylation step is done by LpxC on LpxA product, producing UDP-3-O-(3-hydroxydecanoyl) glucosamine (3). LpxD catalyses the third step by transferring another R-3-hydroxydeconate to the amine at position 2 of product (3), and produces UDP-2,3-diacyl-GlcN (4); adapted from (King et al., 2009).

LpxA is an acyl-carrier protein (ACP) that has fatty acids linked to it as substrates. Unlike *E. coli* whose LpxA has preference for a 14-carbon hydroxymyristoic acid, *P. aeruginosa* LpxA favors the addition of shorter fatty acid chains (King et al., 2009). The next step is the deacylation by LpxC to generate a free amine substrate at position 2. The following step envolves the addition of the 12-carbon 3-hydroxylauroyl group by LpxD enzyme. A longer fatty acid chain is added by LpxD homologues of *E. coli*. The resulting intermediate is UDP-2,3-diacyl-GlcN (Raetz et al., 2008). The next steps involved the biosynthesis of Lipid IVA, an essential intermediate in Gram-negative bacteria.

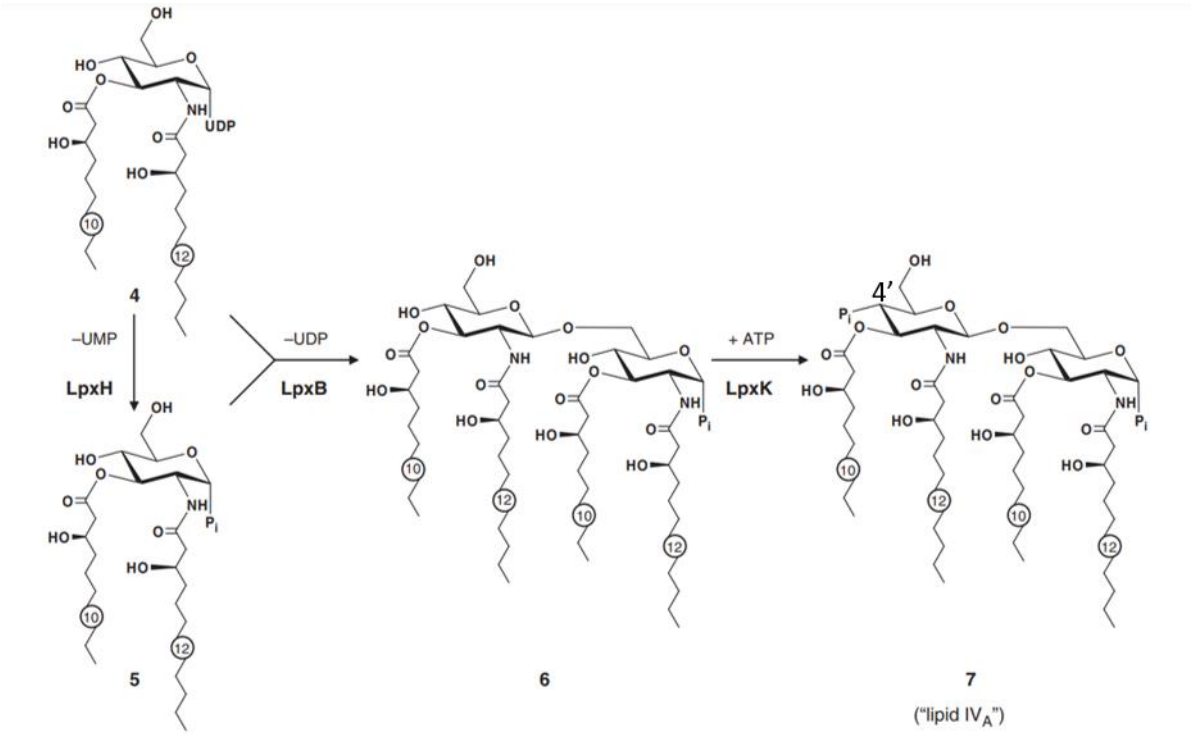

Figure 11: Steps for the synthesis of lipid IVA. UDP-2,3-diacyl-GlcN (4) is cleaved into lipid X (5). Two lipid X molecules are condensed to generate a tetra-acylated disaccharide (6). Finally, a phosphate group is added at carbon 4'. The end product is lipid IVA. P_i: phosphate; adapted from (King et al., 2009).

Once UDP-2,3-diacyl-GlcN is generated, it is then cleaved by the nucleotidase LpxH to form 2,3-diacylglucosamine-1-phosphate, also called lipid X or lipid A precursor (**Figure 11**). A tetra-acylated disaccharide lipid A is then generated by condensing two lipid X molecules by the action of the disaccharide synthase LpxB. The next five steps are catalysed at IM proteins. The tetra-acylated lipid A is further modified by the addition of a phosphate group to the 4'-position of the sugar residue by the action of the kinase LpxK to generate the intermediate lipid IVA (Raetz et al., 2008). Lipid IVA is thought to have endotoxin antagonist function in human cells.

In *E. coli* strains, the next step is the addition of two 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) molecules; this step is obligatory for the completion of lipid A biosynthesis. They derive from arabinose 5-phosphate, and their addition is catalysed by KdtA enzyme. The resulting intermediate is Kdo2-IVA (King et al., 2009). In *P. aeruginose* however, it has been shown that lipid A biosynthesis could be completed without the absolute necessity of Kdo residues addition to lipid-IVA (King et al., 2009). The final step before the completion of lipid A and before its transportion, is its decoration with secondary acyl groups (**Figure 12**).

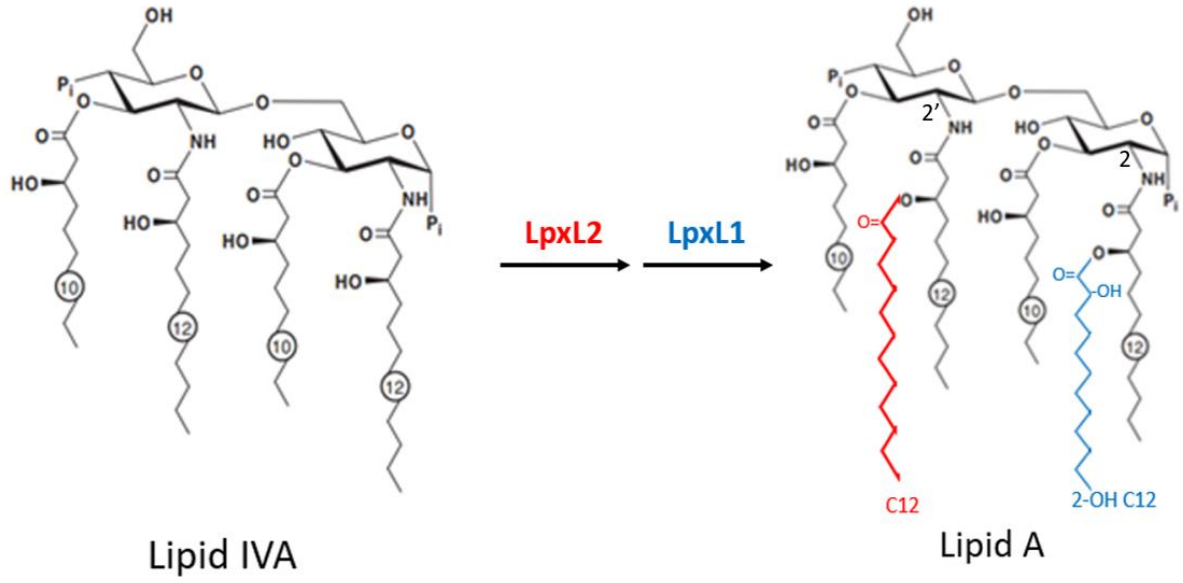

Figure 12: Secondary modifications of LpxL2 and LpxL1 enzymes. LpxL2 is responsible for the addition of laurate group at carbon 2', and LpxL1 adds a 2-hydroxylaurate group at carbon 2 on lipid IVA intermediate. The resulting product is lipid A; adapted from (King et al., 2009).

Acylation patterns of lipid A vary significantly among Gram-negative bacteria. *In E. coli*, the acyltransferase LpxL (HtrB) and LpxM acylate lipid A at carbons C2' and C3' respectively (King et al., 2009). HtrB enzyme adds a 12-carbon laurate group whereas LpxM adds a 14-carbon myristate group. In *P. aeruginosa*, two additional acyl groups are added, a 12-carbon fatty acid at position C2' and a second hydroxylated (2-hydroxylaurate) at position C2. These modifications are mediated by the lauroyltransferase enzymes LpxL1 and LpxL2, which are encoded by the *P. aeruginosa* genes PA0011 and PA3243, respectively. LpxL1 adds a 2-hydroxylaurate group while LpxL2 adds a laurate group at the second carbon of each glucosamine sugar (Hittle et al., 2015; Six et al., 2008). PA0011 and PA3243 share 30% and 47% amino-acid sequence identity with *E. coli* LpxL (HtrB) (Hittle et al., 2015). Even though a putative 3D structure was established, the protein is not well characterized in *P. aeruginosa*.

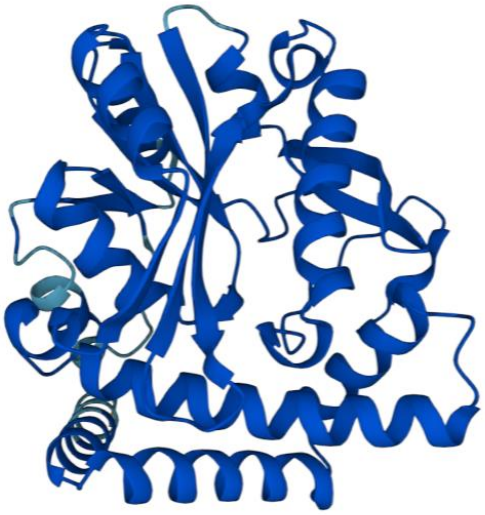

Figure 13: Predicted 3D structure of PAO1 LpxL1. Stucture obtained from AlphaFold Protein Structure Database (https://alphafold.ebi.ac.uk). UniProt ID is Q9I7B5.

LpxL1 is a monomer of 33 kDa (295 amino acids) (*E. coli* LpxL, 36 kDa), and is predicted to be like that of *E. coli* LpxL, to be imbedded in the inner membrane with single N-terminal transmembrane helix (**Figure 13**) (Raetz et al., 2007; Six et al., 2008, uniprot ID Q917B5).

Any defect that occurs in lipid A biosynthesis could alter the membrane integrity and permeability. Hittle et al showed that deleting either *lpxL1* or *lpxL2* increases the membrane permeability compared to parental strains using the ethidium bromide uptake assay, but does not affect growth (Hittle et al., 2015; Wang et al., 2016a). While an increase in membrane permeability was expected to also increase susceptibility to cationic antimicrobial peptides, only the deletion of *lpxL2* increases the susceptiblity to polymyxin B and colistin by 2 and 1.5 folds respectively. In two other studies, deletion of PA0011 gene encoding *lpxL1*, increased susceptibility to polymyxin B by at least 2-fold compared to the reference strain PAO1 (Shen et al., 2012; Wang et al., 2016a). Moreover, an *lpxL1* frameshift mutation was observed *in vitro* selected PAO1 mutants resistant to the newly developed anti-*Pseudomonas* peptidomimetic murepavadin, suggesting that *lpxL1* mutations could be responsible for 16-fold decrease in susceptibility to murepavadin (Díez-Aguilar et al., 2021b). This indicates the important role of LpxL1 in antibiotic resistance and outer membrane integrity (Wang et al., 2016b). Wang et al also showed that PA0011 gene contributes to resistance to carbapenems, gentamicin, and kanamycin with deficient mutants exhibiting a 2-fold increase in susceptibility (Wang et al., 2016a).

PA0011 gene is influenced by external stimuli such as temperature and osmotic changes (Shen et al., 2012). In *E. coli*, *htrB* gene is required for growth above 33°C, but not in *P. aeruginosa* (Clementz et al., 1996; Hittle et al., 2015). In one study, PA0011 deficient mutants were approximately 20 times more likely to die than PAO1 after exposure to 50°C for 20 minutes (Shen et al., 2012). Additionally, measuring PA0011 expression by luminescence with the *luxCDABE* reporter operon or by RT-qPCR showed that PA0011 expression was significantly higher at 21°C rather than 37°C. As a result, PAO1ΔPA0011, which is more susceptible to carbapenems, gentamicin, kanamycin and tetracycline than PAO1, becomes less susceptible when grown at 37°C (Wang et al., 2016b). These results indicate that PA0011 impacts susceptibility to antibiotics in a temperature-dependent manner.

The regulation of PA0011 is not fully understood, but one study showed that PA0011 promoter is activated by antibiotics such as tetracycline, streptomycin, azitromycin, erythromycin and ciprofloxacin (Shen et al., 2012).

2.3.3.2.2 Lipid A modifications

Lipid A is subjected to modifications in Gram-negative bacteria. Indeed, bacteria tend to add modifications or patterns to their lipid A part, like acylation or adding positively charged molecules to phosphate groups as a form of adaptation to external stimuli or to stresses (**Figure 14**).

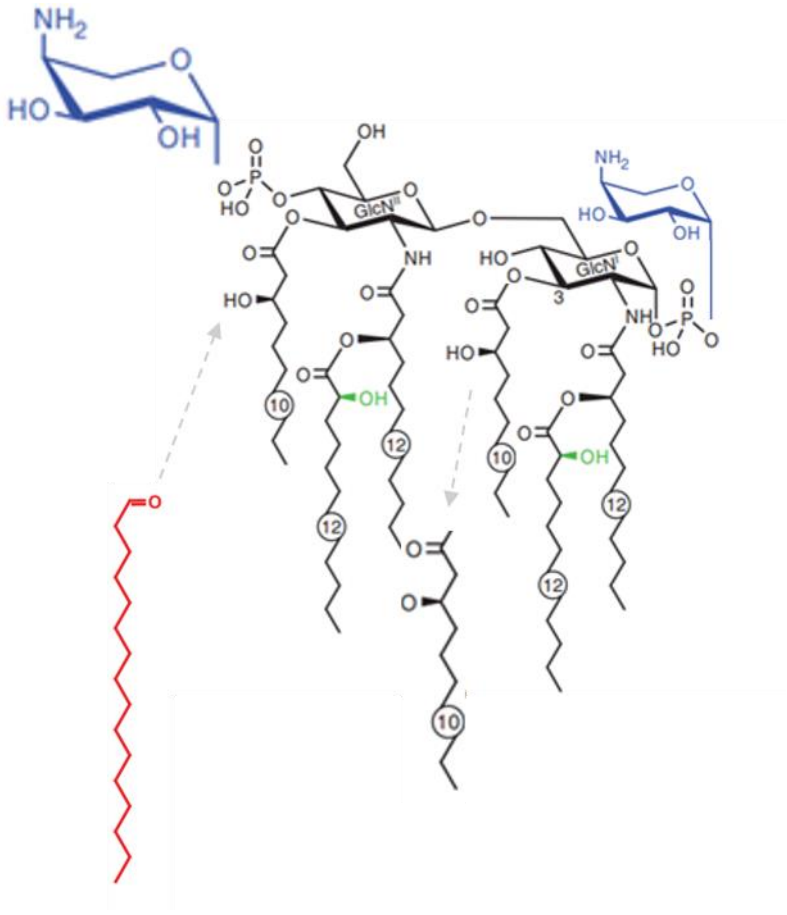

Figure 14: Representation of a hexa-acylated lipid A structure of *P. aeruginosa* and the modifications that could occur. The most common modification is the addition of 4-amino-4-deoxy- L- arabinose (L-Ara4N) substituents at the phosphate groups (blue). Addition of palmitoyl group is possible at C3' and deacetylation at C3. GlcN: diglucoseamine; adapted from (King et al., 2009; Maldonado et al., 2016).

In response to exposure to antimicrobial peptides or bactericidal agents, bacteria can reduce their susceptibility to cationic antimicrobial peptides (cAMPs) by reducing the negative charges of the phosphate substituents in lipid A. This is done by adding positively charged molecules such as 4-amino-4-deoxy-L-arabinose (L-Ara4N) to the phosphate residues. Reducing the negative charge of the OM reduces susceptibility to cAMPs (Maldonado et al., 2016). The *arnBCADTEF-ugd* operon of *P. aeruginosa* is responsible for the biosynthesis and transport of L-Ara4N. Other secondary modifications may occur by varying the number of acyl chains in the lipid A; *P. aeruginosa* lipid A is typically penta-acylated, but, hexa- or hepta-acylated lipid A is found in clinical isolates (Liang et al., 2016). Other modifications involing addition of a palmitoyl group on carbon 3', or deacetylation on carbon 3 have been reported, particularly in CF strains.

2.3.3.2.3 Core oligosaccharides structure and biosynthesis

The core oligosaccharide plays a critical role in pathogenicity as it inhibits the clearance and internalization of *P. aeruginosa*. It consists of an inner core, proximal to lipid A, and an outer core, proximal to the O-antigen, which is less conserved (**Figure 15**) (Sperandeo et al., 2017b).

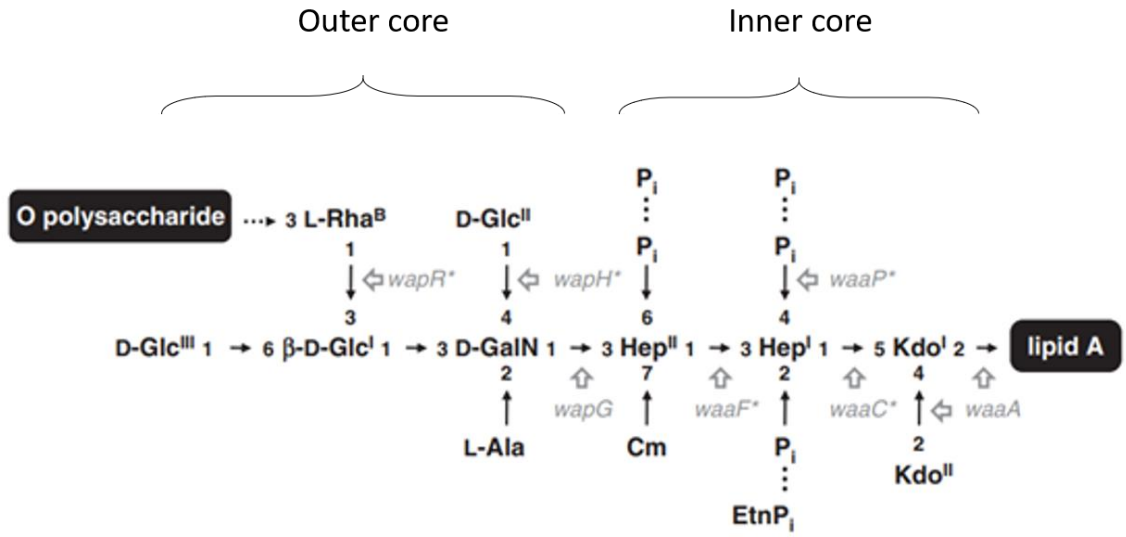

Figure 15: Schematic representation of the core oligosaccharide structure and the genes involved in its assembly. Dotted lines indicate substitutions that could not be found in all strains. Kdo= 3-deoxy-D-manno-oct-2-ulosonic acid, Hep: L-glycero-D-manno-heptose, P_i: phosphate, Etn: ethanolamine, Cm: carbamoyl, GalN: 2-amino-2-deoxy-galactose, Glc: glucose, Ala: alanine, Rha: rhamnose; adapted from (King et al., 2009).

The conserved inner core of Gram-negative bacteria including that of *P. aeruginosa* typically contains two residues of Kdo (Kdo^I and Kdo^{II}) and two L-glycero-D-mannoheptose (Hep^I and Hep^{II}), which serve as phosphorylation sites. The presence of negatively charged phosphate groups on heptoses is associated with increased intrinsic resistance to antibiotics like novobiocin, in addition to a more stable outer membrane (King et al., 2009; Whitfield and Trent, 2014). Core phosphorylation patters can vary between *P. aeruginosa* strains. Phosphorylation occurs on positions 2 and 4 of Hep^I and position 6 on Hep^{II} that could be mono, di or tri phosphate substituents. Some strains might also have diphosphoethanolamine on phosphate 2 of Hep^I. Phosphorylation is essential for *P. aeruginosa* viability, as mutations in the kinases lead to bacterial death. In addition to phosphorylation, other modifications can occur at Hep^I and Hep^{II} such as the addition of ethanolamine (Etn) and O-carbamoyl (Cm) groups respectively. The addition of Cm group was predicted after the identification of PA5005 gene,

homologue to O-carbamoyltransferase of *Streptomyces clavuligerus* by 31% aminoacid identity (King et al., 2009). In contrast, the outer core is more diverse and less conserved as it is more exposed to environmental stresses and selective pressure. In *P. aeruginosa*, it is constituted of 3 sugar types : three D-glucose (Glc) residues, one L-rhamnose (L-Rha) residue, and one 2-amino-2-deoxy-D-galactose (D-galactosamine, GalN) residue (King et al., 2009). Once the core is synthesized and attached to the lipid A, it is transported from the cytoplasmic side to the periplasm by MsbA (King et al., 2009).

The mechanism of core assembly in *P. aeruginosa* was predicted based on homology with *E. coli* and *Salmonella* genes (**Figure 15**). Briefly, the initial step involves WaaA enzyme adding the first two Kdo groups (Kdo^{I} and Kdo^{II}) to the glucosamines of lipid A. Once Kdo residues are added, the heptosyltransferases WaaC and WaaF add Hep^{I} and Hep^{II} to the inner core. The addition of the first outer core sugar has been identified to be accomplished by WapG, homologue to WaaG in *E. coli*. It is responsible for the addition of GalN residue to Hep^{II}. Another glycosyltransferase WapH then adds Glc^{II} to GalN. Next, rhamnosyltransferases WapR catalyses the addition of Rha to Glc^{I}. The final step involved WaaG that adds the last sugar Glc^{III} to Glc^{I}.

2.3.3.2.4 Lipid A-core transport through the inner membrane by MsbA

The MsbA protein is part of the ABC transporter superfamily which is responsible for the transport of the lipid A-core from the cytoplasmic to the periplasmic face of the inner membrane in Gram-negative bacteria. Inactivation of MsbA leads to the accumulation of LPS in the cytoplasmic part of the inner membrane, in addition to causing lethality of strains, indicating that it is an essential gene (**Figure 16**) (Bertani and Ruiz, 2018; Ghanei et al., 2007; King et al., 2009).

MsbA has two transmembrane domains (TMDs) embedded in the inner membrane and two nucleotide-binding domains (NBDs) located in the cytoplasm (Whitfield and Trent, 2014). MsbA uses ATP hydrolysis to flip lipid A-core from the cytoplasmic side to the periplasmic face of the cytoplasmic membrane, where it is further modified and assembled into LPS. Following translocation of lipid A-core to the periplasmic side, it is anchored at the outer leaflet of the IM so that the O-antigen can be incorporated. Once the mature LPS is produced, the

lipopolysaccharide transport protein (LPT) complex translocates it through the outer membrane and incorporates it into the outer leaflet (Sperandeo et al., 2017b).

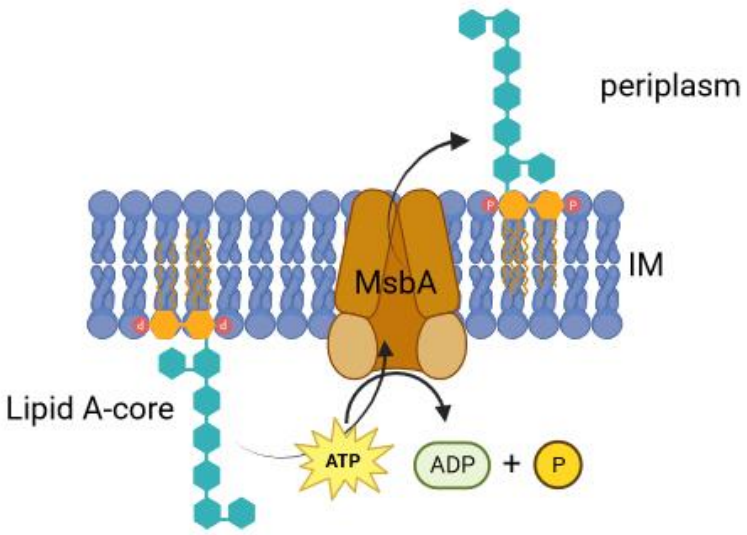

Figure 16: Schematic representation of the mechanism of action of MsbA. MsbA, integrated in the inner membrane, uses ATP hydrolysis to drive the translocation of lipid A-core complex from the cytoplasmic side of the inner membrane towards the periplasmic side of the inner membrane. ATP: adenosine tri-phosphate, ADP: adenosine di-phosphate, P: phosphate, IM: inner membrane; adapted from (Maldonado et al., 2016).

2.3.3.2.5 O-polysaccharide structure and biosynthesis

The O-polysaccharide extends outward from the outer membrane and plays a critical role in the interactions between bacterial strains and their environment. It also triggers a powerful immune response from the host due to its high immunogenicity. In *P. aeruginosa* infections, the O-specific antigen provides protection against phagocytosis and confers resistance to complement-mediated killing in addition to protecting the bacteria against oxidative stress (King et al., 2009). In cases of long-term *P. aeruginosa* infections, strains frequently decrease and eventually eliminate O-antigen production. This is due to selective pressure from the immune system, which targets the O-antigen (King et al., 2009). This outermost part of the LPS is highly diverse and consists of variable number of repeatable oligosaccharide units. Unlike the core oligosaccharide which is synthesized on the lipid A molecule, the O-polysaccharide is synthesized separately from the rest of the LPS molecule (Bertani and Ruiz, 2018).

Two forms of O-antigens can be synthesized, Common Polysaccharide Antigen (CPA) and O-Specific Antigen (OSA). The CPA is a homopolymer of D-rhamnose residues. Most *P.*

aeruginosa isolates produce the CPA form. The OSA is composed of repetitive units of different sugars (King et al., 2009).

The mechanism of ligation of the O-antigen to the lipid A-core is different between the two forms. CPA biosynthesis follows the ABC transporter-dependent pathway (**Figure 17**).

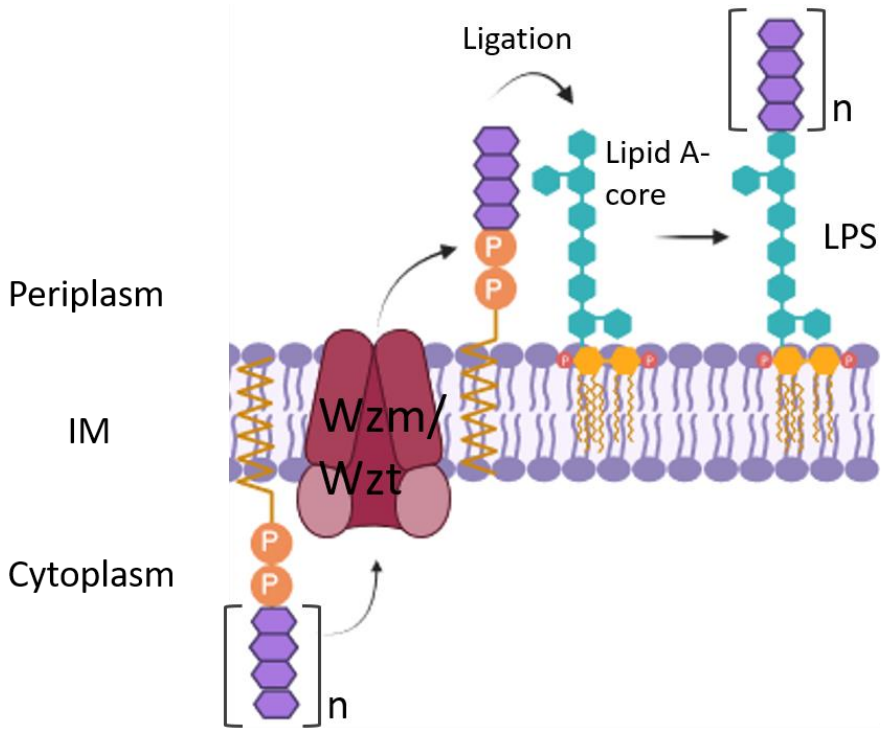

Figure 17: Schematic representation of the ABC-transporter dependent pathway for Common Polysaccharide Antigen (CPA) synthesis. The O-chain of D-rhamnose residues is polymerized on a lipid carrier in the cytoplasm. Once polymerization is terminated, the chain is flipped by the ABC transporter Wzm/Wzt to the periplasmic side and is ligated on lipid A-core complex. IM: inner membrane, LPS: lipopolysaccharide; adapated from (Melamed and Brockhausen, 2021).

Briefly, the O-chain is fully polymerized at the cytoplasmic side of the inner membrane and, once completed, it is transported by the ABC transporter Wzm/Wzt to the periplasmic side of the inner membrane (Melamed and Brockhausen, 2021). In contrast, the OSA biosynthesis follows the Wzy-dependent pathway (**Figure 18**) (King et al., 2009).

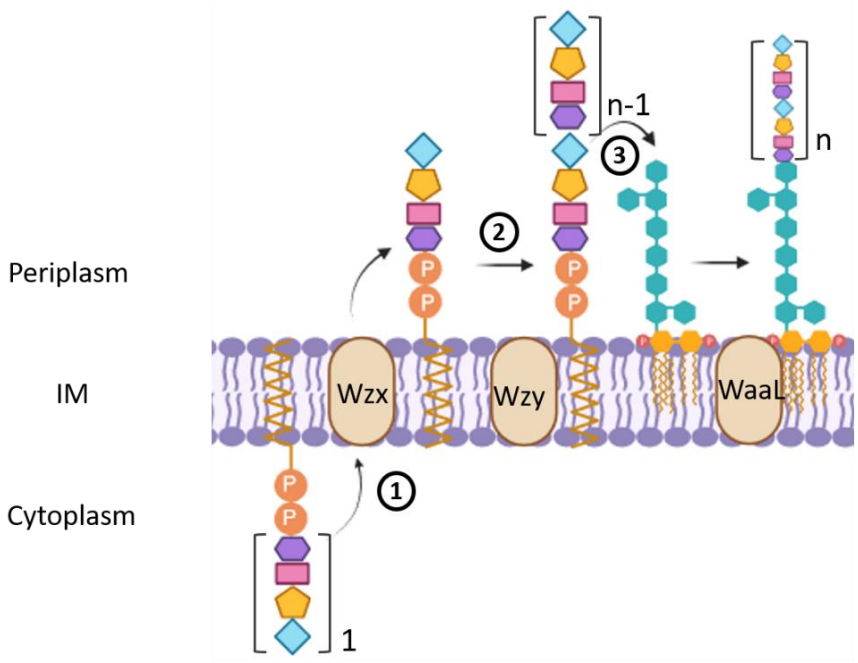

Figure 18: Schematic representation of the Wzy- dependent pathway for O-Specific Antigen (OSA) synthesis. A repeating unit is synthesized sequentially on a lipid carrier in the cytoplasm and is transported by Wzx to the periplasmic side (1). Wzy extends the O-units together to optimal chain length (2). WaaL ligates the elongated O-chain to lipid A-core (3). P: phosphate; adapted from (Hong et al., 2018).

In this mechanism, individual O-repeating units are flipped across the inner membrane and are ligated together with the help of the *wzy* gene product.

2.3.3.3 LPS Transport by the LPT complex

Due to the amphipathic property of the LPS, it needs to be transported through the OM from the periplasm to the outer leaflet of the OM. The Lpt complex protects the hydrophobic acyl chains from the aqueous environment of the periplasmn, and ensures the proper membrane transport. The Lpt complex, composed of seven subunits, LptA, LptB, LptC, LptD, LptE, LptF, LptG, is not present in all Gram-negative bacteria (Sperandeo et al., 2017a). In *P. aeruginosa* and *E. coli*, LPS is an essential component of the outer membrane and the presence of an entire Lpt complex is required for viability (Lo Sciuto et al., 2018). Amino-acid substitutions or the absence of any of the Lpt proteins can impair bacterial growth, susceptibility to antibiotics, and virulence (Lo Sciuto et al., 2018; Steeghs et al., 2001).

During the biogenesis of the outer membrane in Gram-negative bacteria, LptA-LptG proteins assemble to form a complex that spans the entire envelope from the cytoplasm to the OM (**Figure 19**) (Andolina et al., 2018b; Botos et al., 2016; Robinson, 2019; Whitfield and Trent,

2014). The Lpt complex is organised into two sub-assemblies, $LptB_2CFG$ in the IM and LptDE in the outer membrane, connected by the central and periplasmic proteins LptA (Lo Sciuto et al., 2018).

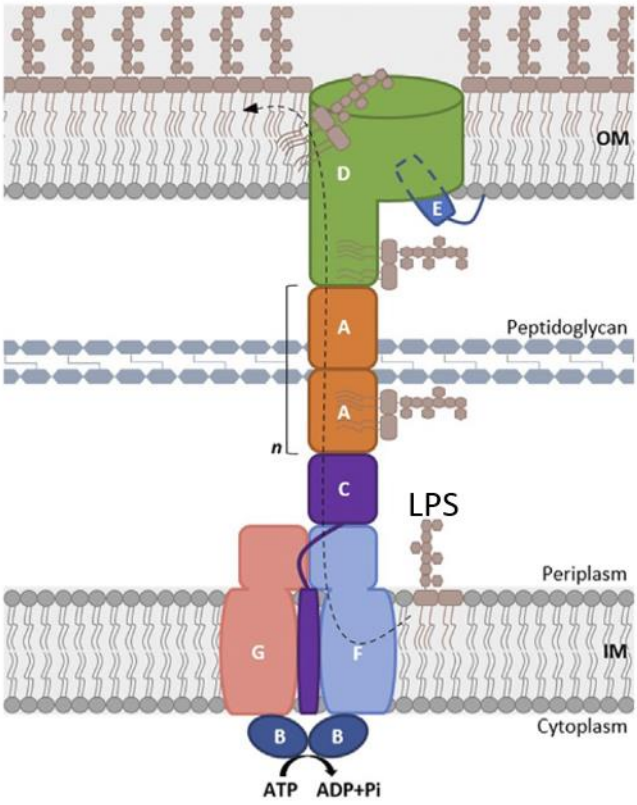

Figure 19: Schematic representation of the Lpt complex. $LptB_2FG$ extract the LPS from the inner membrane and delivers it to the bridge formed of LptC, two subunits of LptA and the N-terminal side of LptD that translocates LPS molecules. A complex formed of the β-barrel LptD and LptE, situated in the outer membrane, transfers and incorporates LPS molecules into the outer leaflet of the outer membrane; adapted from (Martorana et al., 2021).

The transport process begins with the ATP binding cassette (ABC) transporter $LptB_2FG$, which extracts LPS molecules from the inner membrane using the ATP hydrolysis activity of LptB. The LPS molecules are then transferred to a bridge formed of two copies of LptA, which interacts with LptC (Andolina et al., 2018a). LptC is an IM protein with a single TM domain and a large periplasmic region (Villa et al., 2013). The OM, LptD-LptE complex, which is organized as a « plug-and-barrel » structure, (Bollati et al., 2015) translocates LPS from LptA and incorporates it into the outer leaflet of the OM (Andolina et al., 2018a). LptD is an integral outer membrane protein with two important domains: (i) a C-terminal β-barrel domain to which the lipoprotein LptE is anchored in the lumen, and (ii) a N-terminal periplasmic β-jellyroll domain that interacts with LptA (Botos et al., 2016; Sperandeo et al., 2017a). LptE is a

lipoprotein component that interacts with the LptD to form the LPS transport and assembly machinery at the OM in *P. aeruginosa*. It acts as a chaperone to insert and direct LptD into the membrane (Grabowicz et al., 2013; Lo Sciuto et al., 2018). Although the function of LptE is not well understood its loss reduces LptD integration into the outer membrane, suggesting that it plays an important role in LptD function and assembly (Lo Sciuto et al., 2018). Despite being essential for the Lpt complex in *P. aeruginosa*, LptE may be dispensable for growth, but its loss impairs mebrane integrity and drug resistance (Botos et al., 2016; Lo Sciuto et al., 2018).

After synthesis in the cytoplasm, both LptD and LptE pass through the Sec translocon but the completion of their biogenesis pathways diverges at the periplasmic side (**Figure 4**). After signal sequence cleavage, LptD is delivered to the BamABCDE complex by the periplasmic chaperone SurA for assembly into the OM (Chimalakonda et al., 2011). In the case of LptE which is destined for the OM, it gets released from the IM after lipid modifications and delivered to the periplasmic chaperones for assembly into the OM (Chimalakonda et al., 2011; Tokuda, 2009).

The impact of *lpt* genes mutations on Gram-negative bacteria has been discussed extensively in literature (**table 3**). While some mutations may have only a minor effect on the function of the Lpt complex, others may completely abolish its function. Mutations in *lpt* genes can lead to impaired LPS transport due to unfunctional proteins, and to a variety of phenotypes, including impaired growth as shown for LptD mutations in *Yersinia pestis*, increased or decreased susceptibility in *P. aeruginosa* to inhibitors of Lpt complex.

Table 3: List of mutations identified in Lpt complex components in Gram-negative bacteria and their impact.

LpT complex subunit	Mutation	Species	Impact	Reference
LptA	Gene deletion	*P. aeruginosa*	Increased susceptibility to aminoglycosides and increased membrane permeability	(Krahn et al., 2012)
LptC	$G_{56}V$	*E. coli*	Increased susceptibility to polymyxin B and colistin and increased outer membrane permeability	(Fernández et al., 2013)
LptD	Dup$LRDKGM_{210-215}$	*P. aeruginosa*	Resistance to murepavadin	(Srinivas et al., 2010)
LptD	$G_{214}D$	*P. aeruginosa*	Resistance to murepavadin	(Díez-Aguilar et al., 2021b)
LptD	$P_{231}A$, $P_{246}A$	*Yersinia pestis*	Reduced viability	(Botos et al., 2016)
LptD	$\Delta_{330–352}$, $\Delta_{335–359}$	*P. aeruginosa*	Increased susceptibility to antibiotics (rifampicin, ciprofloxacin, tetracycline, novobiocin, azithromycine, colistin) and increased membrane permeability	(Balibar and Grabowicz, 2016)
LptD	$\Delta_{529–538}$	*E. coli*	Increased outer membrane permeability	(Balibar and Grabowicz, 2016; Freinkman et al., 2011)
LptE	$K_{136}D$, $R_{91}D$, $K_{136}D$	*E. coli*	Reduced binding of LPS to LPT complex and reduced LPS export	These BAETA
LptE	$YPISA_{116-120}YRA$	*E. coli*	Increased susceptibility to hydrophobic antibiotics (rifampicin and bacitracin)	(Balibar and Grabowicz, 2016; Chimalakonda et al., 2011)

3. Outer membrane proteins as interesting antibiotic targets

Bacterial outer membrane proteins (OMPs) are a desirable target for designing antibiotics due to their essential roles (Andolina et al., 2018a). Disrupting membrane's essential functions can increase bacterial susceptibility to antibiotics and boost the immune system's ability to clear infections. Additionally, as OMPs are located on the bacterial surface, they are more accessible to therapeutic agents than intracellular targets. For example, Bam complex is currently a target in the development of anti Gram-negative drugs. The heptapeptide Darobactin A, is an antimicrobial peptide produced by *Photorhabdus*. It targets BamA, preventing protein folding and membrane insertion. Darobactin A has shown, *in vitro* and *in vivo*, promising antibacterial activity against several Gram-negative strains including polymyxin-resistant *P. aeruginosa*, yet not on all *P.aeruginosa* strains (Imai et al., 2019). Further attempts to enhance the antibacterial activity of darobactin A lead to the development of two other analog derivatives. Those are darobactin B and darobactin B9. So far, the recently developed darobactin analogs show a stronger activity to reference strain PAO1 than darobactin A, with darobactin B9 being the strongest (MIC of darobactins B and B9 on PAO1 is 2-fold and 4-fold less than that of darobactin A). Interestingly, the two analogs show also a primising antibacterial activity on 66 CF *P. aeruginosa* clinical strains with MIC_{90} for darobactin B and darobactin B9 at 8 mg/L. This observed MIC value is even lower than the recently authorized drugs: ceftazidime-avibactam (CZA) (MIC_{90} >256 mg/L) and ceftolozane-tazobactam (C/T) (MIC = 96 mg/L) (Marner et al., 2023). These results make darobactins B and B9 promising potential candidates for patients infected with multidrug resistant pathogens.

The Lpt complex is another attractive target for the development of new antibiotics. In addition, the Lpt complex is a relatively conserved in most Gram-negative bacteria, suggesting that these compounds are likely effective against a wide range of Gram-negative bacteria, including those that are resistant to other antibiotics. Several antibiotics that target the Lpt complex are currently in clinical development (Robinson, 2019). Among them, thanatin is a peptide produced by the *Podisus maculiventris* insect (Robinson, 2019) that targets LptA, a protein essential for the transport of LPS across the OM. LptA forms a bridge through which LPS is translocated, and thanatin is thought to disrupt this process by binding to the N-terminal β-strand of LptA and blocking its interaction with LptC, another protein involved in LPS transport (Fehlbaum et al., 1996). Murepavadin is a macrocyclic peptidomimetic antibiotic that targets LptD, a β-barrel protein that forms the central pore of the Lpt complex. LptD is essential for the translocation of LPS, and murepavadin is thought to prevent this process by binding to LptD and disrupting its structure. Both thanatin and murepavadin have shown promising activity against a wide range of Gram-negative bacteria, including multidrug-resistant strains. They are also well tolerated in animal models, suggesting that they could be safe and effective antibiotics for the treatment of human infections (Martin-Loeches et al., 2018a).

IMB-881, is a synthetic antibacterial agent that also interferes with the assembly of the subunits of the Lpt complex. IMB-881 was found to inhibit the interaction between LptA and LptC using yeast two-hybrid screening and surface plasmon resonance experiments (Zhang et al., 2019). Exposition of *E. coli* ATCC 25922 strain to IMB-881 resulted in defective morphology and accumulation of membrane material in the periplasm. Overall, the efficacity of OMP targets gives promises for preventing life-threatening bacterial infections.

3.1 Antimicrobial peptides

With the advancement of combinatorial chemistry, the incorporation of aminoacids and peptides into heterocyclic-based molecules has become possible. The presence of aminoacids in small molecules reduces their toxicity, and enhances their pharmacokinetics and potency properties (Wang et al., 2018). As a result, peptide drugs have gained significant interest and are expected to play a major role in the future of drug discovery, especially in the fight against drug resistance (Chen and Jiang, 2023).

Antimicrobial peptides (AMPs) are small, diverse, naturally occurring molecules, found in virtually all life forms. They play a crucial role in the innate immune response of various organisms, including humans, animals, plants, and microorganisms (Chen and Jiang, 2023).

AMPs have potent antimicrobial activity against a broad spectrum of bacteria, fungi, viruses, and even cancer cells (Zhang et al., 2021). They have a selective activity against bacterial membranes, which are typically composed of negatively charged phospholipids. AMPs, which are consituted of 10 to 50 amino acid residues, are typically cationic and amphipathic (i.e, they have both hydrophobic and hydrophilic regions), interact with the negatively charged bacterial cell membranes and insert their hydrophobic regions into the membrane, disrupting its structure and leading to cell death (Chen and Jiang, 2023; Mahlapuu et al., 2016). Only few AMPs have negative charge (Chen and Jiang, 2023). Unlike conventional antibiotics, which target specific proteins and develop resistance rapidly, the evolution of resistance to AMP is much slower (Herzog and Fridman, 2014; Yu et al., 2017). This is because altering the composition or structure of the bacterial membrane in order to acquire resistance is a difficult and costly process in terms of energy requirement, and it poses a significant risk to the survival of the bacteria (Lima et al., 2021). Aditionnally, AMPs are less toxic than antibiotics because they break down into amino acids instead of generating harmful metabolites (Rima et al., 2021). These characteristics make AMPs highly desirable alternatives to antibiotics and promising candidates for addressing the global crisis of drug resistance.

Despite targeting membranes, AMPs are selective towards mammalian cells. This is due to fundamental differences in the membrane composition. Mammalian membranes have negatively charged phospholipids, but they are localized in the inner leaflet facing the cytoplasmic side. Additionally, mammalian membranes are rich in cholesterol, which stabilizes the phospholipid bilayer and reduces the activity of AMPs. Therefore, AMPs interact much weaker with mammalian membranes than with bacterial membranes (Mahlapuu et al., 2016).

Efforts are underway to discover AMPs that target *P. aeruginosa*. Chen and collaborators provide a list of AMPs, in which some are in clinical development (Chen and Jiang, 2023). In additon to the POL7080 peptidomimetic, which is in phase I clinical trials (to be discussed in part 3.1.6), P-113D (Demegen Inc., USA) is a 12-amino acid antimicrobial peptide newly developed for aerosolisation treament for CF patients, to target *Pseudomonas aeruginosa* and *Staphylococcus aureus*. It is derived from its parent histatin-5, a 24-amino acid histidine-rich peptide produced by the salivary glands of higher primates (Sajjan et al., 2001). Lytixar ™

(LTX-109, Lytix Biopharma AS), a synthetic peptidomimetic developed for topical use against *P. aeruginosa* infections is currently in pre-clinical development (Mercer and O'Neil, 2013).

In the following parts, we focus on the polymyxin class of AMPs and on the peptidomimetic murepavadin because our work focuses not only on studying the antibacterial activity of murepavadin, but also on identifying resistance mechanisms to murepavadin and cross-resistance with colistin, the last resort treatment for CF patients.

3.1.1 Polymyxins class of cationic AMPs

The increased reports of resistance to Gram-negative bacteria and the emergence of MDR strains have led to the reconsideration of polymyxin antibiotics, which have been placed aside for several years due to their nephrotoxic effects (Olaitan et al., 2014).

Polymyxins are non-ribosomal cationic, cyclic antimicrobial peptides that were first isolated in the 1940s from the Gram-positive bacterium *Paenibacillus polymyxa*. They are currently being prescribed as rescue treatments for patients infected with MDR Gram-negative strains (Landman et al., 2008; Olaitan et al., 2014; Srinivas and Rivard, 2017).

Of the five polymyxins (A-E), only polymyxin E (colistin) and polymyxin B are used in clinical setting (**Figure 20**). They have a similar structure, differing by only one amino acid, and a similar mode of action (Landman et al., 2008; Srinivas and Rivard, 2017).

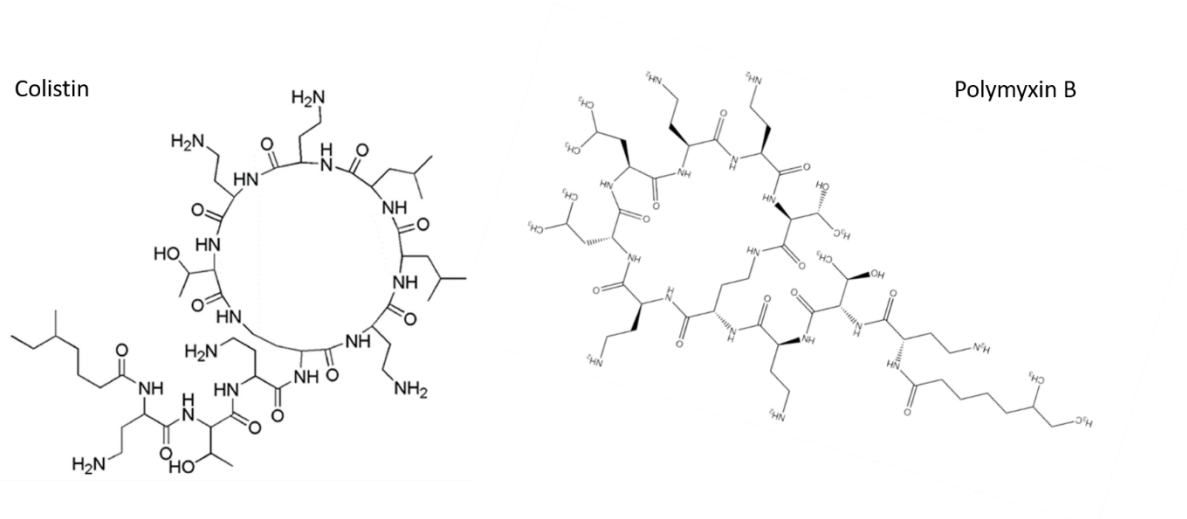

Figure 20: Two dimentional structure of colistin (polymyxin E) and polymyxin B (Alfei and Schito, 2020; Kadar et al., 2013).

3.1.2 Mode of action of polymyxins

Polymyxin B and colistin display antimicrobial activity against Gram-negative bacteria such as *Acinetobacter baumannii*, *P. aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli*, which are nosocomial pathogens known to exhibit resistance to conventional antibiotics. However, despite the susceptibility of these pathogens to polymyxins, and particularly to colistin, the emergence of resistant strains has already begun (Landman et al., 2008).

Like all AMPs, the mechanism of action of polymyxins involves their binding to the negatively charged Gram-negative outer membrane of bacteria, causing damage to the membrane structure by acting as detergent. Polymyxins displace positively charges ions such as Mg^{2+} and Ca^{2+}, which stabilize the LPS from their binding sites, due to their attraction for the negatively charged lipid A of LPS (Moffatt et al., 2019). As a result of membrane destabilisation and permeabilisation, colistin enters the bacterial cell by self-promoted uptake (Dijkmans et al., 2015). This interaction changes the inner membrane integrity and ultimatly causes leakage of the contents and cell lysis. Damage to the membrane integrity upon polymyxin exposure, may make bacteria more vulnerable to hydrophobic antimicrobials such as erythromycin (Landman et al., 2008).

3.1.3 Synthetic antimicrobial peptides

Despite their importance and diversity, natural AMPs may have undesirable side effects, degrade fastly, or exhibit hemolytic activity. In addition, the discovery of new natural AMPs can be difficult due to high production costs, low scalability and low yield (Lima et al., 2021). Therefore, one solution is to develop synthetic AMPs. The goal of designing synthetic AMPs is to retain the antimicrobial properties while excluding or improving undersirable properties. One approach is to start with a natural AMP template and make modifications to obtain structurally related peptides with improved properties. Another approach is to generate libraries of AMPs by combining different AMPs and using *in silico* technologies to predict new AMPs. One example of a synthetic AMP is AamAP1-Lysine, which is derived from scorpion venom. AamAP1-lysine has activity against *S. aureus*, *E. coli*, *Candida albicans* and *P. aeruginosa* (Almaaytah et al., 2014; Lima et al., 2021). Interestingly, the modified sAamAP1-Lysine has much lower minimal inhibitory concentration (MIC) (from 4 to 20 fold) against the same

pathogens than the initial AamAP1, making it a good candidate for future studies (Lima et al., 2021).

3.1.4 Protein epitope mimetic approach and peptidomimetics

Peptides and proteins that occur naturally offer promising starting points for designing and creating biologically active peptidomimetics that serve as new drugs, vaccines or other active molecules. One way to generate mimetics is by the protein epitope mimetic approach (PEM). Starting from naturally occuring peptides of known structure, the peptide epitopes, secondary structure backbone or the side chains could be grafted into alternative macrocyclic scaffolds to create libraries of thousands or peptides (**Figure 21**) (Luther et al., 2017; Zerbe et al., 2017).

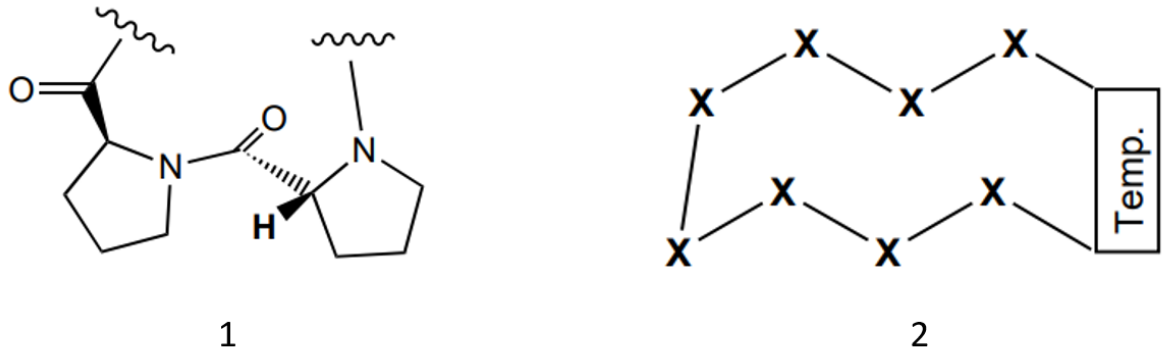

Figure 21: Schema representing the approach for the synthesis of β-hairpin mimetics. The template of 8-residue loop β-hairpin sequence is attached to an organic template like D-Pro-L-Pro (1) to generate a peptidomimetic (2). Temp: template; X: stands for any aminoacid; adapted from (Shankaramma et al., 2002).

β-hairpin cationic host-defense AMPs are a large family of short, positively charged peptides, are interesting starting points for generating protein epitope mimetics due to their important role in immune system of many organisms (Obrecht et al., 2011). Therefore, they are being investigated as potential new antibiotics. The β-hairpin structure is present in several cationic AMPs (CAMPs), all of which are stabilised by disulfide bridges. Therefore, by grafting the cationic hairpin sequences onto template that preserves the β-hairpin geometry to generate libraries of peptidomimetics are considered for further optimization as antibacterial agents (Shankaramma et al., 2002). During this PhD project, we worked principally with murepavadin

peptidomimetic derived from Protegrin-1. For this reason, I will present these two in more details in the following parts.

3.1.5 Protegrin-1 (PG-1)

One famous example to serve as a parent molecule for the generation of peptidomimetics is Protegrin-1 (PG-1). PG-1 is a small cationic cysteine-rich, 18-residue peptide with a β- hairpin structure stabilised by disulfide bridges (**Figure 22**) (Soundrarajan et al., 2019). It is found in porcine leukocytes in which it participates in the first line of non-specific defense in the immune system of pigs (Ranade and Ramalingam, 2020).

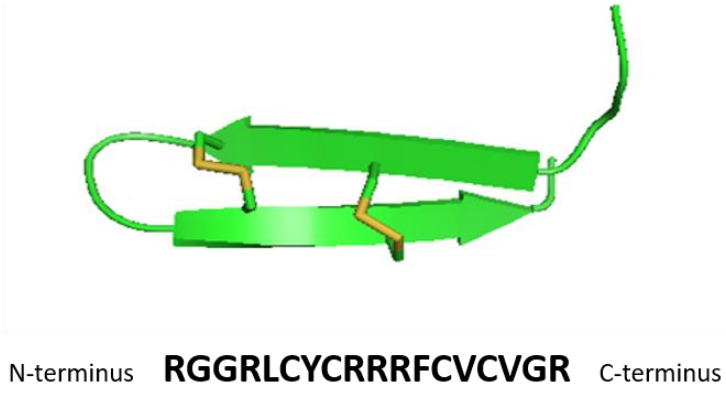

Figure 22: 3D structure of PG-1 (green) and its amino acid sequence indicated in letter code; adapted from (Soundrarajan et al., 2019).

Protegrin-1 is a broad sprectrum antimicrobial peptide with potent activity against MDR Gram-positive and Gram-negative bacteria. It works by binding to the negatively charged bacterial outer membrane, aggregating and permeabilizing the membrane through pore formation (Ranade and Ramalingam, 2020). The disulfide bridges in PG-1 are essential for its ability to form pores (Ranade and Ramalingam, 2020). Despite its strong antibacterial activity, PG-1 is also hemolytic and cytotoxic to human red blood cells (Shankaramma et al., 2002; Soundrarajan et al., 2019; Zerbe et al., 2017). Therefore, researchers have modified PG-1 to enhance its antibacterial activity and reduce its cytotoxicity. One approach has been to design β-hairpin protein mimetics based on the PG-1 structure (Obrecht et al., 2011).

Such mimetics are 14-amino acid β-hairpin PEM molecules that were generated by incorporating PG-1 side-chain residues to a 12-amino acid loop sequence attached to a D-

Proline-L-Proline scaffold. These molecules have remarkable antimicrobial properties, with different activities against different bacteria (**Figures 21** and **23**).

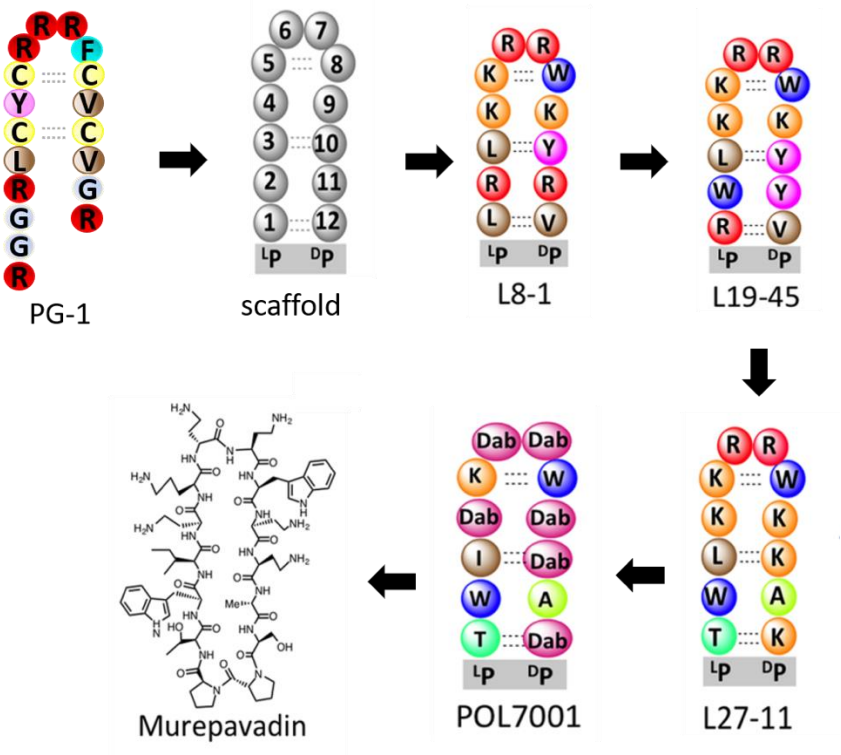

Figure 23: Schematic representation of the development of murepavadin by PEM approach. PG-1 residues are incorporated into a 12-residue β-hairpin scaffold (gray). The peptidomimetics L8-1, L19-45, L27-11, POL7001 resulted after rounds of optimisations and screaning for enhancing antibacterial properties. Dab: L-2,4-diaminobutyric acid, Lp: L-Proline, Dp: D-Proline; adapted from (Zerbe et al., 2017).

The first macrocyclic mimetic derived of PG-1 is L8-1. It displayed little membrane lytic activity to human red blood cells with respect to PG-1 (hemolytic activity of L8-1 was 1% while that of PG-1 was 37% at 100 µg/mL peptide concentration) (**table 4**) (Herzog and Fridman, 2014; Shankaramma et al., 2002).

Further rounds for optimizing structure-function relationship, target-affinity and specificity, drug like properties, plasma stability finally led to the discovery of murepavadin with an antimicrobial activity specific to *P. aeruginosa* (**table 5**).

Table 4: Drawbacks and advantages of the protein epitope mimetics of PG-1 until discovery of murepavadin POL7080.

PG-1 mimetics	Drawback	Advantage	Reference
PG-1	Membrane lytic activity Broad-spectrum activity (against Gram-positive and Gram-negative bacteria and fungi) Cytotoxic effects toward mamalian cells due to high hemolytic activity to red blood (37% at 100 µg/mL peptide concentration)	Rapid bactericidal effect (reduced viable CFU by more than three log units in less than 15 minutes	(Herzog and Fridman, 2014; Shankaramma et al., 2002; Steinberg et al., 1997)
L8-1	Broad spectrum activity (against Gram-positive and Gram-negative bacteria and fungi)	Reduced hemolytic activity to 1% of fresh human red bood cells (at 100 µg/mL peptide concentration)	(Herzog and Fridman, 2014; Shankaramma et al., 2002)
L19-45	Broad spectrum activity (against Gram-positive and Gram-negative bacteria and fungi)	Active against *P. aeruginosa*	(Zerbe et al., 2017)
L27-11	Rapid degradation by trypsin-like enzymes in human serum	Narrower spectrum of antimicrobial activity specific to *P. aeruginosa* Nanomolar range of activity	(Herzog and Fridman, 2014; Zerbe et al., 2017)
POL7001	Toxic effects when introduced intravenously	Clinical candidate More stable toward proteolysis	(Zerbe et al., 2017)
Murepavadin	Toxic effects when introduced intravenously	Cosely related to POL7001 Currently in Phase I clinical trials Retained for use in aerosolisation form	(Zerbe et al., 2017)

Table 5: MIC of PG-1 and its 5 epitope mimetics on *P. aeruginosa*, *S. aureus* and *E. coli* (Shankaramma et al., 2002; Srinivas et al., 2010).

Strain		MIC (mg/L)					
		PG-1	**L8-1**	**L19-45**	**L27-11**	**POL7001**	**Murepavadin (POL7080)**
P. aeruginosa	ATCC 27853	3	8	1	0.1	0.008	0.008
P. aeruginosa	PAO1	ND	8	2	0.004	0.008	0.004
S. aureus	ATCC 25923	6	8	64	64	>64	>64
E. coli	ATCC 25922	3	8	8	64	>64	>64

3.1.6 Murepavadin (POL7080)

Murepavadin, formly known as POL7080, is a 14 amino acid cyclic peptide developed by Polyphor AG by PEM approach (**Figure 24**). This peptidomimetic was fully synthesized and inspired from natural scaffold of PG-1. It has been optimized for drug-like properties and antibacterial activity specific to *P. aeruginosa* (Martin-Loeches et al., 2018a) after several rounds and iterations of optimisations (**Figure 23**).

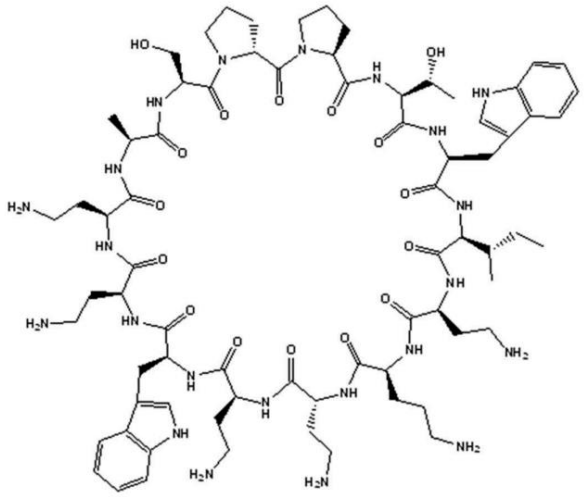

Figure 24: Structure of murepavadin (Martin-Loeches et al., 2018a).

3.1.6.1 Mode of action of murepavadin

Murepavadin is the first-in-class antibiotic called « Outer-Membrane Protein Targeting Antibiotic » OMPTA (Martin-Loeches et al., 2018a). It has a new and unique mechanism of action making it a promising therapeutic agent for cystic fibrosis patients colonized by *P. aeruginosa*.

Unlike other cationic antimicrobial peptides, such as colistin and PG-1, which promote cell lysis, murepavadin works through a non-lytic mechanism of action that is specific to *P. aeruginosa*. Murepavadin binds to the β-barrel protein LptD, which is involved in the transport of LPS to the OM. By blocking the transport of LPS, murepavadin disrupts the outer membrane biogenesis and ultimately leads to cell death. Murepavadin is specific to *P. aeruginosa* because of additional residues in the N-terminal domain of LptD that are not present in other Gram-negative bacteria. This specificity makes murepavadin less likely to harm the human native existing microbiota (**Figure 25**) (Srinivas et al., 2010)

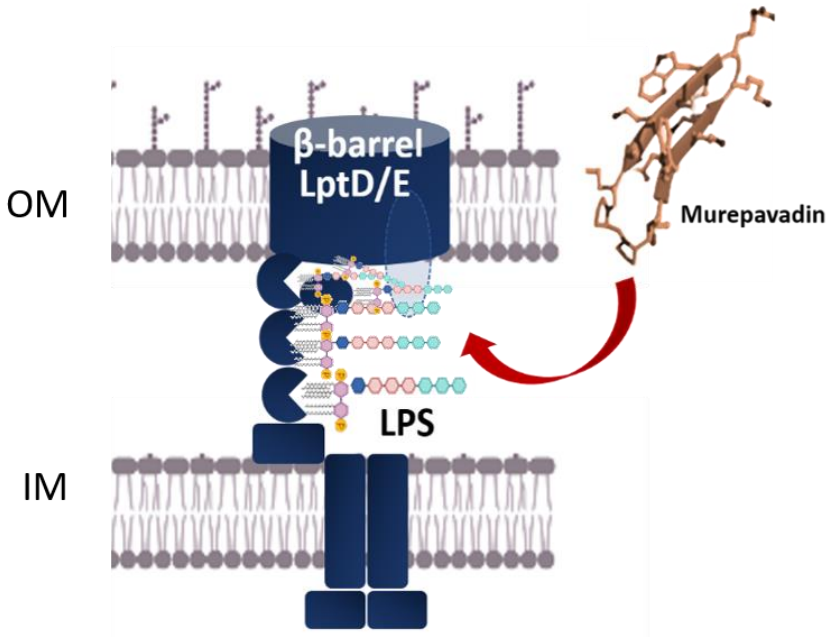

Figure 25: Mode of action of murepavadin. Murepavadin interacts with LptD and blocks the translocation of LPS molecules to the outer membrane. LPS gets accumulated in the periplasmic space leading to cell death. OM: outer membrane, IM: inner membrane, LPS: lipopolysaccharide; adapted from (Andolina et al., 2018a).

3.1.6.2 Murepavadin *in vitro* and *in vivo* activity

Murepavadin is a promising therapeutic antimicrobial peptide for showing important antimicrobial activity against *P. aeruginosa*. It is still currently in Phase I clinical trials for development as aerosolisation treatment before being commercialized to patients. Several studies have been recently published and documented the efficient antimicrobial activity of murepavadin against clinical *P. aeruginosa* strains. Murepavadin have showed to exhibit good antimicrobial activity against both MDR and XDR clinical isolates with an $MIC_{50/90}$= 0.12/0.12 mg/L (Sader et al., 2018a). In addition to that, murepavadin showed the highest activity ($MIC_{50/90}$ = 0.12/0/25) on a collection of 785 XDR clinical *P. aeruginosa* strains in comparison with conventional antibiotics (colistin $MIC_{50/90}$ = ½, meropenem $MIC_{50/90}$ = 16/>32, ceftolozane-tazobactam $MIC_{50/90}$ = 2/>32, tobramycin with $MIC_{50/90}$ = 8/>8) used for treatment of cystic fibrosis patients. It also has an activity against colistin resistant mutants (Sader et al., 2018b).

In addition to its efficacity *in vitro*, murepavadin has a direct relationship between dosage and its pharmacokinetics when tested in living organisms. It also shows strong capability to penetrate the epithelial lung fluid, which emphasizes its effectiveness *in vivo* in treating lung infections, even those caused by extensively drug-resistant isolates (Dale et al., 2018; Luther et al., 2017).

3.1.7 Resistance to antimicrobial peptides

3.1.7.1 Resistance to polymyxins

Gram-negative bacteria have an OM that serve as the first line of interaction with polymyxins and cationic AMPs. Therefore, the majority of resistance mechanisms occur at the level of the OM and specifically the LPS. In several Gram-negative bacteria, including *P. aeruginosa, Salmonella enterica* and *Escherichia coli*, drug resistant strains tend to add substituents like 4-amino-4-deoxy-L-arabinose (L-Ara4N) residues, phosphoethanolamine (PEtN) and/or galactose amine to the lipid A or LPS core (Jeannot et al., 2017; Moffatt et al., 2019). This reduces the net negative charge of the OM, thereby reducing its affinity for cationic polymyxins (Moffatt et al., 2019).

In *P. aeruginosa*, polymyxin resistance mainly occurs when amino-arabinose residues are added to the surface of the OM by attaching to the phosphate groups of the lipid A. The synthesis, translocation and the attachment of the amino-arabinose residues is controlled by a large operon *arnBCADTEF-ugd*. At least four TCS PmrAB, PhoPQ, CprRS and ParRS TCS have been mainly identified to activate this operon and thus cause polymyxin resistance (**Figure 26**) (Jeannot et al., 2017; Jochumsen et al., 2016; Olaitan et al., 2014).

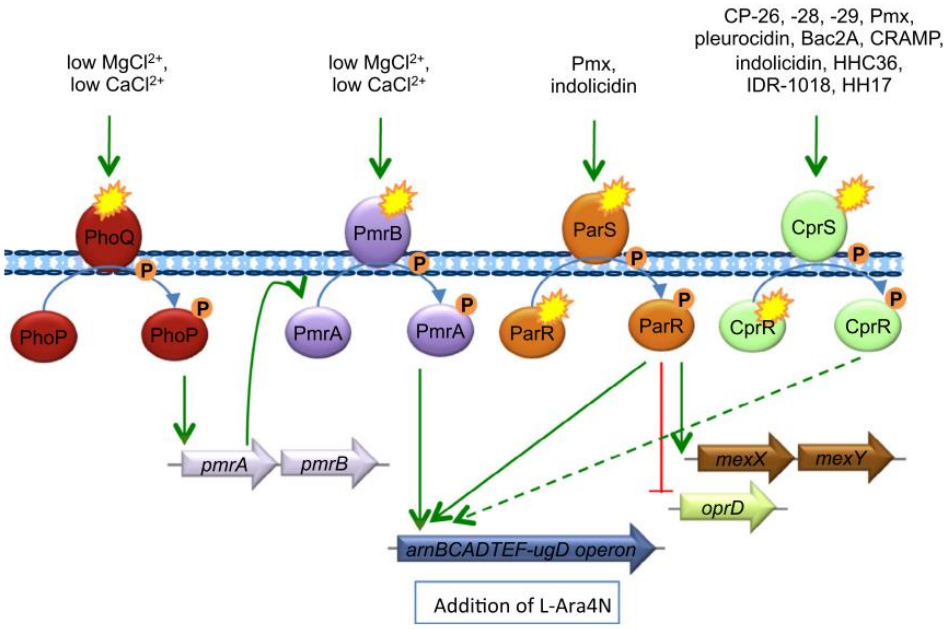

Figure 26: Schematic representation of two-component systems involved in polymyxins resistance in *P. aeruginosa*. The TCSs PhoPQ, PmrAB, ParRS and CprRS contribute to polymyxins resistance. PhoQ, PmrB, ParS and CprS are the sensor kinases situated in the inner membrane. PhoP, PmrA, ParR and CprR are response regulators situated in the cytoplasm. ParRS and CprRS are directly activated by polymyxins and antimicrobial peptides, while PhoPQ and PmrAB are directly activated by low concentration of divalent cations. The activation of these TCSs activates the *arnBCADTEF-ugD* operon that results in the production of amino-arabinose residues and their incorporation on the outer membrane, thus polymyxins resistance. ParRS also activates the expression of the multi-drug efflux pump *mexXY* operon resulting in aminoglycosides resistance. It also inhibits *oprD* expression resulting in carbapenems resistance, adapted from (Jeannot et al., 2017).

Two-component systems (TCS) are a family of global bacterial regulators, located into the inner membrane, that sense environmental signals. They are composed of a sensor transmembrane histidine kinase (HK) and a cytoplasmic or response regulator (RR). In the presence of signal, the HK of a TCS autophosphorylates at a conserved histidine residue by transferring the phosphate group from adenosine triphosphate (ATP). A wide variety of stimuli, such as oxygen levels, nutrient levels, osmolarity, cell envelop stress, antimicrobial peptides, can trigger this autophosphorylation event (Gao and Stock, 2009; Sivaneson et al., 2011). The phosphorylated

HK then transfers the phosphate group to conserved aspartate residue on the RR (**Figure 27**) (Depardieu et al., 2007). As a result, the output domain of the RR corresponding to a DNA binding domain binds to specific promoter region to regulate gene expression (Sivaneson et al., 2011).

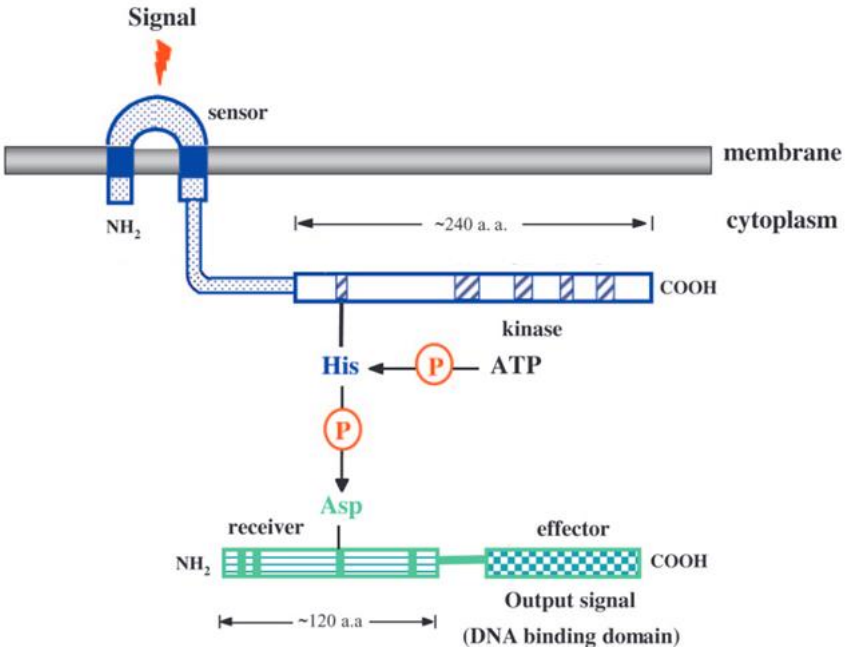

Figure 27: Schematic representation of a classic two-component system. It is composed of a sensor (blue) integrated in the membrane close to its N-terminal, and a response regulator (green) located in the cytoplasm. Conserved motifs are located in the sensor (hatched blue). The conserved histidine residue gets phosphorylated and transfers the phosphate on a conserved aspartate residue located in the receiver domain of the regulator. The effector domain of the regulator is a DNA binding site, that regulate gene expression as a result of response regulator phosphorylation. His: histidine, P: phosphate, Asp: aspartate, a.a: amino acids; adapted from (Depardieu et al., 2007).

The number of TCS in bacterial genomes is variable and is correlated with the bacterium's need to cope with complex environment (Sivaneson et al., 2011). *P. aeruginosa* has approximately 130 TCS genes, which confirms its great capacity for adaptation (Rodrigue et al., 2000).

PmrAB TCS is activated by low concentrations of divalent cations (Ca2+, Mg 2+) and cationic antimicrobial peptides including colistin and polymyxin B (McPhee et al., 2003). Downstream to *pmrA* and *pmrB* genes, three other genes PA4773 (*speD2*), PA4774 (*speE2*) and PA4775 are implicated in polyamines biosynthesis and are activated in response to antimicrobial peptides exposure (Ben Jeddou et al., 2020; Bolard et al., 2019; Johnson et al., 2012). Indeed, *P. aeruginosa* reference strain PA14 exposure to sub-inhibitory concentration of polymyxin B upregulated all the mentioned five genes in addition to the *arnBCADTEF-ugd* operon (Ben Jeddou et al., 2020). In addition to LPS modifications by the activation of the *arnBCADTEF-*

ugd operon, the presence of polyamines ensures a protective role against antimicrobial agents in which the MIC of colistin and polymyxin B increases significantly in the other reference strain PAO1 after the incubation with polyamines (Johnson et al., 2012; Kwon and Lu, 2006).

In addition to activation of TCSs by environmental signals, mutations in the genes encoding TCS are commonly found in colistin-resistant clinical strains isolated from CF patients. Gain-of function mutations of *pmrB, parR* and *parS* genes or loss of function mutation of *phoQ*, lead to constitutive activation of *arnBCADTEF-ugd* operon and the production of amino-arabinose residues (Jochumsen et al., 2016). Resistance to colistin has been mainly attributed to *pmrB* mutations (Moskowitz et al., 2012). Indeed, mutations in *pmrAB* are very common in colistin resistant CF clinical strains and frequently they correspond to amino acid substitutions and contribute to high-resistance levels to colistin (MIC > 256 µg/mL) (Moskowitz et al., 2012).

Besides LPS modification, the activation of the ParRS TCS activates the *mexXY* operon, which encodes for the multidrug efflux pump MexXY/OprM. MexXY/OprM is one of several resistance-nodulation-cell division (RND) family of multi-drug efflux systems that participate significantly in multi-drug resistance. It is considered as a primary player in aminoglycosides resistance (Morita et al., 2012), and has been shown to be implicated in colistin resistance in *pmrB* mutants (Puja et al., 2020). Other forms of polymyxin resistance include the encapsulation of AMPs with anionic bacterial capsule polysaccharides (CPS), resulting in reduced amount of AMPs reaching the bacterial surface (**Figure 28**) (Llobet et al., 2008). The production of CPS has been observed in *Klebsiella pneumoniae*, *Streptococcus pneumoniae* and *P. aeruginosa* and were described to increase resistance to polymyxins (Llobet et al., 2008).

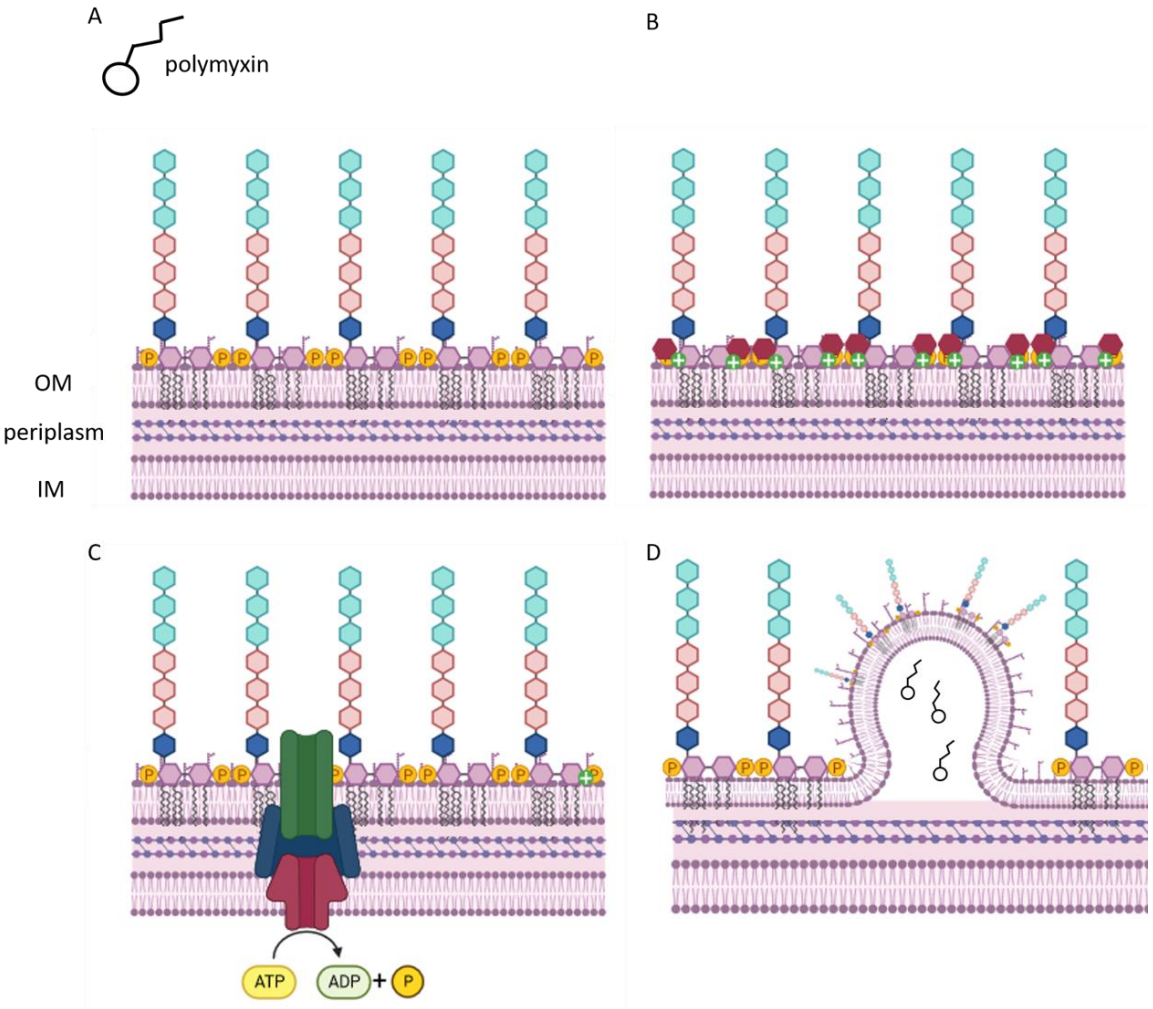

Figure 28: Mechanisms of polymyxins resistance in *P. aeruginosa.* (A) shows the membrane of a susceptible strain to polymyxins. Polymyxins are positively charged and interact with the negatively charged outer membrane. (B) showing a modified outer membrane. The addition of amino-arabinose residues (red shape) on the lipid A of the LPS increases the positive charges and prevents polymyxins from attacking the membrane. (C) *P. aeruginosa* increases the production of efflux pumps that span the membrane like MexXY/OprM. (D) Formation of capsular polysaccharide to trap polymyxins, OM: outer membrane, IM: inner membrane; adapted from (Moffatt et al., 2019).

3.1.7.2 Resistance to murepavadin

Despite the novel mechanism of action of murepavadin, very few mutations confering resistance to murepavadin have been identified so far (**table 6**). These include mutations in the LptD protein and the TCS PmrAB. Since murepavadin's main target is LptD, it is not surprising that mutations in this protein lead to resistance. Indeed, a duplication mutation of 6 residues LRDKGM from the position 210 to 215 in the β-jellyroll periplasmic domain was identified in an *in vitro* spontaneous resistant mutant selected from the PAO1 reference strain exposed at

5xMIC of murepavadin. This mutations was associated with an MIC of 64 mg/L (1064-fold the MIC of the reference PAO1 = 0.06 mg/L) (Andolina et al., 2018a; Srinivas et al., 2010).

Table 6: List of mutations identified in *in vitro* selected resistant mutants to murepavadin.

Gene	Mutation	Reference
acrB2	$V_{909}A$	(Díez-Aguilar et al., 2021b)
crbA	$D_{640}G$	(Díez-Aguilar et al., 2021b)
lpxl1	$frAla_{12}$	(Díez-Aguilar et al., 2021b)
lpxT	$A_{55}T$	(Díez-Aguilar et al., 2021b)
lpxl2	fsE_{110}, fsV_{125}, fsQ_{202}, fsT_{176}, fsE_{63}	(Díez-Aguilar et al., 2021b)
msbA	$Q_{94}R$	(Díez-Aguilar et al., 2021b)
lptD	$InsLRDKGM_{210-215}$, $G_{214}D$	(Díez-Aguilar et al., 2021b; Srinivas et al., 2010)
bamA2	$G_{504}D$	(Díez-Aguilar et al., 2021b)
pmrB	$G_{188}S$, $V_{136}L$, $T_{132}P$, $R_{155}H$, $A_{330}P$, $G_{188}D$, ΔL_{172}	(Romano et al., 2019)

fs**:** frameshift, ins**:** insertion.

Furthermore, mutations in the histidine kinase PmrB of the TCS PmrAB have also been documented to reduce the susceptibility to murepavadin with at least a 4-fold increase in MIC relative to the wild type PA14 strain (Romano et al., 2019). This suggests cross-resistance between colistin and murepavadin through the TCS PmrAB.

In another study attempting to select *in vitro* mutants of *P. aeruginosa* that are resistant to murepavadin to identify potential resistance mechanisms, the authors identified numerous mutated genes that were predicted to participate in reducing the susceptibility of the mutants to murepavadin (Díez-Aguilar et al., 2021b). The majority of those genes are implicated in LPS biosynthesis (*lpxL1*, *lpxL2*, *bamA2*, *lptD and lpxT)* and LPS transport (*msbA*) (Díez-Aguilar et al., 2021b). Other mutations were identified in *acrB2*, and the *cbrA* genes, which encodes the multidrug efflux pump subunit AcrB and the sensor kinase CbrA of the two-component system CbrAB, respectively. Mutations in these genes contributed to a at least 2-fold increase in MIC. However, none of these mutations have been confirmed to reduce the susceptibility to murepavadin in clinical strains. To our knowledge, no other mutations have been documented so far.

III. Results

Chapter 1: Alteration of LpxL1, BamA and PmrB proteins contribute to decrease the murepavadin susceptibility

1.1 Context and objective

Pseudomonas aeruginosa is well-known in being a significant respiratory pathogen in Cystic Fibrosis patients (CF). Early colonisation by this pathogen can be managed through administration of antibiotics essentially by inhalation route (tobramycin), which can postpone the establishment of *P. aeruginosa* infections until older age (Reyne et al., 2023). Nevertheless, once *P. aeruginosa* settles in the respiratory tracts of CF patients, eliminating it becomes an intricate challenge leaving the patients chronically infected.

The commonly inhaled antibiotic colistin, tobramycin and aztreonam are used to treat CF patients chronicaly infected with *P. aeruginosa*. Inhaled antibiotic therapy is often more advantageous over systemic therapy due to a more localized delivery mode of the antibiotic and thus greater airway concentration, in addition to reduced toxicity (Li and Schneider-Futschik, 2023). Moreover, the number of CF patients who are continuously receiving inhaled antibiotics with different combinations is increasing (Taccetti et al., 2021). Despite the major importance of inhaled therapy in maintaining patients with chronic infections, resistance of *P. aeruginosa* to those antibiotics has emerged, which makes the development of alternative treatments urgently needed.

Antimicrobial peptides are one of the possible alternatives to antibiotics because they can be active against drug-resistant bacteria (Wang et al., 2022). Several antimicrobial peptides have been described in literature, each being different from the other in structure, origin, spectrum of activity and mode of action (Rima et al., 2021). Murepavadin is a novel peptidomimetic, positively charged, that is under development (phase 1- ongoing) for patients suffering from CF (Dale et al., 2018). It targets specifically the LptD complex of the outer membrane of *P. aeruginosa* (Martin-Loeches et al., 2018b). Previous published work showed the promising activity of murepavadin on *P. aeruginosa* clinical strains with low MIC (0.125 mg/L) (Díez-Aguilar et al., 2021a). Despite being still in development and not commercialized, some CF clinical strains displayed high MIC value to murepavadin (MIC >4 mg/L). An initial study of the mechanism of action identified *in vitro* mutations in LptD as the mechanism of resistance to this peptidomimetic (Srinivas et al., 2010). The objectives of this work were to study the antibacterial activity of murepavadin on a large collection of *P. aeruginosa* clinical strains

(*n*=230), that were isolated from chronically infected CF patients from 12 different French hospitals, and to characterize the mechanisms affecting the activity of murepavadin. We also tested its activity on 11 colistin resistant strains and aimed at the identification of the cross-resistance with the frequently used inhaled antibiotics. In this work, we demonstrated the excellent activity of murepavadin on *P. aeruginosa* CF clinical strains despite some strains exhibited high MIC values (128 mg/L). We have also identified mechanisms affecting the murepavadin activity through mutations in the *lpxL1* and *bamA* genes, that are involved in LPS biosynthesis and transport. Finally, we have shown that alterations of the histidine kinase of the PmrAB two component system can confer resistance to murepavadin as well to colistin and tobramycin, two other inhaled antibiotics.

1.2 Manuscript accepted in Antimicrobial Agents and Chemotherapy Journal after minor modifications

Mutations in genes *lpxL1*, *bamA* and *pmrB* impair the susceptibility of cystic fibrosis strains of *Pseudomonas aeruginosa* to murepavadin

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Running title: Murepavadin resistance of CF *P. aeruginosa* strains

Keywords : Murepavadin, *Pseudomonas aeruginosa*, Antimicrobial resistance

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Abstract

Murepavadin is a peptidomimetic exhibiting specific inhibitory activity against *Pseudomonas* species. In the present study, its *in vitro* activity was assessed on 230 cystic fibrosis (CF) strains of *P. aeruginosa* isolated from twelve French hospitals, in comparison with twelve other antipseudomonal antibiotics. Although murepavadin is still in pre-clinical stage of development, 9.1% (*n*=21) of the strains displayed a resistance superior to 4 mg/L, a level at least 128-fold higher than the modal MIC value of the whole collection (≤ 0.06 mg/L). Whole-genome sequencing of these 21 strains along with more susceptible isogenic counterparts coexisting in the same patients revealed diverse mutations in genes involved in the synthesis (*lpxL1* and *lpxL2*) or transport of lipopolysaccharides (*bamA*, *lptD*, and *msbA*), or encoding histidine kinases of two-component systems (*pmrB* and *cbrA*). Allelic replacement experiments with wild-type reference strain PAO1 confirmed that alteration of genes *lpxL1*, *bamA* and/or *pmrB* can increase murepavadin resistance from 8- to 32-fold. Furthermore, we found that specific amino-acid substitutions in histidine kinase PmrB (G188D, Q105P, and D45E) reduce the susceptibility of *P. aeruginosa* to murepavadin, colistin and tobramycin, three antibiotics used or intended to be used (murepavadin) in aerosols to treat colonized CF patients. Whether colistin or tobramycin may select mutants resistant to murepavadin or the opposite needs to be addressed by clinical studies.

Introduction

Pseudomonas aeruginosa is a major cause of morbidity and mortality in cystic fibrosis (CF) (1). Because of its ability to survive in multiple environments, this Gram-negative pathogen frequently colonizes the airways of CF individuals generating *in situ* a chronic inflammation itself responsible for a decline of the respiratory function. In an attempt to control such a deleterious lung invasion, international guidelines recommend the administration of repeated cures of inhaled antibiotics to chronically infected patients (2, 3). Aerosols of tobramycin, colistin methane sulfonate, and in a lesser extent aztreonam are thus commonly used with this indication. More recently, murepavadin (POL7080, Spexis), a new peptidomimetic derived from the porcine cationic antimicrobial peptide protegrin-I secreted by neutrophils, has been recognized as potentially useful to treat CF and non-CF bronchiectasis patients by inhalation (upcoming Phase 1 clinical trial) (4, 5). The project of using the intravenous route was abandoned because of significant nephrotoxic effects (6). This 14 amino acid-long, β-hairpin-configured, cationic macrocyclic peptide that is stabilized by a D-proline-L-proline bond, is selectively active on *Pseudomonas* species (4). Unlike colistin which interacts with negatively charged residues born by the lipid A of lipopolysaccharides (LPS), murepavadin targets outer membrane proteins, mainly the β-barrel LPS transport protein D (LptD) (7, 8). This latter forms a complex with the outer membrane anchored protein LptE, to translocate newly synthetized LPS molecules from the periplasmic space to the bacterial surface. Interaction of murepavadin with or near the β-jellyroll periplasmic domain of LptD is believed to prevent the correct insertion of LPS into the outer membrane, leading to detrimental misfunctions (9). In preclinical studies, murepavadin showed an excellent *in vitro* activity (MIC_{90} from 0.12 to 0.25 mg/L) on non-CF clinical strains of *P. aeruginosa*, some of those being multidrug resistant (10, 11). Despite the potential application of the peptide in CF, its activity on CF strains was documented rather scarcely while revealing bacteria with MIC values greater than 4 mg/L (12). Because

P. aeruginosa can adapt easily to most antipseudomonal antibiotics through mutations (13), some studies focused on the emergence of resistant mutants to murepavadin or its peptidomimetic analogue POL7001 *in vitro* (14-16). Thus, tandem duplication of a sequence LRDKGM in protein LptD was associated with a 64-fold increased resistance of reference strain PAO1 (4); whereas Romano et *al*. found that complementation of strain PA14 with *pmrB* alleles encoding altered peptides (G185S, G188D, and L172del) resulted in 2- to 16-fold higher murepavadin MICs (16). Finally, alteration of several genes (*cbrA*, *acrB2*, *lpxL1*, *lpxL2*, *lpxlT*, *msbA*, and *bamA*) by missense or frameshift mutations was predicted to reduce murepavadin susceptibility of PAO1 or its hypermutator mutant PAO1Δ*mutS* in time-kill experiments (15). However, except for some *pmrB* mutants, the impact of these mutations on murepavadin activity was not confirmed further.

The present study was set up to improve our knowledge on the antipseudomonal activity of murepavadin. Its MIC values were compared to that of currently used antibiotics for 230 CF isolates collected from 105 patients in 12 French hospitals. To get an insight into the mechanisms contributing to a decreased activity of the peptide in this particular clinical context, we next compared the genomic sequences of isolates coexisting in a same patient but differing in their resistance levels, and introduced the most prevalent mutations found into wild-type reference *P. aeruginosa* strain PAO1. Thus, we show that some of these mutations generate a cross-resistance between murepavadin and common antibiotics in CF such as tobramycin and colistin. The risk of co-selection of multidrug resistant strains with murepavadin in CF needs to be considered, especially in a hypermutator genetic background.

Results and discussion

In vitro susceptibility of CF strains to murepavadin. Two hundred and thirty isolates of *P. aeruginosa* collected over a three-month period from 105 French CF patients were tested for their resistance levels to 13 antipseudomonal antibiotics including murepavadin (Table 1 and Table S1). According to the EUCAST 2023 breakpoints established for *P. aeruginosa*, 10.9% of these isolates were susceptible or susceptible at increased exposure to all the currently approved antibiotics (no breakpoints have been defined yet for murepavadin), 38.7% were non-susceptible to at least one agent in less than three antimicrobial categories, 37.0% fitted with the definition of MDR, 12.1% were XDR, and 1.3% PDR (17). Among these molecules, colistin (94.4%), ceftazidime plus avibactam (87.4%), ceftolozane plus tazobactam (80.0%), and meropenem (80.0%) were the most frequently active (Table 1). The MIC values of murepavadin ranged from ≤ 0.06 to ≥ 128 mg/L (Table 1). While the murepavadin MIC_{50} value determined on our collection (0.125 mg/L) was identical to that reported previously for non-CF strains, a notable proportion of CF strains appeared to be more resistant (MIC_{90} = 4 mg/L) than the isolates of these studies (0.12 and 0.25 mg/L, respectively) (10, 11). Corroborating this observation, the MIC_{90} of the antibiotic was found equal to 2 mg/L for CF strains collected in Northern Ireland, the Netherlands, Spain, and Australia (12). Because murepavadin is intended to be administrated to CF patients by aerosolization, its activity was compared to that of antibiotics already used under the form of aerosols, such as colistin, aztreonam-lysin, and tobramycin. MIC_{50}/MIC_{90} values of these molecules were equal to 1/2 mg/L, 4/128 mg/L and 2/32 mg/L, respectively (Table 1). Murepavadin retained a good activity on most strains considered as clinically resistant to tobramycin (MIC > 2 mg/L), colistin (MIC > 4mg/L) and aztreonam (MIC > 16 mg/L), thereby suggesting that this new drug could be an alternative to these common treatments. On the other hand, a high resistance to the peptide (arbitrarily fixed > 4 mg/L) was noted in 16 (7%), 8 (3.5%) and 9 (3.9%) isolates resistant to the three antibiotics

110 respectively. Finally, a few strains with such relatively high murepavadin MICs turned out to
111 be susceptible to one or more of these older molecules, mostly tobramycin (*n* = 5) and colistin
112 (*n* = 13) (Table S1).

113 Though none of the CF-patients from this work ever received murepavadin, 21 isolates (9.1%)
114 from 15 individuals exhibited a resistance level greater than 4 mg/L, including eight isolates
115 (3.5%) for which the MIC values were ≥ 128 mg/L (Table S1). To get an insight into the
116 mechanisms involved in these phenotypes, we sequenced the genomes of these 21 bacteria
117 along with those of more susceptible isolates (MIC ≤ 4 mg/L) coexisting in the same sputum
118 samples (13 patients out of 15). Finally, intra-patient comparisons of these genome sequences
119 were carried out in search of the most common SNPs (Table 2). The number of genomic
120 alterations between concomitant clones varied from 2 (patient III-9) to 892 (patient XII-2)
121 (Table S2). CF patients are often initially colonized by a single strain of *P. aeruginosa* which
122 diversifies over the course of the disease to give rise to phenotypically distinct but genotypically
123 related subpopulations well adapted to the lung environment (18). This evolution is usually
124 boosted by the emergence of hypermutator clones deficient in one or several DNA proofreading
125 systems (19, 20). Consistent with the relatively high divergence observed between some intra-
126 patient clones, mutations in the DNA mismatch repair system (genes *mutS*, *mutL*, *uvrD*) and/or
127 8-oxodG system (genes *mutM*, *mutT*, *mutY*) were noticed in 23 out of the 37 sequenced strains
128 (62.2%) (Table S2). On the other hand, a minimal divergence of 22 SNPs was found associated
129 with a large murepavadin MIC difference of 1,024-fold (from 0.125 to ≥ 128 mg/L) between
130 two clones colonizing patient XI-4.

131 ***Mutations in genes* lpxL1 *and* bamA *impact the activity of murepavadin in* P. aeruginosa**
132 ***CF strains*** . Compared with their more susceptible counterparts, isolates with a murepavadin
133 resistance > 4 mg/L (*i.e.*, ≥ 128-fold the modal MIC for the whole population) displayed diverse
134 SNPs in genes *bamA* (*n* =3 strains), *cbrA* (*n* = 3), *lpxL1* (*n* = 4), *lpxL2* (*n* = 1), *lptD* (*n* = 2),

msbA (*n* = 2) and/or *pmrB* (*n* = 4) (Table 2). Genes *cbrA* and *pmrB* encode the sensor histidine kinases of two-component systems CbrA-CbrB, and PmrA-PmrB, respectively while the other loci are involved in the transport (*msbA*, *lptD*, *bamA*) or biosynthesis of LPS (*lpxL1*, *lpxL2*) (21). A first analysis of the distribution of these mutations among the selected strains failed to establish a correlation between murepavadin MICs and the alteration of specific genes or the number of mutated genes per isolate, suggesting that in CF strains murepavadin resistance is multifactorial and likely involves still unidentified loci. Supporting this assumption, several strains turned out not to harbor alterations in the short list of genes cited above, such as III-3-1, XI-4-3, XI-6-2, and XI-6-6 (murepavadin MIC ≥ 128mg/L, Table 2).

Genes *lpxL1* (synonym of *htrB1*) and *lpxL2* (*htrB2*) encode lauryl transferases known to modify the structure of lipid A in a site-specific manner. While LpxL1 mediates the addition of 2-hydroxylaurate at the C-2 position of lipid A, LpxL2 adds laurate at C-2'(22). Defects in either gene result in an increased permeability of the outer membrane, and hypersusceptibility to various antibiotics and polycationic peptides (23). Various patient-specific mutations were noted in the LpxL1-encoding gene resulting in either truncated peptides (W84*, E265*), amino acid substitutions (G30S, T76P, H120N, T60A, R75K, R93K, K181R, E256D), or disruption of the gene *lpxl1* itself (ins_{2nt} 516-517). To assess the impact of some of these alterations on murepavadin susceptibility levels, we replaced the *lpxL1* gene of strain PAO1 with the mutated alleles from clinical strains III-9-1 (inferred amino acid variation T76P), VII-1-1 (E265*) and IX-5-2 (H120N), respectively. These changes resulted in an 8-fold increase in murepavadin resistance (from 0.06 to 0.5 mg/L) (Table 3), in agreement with the reported emergence of a resistant *lpxL1* disruption mutant (MIC > 16 mg/L) along with several *lpxL2* mutants from hypermutator strain PAO1Δ*mutS* during time-kill experiments (15). Though it has been suggested that production of penta-acylated LPS molecules in LpxL1-deficient mutants results in decreased susceptibility to polymyxins, our results did not confirm such effects (Table 3)

(23). In addition to *lpxL1*, the *bamA* gene was found to contain various single point mutations in a subset of CF strains exhibiting diverse resistance levels to murepavadin (from 2 to ≥ 128 mg/L), including the CF strain III-9-3 (Table 2). Its product, the β-barrel outer membrane protein BamA, is a component of the BAM complex (BamA-E). This complex plays an essential role in the folding of β-barrel proteins such as LptD and their insertion into the outer membrane (9). All the mutations identified in the selected CF strains (K291E, D535E, D603G, and T617I) were mapped in the β-strands of the C-terminal β-barrel domain. To assess the impact of the D535E substitution on murepavadin susceptibility, we first replaced the wild-type *bamA* gene of PAO1 with the mutated allele of strain III-9-3. This only resulted in a modest 2-fold increase in the peptide MIC (0.125 mg/L). However, the double replacement of *lpxL1* and *bamA* genes in the reference strain with the mutated alleles from strains III-9-1 and -3 that code for the T76P and D535E variations, respectively, had multiplicative effects on resistance to murepavadin (*e.g.*, 32-fold increase as compared with the wild-type parent) (Table 3). The fact that the resistance of this double mutant (2 mg/L) is far below that of some CF strains (Table 2) reinforces the notion that other loci contribute to higher MIC values. Like with *lpxL1*, colistin susceptibility was unchanged in *bamA* mutants as compared with PAO1.

Cross-resistance between tobramycin, colistin and murepavadin in pmrB clinical mutants. In addition to the genes involved in the transport and synthesis of LPS, single point mutations were identified in *pmrB* and *cbrA* leading to amino acid substitutions in their respective products, PmrB (M48I, R79H, F168L, L172P, P175L, V215A, P254S) and CbrA (A81T, Y457H, G502S, N855S). These histidine kinases sense and transmit stress signals from the cell envelope to their cognate cytoplasmic response regulators PmrA and CbrA, respectively, allowing for an appropriate adaptation of *P. aeruginosa*. Previous studies demonstrated that specific mutations in these phosphor-relays confer a dual resistance to polymyxins and aminoglycosides (24, 25). While inactivation of CbrA caused a modest augmentation of

tobramycin (2-fold) and colistin MICs (4-fold), mutational activation of PmrB had much greater effects on the resistance to these cationic antibiotics (16-fold and 32-fold, respectively) (24, 25).

In the present strain collection, only one isolate (V-5-1) classified as colistin resistant (MIC = 128 mg/L) by reference to the EUCAST 2023 breakpoints, showed a mutation in sensor PmrB (P254S) (Table 2). To investigate on a possible PmrB-mediated cross-resistance to murepavadin, colistin and tobramycin, we selected eleven fully sequenced colistin-resistant *pmrB* mutants from the collection of the French National Reference Center for antibiotic resistance. As indicated in Table 4, these non-CF strains quite highly resistant to colistin (from 16 to > 256 mg/L) showed a susceptibility to murepavadin ranging from 0.25 to 8 mg/L. Reminiscent of the CF strains described in this study, multiple mutations in genes *bamA*, *cbrA*, *lptD, lpxL1* and *lpxL2,* were also present in these bacteria. Constructs of plasmid vector pME6012 carrying the *pmrAB* operons from three strains (3795, 2243, 3890) were used to complement the deletion mutant PAO1Δ*pmrAB*. Confirming the impact of PmrB amino-acid substitutions G188D, Q105P and D45E on the susceptibility to murepavadin, MIC values of the peptide increased from 16 to 64-fold upon complementation, thus reaching 0.5 and 2 mg/L, respectively (Table 3). Colistin MICs varied in parallel (from 32-fold to 256-fold) suggesting that the degree of resistance to both antibiotics is modulated by specific amino acid variations in different regions of PmrB (26). Of note, the G188D and Q105P substitutions are located in the HAMP and periplasmic domains of PmrB, respectively while D45E affects the periplasmic domain of the sensor. Consistent with our results, spontaneous *pmrB* mutants 8- to 32-fold more resistant than parental strain PA14 to murepavadin analogue POL7001 have been previously described (16). As shown by our laboratory, alteration of sensor PmrB can potentially be responsible for a decreased susceptibility of *P. aeruginosa* to aminoglycosides (up to 16-fold) (Table 3) (25). Since mutations may target the *pmrB* gene in the context of CF lung chronic

colonization, the probability that some of them affect the activity of the three molecules, murepavadin, colistin and tobramycin, should be considered. Longitudinal studies enrolling cohorts of CF patients would be necessary to validate this hypothesis, looking at the emergence of cross-resistant mutants under murepavadin, colistin or tobramycin aerosol therapy.

Other mutations identified. Unexpectedly, mutations in the murepavadin target protein LptD were identified in only two CF strains that otherwise displayed multiple alterations in their DNA repair systems (4) (Tables 2 and Table S2). A tandem duplication of the PSDE sequence spanning from positions 151 to 154 was found in V-5-1, a strain with a murepavadin resistance equal to 8 mg/L that also harbored mutations in PmrB (P254S) and BamA (D603G). Though the impact of this structural change on the function of LptD was not investigated further, it is interesting to note that a tandem duplication of residues LRDKGM at positions 210 to 215 together with a G214D change was reported previously for an *in vitro* selected mutant showing a 64-fold higher murepavadin resistance than its parent PAO1 (4, 15). The second isolate (IX-3-5) of this study displaying a LptD variant (M261T) contained a concomitant V215A change in PmrB, for a resistance level to murepavadin equal to 32 mg/L. Again, highlighting the multifactorial nature and complexity of mechanisms contributing to elevated MICs of the peptide, 511 SNPs were identified between the susceptible isolate IX-3-3 (MIC = 0.125 mg/L) and its counterpart, IX-3-5 (Table S2).

ATPase MsbA is a member of the ABC-transporter superfamily. The role of this transmembrane protein is to flip complete lipid A-core molecules from the inner to the outer side of the cytoplasmic membrane before their modification and subsequent transport by the Lpt machinery to the cell surface (27). Five CF strains of our collection produced MsbA proteins with single amino acid substitutions (V419I in I-5-4, I-5-2, I-5-1; H135R in VI-7-2; I39V in X-6-2). In contrast to the other isolates, strain VI-7-2 did not appear to contain alterations in genes *bamA, cbrA, lptD, lpxL1, lpxL2* and *pmrB*. Interestingly, its genetic

divergence from its susceptible counterpart VI-7-1 (MIC ≤ 0.06 mg/L) was limited to 26 SNPs, which suggests a contribution of the H135R mutation to the resistance of VI-7-2 to murepavadin (8 mg/L). Little is known about the impact the alteration of MsbA may have on the fitness of *P. aeruginosa.* In *Escherichia coli,* experiments demonstrated that the deletion of the MsbA-encoding gene drastically decreases cell viability both *in vitro* and *in vivo* (28). However, amino acid substitutions in the protein, which shares 40.3% sequence identity with its homologue in *P. aeruginosa*, can be tolerated as those conferring resistance to quinoline compounds targeting MsbA (28). Further experiments are required to clarify to which extent mutations in MsbA and LptD reduce the susceptibility of CF strains to murepavadin and may affect the fitness of *P. aeruginosa*.

Conclusion. The present study confirms the good *in vitro* activity of murepavadin on CF strains, making this original antipseudomonal peptide endowed with a unique mode of action an interesting alternative to older antibiotics currently used by inhalation, such as colistin, tobramycin and aztreonam. However, although murepavadin is still under development, a notable proportion of the *P. aeruginosa* strains that already colonize CF patients display various degrees of resistance to the drug, including some isolates for which MICs are > 4 mg/L (9.1%) (*i.e.,* ≥ 128-fold the modal value of our strain population). It is now well established that long-term and repeated administration of antibiotics to CF patients select bacterial subpopulations increasingly resistant to one or more antimicrobials (29). A trivial explanation for the presence of *P. aeruginosa* relatively resistant to murepavadin in patients never treated with this drug could be that current treatments by aminoglycosides (mostly tobramycin) and/or polymyxins (mostly colistin) select mutation-based mechanisms of cross-resistance implying global regulators or two-components such as PmrAB. Aminoglycosides, polymyxins and murepavadin have in common to interact with components of the bacterial outer membrane. Thus, it is tempting to speculate that still unknown mechanisms impairing the activities of these antibiotic

families are related to the structure or physiology of the cell envelope, as for the resistant strains from patients III-3, X-6, XI-4 and XI-6. However, it remains unclear which selective pressure in the CF lung can lead to the emergence of mutants specifically resistant to murepavadin while the drug has never been used (*e.g*, *lpxL1*). Mutants exhibiting various modifications in the structure of the lipid A have already been reported in the context of CF, which could reflect a phenotypic adaption of *P. aeruginosa* to this particular lung environment, not necessarily linked to the presence of antibiotics (30). Understanding the phenotypic and genotypic evolution of CF strains under murepavadin therapy will be key to the positioning of this new agent among the antibiotic resources available against *P. aeruginosa.*

Materials and methods

Bacterial strains, culture media, and growth conditions. The strains and plasmids used in this study are described in Table S3. During a four-month multicenter national survey (GERPA MUCO II, from October 2019 to January 2020) involving twelve French hospitals (Besançon, Brest, Limoges, Lyon La Croix Rousse, Nantes, Paris Foch, Paris Necker, Paris Robert Debré, Toulon, Toulouse, Reims and Rennes), 718 isolates of *P. aeruginosa* were collected from 120 CF patients (ten patients per participating center, and six colonies randomly picked from a single sputum sample per individual). A subcollection of 230 strains from 105 CF patients aged 1 to 52-years (median 24-years) was selected for the present study, to retain only those isolates exhibiting different antibiotic susceptibility profiles (at least a 2-fold MIC difference for at least 2 antibiotics, data not shown). The collection was enriched with 11 non-CF colistin-resistant clinical strains of *P. aeruginosa* (MIC > 4 mg/L) harboring a PmrB mutation, isolated between 2014 and 2019 in eleven French hospitals (repository of the French National Reference Center for antibiotic resistance, Besançon hospital). All strains were grown at 35 +/-2°C in Mueller-

Hinton broth (MHB) (Dickinson Microbiology Systems, Cockeysville, Md, United States) with adjusted concentrations of divalent cations Ca^{2+} and Mg^{2+} or on Mueller-Hinton Agar (MHA) plates (Bio-Rad, Marnes-la-Coquette, France). In conjugation experiments, transconjugants were selected on *Pseudomonas* Isolation Agar (PIA, Becton Dickinson) supplemented with 2,000 mg/L streptomycin. The plasmid has been excised after culture of the transconjuguants on a M9 minimal medium (42 mM Na_2HPO_4, 22 mM KH_2PO_4, 19 mM NH_4Cl, 8.5 mM NaCl) with 5% sucrose as a source of carbon and energy.

Antimicrobial susceptibility testing. Minimum inhibitory concentrations (MICs) of ticarcillin, piperacillin plus 4 mg/L tazobactam, aztreonam, ceftazidime, ceftolozane plus 4 mg/L tazobactam, ceftazidime plus 4 mg/L avibactam, cefepime, imipenem, meropenem, amikacin, tobramycin and ciprofloxacin were determined in MHB by using customized microplates containing lyophilized antibiotic powders (Thermo Fisher, Illkirch-87 Graffenstaden, France). MICs of colistin (from 0.12 to 256 mg/L) and murepavadin (from 0.06 to 128 mg/L) were determined by the standard microdilution method in MHB using titrated powders of colistin sulfate (Sigma-Aldrich) and murepavadin (ProbeChem, China) (31). The strains were categorized as susceptible (S), susceptible at increased exposure (I) or resistant (R) according to the EUCAST 2023 clinical breakpoints (32). Quality controls in MIC experiments were performed on a regular basis with *P. aeruginosa* strains ATCC 27853 and PAO1, and *E. coli* NCTC 13846.

SNP identification. Twenty-one CF strains with a murepavadin MIC value > 4 mg/L were submitted to complete genome sequencing along with 17 more susceptible isolates (≤ 4 mg/L) coexisting in the same sputum samples. Whole DNA was extracted from overnight cultures by using the PureLink Genomic DNA mini kit (Thermo Fisher Scientific). Libraries were prepared (Nextera XT DNA Library Preparation kit) and sequenced on an Illumina NextSeq 500 platform (Illumina, San Diego, CA; P2M platform, Institut Pasteur, Paris, France). Fastq files were

generated and demultiplexed with bcl2fastq Conversion Software (v2.20; Illumina). The final average sequencing depth was > 80 X for all of the strains. The reads were assembled using Shovill-Spades (v3.14.0) and the contigs annotated with Prokka (v1.14.5). Single Nucleotide Polymorphisms (SNPs) (accession number NC_002516.1) were detected by mapping the reads against the reference strain PAO1 sequence, by using BioNumerics (v7.6.3) software (Applied Maths) with a minimum sequencing depth of 10 X. Sequence Types (STs) were determined according to the MLST scheme available at PubMLST (https://pubmlst.org).

Allelic replacement. Respective sequences of genes *lpxL1* (888-bp in length) and *bamA* (2,394-bp) were amplified by PCR from whole DNA extracts of strains III-9-1, III-9-3, VII-1-1 and IX-5-2 (Genomic DNA extraction kit, Macherey-Nagel, Hoerdt) by using the primers listed in Table S4. The resultant fragments were cloned into plasmid pKNG101 by using the NEBuilder® HiFi DNA Assembly Cloning kit (New England Biolabs, Ipswich, MA, USA) (33). The recombinant plasmids were next transferred to *E. coli* CC118λ*pir* by transformation and then to strain PAO1 by triparental mating with helper strain *E. coli* HB101(pRK2013) (34). Transconjugants were selected on PIA medium containing 2,000 mg/L streptomycin. Excision of integrated plasmids was obtained by replica plating on M9 minimal agar medium supplemented with 5% sucrose. The allelic replacement of genes *lpxL1* and *bamA* in PAO1 was checked PCR sequencing (RUO3500 Genetic Analyzer, Applied Biosystems) with specific primers (Table S4).

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Transparency declarations

None to declare.

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TABLE 1 Antibiotic susceptibility levels of the 230 CF strains of *P. aeruginosa* selected for this study

Antibiotic	MIC (mg/L)														MIC_{50}	MIC_{90}	Percentage of susceptibility (%)[a]
	≤0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512			
Piperacillin/tazobactam[b]							109*	33	20	17	10	10	15	16**	8	128	70.5
Aztreonam						87*	32	30	19	19	12	15	16**		4	128	73.0
Ceftazidime					45*	51	35	23	12	13	10	41**			4	64	66.9
Ceftolozane/tazobactam[b]				44*	72	48	20	16	10	7	13**				1	16	71.3
ceftazidime/avibactam[c]				38*	51	59	36	17	9	6	14**				2	16	80.0
Cefepime					12*	18	47	58	39	16	18	22**			8	64	58.7
Amikacin						27*	35	40	45	**28**	**25**	**19****	**11****		16	128	63.9
Tobramycin			23*	29	48	37	**20**	**22**	**22**	**9**	**20****				2	32	68.3
Imipenem				48*	47	29	18	22	28	29	9				2	32	61.7
Meropenem				105*	30	17	15	17	29	14	2	1			1	32	80.0
Colistin			11*	48	89	66	3	**6**	**3**	**0**	**0**	**2**	**1**	**1**	1	2	94.3
Ciprofloxacin		44*	26	32	44	34	19	12	16	3					1	8	44.3
Murepavadin	69	47	22	18	30	12	11	7	1	1	4	8**			0.12	4	NA

* MIC values ≤ to the concentration indicated in the column, ** MIC values ≥ to the concentration indicated in the column

[a] Interpretation according to EUCAST 2023 clinical breakpoints

[b] With a fixed concentration of 4 mg/L tazobactam

[c] With a fixed concentration of 4 mg/L avibactam

NA, not applicable

The values highlighted in grey and in bold correspond to strains categorized as resistant according to the clinical breakpoints and Epidemiological Cut-Off values (ECOFF) of EUCAST 2023 recommendations, respectively.

TABLE 2 Protein or gene alterations identified at least two times in CF-strains with murepavadin MIC > 4 mg/l.

Patient	Strain	MIC (mg/L)		Mutations identified[a]						
		MUR	**CST**	BamA	CbrA	LpxL1	LpxL2	LptD	MsbA	PmrB
I-5	4	2	1	K291E	-	G30S, W84*	-	-	V419I	F168L
	2	**16**	1	K291E	-	G30S, W84*	-	-	V419I	F168L
	1	**64**	2	K291E	-	G30S, W84*	-	-	V419I	**L172P**
III-3	2	0.25	1	-	-	-	-	-	-	-
	1	**≥128**	≤0.25	-	-	-	-	-	-	-
III-9	**1***	4	4	-	-	T76P	-	-	-	-
	3*	**64**	**8**	**D535E**	-	T76P	-	-	-	-
	4	**≥128**[b]	**8**	D535E	-	T76P	-	-	-	-
V-5	4	0.25	2	-	-	-	-	-	-	
	1	**8**	**128**	**D603G**	**-**	**-**	**-**	**PSDE**[c]	**-**	**P254S**
VI-7	1	≤0.06	**8**	-	-	-	-	-	-	-
	2	**8**	≤0.25	-	-	**-**	**-**	**-**	**H135R**	-
VII-1	2	≤0.06	2	-	-	-	-	-	-	-
	1*	**64**	≤0.25	-	-	**E265***	**-**	**-**	-	-
IX-3	2	0.125	≤0.5	-	-	-	-	-	-	-
	3	**8**	1	-	-	-	**-**	**-**	-	**V215A**

	5	**32**	1	-	-	-	**-**	**M261T**	-	V215A
IX-5	5	0.125	0.5	-	-	-	-	-	-	
	3	1	0.5	-	-	-	-	-	-	**M48I**
	2*	**≥128**	4	-	-	**H120N**	-	-	-	M48I
	6	**≥128**	**16**	-	-	**Δ245-248**[d]	-	-	-	M48I
X-6	4	0.25	≤0.25	-	-	-	-	-	-	-
	5	1	1	-	-	-	-	-	-	-
	1	4	1	-	-	-	-	-	-	-
	2	**64**	1	-	**A81T**	**Ins$_{2nt}$**[e]	**-**	**-**	**I39V**	**-**
X-9	1	4	2	-	-	-	-	-	-	**R79H**
	2	**8**	2	-	-	-	-	-	-	R79H
XI-4	1	0.125	1	-	-	-	-	-	-	-
	3	**≥128**	0.5	-	-	-	-	-	-	
XI-6	4	4	4	-	-	-	-	-	-	**P175L**
	6	**≥128**	**128**	-	-	-	-	-	-	-
	2	**≥128**	**>256**	-	-	-	-	-	-	-
XII-2	1	≤0.06	1	-	-	-	-	-	-	-
	3	1	2	-	-	-	-	-	-	-
	2	**8**	≤0.25	-	**N855S**	-	**R298C**	-	-	-
Single strains										
II-2	2	**8**	**256**	T617I	Y457H, G502S	-	R279H	-	K469N	-

III-6	1	**8**	2	-	-	T60A, R75K, R93K, K181R, E256D	-	-	-	-

MUR, Murepavadin, CST, colistin

[a] In comparison with the amino-acid sequences of the PAO1 strain (-)

[b] Two strains with a murepavadin MIC ≥ 128 mg/L showed strictly identical genomic sequences

[c] Duplication of PSDE amino-acid sequence at position 151-154

[d] Deletion of 10 nucleotides from position 736 to 745

[e] Insertion of 2 nucleotides (CC) at positions 516-517

Values in bold correspond to murepavadin MIC > 4 mg/L. Proteins in bold contained alterations which were not present in isolates with murepavadin MIC ≤ 4 mg/L. The nucleotide sequence of strains marked with an asterix were used for allelic replacement experiments.

484 **TABLE 3** Impact of the replacement of genes *lpxL1*, *bamA* and *pmrB* with mutated alleles on
485 susceptibility of strain PAO1 to inhaled antibiotics

Strains	Alterations in proteins			MIC (mg/L)		
	Lpxl1	**BamA**	**PmrB**	**Murepavadin**	**Colistin**	**Tobramycin**
PAO1 wild-type	_	_	_	≤ 0.06	0.5	0.5
PAO1::*lpxL1*$_{\text{III-9-1}}$	T76P	_	_	**0.5**	0.5	0.25
PAO1::*lpxL1*$_{\text{VII-1-1}}$	E265*	_	_	**0.5**	0.5	0.25
PAO1::*lpxL1*$_{\text{IX-5-2}}$	H120N	_	_	**0.5**	0.5	0.25
PAO1::*bamA*$_{\text{III-9-3}}$	_	D535E	_	0.125	0.5	0.5
PAO1::*lpxL1*$_{\text{III-9-1}}$-*bamA*$_{\text{III-9-3}}$	T76P	D535E	_	**2**	0.5	0.5
PAO1Δ*pmrAB*	_	_	_	0.03	0.5	0.25
PAO1Δ*pmrAB(*pME6012)	_	_	_	0.03	0.5	0.25
PAO1Δ*pmrAB(*pAB3795)	_	_	G188D	**2**	**128**	**2**
PAO1Δ*prmAB(*pAB2243)	_	_	Q105P	**2**	**128**	**4**
PAO1Δ*pmrAB(*pAB3890)	_	_	D45E	**0.5**	**16**	**2**

486

487 Values in bold correspond to MICs increased at least 4-fold as compared with those for wild-
488 type PAO1

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TABLE 4 Murepavadin susceptibility of colistin-resistant clinical strains harboring mutations in gene pmrB

Clinical strains	Mutations identified	MIC (mg/L)	
		Colistin	Murepavadin
5115	PmrB (R92H, G123S), LpxL1 (K145N, P87A), BamA (Q533R)	>256	**8**
3890	PmrB (**D45E**), LpxL1 (P191L)	>256	4
5101	PmrB (R92H, G123D), BamA (D494A), CbrA (N225S), LpxL2 (Δ45-311)[a], LpxL1 (P87A)	>256	4
3795	PmrB (**G188D**), BamA (Q533R), LptD (D593N, K785R), LpxL2 (R3 frameshift, K304R)[b]	>256	4
5071	PmrB (F168L), LpxL1 (R96H), BamA (V30L)	>256	2
2243	PmrB (**Q105P**), BamA (T657R), LpxL2 (Δ45-311) [a]	256	2
3038	PmrB (D47N), BamA (T743Q)	128	0.5
2739	PmrB (V6A, V264A), BamA (T743Q)	64	4
4660	PmrB (G121D, V313A), BamA (Q533R, T743Q), LpxL1 (V11 frameshift)[c], CbrB (V125A)	32	**> 64**
6305	PmrB (A256V)	32	0.25
6369	PmrB (V28G), BamA (Q533R)	16	1

Amino acid substitutions refer to the protein sequences encoded by both wild-type strains PAO1 and PA14

[a] Deletion of 801 nucleotides from the position 133 to 933

[b] Insertion of 1 nucleotide (C) at position 8

[c] Deletion of 1 nucleotide (G) at position 34

Murepavadin MICs indicated in bold correspond to values > 4 mg/L. Alleles encoding amino acid variations noted in bold were cloned in plasmid pME601

Supplemental data

Table S1 Antibiotic susceptibility of 230 CF strain

CF-Strain	MIC (mg/L)													
	TIC	PTZ	ATM	CZD	CT	CZA	FEP	AKN	TMN	IPM	MEM	CST	CIP	MUR
I-1-1	16	≤4/4	4	2	1/4	1/4	8	4	1	4	1	2	≤0.12	≤0.06
I-2-1	32	≤4/4	4	2	1/4	2/4	4	4	0.5	2	1	≤0.5	0.25	≤0.06
I-3-1	256	16/4	32	32	32/4	32/4	8	≤2	≤0.25	1	16	≤0.5	≤0.12	0.125
I-3-2	32	≤4/4	4	2	≤0.5/4	2/4	2	4	0.5	1	≤0.5	2	≤0.12	≤0.06
I-4-1	≤8	≤4/4	≤2	≤1	≤0.5/4	1/4	4	4	0.5	2	≤0.5	2	≤0.12	4
I-4-4	≤8	≤4/4	≤2	≤1	1/4	1/4	2	4	0.5	8	≤0.5	2	≤0.12	2
I-5-1	>512	256/4	128	>64	16/4	8/4	>64	64	16	32	32	2	4	64
I-5-2	>512	64/4	>128	>64	16/4	4/4	64	8	1	32	32	1	4	16
I-5-4	512	32/4	128	>64	8/4	4/4	64	8	1	32	16	1	8	2
I-6-1	32	64/4	8	32	1/4	4/4	16	32	4	8	2	2	≤0.12	0.125
I-7-1	64	32/4	8	8	1/4	2/4	8	64	4	≤0.5	≤0.5	2	0.5	0.125
I-7-2	128	64/4	16	16	2/4	4/4	8	4	1	1	1	2	≤0.12	≤0.06
I-8-1	≤8	≤4/4	≤2	≤1	1/4	1/4	4	8	1	≤0.5	≤0.5	1	1	0.25
I-9-1	≤8	≤4/4	≤2	2	2/4	1/4	8	32	4	2	1	1	2	2
I-10-1	32	8/4	8	2	1/4	2/4	2	≤2	0.5	2	≤0.5	2	≤0.12	≤0.06

II-1-2	>512	>256/4	128	>64	>32/4	>32/4	>64	>128	>32	16	64	2	0.5	0.25
II-1-4	128	16/4	32	8	2/4	8/4	16	4	0.5	32	16	1	1	0.25
II-2-2	≤8	≤4/4	≤2	≤1	≤0.5/4	≤0.5/4	4	>128	>32	≤0.5	≤0.5	256	16	8
II-3-1	>512	128/4	32	64	4/4	16/4	64	64	8	16	16	2	8	4
II-4-1	>512	>256/4	>128	>64	>32/4	>32/4	>64	32	4	64	32	2	4	1
II-5-4	>512	256/4	128	>64	16/4	>32/4	>64	32	4	32	16	≤0.5	2	0.125
II-6-1	64	16/4	16	4	2/4	4/4	8	16	2	1	1	2	1	≤0.06
II-6-4	≤8	≤4/4	≤2	≤1	≤0.5/4	≤0.5/4	≤1	8	1	2	≤0.5	≤0.5	≤0.12	≤0.06
II-7-6	256	256/4	32	64	4/4	2/4	32	64	2	2	1	1	0.5	0.125
II-8-3	≤8	≤4/4	≤2	≤1	≤0.5/4	≤0.5/4	≤1	≤2	≤0.25	2	≤0.5	1	≤0.12	0.5
II-8-5	≤8	8/4	8	4	2/4	4/4	2	4	8	1	≤0.5	1	0.25	≤0.06
II-9-4	256	16/4	16	8	4/4	4/4	32	16	1	32	16	1	1	0.25
II-9-6	64	64/4	16	32	4/4	8/4	16	32	4	32	32	1	2	0.125
II-10-1	128	>256/4	32	>64	16/4	>32/4	64	64	4	64	16	1	8	1
II-10-3	>512	128/4	32	>64	4/4	32/4	>64	64	8	64	32	1	16	4
III-1-1	128	≤4/4	8	8	≤0.5/4	≤0.5/4	8	32	4	8	2	≤0.5	≤0.12	2
III-1-5	>512	≤4/4	128	>64	8/4	2/4	64	32	4	16	8	≤0.5	≤0.12	0.5
III-2-1	64	≤4/4	≤2	4	1/4	2/4	2	≤2	16	≤0.5	≤0.5	≤0.5	≤0.12	0.125
III-2-2	512	>256/4	128	>64	8/4	8/4	64	16	32	4	4	1	≤0.12	≤0.06
III-3-1	≤8	≤4/4	≤2	2	2/4	2/4	8	>128	16	1	≤0.5	≤0.5	0.5	>64

III-3-2	≤8	≤4/4	≤2	2	1/4	2/4	8	16	2	1	≤0.5	1	0.5	0.25
III-4-1	≤8	≤4/4	≤2	2	1/4	≤0.5/4	4	8	1	≤0.5	≤0.5	1	≤0.12	≤0.06
III-5-1	32	8/4	8	4	1/4	2/4	2	8	1	2	≤0.5	2	≤0.12	0.25
III-6-1	64	8/4	8	4	1/4	4/4	4	≤2	0.5	2	≤0.5	2	≤0.12	8
III-7-1	≤8	≤4/4	≤2	≤1	≤0.5/4	≤0.5/4	≤1	≤2	≤0.25	1	≤0.5	2	≤0.12	0.125
III-8-1	16	8/4	4	4	1/4	1/4	8	8	1	32	4	2	0.5	0.25
III-8-2	128	32/4	8	32	2/4	4/4	16	16	2	16	16	≤0.5	2	0.125
III-8-5	512	>256/4	128	32	8/4	16/4	>64	128	8	64	64	≤0.5	4	4
III-9-1	≤8	≤4/4	≤2	≤1	≤0.5/4	≤0.5/4	4	16	>32	8	1	4	16	4
III-9-2	≤8	≤4/4	≤2	≤1	≤0.5/4	≤0.5/4	4	16	16	8	1	16	16	2
III-9-3	≤8	≤4/4	≤2	≤1	≤0.5/4	≤0.5/4	4	16	>32	8	1	8	16	64
III-9-4	≤8	≤4/4	≤2	≤1	≤0.5/4	≤0.5/4	4	16	>32	8	1	8	16	>64
III-9-5	≤8	≤4/4	≤2	≤1	≤0.5/4	≤0.5/4	4	16	>32	8	1	8	16	>64
III-9-6	≤8	≤4/4	≤2	≤1	≤0.5/4	≤0.5/4	4	16	>32	8	1	8	16	1
III-10-1	16	16/4	4	4	1/4	2/4	2	8	2	2	1	2	≤0.12	≤0.06
III-10-6	32	16/4	8	4	2/4	4/4	4	≤2	≤0.25	8	4	≤0.5	≤0.12	≤0.06
IV-1-1	≤8	≤4/4	≤2	≤1	1/4	1/4	8	8	1	1	≤0.5	2	2	1
IV-2-2	≤8	≤4/4	≤2	≤1	1/4	1/4	4	4	0.5	8	≤0.5	≤0.5	1	1
IV-2-3	≤8	≤4/4	≤2	≤1	2/4	1/4	8	64	8	1	≤0.5	1	≤0.12	0.5
IV-3-1	512	128/4	64	32	8/4	4/4	64	128	16	32	32	1	2	0.125

IV-3-5	64	32/4	4	2	1/4	1/4	8	16	2	4	4	1	4	4
IV-3-6	256	≤4/4	16	16	≤0.5/4	1/4	32	16	1	2	2	≤0.5	1	0.125
IV-4-1	16	8/4	4	8	≤0.5/4	≤0.5/4	4	4	32	≤0.5	≤0.5	1	16	1
IV-4-2	64	64/4	16	16	1/4	≤0.5/4	8	4	32	1	≤0.5	1	16	≤0.06
IV-5-1	32	8/4	8	4	1/4	4/4	2	4	1	2	≤0.5	2	0.25	≤0.06
IV-6-1	≤8	≤4/4	≤2	≤1	≤0.5/4	1/4	4	4	0.5	1	≤0.5	2	≤0.12	≤0.06
IV-7-1	≤8	8/4	4	2	≤0.5/4	≤0.5/4	≤1	≤2	0.5	≤0.5	≤0.5	≤0.5	≤0.12	≤0.06
IV-8-3	32	8/4	8	4	2/4	2/4	4	4	1	2	1	2	≤0.12	0.125
IV-8-4	≤8	≤4/4	≤2	2	1/4	1/4	≤1	≤2	0.5	4	≤0.5	2	0.25	≤0.06
IV-9-2	32	8/4	8	4	1/4	4/4	8	≤2	0.5	1	≤0.5	≤0.5	≤0.12	≤0.06
IV-10-1	≤8	≤4/4	≤2	2	1/4	2/4	4	4	1	≤0.5	≤0.5	1	2	≤0.06
IV-10-2	≤8	≤4/4	≤2	2	2/4	2/4	8	16	1	1	≤0.5	2	4	0.125
V-1-1	≤8	≤4/4	≤2	8	2/4	1/4	8	8	1	8	2	1	0.5	0.125
V-1-4	≤8	≤4/4	≤2	4	4/4	1/4	8	16	1	16	16	≤0.5	0.5	1
V-2-1	≤8	≤4/4	≤2	≤1	≤0.5/4	≤0.5/4	4	4	0.5	≤0.5	≤0.5	2	2	0.125
V-2-5	≤8	≤4/4	≤2	2	1/4	1/4	8	16	2	≤0.5	≤0.5	2	4	0.5
V-3-1	>512	128/4	128	>64	16/4	>32/4	>64	64	8	32	32	1	2	2
V-3-4	>512	8/4	64	>64	>32/4	>32/4	64	64	8	16	16	2	1	≤0.06
V-3-5	>512	≤4/4	16	32	8/4	8/4	32	32	4	2	4	2	1	≤0.06
V-4-1	≤8	≤4/4	≤2	4	4/4	2/4	8	128	16	1	≤0.5	1	2	0.25

V-4-4	≤8	≤4/4	≤2	≤1	≤0.5/4	≤0.5/4	≤1	≤2	≤0.25	≤0.5	≤0.5	1	1	≤0.06
V-4-5	≤8	≤4/4	≤2	≤1	1/4	1/4	2	16	2	1	≤0.5	1	1	≤0.06
V-5-1	>512	32/4	32	>64	>32/4	>32/4	64	128	8	16	16	128	4	8
V-5-4	≤8	≤4/4	≤2	2	2/4	1/4	8	128	16	≤0.5	≤0.5	2	2	0.25
V-6-1	32	≤4/4	4	2	1/4	2/4	2	≤2	1	≤0.5	≤0.5	1	≤0.12	0.125
V-6-2	64	16/4	16	4	2/4	4/4	4	≤2	≤0.25	≤0.5	≤0.5	1	≤0.12	≤0.06
V-6-3	16	≤4/4	4	≤1	≤0.5/4	1/4	≤1	≤2	≤0.25	≤0.5	≤0.5	1	≤0.12	≤0.06
V-7-2	≤8	≤4/4	≤2	≤1	≤0.5/4	≤0.5/4	4	8	2	4	≤0.5	1	1	≤0.06
V-7-4	32	16/4	>128	>64	>32/4	>32/4	16	8	0.5	16	8	2	16	0.125
V-7-6	>512	256/4	128	>64	32/4	4/4	>64	32	>32	64	16	1	8	0.25
V-8-1	256	256/4	64	64	8/4	16/4	32	16	1	16	2	1	1	0.125
V-8-2	≤8	≤4/4	≤2	2	1/4	≤0.5/4	4	4	0.5	16	1	2	1	0.25
V-8-3	16	8/4	4	>64	≤0.5/4	2/4	4	4	≤0.25	16	8	1	0.5	0.125
V-8-6	≤8	≤4/4	≤2	≤1	≤0.5/4	≤0.5/4	8	≤2	≤0.25	8	≤0.5	1	0.25	≤0.06
V-9-1	32	16/4	8	16	2/4	2/4	8	8	1	2	2	2	0.25	0.125
V-10-1	>512	>256/4	>128	>64	16/4	8/4	64	32	>32	32	8	1	>16	1
V-10-4	>512	>256/4	>128	>64	>32/4	32/4	>64	32	>32	64	32	1	>16	0.25
VI-1-2	≤8	≤4/4	≤2	2	1/4	≤0.5/4	8	16	2	1	≤0.5	1	0.5	1
VI-1-6	≤8	≤4/4	4	≤1	≤0.5/4	≤0.5/4	4	8	1	1	1	1	0.25	0.25
VI-2-2	≤8	≤4/4	≤2	≤1	1/4	1/4	4	8	1	≤0.5	≤0.5	2	0.5	≤0.06

VI-3-1	≤8	8/4	4	2	1/4	1/4	4	128	>32	≤0.5	≤0.5	1	16	0.125
VI-4-1	>512	>256/4	>128	>64	>32/4	>32/4	>64	64	8	32	16	≤0.5	4	≤0.06
VI-5-3	128	16/4	32	8	2/4	8/4	16	8	1	16	>64	1	1	0.125
VI-5-4	64	32/4	8	16	2/4	2/4	16	4	≤0.25	1	1	1	0.25	≤0.06
VI-6-2	>512	64/4	128	>64	16/4	4/4	16	32	2	8	8	≤0.5	1	≤0.06
VI-7-1	32	8/4	8	2	1/4	2/4	2	8	>32	2	1	8	0.5	≤0.06
VI-7-2	≤8	≤4/4	≤2	≤1	≤0.5/4	1/4	4	4	32	≤0.5	≤0.5	≤0.5	0.25	8
VI-8-1	64	32/4	8	8	1/4	2/4	8	32	8	16	8	≤0.5	1	2
VI-8-3	64	32/4	8	8	1/4	2/4	8	16	2	4	4	≤0.5	0.5	1
VI-9-1	128	64/4	32	16	8/4	8/4	16	8	2	32	8	2	1	1
VI-9-3	128	128/4	64	32	4/4	32/4	16	32	2	16	8	1	0.5	0.125
VI-9-4	128	64/4	16	16	1/4	2/4	8	16	1	32	4	1	2	≤0.06
VI-9-6	16	16/4	4	4	1/4	1/4	8	8	1	4	4	1	1	0.125
VI-10-1	≤8	16/4	8	4	4/4	4/4	8	≤2	≤0.25	≤0.5	≤0.5	≤0.5	0.5	0.25
VI-10-2	512	128/4	128	>64	16/4	32/4	64	128	16	16	16	≤0.5	2	≤0.06
VI-10-3	512	256/4	>128	>64	32/4	32/4	>64	>128	16	16	16	8	2	≤0.06
VII-1-1	≤8	≤4/4	≤2	≤1	≤0.5/4	≤0.5/4	4	16	1	2	≤0.5	≤0.5	0.25	64
VII-1-2	32	64/4	8	8	1/4	1/4	8	8	1	4	1	2	0.5	≤0.06
VII-2-1	32	≤4/4	4	2	≤0.5/4	2/4	4	≤2	≤0.25	1	≤0.5	2	0.25	≤0.06
VII-4-1	≤8	≤4/4	≤2	2	1/4	2/4	8	4	0.5	1	≤0.5	1	4	≤0.06

VII-4-2	32	8/4	4	2	1/4	2/4	16	4	0.5	1	≤0.5	1	1	≤0.06
VII-6-1	32	8/4	8	2	1/4	2/4	2	≤2	≤0.25	≤0.5	≤0.5	1	≤0.12	0.125
VII-6-2	32	8/4	8	2	≤0.5/4	2/4	2	4	≤0.25	≤0.5	≤0.5	1	≤0.12	0.5
VII-6-6	64	8/4	8	4	1/4	2/4	16	4	0.5	1	≤0.5	2	0.25	≤0.06
VII-7-1	128	32/4	32	64	2/4	8/4	16	4	1	16	16	1	0.5	≤0.06
VII-7-2	128	16/4	16	4	1/4	4/4	4	≤2	0.5	8	8	1	0.5	≤0.06
VII-8-1	32	8/4	4	4	1/4	2/4	16	8	1	16	2	2	0.25	≤0.06
VII-9-2	>512	>256/4	>128	>64	16/4	8/4	>64	64	16	32	32	1	4	1
VII-9-3	16	≤4/4	4	≤1	≤0.5/4	≤0.5/4	2	16	2	4	4	≤0.5	≤0.12	0.5
VII-10-1	≤8	≤4/4	≤2	4	2/4	2/4	16	16	>32	≤0.5	≤0.5	2	≤0.12	0.5
VII-10-2	16	8/4	8	8	2/4	4/4	16	8	>32	1	≤0.5	1	≤0.12	0.125
VIII-1-3	32	≤4/4	4	2	≤0.5/4	2/4	2	4	0.5	2	1	1	≤0.12	≤0.06
VIII-2-2	≤8	≤4/4	4	32	1/4	2/4	8	16	1	8	4	≤0.5	2	≤0.06
VIII-2-3	512	256/4	128	>64	8/4	4/4	>64	32	2	32	16	≤0.5	2	0.125
VIII-3-1	16	≤4/4	4	2	≤0.5/4	1/4	4	≤2	≤0.25	1	≤0.5	1	0.25	≤0.06
VIII-4-2	>512	>256/4	>128	>64	>32/4	16/4	>64	>128	>32	≤0.5	≤0.5	≤0.5	16	0.5
VIII-4-3	>512	>256/4	>128	>64	>32/4	>32/4	>64	>128	>32	1	4	1	1	1
VIII-5-1	16	≤4/4	≤2	4	1/4	≤0.5/4	8	32	4	4	16	1	2	0.125
VIII-5-3	64	>256/4	>128	>64	32/4	16/4	64	64	4	16	16	1	2	1
VIII-6-1	≤8	≤4/4	≤2	≤1	≤0.5/4	≤0.5/4	≤1	≤2	≤0.25	≤0.5	≤0.5	≤0.5	≤0.12	0.125

VIII-7-1	32	32/4	32	64	1/4	2/4	8	4	0.5	≤0.5	≤0.5	1	4	≤0.06
VIII-7-3	64	16/4	16	>64	8/4	4/4	16	8	1	2	1	1	16	2
VIII-7-5	≤8	≤4/4	≤2	≤1	≤0.5/4	1/4	4	8	1	≤0.5	≤0.5	1	1	≤0.06
VIII-8-1	16	≤4/4	4	2	≤0.5/4	1/4	4	≤2	≤0.25	1	1	1	1	≤0.06
VIII-9-1	64	16/4	32	64	16/4	4/4	>64	>128	>32	16	16	≤0.5	>16	≤0.06
VIII-9-4	≤8	≤4/4	≤2	≤1	≤0.5/4	≤0.5/4	2	≤2	≤0.25	≤0.5	1	≤0.5	0.25	≤0.06
VIII-10-1	≤8	≤4/4	≤2	≤1	1/4	1/4	4	4	0.5	≤0.5	≤0.5	1	0.25	≤0.06
VIII-10-2	64	8/4	8	4	2/4	4/4	8	8	1	1	≤0.5	2	1	0.125
IX-1-1	≤8	≤4/4	64	16	4/4	2/4	32	128	16	2	1	≤0.5	1	1
IX-1-3	32	32/4	64	>64	8/4	4/4	64	128	16	2	2	≤0.5	1	1
IX-1-4	128	128/4	>128	32	≤0.5/4	≤0.5/4	16	32	4	64	32	≤0.5	2	≤0.06
IX-1-5	≤8	≤4/4	≤2	16	4/4	4/4	>64	128	16	4	2	≤0.5	1	1
IX-2-1	≤8	≤4/4	≤2	≤1	1/4	1/4	4	64	16	≤0.5	≤0.5	≤0.5	0.25	≤0.06
IX-3-2	≤8	≤4/4	≤2	2	≤0.5/4	≤0.5/4	8	8	1	16	≤0.5	≤0.5	2	0.125
IX-3-3	>512	>256/4	>128	>64	>32/4	>32/4	>64	128	32	32	32	1	8	8
IX-3-4	≤8	≤4/4	≤2	8	4/4	1/4	8	16	2	≤0.5	≤0.5	≤0.5	2	0.125
IX-3-5	256	128/4	32	>64	8/4	8/4	64	32	2	64	16	1	4	32
IX-4-1	≤8	≤4/4	≤2	≤1	≤0.5/4	≤0.5/4	2	4	≤0.25	8	1	2	1	2
IX-4-4	128	32/4	16	8	1/4	4/4	8	16	1	32	16	2	8	0.5
IX-5-2	≤8	≤4/4	≤2	2	2/4	2/4	16	16	2	1	≤0.5	4	0.5	>64

IX-5-3	≤8	≤4/4	≤2	2	2/4	2/4	16	16	2	1	≤0.5	≤0.5	0.5	1
IX-5-5	≤8	≤4/4	≤2	2	2/4	2/4	16	8	2	2	≤0.5	≤0.5	0.25	0.125
IX-5-6	≤8	≤4/4	≤2	≤1	2/4	1/4	8	128	16	≤0.5	≤0.5	16	4	>64
IX-6-1	≤8	≤4/4	≤2	≤1	2/4	1/4	16	4	0.5	≤0.5	≤0.5	≤0.5	4	0.5
IX-7-4	≤8	≤4/4	≤2	2	1/4	1/4	8	8	1	≤0.5	≤0.5	2	0.5	0.25
IX-8-1	128	16/4	32	8	1/4	8/4	8	16	2	8	16	1	1	≤0.06
IX-8-2	256	32/4	32	8	2/4	8/4	16	16	1	≤0.5	2	1	1	≤0.06
IX-8-5	128	16/4	16	4	1/4	4/4	8	8	1	8	16	1	1	0.125
IX-10-1	≤8	≤4/4	≤2	2	1/4	2/4	≤1	8	0.5	1	≤0.5	2	≤0.12	≤0.06
IX-10-2	≤8	≤4/4	≤2	4	2/4	2/4	16	16	2	1	≤0.5	2	0.5	1
X-1-1	16	8/4	4	4	2/4	≤0.5/4	8	32	4	1	≤0.5	≤0.5	2	1
X-1-2	16	≤4/4	≤2	2	1/4	1/4	4	64	8	8	8	16	8	1
X-2-1	≤8	≤4/4	≤2	2	2/4	1/4	16	16	2	16	2	2	0.5	≤0.06
X-2-3	64	8/4	8	2	1/4	2/4	8	8	1	≤0.5	1	2	2	0.125
X-3-2	32	≤4/4	≤2	4	2/4	2/4	16	16	2	1	≤0.5	1	8	0.125
X-3-6	≤8	≤4/4	≤2	2	1/4	1/4	8	8	2	≤0.5	≤0.5	1	4	0.125
X-4-1	≤8	≤4/4	≤2	2	1/4	1/4	4	16	1	1	≤0.5	2	8	2
X-5-1	128	8/4	16	8	1/4	≤0.5/4	32	16	1	32	8	1	1	0.5
X-5-3	256	≤4/4	8	64	32/4	16/4	8	≤2	1	1	1	1	1	1
X-5-4	>512	256/4	>128	>64	>32/4	>32/4	>64	64	8	2	2	≤0.5	2	1

X-6-1	>512	>256/4	128	>64	32/4	16/4	>64	128	16	32	16	1	0.5	4
X-6-2	256	256/4	64	>64	8/4	4/4	32	128	16	16	8	1	8	64
X-6-4	16	8/4	4	8	2/4	2/4	16	64	8	4	≤0.5	≤0.5	≤0.12	0.25
X-6-5	64	256/4	16	64	2/4	2/4	32	128	16	16	16	1	1	1
X-6-6	32	128/4	16	32	4/4	2/4	32	128	16	2	2	≤0.5	≤0.12	0.5
X-7-2	16	≤4/4	4	2	1/4	2/4	4	8	0.5	≤0.5	≤0.5	2	2	0.125
X-7-3	32	≤4/4	4	2	1/4	2/4	8	32	2	1	≤0.5	2	4	0.125
X-7-5	128	32/4	32	8	4/4	8/4	32	64	4	2	2	1	1	≤0.06
X-8-3	≤8	≤4/4	≤2	2	1/4	1/4	4	≤2	≤0.25	≤0.5	≤0.5	≤0.5	4	0.25
X-9-1	128	32/4	64	16	4/4	4/4	32	32	4	32	32	2	8	4
X-9-2	256	64/4	64	32	8/4	8/4	64	32	4	32	32	2	8	8
X-9-3	128	8/4	16	8	2/4	2/4	32	32	8	16	8	2	16	4
X-10-1	≤8	8/4	≤2	2	1/4	1/4	8	≤2	≤0.25	≤0.5	≤0.5	1	0.5	≤0.06
XI-1-4	16	≤4/4	4	4	2/4	2/4	16	16	2	1	≤0.5	2	0.25	0.125
XI-1-5	≤8	≤4/4	≤2	≤1	1/4	2/4	8	8	1	2	≤0.5	2	≤0.12	0.125
XI-2-2	≤8	≤4/4	≤2	2	≤0.5/4	≤0.5/4	4	16	2	1	≤0.5	≤0.5	1	≤0.06
XI-2-3	64	8/4	8	4	2/4	4/4	8	8	1	16	2	2	0.5	≤0.06
XI-2-6	16	8/4	4	16	1/4	1/4	8	32	8	8	4	≤0.5	2	0.125
XI-3-1	256	256/4	64	>64	8/4	2/4	64	64	8	1	2	≤0.5	2	0.25
XI-3-2	16	≤4/4	≤2	≤1	≤0.5/4	1/4	≤1	4	0.5	1	≤0.5	2	≤0.12	0.125

XI-3-4	256	8/4	128	>64	4/4	≤0.5/4	16	16	2	≤0.5	≤0.5	≤0.5	2	1
XI-4-1	16	≤4/4	4	2	1/4	1/4	2	32	4	2	≤0.5	1	0.25	0.125
XI-4-3	256	32/4	32	64	2/4	2/4	16	64	16	≤0.5	≤0.5	≤0.5	≤0.12	>64
XI-5-2	≤8	≤4/4	≤2	≤1	1/4	1/4	≤1	4	0.5	4	≤0.5	2	1	≤0.06
XI-6-2	≤8	≤4/4	≤2	4	2/4	1/4	8	64	8	≤0.5	≤0.5	>256	1	>64
XI-6-4	16	≤4/4	≤2	4	1/4	1/4	8	16	2	4	≤0.5	4	2	4
XI-6-6	≤8	≤4/4	≤2	4	4/4	2/4	16	64	8	2	≤0.5	128	2	>64
XI-7-1	16	≤4/4	4	16	2/4	2/4	32	64	8	≤0.5	≤0.5	≤0.5	0.5	1
XI-7-2	64	16/4	8	8	2/4	4/4	16	128	16	16	≤0.5	2	0.25	0.5
XI-7-3	16	≤4/4	4	8	1/4	2/4	16	64	8	64	8	2	0.25	1
XI-8-4	≤8	≤4/4	≤2	≤1	≤0.5/4	≤0.5/4	4	4	0.5	≤0.5	≤0.5	1	2	0.125
XI-8-5	≤8	≤4/4	≤2	4	1/4	1/4	4	4	0.5	8	2	1	1	0.125
XI-9-1	32	8/4	8	4	2/4	4/4	16	16	2	1	≤0.5	1	2	1
XI-9-4	512	256/4	64	>64	4/4	16/4	64	>128	32	4	4	≤0.5	4	4
XI-9-5	≤8	8/4	≤2	2	4/4	2/4	16	64	8	2	≤0.5	≤0.5	1	0.5
XI-10-1	>512	>256/4	>128	>64	>32/4	16/4	64	16	2	32	16	1	0.5	0.5
XI-10-2	256	256/4	64	>64	8/4	4/4	32	16	1	32	8	1	0.25	1
XII-1-1	16	≤4/4	≤2	2	≤0.5/4	≤0.5/4	4	4	≤0.25	1	≤0.5	1	2	≤0.06
XII-1-2	>512	256/4	>128	>64	>32/4	>32/4	>64	64	8	32	8	≤0.5	16	0.25
XII-2-1	64	16/4	8	8	2/4	2/4	16	16	2	32	4	1	≤0.12	≤0.06

XII-2-2	>512	256/4	128	>64	32/4	>32/4	>64	32	4	32	32	≤0.5	2	8
XII-2-3	128	>256/4	32	64	4/4	8/4	32	16	2	32	16	2	0.25	1
XII-2-6	64	128/4	16	32	2/4	4/4	16	8	2	32	8	2	0.25	0.5
XII-3-1	≤8	≤4/4	≤2	≤1	1/4	≤0.5/4	16	>128	32	1	≤0.5	≤0.5	0.25	0.25
XII-3-2	≤8	≤4/4	≤2	≤1	≤0.5/4	1/4	4	>128	32	≤0.5	≤0.5	≤0.5	0.5	2
XII-3-3	≤8	≤4/4	≤2	2	2/4	2/4	8	>128	>32	4	≤0.5	1	1	≤0.06
XII-4-1	≤8	≤4/4	≤2	2	1/4	1/4	4	32	4	1	1	2	1	0.125
XII-5-1	32	8/4	4	2	2/4	2/4	8	128	32	4	4	2	1	2
XII-5-3	32	16/4	16	4	2/4	4/4	16	32	2	1	1	1	1	0.5
XII-6-1	≤8	≤4/4	≤2	≤1	1/4	1/4	4	8	>32	≤0.5	≤0.5	1	0.5	0.25
XII-6-2	≤8	≤4/4	≤2	2	1/4	1/4	4	8	1	4	1	≤0.5	0.5	0.25
XII-7-1	128	32/4	32	8	1/4	8/4	8	8	1	16	16	2	1	≤0.06
XII-8-1	≤8	≤4/4	≤2	≤1	≤0.5/4	≤0.5/4	≤1	≤2	≤0.25	≤0.5	≤0.5	1	≤0.12	≤0.06
XII-9-1	≤8	≤4/4	≤2	≤1	2/4	1/4	8	8	>32	1	≤0.5	1	0.5	0.5
XII-10-1	≤8	≤4/4	≤2	4	2/4	4/4	8	128	16	≤0.5	≤0.5	2	≤0.12	≤0.06

TIC, ticarcillin; PTZ, piperacillin/tazobactam; ATM, aztreonam; CZD, ceftazidime; CT, ceftolozane/tazobactam ; CZA, ceftazidime/avibactam ; FEP, cefepim ; AKN, amikacin ; TMN, tobramycin ; IPM, imipenem ; MEM, meropenem ; CST, colistin ; CIP, ciprofloxacin ; MUR, murepavadin.

505 **TABLE S2**

506 **TABLE S1** Number of SNPs identified in CF-clinical strains with murepavadin MIC > 4 mg/L compared with the PAO1 reference sequence

Patient	Strain	Murepavadin MIC (mg/L)	SNPs				Mutations in genes linked to hypermutability
I-5	I-5-4	2	108		375		MutL (R251C, R287W), MutT (E236D), UvrD (T547)
	I-5-2	16		358			MutL (R251C, R287W), MutT (E236D), UvrD (T547)
	I-5-1	64					MutL (R251C, R287W), MutT (E236D), UvrD (T547)
III-3	III-3-2	0.25	49				MutL (A406T), MutM (A66T)
	III-3-1	≥ 128					MutL (A406T), MutM (A66T)
III-9	III-9-1	4	7		7		
	III-9-3	64		2			
	III-9-4	≥ 128 [a]					MutY (A354T), UvrD (S662N, N666S)
V-5	V-5-4	0.25	23				MutS (I579V), MutY (G311S), UvrD (A96T)
	V-5-1	8					MutS (I579V), MutY (G311S), UvrD (A96T)
VI-7	VI-7-1	≤ 0.06	26				
	VI-7-2	8					
VII-1	VII-1-2	≤ 0.06	26				MutL (E428D)
	VII-1-1	64					MutL (E428D)

IX-3	IX-3-2	0.125	541	218	511			MutL (ΔV383, S386A), MutM (A66T), MutS (Q155*), MutT (E55A, G289A, MutY (H100Y, L164P, L268I, A301S, A308E)
	IX-3-3	8						MutL (ΔV383, S386A), MutM (A66T), MutS (E509, frameshift [a]), MutT (E55A, G289A), MutY (H100Y, L268I, A301S, A308E)
	IX-3-5	32						MutL (ΔV383, S386A), MutM (A66T), MutS (E509X), MutT (E55A, G289A), MutY (H100Y, L268I, A301S, A308E)
IX-5	IX-5-5	0.125	69	44	56	88	89	
	IX-5-3	1						
	IX-5-2	≥ 128						
	IX-5-6	≥ 128						
X-6	X-6-4	0.25	26	26	529	33	529	
	X-6-5	1						
	X-6-1	4						
	X-6-2	64						MutS (E729, frameshift)
X-9	X-9-1	4	11					
	X-9-2	8						
XI-4	XI-4-1	0.125	22					MutL (N595S)
	XI-4-3	≥ 128						MutL (N595S)
XI-6	XI-6-4	4	367	5	366			
	XI-6-6	≥ 128						MutY (Y31, frameshift)
	XI-6-2	≥ 128						MutY (Y31, frameshift)

XII-2	XII-2-1	≤ 0.06	11	892	889		MutL (R370H, S515N)
	XII-2-3	1					MutL (R370H, S515N)
	XII-2-2	8					MutL (Q17*), MutS (L80P), MutT (V4A)
Single strains							
II-2	II-2-2	8					MutL (Y560, frameshift)
III-6	III-6-1	8					MutL (S43A, I55V, S389A, A391S, G394A, S515G, S531T), MutM (I27V, A103S), MutS (A781S, M803V), MutT (D42E, A48V, A94P, H95D, V105A, A120V, G138S, V152L, A154S, E263A), MutY (G27D, A57E, E63Q, Q94R, V205L, S206A, A217T, D242E, G270D, E272A, G311T, R314P, G319A), UvrD (E227K, S494G, T659A)

[a] Two isolates from patient III-9 had strictly identical genomes and a murepavadin resistance ≥ 128 mg/L)

509 **TABLE S3 List of strains and plasmids used in this study**

Strains or plasmids	Characteristics	Reference
Pseudomonas aeruginosa		
PAO1	Wild-type susceptible strain	(1)
2243	Clinical strain, CS^r, PmrB (Q105P, Y345H)	(2)
3795	Clinical strain, CS^r, PmrB (G188D, Y345H)	(2)
3890	Clinical strain, CS^r, PmrB (S2P, A4T, D45E, Y345H)	(2)
III-9-1	CF clinical strain, murepavadin MIC= 4 mg/L	This study
III-9-3	CF clinical strain, murepavadin MIC= 64 mg/L	This study
IX-5-2	CF clinical strain, murepavadin, MIC ≥128 mg/L	This study
VII-1-1	CF clinical strain, murepavadin, MIC= 64 mg/L	This study
PAO1::*lpxL1*$_{\text{III-9-1}}$	Allelic replacement of the *lpxL1* gene by that of strain III-9-1 (T76P) in strain PAO1	This study
PAO1::*lpxL1*$_{\text{IX-5-2}}$	Allelic replacement of the *lpxL1* gene by that of strain IX-5-2 (H120N) in strain PAO1	This study
PAO1::*lpxL1*$_{\text{VII-1-1}}$	Allelic replacement of the *lpxL1* gene by that of strain VII-1-1 (E265*) in strain PAO1	This study
PAO1::*bamA*$_{\text{III-9-3}}$	Allelic replacement of the b*amA* gene by that of strain III-9-3 (D535E) in strain PAO1	This study
PAO1::l*pxl1*$_{\text{III-9-1}}$*bamA*$_{\text{III-9-3}}$	Allelic replacement of the b*amA* gene by that of strain III-9-3 (D535E) in mutant PAO1::*lpxL1*$_{\text{III-9-1}}$	This study
PAO1Δ*pmrAB*	PAO1 mutant with in-frame deletion of operon *pmrAB*	(2)
E. coli		
CC118 λ*pir*	CC118 lysogenized with phage λ*pir*	(3)
HB101	*subE44 hsdS20(rB- mB-) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	(4)
Plasmids		
pKNG101	Suicide vector in *P. aeruginosa ; sacB*^{r} *mobRK2 oriR6K*; Str^r	(5)
pKNG101-III-9-1	pKNG101 with *lpxl1* gene of III-9-1 clinical strain cloned, Str^r	This study
pKNG101-IX-5-2	pKNG101 with *lpxl1* gene of IX-5-2 clinical strain cloned, Str^r	This study

pKNG101-VII-1-1	pKNG101 with *lpxl1* gene of VII-1-1 clinical strain cloned, Str^r	This study
pKNG101-III-9-3	pKNG101 with *bamA* gene of III-9-3 clinical strain cloned, Str^r	This study
pRK2013	Helper plasmid, *mob1*, *tra1*, *colE1*, Kan^r	(6)
pME6012	Broad host-range expression plasmid; Tet^r	(7)
pAB2243	pME6012 carrying genes *pmrAB* from clinical strain 2243	(2)
pAB3795	pME6012 carrying genes *pmrAB* from clinical strain 3795	(2)
pAB3890	pME6012 carrying genes *pmrAB* from clinical strain 3890	(2)

510 CS^r, colistin resistance; Tet^r, tetracycline resistance; Str^r, streptomycin resistance; Kan^r, kanamycin resistance.

511

TABLE S4 List of the primers used in this study

Primer name	Nucleotide sequence (5'- 3')	Reference
Gene cloning		
Lpxl1-for-NEB	CCCCCCCCTGCAGGTCGACGGAGCGAGGAGTGTTTCGTG	This study
Lpxl1-rev-NEB	TTCTACTTATGGTACCCGGGTGAAAAAGGCGTCTTCCG	This study
BamA-fwd-NEB	CCCCCCCCTGCAGGTCGACGATGAAACGCTTTCTGCTACC	This study
BamA-rev-NEB	TTCTACTTATGGTACCCGGGTCAGAAGGTCTGGCCCAG	This study
Gene replacement		
Lpxl1-for-NEB	CCCCCCCCTGCAGGTCGACGGAGCGAGGAGTGTTTCGTG	This study
Lpxl1-rev-NEB	TTCTACTTATGGTACCCGGGTGAAAAAGGCGTCTTCCG	This study
BamA-F3	AGAGCAACCGTCCGGCTCGA	This study
BamA-R3	CCGTCTGGATTACGAGCCAC	This study

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1.3 Additional results

1.3.1 Susceptibility of murepavadin in colistin resistant clinical strains

The susceptibility of murepavadin was determined on two colistin resistant clinical strains that does not have PmrB mutations with minimum colistin MIC value ≥ 8 mg/L (**table 7**). The strain 543 had a mutation in *pmrA* gene encoding for the response regulator PmrA. We have previously shown the cross-resistance between colistin and murepavadin through the two-component system PmrAB. In addition to that, we have shown that the deletion of *pmrAB* operon in PAO1 reduces by 2-folds the MIC of murepavadin. Furthermore, in a *pmrB* PAO1 mutant AB8.2, murepavadin MIC was 16-folds higher (1 mg/L) with respect to PAO1 (≤0.06 mg/L). Therefore, PmrA mutation could explain the increased resistance to colistin and to reduced susceptibility to murepavadin. In addition to that, the strain 5934 had mutations in *cbrA* and *msbA* genes. As shown previously, alterations of CbrA and MsbA proteins could reduce the susceptibility to murepavadin and colistin.

Table 7: Mutations identified in two non-*pmrB* colistin resistant clinical strains and their susceptibility to murepavadin

Clinical strain	**Alterations identified**	**MIC (mg/L)**	
		Colistin	Murepavadin
543	OprD (frN$_{112}$), GyrA(T$_{83}$I), PmrA (G$_{19}$E), ParC (S$_{87}$L)	>256	1
5934	CbrA (A$_{321}$D), MsbA (D$_{411}$S)	8	8

fs: frameshift

1.3.2 Impact of BamA and LpxL1 mutations on the membrane permeability

Since alteration of the outer membrane constituents (BamA, and/or lipid A) may compromise its permeability, we measured N-phenyl-1-naphtylamine (NPN) dye fluorescence kinetic for 90 seconds in the *lpxL1*, *bamA* mutant, *lpxL1-bamA* double mutant as well in the PAO1 reference strain. Because colistin is known to permeabilize the outer membrane, we added colistin to PAO1 at 16xMIC as our positive control (gray line). NPN is a hydrophobic fluorescent probe, which fluoresce weakly in aqueous environment and strongly in hydrophobic environments. Increased penetration of the NPN molecules into the membrane is reflected by increased and rapid emission of fluorescence. Among all the tested mutants, the *bamA* mutant had the highest

fluorescence levels (violet line) (**Figure 29**). This indicates that the alteration of BamA mutation has a significant effect on the membrane permeability. Due to the important role of BamA in the proper folding and insertion of OMPs into the outer membrane, a compromised BamA might strongly impact the incorporation of OMPs including LptD (the target of murepavadin), but also other OMPs, into their proper location. However, no significant difference was found between our reference strain PAO1 (red line), the three *lpxL1* mutants (blue, green and yellow) and surprisingly, the double *lpxL1-bamA* mutant (navy line), in which their fluorescence levels were weak compared to the positive control (PAO1 exposed to colistin). The difference between the *bamA* mutant and the double mutant *bamA-lpxL1* may be due to counterbalance of *lpxL1* on membrane permeability. Interestingly, in *Bordetella bronchiseptica*, the loss of the secondary acyl group added by LpxL1 enzyme reduces the susceptibility of the strains to hydrophobic antibiotic rifampicin by 10-fold with respect to wild type strain, and renders the membrane less permeable (Pérez-Ortega et al., 2023). Unlike, in *E. coli*, the loss of secondary acyl chains increases the membrane permeability, suggesting that the impact of LpxL1 on membrane permeability depend on the bacterial species (Hittle et al., 2015).

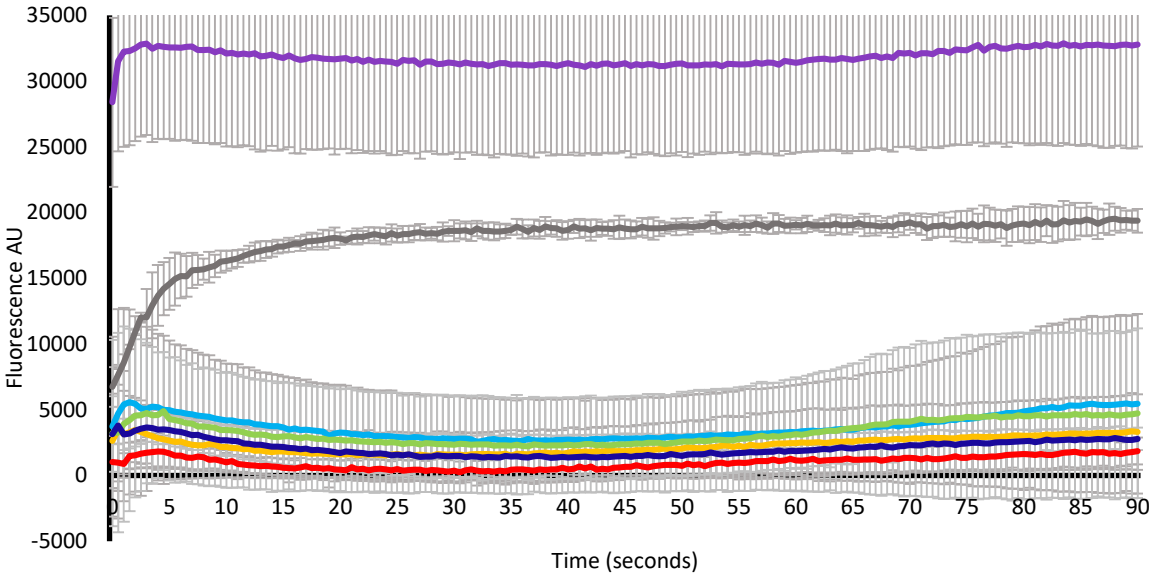

Figure 29: Membrane permeability of PAO1 reference strain and its *lpxL1* and *bamA* mutant derivatives. The fluoresence of NPN was measured for each strain, during 90 seconds. The membrane permeability was measured in PAO1 strain exposed to 16 xMIC of colistin as a positive control (grey line), PAO1 reference strains (line red), PAO1 ::*lpxL1* $_{IX\text{-}5\text{-}2}$ (blue line), PAO1 ::*lpxL1* $_{VII\text{-}1\text{-}1}$ (green line), PAO1 ::*lpxL1* $_{III\text{-}9\text{-}1}$ (yellow line), PAO1 ::*bamA* $_{III\text{-}9\text{-}3}$ (violet line) and PAO1 ::*lpxL1* $_{III\text{-}9\text{-}1}$-*bamA* $_{III\text{-}9\text{-}3}$ (dark blue line).

To study the impact of murepavadin on the membrane permeability in PAO1 strain, *lpxL1*, *bamA*, and *lpxL1-bamA* mutants, we exposed the strain and mutants to 16 x MIC of murepavadin using an injector (TECAN), and measured the NPN fluorescence during 90 seconds. The addition of murepavadin did not cause significant increase in membrane permeability regardless of the strain and mutants tested except for the double mutant *bamA-lpxL1* (**Figure 30**). Our results for the PAO1 strain were similar to that previously reported, where murepavadin does not permeabilize the outer membrane in contrast to several antimicrobial peptides such as colistin. Further experiments are needed to confirm this observation in particular by analysing the lipid A structure in *lpxL1*, *bamA* and the double mutant *lpxL1-bamA*.

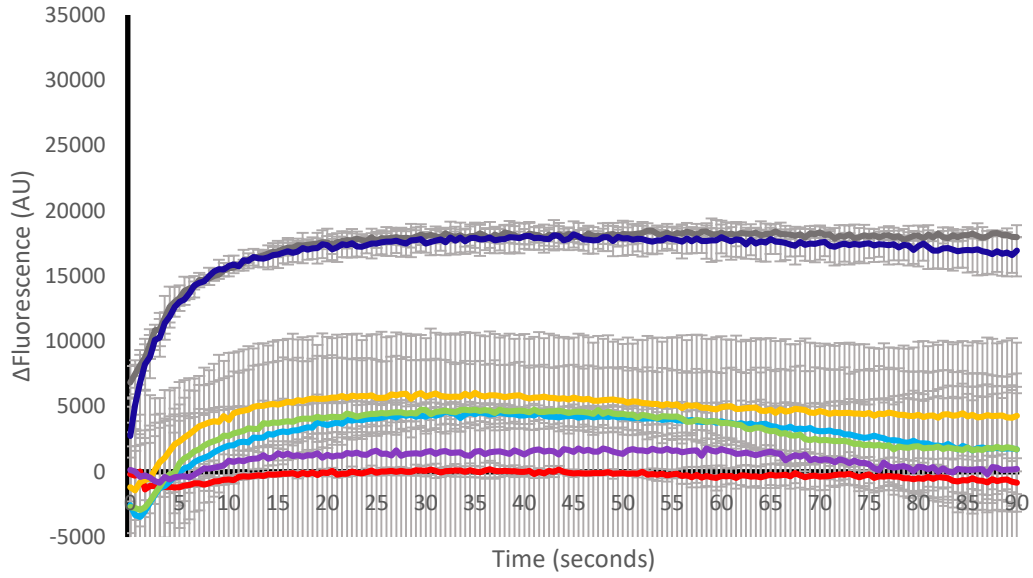

Figure 30: Difference between the membrane permeability measurement of PAO1 reference strain and its *lpxL1* and *bamA* mutant derivatives grown in the presence of 16 x MIC of murepavadin or not. The fluoresence of NPN was measured during 90 seconds. The membrane permeability was measured in PAO1 strain exposed to 16 xMIC of colistin as a positive control (grey line), PAO1 reference strains (red line), PAO1 ::*lpxL1* $_{\text{IX-5-2}}$ (blue line), PAO1 ::*lpxL1* $_{\text{VII-1-1}}$ (green line), PAO1 ::*lpxL1* $_{\text{III-9-1}}$ (yellow line), PAO1 ::*bamA* $_{\text{III-9-3}}$ (violet line) and PAO1 ::*lpxL1* $_{\text{III-9-1}}$*-bamA* $_{\text{III-9-3}}$ (dark blue line).

Chapter 2: Response to murepavadin in *Pseudomonas aeruginosa*

Resistance to polymyxins is governed by two-component regulatory systems, ParRS, CprRS and PmrAB, which activate the expression of the *arnBCADTEF* operon responsible for modifying the lipopolysaccharide (LPS) (Jeannot et al., 2017). The *arn* gene products facilitate the addition of 4-aminoarabinose to the lipid A component of LPS, leading to a reduction in its

overall negative charge. Consequently, the altered LPS limits the interaction with the outer membrane and uptake of polycationic antibiotics, including polymyxins, host defense peptides, and aminoglycosides (Fernández et al., 2010). In addition to LPS modifications, a recent publication documented the increase in polyamine biosynthetic genes in PA14 reference strain upon exposure to sub-inhibitory concentrations to polymyxin B (Ben Jeddou et al., 2020). Polyamines were also shown to contribute to aminoglycoside tolerance in *P. aeruginosa* (Bolard et al., 2019; Puja et al., 2020). The production of polyamines might confer protective roles to the strain by binding to the negatively charged outer membrane. So far, nothing has been described concerning the response of *P. aeruginosa* to murepavadin. The aim of this work was to decipher the molecular response of *P. aeruginosa* upon exposure to murepavadin (¼ MIC). To identify genes involved in the response to murepavadin, we compared the transcriptomes of the PA14 reference strain grew in the presence of murepavadin with that of the strain unexposed. The results were controlled by RT-qPCR. Finally, we quantified by mass spectrometry (ESI-MS) the amount of polyamines present on cell-surface and in the supernatent of the PAO1 reference strain exposed or not to murepavadin.

2.1 Transcriptome analysis of PA14 strain exposed to ¼ MIC of murepavadin

To gain insight into the response of *Pseudomonas aeruginosa* upon exposure to murepavadin, we compared the transcriptome of the reference strain PA14 not exposed to murepavadin to that of PA14 exposed to ¼ MIC of murepavadin, grown in MH broth. Overall, a total of 201 genes were differentially expressed in PA14 exposed to subinhibitory concentration of murepavadin, from which 70.38% were upregulated and 29.62% were downregulated (appendix, table S1). Among the most upregulated genes in presence of murepavadin, three genes were involved in the polyamines biosynthesis pathways. These genes belong to the same locus PA14_63110, PA14_63120, and PA14_63130, which were homologous of genes *speD2* (PA4773), *speE2* (PA4774) and a hypothetical protein encoded by PA4775 in the PAO1 strain, respectively. Interestingly, the same genes were previously shown to be upregulated in PA14 in response to exposure to polymyxin B at sub-inhibitory concentration (Ben Jeddou et al., 2020). These genes convert amino-acid precursors to different polyamines such as spermidine, spermine and norspermidine. SpeD2 is an S-adenosyl methionine decarboxylase while SpeE2 is an aminopropyl transferase that transfers the aminopropyl group from the decarboxylated S-adenosyl methionine and incorporates it into an existing polyamine molecule (Bolard et al., 2019). The *speD2-speE2* operon is known to be activated by and co-transcribed with the TCS

PmrAB. Additionally, *spdH* was slightly downregulated (-2.5-fold). SpdH is a spermidine dehydrogenase, which is involved in the degradation of spermine and spermidine, suggesting that the latter two polyamines are not degraded. Interestingly, a PA14_24360 gene, with no homologue in PAO1 strain, located just downstream of genes encoding the two-component CprRS, was significantly upregulated (16-fold) and was the second most upregulated gene among the 201 differentially expressed gene. PA14_24360 encodes for a putative serine-type endopeptidase outer membrane autotransporter protein (Ben Jeddou et al., 2020). This gene PA14_24360 was also slightly upregulated (2-fold) in PA14 strain exposed to a sub-inhibitory concentration (0.25xMIC) of polymyxin B (Ben Jeddou et al., 2020). As PA14_24360, PA1797 homologue in PA14, located just downstream the ParRS-two component operon was upregulated (6.5 fold), suggesting that ParRS was activated (Fernández et al., 2010), as in the presence of polymyxins (Ben Jeddou et al., 2020). Finally, the response regulator encoding gene *phoP* (2.6-fold), the efflux pump gene *mexY* (2.5-fold) and *arnA* (7.4 fold), one gene of the large operon *arnBCADTEF-ugd,* were overexpressed after murepavadin exposure. Collectively, transcriptomic results suggest the activation of several TCSs including ParRS, CprRS and PhoPQ that result in the activation of *arnBCADTEF-ugd* operon, in addition to the efflux pump MexXY/OprM and the activation of polyamine biosynthetic genes.

In order to confirm the transcriptomic data, the expression of a set of genes was measured by RT-qPCR in PA14 and PAO1 reference strains unexposed and exposed to a sub-inhibitory concentration of murepavadin (1/4 MIC). As MH broth contains polyamines, measurements were also determined in M63 minimal medium supplemented with 20% glucose. The reason for also culturing the strains in minimal medium is because MH is rich in polyamines, and that might cause a bias in interpreting the results. In the case of minimal medium, due to absence of polyamines, we might see even the real impact of the expression levels of polyamines biosynthetic genes. Surprisingly, all the relative expression values were weak in comparison with those of transcriptomic data, even for genes whose expression was high in transcriptomic data (*spE2*, and *speD2*). The response to murepavadin differed between the PAO1 and PA14 strains in MH and in M63 minimal medium (**table 8**). While some genes were slightly upregulated in PAO1 strain in minimal medium (i.e PA4114, the homologue of spermidine acyltransferase PA14-10740 in PA14, 2.63-fold), they were slightly downregulated in PA14 strain (- 2,11-fold). RT-qPCR data confirmed only the overexpression of genes *mexX* and *arnB* (3.73 and 3.93-fold, respectively) in PA14 strain cultured in minimal medium exposed to murepavadin. This suggests that the response to murepavadin involves the modification of the

LPS through the addition of aminoarabinose residues and the overproduction of the RND efflux pump MexXY/OprM. This is aligning with the work of Romano et al, that found upregulation of *arn* operon genes in PA14 subjected to 2 x MIC to POL7000, a homologue peptidomimetic to murepavadin (Romano et al., 2019).

Table 8: Relative expression of genes in the reference strains PAO1 and PA14 exposed to ¼ murepavadin MIC, grown in minimal medium M63 supplemented with glucose 20%, or in MH.

	M63+ glucose 20% medium		**Mueller Hinton medium**	
Gene	**Strain PAO1**	**Strain PA14**	**Strain PAO1**	**Strain PA14**
PA4114	2.63	-2.11	-1.18	1.05
oprM	2.20	1.53	-1.60	1.00
mexX	-1.37	3.73	-1.18	1.05
mexY	1.18	1.25	2.18	1.13
PA1797	1.82	2.58	2.03	1.90
speE2	2.10	1.60	1.68	1.60
speD2	1.60	1.55	1.75	1.63
PA4775	1.40	-1.48	1.15	1.70
pmrA	1.93	2.83	1.15	1.10
pmrB	1.45	1.18	1.35	1.03
arnB	2.03	3.93	1.78	1.95
cprR	1.50	1.43	1.25	1.05
cprS	1.58	2.73	1.15	-1.25
phoP	1.58	1.30	1.23	1.00
phoQ	1.78	1.95	1.30	1.00
speC	-1.11	1.20	-1.14	-1.25

2.2 Murepavadin influences the polyamine content of the culture supernatant

Polyamines were previously found on the cell surface of PAO1 *pmrB* mutants and were shown to increase resistance to aminoglycosides (Bolard et al., 2019). Therefore, we were interested in quantifying polyamines in the supernatant and on the cell surface PAO1 and compare with that exposed to murepavadin at ½ MIC of murepavadin. Five polyamines (1,3-diaminopropane, norspermidine, spermidine, cadaverine and putrescine), were quantified by LC-ESI-MS in collaboration with the plateform of Archamps, Biopark (Dr P.Bulet), in the supernatent of cultures and on the cell surface of the strain PAO1 exposed or not ½ murepavadin MIC concentration. As shown in the **Figure 31**, all polyamines tested were detected in significant quantities in Mueller Hinton broth (hatched bars) indicating that Mueller-Hinton contains a significant amount of polyamines. The amount of 1,3-diaminopropane, putrescine, and

cadaverine in the supernatant and at the cell surface of the PAO1 strain (**Figure 31**, **A** and **B**) was higher than those of the control suggesting that the strain produced and secreted these polyamines (black bars). In opposite, the norspermidine seems to be catabolized by the strain PAO1. Addition of murepavadin led to a reduction of all polyamines tested in the supernatent in comparison with the strain unexposed (grey bars). At the cell-surface, addition of murepavadin did not change the number of polyamines. All these data suggest that murepavadin might influence the polyamines metabolism (production and secretion). However, further experiments are needed to confirm these preliminary data.

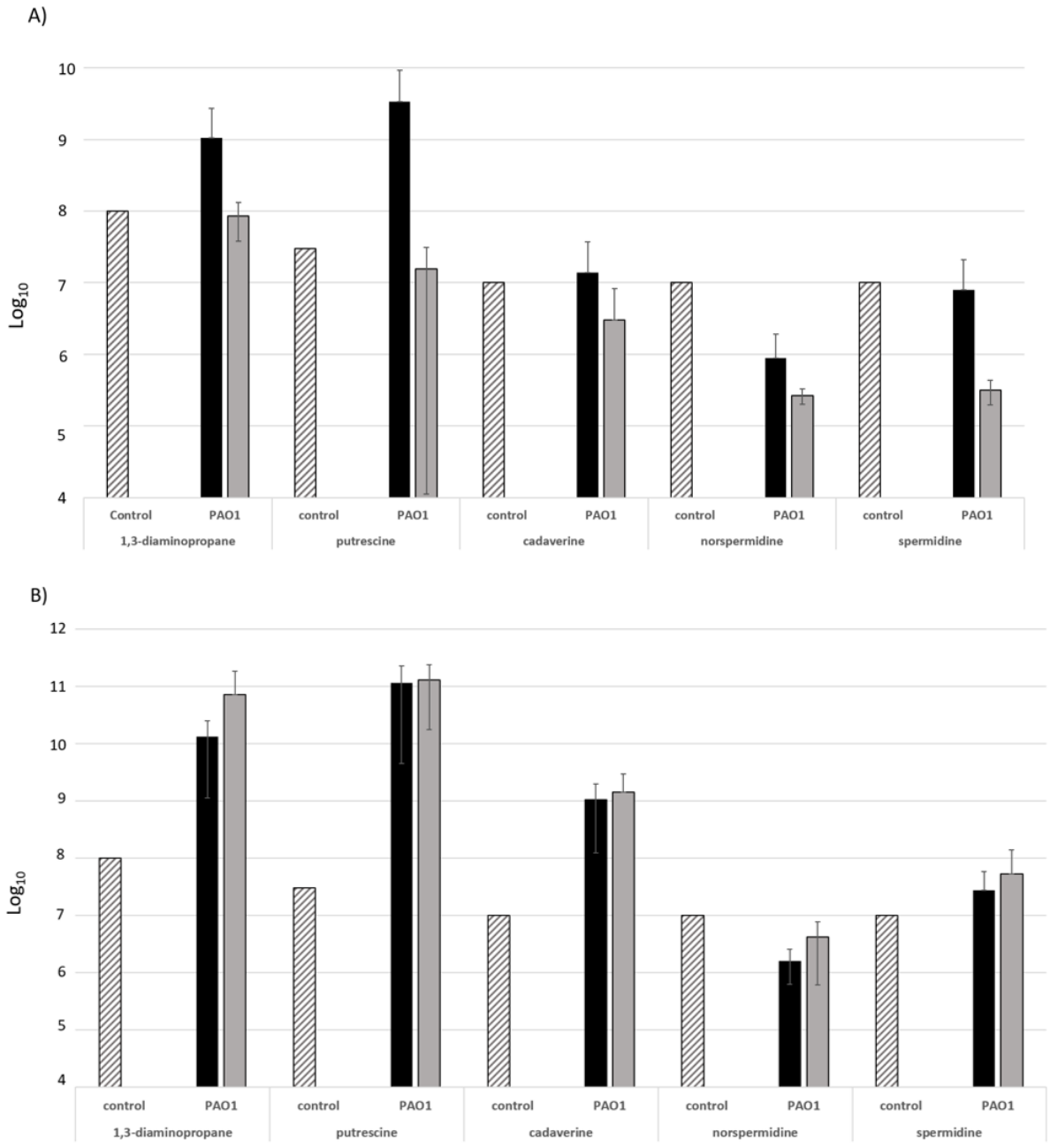

Figure 31: Amount of polyamines identified in the supernatant of culture (A) and cell surface extracts (B) from the PAO1 reference strain (black bars). The quantities of polyamines after the addition of murepavadin at 1/2MIC are represented by (grey bars). The hatched bars represent the quantities of polyamines in the control, MH.

Chapter 3: MexXY/OprM efflux pump confers resistance to murepavadin in *pmrB* mutants

This work is achieved in collaboration with Xavier Vuillemin (PhD student), who is interested in resistance mechanisms to polymyxins through the efflux pump MexXY/OprM. In *Pseudomonas aeruginosa*, four RND (MexXY/OprM, MexAB-OprM, MexCD-OprJ and

MexEF-OprN) multidrug efflux systems contribute significantly to antimicrobial resistance in both laboratory and clinical isolates (Puzari and Chetia, 2017). RND efflux pumps are tripartite structures, composed of a RND transporter located into the inner membrane (MexB, MexD, MexY, MexF), a periplasmic membrane fusion protein (MexX, MexA, MexC and MexE) (MFP), and an outer membrane protein (OMF) (OprM, OprJ and OprN) located into the outer membrane (Morita et al., 2012).

MexXY/OprM efflux pump has initially been known in the efflux of aminoglycosides (Aires et al., 1999; Morita et al., 2012). The upregulation of MexXY/OprM efflux pump has been shown to be a common mechanism of low susceptibility to aminoglycosides in CF clinical isolates due to the alteration of the local repressor MexZ (Morita et al., 2012). More recently, it was shown that it participates in colistin resistance, although polymyxins (antimicrobial peptides) seem not to be substrates of the pump (Puja et al., 2020). Given that murepavadin is a peptidomimetic derived from antimicrobial peptide, the role of MexXY/OprM in resistance to murepavadin has been explored.

3.1 MexXY/OprM impact the susceptibility to murepavadin

MexXY has been shown to be implicated in the reduced susceptibility to colistin in PmrB mutant background (Bolard et al., 2019). In order to evaluate the impact of MexXY/OprM efflux pump on the susceptibility to murepavadin, we determined the MIC of murepavadin in several *in vitro* and clinical strains (**table 9**). As previously observed for colistin, MexXY/OprM did not change the MIC of murepavadin in the parental strain PAO1 after deletion of the operon *mexXY*, nor after complementation with pAGH97 plasmid carrying *mexXY* operon. However, in AB8.2 mutant, a PAO1 derived *pmrB* mutant (substitution V28G in PmrB) that constitutively express the *arnBCADTEF* operon responsible for the addition of 4-aminoarabinose residues to the lipid A, the deletion of *mexXY* operon resulted in a decreased of one dilution of the MIC of murepavadin (from 1 to 0.5 mg/L). And, the complementation with a plasmid encoding the *mexXY* operon (pAGH97), led to a significant increase of the MIC of murepavadin (4-fold). This effect was similar to that observed with the deletion of the *mexZ* gene encoding the local repressor of *mexXY* operon. The susceptibility of murepavadin was also evaluated in two *pmrB* mutated clinical strains (substitution G188D and Q105P in strains 3795 and 2243 respectively). Interestingly, the impact of the deletion of *mexXY* operon was more important in clinical strains (from 16 to 64-fold). Like colistin, the efflux pump MexXY/OprM, seems to participate in reducing susceptibility to murepavadin in both laboratory and clinical *pmrB* mutants. These

data suggest that resistance to murepavadin, similarly to colistin, through the efflux pump MexXY/OprM might depend on the TCS PmrAB and/or the membrane permeability subsequent to the addition of Ara4N to the LPS. In contrast, the contribution of MexXY/OprM was observed in resistance to synthetic antimicrobial peptides independently to *pmrB* mutations (1037, HHC-36, HHC-10, Bac2A) (Neidig et al., 2023).

Table 9: MIC of murepavadin tested on AB8.2 mutant and its derivatives and also in the clinical strains 3795 and 2243 and their derivatives.

Mutant	**Murepavadin (mg/L) MIC**
PAO1	≤0.03
PAO1Δ*mexXY*	≤0.03
PAO1Δ*mexXY*(pAGH97)	≤0.03
AB8.2	1
AB8.2Δ*mexXY*	0.5
AB8.2Δ*mexXY*(pAGH97)	4
AB8.2Δ*mexZ*	4
3795	2
3795Δ*mexXY*	≤0.03
2243	2
2243Δ*mexXY*	0.125

3.2 MexXY/OprM efflux pump exports a small peptide

To approach the molecules that could be exported by the efflux pump MexXY/OprM, we performed the global metabolome of the supernatant of AB8.2Δ*mexXY* mutant in comparison with AB8.2(pAGH97) mutant overproducing the MexXY/OprM efflux pump. We also exposed each mutant to murepavadin at ½ MIC, to look for differences in the metabolome in the presence of murepavadin. The preparation of the samples was carried out as part of an internship at the National Museum of Natural History (MNHN, Paris, Dr S Zirah, Unité "Molécules de

Communication et Adaptation des Micro-organismes"). Six biological replicates were performed in total for each mutant, either exposed or not to ½ murepavadin MIC and they were cultured in minimal medium M63 supplemented with glucose 20%. For each mutant in each condition, we also prepared cell-surface extracts in addition to supernatant samples. The data were statistically processed based on Partial Least Square-Discriminant Analysis (PLS-DA). In metabolomics, PLS-DA method is used to analyze metabolomic data with the goal of distinguishing between different experimental groups or conditions based on their metabolic profiles. **Figure 32** shows the discrimination of our data by setting three variables: x-variate 1 discriminates the data representing the sterile culture medium from that of bacterial cultures and explains 11% of the variability, x-variate 2 separates our data based on the mutant type (AB8.2Δ*mexXY* or AB8.2(pAGH97)) and explains 4% the variability, and x-variate 3 that separates the data based on the nature of the sample (supernatant or cell surface) and explains 20% the variability.

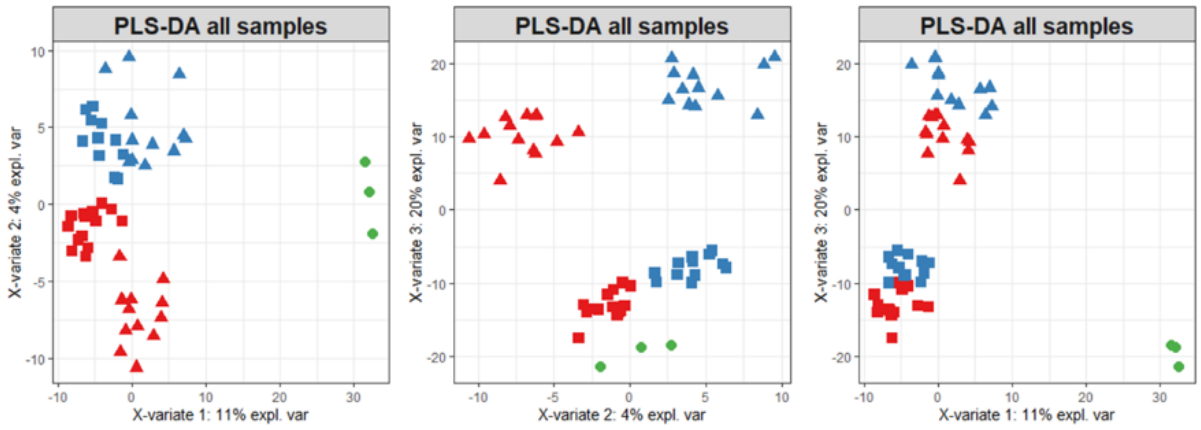

Figure 32: PLS-DA of the metabolomic data. Three variables were applied to discriminate the samples from the type of mutants and from the origin of sample (supernatant or cell-surface extracts). Variable 1 discriminates the sterile culture M63+glucose 20% (green circles) from the bacterial cultures (red and blue symbols), variable 2 discriminates samples from the mutant AB8.2Δ*mexXY* (red color), and AB8.2(pAGH97) (blue color), and variable 3 discriminates supernatant samples (triangle symbols) from cell-surface samples (square symbols).

First analysis did not reveal significant difference between murepavadin exposure and non-exposure, neither in the supernatant samples and in the cell-surface extracts. However, the comparison of secretomes of the AB8.2(pAGH97) and AB8.2Δ*mexXY* mutants revealed the presence of a small peptide of a Mw 1350.7 Da only in the 12 replicates of the mutant AB8.2(pAGH97) and lacking from the AB8.2Δ*mexXY* mutant. The identification of this unknown peptide exclusively in MexXY overproducing mutants suggest that its exported by MexXY/OprM, and it is not influenced by murepavadin presence. Interestingly, we did not

identify murepavadin in the supernatant suggesting that murepavadin is not exported by the pump. However, further analysis is needed to finish the analysis of the metabolomic data.

3.3 Relation between the efflux pump MexXY/OprM and polyamines

In order to understand whether MexXY/OprM is responsible for the export of polyamines, we measured the area under the peaks of five polyamines (1,3-diaminopropane, putrescine, cadaverine, norspermidine and spermidine) by LC-ESI-MS in the supernatant and on the cell surface of strain PAO1, mutant AB8.2, AB8.2Δ*mexXY* grown in MH. No significant differences were observed between the PAO1 strain and its *pmrB* mutant in the supernatant, and at the cell surface except for norspermidine, and spermidine. These latter were increased in the *pmrB* mutant in comparison with the parental strain (**Figure 33**, **A** and **B**). Interestingly, inactivation of the MexXY/OprM led to a significant reduction of the polyamine amount in the supernatant as well on the cell surface for the five polyamines, except for norspermidine at the cell surface indicating that MexXY/OprM plays a role in polyamines export to the supernatant.

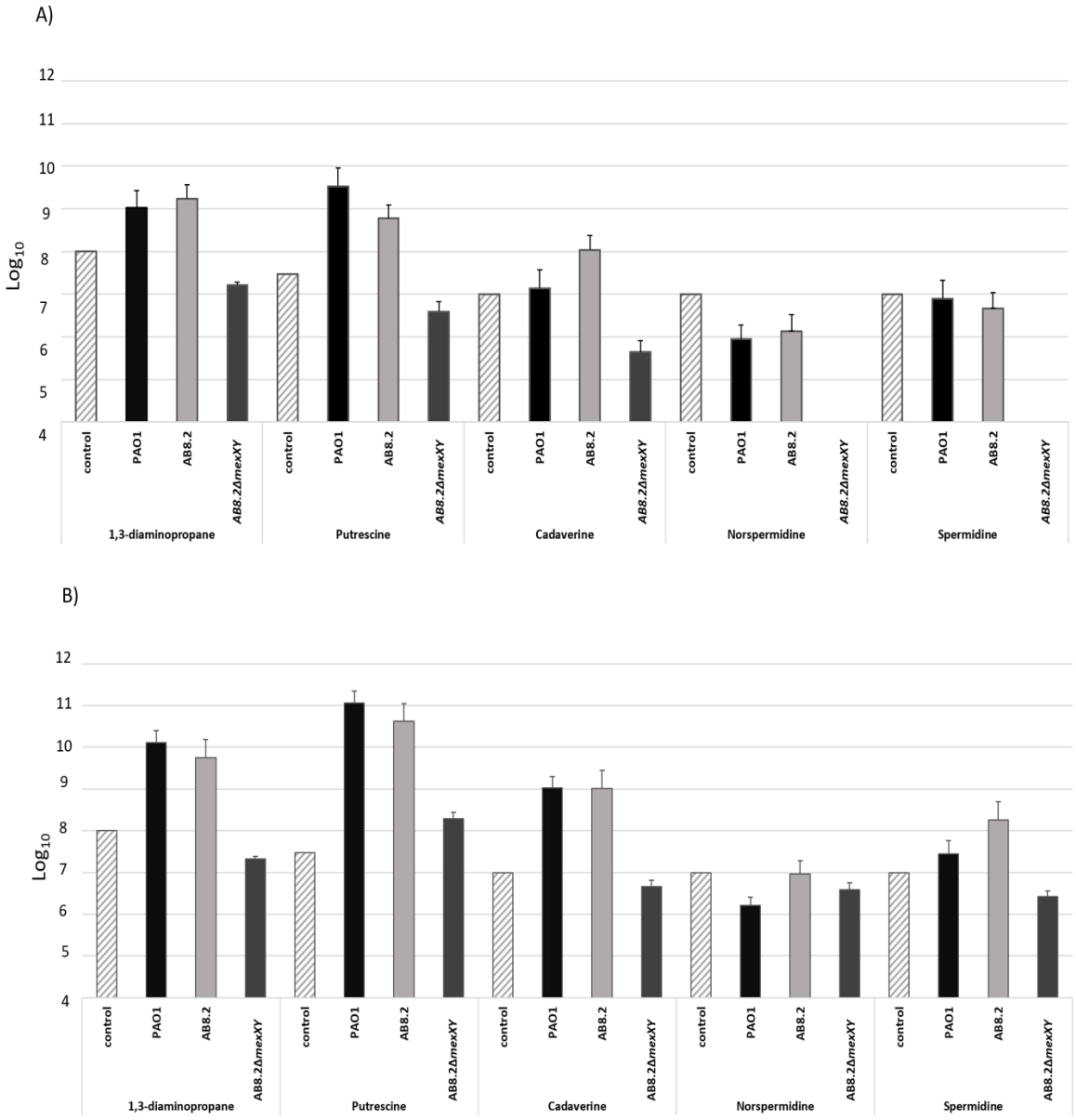

Figure 33: Amount of polyamines identified in supernatants (A) and cell surface extracts (B) of PAO1 reference strain, AB8.2 and AB8.2Δ*mexXY*. Hashed bars represent the quantities of polyamines in the control, MH.

IV. Discussion and Perspectives

Chapter 1: Alteration of LpxL1, BamA and PmrB proteins contribute to decrease the murepavadin susceptibility

The main objective of this study was to evaluate the antimicrobial activity of murepavadin on CF clinical strains isolated from patients in France. Even though murepavadin showed an excellent antimicrobial activity, high MIC values (> 4mg/L) were determined in 21 clinical strains. Genome sequencing of these strains revealed mutations in several genes involved in biosynthesis and transport of LPS (*msbA, lptD, bamA, lpxL1*, *lpxL2*), and in histidine kinases of two-component systems CbrA-CbrB, and PmrA-PmrB. The most mutated gene in our collection was *lpxL1*, which encodes an acyl transferase that incorporate secondary modifications to the lipid A. These modifications correspond to the first steps of the LPS synthesis. Evaluation of the impact of mutations using the Pholyphen-2 software, which predicted probably/possibly damage (score ≥0.784) on the function of LpxL1, suggested that the protein was inactive. Indeed, several clinical strains presented a truncated protein (e.g. W84*, E265*, Δ245-248) by a premature stop codon or by a frameshift (e.g. I172 frameshift). Thus, in the *P. aeruginosa* PAO1 strain and in clinical isolates, loss of function of the LpxL1 protein was associated with a decrease in susceptibility to murepavadin but not to colistin. However, it appears that the impact of mutations in LpxL1 differs on susceptibility to cationic peptides depending on the *P. aeruginosa* strain studied and the bacterial species. Indeed, in the reference *P. aeruginosa* strain PAK, a slight increase of 1.3-fold to colistin was observed in a mutant deleted of the *lpxL1* gene but not to polymyxin B (Hittle et al., 2015). In a different genetic background, in the PAO1 strain, this same deletion resulted in a decrease of MIC of polymyxin B (2-fold), vancomycin, erythromycin and rifampicin (Wang et al., 2016). Finally, in *Bordetella bronchiseptica* and *Haemophilus influenzae*, deletion of *htrB* gene, the homologue of *lpxL1*, led to a slight increase (2-fold) in susceptibility to cationic peptides such as polymyxin B, colistin (Pérez-Ortega et al., 2023), and significant increase to human defensin HBD-2 (45-fold) (Anaya-López et al., 2012). Because LPS partly conditions the fluidity of the outer membrane, the change in structure of LPS from a hexa-acylated to a penta-acylated form could modify the permeability of the outer membrane. The measurement of membrane permeability using the NPN fluorescence probe confirms that murepavadin does not permeabilize the outer membrane in comparison with colistin, which is a powerful membrane permeabilizer (Mohamed, 2016; Srinivas et al., 2010) (**Figure 29**). The three LpxL1 mutants tested were indeed all very slightly more permeable than the reference strain PAO1 (**Figures 29** and **30**). These results are in agreement with those previously described by Hittle et al,

indicating an increase in membrane permeability using ethidium bromide after deletion of the *htrb1* (older name of *lpxL1*) gene in the PAO1 strain (Hittle et al., 2015; Wang et al., 2016b). Therefore, although the membrane permeability was increased, the strains were less susceptible to murepavadin, suggesting an interaction of murepavadin with the lipid A or a change in the conformation of LptD in the presence of a modified lipid A, decreases the affinity of murepavadin with LptD. Thus, it would be interesting to study other forms of lipid A to evaluate the impact on the susceptibility to murepavadin, as well as modifications of LptD on the susceptibility of murepavadin by directed mutagenesis. Given that CF strains frequently have modifications at the lipid A level (acylation, hydroxylation) (Moskowitz and Ernst, 2010), a study of these modifications on the susceptibility to murepavadin is important.

We also showed that mutations localized in the BamA protein, whose role is essential for the insertion of outer membrane proteins such as LptD, modified the susceptibility to murepavadin. It is likely that the modification of BamA, decreases or modifies the targeting of LptD to the outer membrane or that BamA is also a target of murepavadin, as has been suggested by (Díez-Aguilar et al., 2021b). It would also be interesting to test other molecules in development such as darobactin A, B, B9 or thanatin, which have BamA as their main target. The identification of cross-resistance in clinical strains before these molecules are even available would improve the development of these molecules. Without much surprise, we found that the outer membrane of the mutant PAO1::*bamA* III-9-3 was more permeable than the reference strain PAO1 and even at a level significantly higher than the strain grown in the presence of colistin, highlighting the major impact of the mutation on BamA (**Figure 29**). However, the mutant did not show any change in its permeability in the presence of murepavadin. This result was surprising and further investigations seem necessary to ensure the validity of our results. In the first place, we want to reproduce the experiments performed. These results having been obtained late in the PhD work, we did not have time to reproduce the experiments of NPN. In addition, it would be interesting to use another fluorescent molecule, such as the fluorescent nucleic acid stain SYTOX to measure the permeability of the outer membrane to confirm these results. SYTOX green stain labels nucleic acids and gives as a result an intense green fluorescence (Roth et al., 1997).

We confirmed that PmrB mutations in CF clinical strains decrease susceptibility to murepavadin (Romano et al., 2019). This is important as *pmrB* mutants are frequent among CF strains, and in addition to the decrease in susceptibility to murepavadin, they are resistant to colistin and aminoglycosides, antibiotics used in aerosol therapy in CF patients. However, the mechanism responsible for the decrease in susceptibility to murepavadin of CF clinical strains

dependent on the two-component PmrA-PmrB system remains to be elucidated. The response of the reference strain PA14 to murepavadine (transcriptomic data) should allow us to advance on this question.

Finally, in 4 CF clinical strains that had a high-level of resistance to murepavadin (MIC≥128 mg/L), we did not identify mutations in the selected genes, suggesting that other genetic determinants are involved in the decrease in susceptibility to murepavadin. If some CF clinical strains had many mutations in their genome compared to the strain with an MIC of less than 4 mg/L, others had a low number of SNPs (7 SNPs) allowing the search for genes potentially involved in the decrease in susceptibility to murepavadin. A similar strategy to that used for the genes *bamA, lpxL1* and *pmrB* will be used for the candidate genes. Thus, among the candidate genes are other genes involved in LPS synthesis such as *msbA, lpxL2* whose role in murepavadin resistance needs to be elucidated.

In total, the decrease in the susceptibility to murepavadin in CF clinical strains is complex and involves several mechanisms of resistance, the majority of which are linked to the synthesis of LPS. The accumulation of mutations in these genetic determinants leads to high levels of resistance to murepavadin (MIC>128 mg/L). Finally, a side that has not been mentioned and which is no less interesting is the response of the immune system and the fitness of these strains. Indeed, the alteration of LPS could be associated with a decrease in fitness and a decrease in the immune response. In both *P. aeruginosa* and *Acinetobacter baumannii*, reduced acylation has been shown to reduce the immune potency of the LPS and thus helping the strain to escape the immune system (Moskowitz and Ernst, 2010; Nijland and Hofland, 2014). Therefore, it would be important to analyse the impact of LPS modification on the immune system response.

Chapter 2: Response of *Pseudomonas aeruginosa* to murepavadin

Given that the alteration of the PmrA or PmrB proteins belonging to the TCS PmrAB complex, confers cross-resistance to polymyxins, aminoglycosides, and murepavadin, it is interesting to better understand the response of *P. aeruginosa* to these molecules including murepavadin. It is likely that part of this response is common for these different antibiotics. Thus, among the most highly overexpressed genes were the genes *speD2, speE2*, and PA4775 when the reference strain PA14 was cultured in the presence of a sub-inhibitory concentration of murepavadin. These were also overexpressed in the presence of polymyxin B or colistin, as well as antimicrobial peptides such as T7 and G3KL dendrimers (Ben Jeddou et al., 2020; Johnson et

al., 2012). The genes *speD2* and *speE2* encode enzymes which are implicated in accessory polyamines biosynthesis, while the gene PA4775 is of unknown function. In *P. aeruginosa*, there are homologous of the genes *speD2* (24.2 % sequence identity with PA0654) and *speE2* (38.3 % sequence identity with PA1687) involved in the synthesis of polyamines such as putrescine, cadaverine, or spermidine. However, these latter were not overexpressed in the PA14 strain. SpeD2 is a probable S-adenosylmethionine (SAM) decarboxylase, while SpeE2 is a probable aminopropyl transferase that extracts the aminopropyl group from dSAM and incorporates it to existing polyamines. These two enzymes can accept several substrates and synthesize various polyamines. Previously, we showed in the laboratory that these three genes were also highly overexpressed in a *pmrB* mutant, resistant to colistin and aminoglycosides. While the deletion of this gene locus abolished resistance to aminoglycosides, it did not lead to any change in the susceptibility to colistin, suggesting that the resistance mechanisms were different for these two families of antibiotics. Moreover, we found that the amount of norspermidine present on the membrane surface of the *pmrB* mutant was significantly higher than that of mutants whose *speD2*, *speE2*, and PA4775 genes were inactive, suggesting that norspermidine synthesis was associated with resistance to aminoglycosides (Bolard et al., 2019).

Interestingly, we observed that the deletion of this locus in a *pmrB* mutant having MIC to murepavadin at 1mg/L decreased the MIC by 16-fold (MIC= 0.06 mg/L), suggesting that the synthesis of a polyamine could contribute to the resistance of this molecule as well as for aminoglycosides. Polyamines being positively charged molecules; they could decrease the interaction of murepavadin with the outer membrane and protect LptD protein. Other work done in *Neisseria gonorrhoeae* and *Haemophilus influenzae* has shown that spermine or putrescine contributed to increasing resistance to polymyxin B and LL-37 (Goytia and Shafer, 2010) (Banerji et al., 2021). However, mass spectrometry data in the presence of murepavadin did not confirm these results. Indeed, the data showed only a slight increase in the amount of norspermidine and 1-3 diaminopropane on the surface of the bacteria. However, our work has limitations. First, the reference strain used for the transcriptomic analysis (PA14) and mass spectrometry (PAO1) was different. During the PhD work, the publication of Gutu et al. highlighted that the genes PA1559-PA1560 corresponded to only one gene called *cprA* which was involved in polymyxin resistance and is non-functional in strain PAO1, which is why we performed the transcriptome with the reference strain PA14 (Gutu et al., 2015). It would be interesting to reproduce the mass spectrometry results with strain PA14 and use a culture

medium with minimal amounts of polyamines, such as a M63 medium supplemented with glucose, as that used for the metabolomics analysis. The MH medium containing a large amount of polyamines, variations could be masked by bacterial metabolism.

Among bacteria, other species such as *Thermus thermophilus*, an extreme thermophile bacterium, is capable of synthesizing more than 15 polyamines including unusual polyamines (Oshima, 2010). *T. thermophilus* exhibits SpeD2 and SpeE enzymes that are close homologues to those of *P. aeruginosa* (56% and 61% aminoacid sequence identities with PA4773 and PA4774 respectively). SpeE and SpeD2 of *T. thermophilus* are capable of extending further putrescine, spermidine, thermine by adding aminopropyl groups (Oshima, 2010). Based on this observation, we propose the model presented in **Figure 34** showing the possible polyamines that could be synthesized by SpeE2 and SpeD2. The model is based on the uptake of excess polyamines present in MH broth as substrates for the production of other polyamines, and maintaining polyamines on the cell surface polyamines to guarantee protection against murepavadin. Another hypothesis that could explain the decrease in polyamines in the supernatant is their uptake by cells and their catabolism into other products that could participate to resistance.

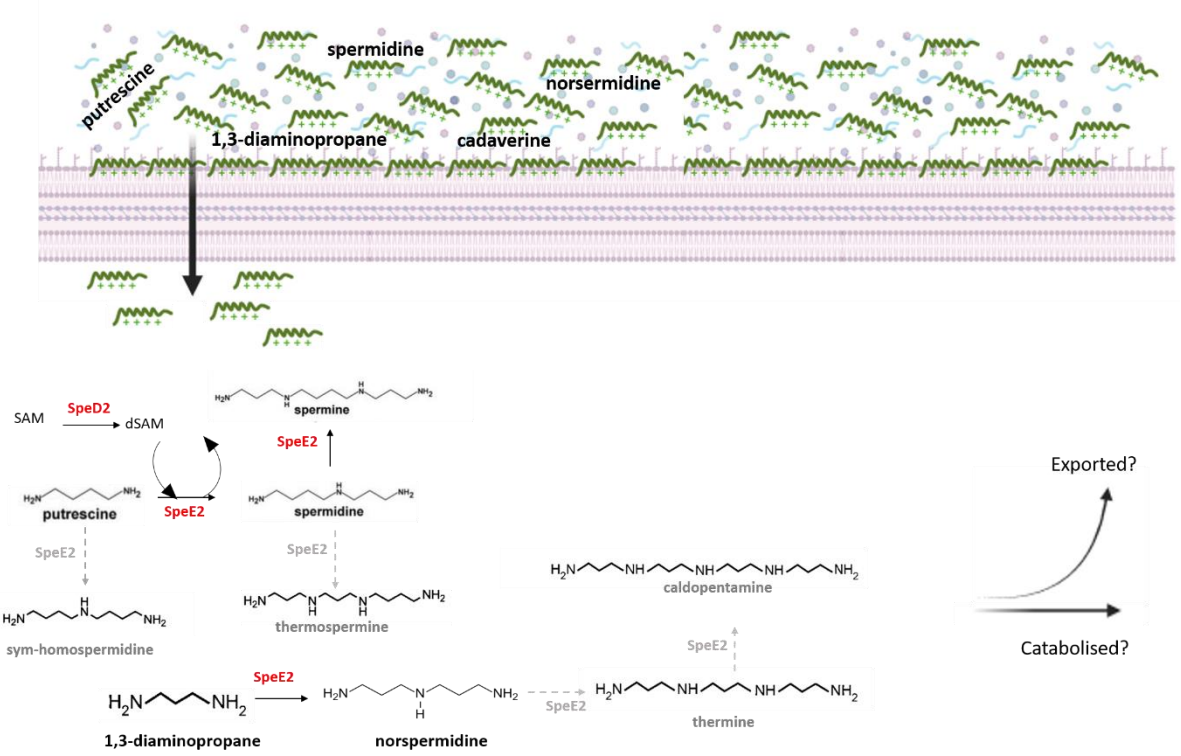

Figure 34: Proposed model for the biosynthesis of unusual polyamines (sym-homospermidine, thermospermine, thermine and caldopentamne) from existing polyamines. *P. aeruginosa* could import standard polyamines present in the extracellular space (putresceine, spermidine, cadaverine, norspermidine and 1,3 diaminopropane), while keeping th cell surace polyamines intact. The imported polyamines are used as substrated for the synthesis of oher longer polyamines. Once synthesized, we don't know if they are further catabolised or exported again to the extracellular space; adapted from (Bolard et al., 2019; Oshima, 2010).

In this model, only the commonly known polyamines are used as starting points for the synthesis of other polyamines. Further addition of aminopropyl group to putrescine, spermidine and norspermidine, results in sym-homospermidine, thermospermine, the isomer of spermine, and thermine respectively. Further extension of thermine can also results in caldopentamine (Oshima, 2007). These hypotheses need to be validated by studying *in vitro* enzymatic reactions of SpeE2 and SpeD2. This is achieved by expressing the enzymes in *E. coli*, performing protein purification, running *in vitro* liquid cultures in the presence of different polyamine substrates, and analysing the polyamines produced by mass spectrometry. As a next step, quantification of those new polyamines (sym-homospermidine, thermospermine, thermine, caldopentamine) again on the cell-surface and in the supernatant, but also in the intracellular space in PAO1 exposed or not to ¼ MIC of murepavadin would be interesting. This will reconfirm our previous quantification and also give us information about the localisation of those polyamines, whether they are exported to provide a protection against murepavadin extracellularly or catabolised. Comparison of the amount of sym-homospermidine, thermospermine, thermine,

caldopentamine polyamines in samples coming from mutants PAO1Δ4773 and PAO1Δ4774 with that of PAO1 will also provide elements helping to confirm the ability of PA4773 (SpeD2) and PA4774 (SpeE2) to synthesize those polyamines. In addition to MH, it would be interesting to carry out exactly the same quantifications in minimal medium M63+ glucose 20% lacking polyamines. This should allow us to compare the response of the strains, and to determine whether they still produce polyamines or not. One way to also confirm the protective role of polyamine to murepavadin, is by realizing MIC of murepavadin in the presence of exogenous polyamines, or by analysing the bactericidal impact of murepavadin in the presence of exogenous polyamines. Collectively, by performing this set of experiments we will have a clearer answer about the response to PAO1 to murepavadin and about the implication of polyamines in resistance to murepavadin. Doing the same set of experiment in PA14 is also important, inorder to link our quantification results with transcriptomic results.

In addition to polyamine biosynthesis, the PA14 strain exposed to murepavadin has an upregulation of the *arn* operon, which contributes to decrease the polymyxin susceptibility through the addition of L-Ara4N residues on the lipid A. This is particularly interesting as it suggests that murepavadine may antagonize colistin activity. In order to verify this hypothesis, we will be able to determine the sensitivity of colistin in the presence of murepavadine. PhoPQ, ParRS and CprRS seemed to be activated based on transcriptomic results, but not reconfirmed with RT-qPCR. If those TCSs respond to murepavadin exposure, the use of murepavadin should be reconsidered due to the common response with aminoglycosides and polymyxins leading to cross-resistance between the mentioned antibiotics.

Chapter 3: MexXY/OprM efflux pump confers resistance to murepavadin in *pmrB* mutants

The last part of this work was to better understand the role of the MexXY/OprM active efflux system. This work, which began during my PhD, is now the PhD subject of Xavier Vuillemin, who is in his second year. Since murepavadin was able to activate the TCS ParR-ParS (previous chapter) and since this positively regulates the MexXY/OprM active efflux system, we evaluated its role in murepavadin susceptibility. Thus, as with colistin, MexXY/OprM contributes to acquired resistance to murepavadin in a *pmrB* mutant derived from the reference (PAO1) and in clinical isolates with a mutated *pmrB* gene (strains 2243 and 3795) (Puja et al., 2020). However, how the MexXY/OprM efflux system participates in resistance to murepavadine and colistin remains to be elucidated. To evaluate whether the system was able

to export molecules capable of modifying the surface of *P. aeruginosa*, we performed a non-targeted metabolomic approach with *pmrB* mutants in which the MexXY/OprM system was inactivated (AB8.2Δ*mexXY*) or overexpressed in an expression vector AB8.2(pAGH97). We did not identify any significant differences between the two mutants, with the exception of a 1350.7 Da peptide present only in the supernatant of the AB8.2(pAGH97) mutant and absent from the mutant in which the system was absent. The characterization of this peptide by mass spectrometry MS/MS is ongoing in collaboration with the team of Dr. S. Zirah (Molecules of Communication and Adaptation of Microorganisms, UMR7245, National Museum of Natural History).

One possibility is that MexXY could export either a positively charged molecule that could attach to the negatively charged outer membrane, or a negatively charged molecule that could sequester the positively charge polymyxins and murepavadin. Measurement of Zeta potential (difference in the electric potential between the aqueous layer and the fluid layer surrounding the bacterial surface) for the mutant AB8.2Δ*mexXY* and AB8.2(pAGH97) cultivated in the presence of colistin or murepavadin will help in knowing the charge of the exported molecule.

We hypothesized the export of polyamines by MexXY/OprM, so we quantified cadaverine, putrescine, spermidine, spermine, norspermidine and 1,3-diaminopropane in the supernatant and cell surface of AB8.2Δ*mexXY* and AB8.2(pAGH97). The decrease of both supernatant and cell surface polyamines upon MexXY inhibition might indicate the possibility of MexXY to export them. In the same mutant AB8.2Δ*mexXY*, transcriptomic data revealed overexpression of polyamines catabolism genes (data not shown). Indeed, the concentration of polyamines is strictly balanced, and accumulation might be toxic rather beneficial. The overexpression of catabolism genes could be a result of accumulation of polyamines in intracellular space. This observation reinforces our hypothesis about MexXY/OprM export to polyamines. This preliminary work also supports the idea of the implication of polyamines in murepavadin resistance. Indeed, MIC of murepavadin in PAO1 *pmrB* mutant AB16.2 and AB16.2Δ4773, AB16.2Δ4774, AB16.2Δ4774 (mutants inactivated in genes implicated in polyamines synthesis), was reduced 16-fold (data not shown). In both Gram negative and Gram positive bacteria, certain efflux pumps that belong to major facilitator superfamily (MFS) of transporters, small multi-drug resistance (SMR) and RND efflux pumps (Teelucksingh et al., 2020) are implicated in polyamines transport precisely spermidine and also confer resistance to aminoglycosides, as MexXY/OprM. The SMR excretion complex MdtJI in *E. coli*, the MFS Blt in *Bacillus subtilis* and BpeAB-OprB in *Burkholderia pseudomallei* have been shown to

export spermidine (Higashi et al., 2008; Teelucksingh et al., 2020; Woolridge, 1997). In addition to that, the putative proteobacterial antimicrobial compound efflux (PACE) efflux pumps AceI of *Acinetobacter baumannii* and its homologue in *Pseudomonas protegens* suggested the efflux of cadaverine polyamines. This was concluded after obtaining a significant overexpression (at least 20-fold) of the efflux pump AceI of *Acinetobacter baumannii*, in addition to reduced accumulation of intracellular cadaverine in comparison with the mutant deprived of AceI (Hassan et al., 2019). Moreover, the expression of the homologue of AceI of *Pseudomonas protegens* in *E. coli* also reduced the accumulation of intracellular cadaverine. None is published about RND efflux pumps so far. Therefore, it will be interesting to further identify the impact of polyamines on murepavadin resistance by (i) identifying the molecules exported by the MexXY/OprM, and (ii) analysing transcriptomic data of mutants AB8.2 and AB8.2Δ*mexXY* grown in presence of murepavadin or colistin.

V. Materials and Methods

1. Microbiology

1.1 Bacterial strains and plasmids

Table 10: List of bacterial strains.

Strain	Characteristics	Reference
Escherichia coli		
CC118 *λpir*	Strain CC118 lysogenized with phage *λpir*	(Herrero et al., 1990)
HB101	*subE44 hsdS20(rB- mB-) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	(Lacks and Greenberg, 1977)
Pseudomonas aeruginosa		
PAO1	Wild type reference strains	(Stover et al., 2000)
PA14	Wild type reference strains	(Liberati et al., 2006)
AB8.2	PAO1 spontaneous colistin-resistant mutant with V28G substitution in *pmrB* gene	(Bolard et al., 2019)
AB8.2(pAGH97)	Mutant AB8.2 with pAGH97 plasmid overexpressing *mexXY* operon, Tic^r	This study
AB8.2Δ*mexXY*	Mutant AB8.2 with deletion of *mexXY* operon	(Puja et al., 2020)
AB8.2Δ*mexXY*(pAGH97)	Mutant AB8.2Δ*mexXY* with pAGH97 plasmid overexpressing *mexXY* operon, Tic^r	(Puja et al., 2020)
AB8.2Δ*mexZ*	PAO1 with in-frame deletion of 627-bp in gene *mexZ*	(Bolard et al., 2019)

PAO1Δ*mexXY*	PAO1 with in-frame deletion of 4,185-bp in operon *mexXY*, Tic^r	(Guénard et al., 2014)
PAO1Δ*mexXY*(pAGH97)	PAO1Δ*mexXY* complemented with plasmid pAGH97, Tic^r	(Guénard et al., 2014)
2243	Clinical strain, CS^r, PmrB (Q105P, Y345H)	(Bolard et al., 2019)
3795	Clinical strain, CS^r, PmrB (G188D, Y345H)	(Bolard et al., 2019)
3890	Clinical strain, CS^r, PmrB (S2P, A4T, D45E, Y345H)	(Bolard et al., 2019)
2243Δ*mexXY*	2243 clinical strain with *mexXY* operon deletion	(Bolard et al., 2019)
3795Δ*mexXY*	3795 clinical strain with *mexXY* operon deletion	(Bolard et al., 2019)
III-9-1	CF clinical strain with T76P substitution mutation in LpxL1, murepavadin MIC= 4 mg/L	This study
III-9-3	Clinical strain with T76P substitution mutation in LpxL1 and D535E substitution mutation in BamA, murepavadin MIC= 64 mg/L	This study
IX-5-2	Clinical strain with H120N substitution mutation in LpxL1, murepavadin MIC ≥128 mg/L	This study
VII-1-1	Clinical strain with premature stop codon E265* in LpxL1, murepavadin, MIC= 64 mg/L	This study
PAO1::$lpxL1_{III\text{-}9\text{-}1}$	PAO1 with allelic replacement of *lpxL1* gene by that of strain III-9-1 (T76P)	This study
PAO1::$lpxL1_{IX\text{-}5\text{-}2}$	PAO1 with allelic replacement of *lpxL1* gene by that of strain IX-5-2 (H120N)	This study
PAO1::$lpxL1_{VII\text{-}1\text{-}1}$	PAO1 with allelic replacement of the *lpxL1* gene by that of strain VII-1-1 (E265*)	This study

PAO1::*bamA*$_{\text{III-9-3}}$	PAO1 with allelic replacement of the b*amA* gene by that of strain III-9-3 (D535E)	This study
PAO1::l*pxl1*$_{\text{III-9-1}}$*bamA*$_{\text{III-9-3}}$	PAO1::*lpxL1*$_{\text{III-9-1}}$ with allelic replacement of the b*amA* gene by that of strain III-9-3 (D535E)	This study
PAO1Δ*pmrAB*	PAO1 with in-frame deletion of operon *pmrAB*	(Bolard et al., 2019)
PAO1Δ*pmrAB*(pME6012)	PAO1Δ*pmrAB* with broad host-range expression plasmid, Tet^{r}	(Bolard et al., 2019)
PAO1Δ*pmrAB*(pB2243)	PAO1Δ*pmrAB* complemented with pME6012 carrying genes *pmrAB* from clinical strain 2243, Tet^{r}	(Bolard et al., 2019)
PAO1Δ*pmrAB*(pB3795)	PAO1Δ*pmrAB* complemented with pME6012 carrying genes *pmrAB* from clinical strain 3795, Tet^{r}	(Bolard et al., 2019)
PAO1Δ*pmrAB*(pME3890)	PAO1Δ*pmrAB* complemented with pME6012 carrying genes *pmrAB* from clinical strain 3890, Tet^{r}	(Bolard et al., 2019)

Kan^{r}: kanamycin resistant, Tet^{r}: tetracycline resistant, Tic^{r}: ticarcilline resistance

Table 11: List of plasmids used.

Plasmid	Characteristics	Reference
pKNG101	Suicide vector in *P. aeruginosa; sacB*^{r} *mobRK2 oriR6K*; Str^r	(Kaniga et al., 1991)
pKNG101-III-9-1	pKNG101 with gene *lpxL1* of clinical strain III-9-1, Str^r	This study
pKNG101-IX-5-2	pKNG101 with gene *lpxL1* of clinical strain IX-5-2, Str^r	This study
pKNG101- VII-1-1	pKNG101 with gene *lpxL1* of clinical strain VII-1-1, Str^r	This study
pKNG101- III-9-3	pKNG101 with gene *bamA* of clinical strain III-9-3, Str^r	This study
pAGH97	pAK1900-derived *mexXY* expression vector, Tic^r	(Aires et al., 1999)
pRK2013	Helper plasmid, *mob1*, *tra1*, *colE1*, Kan^r	(Ditta et al., 1980)
pME6012	Broad host-range expression plasmid; Tet^r	(Heeb et al., 2000)
pAB2243	pME6012 carrying genes *pmrAB* from clinical strain 2243	(Bolard et al., 2019)
pAB3795	pME6012 carrying genes *pmrAB* from clinical strain 3795	(Bolard et al., 2019)
pAB3890	pME6012 carrying genes *pmrAB* from clinical strain 3890	(Bolard et al., 2019)

1.2 Culture media

Table 12: List of culture media used.

Culture medium	Composition	Reference
Mueller-Hinton agar (MHA)	0.2% beef extract, 1.75% acid hydrolysate of casein, 0.15% starch, 1.7% agar	Becton, Dickinson and Company
Mueller-Hinton broth cation-adjusted (cMHB)	0.3% beef extract, 1.75% acid hydrolysate of casein, 0.15% starch with adjusted concentrations of the divalent cations Ca2+ (20 to 25 µg/ml) and Mg2+ (10 to 12.5 µg/ml)	Becton, Dickinson and Company
LB broth	1% tryptone, 0.5% yeast extract, 1% NaCl	Becton, Dickinson and Company
Pseudomonas isolation agar PIA	2% bacto peptone, 14.70 mM MgCl2, 57.39 mM K2SO4, 25 µg/ml Irgasan, 1.36% agar, 2% glycerol	Becton, Dickinson and Company
M63 + 20% Glucose	10 g (NH4)2 SO4, 68 g KH2 PO4, 2.5 mg FeSO4·7H2 O in 800 mL H 2O, adjust pH to 7.0 with KOH, add H2O to 1 L	Current protocols in molecular biology, volume 1
M9 minimal broth medium with 5 % sucrose	8.54 mM NaCl, 25.18 mM NaH2PO4, 18.68 mM NH4Cl, 22 mM KH2PO4, 2 mM MgSO4, 5% sucrose, 0.8% agar	(Kaniga et al., 1991)

1.3 Determination of antibiotic susceptibility, Minimum inhibitory concentration (MIC) method

Minimal inhibitory concentration (MIC) of selected antibiotics including colistin and murepavadin was determined by the standard serial 2-fold dilution method in calibrated Mueller-hinton broth (cMHB) with inocula of final concentration 5 x 10^5 CFU/ml, following the recommendations of European Committee on Antimicrobial Susceptibility Testing

(EUCAST 2021). Growth was visually assessed after 18 h of incubation at 37°C. Strains were categorised as « susceptible » or « resistant » to the different antibiotics according to EUCAST breakpoints.

1.4 Membrane permeability measurement

Bacterial membrane permeability was measured by measuring the fluorescence of the nonpolar probe 1-N-phenylnapthylamine (NPN) which fluoresce strongly in hydrophobic environment and weekly in acqueous environment (Loh et al., 1984). Starting from 5ml overnight culture cultivated at 37°C, 250 rpm, a fresh culture was prepared the next day in 50 ml total volume by diluting 50 times in cMHB and incubated until OD reaches OD_{600}=0.5 (250 rpm, 37°C). 1ml from the culture is captured and cetrifuged at 7000 rpm for 30 seconds. The supernatent was discarded and the pellet was resuspended in 1 mL Hepes Sodium buffer (5mM, pH= 7.2). 200µl of the suspension was diposited in a well of black 96-well plate that permits the reading of fluorescence. The fluorescence was measured by Tecan robot every 500 ms during 90 s, after the injection of 10 µM of NPN by the integrated injector. Murepavadin and colistin were injected at a final concentration = 16x MIC (excitation at 350 nm and emission at 420 nm) and the fluorescence was measured again. Two biologycal replicates were performed, and the results were processed by substracting the background noise (fluorescence of Hepes buffer) from each sample.

2. Molecular biology

2.1 Primers

Table 13: List of primers used for allelic replacement.

Name	Sequence (5’ > 3’)	Reference
Allelic replacement		
lpxL1		
Lpxl1-for3-NEB	CCCCCCCCTGCAGGTCGACGGAGCGAGGAGTGTTTCGTG	This study
Lpxl1-rev3-NEB	TTCTACTTATGGTACCCGGGTGAAAAAGGCGTCTTCCG	This study
bamA		
bamA-fwd-neb	CCCCCCCCTGCAGGTCGACGATGAAACGCTTTCTGCTACC	This study
bamA-rev-neb	TTCTACTTATGGTACCCGGGTCAGAAGGTCTGGCCCAG	This study
Sequencing		
bamA		
BamA-fwd	GCCCTTTGCGGCAGATTCTCT	This study
BamA-R1	AGGTCGCCGGAGAGCTTTT	This study
BamA- F2	TGGGCAACACCGTCTTCTCC	This study
BamA-R2	TGTTCTGGCTGATCGAGCCG	This study
BamA-F3	AGAGCAACCGTCCGGCTCGA	This study
BamA-R3	CCGTCTGGATTACGAGCCAC	This study
BamA F4	TCAAGGACAGCACGCTGGG	This study
BamA-R4	TGATGGCTGCGGTGATCAGG	This study

lpxL1		
Lpxl1-for3-NEB	CCCCCCCCTGCAGGTCGACGGAGCGAGGAGTGTTTCGTG	This study
Lpxl1-rev3-NEB	TTCTACTTATGGTACCCGGGTGAAAAAGGCGTCTTCCG	This study

Table 14: Primers used for RT-qPCR.

Name	**Sequence (5' > 3')**	**Reference**
RT-qPRC		
rpsl		
RT_rpsl_Fw	GCAACTATCAACCAGCTGGTG	(Dumas et al., 2006)
RT_rpsl_Rv	GCTGTGCTCTTGCAGGTTGTG	(Dumas et al., 2006)
pmrA		
pmrA RTAB1	GTTCGACCTGCTGGTGCT	This study
pmrA RTAB2	TCGAGATCGAAGGGCTTG	This study
pmrB		
pmrB1	CCTCTCGCTGAAGCAGGTGA	(Ben Jeddou et al., 2020)
pmrB2	CTGGTCTTCGGTGGCAAGGT	(Ben Jeddou et al., 2020)
speD2 -63110		
SpeD2 RT1	CAAGCAACTGGTCATCGAGC	This study
SpeD2 RT2	ATCGGGCTGAACTTGTGGAA	This study
speE2 63120		
SpeE RT- fwd	CGATCGAGGACAGCGACCC	This study
SpeE RT- rev	GCACGCGGTCGTAGTTGT	This study
cprR		
CprR RT- fwd	CACCGTCATCGACTACCTCGA	This study

CprR RT- rev	TCACGTCGTAGCGGTTGGCG	This study
cprS		
cprS RT1	CGAAGAGGAGGATGACGACG	(Puja et al., 2020)
cprS RT2	ACGATGAACCAGACCAGCAG	(Puja et al., 2020)
phoQ		
PhoQ RT1	GAACACGAAGAACTCCTCGC	This study
PhoQ RT2	CCAAGGTCCTCGGCTATATCT	This study
phoP		
phoP RT-Fwd	TAGTGGAAGACGAGGCGCTGT	This study
phoP RT- rev	ATTCGCTGACCCGGTAGAGG	This study
mexX		
MexX RT-Fwd	CACATCCAATGGACCGGCTCG	This study
MexX RT- rev	GGCGGTACGCTTCCAGGCG	This study
mexY		
RT_mexY_AB1	ATCAGGAAGGTGGTCAGCAC	(Bolard et al., 2019)
RT_mexY_AB2	CGAACATGGTCAGCACGTTG	(Bolard et al., 2019)
arnB		
arnB RT1	AAGTGATCACGCCGTCGCT	This study
arnB RT2	AGTGCACCGGCACGATGG	This study
speC		
speC RT1	CTCGGTGATTTCGGTAGCGGG	This study
speC RT2	TCGCCGACAAGCAGGAAACC	This study
PA1797		
PA1797 RT1	AGCCAGACACCGGATACTTT	This study
PA1797 RT2	CTTGCGCGAGTTCTACGTAC	This study
PA4775		

PA4775 RTAB1	ACGAGCTGCTGGTGATCTTC	(Bolard et al., 2019)
PA4775 RTAB2	CAGGTGACCTTCGTGCCAG	(Bolard et al., 2019)
oprM		
OprM-RT1	AGTTGCAGCTGACCAAGGAC	(Puja et al., 2020)
OprM-RT2	GCATTCTGGTCCTGGGCTAC	(Puja et al., 2020)
PA4114		
PA4114 RT-fwd	CCATCACCCGCGACGACTG	This study
PA4114 RT-rev	GGCGAAGCCGACCATGCG	This study

2.2 Nucleic acids purification

2.2.1 Genomic DNA extraction

Bacterial genomic DNA was extracted using Nucleospin Microbial DNA mini kit (Macherey-Nagel, Hoerdt) following the manufacturer's recommendations. For the purpose of whole genome sequencing, Genomic DNA was extracted with the PureLink Genomic DNA mini kit (ThermoFisher Scientific).

2.2.2 Plasmid DNA extraction

Plasmid DNA was extracted and purified using NucleoSpin plasmid kit (Macherey-Nagel, Hoerdt) following the manufacturer's instructions.

2.3 Polymerase chain reaction (PCR)

Genes were amplified in thermocycler Veriti (thermofischer scientific, USA), using either Mytaq Red DNA Polymerase (Bioline, London, UK) or iProof High Fidelity Master Mix (Bio-Rad). With Mytaq Red DNA polymerase, 50µl final reaction volume was prepared by mixing the following components: 10µl of 5X Mytaq Red reaction buffer, 2µl of each primer at 10µM, Mytaq Red DNA polymerase 0.25µl, 100 ng of template DNA and 50µl qsp water. With iProof High Fidelity Master Mix, 50µl final was prepared from the following components: 25µl iProof High Fidelity Master Mix, 2.5µl from each primer at 10µM, 1.5µl of 3% dimethyl sulfoxide

(DMSO), and 50-500ng DNA, water at qsp 50µl. The thermocycler was programmed according to the following conditions : 1 cycle of initial denaturation step at 98°C for 3 minutes, 35 cycles of denaturation step at 95°C for 15seconds, 15 seconds of annealing and 30seconds/kb of extention at 72°C. Finally a final extention cycle of 7 minutes at 72°C.

2.4 Gel electrophoresis

DNA fragments resulted from restriction digestion or PCR amplification, were analysed based on their size by migrating them on agarose gel. The gels were prepared in TAE 1X buffer (Tris-acetate 40 mM, EDTA 1 mM, pH = 8.0) with 0.5µg/ml ethidium bromide (BET) added to the buffer. The percentage of agarose used was either 0.8% or 1.3% depending on the fragment size. Migration was achieved during 25 minutes at 110v. The bands were visualized under UV light UV ChemiDoc XRS system (BioRad).

2.5 Purification of PCR product

PCR fragments were purified either directly from the agarose gel or from the PCR product using kit NucleoSpin Gel and PCR clean up (Qiagen) following the manufacturer's recommendations.

2.6 DNA cloning

2.6.1 Digestion by restriction enzymes

Plasmid DNA were digested with specific and suitable restriction enzymes following manufacturer's recommendation. A total volume of reaction mixture of 25µl was prepared by mixing 500 ng plasmid DNA, 2.5µl of cutsmart buffer 10X (New England Biolabs, France) and 1µl HIFI restriction enzyme (New England Biolabs, France). The reaction mix was incubated at 37°C for 1 hour.

2.6.2 NEB cloning

Amplified and purified PCR product of the genes of interest were mixed with plasmid DNA after being linearized with the suitable restriction enzyme. A total volume of 20µl recombinant plasmid DNA resulted after using the NEBuilder HIFI DNA Assembly Cloning Kit (New

England Biolabs, France) by mixing 10µl NEBuilder HIFI DNA assembly master mix (New England Biolabs, France) with the linearized plasmid and DNA fragment at a ratio 1 :2 of insert:vector. The mix was incubated at 50°C for 30 minutes.

2.7 Bacterial transformation

2.7.1 By thermic shock

Chemically competent cells *E. coli* CC118*λpir* previously rendered competent upon treatment with rubidium chloride were used (Hanahan, 1983). 50µl of competent cells were mixed with 2µl of ligated plasmid and incubated on ice for 30 min. The bacteria were then subjected to a thermic shock at 42°C for 30 seconds and then incubated again on ice for 2 minutes, then cultivated at 37°C for 1 hour under constant agitation (225rpm) after being diluted with 950ml room temperature cMHB. *E. coli* CC118*λpir* transformants were selected on MH agar supplemented with streptomycine 50µg/ml in case of pKNG101.

2.7.2 By electroporation

Pseudomonas aeruginosa strains were rendered electrocompetent after being treated with 300mM sucrose following the protocol described by Choi et al. (Choi et al., 2006). 100µl of electrocompetent cells were mixed with 50-500 ng plasmid DNA and transferred to a 2mm electroporation cuvette. A pulse is applied : 25 AF; 200 V; 2.5 kV using the electroporator MicroPulser (Bio-Rad). After that, 1 ml of warm cMHB was added over the cells and they were cultivated for 1 hour at 37°C under constant agitation (250rpm). 100µl were finally spread on MH agar plate with the corresponding selection antibiotic.

2.7.3 Bacterial conjugation

Tri-partite conjugation was performed in the presence of the recepient *Pseudomonas aeruginosa* strain, the helper *E. coli* HB101 strain containing plasmid pRK2013, and the donor strain *E. coli* CC118*λpir* containing the recombinant pKNG101 plasmid. 40µl of overnight cultures of each of the donor and helper strains were placed as spots superposing each other on the surface of MHA and incubated for 2 hours at 37°C. In the meantime, the recipient strain was incubated at 42°C without agitation. After that, another 40µl of the recepient strain is

spotted over the previous bacterial spots. After 4 hours of incubation at 37°C, the bacterial spots are collected in 1ml cMHB and cultured for 1 hour at 37°C under constant agitation. Transconjugants were selected on PAI agar plates supplemented with streptomycin 2000µg/ml.

2.8 Gene mutation by allelic replacement

Allelic replacement of *lpxL1* or *bamA* genes of PAO1 by mutated *lpxL1* or *bamA* genes was achieved by homologous recombination events. After cloning the gene of interest in pKNG101 vector, the vector was transformed in *E. coli cc118λpir.* Transformants were selected on MH agar plates supplemented with streptomycin 50µg/ml. After selecting the good transformant by verifying the good plasmid, the vector was then transferred to *Pseudomonas aeruginosa* by tri-partite conjugation in the presence of the helper strain *E. coli* HB101(pRK2013) and *P. aeruginosa* transconjugants were selected on PAI strep 2000µg/ml. Transconjugants were then isolated on M9 minimal medium supplemented with 5% sucrose in order to allow the excision of pKNG101. Finally, the clones that become sensitive to streptomycin are selected. The successful allelic replacement of *lpxL1* or *bamA* genes was confirmed by amplifying the gene and verifying the gene sequence by Sanger sequencing.

2.9 DNA sequencing

2.9.1 Sanger sequencing

Sanger sequencing was done after amplifying the genes with suitable primers (table 13) based on the method dideoxyribonucleotides, at the Sequencing platform of UFR Santé of Besançon and CHRU Besançon.

2.9.2 Whole genome sequencing (WGS)

WGS was performed in the P2M platform (Institut Pasteur, Paris, France). In brief, the Nextera XT DNA Library Preparation kit (Illumina, San Diego, CA, USA) was used for library construction. The pooled libraries were sequenced on an Illumina NextSeq 500 platform using paired-end 150-bp runs. The fastq files were generated and demultiplexed with bcl2fastq Conversion Software (v2.20; Illumina). The final average sequencing depth was around 80 X. The reads were asssembled using shovill-spades (v3.14.0), and the contigs were annotated with

Prokka (v1.14.5). Single nucleotide polymorphism (SNP) analysis was conducted using the reference strain PAO1 genome (accession number NC_002516.1) and BioNumerics (v7.6.3) software (Applied Maths) with a minimum sequencing depth of 10. Others genome analysis were performed using CLC Genomics Workbench (v20.0.4) and benchling.

3. mRNA transcripts quantification by RT-qPCR

3.1 RNA extraction and reverse transcription (RT)

Overnight bacterial cultures were diluted the next morning in cMHB or in minimal medium M63 supplemented with 20% glucose at an absorbance OD_{600}= 0.1 either with or without ¼ murepavadin MIC. Bacterial cultures were cultivated at 37°C under constant shaking (250 rpm) until the absorbance reaches OD_{600}=0.8. 500µl of the culture were mixed with 1mL RNA protect (Qiagen), then centrifuged inorder to collect the pellet. Collected pellets were dryed and then conserved at -20°C. The extraction of total RNA was performed using the RNeasy Plus Mini Kit (Qiagen) following the supplier's recommendations. 2µg of the total RNA were reverse transcribed using Kit ImProm-II Reverse Transcription System (Promega). A control free non reverse transcritptase sample (NRT) was made in parallel. Resulting complementary DNA (cDNA) were stored at -20°C for further use.

3.2 Quantification of mRNA transcripts

The expression levels of the genes of interests was measured using RotorGene RG6000 Real Time PCR Instrument (QIAGEN) using the primers listed primers listed in table 14. Each series of measurements included NRT control, the dilution range of the cDNA of PA14 strain (1/10, 1/50, 1/100, 1/500, 1/1000) and a negative control NTC with RNase free water and without cDNA. A 15 µl reaction mix was reconstituted using 3µl cDNA diluted at 1/10, 7.5µl QuantiTect SYBR Green PCR Kit (Qiagen) and 1.5 mM of each primer. Expression levels of messenger RNA (mRNA) transcripts were compared to that of PA14 or PAO1 after normalizing the expression levels with house-keeping gene *rpsl*. The expression levels were measured in duplicates for two independent biologycal replicates.

4. RNA sequencing

RNA sequencing was performed at the Illumina NextSeq 500 platform by Microsynth (Balgach, Switzerland).

5. Polyamines

5.1 Cell-surface and supernatent polyamines samples preparation

Supernatent and cell surface polyamines were extracted based on the protocol established by (Johnson et al., 2012). 100mL of bacterial fresh cultures from PAO1, AB8.2, AB8.2Δ*mexXY*, and AB8.2(pAGH97) mutants, were prepared either with or without murepavadin at ½ MIC starting from overnight cultures (1/100 dilution) in cMHB and cultivated with constant shaking (250 rpm) at 37°C until OD600= 0.8. 500µl of the supernatents were conserved after centrifuging the 50ml bacterial cultures for 10 mins (RT, 5000g). This volume contains the supernatent polyamines. The pellets were then resuspended in 250µL of 10 mM HEPES sodium salt, then incubated again for 10 minutes at 37°C with shaking at 250 rpm. Finally, the suspension was centrifuged and suspended with the same buffer for 3 times (10 min, RT, 5 000 g) to remove whole cells and conserve 250 µl of supernatant. This supernatant volume contains the cell-surface polyamines. Three biological replicates were perfomed for each mutant.

5.2 Polyamines analysis and quantification

Polyamines analysis was perfomed by Liquid Chromatography-ElectroSpray Ionization-Mass Spectrometry (LC-ESI-MS) at the platform BioPark at Archamps Technopole located in St-Julien en Genevois, France.

6. Global metabolomic analysis

Overnight cultures were prepared from AB8.2Δ*mexXY* and AB8.2(pAGH97) and incubated overnight at 37°C. The next day, the 15 mL liquid cultures were centrifuged for 10 min, RT, 3 500 g, and pellets were resuspended in 4 mL of M63+20% glucose minimal media. Fresh cultures of total volume of 15mL in M63+20% glucose minimal media were prepared, either with or without ½ MIC of murepavadin, by calibrating the OD_{600}= 0.1 and incubating them with constant shaking 250 rpm, 37°C until OD_{600}=0.8. The cultures were then centrifuged for 10 min, RT, 5000 g. 2 mL of the filtered supernatant were conserved for further analysis. This volume represents the polyamines present in the supernatant. The pellets were then resuspended in 1.5 mL of 10 mM HEPES sodium salt, then incubated again for 10 minutes at 37°C with shaking at 250rpm. Finally, the suspensions were centrifuged and resuspended with the same buffer for 3 times (10min, RT, 5 000 g) to remove whole cells and conserve 1 mL of supernatant. This supernatant volume contains the cell-surface polyamines. Six biological replicates were perfomed for each strain and for each condition. The polyamines were analysed by mass spectrometry at the mass spectrometry platform of the Museum National d'Histoire Naturelle, Paris.

VI. Appendix

Table S1: List of genes identified by RNAseq to be differentially expressed in PA14 +1/4 MIC of murepavadin in comparison with the PA14 strain unexposed.

PA14_locus	Gene (PAO1 orthologue)	description	Fold change
PA14_00440	*trpA* (PA0035)	tryptophan synthase subunit alpha	-3,9
PA14_00490	PA0040 PA0692 PA2463 PA4540 PA4624	hemolysin activation/secretion protein	2,7
PA14_00510	PA0041 PA2462	hemagglutinin	2,2
PA14_00560	*exoT* (PA0044)	exoenzyme T	-3,1
PA14_00990	PA0082 PA1652 PA2360	TssA1	2,5
PA14_01400	PA0115	hypothetical protein	3,4
PA14_02010	PA0161	hypothetical protein	2,4
PA14_02810	*pcaT* (PA0229)	dicarboxylic acid transporter PcaT	3,8
PA14_03290	no orthologue	hypothetical protein	2,3
PA14_03400	no orthologue	hypothetical protein	2,5
PA14_04270	*carP* (PA0327)	transcriptional regulator	3,2
PA14_04710	no orthologue	hypothetical protein	-2,5
PA14_04780	PA0364	LaoA	3,1
PA14_05340	PA0410	twitching motility protein PilI	-2,3
PA14_05380	PA0412 PA3706	methyltransferase PilK	-3,0
PA14_05460	*bioA* (PA0420)	adenosylmethionine-8-amino-7-oxononanoate aminotransferase	-1,9
PA14_05600	PA0431	hypothetical protein	-3,4
PA14_07560	*rpsU* (PA0579)	30S ribosomal protein S21	1,9
PA14_08350	*trpD* (PA0650)	anthranilate phosphoribosyltransferase	-2,4
PA14_08860	*rplD* (PA4262)	50S ribosomal protein L4	-2,4
PA14_08950	PA4253	50S ribosomal protein L14	-2,2
PA14_09010	*rplR* (PA4247)	50S ribosomal protein L18	-2,6
PA14_09020	*rpsE* (PA4246)	30S ribosomal protein S5	-2,2
PA14_09610	PA4200 PA3037	hypothetical protein	-4,2
PA14_10220	PA4154	SH3 domain-containing protein	4,2
PA14_10530	*mpaR* (PA4132)	GntR family transcriptional regulator	3,1
PA14_10740	PA4114	diamine acetyltransferase	2,1
PA14_11140	PA4078	nonribosomal peptide synthetase	2,8
PA14_11480	PA1531 PA3261 PA3959 PA4049	hypothetical protein	-2,9
PA14_12130	*lis* (PA3996)	lipoyl synthase	-2,2
PA14_12390	*hemL* (PA3977)	glutamate-1-semialdehyde aminotransferase	-2,9
PA14_12900	PA3940	DNA binding protein	2,0

PA14_13140	PA3421 PA3922	hypothetical protein	3,3
PA14_14860	PA3804	hypothetical protein	-2,3
PA14_15700	PA3766	amino acid permease	-2,6
PA14_16250	PA3724	elastase LasB	4,1
PA14_16260	PA2716 PA3723	FMN oxidoreductase	4,4
PA14_16360	*spdH* (PA3713)	hypothetical protein	-2,5
PA14_16380	PA1138 PA3711	LysR family transcriptional regulator	-5,2
PA14_16450	PA0412 PA3706	methyltransferase	-3,2
PA14_16820	PA0158 PA3676	efflux transmembrane protein	2,7
PA14_16950	*dapD* (PA3666)	tetrahydrodipicolinate succinylase	-2,1
PA14_17370	PA3631 PA5430	inner membrane protein	-2,6
PA14_17450	*surE* (PA3625)	stationary phase survival protein SurE	-2,7
PA14_17930	*glpD* (PA3584)	glycerol-3-phosphate dehydrogenase	2,3
PA14_18300	PA3559	Probable nucleotide sugar dehydrogenase	7.7
PA14_18350	*arnA* (PA3554)	UDP-4-amino-4-deoxy-L-arabinose formyltransferase	7.4
PA14_18800	PA3520	Hypothetical protein	5.2
PA14_19110	PA3478	rhamnosyltransferase chain B	3,7
PA14_19130	*rhlI* (PA3476)	autoinducer synthesis protein RhlI	1,9
PA14_20740	PA3350	flagellar basal body P-ring biosynthesis protein FlgA	2,4
PA14_21030	*clpP2* (PA3326)	ATP-dependent Clp protease proteolytic subunit	2,6
PA14_21830	no orthologue	hypothetical protein	2,7
PA14_22400	PA3231	hypothetical protein	3,9
PA14_22740	PA3205	hypothetical protein	4,2
PA14_22980	PA3190	sugar ABC transporter substrate-binding protein	2,8
PA14_22990	PA3189	ABC sugar transporter permease	2,7
PA14_23000	PA3188	ABC sugar transporter permease	3,1
PA14_23010	PA3187	ABC transporter ATP-binding protein	2,5
PA14_23030	PA2291 PA3186	glucose/carbohydrate outer membrane porin OprB precursor	2,3
PA14_23050	PA3185	hypothetical protein	2,4
PA14_23360	wzz (PA0938 PA3160)	O-antigen chain length regulator	2,1
PA14_23400	no orthologue	hypothetical protein	2,2
PA14_23980	*xcpP* (PA3104)	secretion protein XcpP	2,2
PA14_23990	PA0686 PA3103	general secretion pathway protein E	2,8
PA14_24360	no orthologue	Hypothetical protein	16.0
PA14_24370	PA3076	hypothetical protein	-4,6
PA14_24770	PA1945	hypothetical protein	3,4
PA14_24860	*snr1* (PA3032)	cytochrome c Snr1	5.7
PA14_25790	PA0813 PA2958	hypothetical protein	2,9
PA14_25880	*etfA* (PA2951)	electron transfer flavoprotein subunit alpha	-2,1
PA14_27990	PA2794	sialidase	2,2
PA14_28360	no orthologue	hypothetical protein	3,2
PA14_28530	PA2751 PA0951	hypothetical protein	2,8
PA14_28600	PA2747	hypothetical protein	2,3
PA14_28790	no orthologue	hypothetical protein	2,1
PA14_28850	no orthologue	hypothetical protein	2,1

PA14_29260	PA2696 PA5571 PA3898	transcriptional regulator	4,8
PA14_29470	PA0558 PA2679	hypothetical protein	3,0
PA14_29590	*mvaU* (PA2667)	transcriptional regulator	2,2
PA14_30200	*cspD* (PA2622)	cold-shock protein CspD	2,3
PA14_30210	PA2621	ATP-dependent Clp protease adaptor protein ClpS	2,3
PA14_30240	*infA* (PA2619)	translation initiation factor IF-1	1,8
PA14_30620	PA2588	AraC family transcriptional regulator	2,1
PA14_30690	no orthologue	hypothetical protein	2,2
PA14_31150	no orthologue	hypothetical protein	2,2
PA14_31300	PA2569	hypothetical protein	3,5
PA14_31580	PA2550	acyl-CoA dehydrogenase	2,6
PA14_31610	PA2549	TerC family protein	2,0
PA14_31870	*muxA* (PA2528)	RND efflux membrane fusion protein	3,5
PA14_32820	no orthologue	hypothetical protein	2,2
PA14_33360	no orthologue	hypothetical protein	3,3
PA14_33530	*fpvF* (PA2410)	hypothetical protein	-2,7
PA14_35700	no orthologue	hypothetical protein	2,5
PA14_35770	no orthologue	hypothetical protein	2,2
PA14_35780	no orthologue	hypothetical protein	2,2
PA14_35790	no orthologue	homospermidine synthase	2,4
PA14_36330	*hcnA* (PA2193)	hydrogen cyanide synthase HcnA	3,2
PA14_36470	PA2174	hypothetical protein	6.8
PA14_37060	PA5284 PA0992 PA2128 PA4081 PA4086	fimbrial subunit CupA1	2,6
PA14_37690	PA2072	sensory box protein	4,3
PA14_38360	PA2022 PA3559	nucleotide sugar dehydrogenase	4,6
PA14_38440	*liuA* (PA2015)	citronelloyl-CoA dehydrogenase, GnyD	2,3
PA14_38580	PA2004	Hypothetical protein	5.7
PA14_38825	pqqA (PA1985)	coenzyme PQQ synthesis protein PqqA	5.2
PA14_39100	PA1966	hypothetical protein	-6,3
PA14_39360	PA1945	sigma-54 dependent transcriptional regulator	3,7
PA14_39470	no orthologue	hypothetical protein	2,3
PA14_39790	PA1913	hypothetical protein	-2,2
PA14_39980	PA1898 PA3477 PA1136 PA1430	transcriptional regulator	2,5
PA14_40290	*lasA* (PA1871)	LasA protease	3,5
PA14_40780	PA1837	hypothetical protein	-2,4
PA14_40850	PA1831	hypothetical protein	2,3
PA14_41280	PA1797	endo peptidase	6.5
PA14_41980	PA1745	hypothetical protein	8.5
PA14_42270	PA1723	pscJ type III export protein	-2,8
PA14_42280	*pscL* (PA1722)	type III export protein PscI	-2,7
PA14_42290	*pscH* (PA1721)	type III export protein PscH	-6,8
PA14_42300	PA1720	type III export protein PscG	-2,7
PA14_42430	*exsC* (PA1710)	exoenzyme S synthesis protein C	-2,3

PA14_42440	*popD* (PA1709)	translocator outer membrane protein PopD precursor	-3,7
PA14_42460	*pcrH* (PA1707)	regulatory protein PcrH	-3,1
PA14_42470	*pcrV* (PA1706)	type III secretion protein PcrV	-2,6
PA14_42500	*pcrD* (PA1703)	type III secretory apparatus protein PcrD	-2,4
PA14_42570	*pscN* (PA1697)	type III secretion system ATPase	-3,0
PA14_42610	*pscQ* (PA1694)	type III secretion system protein	-5,1
PA14_42620	*pscR* (PA1693)	type III secretion system protein	-3,3
PA14_43050	PA0082 PA1656 PA2360	HsiA2	2,1
PA14_43100	no orthologue	RhsP2	2,1
PA14_43350	*kdpD* (PA1636)	two-component sensor KdpD	-2,9
PA14_43640	*gpsA* (PA1614)	NAD(P)H-dependent glycerol-3-phosphate dehydrogenase	-2,2
PA14_43680	*fabA* (PA1610)	3-hydroxydecanoyl-ACP dehydratase	-2,3
PA14_43690	*fabB* (PA1609)	3-oxoacyl-ACP synthase	-2,2
PA14_43900	PA1592	hypothetical protein	3,8
PA14_44000	*sucB* (PA1586)	dihydrolipoamide succinyltransferase	-2,3
PA14_44311	PA1559	hypothetical protein	9.5
PA14_44670	*zipA* (PA1528)	cell division protein ZipA	1,9
PA14_44800	PA1519	transporter	4,5
PA14_44920	PA1509	hypothetical protein	2,1
PA14_44950	PA1507	transporter	3,3
PA14_45950	*rsaL* (PA1431)	regulatory protein RsaL	3,3
PA14_46080	PA1420	hypothetical protein	-9,8
PA14_46110	PA0287 PA1418	sodium:solute symport protein	-9,2
PA14_46530	no orthologue	hypothetical protein	3,4
PA14_46540	no orthologue	hypothetical protein	4,0
PA14_47110	PA1325	hypothetical protein	2,8
PA14_47130	PA1323	hypothetical protein	6.0
PA14_47440	PA1296	2-hydroxyacid dehydrogenase	1,9
PA14_48140	*aprX* (PA1245)	hypothetical protein	2,3
PA14_48790	PA1199	lipoprotein	2,1
PA14_49020	no orthologue	Pf5 repressor C	1,9
PA14_49180	*phoP* (PA1179)	response regulator of PhoPQ TCS	2,6
PA14_50770	PA1051 PA2311	transporter	-4,4
PA14_50810	PA1048	hypothetical protein	2,4
PA14_51530	no orthologue	ExoU	-2,2
PA14_51570	no orthologue	hypothetical protein	2,0
PA14_51580	no orthologue	hypothetical protein	2,9
PA14_52530	PA0611 PA0906 PA1359	transcriptional regulator	-3,9
PA14_53630	PA0825	hypothetical protein	2,6
PA14_53840	PA0805	hypothetical protein	2,1
PA14_54420	*mucA* (PA0763)	anti-sigma factor MucA	3,4
PA14_54430	PA0762	RNA polymerase sigma factor AlgU	2,1
PA14_54620	PA0747 PA3570	aldehyde dehydrogenase	4,9
PA14_54640	PA0745 PA1629 PA3591	DspI	3,2

PA14_55170	PA0706 PA3156 PA3853	chloramphenicol acetyltransferase	2,1
PA14_56300	PA4333	fumarase	-1,9
PA14_56390	PA4338	hypothetical protein	2,9
PA14_56930	*warA* (PA4379)	hypothetical protein	2,4
PA14_57670	*trpS* (PA4439)	tryptophanyl-tRNA synthetase	-2,1
PA14_57800	*hisG* (PA4449)	ATP phosphoribosyltransferase	-2,9
PA14_58610	PA1972 PA4517	hypothetical protein	2,8
PA14_58630	*speC* (PA4519)	ornithine decarboxylase	1,8
PA14_59190	no orthologue	hypothetical protein	2,6
PA14_59390	no orthologue	hypothetical protein	2,4
PA14_59590	no orthologue	hypothetical protein	2,3
PA14_59845	no orthologue	hypothetical protein	2,6
PA14_60030	no orthologue	hypothetical protein	2,7
PA14_60830	*mexY*	cytoplasmic protein of the MexXY/OprM	2,5
PA14_61190	PA0040	hypothetical protein	4,3
PA14_61200	PA4625	hypothetical protein	4,9
PA14_61350	no orthologue	hypothetical protein	3,4
PA14_61380	no orthologue	hypothetical protein	6.8
PA14_61750	*ipK* (PA4669)	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase	2,1
PA14_62590	*panC* (PA4730)	pantoate--beta-alanine ligase	-2,3
PA14_63110	*speD2* (PA4773)	S-adenosylmethionine decarboxylase	16.7
PA14_63120	*speE2* (PA4774)	hypothetical	9.5
PA14_63130	PA4775	hypothetical protein	3,1
PA14_63570	PA4810 PA4883	nitrate-inducible formate dehydrogenase subunit gamma	-2,7
PA14_64900	PA4913	ABC transporter substrate-binding protein	2,7
PA14_66850	PA5059	TetR family transcriptional regulator	5.8
PA14_66875	no orthologue	polyhydroxyalkanoate synthesis protein PhaF	2,2
PA14_67190	PA5088	type VI secretion lipase immunity protein, Tli5b3	2,1
PA14_67990	PA5147	A/G-specific adenine glycosylase	-3,0
PA14_68400	PA5178	LysM domain/BON superfamily protein	3,3
PA14_68450	PA5182	hypothetical protein	3,1
PA14_69040	PA5228	5-formyltetrahydrofolate cyclo-ligase	1,9
PA14_70930	PA5371	hypothetical protein	2,1
PA14_72660	PA1902 PA3066 PA4213 PA5507	hypothetical protein	-7,1
PA14_72900	PA5526	lipoprotein	3,1
PA14_73170	*glmS* (PA5549)	glucosamine--fructose-6-phosphate aminotransferase	-2,4
PA14_73320	*atpI* (PA5561)	F0F1 ATP synthase subunit I	3,1

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ÉCOLE DOCTORALE
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Title: Study of the activity of murepavadin on *Pseudomonas aeruginosa* cystic fibrosis (CF) clinical strains and identification of resistance mechanisms.

Keywords: *Pseudomonas aeruginosa*, cystic fibrosis, antibiotic resistance, antimicrobial peptides

Abstract: Murepavadin, is a peptidomimetic derived from protegrin-1, that shows so far a promising activity against multi-drug resistant *P. aeruginosa*. In this project, we improved our knowledge on murepavadin activity and demonstrated once again its promising activity when tested on a large panel of clinical strains isolated from CF patients. However, low susceptibility levels to murepavadin still appeared. By analysing whole genome sequences of isolates with low susceptibility in comparison with those with higher susceptibility, we could identify several determinants and confirm their implication in reducing susceptibility, with some causing cross-resistance to commonly used antibiotic treatments. A second part of the project was focued on understanding the molecular response of *P. aeruginosa* following the exposure to murepavadin. We could show the activation of pathways implicated in LPS modification as a response to murepavadin. A third part, was focued on studying the role of the multi-drug efflux pump MexXY/OprM, which is frequently found to be upregulated in CF clinical isolates. We could show that it also plays part in reducing the susceptibility to murepavadin in PAO1 *pmrB* mutants. Global metabolomic analysis to approach the molecule exported by this pump identified a small potential peptide that could be exported by MexXY/OprM. However more analysis to identify this peptide are still ongoing. Finally, we also demonstrated a relation between this efflux pump and polyamines also in PAO1 *pmrB* mutants, suggesting that polyamines might also participate in reducing the susceptibility to murepavadin.

Titre : Etude de l'activité de la murepavadine sur des souches cliniques de *Pseudomonas aeruginosa* dans le contexte de la mucoviscidose et identification des mécanismes de résistance.

Mots clés : *Pseudomonas aeruginosa*, mucoviscidose, résistance aux antibiotiques, peptides antimicrobiens

Résumé : La murepavadine est un peptidomimétique dérivé de la protégrine-1, qui montre jusqu'à présent une activité prometteuse contre la *P. aeruginosa* multirésistante. Dans ce projet, nous avons amélioré nos connaissances sur l'activité de la murepavadine et démontré une fois de plus son activité prometteuse lorsqu'elle a été testée sur un large panel de souches cliniques isolées de patients atteints de mucoviscidose. Cependant, de faibles niveaux de sensibilité à la murepavadine sont toujours apparus. En analysant les séquences du génome entier des isolats peu sensibles par rapport à ceux plus sensibles à la murepavadine, nous avons pu identifier plusieurs déterminants et confirmer leur implication dans la réduction de la sensibilité, certains entraînant une résistance croisée aux traitements antibiotiques couramment utilisés. Une deuxième partie du projet a été consacrée à la compréhension de la réponse moléculaire de *P. aeruginosa* après l'exposition à la murepavadine. Nous avons pu montrer l'activation des voies impliquées dans la modification du LPS en réponse à la murepavadine. Une troisième partie a été consacrée à l'étude du rôle de la pompe d'efflux MexXY/OprM, qui est fréquemment sur-exprimée dans les isolats cliniques de la mucoviscidose. Nous avons pu montrer qu'elle joue également un rôle dans la réduction de la sensibilité à la murepavadine chez les mutants *pmrB* de PAO1. Une approche métabolomique globale de la molécule exportée par cette pompe a permis d'identifier un petit peptide potentiel qui pourrait être exporté par MexXY/OprM. Cependant, d'autres analyses visant à identifier ce peptide sont encore en cours. Enfin, nous avons également démontré une relation entre cette pompe d'efflux et les polyamines dans les mutants PAO1 *pmrB*, ce qui suggère que les polyamines pourraient également participer à la réduction de la sensibilité à la murepavadine.

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