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Clonal epidemiology and antimicrobial resistance in

Pseudomonas aeruginosa chronic respiratory infections:

interpatient transmission and resistome evolution of an

international cystic fibrosis clone.

Carla López Causapé

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Doctoral Degree in Environmental and Biomedical Microbiology

Clonal epidemiology and antimicrobial resistance in *Pseudomonas aeruginosa* chronic respiratory infections: interpatient transmission and resistome evolution of an international cystic fibrosis clone.

Thesis Supervisor and Tutor: PhD Candidate:

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To obtain de Degree of Doctor by the Universitat de les Illes Balears

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DECLARO:

Que la tesis doctoral que lleva por título "Clonal epidemiology and antimicrobial resistance in *Pseudomonas aeruginosa* chronic respiratory infections: interpatient transmission and resistome evolution of an international cystic fibrosis clone", presentada por Carla López Causapé para la obtención del título de doctor, ha sido dirigida bajo mi supervisión y cumple con los requisitos necesarios para optar al título de Doctor Internacional.

Y para que quede constancia de ello firmo el presente documento.

Dr. Antonio Oliver Palomo

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Index ··

I. SUMMARY

Chronic respiratory infection (CRI) by *Pseudomonas aeruginosa* is the main cause of morbidity and mortality in cystic fibrosis (CF). During the progression from early infection to chronic non-eradicable colonization *P. aeruginosa* undergoes a complex evolutionary adaptation and diversification process which eventually leads to a mixed and persistent infecting population in which multidrug resistant variants frequently rise compromising the selection of appropriate antibiotic therapies.

In this work the interplay between three key microbiological aspects of these infections was investigated: the occurrence of transmissible and persistent strains, the emergence of variants with enhanced mutation rates (mutators) and the evolution of resistance to antibiotics. Clonal epidemiology, antibiotic susceptibility profiles, contribution of *P. aeruginosa* classical resistance mechanisms and the role of mutator variants were investigated in two large collections of CF *P. aeruginosa* isolates from the Balearic Islands and Spain. As well, whole genome sequencing (WGS) was used to decipher the phylogeny, interpatient dissemination, within-host evolution, WGS mutator genotypes (*mutome*) and resistome of widespread *P. aeruginosa* clonal complex 274 (CC274), in isolates from two highly-distant countries, Australia and Spain, covering an 18-year period. Finally, due to the relevance of aminoglycosides in the management of CF-CRI, the dynamics of *P. aeruginosa* resistance development to aminoglycosides was also studied *in vitro* by WGS approaches.

Despite discrepancies between molecular genotyping methods, a high degree of genetic diversity was documented among CF isolates from the Balearic Islands and Spain with scarce representation of CF epidemic strains. However, for the first time in Spain, we documented a superinfection with the multidrug resistant Liverpool Epidemic Strain (LES) in a chronically colonized patient. As well, *P. aeruginosa* CC274, previously detected in several CF individuals from Austria, Australia and France, was detected in 5 unrelated chronically colonized patients from the Balearic Islands and, therefore, this clone-type should be added to the growing list of CF epidemic clones. Subsequent analysis of the whole genomes sequences of *P. aeruginosa* isolates from the CC274 *P. aeruginosa* collection provides evidence of interpatient dissemination of mutator sublineages and denotes their potential for unexpected short-term sequence type (ST) evolution and antibiotic resistance spread, illustrating the complexity of *P. aeruginosa* population biology in CF. As well, epidemiological studies demonstrated the coexistence of two divergent lineages but without evident geographical barrier.

Antibiotic resistance significantly accumulated overtime accompanied by hypersusceptibility to certain antibiotics such as aztreonam, which can be explained in terms of collateral susceptibility. Correlation between phenotypes and WGS genotypes of clonal isolates from the CC274 collection allowed us to define the mutational resistome of CF *P. aeruginosa*

Summary··

which extends beyond the classical mutational resistance mechanisms. Among the new chromosomic resistance determinants encountered, mutations within the penicillin-binding-protein 3 (PBP3), shaping up β-lactam resistance, are noteworthy as well as mutations within the *fusA1* gene, coding for elongation factor G, which along with MexXY overexpresion contribute to high-level aminoglycoside resistance. Strikingly, we encountered that MexXY overexpression is dispensable for *in vitro* resistance development to aminoglycosides which suggests an evolutionary advantage of its overexpression in the CF respiratory tract.

Altogether this work demonstrates that clonal epidemiology and antibiotic resistance evolution in the CF setting results from the complex interplay among mutation-driven resistance mechanisms, within host diversification and interpatient transmission of epidemic strains.

II. RESUMEN EN LENGUA CASTELLANA

La infección respiratoria crónica por *P. aeruginosa* es la principal causa de morbilidad y mortalidad en pacientes con fibrosis quística (FQ). Durante la progresión desde la infección temprana a la colonización crónica, *P. aeruginosa* experimenta un complejo proceso adaptativo y de diversificación que resulta en una población heterogénea y persistente en la que la aparición de resistencias a los antibióticos comprometen la selección de terapias apropiadas.

En este trabajo se investigó la interacción entre tres aspectos microbiológicos clave de estas infecciones: la presencia de cepas transmisibles y persistentes, la aparición de variantes con tasas de mutación incrementadas y la evolución de la resistencia a los antibióticos. La epidemiología clonal, los perfiles de sensibilidad antibiótica, la contribución de los mecanismos clásicos de resistencia de *P. aeruginosa* y el papel de las variantes hipermutadoras se estudiaron en dos grandes colecciones de aislados procedentes de pacientes con fibrosis quística de las Islas Baleares y España. Asimismo, mediante secuenciación de genoma completo, se determinó la filogenia, diseminación interpaciente, evolución intrapaciente, genotipo hipermutador y resistoma de una colección de aislados clonales pertenecientes al complejo clonal 274 (CC274), proviniendo dichos aislados de dos países muy distantes, Australia y España, y cubriendo un período de 18 años. Finalmente, dada la relevancia de los aminoglucósidos en el manejo de estos pacientes, se estudió la dinámica del desarrollo de resistencia a aminoglucósidos *in vitro* mediante secuenciación de genoma completo.

A pesar de encontrarse discrepancias entre los métodos de genotipado molecular, se documentó un alto grado de diversidad genética en las colecciones de las Islas Baleares y España, siendo escasa la representación de cepas epidémicas. No obstante, por primera vez en España, se documentó un caso de sobreinfección con el clon epidémico multirresistente de Liverpool. Además, en 5 pacientes de Baleares, crónicamente colonizados y sin aparente relación epidemiológica, se detectó el CC274. Puesto que este complejo clonal también ha sido detectado en pacientes de países como Austria, Australia y Francia, éste debería incluirse en la creciente lista de cepas epidémicas. El análisis posterior de las secuencias de genoma completo de los aislados del CC274 evidenció la diseminación interpaciente de un sublinaje hipermutador, denotando además el potencial de estas variantes para la inesperada evolución a corto plazo del secuenciotipo y la rápida diseminación de resistencias. Además, los estudios epidemiológicos demostraron la coexistencia de dos linajes divergentes, no evidenciándose barrera geográfica.

Asimismo se documentó una tendencia generalizada a la acumulación de resistencias a los antibióticos en el tiempo, acompañada de hipersensibilidad a ciertos antibióticos como aztreonam, lo cual se puede explicar en términos de sensibilidad colateral. La correlación

entre los fenotipos y genotipos determinados mediante secuenciación del genoma completo de los aislados pertenecientes al CC274 nos permitió definir el resistoma mutacional de *P. aeruginosa* en la FQ, el cual se extiende más allá de los mecanismos mutacionales clásicos. Entre los nuevos determinantes de resistencia cromosómica encontrados caben destacar tanto las mutaciones en la proteína fijadora de penicilina PBP3, que confieren resistencia a betalactámicos, como las mutaciones en *fusA1*, que codifica para el factor de elongación G, y que junto con la hiperexpresión de MexXY contribuyen a la resistencia de alto nivel a aminoglucósidos. Paradójicamente, encontramos que la hiperexpresión de MexXY es prescindible para el desarrollo de resistencia *in vitro* a aminoglucósidos, lo que sugiere que dicha hiperexpresión confiere una ventaja evolutiva *in vivo.*

En conjunto, este trabajo demuestra que, en la FQ, la epidemiología clonal y la evolución de la resistencia a los antibióticos son el resultado de una compleja interacción entre los mecanismos de resistencia mutacionales, la diversificación de la población infectante y la transmisión interpaciente de cepas epidémicas.

III. RESUM EN LLENGUA CATALANA

La infecció respiratòria crònica per *P. aeruginosa* és la principal causa de morbiditat i mortalitat en els pacients amb fibrosi quística (FQ). Durant la progressió des de la infecció primerenca a la colonització crònica, *P. aeruginosa* experimenta un complexe procés adaptatiu i de diversificació que resulta en una població heterogènia i persistent en la qual l'aparició de variants resistents a múltiples antibiòtics comprometen la selecció de teràpies antibiòtiques apropiades.

En aquest treball es va investigar la interacció entre tres aspectes microbiològics clau: la presència de soques transmissibles i persistents, l'aparició de variants amb taxes de mutació incrementades i l'evolució de la resistència als antibiòtics. L'epidemiologia clonal, els perfils de sensibilitat antibiòtica, la contribució dels mecanismes clàssics de resistència i el paper de les variants hipermutadores es van estudiar en dos grans col·leccions d'aïllats procedents de pacients amb FQ de les Illes Balears i Espanya. Així mateix, mitjançant seqüenciació del genoma complet, es va determinar la filogènia, disseminació interpacient, evolució intrapacient, genotip hipermutador i resistoma d'una col·lecció d'aïllats pertanyents al complexe clonal 274 (CC274), provenint de dos països molt distants, Austràlia i Espanya, i cobrint un període de 18 anys. Finalment, donada la rellevància dels aminoglicòsids en el maneig d'aquests pacients, es va estudiar la dinàmica del desenvolupament de resistència a aminoglicòsids *in vitro* mitjançant seqüenciació de genoma complet.

Tot i trobar discrepàncies entre els mètodes de genotipat molecular, es va documentar un alt grau de diversitat genètica en les col·leccions de les Illes Balears i Espanya, sent escassa la representació de soques epidèmiques. No obstant això, per primera vegada a Espanya, es va documentar un cas de sobreinfecció amb el clon epidèmic multiresistent de Liverpool. A més, en 5 pacients de les Illes Balears, crònicament colonitzats i sense aparent relació epidemiològica, es va detectar el CC274. Ja que aquest complexe clonal també ha estat detectat en països com Àustria, Austràlia i França, aquest clon hauria d'incloure a la creixent llista de soques epidèmiques. L'anàlisi posterior de les seqüències de genoma complet dels aïllats pertanyents al CC274, va evidenciar la disseminació interpaciente d'un subllinatge hipermutador, denotant a més el potencial d'aquestes variants per a la inesperada evolució a curt termini del sequenciotip i per a la ràpida disseminació de la resistència antibiòtica. A més, els estudis epidemiològics van demostrar la coexistència de dos llinatges divergents, no existint barrera geogràfica.

Així mateix es va evidenciar una tendència generalitzada a l'acumulació de resistències en el temps, acompanyada d'hipersensibilitat a certs antibiòtics com l'aztreonam, la qual cosa es pot explicar en termes de sensibilitat col·lateral. La correlació entre els fenotips i genotips determinats mitjançant seqüenciació del genoma complet dels aïllats pertanyents al CC274 ens va permetre definir el resistoma mutacional de *P. aeruginosa* en la FQ, el qual s'estén

més enllà dels mecanismes de resistència mutacionals clàssics. Entre els nous determinants de resistència cromosòmica trobats cal destacar tant les mutacions en la proteïna fixadora de penicil·lina PBP3, que confereixen resistència a betalactàmics, així com les mutacions en *fusA1*, que codifica per al factor d'elongació G, i que juntament amb la hiperexpressió de MexXY contribueixen a la resistència d'alt nivell a aminoglucòsids. Paradoxalment, vam trobar a més que la hiperexpressió de MexXY és prescindible per al desenvolupament de resistència *in vitro* a aminoglucòsids, el que suggereix que aquesta hiperexpressió suposa un avantatge evolutiu *in vivo*.

En conjunt, aquest treball demostra que l'epidemiologia clonal i l'evolució de la resistència als antibiòtics en el context de la FQ són el resultat d'una complexa interacció entre els mecanismes de resistència mutacionals, la diversificació de la població infectant i la transmissió interpaciente de ceps epidèmiques.

IV. LIST OF ABBREVIATIONS

AK: amikacin

AMG: aminoglycosides

AT: aztreonam

CC: clonal complex

CF: cystic fibrosis

CFTR: cystic fibrosis transmembrane conductance regulator

CFU: colony forming unit

CI: ciprofloxacin

CO: colistin

COPD: chronic obstructive pulmonary disease

CRI: chronic respiratory infection

DGCs: diguanylate cyclases

DNA: Deoxyribonucleic acid

EUCAST: European Committee on Antimicrobial Susceptibility Testing

FO: fosfomycin

FQ: fluoroquinolones

GM: gentamycin

GlcNAc: N-acetil-glucosamine

ID: identification

IP: imipenem

LB: Luria-Bertani

LE: levofloxacin

LES: Liverpool Epidemic Strain

LPS: lipopolysaccharide

MDR: multidrug resistant

MH: Mueller Hinton

MHA: Mueller Hinton agar

MHB: Mueller Hinton broth

MIC: Minimun Inhibitory Concentrations

min: minutes

MLST: Multilocus Sequence Typing

MMR: Mismatch Repair

MP: meropenem

MST: Minimum Spanning Tree

mRNA: messenger ribonucleic acid

MurNAc: N-acetyl-muramic-acid

nm: nanometre

List of abbreviations ···

OMP: outer membrane protein

PBP: penicillin-binding protein

PCR: polymerase chain reaction

PDR: pandrug resistant

PFGE: Pulsed Field Gel Electrophoresis

PGN: peptidoglycan

PM: cefepime

PMN: polymorphonuclear phagocyte

PPT: piperacillin/tazobactam

QS: Quorum-sensing

qRT-PCR: real-time quantitative Reverse Transcription-PCR

RIF: rifampicin

RNA: ribonucleic acid

RND: resistance-nodulation-division

ROS: reactive oxygen species

SCV: small colony variants

sec: seconds

SNP: single nucleotide polymorphism

ST: sequence type

TI: ticarcillin

TM: tobramycin

TOL/TAZ: ceftolozane/tazobactam

TZ: ceftazidime

TZ/AVI: ceftazidime/avibactam

WGS: whole genome sequencing

WT: Wild-type

XDR: extensively drug resistant

1. INTRODUCTION

La levedad y el peso

1.1. *Pseudomonas aeruginosa* GENERAL MICROBIOLOGICAL ASPECTS

Pseudomonas aeruginosa is the major pathogenic species in the family *Pseudomonadaceae*. It is a non-spore-forming, Gram-negative straight or slightly curved rod with a length ranging from 1 to 3 µm and a width of 0.5 to 1.0 µm. *P. aeruginosa* produces many cell surface fimbriae or pili and a polar flagellum which confers its motility.

In the laboratory, *P. aeruginosa* is able to grow on a wide variety of media, ranging from minimal to complex. Most isolates are easily recognizable on primary isolation media on the basis of colonial morphology, a grape-like odor and production of hydrosoluble pigments such as pyocyanin (blue), pyorubin (red), pyomelanin (brown-black) and/or pyoverdin (yellow-green or yellow-brown). In fact, the name *aeruginosa* (from Latin *aerūgō* "copper rust or verdigris" plus -*ōsus*, added to a noun to form an adjective indicating an abundance of that noun) stems from the greenish-blue color of bacterial colonies when pyocyanin and pyoverdin pigments are co-produced. Colonies are usually flat and spreading and have a serrated edge, but other morphologies can exist, including, among others, the mucoid or the small colony variants (SCV, section 1.5.).

P. aeruginosa can metabolize a large array of carbon sources. It does not ferment carbohydrates but produces acid from sugars such as glucose, fructose and xylose, but not from lactose or sucrose. Additionally, it is strongly positive in indophenol oxidase, catalase and arginine tests. *P. aeruginosa* grows best aerobically but can also be grown anaerobically in the presence of nitrate as a terminal electron acceptor. As well, although optimal temperature for growing is 37º C, it can also grow at 42ºC, characteristic that differentiates this species from other rarely pathogenic fluorescent *Pseudomonas* such as *P. fluorescens* or *P. putida*.

1.2. NATURAL HABITATS AND CLINICAL SIGNIFICANCE

P. aeruginosa possesses a complex and large genome (5-7 Mb), including a large proportion of regulatory genes (>8%). These features, along with its metabolic versatility, the large number of genes involved in transport and efflux and the documented genome plasticity of individual strains, explain the ability of this opportunistic pathogen to adapt, survive and persist in virtually any environment.

Related to the persistence of *P. aeruginosa* in nature it should be highlighted its ability to form polysaccharide-encased surface-attached communities, known as biofilms (section 1.6.). Moreover, its genome encodes a remarkable repertoire of virulence determinants and outstanding intrinsic antibiotic resistance machinery that confers *P. aeruginosa* an impressive capacity to cause opportunistic infections in humans and evade the activity of antimicrobial treatments [Breidenstein EB *et al*, 2011; Gellatly SL & Hancock RE, 2013; Silby MW *et al*, 2011].

Within the hospital setting, *P. aeruginosa* can be isolated from moist inanimate environments including water in sinks and drains, toilets, showers and hospital equipment that come in contact with water such as mops, respiratory therapy equipment, antiseptics, cleaning solutions, etc. [Pier GB & Ramphal R, 2005]. On the community, its reservoirs include swimming pools, whirlpools and hot tubes, home humidifiers, contact lens solutions, vegetables and soil, among others [Pier GB & Ramphal R, 2005]. Additionally, although not considered part of the resident human microbiota, gastrointestinal, upper respiratory tract or cutaneous colonization may occur, especially in hospitalized and immunocompromised patients [Pier GB & Ramphal R, 2005], and can be an important preliminary step before infection [Taconneli *et al*, 2009]. Representative colonization rates for specific sites in non-hospitalized humans are 0 to 2% for skin, 0 to 3.3% for the nasal mucosa, 0 to 6.6% for the throat, and 2.6 to 24% for fecal samples; rates that may exceed 50% during hospitalization, especially among patients who have experienced trauma or a breach in cutaneous or mucosal barriers by mechanical ventilation, tracheostomy, catheters, surgery, or severe burns [Lister *et al*, 2009]. As well, disruption in the normal microbial flora as a result of antimicrobial therapy has also been shown to increase colonization by *P. aeruginosa* [Lister *et al*, 2009].

Thus, *P. aeruginosa* is a ubiquitous microorganism that can be implicated in both, hospital and community acquired infections. Indeed, it is recognized as one of the most frequent and severe causes of acute nosocomial infections, accounting for about 10% of all such infections in most European Union hospitals [de Bentzmann *et al*, 2011] and particularly affecting patients with compromised immune systems (especially neutropenic) or those who are admitted to the Intensive Care Units. *P. aeruginosa* is the number one pathogen causing ventilator associated pneumonia and burn wound infections, being both entities associated

with a very high (>30%) mortality rate [Vincent JL, 2003]. Likewise, it is the most frequent and severe driver of CRI in patients suffering from CF or other chronic underlying respiratory diseases such as bronchiectasis or chronic obstructive pulmonary disease (COPD) [Oliver A *et al*, 2009]. As well, this opportunistic pathogen may also be implicated in bloodstream infections, septic shocks, urinary tract or gastrointestinal infections, keratitis, endophthalmitis, otitis, enterocolitis, osteomyelitis, meningitis or folliculitis, among other types of infections [Pier GB & Ramphal R, 2005].

1.3. INTRINSIC ANTIBIOTIC RESISTANCE

As abovementioned, *P. aeruginosa* is genetically equipped with outstanding intrinsic antibiotic resistance machinery [Breidenstein EB *et al*, 2011; Lister *et al*, 2009; Poole, 2011]. Indeed, *P. aeruginosa* wild-type (WT) susceptible strains exhibit a basal reduced susceptibility to a wide variety of antibiotic classes, including β-lactams, aminoglycosides and fluoroquinolones. Specifically, it is naturally resistant to many β-lactams compounds, such as benzylpenicillin and oxacillin, aminopenicillins (including those with β-lactamase inhibitors), 1st and 2nd generation cephalosporins (e.g. cephalotin, cefoxitin and cefuroxime), several 3rd generation cephalosporins (e.g. cefotaxime) and to the carbapenem ertapenem. As well, it shows natural resistance to the aminoglycoside kanamycin and lower susceptibility to fluoroquinolones.

P. aeruginosa intrinsinc antibiotic resistance has been shown to be combinatorial and results from the interplay of several chromosomally-encoded resistance mechanisms, including the production of a narrow spectrum oxacillinase (PoxB/OXA-50) [Girlich D *et al*, 2004; Kong KF *et al*, 2005] and a more recently described imipenemase (PA5542) [Fajardo A *et al*, 2014], the inducible chromosomal AmpC cephalosporinase [Nordmann P & Guibert M, 1998], the constitutive expression of MexAB-OprM efflux pump [Livermore DM, 2001], the inducible expression of MexXY efflux pump [Aires JR *et al*, 1999] and the reduced permeability of its outer membrane [Livermore DM, 1984]. Whereas its outer membrane acts as a first barrier reducing the penetration of antibiotic compounds into the bacterial cell, its chromosomally-encoded oxacillinase, its imipenemase, its cephalosporinase AmpC and its efflux pumps act removing efficiently the antibiotics that do penetrate into the cell.

Moreover, in addition to the abovementioned resistance mechanisms, recent works have demonstrated that inactivation of a large number of genes, mainly involved in basic functions of the physiology of *P. aeruginosa*, are also involved in antibiotic susceptibility changes [Breidenstein EB *et al*, 2008; Fajardo A *et al*, 2008; Schurek KN *et al*, 2008; Dötsch A *et al*, 2009; Alvarez-Ortega C *et al*, 2010; Khran T *et al*, 2012; Fernandez L *et al*, 2013]. On the whole, these works have demonstrated that intrinsic resistance to antibiotics involves a complex network of elements. Of note, although inactivation of many of these genes just lead to slight decreases in susceptibility (1-2 fold), an overlap between them and genes dysregulated upon antibiotic exposure has been observed, which indicates that *P. aeruginosa* adaptively activates resistance mechanisms to combat the inhibitory effects of antibiotics.

1.3.1. A first barrier to antibiotics: the outer membrane

When compared to other Gram-negative bacteria, *P. aeruginosa* exhibits a lower outer membrane permeability (approximately 8% that of *E. coli* outer membrane) [Nikaido H, 1985]

but a large exclusion limit allowing the entrance of large compounds (3000 molecular weight vs 500 in *E. coli*) [Bellido F *et al*, 1992].

However, in order to survive, *P. aeruginosa* must allow the entrance of nutrients into the cell and this exchange is accomplished through a collection of β-barrel proteins producing water-filled diffusion channels called porins. Up to 163 known or predicted outer membrane proteins (OMPs) have been described within *P. aeruginosa* genomes, of which 64 are found as part of 3 families of porins: the OprD-specific porin family, the TonB-dependent gated porin family, and the OprM efflux/secretion family. Most of these porins have low molecular masses, being the OprF porin the largest one (37.6 kDa). Thus, the low permeability documented for *P. aeruginosa* strains can be explained in terms of a limited number of large general diffusion porins [Hancock RE & Brinkman FS, 2002].

Porins play an important physiological role in the transport of sugars, aminoacids, phosphates, divalent cations and siderophores [Hancock RE & Brinkman FS, 2002] and they have also be implicated in the transport of certain hydrophilic antibiotics such as β-lactams, aminoglycosides, tetracyclines and some fluoroquinolones [Nikaido H *et al*, 1991; Yoshimura F & Nikaido H, 1985]. Therefore, in addition to their contribution to the intrinsic antibiotic resistance, porins can further diminish *P. aeruginosa* susceptibility by regulating their expression or by acquiring mutations with effects onto their structures and functionality (section 1.8).

1.3.2. AmpC-inducible expression

P. aeruginosa possesses an inducible chromosomally-encoded AmpC cephalosporinase which is similar to that found in several members of the *Enterobacteriaceae* [Jacoby GA, 2009]. According to the Bush-Jacoby-Medeiros classification, AmpC is a serine β-lactamase belonging to group I and, based on the Ambler structural classification, to class C β-lactamases. Possibly, AmpC is the most relevant antibiotic resistance mechanism of this opportunistic pathogen.

WT *P. aeruginosa* strains produce only low basal amounts of this enzyme remaining susceptible to antipseudomonal penicillins, penicillin-inhibitor combinations, antipseudomonal cephalosporins (ceftazidime and cefepime) and carbapenems. Nevertheless, AmpC production can significantly be increased under particular circumstances, conferring resistance to all β-lactams. AmpC increased production can occur either, through mutations within its regulatory genes (section 1.8.) or by induction of the *ampC* gene. AmpC induction is a reversible process which occurs under exposure to specific β-lactams and β-lactamase inhibitors such as cefoxitin, imipenem and/or clavulanate [Lister PD *et al*, 2009]. As following detailed, AmpC induction is a complex process intimately linked with peptidoglycan (PGN) recycling (Figure 1.1.).

Introduction ···

The PGN of *P. aeruginosa* is built up of chains with n repeats of the disaccharide monomer N-acetyl-glucosamine-N-acetyl-muramic-acid (GlcNAc-MurNAc) connected to other identical chains by stem peptides linked to the MurNac units. The stem peptide from a disaccharide monomer is originally a pentapeptide (L-Alanine-D-Glutamicacid-diaminopimelicacid-D-Alanine-D-Alanine) and connects to a second stem peptide from another disaccharide monomer located on a different chain thanks to the transpeptidase activity of the high molecular mass penicillin-binding proteins (PBP1, PBP2 and PBP3). These PBPs cleave the terminal D-Alanine from the first pentapeptide (carboxypeptidase activity), converting it into a tetrapeptide which eventually binds to the diaminopimelic acid from other pentapeptide (transpeptidation). Thus, these bonds allow for the crosslinking of disaccharide chains which constitute the essential PGN architecture. Once the basic PGN structure is built, some other PBPs, mainly the low molecular mass PBPs (PBP4, PBP5 and PBP6) are thought to finely shape it. These PBPs exert D-carboxypeptidase activities and are known to release the terminal D-Ala from pentapeptides not destined to be cross-linked converting them into tetrapeptides not suitable for transpeptidation [Juan C *et al*, 2017].
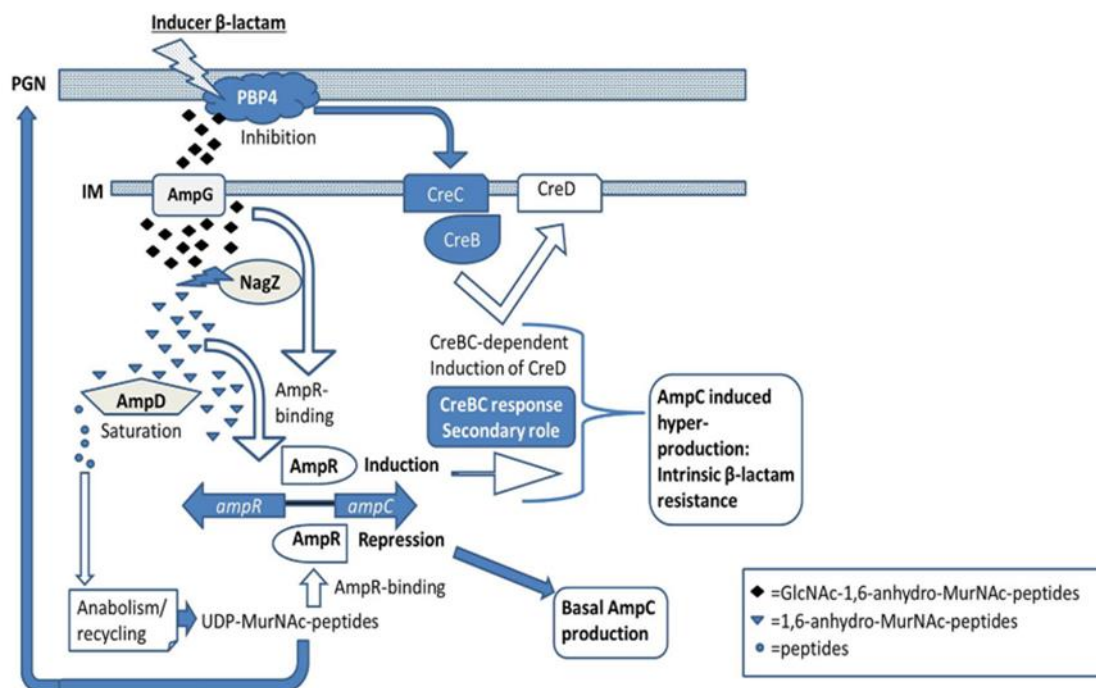
Figure 1.1. Schematic representation of the interplay between PGN recycling, ampC regulation (induction) and intrinsic β-lactam resistance in *P. aeruginosa*. From: Juan C *et al*, 2017.

On each generation *P. aeruginosa* naturally degrades about 50% of its PGN mainly thanks to the action of the periplasmic autolysins (endopeptidases), which break the abovementioned bonds originating not cross-linked peptides, and to the action of the lytic transglycosylases, which break the bonds between the disaccharide units. Up to 90% of the degraded PGN is thought to be recycled, which supposes an outstanding resource-saving strategy. The action of the cited periplasmic enzymes results mainly in GlcNAc-1,6-anhydro-

MurNAc tri-, tetra- and penta- peptides [Vollmer W & Höltje JV, 2001], resulting fragments that are transported through the permease AmpG into the cytosol [Korfmann G & Sanders CC, 1989; Dietz H & Wiedemann B, 1996; Cheng Q & Park JT, 2002]. This is a key step for the downstream AmpC regulation and recycling events, as AmpG is the specific door for the entrance of PGN-derived mediators with AmpC regulator capacity [Zamorano L, 2011]. Once in the cytosol, the cytosolic L, D-carboxypeptidase LdcA cleaves the D-Ala from the tetrapeptides units, avoiding the potential accumulation of UDP-MurNAc tetrapeptides which are thought to be toxic for the bacterial cell [Templin MF *et al*, 1999]. As well, a glycoside hydrolase called NagZ removes the GlcNAc residues [Zamorano L *et al*, 2010] resulting in a pool of cytosolic GlcNAc units plus 1,6-anhydro-MurNAc peptides [Cheng Q *et al*, 2000; Vötsch W & Templin MF, 2000] that, in non-inducer standard conditions, would eventually be recycled into UDP-MurNAc pentapeptides and exported to the nascent PGN.

Classically, it has been believed that the 1,6-anhydro-MurNAc tri- and penta- peptides units [Jacobs C *et al*, 1994; Dietz H *et al*, 1997] are signal molecules that induce ampC transcription and, indeed, the UDP-MurNAc pentapeptide has been identified as a repressor of ampC transcription to basal levels. Thus, these metabolites have been suggested to competitively regulate *ampC* transcription by directly binding to the LysR-type transcriptional regulator AmpR [Jacobs C *et al*, 1994]. AmpR and AmpC coding genes are located next to each other within the genome, divergently codified and with overlapping promoter regions to which AmpR binds to regulate their transcription [Lindquist S *et al*, 1989; Bartowsky E & Normark S, 1993]. Under non-inducer standard conditions, the cytosolic AmpD, through its N-acetyl-muramyl-L-alanine amidase activity, cleaves the stem peptide from both the GlcNAc-1,6-anhydro-MurNAc and the 1,6-anhydro-MurNAc peptides [Höltje JV & Glauner B, 1990; Jacobs C *et al*, 1994], which results in low amounts of activation ligands. On the contrary, the amount of UDP-MurNAc pentapeptides can be increased thanks to the anabolic pathways starting from the pool of AmpD cleaved peptides and, thus, can both, enter into the PGN recycling route and bind to AmpR promoting the formation of an AmpR-deoxyribonucleic acid (DNA) complex that represses ampC transcription to basal levels.

In this sense, it has been proposed that exposure to certain β-lactams known to be AmpC inducers, such as cefoxitin and imipenem, triggers the accumulation of 1,6-anhydro-MurNAc peptides within the cytosol, reaching levels that cannot be efficiently processed by AmpD [Dietz H & Wiedemann B, 1996; Wiedemann B *et al*, 1998; Vollmer W & Höltje JV, 2001]. This accumulation would presumably displace the UDP-MurNAc pentapeptide from AmpR, generating a new complex that would act as an activator of *ampC* transcription and, thus, leading to clinically significant resistance against the inducer and other hydrolysable β-lactams [Jacobs C *et al*, 1994]. The molecular basis for the mentioned increase in the 1,6-anhydro-MurNAc pentapeptides amount during induction is believed to be related with the capacity of the inducer β-lactams to inhibit the DD-carboxypeptidase activity of the low

molecular mass PBPs [Sanders CC *et al*, 1997; Tayler AE *et al*, 2010; Fisher JF & Mobashery S, 2014]. In this sense, *Moyà et al.* showed that the inducer β-lactams can inhibit the non-essential low molecular mass PBP4 (dacB) [Moyà B *et al*, 2009], affecting the PGN composition and favouring the entrance of activation ligands through AmpG. Interestingly, the authors also showed that PBP4 inducer-inhibition additionally triggers the activation of the two-component system CreBC which plays a collateral and minor role during the process. Thus, it has been proposed that PBP4 acts as a sentinel for the cell wall damage caused by the inducers, triggering an AmpR-dependent overproduction of AmpC and activating the CreBC system. The induction mechanism is a reversible process and *ampC* expression returns to basal levels in the absence of the antibiotic inducers [Mark BL *et al*, 2012]. Also it should be highlighted that the hydrolytic effect of AmpC onto a β-lactam will not only depend on the antibiotic inducer capacity but also on the hydrolysing efficiency of the cephalosporinase. Therefore, the inducible expression of AmpC plays a major role in the intrinsic resistance of *P. aeruginosa* to aminopenicillins and most cephalosporins (particularly cephamycins such as cefoxitin) since these molecules are potent inducers of the expression and efficiently hydrolyzed by this enzyme. Likewise, the inducible AmpC plays a major role in the basal reduced susceptibility level of *P. aeruginosa* to the carbapenem imipenem, as the relatively stability of this molecule to the hydrolysis by the cephalosporinase is to some extent compromised by its extremely high potency as inducer [Livermore DM, 1992].

Non-reversible mutational derepression leading to constitutive high-level expression of AmpC will be discussed later in section 1.8.

1.3.3. Efflux-pumps systems: constitutive and inducible expression

Efflux pumps play an important role in antibiotic resistance. These pumps may be specific for a substrate or may extrude a broad range of compounds including dyes, detergents, fatty acids and antibiotics of multiple classes structurally unrelated. Thus, it is probable that efflux pumps were created so that harmful substances could be transported out of the bacterial cell, thus, allowing for survival.

Based primarily on amino acid sequence identity, on the energy source required to drive export and on substrate specificities, efflux pumps have been categorized in five superfamilies including (i) the ATP-binding cassette family, (ii) the small multidrug resistance family, (iii) the major facilitator superfamily, (iv) the resistance-nodulation-division (RND) family, and (v) the multidrug and toxic compound extrusion family. In *P. aeruginosa*, genome sequence analysis has revealed the presence of efflux systems from all five superfamilies, being the RND family the most prevalent with 12 different systems identified.
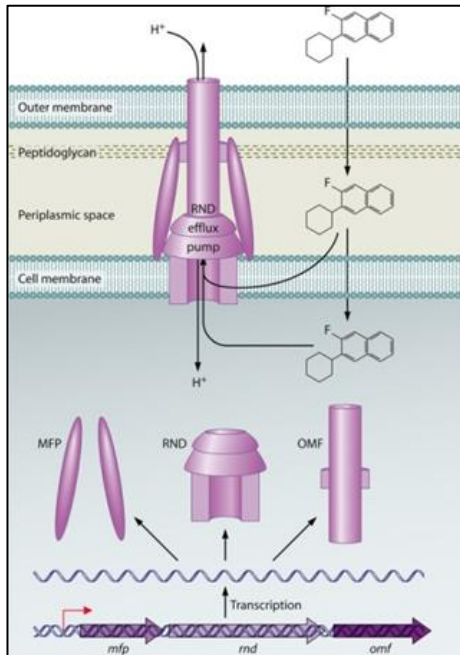
Figure 1.2. Location of RND-type efflux pumps components across the outer and inner membrane in *P. aeruginosa*. MFP: membrane fusion protein, RND: transporter protein, and OMF: outer membrane factor.

The RND-type efflux pumps are secondary active transporters that derive the energy required for compound extrusion by proton motive force and are typically organized as a tripartite consisting of a periplasmic membrane fusion protein, a transporter protein in the inner membrane and an outer membrane factor.

Within this complex, the inner membrane protein captures the substrates from either, the phospholipid bilayer of the inner membrane of the bacterial cell envelope or the cytoplasm, and transports them into the extracellular medium via the OMP, being the cooperation between these proteins mediated by the periplasmic protein (Figure 1.2.) [Lister PD *et al*, 2009; Li XZ *et al*, 2015].

The genes coding for the RND efflux pumps components are organized into operons in the *Pseudomonas aeruginosa* chromosome. Not all of them code for an outer membrane factor and, thus, the tripartite efflux pump is completed by taking this protein from a different efflux pump system (e.g. MexXY). As well, some of them harbor an adjacent regulatory gene transcribed in the same orientation or divergently from the operon and whose products act repressing or activating the operon expression (Figure 1.3. and Table 1.1.) [Lister PD *et al*, 2009].
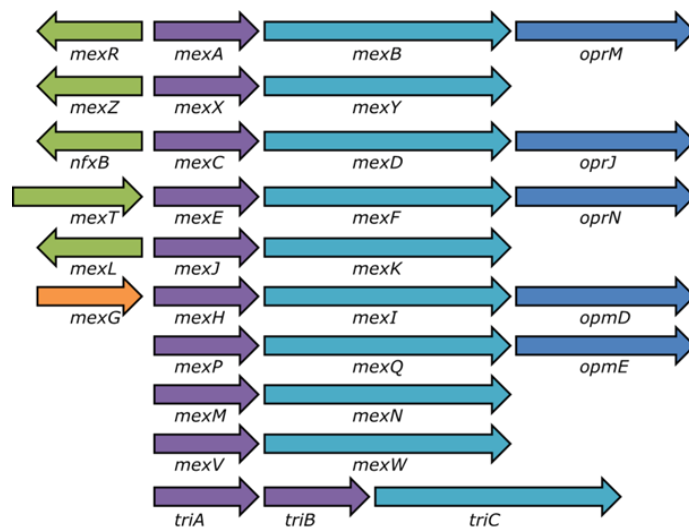
Figure 1.3. RND efflux operons in *P. aeruginosa*. Operons encoding the 10 RND pumps (excluding the 2 metal cation transporters) are represented. Color scheme: green, transcriptional regulator; purple, membrane fusion protein; light blue, RND transporter; dark blue, OMP; and orange, protein with unknown function.

Introduction

Most RND efflux systems in *P. aeruginosa* exhibit broad substrate specificity and recognize many structurally dissimilar compounds (Table 1.1). Of all systems, MexAB-OprM and MexXY contribute to its intrinsic antibiotic resistance as all the others are not expressed in WT strains.

Table 1.1. Substrates for the RND efflux systems of *P. aeruginosa*

Efflux system	Substrates	
	Antibiotics	Additional compounds
MexAB-OprM	β-lactams (not imipenem), β-lactamase inhibitors, fluoroquinolones (FQ), chloramphenicol, macrolides, novobiocin, tetracyclines, trimethoprim, sulfonamides	Biocides, detergents, dyes, homserin lactones, aromatic hydrocarbons
MexXY-OprM/Opm-[a]	Penicillins (not carbenicillin and sulbenicillin), cephalosporins (not ceftazidime), meropenem, FQ, aminoglycosides (AMG), tetracyclines, macrolides, chloramphenicol	
MexCD-OprJ	Penicillins, cephalosporins (not ceftazidime), meropenem, FQ, chloramphenicol, macrolides, novobiocin tetracyclines, trimethoprim	Biocides, detergents, dyes, aromatic hydrocarbons
MexEF-OprN	FQ, cloranphenicol, trimethoprim	Biocides, aromatic hydrocarbons
MexJK-OprM/OpmH	Tetracyclines, erythromycin	Biocides
MexGHI-OpmD	FQ	Vanadium
MexVW-OprM	FQ, tetracyclines, chloramphenicol, erythromycin	
MexPQ-OpmE	FQ, tetracyclines, chloramphenicol, macrolides	
MexMN-OprM	Chloramphenicol, thiamphenicol	
TriABC-OpmH		Triclosan

[a] MexXY may utilize OpmB, OpmG, OpmH and/or OmpI as OMFs.

1.3.3.1. Constitutive expression of MexAB-OprM

MexAB-OprM was the first RND multidrug efflux system to be described in *P. aeruginosa* [Poole K *et al*, 1993; Li XZ *et al*, 1995]. As shown in Table 1.1., this pump is able to export antibiotic compounds from different families and exhibits the broadest substrate profile for the β-lactam class including carboxypenicillins, aztreonam, cefotaxime, ceftazidime and meropenem.

This system is expressed constitutively in cells grown under standard laboratory conditions [Poole K & Srikumar R, 2001] and laboratory-constructed MexAB-OprM knockout mutants have been shown to be hypersensitive to its substrates [Li XZ *et al*, 1995; Masuda N *et al*, 1999; Morita Y *et al*, 2001]. In WT *P. aeruginosa* strains, MexAB-OprM expression is growth-phase-dependent, reaching its maximum in late log-phase/early stationary phase. This dependency led to the suggestion that MexAB-OprM expression could be regulated by the

quorum sensing (QS) system (cell to cell communication) and, in this sense, it has been demonstrated that N-butyryl-L-homoserin-lactones enhance its expression.

All three components of this efflux pump are encoded within the same operon (Figure 1.3.), which additionally harbors a regulatory protein (MexR) located directly upstream but transcribed divergently from MexA-MexB-OprM coding genes. MexR belongs to the MarR family member and is the major regulator of this efflux pump system. It binds as a stable homodimer to two sites within the *mexR-mexA* intergenic region overlapping the promoters for mexR and mexAB-oprM and, thus, repressing their expression. Recently, it has been demonstrated that MexR repressor capacity depends on its redox state as, within the stable homodimer, MexR-Cys residues form intermonomer disulfide bonds whose oxidation eventually lead to its dissociation from the promoter DNA [Chen H *et al*, 2008; Chen H *et al*, 2010]. MexR activity has been found to be additionally controlled by *armR* encoded product, as it binds to MexR diminishing its repressor activity [Daigle DM *et al*, 2007; Wilke MS *et al*, 2008]. Finally, MexAB-OprM expression is controlled by *nalD*, which encodes a TetR family repressor-like protein that binds to a second promoter upstream of *mexA-mexB-oprM* [Morita Y *et al*, 2006a]. Also of note, it has been shown that *oprM* expression can occur independently of *mexA-mexB*, through an alternative weak promoter within *mexB* [Zhao Q *et al*, 1998], which ensures sufficient levels of this OMP to other *P. aeruginosa* efflux systems (MexXY, MexJK, MexVW and MexMN) even when *mexA-mexB-oprM* expression is compromised.

Mutation-driven overexpression of this efflux system will be discussed later in section 1.8.

1.3.3.2. Inducible expression of MexXY

The MexXY efflux system was discovered several years later, in 1999, being the fourth efflux system to be identified in *P. aeruginosa* PAO1 [Aires JR *et al*, 1999; Mine T *et al*, 1999]. It is able to extrude a wide variety of substrates (Table 1.1.) and, of note, is the only efflux pump encoded in *P. aeruginosa* chromosome with the ability to mediate aminoglycoside resistance.

MexXY expression is induced when bacterial cells are grown in the presence of sub-inhibitory concentrations of some of its antibiotic substrates such as tetracycline, erythromycin or aminoglycosides. Additionally, *P. aeruginosa* PAO1 mutants lacking this efflux system are hypersusceptible to its substrates which suggests that it contributes to the intrinsic antibiotic resistance to these agents [Aires JR *et al*, 1999; Masuda N *et al*, 2000].

Genetically, the operon coding for MexXY lacks an outer membrane factor (Figure 1.3.). Therefore, it takes the OMF protein from other operons to complete the tripartite system. Mainly, OprM completes the tripartite system but other porins such as OpmB, OpmG, OpmH or OpmI can also be implicated (Table 1.1.) [Chuanchuen R *et al*, 2005; Murata T *et al*,

2002]. Located upstream but transcribed divergently from *mexX-mexY*, is encountered *mexZ* which encodes a protein that belongs to the TetR family of transcriptional regulators and negatively regulates its expression (Figure 1.3.). Similar to MexR (section 1.3.3.1.), MexZ binds as a homodimer to an inverted repeated sequence within the intergenic region *mexZ-mexX,* overlapping the putative *mexX-mexY* promoter [Matsuo Y *et al*, 2004] and repressing its expression.

In contrast to other drug-inducible multidrug efflux systems, MexXY inducers do not alter MexZ and *mexZ-mexX* interactions. Instead, induction has been shown to be dependent on drug-ribosome interactions and to occur, although in a lesser extent, even in the *mexZ* mutant [Jeannot K *et al*, 2005]. Therefore, these data suggest an alternative biological role for the MexXY system beyond antibiotics efflux. Multiple pathways participate in the regulation of *mexX-mexY* induction. Although ribosome disruption has been shown to impact the expression of a myriad of genes, by using a transposon insertion mutant library PA5471 was found to be not only drug-inducible but also required for *mexX-mexY* induced expression [Morita Y *et al*, 2006b]. Later on, it was demonstrates that the antimicrobial-inducible PA5471 gene product has interacts with the repressor MexZ and, thus, interfere with its DNA binding activity [Yamamoto M *et al*, 2009].

More recently, it has been also demonstrated the involvement of *parR,* a gene coding for the response regulator of the two-component regulatory system ParR-ParS, in promoting either induced or constitutive *mexX-mexY* upregulation. In addition, this gene was demonstrated to be also implicated in OprD porin downregulation and in lipopolysaccharide (LPS) modification in a MexZ-independent manner [Muller C *et al*, 2011].

Mutation-driven overexpression of this efflux system will be discussed later in section 1.8.

1.4. CHRONIC RESPIRATORY INFECTIONS

On average, about 10,000 L of air are inhaled per person per day and, thus, the respiratory tract is continuously exposed to a wide variety of potential pathogenic microorganisms. However, and due to sophisticated host defence mechanisms at the lung mucosa, infections are rare among healthy individuals. Airway bronchial and alveolar epithelial cells constitute the first line of defense against invading bacteria, providing not only a physical barrier and exhibiting local antimicrobial activity but also acting as sentinels stimulating downstream recruitment and activation of immune cells which clear invading bacteria. As well, resident alveolar macrophages and occasionally dendritic cells are also found in the alveolar epithelium and are key mediators of innate and adaptive immunity [Eisele NA & Anderson DM, 2011]. On the opposite, the immune response within the respiratory airway of patients suffering chronic respiratory underlying diseases such as CF, non-CF bronchiectasias or COPD, is impaired and, therefore, these disorders are characterized by repeated cycles of inflammation, tissue damage and bacterial infections that may eventually lead to the establishment of chronic non-eradicable respiratory infections and a rapid decline of the pulmonary function [Döring G *et al*, 2011]. In fact, *P. aeruginosa* CRI acquired a major relevance within the CF setting, being the most frequent and severe driver of morbidity and mortality.

CF is the most prevalent autosomal recessive hereditary disease affecting Caucasian populations, with approximately 70,000 people affected worldwide and with an estimated incidence of 1 per 2500-5000 newborns in white populations from Europe, Canada and USA [O'Sullivan BP & Freedman SD, 2009]. This chronic respiratory disease is caused by mutations (two-thirds F508Δ) disrupting the function of the CF transmembrane conductance regulator (CFTR) gene, which encodes a chloride channel that is expressed on the apical surface of many epithelial and blood cells. The clinical spectrum of the CF disease is wide; however, pulmonary insufficiency is the first cause of morbidity and mortality among CF patients being approximately 80% of CF deaths related with chronic lung infection [O'Sullivan BP & Freedman SD, 2009]. Fortunately, over the past decades, CF respiratory infections management has considerably been improved and median age of survival of CF patients is now set in more than 40 years in developed countries [McCormick J *et al*, 2010]. As a result, the number of CF adults (age ≥18 years) is larger than the number of children in several EU countries with well-established healthcare systems [McCormick J *et al*, 2010] and forecasts predict a large increase in the number of CF adults by 2025 [Burgel PR *et al*, 2015].

By the time of birth, the respiratory tract of CF children is normal but, soon after, becomes inflamed and infected. Mechanisms underlying the early acquisition of infection and the establishment of *P. aeruginosa* CRI are complex and several factors participate as following described.

Introduction

One contributing factor is the inability of mutated CFTR to effectively secrete chloride from respiratory epithelial cells into the airway surface liquid which eventually causes excessive water absorption from the airway surface liquid, leading to an impaired mucociliary clearance. Likewise, the viscosity of the secretions may impair the transport of antimicrobial oligopeptides onto the epithelium and, thus, may also negatively affect the migration of neutrophils towards the pathogens. Furthermore, within the highly viscous mucus, a microaerobic/anaerobic milieu prevails due to oxygen consumption by bacterial pathogens or invading neutrophils which abolish the generation of reactive oxygen species (ROS) by neutrophils and other cells impairing bacterial killing. As well, other investigators have demonstrated that the abnormal accumulation of ceramide in the lungs of CF mice and in the epithelial cells from CF patients, results in an increased death rate of respiratory epithelial cells and DNA deposits on the respiratory epithelium, which in turns facilitates bacterial adherence. Finally, *P. aeruginosa* infection may also be facilitated directly by defective CFTR, as in its functional state can bind the pathogen within lipid rafts removing it from the epithelial surface via internalization [Döring G *et al*, 2011]. Of course, *P. aeruginosa* also plays a major role as, thanks to the enormous armamentarium of immunoevasive strategies encoded within its genome, is capable of evading not only host defenses but also repeated courses of antibiotics.

All the above mentioned alterations in the CF airway surface provide an ideal environment for infection/colonization which is not only exploited by *P. aeruginosa*. In fact, CF patients experience multiple bacterial infections throughout their life and whereas, overall, *Staphylococcus aureus* and *Haemophilus influenzae* are typically first cultured in young children, *P. aeruginosa* and other opportunistic multidrug resistant pathogens such as *Achromobacter spp.*, *Stenotrophomonas maltophilia* or *Burkholderia cepacia complex* are first cultured during adolescence and young adulthood (Figure 1.4.) [CFF Patient Registry, 2016 Annual Data Report].
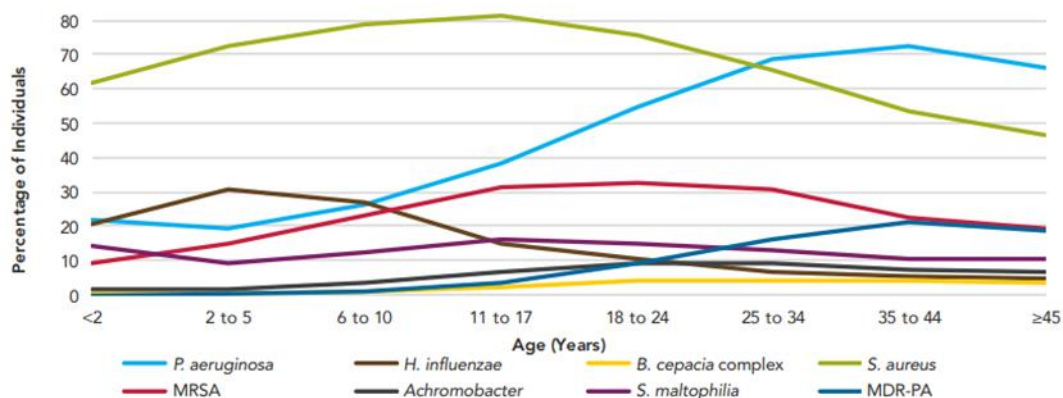
Figure 1.4. Prevalence of respiratory microorganisms by age cohort, 2016. From: Cystic Fibrosis Foundation Patient Registry, 2016 Annual Data Report. Bethesda, Maryland, ©2017 Cystic Fibrosis Foundation.

As shown, *P. aeruginosa* is by far the most significant CF pathogen. Early infection occurs in a large number of patients before the age of 3 years [Speert DP *et al*, 2002], after, and for a variable period of time, *P. aeruginosa* isolation from CF respiratory samples can be intermittent and, usually, involving multiple strains. Eventually, by the age of 25, over 70% of the patients are chronically colonized and a single well adapted strain (or clonal lineage) predominates [Cystic Fibrosis Foundation Patient Registry, 2016 Annual Data Report].

1.5. EVOLUTION AND ADAPTATION TO THE CYSTIC FIBROSIS AIRWAYS

During the progression from early infection to chronic non-eradicable colonization, *P. aeruginosa* undergoes a complex evolutionary adaptation and diversification process that implies both phenotypic and genotypic variations.

Usually, first *P. aeruginosa* CF isolate resembles those from the environment or from acute infections in terms of phenotype and genotype. Thus, during long time, it was extensively accepted that early *P. aeruginosa* acquisition occurs from diverse environmental reservoirs so, generally, each patient harbors its unique non-clonal unrelated strain. However, this classical perception changed in 1986, when an outbreak caused by a *P. aeruginosa* strain resistant to several antibiotics was reported in a CF center in Denmark [Pedersen SS *et al*, 1986]. Since this first description, other strains infecting a large proportion of CF patients have been detected and, in some cases, strongly associated with multidrug resistant profiles. Therefore, nowadays "person-to-person" transmission is also an accepted route of *P. aeruginosa* acquisition among CF patients and certain clones are extensively recognized as epidemic and/or transmissible being worldwide distributed (section 1.8.).

Regardless to the source of infection, if not eradicated, the cell density and the collective growth pattern of *P. aeruginosa* change and a complex diversification process occurs within the bacterial population which, in turns, improve its capacity for survive and persist in the CF airways throughout the lifespan of a CF patient [Renders N *et al*, 2001; Munck A *et al*, 2001].

Overtime, during the course of infection, the genome of *P. aeruginosa* can be modified by either acquiring new mutations or by the acquisition and/or loose of genomic DNA. Whereas few works have focused their attention on DNA acquisition/loose and obtained results are not consistent, different authors have focused on genomic modification based on the acquisition of SNPs and small insertions stablishing mutation rates from 1 to 3 SNPs/year for non-mutator isolates [Marvig RL *et al*, 2015a]. As well, since whole-genome sequencing technologies have become more affordable, many efforts have been put in identify those genes that are recurrent mutated among CF isolates and, therefore, directly implicated in the adaptation to the CF lungs, the so-called pathoadaptative genes. Whereas some genes have been found to be frequently mutated, others have just been found to be mutated in single studies which suggest that different evolutionary pathways exist [Marvig *et al*, 2015a]. Addtionally, the study of longitudinal isolates within single patients have provided evidence that population diversification and stable maintenance of these genetically distinct subpopulations frequently occurs, even including mutator and non-mutator sublineages [Chung JC *et al*, 2012; Marvig RL *et al*, 2013; Feliziani S *et al*, 2014]; diversification procces that have been suggested to be triggered by the spatial heterogeneity of the CF airways [Markussen T *et al*, 2014].

Indeed, in patients in advanced infection-colonization stages, this impressive process of genotypic diversification can be easily recognized in the microbiological cultures of their respiratory samples which are characterized by a mixture of phenotypic varieties following described.

Mucoid variants. One of the most common features of *P. aeruginosa* isolates causing CRI is the frequent conversion to a mucoid phenotype. In fact, the appearance of mucoid colonies within the microbiological culture can be used as a marker of infection chronicity and poor clinical outcomes. This phenotype results from the constitutive production of the extracellular polysaccharide alginate, a polymer of D-mannuronic and L-guluronic acid, which forms a glycocalyx that encapsulates the bacteria, protecting them from adverse environmental stresses such as dessication, oxidizing agents and host defence [Franklin MJ *et al*, 2011]. As well, this extracellular polysaccharide is one of the major components of the biofilms matrix (section 1.6.).

The genetic mechanisms underlying the switch to mucoidity in *P. aeruginosa* have been largely studied and mainly results from the mutational inactivation of the *mucA* gene, which codes for an anti-σ-factor [Govan JR & Deretic V, 1996; Boucher JC *et al*, 1997]. All the enzymes required for alginate production are encoded in the operon *algD-algA* and, in the absence of mutations, the *algD* operon expression is limited by the *mucA* gene product that binds to the alternative ribonucleic acid (RNA) polymerase σ-factor σ22 encoded in *algU* [Folkesson A *et al*, 2012].

Figure 1.5. Mucoid *P. aeruginosa* on Mueller-Hinton Agar (MHA).

In addition to the *algD* cluster, σ22 is known to regulate, directly and indirectly, a large number of stress response and virulence-associated genes in *P. aeruginosa* which suggests that the importance of the *mucA* mutations goes beyond the conversion to a mucoid phenotype [Folkesson A *et al*, 2012]. Despite the success and theoretical advantages of this variant, the most common situation during late chronic stages is the coexistence of mucoid and non-mucoid variants but with different zonal distribution [Bjarnsholt T *et al*, 2009]; situation that reflects the advantage of diversification for persistence.

Small colony variants (SCV). Another frequent phenotypic variant of chronic stages are the named SCV, which are characterized by their reduced colony size of 1-3 mm. These slow-growing variants have been associated with increased antimicrobial resistance, in particular to aminoglycoside compounds, and a poorer lung function in CF patients [Häussler S *et al*,

1999]. Besides increased aminoglycoside resistance, *P. aeruginosa* SCV can exhibit hyperadherent and autoaggregative behaviors (named rugose SCV, RSCV). These variants can act favoring biofilm formation as showed an increase expression of the *pel* and *psl* exopolysaccharide gene clusters and a decreased expression of flagellum and pilus coding genes [Häussler S *et al*, 2003; Cullen L & McClean S, 2015].

Genetically, the most commonly identified SCV-inducing mutations are loss-of-function mutations in repressor proteins that control the activity of diguanylate cyclases (DGCs) [Malone JG, 2015]. DGCs participate in the production of the ubiquitous bacterial signaling molecule bis-(3',5')-cyclic diguanosine monophosphate which controls a wide range of cellular processes involved in the transition between motile, virulent, and sessile biofilm forming lifestyles [Hengge R, 2009].

Non-motile variants. Early infection of CF airways requires bacterial adhesion to host epithelial cell surfaces, a process that is mediated by flagellum and pilli. By contrast, chronic *P. aeruginosa* isolates are characterized by the lack of twitching and swimming motility due to non-pilation and loss of flagellum, respectively [Mahenthiralingam E *et al*, 1994].

It has been pointed out that this mechanism can enable *P. aeruginosa* to better evade the host immune response, as isolates lacking the flagellum are less effective phagocytosed by alveolar macrophages and polymorphonuclear phagocytes (PMNs). At the genetic level, these variants have been linked to mutations within the *rpoN* gene or to genes participating in flagellum sysnthesis [Mahenthiralingam E *et al*, 1994].

Loss of the Quorum-Sensing (QS) system. In general, *P. aeruginosa* behaves as single cellular organisms in low population densities. However, as cell density increases, bacterial cells can communicate to each other using small signaling molecules inducing changes in gene expression with community purposes. This communication system is known as the QS system and it has been demonstrated to be frequently impaired in late CF isolates [Smith EE *et al*, 2006].

The loss of the QS signaling in *P. aeruginosa* is associated with the presence of mutations in LasR and RhlR. These QS mutants have demonstrated a growth advantage in the presence of low amino acids amount, which is particularly relevant in the CF lungs. Additionally, QS controls the expression of a variety of virulence factors that are generally selected against during CRI [Cullen L & McClean S, 2015]. As well, an increased β-lactamase activity *in vitro* has been documented for these QS mutants, which could be another potential benefit in down regulation of QS mechanisms [D'Argenio DA *et al*, 2007].

Other chronic variants. *P. aeruginosa* variants presenting a modified LPS are also frequent in CF chronic stages of infection [Hancock RE *et al*, 1983; Ernst RK *et al*, 1999]. In Gram-negative bacteria, the LPS is the major component of the outer membrane giving not only

structural integrity but also protecting the bacterial cell from environmental factors and, of course, contributing to cell impermeability. The LPS induces a variety of host immune responses, so its modification may participate in survival and persistance [Hauser AR, 2011]. Within the LPS, three components can be differentiated: (1) the toxic highly acylated lipid A, (2) the central core oligosaccharides and (3) the O-antigen. Structural modification in late CF isolates frequently implies the loss of the O-antigen (as a result of the accumulation of inactivating mutations within the cluster of genes responsible for its production) or an altered lipid A portion (in terms of its pattern of acylation or by the addition of aminoarabinose) [Hauser AR, 2011]. These modifications have important clinical implications as, for instance, it has been demonstrated that the addition of aminoarabinose enhances resistance to antimicrobial peptides and some antibiotics [Ernst RK *et al*, 1999] or that the acylation pattern influences the induced proinflammatory response [Alexander C & Rietschel ET, 2001].

Other adaptive variants that commonly emerge include: auxotrophic variants, pyomelanin hyperproducers, variants which have lost the type III secretion system, variants deficient in pyoverdine and/or pyocianine production, variants resistant to multiple antibiotic compounds and hypermutable variants (sections 1.7. and 1.8.).

1.6. PHYSIOLOGICAL RESISTANCE DURING CYSTIC FIBROSIS CHRONIC RESPIRATORY INFECTIONS

1.6.1. From the planktonic to the biofilm mode of growth

It has been set that the CF lung is a heterogeneous, hostile and stressful environment for invading bacteria. In order to overcome all these challenges, and apart from population diversification, *P. aeruginosa* shifts its mode of growth from free-living cells (planktonic state) to biofilm-forming cells, change that is currently recognized as one of the hallmarks of chronic infections. In fact, both processes are part of the same evolutionary path as all the above described variants (section 1.5.) live together within the biofilm community and contribute to its formation and existence, which constitutes an amazing example of how bacterial populations can enhance its survival and persistence in hostile environments by acting in a cooperative manner.

Biofilms are defined as organized bacterial communities surrounded by an extracellular polymeric matrix that confers resistance against the hostile environment. Biofilm formation classically involves the following stages: attachment, microcolonies formation, biofilm maturation and dispersal or detachment (Figure 1.6.) [O'Toole G *et al*, 2000].
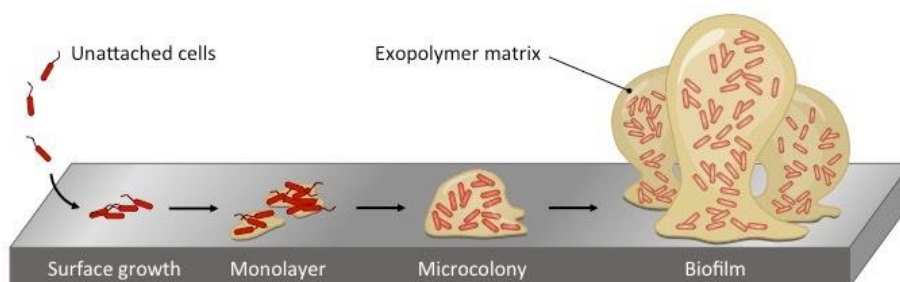
Figure 1.6. Stages of Biofilms formation

As shown, its development starts with the adherence of individual planktonic bacterial cells to a surface with the help of pili and flagella [O'Toole G *et al*, 2000]. Although most biofilm-related infections generally require an attachment to a solid surface, in the case of CF, some studies indicate that the biofilm found in the lung is directly formed on the mucus instead of being in contact with the lung epithelium [Bjarnsholt T *et al*, 2009; Worlitzsch D *et al*, 2002].

Attachment is then followed by bacteria multiplication, thus forming microcolonies, matrix building and eventual biofilm maturation. The extracellular polymeric matrix plays an important role during CRI not only giving cohesion to the structure and acting as a nutrient source but also providing a protective barrier against host defense, desiccation, ROS and antibiotics [Flemming HC & Wingender J, 2010]. This matrix is mainly composed of a conglomerate of exopolyscharides (including alginate provided by mucoid variants), extracellular DNA, proteins, surfactants, lipids, bacterial lytic products and host compounds.

Finally, once matured, biofilm population ensure its persistence in the hostile environment by releasing or dispersing small aggregates or even individual cells to uncolonized sites and reinitiating the biofilm lifecycle during the dispersal stage [O'Toole G *et al*, 2000].

Such a tangled process is known to be closely regulated by intra- and extracellular cues that modulate the levels of diffusible signal molecules, second messengers and small RNAs [Bjarnsholt T, 2013]. QS systems detect these signals as cell density evidences and trigger changes in bacterial gene transcription, including virulence factors and diverse proteins involved in the innate resistance of biofilms to antibiotics and the immune system. In this sense, *P. aeruginosa* biofilms are known to be able to initiate detachment on their own and this process can be mediated by either, alginate lyase overexpression [Boyd A & Chakrabarty AM, 1994] or by up-regulation of motility factors such as the rhamnolipid and type IV pili [Pamp SJ & Tolker-Nielsen T, 2007].

1.6.2. Inherent antimicrobial tolerance of biofilms

One of the most relevant aspects of biofilms is that they determine the persistence of the infection despite long-term antimicrobial treatment. In fact, it is estimated that biofilms can tolerate up to 100–1000 fold higher concentrations of antibiotics than the planktonic cells [Høiby N *et al*, 2010]. As following described, the documented inherent biofilm antimicrobial tolerance is multifactorial (Figure 1.7.).
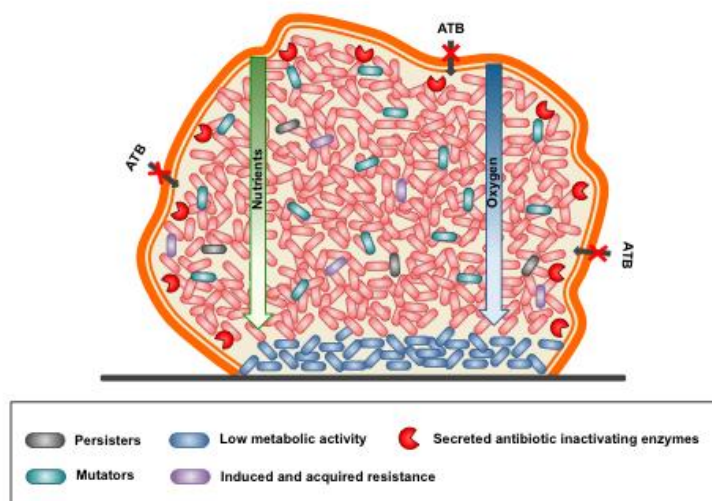
Figure 1.7. Schematic representation of the factors contributing to inherent biofilm antimicrobial resistance. ATB: antibiotics. From: The Problems of antibiotic resistance in CF and soultions. López-Causapé *et al*., Expert Rev Respir Med 2015

Limited antibiotic penetration. The biofilm matrix acts as a primary barrier preventing the entrance of some compounds such as polar and charged antibiotics [Lewis K, 2008]. This restricted penetration has been linked to some components of the matrix such as alginate or eDNA which have shown antibiotic chelating activity [Alipour M *et al*, 2009] or to the

presence of antibiotic-inactivating enzymes within the matrix. As well, eDNA behaves as an antimicrobial shield and contributes to aminoglycoside tolerance [Chiang WC *et al*, 2013; Mulcahy H *et al*, 2008; Walters MC 3rd *et al*, 2003].

Growth rate and nutrient gradients. Internal gradients of biofilms give rise to anaerobic and nutrient-deficient areas, leading to a slowing down of the metabolism. Indeed, several studies have provided evidence that bacterial metabolic activity is high in the outer part of the biolfilm which compares with the inner parts [Walters MC 3rd *et al*, 2003; Bagge N *et al*, 2004; Werner E *et al*, 2004]. The lack of oxygen and the reduced multiplication rates contribute to fluoroquinolones and aminoglycosides' tolerance as these antibiotics targets processes that occur in growing bacteria [Walters MC 3rd *et al*, 2003]. Furthermore, osmotic stress response may also contribute to antibiotic resistance inducing a change in the proportions of porins [Stewart PS & Costerton JW, 2001].

Persister phenomenon. Persisters are defined as a dormant phenotypic state of bacteria within biofilms, characterized by a high tolerance to antibiotics including compounds that kill non-growing cells. Also, this latent bacterial state behaves as a bumper to host defense and may cause a relapse of infection, being a source of recalcitrant biofilm infection [Lewis K, 2010].

Induction of antimicrobial resistance mechanisms. Induction of resistance mechanisms can significantly differ between biofilm and planktonic growth. Indeed, various studies have found a differential expression of several conventional and biofilm-resistance genes in biofilms [Whiteley M *et al*, 2001; Mulet X *et al*, 2011].

Biofilms and mutation-driven resistance. The antibiotic gradient driven by biofilm physiology favors gradual development of mutational resistance during antimicrobial treatment, which is of particular significance when involving mutator strains (section 1.7.) [Oliver A *et al*, 2000; Macià MD *et al*, 2005; Henrichfreisse B *et al*, 2007]. Also, endogenous oxidative stress [Driffield K *et al*, 2008] and mutagenic ROS released from PMNs are likely to induce mutability in biofilm cells. In fact, recent findings have shown that mutagenesis is intrinsically increased in biofilms [Driffield k *et al*, 2008; Boles BR & Singh PK, 2008].

Horizontal gene transfer. Bacterial proximity within a biofilm allows an effective horizontal gene transfer [Bagge N *et al*, 2004]. Moreover, bacterial eDNA may represent a reservoir for the acquisition of exogenous resistance determinants.

All the above described tolerance mechanisms contribute to the persistence of biofilms, which therefore provide a fertile ground for the emergence and selection of antibiotic-resistant mutants.

1.7. HYPERMUTATION: A MARKER OF CYSTIC FIBROSIS CHRONIC RESPIRATORY INFECTIONS

Hypermutable (or mutator) microorganisms are defined as those that have an increased spontaneous mutation rate as a result of defects in DNA repair or error avoidance systems [Miller JH, 1996]. Although the optimal mutation rate for a bacterial population for a perfectly adapted clonal population is close to zero, in the absence of mutations the population could not adapt to environmental changes. Conversely, a high mutation rate is optimal for populations under strong selective pressure but too many mutations would cause a genetic breakdown (Figure 1.8).
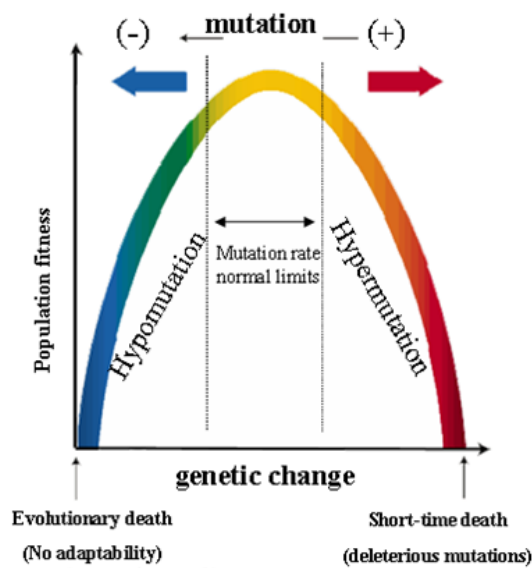
Figure 1.8. Mutation rates and genetic adaptability (fitness). Modified from: Radman 1999.

So, in regular bacterial populations, mutators are present at a rate of the order of 10^5 as a consequence of spontaneous mutations within DNA repair genes or error avoidance systems. Moreover, several investigations have demonstrated that these mutator variants can confer an evolutionary advantage during bacterial adaptation to new or stressful environments as the mutator subpopulation can be dramatically amplified by co-selection (hitchhiking) with other adaptive mutations such as those conferring antibiotic resistance [Cox B & Game J, 1974, Taddei F *et al*, 1997; Mao EF *et al*, 1997; Giraud A *et al*, 2001; Macià MD *et al*, 2006].

1.7.1. Genetic basis for hypermutation

So, in order to adapt and survive in new stressful environments, bacteria can increase their mutation rate and this increase can be either stable or transient.

The stable mutator phenotype is consequence of a defect in one of the several DNA repair or error avoidance systems, the so-called (anti) mutator genes. Several mutator genes with different effects onto the mutation rate have been described (Table 1.2.).

By far, the most frequent cause of hypermutation in natural bacterial populations is the presence of defects on the methyl-directed mismatch repair (MMR) system [Miller JH, 1996; Oliver A, 2010; Oliver A & Mena A, 2010]. The MMR system, which is present in all organisms, detects and repairs DNA replication errors including any kind of mispairs and short insertions or deletions. Besides, this system is the most potent inhibitor of recombination between weekly and moderately diverged sequences. Key components of the

system include the proteins encoded in *mutS* (PA3620), *mutL* (PA4946), *mutH* and *uvrD/mutU* (PA5443) genes. MutS first recognizes and binds to the mismatched DNA initiating the MMR machinery. Then, MutL interacts with MutS and together activate the endonuclease MutH that cleaves the non-methylated strand containing the mismatch. As well, MutL loads the DNA helicase II (UvrD/MutU) onto the DNA which is finally unwinded, excised and repaired by other MMR components. In the particular case of *P. aeruginosa*, mutH homologues do not exist and the recognition of the daughter DNA strain is therefore not based on methylation. Thus, in natural *P. aeruginosa* populations, inactivating mutations within *mutS*, *mutL* and *uvrD* genes can lead to higher mutation rates (from 100- to 1,000-fold) and increased rates of homologous recombination. In the CF setting, up to 60–90% of the mutator variants have a defective MMR system, mainly caused by mutation within *mutS* or *mutL* [Mena A *et al*, 2008; Montanari S *et al*, 2007; Oliver A *et al*, 2002a; Ciofu O *et al*, 2010].

Table 1.2. Principal mutator genes, most functionally characterized in *Escherichia coli*. From: Oliver A & Mena A. Bacterial hypermutation in cystic fibrosis, not only for antibiotic resistance. *Clin Microbiol Infect*. 2010; 16(7):798-808.

Gene	Product activity	Mutations produced	Mutator effect
mutD (*dnaQ*)	E subunit of DNA pol III, proofreading activity	All base substitutions, frameshifts	Very strong
MMR system			
mutS	DNA mismatch recognition, binds mismatches	GC → AT, AT → GC, frameshifts	Strong
mutL	Interacts with MutS and MutH		
mutH	Endonuclease, nicks hemi-methylated GATC sequences		
uvrD	DNA Helicase II, strand displacement		
GO system			
mutT	Nucleoside triphosphatase, prevents incorporation of 8-oxoG to DNA	AT → CG	Strong
mutM	DNA glycosylase, removes 8-oxoG from 8-oxoG-C mispairs	G:C → T:A	Weak
mutY	DNA glycosylase, removes A from 8-oxoG-A or A-G mispairs	G:C → T:A	Moderate
Prevention of oxidative damage			
mutA	GlyV, glycyl tRN	AT → TA, GC → TA, AT → CG	Weak-moderate
mutC	GlyW, glycyl tRNA	AT → TA, GC → TA, AT → CG	Weak-moderate
ung	Uracil glicosylase, removes U from U-G mispair	GC → TA	Weak-moderate
sodA, sodB	Superoxide dismutase, removes superoxide radicals	AT → TA	Weak
oxyR	Regulates hydrogen peroxide inducible genes	AT → TA	Weak
polA	DNA polymerase I	Frameshifts, deletions	Weak-moderate

As well, although not found in natural populations, mutations in *dnaQ/mutD* (PA1816) can lead to strong mutator phenotypes (up to 10,000 fold) and reduced growth rates [Miller JH *et al*, 1996; Oliver A, 2010; Oliver A & Mena A, 2010].

Other mutator genes in *P. aeruginosa* include those of the GO system, which prevent mutations caused by the oxidative lesion mediated by the 7,8-dihydro-8-oxo-deoxyguanosine (8-oxodG or GO) (Table 1.2.) [Oliver A *et al*, 2002b].

Finally, mutations within those genes involved in the prevention of oxidative damage produced by ROS, such as *oxyR* (PA5344), *sodA/sodM* (PA4468), *sodB* (PA4366), mutator tRNAs (*mutA* and *mutC*), *pfpI* (PA0355), *ung* (PA0750), *mfd* (PA3002), *radA* (PA4609) and *polA* (PA5493) genes have also been linked with mutator phenotypes (Table 1.2.) [Oliver A & Mena A, 2010; Oliver A, 2010].

In addition to the stable mutator phenotype, under particular circumstances, such as when DNA is damaged, a transient mutator phenotype can rise by the induction of the of the error-prone DNA polymerases (IV and V) as part of the SOS response [Friedberg EC & Gerlach VL, 2002; Foster PL, 2007]. Of note, some antibiotics compounds may induce this phenotype which in turns promotes the appearance of antibiotic resistance [Blázquez J *et al*, 2002; Pérez-Capilla T *et al*, 2005].

1.7.2. Prevalence of *P. aeruginosa* mutators in the CF airways

CRI by *P. aeruginosa* in CF patients was the first natural model to reveal a high and unusual prevalence of mutator variants in natural bacterial populations [Oliver A *et al*, 2000].

Prevalence of *P. aeruginosa* hypermutable variants in the CF airways is extremely high, ranging from 30% to 60% [Oliver A, 2010; Montanari S *et al*, 2007; Mena A *et al*, 2008; Ciofu O *et al*, 2005; Marvig RL *et al*, 2013]. Moreover, their proportion significantly increases during the course of CRI as was demonstrated in a 25-year longitudinal study in which the proportion of hypermutable isolates increased from 0% at the onset/early colonization to 65% in late stages [Ciofu O *et al*, 2005].

This unusual high prevalence among *P. aeruginosa* isolates from CF patients compares with the documented prevalence among isolates from environmental sources (6%) or from acute infections (<1%) [Kenna DT *et al*, 2007; Oliver A *et al*, 2000; Gutiérrez O *et al*, 2004; Mulet X *et al*, 2013] and can be explained in terms of hitchhiking as an important role of mutators in adaptive mechanisms [Mena A *et al*, 2008] and in the development of antimicrobial resistance has already been proven [Oliver A *et al*, 2000; Macià MD *et al*, 2005; Henrichfreise B *et al*, 2007].

1.7.3. Hypermutation drivers in the CF airways

Because of the high prevalence of mutator variants encountered in the CF airways, nowadays the chronically infected CF airways are considered to be a mutagenic context, in which both, intrinsic and extrinsic factors are implied [Rodríguez-Rojas A *et al*, 2012; Oliver A, 2010; Oliver A & Mena A, 2010].

In the CF airways, the level of ROS is high mainly due to the increased availability of iron and because the antioxidant mechanisms in CF patients are highly diminished. ROS cause DNA damage and can further increase the inflammatory response, which eventually lead to the establishment of a vicious cycle of inflammation and hypermutation [Rodríguez-Rojas A *et al*, 2012; Oliver A, 2010; Oliver A & Mena A, 2010].

Also the biofilm mode of growth may itself be directly implied in the documented increase mutability. In this sense, *Driffield* and colaborators demonstrated that *P. aeruginosa* antioxidant enzymes coding genes (*katA*, *sodB*, *ahpC* and PA3529) are down-regulated when growing in biofilms compared to planktonic cells [Driffield K *et al*, 2008], which eventually lead to a decrease protection against oxidative mutagenesis. In addition, *Boles* and *Singh* documented that double-strand break mutations tend to occur more frequently within biofilms [Boles BR & Singh PK, 2008]. Besides, through competition experiments it has been demonstrated that *P. aeruginosa* MMRS-deficient variants exhibit enhanced adaptability over WT strains when grown in structured biofilms [Luján AM *et al*, 2011]. As well, Conibear *et al*. demonstrated that the presence of mutator variants can enhance microcolony-based growth initiation and, therefore, the biofilm development [Conibear TC *et al*, 2009]. All these findings suggest a strong and bidirectional link between the biofilm mode of growth and hypermutation.

Finally, the wide use of antibiotics in CF patients also contributes to mutagenesis. Several studies have demonstrated that, when administered at sublethal concentrations, antimicrobials can drive bacterial mutation [Rodríguez-Rojas A *et al*, 2012; Oliver A, 2010; Oliver A & Mena A, 2010]. Within the biofilm sublethal antibiotic concentrations are not rare as the extracellular polymeric matrix acts as a primary barrier preventing the entrance of polar and charged antibiotics [Lewis K, 2008] and, additionally, some of the matrix components such as the alginate or the extracromosomic DNA have shown antibiotic chelating activity [Alipour M *et al*, 2009].

1.7.4. Mutators and antibiotic resistance

Since the first description of hypermutable *P. aeruginosa* strains was made in CF CRI, a strong linkage between mutators and increased antibiotic resistance was noticed as mutators were encountered to be much more resistant than non-mutator CF isolates to each of the eight antipseudomonal agents tested. For instance, the percentage of ceftazidime

resistance reached 80% in hypermutable strains in contrast to a 30% documented for non-mutators; likewise, fluoroquinolone resistance increased from 5% in non-mutators to 40% in mutator variants [Oliver A *et al*, 2000].

Subsequent studies have confirmed and extended this observation, establishing a clear link between mutator phenotypes and multidrug resistant (MDR) profiles [Ciofu O *et al*, 2005; Henrichfreisse B *et al*, 2007; Hogardt M *et al*, 2007; Ferroni A *et al*, 2009]. Current management of CF patients include wide use of antibiotics, so this finding supports that amplification of mutators during CRI occurs along with the selection of antibiotic resistance mutations (adaptive mutations).

Given the high prevalence of P. aeruginosa mutator variants in the CF setting, one of the aims of this work was to define their impact in molecular epidemiology and in antibiotic resistance evolution and spread.

1.8. ACQUIRED ANTIBIOTIC RESISTANCE

In addition to its remarkable intrinsic resistance, *P. aeruginosa* shows an extraordinary capacity for further developing resistance to all available antibiotics. In general, bacteria can increase its intrinsic resistance by either acquiring horizontal resistance determinants and/or through the selection of certain chromosomal mutations that alter their expression and/or function.

1.8.1. Transferable resistance determinants in CF isolates

The CF airway hosts a complex microbiome [Lim YW *et al*, 2014] where genetic exchange could theoretically occur effectively, thus theoretically contributing to the emergence of antibiotic resistance. Most mobile antibiotic resistance genes are encoded on plasmids and transposons, but a recent study have also suggested that phages may also play an important role in the CF setting as the CF virome encodes more antimicrobial resistance sequences than the non-CF virome [Fancello L *et al*, 2011].

Among the transferable resistance determinants, extended spectrum b-lactamases and carbapenemeses are widely distributed worldwide but, with some exceptions, horizontal gene transfer of resistance determinants seems not to be frequent in *P. aeruginosa* [Oliver A *et al*, 2015], especially among CF isolates. Although biofilms are known to provide cell-to-cell contact and stabilise mating pair formation, biofilms theirselves appear to limit the horizontal plasmid spread through a combination of physicochemical and biological factors inherent to the spatial structure and heterogeneity of these structures [Stalder T & Top E, 2016]. However, it should be mentioned that although rare, in late years some authors have reported several cases of CF patients infections with *P. aeruginosa* isolates producing ESBL and/or carbapenemases, including IMP and VIM metallo- β-lactamases [Agarwal G *et al*, 2005; Cardoso O *et al*, 2008; Pollini S *et al*, 2011].

1.8.2. Mutation-driven resistance

In comparison with *P. aeruginosa* isolates causing acute infections, mutation-driven resistance has been shown to be the major contributor to antimicrobial resistance development in CF *P. aeruginosa* isolates [Ferroni A *et al*, 2009], development which is indeed catalyzed by the unusual high prevalence of mutators in the CF airways.

All antibiotics compounds are prone to being compromised by acquiring mutations that eventually lead to alter the expression of chromosomally-encoded resistance mechanisms or that modify the function of its encoded-protein. In *P. aeruginosa*, major mutational resistance mechanisms include overexpression of the chromosomal AmpC cephalosporinase, efflux pumps overexpression, porin loss or altered antibiotic targets (Table 1.3.).

Table 1.3. Mutation-driven resistance mechanisms in *P. aeruginosa*.

Mutation	Resistance mechanisms / Altered target	Antibiotics affected[a]
gyrA, gyrB	DNA gyrase	FQ
parC, parE	DNA topoisomerase IV	FQ
pmrAB	LPS (lipid A)	CO
phoQ	LPS (lipid A)	CO
colRS	LPS (lipid A)	CO
cprS	LPS (lipid A)	CO
parRS	LPS (lipid A)	CO
	OprD downregulation	IP, MP
	MexEF-OprN hyperproduction	FQ
	MexXY-OprM hyperproduction	FQ, AMG, PM
mexR	MexAB–OprM hyperproduction	FQ, TZ, PM, PPT, MP
nalC	MexAB-OprM hyperproduction	FQ, TZ, PM, PPT, MP
nalD	MexAB-OprM hyperproduction	FQ, TZ, PM, PPT, MP
nfxB	MexCD-OprJ Hyperproduction	FQ, PM
mexS	MexEF-OprN hyperproduction	FQ
	OprD downregulation	IP, MP
mexT	MexEF-OprN hyperproduction	FQ
	OprD downregulation	IP, MP
mvaT	MexEF-OprN hyperproduction	FQ
mexZ	MexXY –OprM hyperproduction	FQ, AMG, PM
PA5471.1	MexXY –OprM hyperproduction	FQ, AMG, PM
amgS	MexXY –OprM hyperproduction	FQ, AMG, PM
oprD	OprD porin inactivation	IP, MP
ampC	AmpC structural modification	PPT, TZ, PM, IP, MP
ampD	AmpC hyperproduction	TZ, PM, PPT
ampDh2	AmpC hyperproduction	TZ, PM, PPT
ampDh3	AmpC hyperproduction	TZ, PM, PPT
ampR	AmpC hyperproduction	TZ, PM, PPT
dacB	AmpC hyperproduction	TZ, PM, PPT
glpT	Transporter protein GlpT	FO
rpoB	RNA polymerase β-chain	RIF

β-lactam resistance mechanisms. Development of resistance to antipseudomonal penicillins (ticarcillin and piperacillin), cephalosporins (ceftazidime and cefepime) and monobactams (aztreonam) is the selection of mutations within PGN-recycling genes (*ampD, dacB, ampR*) that eventually leads to the constitutive overexpression of the chromosomal cephalosporinase AmpC [Cabot G *et al*, 2011; Juan C *et al*, 2005; Moyà B *et al*, 2009]. Besides *ampC* overexpression, recent studies have revealed that β-lactam resistance development, including novel β-lactam-β-lactamase inhibitor combinations such as ceftolozane/tazobactam, may also result from mutations leading to the structural modification of AmpC [Cabot G *et al*, 2014; Lahiri SD *et al*, 2014].

Introduction ···

Beyond the chromosomal cephalosporinase AmpC, another contributing factor to β-lactam resistance is MexAB-OprM overexpression. This efflux system displays the broadest substrate profile (Table 1.1. and 1.3.) and its mutational overexpression determines reduced susceptibility to all β-lactams with the single exception of imipenem. MexAB-OprM-overproducing mutants can be readily generated *in vitro* in the presence of antibiotic by the selection of any mutational event leading to the inactivation or impairment of the *mexR*, *nalC* or *nalD* regulator genes. As well, these mutants have been shown to be very prevalent among multiresistant non-CF strains, and, of note, rates of MexAB-OprM overproducers of near 50% have been recorded in subpopulations of isolates exhibiting a reduced susceptibility to ticarcillin (≥32 μg/ml) [Li XZ *et al*, 2015].

Likewise, the mutational overexpression of MexXY or MexCD-OprJ can also confer resistance to cefepime. MexCD-OprJ overexpression, which is more frequent among *P. aeruginosa* isolates recovered from CRI not only confers increased cefepime resistance but have also been shown to determine hypersusceptibility to most β-lactams and aminoglycosides [Mulet X *et al*, 2011].

Finally, screenings of transposon mutant libraries have shown that inactivation of *galU*, a gene which code for an enzyme involved in the LPS core, increases ceftazidime and meropenem minimum inhibitory concentrations (MICs) [Dötsch A *et al*, 2009; Álvarez-Ortega C *et al*, 2010].

Carbapenem resistance mechanisms. Mutational inactivation of the porin OprD, together with the inducible expression of AmpC, confers resistance to imipenem and reduced susceptibility to meropenem [Livermore DM *et al*, 1992]. Indeed, the prevalence of imipenem resistant isolates frequently exceeds 20%, and nearly all them are OprD deficient [Cabot G *et al*, 2011; Riera E *et al*, 2011]. As well, MexAB-OprM mutational overexpression determines reduced susceptibility to meropenem and its overexpression plus OprD inactivation is one of the most relevant causes of clinical resistance to this carbapenem [Riera E *et al*, 2011].

Finally, although less frequent, mutation-driven resistance to carbapenems can also result from MexEF-OprN overexpression, as mutations within *mexT, mexS* and/or the ParRS two-component system not only lead to MexEF-OprN overexpression but also to OprD downregulation, which in turns determine a reduced susceptibility to carbapenems [Köhler T *et al*, 1999; Li XZ *et al*, 2015].

Aminoglycoside resistance mechanisms. Resistance to this antibiotic class has been clasically linked to the mutational overexpression of MexXY efflux pump, being its overexpression very frequent among clinical isolates and mainly caused by *mexZ*, *amgS*, or *parRS* mutations [Guénard S *et al*, 2014]. However, recent studies have revealed that the aminoglycoside mutational resistome extends far beyond MexXY overexpression and

several novel resistance determinants have been described; moreover accumulation of mutations within these genes can eventually lead to high-level antibiotic resistance [El'Garch F *et al*, 2007; Schurek KN *et al*, 2008].

Fluoroquinolone resistance mechanisms. Fluoroquinolone resistance in *P. aeruginosa* frequently results from gain-of-function mutations in topoisomerases, including DNA gyrases (GyrA/GyrB) and type IV topoisomerases (ParC/ParE) [Bruchmann S *et al*, 2013].

Besides, overexpression of all 4 major efflux-pumps systems also contributes to fluoroquinolone resistance (Table 1.1. and 1.3.). The overexpression of MexAB-OprM and MexXY-OprM is globally more frequent among clinical strains but its contribution to clinical fluoroquinolone resistance is likely more modest [Bruchmann S *et al*, 2013]. On the other hand, mutational overexpression of MexEF-OprN or MexCD-OprJ efflux pump is associated with high-level fluoroquinolone resistance, and although their prevalence is considered low except in the CF-CRI setting, recent data show that it might be higher than expected [Richardot C *et al*, 2015].

Polymyxin resistance mechanisms. The prevalence of polymyxin (polymyxin B and colistin) resistance is still very low (<5%) among *P. aeruginosa* isolates. Resistance to polymyxins most frequently results from the modification of the LPS caused by the addition of a 4-amino-4-deoxy-l-arabinose moiety in the lipid A structure [Olaitan AO *et al*, 2014] and the underlying mutations are frequently tracked to the PmrAB or PhoPQ two-component regulators, which in turns lead to the activation of the arnBCADTEF operon [Barrow K & Kwon DH, 2009]. More recent studies have also revealed that mutations within the two-component regulator ParRS, in addition to conferring colistin resistance due to the activation of the arnBCADTEF operon, lead to a MDR profile caused by the overprexpression of MexXY, MexEF and the repression of OprD [Muller C *et al*, 2011]. Finally, two additional two-component regulators, ColRS and CprRS, have also been shown to play a role in polymyxin resistance [Gutu AD *et al*, 2013]. Moreover, recent *in vitro* evolution assays have revealed, through WGS, the implication of additional mutations in high level colistin resistance, facilitated by the emergence of mutator (*mutS* deficient) phenotypes [Döβelmann B *et al*, 2017]. Particularly noteworthy among them are those occurring in LptD (essentaial OMP involved in LPS transport), LpxC (UDP-3-O-[hydroxymyristoyl]-N-acetylglucosamine deacetylase involved in lipid A biosynthesis) or MigA (α-1,6-rhamnosyltransferase, involved in the synthesis of the LPS core region [Döβelmann B *et al*, 2017].

P. aeruginosa possesses a complex and large genome, thus, and given the current gaps and the crutial role of mutatrion-driven resistance mechanisms for acquiring antibiotic resistance, one of the aims of this work was to decipher the P. aeruginosa mutational resistome.

1.9. *P. aeruginosa* POPULATION STRUCTURE: CF EPIDEMIC CLONES

Bacterial population structures can range from panmintic or fully sexual, showing random association between loci due to unrestricted recombination (such as *Neisseria gonorrhoeae*), to clonal, characterized by non-random association of alleles and evolving mainly through mutation (such as *Salmonella enterica*) [Smith JM *et al*, 1993].

Early studies suggested a panmintic population structure for *P. aeruginosa* [Denamur E *et al*, 1993; Picard B *et al*, 1994]. Kiewitz and Tümmler later described that *P. aeruginosa* shows a net-like population structure with a high frequency of recombination between isolates [Kiewitz C & Tümmler B, 2000]. Wide consensus was finally reached to conclude that *P. aeruginosa* has a non-clonal epidemic population structure [Curran B *et al*, 2004; Kidd TJ *et al*, 2012; Maâtallah M *et al*, 2011; Pirnay JP *et al*, 2009; Pirnay JP *et al*, 2002]. This means that the population structure of *P. aeruginosa*, similarly to that described in *N. meningitidis*, is composed of a limited number of widespread clones which are selected from a background of a large number of rare and unrelated genotypes that are recombining at high frequency.

Population structure analysis have also revealed that *P. aeruginosa* contains a conserved core and an accessory genome made up of extrachromosomal elements, such as plasmids and blocks of DNA inserted into the chromosome at several loci [Klockgether J *et al*, 2011]. The accessory genome is believed to be acquired through horizontal gene transfer (frequently phage-mediated) from different sources including other species.

Several experimental approaches have been used to define the population structure of *P. aeruginosa,* ranging from single loci to whole genome, mapping or sequencing [Curran B *et al*, 2004; Denamur E *et al*, 1993; Maâtallah M *et al*, 2013; Pirnay JP *et al*, 2009; Wiehlmann L *et al*, 2007]. Strategies based in the combined analysis of up to eight different genomic markers [O-serotype, total genome profile by fluorescent amplified-fragment length polymorphism analysis, nucleotide sequence of the OMP genes (*oprI, oprL*, and *oprD*), pyoverdine receptor gene profile (*fpvA* type and *fpvB* prevalence), prevalence of exoenzyme genes *exoS* and *exoU* and prevalence of group I pilin glycosyltransferase gene *tfpO*] [Pirnay JP *et al*, 2009] or a 58-binary genotypic markers microarray [Cramer N *et al*, 2012; Wiehlmann L *et al*, 2007] have provided very useful and complete information on the core and accessory genomes to define *P. aeruginosa* population structure. *P. aeruginosa* widespread C or PA14 clones, O11/O12 MDR nosocomial clones, or the LES were likely the most notorious successful epidemic lineages identified. Increasing access to WGS data is providing even more detailed information on the population structure and dynamics of epidemic and nonepidemic strains [Cramer N *et al*, 2011; Dettman JR *et al*, 2013; Jeukens J *et al*, 2014; Yang L *et al*, 2011]. However, despite only providing information on the core genome, the likely most popular standardized approach for the analysis of *P. aeruginosa* populations is still the multilocus sequence typing (MLST) scheme developed by *Curran et*

al. in 2004, based in the sequencing of 7 locci evenly distributed in the core genome of *P. aeruginosa*. They include *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE*, considered housekeeping genes, and therefore not subjected to positive selection. However, mutation of *mutL*, encoding a component of the DNA MMR system, is a frequent cause of the mutator phenotypes, positively selected in CRI [García-Castillo M *et al*, 2012; Kidd TJ *et al*, 2012; Mena A *et al*, 2008; Oliver A *et al*, 2002a].

The MLST database (http://pubmlst.org/paeruginosa), although biased by the deposition of only a small fraction of the isolates, is a source of very valuable epidemiological information. As shown in Figure 1.9., most of the registered STs (June 4th 2015) are represented by single isolates but up to 18 of them are represented by more than 10 isolates from at least three different countries, thus likely indicating that they are successful clones. Among others they include the wide spread clone C (ST17) and PA14 (ST253) clones, the high-risk clones associated with MDR or extensively drug resistant (XDR) nosocomial infections (such as ST111, ST175, or ST235) or the CF epidemic clone ST146 (LES). Of note, many of these frequent clones are the founder clones of one of the 297 clonal groups or complexes detected by eburst analysis, each including from 2 to 116 STs [Oliver A *et al*, 2015].

As mentioned previously in section 1.5., during long time it was extensively accepted that early *P. aeruginosa* acquisition occurs from environmental sources. However, successful strains infecting a large proportion of CF patients are nowadays recognized and, in some cases, strongly associated with MDR profiles.
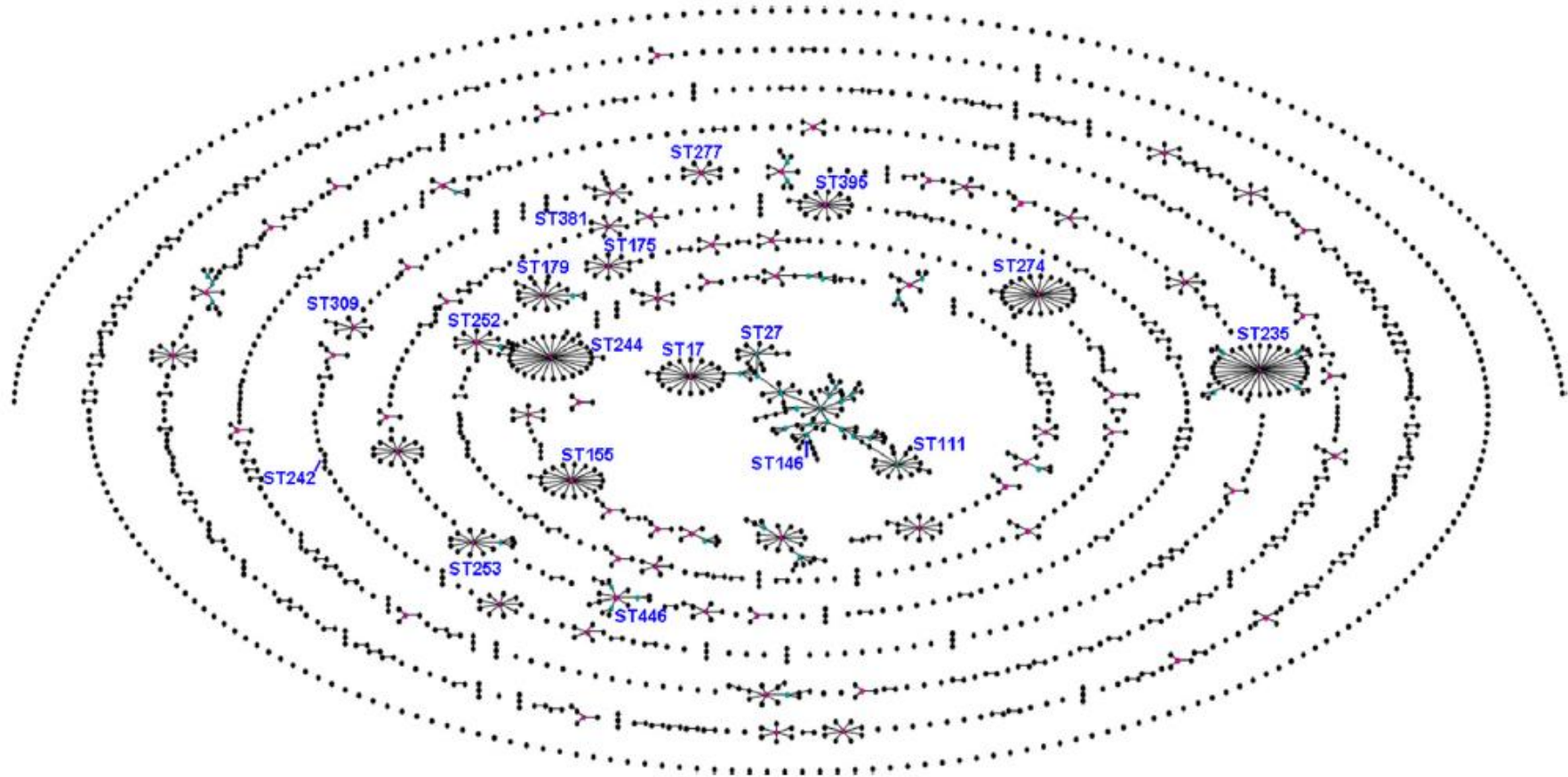
Figure 1.9. Population snapshot of *P. aeruginosa*. The 2106 STs listed on the *P. aeruginosa* PubMLST database (http://pubmlst.org/paeruginosa, 2015/06/03) are displayed in a single eBURST diagram by setting the group definition to zero of seven shared alleles. Each dot representes a ST, and lines connect single-locus variants. In each group of related STs the predictive primary founder is shown in pink, and subgroup founders are shown in blue. STs detected in at least 3 different countries with more than 10 recorded isolates are indicated; note that the ST corresponds to the primary founder or subgroup founder of the CC.

1.9.1. The Liverpool Epidemic Strain: a new paradigm in the CF setting

The LES is likely the most prominent epidemic clone infecting and chronically colonizing CF patients. It was originally described in the mid-1990s affecting a unique CF center in Liverpool [Cheng K *et al*, 1996] but it was soon detected in other CF centers across England [Scott FW & Pitt TL, 2004] and Scotland [Edenborough FP *et al*, 2004], and more recently, it has also been detected in CF patients from Canada [Aaron SD *et al*, 2010].

LES isolates show some unique characteristics. For instance, it cannot only infect previously uncolonized patients but also patients already colonized with unique *P. aeruginosa* strains [McCallum SJ *et al*, 2001] as well as non-CF patients [McCallum SJ *et al*, 2002]. Moreover, several studies have demonstrated that patients chronically infected with LES strains have a worse prognosis when compared with patients chronically colonized with unique non-clonal strains [Aaron SD *et al*, 2010; Al-Aloul M *et al*, 2004; Ashish A *et al*, 2012]; however, fortunately, being infected with this clone was not associated with poorer post-transplant outcomes [Srour N *et al*, 2015]. When compared with other CF strains, LES isolates are more frequently resistant to antibiotics and further resistance is also more likely to be developed over time [Ashish A *et al*, 2012; Salunkhe P *et al*, 2005; Tomás M *et al*, 2010].

In order to determine the underlying factors explaining the success of this epidemic clone among CF patients, environmental surveys have been performed but, to date, environmental LES isolates have only been detected in close temporospatial proximity to LES-colonized patients and therefore its high transmissibility cannot be explained by long-term environmental persistence [Panagea S *et al*, 2005]. Therefore, its successful transmission and lung colonization might be due to intrinsic phenotypic and genotypic features.

An unusual phenotype characterized by the overproduction of QS-regulated exoproducts, including pyocyanin and elastase A, is common among LES isolates and can persist within CF patients for several years. As this virulence-related exoproducts have a number of toxic effects directly relevant to CF, this unusual phenotype could explain the greater morbidity and mortality associated LES isolates and may play an important role in the success of this clone [Fothergill JL *et al*, 2007]. Moreover, it has been demonstrated that LESB58 produced more biofilm but was less motile than PAO1 and PA14, properties that can favor its persistence in the CF airway [Kukavica-Ibrulj I *et al*, 2008].

At the genomic level, this epidemic strain also exhibits some unique and relevant features. LESB58 was the first LES isolate sequenced and an accessory genome encoding many large genomic islands and prophages not previously found in other sequenced *P. aeruginosa* strains was revealed [Winstanley C *et al*, 2009]. At first, the presence of this accessory genomic material was thought to be essential for *in vivo* competitiveness [Winstanley C *et al*, 2009] but further comparative genomic studies with other representative LES isolates have

revealed a wide diversity in prophage and genomic islands among isolates [Carter ME *et al*, 2010; Jeukens J *et al*, 2014] even during very short episodes of exacerbations and antibiotic therapy [Fothergill JL *et al*, 2010; Fothergill JL *et al*, 2011]. Recently, it has been also suggested that LES phages may play an important role in host invasions and may confer a large fitness advantage during mixed infections by mediating bacteria-bacteria competition [Burns N *et al*, 2015]. Thus, this accessory genomic material seems to play an important role but further research is needed.

Another interesting point is the common coexistence of distinct LES lineages, exhibiting widely variable phenotypic and genotypic characteristics, within individuals [Williams D *et al*, 2015]. Nevertheless, this characteristic is not exclusive for this lineage and some authors have already demonstrated the coexistence of distinct lineages in the respiratory tract for other successful CF strains [Feliziani S *et al*, 2014, Marvig RL *et al*, 2013]. Thus, it seems that the phenotypic and the genetic diversity observed among LES isolates play a key role in the successful spread of this lineage throughout the CF population, diversity that is indeed enhanced by the frequent presence of mutators in the CF lung [Oliver A *et al*, 2000].

1.9.2. Other successful CF strains

Epidemic strains have also been detected in Australia, the so-called Australian Epidemic Strains AES-1 (also denominated Melbourne Epidemic Strain), AES-2, a cluster of related strains [Anthony M *et al*, 2002; Armstrong D *et al*, 2003; Armstrong DS *et al*, 2002] and, the more recently described, AES-3 [Bradbury R *et al*, 2008]. AES show increased antibiotic resistance, increased virulence gene expression and higher morbidity and mortality during CRI [Armstrong D *et al*, 2003; Griffiths AL *et al*, 2012; Hare NJ *et al*, 2012; Manos J *et al*, 2009; Naughton S *et al*, 2011; Tingpej P *et al*, 2010]. As occurs for LES, AES has not been recovered from any source other than the respiratory secretions of CF patients [Bradbury RS *et al*, 2009; Cramer N *et al*, 2012].

Besides these well-recognized CF epidemic strains, other *P. aeruginosa* MDR transmissible strains have been reported in European countries or Canada [Fluge G *et al*, 2001; Jelsbak L *et al*, 2007; Jones AM *et al*, 2001; Logan C *et al*, 2012; Luna RA *et al*, 2013; Parkins MD *et al*, 2014; Pedersen SS *et al*, 1986; Scott FW & Pitt TL, 2004; van Mansfeld R *et al*, 2009]. Overall, these strains show increased antibiotic resistance, long-term persistence within individuals, and are frequently associated with higher morbidity and mortality. Worldwide distribution of these strains is shown in Figure 1.10.
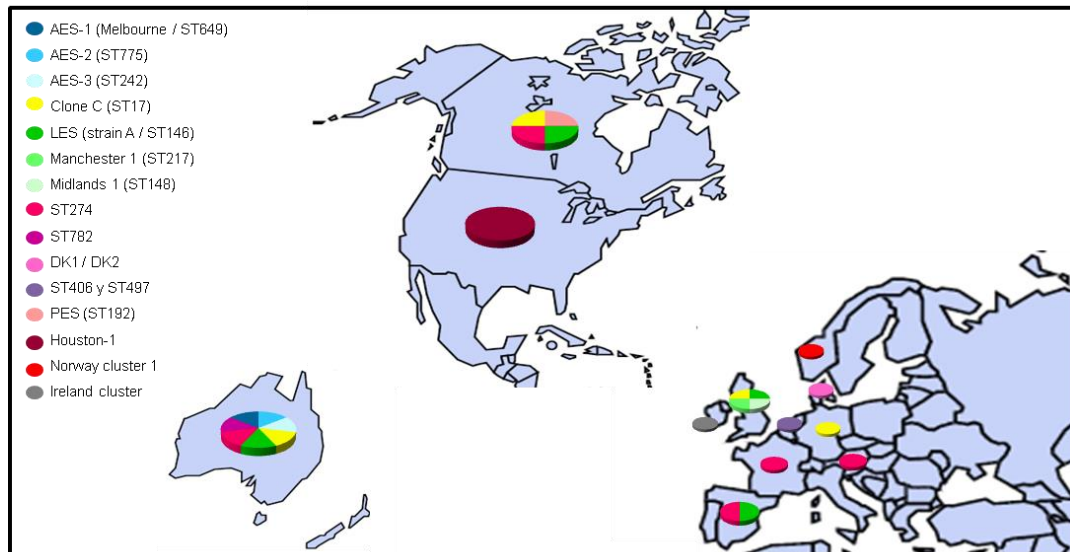
Figure 1.10. Worldwide distribution of *P. aeruginosa* CF epidemic/transmissible strains.

P. aeruginosa CF clonal epidemiology has remained unexplored among CF patients from the Balearic Islands and Spain. Thus, given the relevance of epidemic clones for CF patients outcome and management, one of the aims of this work was to define the *P. aeruginosa* population structure and to explore the presence of highly transmissible *P. aeruginosa* clones in our setting.

2. HYPOTHESIS AND OBJECTIVES

El alma y el cuerpo

P. aeruginosa CRI is the main cause of morbidity and mortality in CF individuals. One of the hallmarks of these infections, led by the opportunistic pathogen *P. aeruginosa*, is their long-term (lifelong) persistence despite of the host immune response and intensive antimicrobial therapies. Naturally equipped with a set of chromosomal genes that confer resistance to some antibiotic compounds (intrinsic resistome), *P. aeruginosa* can further develop resistance to virtually all available antimicrobials. Antimicrobial resistance in CF is a multifactorial problem which not only includes bacterial physiological changes, represented by the transition from the planktonic to the biofilm mode of growth, but also the acquisition of multiple chromosomal antibiotic resistance (mutational resistome) and adaptive mutations that eventually lead to a diversified infecting *P. aeruginosa* population. As well, in late years, there is increasing evidence suggesting that adaptation to the CF respiratory tract and antimicrobial resistance development may escape from the scale of the individual patients (epidemic strains).

Besides, recent advances in sequencing technologies have made it possible to obtain the whole genome of bacterial pathogens shaping up a new dimension to explore CF *P. aeruginosa* clonal epidemiology and antimicrobial resistance evolution. Therefore, the objectives of this thesis were:

1. To define the population structure of *P. aeruginosa* isolates infecting CF patients from the Balearic Islands and Spain.

2. To perform a longitudinal and cross-sectional analysis of the antibiotic susceptibility profiles and resistance mechanisms of CF *P. aeruginosa*.

3. To determine the role of mutators onto CF *P. aeruginosa* clonal epidemiology and onto the evolution and spread of antibiotic resistance.

4. To characterize by WGS approaches the phylogeny and evolution of widespread *P. aeruginosa* CC274.

5. To decipher the *P. aeruginosa* CC274 resistome evolution.

6. To assess the evolutionary dynamics and mechanisms of aminoglycoside resistance development *in vitro* and *in vivo*, given their key role in the management of CF patients.

3. MATERIALS AND METHODS

Palabras incomprendidas

3.1. LABORATORY STRAINS, PLASMIDS AND PRIMERS

Table 3.1. Laboratory strains and derived mutants used in this work.

Strain	Genotype / relevant characteristics	Reference
P. aeruginosa		
PAO1	Laboratory reference strain fully sequenced	Stover CK *et al*, 2000
PA14	Laboratory reference strain fully sequenced	He J *et al*, 2004
ATCC®27853™	Laboratory reference strain	
PAOAD	PAO1 Δ*ampD*::lox AmpD is an N-acetyl-anhydromuramyl-L-alanine amidase involved in PGN recycling as well as a negative transcriptional regulator of the *P. aeruginosa* chromosomal β-lactamase AmpC. Its inactivation increases the AmpC expression level.	Juan C *et al*, 2006
PAOMxR	PAO1 Δ*mexR*::lox MexR is a negative transcriptional regulator of *P. aeruginosa* efflux pump MexAB-OprM. Its inactivation results in overexpression.	Mulet X *et al*, 2011
PAOMxZ	PAO1 Δ*mexZ*::lox MexZ is a negative transcriptional regulator of *P. aeruginosa* efflux pump MexXY-(OprM). Its inactivation results in overexpression.	Martínez-Ramos *et al*, 2014
PAONB	PAO1 Δ*nfxB*::lox NfxB is a negative transcriptional regulator of *P. aeruginosa* efflux pump MexCD-OprJ and its own. Its inactivation increases overexpression.	Mulet X *et al*, 2009
PAOD1	PAO1 OprD null spontaneous mutant (W65X) OprD is an OMP related with carbapenems extrusion.	Moyà B *et al*, 2010
PAOMS	PAO1 Δ*mutS*::lox MutS is a component of the DNA MMR system. Its inactivation increases the spontaneous mutation rate by 2-3 log.	Mena A *et al*, 2008

Table 3.2. Plasmids used in this work

Plasmids	Genotype /relevant characteristics	Reference
pUCP24	Gentamycin[R], based in pUC18, *Escherichia-Pseudomonas* shuttle vector.	West SE *et al*, 1994
pUCP*mutS*	Gentamycin[R], pUCP24 harbouring the PAO1 WT *mutS* gene.	Oliver A *et al*, 2004
pUCP*mutL*	Gentamycin[R], pUCP24 harbouring the PAO1 WT *mutL* gene	Mena A *et al*, 2008

Materials and methods

Table 3.3. Primers used in this work

Primer	Sequence 5'→3'	Product size (bp)	Use	Reference
acsA-F	ACCTGGTGTACGCCTCGCTGAC	842	MLST amplification	Curran B *et al*, 2004
acsA-R	GACATAGATGCCCTGCCCCTTGAT			
aroE-F	TGGGGCTATGACTGGAAACC	1053	MLST amplification	Curran B *et al*, 2004
aroE-R	TAACCCGGTTTTGTGATTCCTACA			
guaA-F	CGGCCTCGACGTGTGGATGA	940	MLST amplification	Curran B *et al*, 2004
guaA-R	GAACGCCTGGCTGGTCTTGTGGTA			
mutL-F	CCAGATCGCCGCCGGTGAGGTG	940	MLST amplification	Curran B *et al*, 2004
mutL-R	CAGGGTGCCATAGAGGAAGTC			
nuoD-F	ACCGCCACCCGTACTG	1042	MLST amplification	Curran B *et al*, 2004
nuoD-R	TCTCGCCCATCTTGACCA			
ppsA-F	GGTCGCTCGGTCAAGGTAGTGG	989	MLST amplification	Curran B *et al*, 2004
ppsA-R	GGGTTCTCTTCTTCCGGCTCGTAG			
trpE-F	GCGGCCCAGGGTCGTGAG	811	MLST amplification	Curran B *et al*, 2004
trpE-R	CCCGGCGCTTGTTGATGGTT			
acsA-F2	GCCACACCTACATCGTCTAT	390	MLST sequencing	Curran B *et al*, 2004
acsA-R2	AGGTTGCCGAGGTTGTCCAC			
aroE-F2	ATGTCACCGTGCCGTTCAAG	495	MLST sequencing	Curran B *et al*, 2004
aroE-R2	TGAAGGCAGTCGGTTCCTTG			
guaA-F2	AGGTCGGTTCCTCCAAGGTC	372	MLST sequencing	Curran B *et al*, 2004
guaA-R2	GACGTTGTGGTGCGACTTGA			
mutL-F2	AGAAGACCGAGTTCGACCAT	441	MLST sequencing	Curran B *et al*, 2004
mutL-R2	GGTGCCATAGAGGAAGTCAT			
nuoD-F2	ACGGCGAGAACGAGGACTAC	366	MLST sequencing	Curran B *et al*, 2004
nuoD-R2	TGGCGGTCGGTGAAGGTGAA			
ppsA-F2	GGTGACGACGGCAAGCTGTA	369	MLST sequencing	Curran B *et al*, 2004
ppsA-R2	GTATCGCCTTCGGCACAGGA			
trpE-F2	TTCAACTTCGGCGACTTCCA	441	MLST sequencing	Curran B *et al*, 2004
trpE-R2	GGTGTCCATGTTGCCGTTCC			
exoS-F	TCAGGTACCCGGCATTCACTACGCGG	534	*exoS* amplification	Feltman H *et al*, 2001
exoS-R	TCACTGCAGGTTCGTGACGTCTTTCTTTTA			
exoU-F	AGCGTTAGTGACGTGCG	1546	*exoU* amplification	Feltman H *et al*, 2001
exoU-R	GCGCATGGCATCGAGTAATG			
rpsl-1	GCTGCAAAACTGCCCGCAACG	250	mRNA *rpsL* qRT-PCR	Oh H *et al*, 2003
rpsl-2	ACCCGAGGTGTCCAGCGAACC			
acrna-F	GGGCTGGCCTCGAAAGAGGAC	246	mRNA *ampC* qRT-PCR	Juan C *et al*, 2006
acrna-R	GCACCGAGTCGGGGAACTGCA			
mexB-U	CAAGGGCGTCGGTGACTTCCAG	273	mRNA *mexB* qRT-PCR	Oh H *et al*, 2003
mexB-L	ACCTGGGAACCGTCGGGATTGA			
mexD-U	GGAGTTCGGCCAGGTAGTGCTG	236	mRNA *mexD* qRT-PCR	Oh H *et al*, 2003
mexD-L	ACTGCATGTCCTCGGGGAAGAA			
mexF-U	CGCCTGGTCACCGAGGAAGAGT	254	mRNA *mexF* qRT-PCR	Oh H *et al*, 2003
mexF-L	TAGTCCATGGCTTGCGGGAAGC			

Table 3.3. Primers used in this work. (Cont.)

Primer	Sequence 5'→3'	Product size (bp)	Use	Reference
mexY-F	TGGAAGTGCAGAACCGCCTG	270	mRNA *mexY* qRT-PCR	Peña C *et al*, 2009
mexY-R	AGGTCAGCTTGGCCGGGTC			
mexZ-F	ATTGGATGTGCATGGGTG	980	*mexZ* amplification and sequencing	Sobel ML*et al*, 2003
mexZ-R	TGGAGATCGAAGGCAGC			
AD-F	GTACGCCTGCTGGACGATG	910	*ampD* amplification and sequencing	Juan C *et al*, 2006
AD-R	GAGGGCAGATCCTCGACCAG			
dacB-F	CGACCATTCGGCGATATGAC	1400	*dacB* amplification and sequencing	Moyà B *et al*, 2009
dacB-R	CGCGTAATCCGAAGATCCATC			
dacB-IF	GCCAGGGCAGCGTACCGC		*dacB* sequencing	Moyà B*et al*, 2009
dacB-IF2	GTGCTCAACGGCAACCTCTAC			
dacB-IR	GTCGCGCATCAGCAGCCAG			
oprD-F	CGCCGACAAGAAGAACTAG	1413	*oprD* amplification and sequencing	Juan C *et al*, 2010
oprD-R	GTCGATTACAGGATCGACAG			
oprD-F1	ATGCTGAAGTGGGGCGAGATG		*oprD* sequencing	Juan C *et al*, 2010
oprD-F2A	GCAGGCCACTTCACCGAGG			
oprD-F3A	GATTATATCGGCTTCGGC			
oprD-R2	GTCGAGCCCTTCGAATTCGC			
gyrA-1	TTATGCCATGAGCGAGCTGGGCAACGACT	364	*gyrA* QRDR amplification and sequencing	Juan C *et al*, 2010
gyrA-2	AACCGTTGACCAGCAGGTTGGGAATCTT			
gyrB-3	AGCTCGCAGACCAAGGACAAG	600	*gyrB* QRDR amplification and sequencing	Juan C *et al*, 2010
gyrB-4	GGGCTGGGCGATGTAGATGTA			
parC-1	ATGAGCGAACTGGGGCTGGAT	208	*parC* QRDR amplification and sequencing	Juan C *et al*, 2010
parC-2	ATGGCGGCGAAGGACTTGGGA			
parE-1	CGGCGTTCGTCTCGGGCGTGGTGAAGGA	592	*parE* QRDR amplification and sequencing	Juan C *et al*, 2010
parE-2	TCGAGGGCGTAGTAGATGTCCTTGCCGA			
mutS-F1	TTAACATTACCCTCTTTTGCAC	2687	*mutS* amplification and sequencing	Mena A *et al*, 2008
mutS-R1	TCATTTTCTAGTTCTCTCCTCA			
mutS-F4	CGCTCCGCTCCAGGACAGCGC		*mutS* sequencing	Mena A *et al*, 2008
mutS-F5	CGGCTGCCTGCTCGCCTAC			
mutS-F6	CATTCGGCGGAGGGCTACCTG			
mutS-R6	TGGCGGTTTCGCTCATCTCCAC			
mutS-F11	TTCCTGATGGACCTGGAAGCG			

Table 3.3. Primers used in this work. (Cont.)

Primer	Sequence 5'→3'	Product size (bp)	Use	Reference
mutL-F	CGATGATCGCCCAGCGCT	2299	*mutL* amplification and sequencing	Mena A *et al*, 2008
mutL-R	TCCGCCGGGTCGCGGATA			
mutL-F2	TAGCGCGCCTGACCATGA		*mutL* sequencing	Mena A *et al*, 2008
mutL-F3	GCGCATGGTGCGCGACAA			
mutL-F4	GCCTCCGGCGGCTCCTCCG			
mutL-R2	GCAGGTCGGCGATGACAT			

3.2. *Pseudomonas aeruginosa* CYSTIC FIBROSIS ISOLATES

3.2.1. The Balearic Islands *P. aeruginosa* collection.

Since 2003, all *P. aeruginosa* isolates recovered from routine respiratory cultures from CF patients attending the Son Espases University Hospital in Palma de Mallorca (former Son Dureta), reference hospital of the Balearic Islands, have been regularly stored frozen at -80°C. Culture, isolation and identification of *P. aeruginosa* from respiratory samples have always been carried out following the current established microbiological diagnostic procedures and expert recommendations. In this work, different subsets from this huge collection were used as detailed below.

In 2010, with the aim of determine the long-term clonal epidemiology and antibiotic resistance evolution of *P. aeruginosa* CRI in CF patients from the Balearic Islands, 10 sequential isolates from each of 10 chronically colonized CF patients were studied. The 10 included patients were selected based on the fulfilment of the following criteria: (i) wider follow-up period, (ii) higher temporal distribution of isolates and (iii) inclusion of the first *P. aeruginosa* isolate within the 8-year studied period (2003-2010). Likewise, each of the sequential isolates included per patient were separated by at least a 6-month interval.

Occasioned by the results obtained when long-term clonal epidemiology was explored, in 2013 we decided to extend the molecular epidemiology studies to all CF patients, including both children and adults, which had been attended at Son Espases CF Units since 2003. For this purpose, last available *P. aeruginosa* isolate of each CF patient was included. From 2003 to 2013, more than 50 CF patients had been attended at Son Espases University Hospital adult and paediatric CF Units and about the 80% had had a positive sputum culture for *P. aeruginosa* at some time point; thus, a total of 40 isolates were studied. Infection-colonization patterns and basic demographic data were recorded.

3.2.2. The Spanish *P. aeruginosa* collection.

From 2013 to 2014 the first Spanish multicentre study on the microbiology of CF was conducted. The study involved 24 CF Units, 12 paediatric and 12 adult, from 17 different Spanish hospitals. In Spain no national CF patient registry exists and, therefore, the precise number of people suffering from CF is still unknown. Nevertheless, participating hospitals are the reference ones within their geographical areas, attending the majority of the Spanish CF population, thus, a representative patient population from across Spain was included (Figure 3.1.) [de Dios Caballero *et al*, 2016].
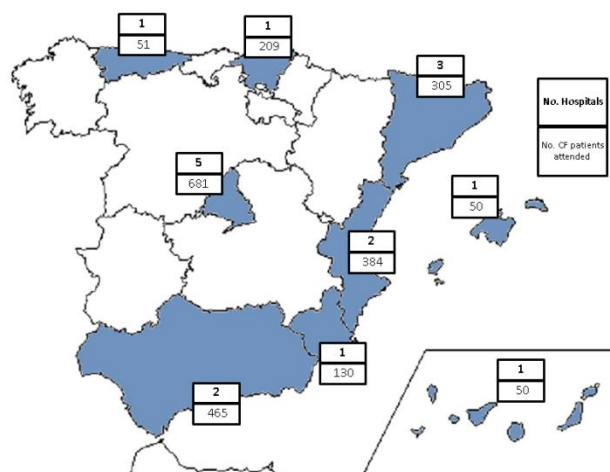
Figure 3.1. Geographical distribution of the participating hospitals and number of CF patients attended.

Fifteen consecutive patients per CF Unit were recruited and a single sputum sample from each was immediately frozen after collection at −80°C and sent to the Ramon y Cajal University Hospital in Madrid for microbiological culture. Samples were plated in appropiate culture media and plates were examined at 24 and 48 h. In order to identify potentially slow-growing bacteria, the incubation time was extended to 5 days [see details in *de Dios Caballero et al*, *2016*]. Colonies with compatible *P. aeruginosa* morphology were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Daltonics GmbH, Leipzig, Germany). Finally, all recovered *P. aeruginosa* isolates were collected and frozen for further studies.

From a total of 341 respiratory samples cultured, 79 *P. aeruginosa* were recovered from 75 different CF patients, setting a global colonization rate of 22%, and being higher in the adult population (33%) than in the pediatric population (10%). Infection-colonization patterns, basic demographic data and main patients' characteristics were recorded.

3.2.3. The 274 clonal complex *P. aeruginosa* collection.

The CC274 *P. aeruginosa* collection included 29 isolates, 28 of which had been recovered from 18 different CF patients from two highly-distant countries, Australia and Spain, and 1 blood culture isolate from a Spanish non-CF patient, covering up to an 18-year period from 1995 to 2012.

All isolates had been previously classified within the CC274 (defined as sharing at least 5 alleles with ST274) based on MLST available protocols and databases (http://pubmlst.org/paeruginosa/). All the Australian and 4 CF Spanish isolates were single isolates recovered from different patients attending clinical settings located in different geographical areas, selected randomly from those available. In addition, we included 4 sequential *P. aeruginosa* isolates, each separated by at least 6-month intervals, from each of 4 chronically colonized CF patients attended at Son Espases University Hospital, thus representing intrapatient clone evolution. Sampling time and geographic origin of CC274 *P. aeruginosa* isolates is represented in Figure 3.2.

Figure 3.2. CC274 *P. aeruginosa* **collection.** Sampling time from the 29 studied isolates can be inferred from the X axis. Isolates are labelled according to the following format: Patient identification - Country (AUS: Australia; SPA: Spain), Region

3.2.4. Colony morphotype

Morphotype was assessed plating *P. aeruginosa* isolates onto MHA and by visual examination after 24 and 48 hours of aerobic incubation at 37ºC. Afterwards, isolates were classified as regular, mucoid or SCV morphotype.

Morphotype was investigated in all isolates from the Spanish CF collection and in those from the Balearic Islands collection collected between 2003 and 2010.

3.3. PAO1 *P. aeruginosa IN VITRO* EVOLUTION EXPERIMENT UNDER AMINOGLYCOSIDE PRESSURE

To determine and in-depth study the dynamics of *P. aeruginosa* resistance development to aminoglycosides, 10-ml Mueller-Hinton broth (MHB) tubes (Annex 1) containing 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512 and 1024 mg/l of tobramycin were inoculated with approximately 10^6 CFU/ml of exponentially growing *P. aeruginosa* PAO1 reference strain and incubated for 24 h at 37°C and 180 r.p.m. After incubation, the tubes from the highest antibiotic concentration showing growth were reinoculated, at a 1:1,000 dilution, in fresh 10-ml MHB tubes containing tobramycin concentrations up to 1024 mg/l and incubated again for 24 h at 37ºC and 180 r.p.m. This step was repeated during 14 consecutive days in order to get PAO1-derived tobramycin high-resistant mutants. This procedure was performed in quintuplicate.

At days 1, 7 and 14, two colonies from the highest antibiotic concentration tubes showing growth were purified in antibiotic-free MHA plates (Annex 1) and frozen at -80ºC for further characterization.

3.4. MOLECULAR EPIDEMIOLOGY STUDIES

3.4.1. Pulsed-field gel electrophoresis

Pulsed field gel electrophoresis (PFGE) is a highly discriminatory and reproducible typing method used to determine the genetic relationship between microbial isolates from the same species level which is based on the analysis of the chromosomic DNA. Basically, this genotyping method consists in fragmenting the bacterial chromosomic DNA by using appropriate restriction endonucleases of low cut frequency and then separate the resulted DNA fragments by PFGE in order to obtain a unique macrorestriction pattern for each microbial isolate and, eventually, compare all these unique molecular fingerprints to determine their genetic relationship. In this work, previously defined protocols [Kaufmann ME, 1998] with slight modifications were used, as set forth in detail below.

P. aeruginosa isolates were grown in suspension on 5 ml of Brain Heart Infusion (BHI) broth (Annex 1) under aerobic conditions for 16-20 hours at 37ºC and shaking at 180 r.p.m. Afterwards, a volume of 250 µl (500 µl for mucoid phenotype isolates) was centrifuged at 13000 r.p.m. for 5 minutes and bacterial pellets were washed twice with PIV solution (Annex 1). Then, bacterial pellets were resuspended in 200 µl of this PIV solution, mixed with an equal volume of 1.6% low-melting temperature agarose (Annex 1) at 42ºC and, finally, this molten mixture was used to prepare plugs in adequate molds (Bio-Rad). Once the agarose was set, the plugs were collected and incubated within 1 ml of EC-Lysis solution (Annex 1) at 37ºC for at least 5 hours and, then, in 1 ml of ESP solution (Annex 1) at 50ºC for 16-20 hours in order to release DNA from bacteria embebbed in the agarose plugs. After overnight incubation, ESP solution was removed and plugs were washed in quintuplicate with TE buffer (Annex 1), rinsed with 1 ml of sterile distilled water at 37ºC for at least 10 minutes, transferred to microfuge tubes containing 20 units of *SpeI* restriction enzyme (New England BioLabs) and incubated in appropriate conditions (20 hours, 37ºC). Then, each plug was soaked in 1 ml of TE buffer at 37ºC for 1 hour. In the meantime, a 1% megabase agarose gel (Annex 1) was prepared and, once soaked, plugs were placed and sealed in individual wells of the gel. Right after, fragments were separated in a CHEF- DR III contour-clamped homogeneous-electric-field apparatus (Bio-Rad, La Jolla, CA) in standard 0.5X TBE running buffer (Annex 1) chilled at 14ºC for 26 hours at 6V/cm with pulse times of 5 to 40 s and using a 120º included angle. After electrophoresis, the gel was stained in 0.5X TBE buffer with either a 1 µg/ml solution of ethidium bromide or with an appropriate solution of RedSafe 20.000X and photographed under UV light.

This genotyping method was used to infer clonal relatedness among isolates within the two studied subsets of the Balearic Islands *P. aeruginosa* collection (longitudinal and cross sectional), as well as for isolates from the Spanish and the CC274 collections. For this purpose, the criteria established by *Tenover et al.* [Tenover FC *et al*, 1995] were applied with

the single exception of inferring the genetic relationship within the CC274 *P. aeruginosa* collection in which the Unweighted Pair Group Method with Arithmetic mean (UPGMA) clustering method was used [CLIQS 1D Pro, Totallab].

3.4.2. Multilocus sequence typing

Multilocus Sequence Typing (MLST) is a molecular genotyping tool that not only results into highly discriminatory but also electronically portable data between laboratories worldwide. This unambiguous typing method is based in the use and comparison of the nucleotidic sequences of internal fragments of approximately 450-500 bp of seven established house-keeping genes. For each house-keeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the seven loci define the allelic profile or ST.

In this work the MLST scheme for *P. aeruginosa* proposed by *Curran B et al.*, which is based in the house-keeping genes *acsA* (acetil coenzyme A synthetase), *aroE* (shikimate dehydrogenase), *guaA* (GMP synthase), *mutL* (DNA MMR protein), *nuoD* (NADH dehydrogenase I chain C, D), *ppsA* (phosphoenolpyruvate synthase) and *trpE* (anthralite synthetase component I), was applied [Curran B *et al*, 2004; http://pubmlst.org/paeruginosa/]. Briefly, workflow was as follows.

Genomic DNA was obtained by using a commercially available extraction kit (DNeasy Blood & Tissue kit, Qiagen or High Pure PCR template preparation kit, Roche Diagnostics) and polymerase chain reaction (PCR) amplification of the seven house-keeping genes was performed (primers and PCR Master Mix details in Table 3.3 in section 3.1. and Annex 1, respectively) under the next reaction conditions: initial denaturation at 94ºC for 12 min; 35 cycles of denaturation at 94ºC for 1 min, primer annealing at 59ºC (60ºC for *mutL* amplification) for 1 min, extension at 72ºC for 1 min; followed by a final extension step of 72ºC for 10 min. Hereafter, PCR amplification products were purified and sequenced with the BigDye Terminator kit (PE Applied Biosystems) on an ABI Prism 3100 DNA sequencer (PE Applied Biosystems, Foster City, CA). The *P. aeruginosa* MLST database (https://pubmlst.org/paeruginosa/) was used to assign an allele to each resulting sequence and to assign a ST.

This technique was performed within the two studied subsets of the Balearic Islands collection (see above), as well for the isolates from the Spanish CF *P. aeruginosa* collection. Phylogenetic relationship was assessed by constructing a Minimum Spanning Tree (MST) using the goeBURST algorithm, available at www.phyloviz.net ST were considered to belong to a same CC when sharing at least five of the seven sequenced loci.

3.4.3. Array-tube genotyping

Wiehlmann and collaborators recently developed a species-specific oligonucleotide-microarray for genotyping *P. aeruginosa* isolates [Wiehlmann L *et al*, 2007] based on the Array Tube platform (Alere Technologies GmbH, Jena, Germany) which is currently commercially available. This microarray enables *P. aeruginosa* genotyping by using 13 informative single nucleotide polymorphisms (SNP) at conserved loci, the *fliCa/fliCb* multiallelic locus and the presence or absence of the *exoS/exoU* marker gene; SNP pattern which is eventually converted into a 4 letter-code that can easily be used and exchanged for the identification of a clone. Additionally, this system also includes 38 genetic markers from the accessory genome for analysis of microevolutionary events within a clone or Array Tube genotype, thus allowing definition of intraclonal diversity. Up to 10 genome islets and 6 genomic islands can be detected, including the ferripyoverdine receptor genes (*fpvA*) type I, IIa, IIb and III; the alternative type-I ferripyoverdine receptor gene *fpvB*; the flagellin glycosylation island; the *P. aeruginosa* Genomic Island type 1 (PAGI-1); the genomic islands of the CLC family PAGI-2/3, the *P. aeruginosa* Pathogenicity Islands type 1 and type 2 (PAPI-1 and PAPI-2) as well as other pKLC102-like islands (Figure 3.3.).

M	C-45	C-46	C-47	PAGI-3-1	PAGI-3-8	PAGI-2-1	M
	PAGI-2/3-1	PAGI-2/3-4	PAGI-2/3-5	PAGI-2/3-6			
M	pKL-1	pKL-3	TB-C47-1	TB-C47-2	PAPI-1 pili chaperone	PAPI-1 luminal binding protein	
M	pKLC conserved hypothetical	pKLC adhesin	pKLC fatty acid synthase				
	fpvA type I	fpvA type IIa	fpvA type IIb	fpvA type III	fpvB	LES	
	PA0636	PA0722			PAGI-1	PA0980	
	PA0728	PA2185	fla-island	orfA	tRNA^Pro - island 1	XF1753	
	PA2221	PA3835	orfI	orfJ	tRNA^Pro - island 2	acetyl-transferase	
	PAO ampC-7 non-PAO		fliCa	fliCb	exoS	exoU	
	PAO ampC-4 non-PAO		PAO ampC-5 non-PAO		PAO ampC-6 non-PAO		
	PAO oprI (2) non-PAO		PAO ampC-1 non-PAO		PAO ampC-3 non-PAO		
	PAO citS-1 non-PAO		PAO citS-2 non-PAO		PAO oprI (1) non-PAO		
	PAO fliCa (1) non-PAO		PAO fliCa (2) non-PAO		PAO alkB2 non-PAO		
M	PAO oriC non-PAO		PAO oprL (1) non-PAO		PAO oprL (2) non-PAO		M

Figure 3.3. Core and accessory genome markers disposition in the Alere™ Array Tube *P. aeruginosa* species-specific microarray.

This microarray was employed for further characterization of the Spanish CF collection. All procedures were performed according to the manufacturer's protocol. Briefly, RNA-free unfragmented genomic DNA from pure and monoclonal *P. aeruginosa* isolates were obtained by using a commercially available extraction kit (High Pure PCR template preparation kit, Roche Diagnostics) and after digestion with 4 µl of RNase A 100 mg/ml solution (Qiagen). After, obtained genomic DNA was amplified approximately 50-fold and internally labelled with biotin-11-dUTP using a linear amplification protocol. Then, a multiplex primer extension reaction was performed with two nested primers per target in each cycle and resulting biotin labelled single-strand DNA (ssDNA) was transferred and hybridised to the DNA oligonucleotide microarrays and red by using the ArrayMate Reader (Alere).

Extended information about probes and primers has been collected in Annex 2. For further details please see literature [Wiehlmann L *et al*, 2007] or the manufacturer's *P. aeruginosa* Genotyping Kit 2 manual available at https://alere-technologies.com/.

The Type 3 Secretion System (T3SS). To further characterize the type 3 Secretion System (T3SS) protein effectors (ExoS, ExoU) independent specific PCR assays were performed. Protocols previously described by *Feltman H* and collaborators were used with slight modifications [Feltman H *et al*, 2001]. Primers and PCR Master Mix details are collected in Table 3.3. and Annex 1, respectively. Reaction conditions were as follow: initial denaturation at 94°C for 12 min, followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec and a final extension step of 10 min at 72°C. Amplified DNA products were resolved by electrophoresis on agarose 1% w/v gels.

3.5. ANTIMICROBIAL SUSCEPTIBILITY PROFILES AND RESISTANCE MECHANISMS

3.5.1. Antimicrobial susceptibility testing

3.5.1.1. *P. aeruginosa* clinical isolates

One of the most important tasks in a Clinical Microbiology Laboratory is to determine the antimicrobial susceptibility profiles of significant bacterial isolates. Currently, there is not a universal standardized method that perfectly reproduces the conditions in which infecting bacteria are grown *in vivo* and different technical approaches can be used. This point is particularly striking in the CF setting, especially at chronic stages in which bacteria change their mode of growth from planktonic to biofilm and in which there is, indeed, a high prevalence of mucoid and hypermutable isolates and, therefore, mutant resistant subpopulations frequently rise.

In this work different susceptibility testing methods were used. Regardless of the technical approach used, it should be highlighted that the following CF expert recommendations were in all cases taken into account: (i) the incubation time was extended up to 24 hours (36-48 hours for slow growing variants) and (ii) a 0.5 or 1 McFarland standard suspension was used for inoculum standardization of non-mucoid or mucoid isolates, respectively.

MICs for all isolates belonging to both, the Balearic Islands and the CC274 *P. aeruginosa* collection, were determined by using commercially available strips containing a gradient of antibiotic concentrations in MHA plates. Standard suspensions were prepared in accordance with morphotypes and MICs were recorded after aerobic incubation of plates at 37°C. The antimicrobials compounds tested included the antipseudomal cephalosporins ceftazidime (TZ) and cefepime (PM), the carbapenems imipenem (IP) and meropenem (MP), the quinolone ciprofloxacin (CI), the aminoglycoside tobramycin (TM) and the polymyxin colistin (CO) (AB bioMèrieux, Solna, Sweden). Besides, for selected subsets of these *P. aeruginosa* collections, MICs for the monobactam aztreonam (AT) (AB bioMèrieux, Solna, Sweden), for amikacin (AK) and for the β-lactam β-lactamase combinations piperacillin/tazobactam (PPT) and ceftolozane/tazobactam (TOL/TAZ) (Liofilchen) were also determined. *P. aeruginosa* PAO1 reference strain was used as control.

In the case of the Spanish *P. aeruginosa* CF collection the disk-difussion method was used to determine the antibiotic susceptibility profiles, except for fosfomycin (FO) for which the agar dilution method was performed [Díez-Aguilar M *et al*, 2013]. The antimicrobials tested included the antipseudomal cephalosporins ceftazidime and cefepime, the monobactam aztreonam, the penicillin- β-lactamase combination piperacillin/tazobactam, the carbapenems imipenem and meropenem, the quinolones ciprofloxacin and levofloxacin (LE), the aminoglycosides tobramycin, gentamicin (GM) and amikacin, and the polymyxin colistin.

Materials and methods

Procedures were performed in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST, www.eucast.org). *P. aeruginosa* PAO1 and ATCC27853 reference strains were used as controls.

Analysis of the data from the Balearic Islands and the Spanish CF *P. aeruginosa* collections was additionally performed taking into account patients' infection-colonization pattern and colony morphotype. Temporal evolution of antibiotic resistance was also explored within the Balearic Islands collection. It should also be mentioned that in the data analysis of the Balearic Islands collection just one isolate per patient, semester, morphotype and antibiotype was included to avoid any bias.

3.5.1.2. *P. aeruginosa* laboratory strains

MICs for *P. aeruginosa* PAO1 reference strain and its evolved derivatives obtained from the *in vitro* experiment under aminoglycoside treatment pressure (section 3.3.) were determined by broth microdilution using customized Sensititre® plates (reference FRCNRP; Thermo Fisher Scientific). These customized plates included the following compounds: ceftolozane/tazobactam, ceftazidime, cefepime, piperacillin/tazobactam, aztreonam, imipenem, meropenem, ciprofloxacin, ticarcillin, tobramycin, amikacin and colistin. Aminoglycosides MICs (GM, AK, TM) were also determined by broth microdilution according to EUCAST (www.eucast.org) and the International Standards Organisation guidelines. *P. aeruginosa* PAO1 and ATCC27853 reference strains were used as controls.

3.5.1.3. Clinical breakpoints and definitions

EUCAST clinical breakpoints for systemic infections were applied (www.eucast.org) for both, MICs and zone diameter inhibition, interpretation. Different versions have been applied in this work, the specific version used in each experiment/analysis is detailed within the results section (Annex 3).

Antibiotic susceptibility profiles were classified in accordance with Magiorakos *et al.* [Magiorakos AP *et al*, 2012] proposed criteria. Subsequently, MDR *P. aeruginosa* was defined as non-susceptibility to at least 1 antibiotic agent in at least 3 antipseudomonal antibiotic classes; XDR *P. aeruginosa* was defined as non-susceptible to at least 1 agent in all but 1 or 2 antipseudomonal antimicrobial categories and, finally, *P. aeruginosa* isolates non-susceptible to all antipseudomonal antibiotics classes were classified as pan-drug resistant (PDR). Likewise, an isolate was considered to be hypersusceptible to an antibiotic compound when the determined MIC was at least two-fold lower than *P. aeruginosa* PAO1 reference strain MIC.

3.5.2. Relative expression of chromosomically encoded *P. aeruginosa* resistance genes by real time qRT-PCR

The expression of the genes encoding the chromosomal β-lactamase AmpC (*ampC*) and four *P. aeruginosa* efflux pumps representative components, MexAB-OprM (*mexB*), MexCD-OprJ (*mexD*), MexXY (*mexY*), and MexEF-OprN (*mexF*), were determined by real-time quantitative Reverse Transcription-PCR (qRT-PCR).

P. aeruginosa isolates were grown in 10 ml of Luria-Bertani (LB) broth (Annex 1) at 37°C and 180 r.p.m. to the late-log-phase (optical density at 600 nm [OD$_{600}$] of 1) and collected by centrifugation. Total RNA was obtained by using the RNeasy minikit (Qiagen), dissolved in RNAse-free water and treated with 2 units of TURBO DNA-free™ (Ambion) during 30 minutes at 37ºC for remove any contaminating DNA trace. Reaction was then stopped with 5 µl of DNase inactivation reagent (Ambion) and adjusted to a final RNA concentration of 50 ng per µl. A 50 ng sample of purified RNA was then used for one-step reverse transcription and qRT-PCR amplification using the QuantiTect SYBR green RT-PCR kit (Qiagen, Hilden, Germany) on either a SmartCycler II instrument (Cepheid, Sunnyvale, CA) or an Eco real-time PCR system (Illumina). Previously described primers were used for the amplification of *ampC*, *mexB*, *mexY*, *mexD*, *mexF* and *rpsL* (gene used as a reference to normalize the relative amount of mRNA). Primers and PCR Master Mix details are collected in Table 3.3. and Annex 1, respectively. Real time qRT-PCR conditions were as follows: reverse transcription at 50ºC for 20 min, followed by Taq activation at 95ºC for 15 min and 40 cycles of 95°C for 15 sec, 62°C for 30 sec and 72°C for 30 sec, measuring fluorescence emission in the second step of each amplification cycle. As controls, previously characterized knockout mutants and/or clinical strains overexpressing these antibiotic resistance mechanisms were used.

Isolates were considered positive for AmpC, MexCD-OprJ, MexEF-OprN or MexXY(-OprM) overexpression when the corresponding mRNA levels (*ampC*, *mexD*, *mexF* or *mexY*) were at least 10-fold higher than that of *P. aeruginosa* PAO1 reference strain, negative if lower than 5-fold, and borderline if between 5- and 10-fold. Likewise, isolates were considered positive for *mexB* overexpression when the corresponding mRNA level was at least 3-fold higher than that of PAO1, negative if lower than 2-fold and borderline if between 2- and 3-fold. All real time qRT-PCRs were performed in duplicate and mean values (± standard deviations) of mRNA levels were obtained from three independent experiments.

Relative expression of *ampC*, *mexB*, *mexD*, *mexF* and *mexY* was investigated in the subset of 100 CF *P. aeruginosa* isolates (10 sequential isolates from each of 10 chronically colonized patients) of the Balearic Islands and in all isolates from the CC274 collection. As well, *mexY* relative expression was investigated in all PAO1 derivatives isolates obtained in the *in vitro* experiment evolution under aminoglycoside treatment pressure.

3.5.3. Isolation and analysis of the outer membrane protein OprD

The presence or absence of the carbapenem porin OprD was evaluated by analyzing the OMPs profiles. For this purpose, a protocol adapted from those previously described by Filip *et al* was followed [Filip C *et al*, 1973]. First, *P. aeruginosa* isolates were grown in 10 ml of LB broth (Annex 1) at 37°C and 180 r.p.m. to the late-log-phase (optical density at 600 nm [OD_{600}] of 1) and about 3 ml were collected by centrifugation, washed and resuspended in 5ml of cold Tris-Mg buffer pH 7.3 (Annex 1). Cells were then sonicated (4 cycles of 35 sec, ON/OFF pulses of 5/2 sec, amplitude 10%) and centrifuged at 5000 r.p.m. for 2 min to eliminate unbroken cells. Carefully supernatant fluids were transferred and centrifuged at full speed for 30 min at 4ºC to pellet cell envelopes. Afterwards, the obtained pellets were twice resuspended in 1.5 ml of 1% sodium lauryl sarcosinate in Tris-Mg buffer, incubated for 30 min at room temperature and centrifuged again at full speed for 30 min at room temperature. These pellets were finally resuspended in 40 µl of Laemmli's electrophoresis sample buffer (BioRad), boiled for 5 min, centrifuged for remove any insoluble material and stored at -4ºC. Once isolated, OMPs were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized using Coomassie staining. Stacking and separating gel compositions as well as running buffer details are collected in Annex 1. An unstained protein standard ladder (Precision Protein™ Standards, BioRad) was also loaded to detect the presence or absence of OprD (45 kDa). *P. aeruginosa* reference strain PAO1 and its OprD null spontaneous mutant PAOD1 were used as controls (Table 3.1.).

The presence or absence of the carbapenem porin OprD was evaluated within the 100 *P. aeruginosa* CF isolates chronically colonizing 10 CF patients from the Balearic Islands.

3.5.4. DNA sequencing of *P. aeruginosa* antibiotic-resistance related genes

When needed, for isolates showing AmpC or MexXY overexpression, genes encoding their main transcriptional regulators (*ampD dacB*, *mexZ*) were fully sequenced. Briefly, workflow was as follows. Genomic DNA was obtained by using a commercially available extraction kit (DNeasy Blood & Tissue kit, Qiagen or High Pure PCR template preparation kit, Roche Diagnostics) and PCR amplification of the regulatory genes was performed (primers and PCR Master Mix details in Table 3.3. and Annex 1, respectively) under the following reaction conditions: initial denaturation at 94ºC for 12 min; 35 cycles of denaturation at 94ºC for 1 min, primer annealing at 60ºC or 64ºC (for *mexZ* or *ampD*/*dacB*, respectively) for 1 min, extension at 72ºC for 1 min; followed by a final extension step of 72ºC for 10 min. Hereafter, PCR amplification products were purified and sequenced with the BigDye Terminator kit (PE Applied Biosystems, Foster City, CA) on an ABI Prism 3100 DNA sequencer (PE Applied Biosystems) using appropiate primers (Table 3.3.).

As well, other antibiotic resistance related genes such as *oprD*, *gyrA*, *gyrB*, *parC* and/or *parE*, were sometimes sequenced in order to explain or confirm the documented antibiotic

susceptibility profiles. The procedure was exactly as it has been described above but using appropriate primers and adjusting PCR conditions (Table 3.3.)

In all cases, obtained DNA sequences were compared with PAO1 DNA and protein sequences using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST.)

3.6. MUTATOR PHENOTYPE AND GENETIC BASIS FOR HYPERMUTATION

3.6.1. Estimation of mutation frequencies

In order to determine which *P. aeruginosa* isolates had an increased spontaneous mutation rate, rifampicin (RIF) resistance mutant frequencies were determined following previously described protocols with slight modifications [Mena A *et al*, 2008; Oliver A *et al*, 2000].

First, a screening was performed in order to detect all *P. aeruginosa* isolates showing an increased spontaneous mutation rate. For this purpose, bacterial suspensions containing approximately $5 \cdot 10^8$ CFU/ml were prepared and a volume of 100 µl was plated on MHA plates supplemented with 300 mg/l of RIF (MHA-RIF) (Annex 1). After 36 h (48 h for slow growing variants) of incubation at 37ºC, plates were carefully examined. In the absence of *P. aeruginosa* colonies, the corresponding isolate was considered to have a normal mutation rate; otherwise, isolates were considered to have an increased spontaneous mutation rate and, therefore, its mutation frequency was estimated as following described.

For each *P. aeruginosa* isolate, 5 independent 10 ml MHB-containing tubes (Annex 1) were inoculated with approximately 10^3 bacterial cells and incubated overnight at 37ºC and 180 r.p.m. in aerobic conditions. Then, cells were recovered by centrifugation (4ºC, 10 min, 3000 r.p.m.) and pellets were resuspended in 1 ml of saline sterile solution. Finally, 1:10 serial dilutions were prepared and plated onto MHA plates and onto RIF-supplemented MHA plates (MHA-RIF) (Annex 1). MHA and MHA-RIF plates were incubated at 37ºC for 24 h and 36h, respectively (36-48 h for slow growing variants). Once incubated, colonies were counted and mutation frecuencies were estimated as the median number of mutant colonies (colonies grown in MHA-RIF/ml) divided by the median number of total cells (colonies grown in MHA/ml). Following previous recommendations [Mena A *et al*, 2008; Oliver A *et al*, 2000], isolates with a RIF resistance mutant frequency higher than $2 \cdot 10^{-7}$ were classified as hypermutators.

In all these procedures, both, *P. aeruginosa* PAO1 reference strain and its derivative hypermutator knockout mutant defective in *mutS*, PAOMS, were used as controls (see Table 3.1. section 3.1.).

Screening was performed in all CF *P. aeruginosa* isolates from the Spanish and the CC274 collection, as well as in the 100 CF isolates chronically infecting the 10 selected patients of the Balearic Islands. Mutation frequencies were determined for all mutator isolates detected in both, the Spanish CF collection and the CC274 collection.

3.6.2. Mismatch repair system deficiency complementation assays

Genetic basis for hypermutation were explored in all *P. aeruginosa* isolates with a demonstrated increased spontaneous mutation rate.

Deficiencies within the MMR system are the most frequent cause of bacterial hypermutation [Mena A *et al*, 2008; Oliver A *et al*, 2000]. Thus, to characterize the mutator phenotype, complementation assays using WT MMR genes *mutS* and *mutL* were performed.

For this end, gentamycin resistant plasmids pUCP*mutS* and pUCP*mutL*, harbouring *P. aeruginosa* PAO1 WT *mutS* and *mutL* genes respectively, were electroporated into the mutator isolates as follows. Plasmid pUCP24 was also electroporated as control cloning vector (Table 3.2.). First, isolates were inoculated in 50ml-tubes containing 5 ml of LB broth (Annex 1) and incubated overnight at 37ºC with shaking at 180 r.p.m. in aerobic conditions. Then, 1 ml of the overnight culture was inoculated into 50 ml of fresh LB broth (Annex 1) and incubated at 37ºC and 180 r.p.m. until the log-phase (optical density at 600 nm [OD_{600}] of 0.5). After, the flask was chilled on ice for 10 min and cells were collected by centrifugation (4ºC, 15 min, 3000 r.p.m.), washed and finally resuspended in 500 µl of sterile chilled Sucrose Magnesium Electroporation Buffer (SMEB) buffer (Annex 1).

Electrocompetent *P. aeruginosa* cells were aliquoted (100 µl), incubated on ice with each of the purified plasmids (5 µl) for 10 min and electroporated on a Gene Pulser Xcell (BioRad) under the following conditions: voltage 2.5 kV, pulse time 2 min, resistance 200 Ω, capacitance 25 µF. Finally, electroporated cells were recovered, incubated for 1h at 37ºC and 180 r.p.m. in 1 ml of Super Optimal broth with Catabolite repression (SOC, Annex 1) and plated onto MHA-GEN plates (Annex 1). After 36-48 h of incubation at 37ºC, independent transformant colonies harbouring pUCP24, pUCP*mutS* and pUCP*mutL* were selected and their mutation frequencies were estimated as previously described (section 3.6.1). A positive complementation result was obtained when the mutation frequency decreased back to basal levels, $1·10^8$ approximately.

3.6.3. *mutS* and *mutL* sequencing

To complete the genetic characterization of hypermutation and, based on complementation assays results, *mutS* or *mutL* genes were fully sequenced. Briefly, after obtaining genomic DNA by using a commercially available extraction kit (DNeasy Blood & Tissue kit, Qiagen or High Pure PCR template preparation kit, Roche Diagnostics), PCR amplification was performed (see primers and PCR Master Mix details in Table 3.3. and Annex 1, respectively) under the following reaction conditions: initial denaturation at 94ºC for 12 min; 35 cycles of denaturation at 94ºC for 1 min, primer annealing at 60ºC/62ºC for 3 min (*mutS*/*mutL*, respectively), extension at 72ºC for 1 min; followed by a final extension step of 72ºC for 10 min. Then, PCR amplification products were purified and sequenced with the BigDye

Terminator kit (PE Applied Biosystems, Foster City, CA) on an ABI Prism 3100 DNA sequencer (PE Applied Biosystems) and appropiate primers (Table 3.3.).

Obtained DNA and protein sequences were compared with *P. aeruginosa* PAO1 DNA sequence using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST).

All *P. aeruginosa* isolates with a demonstrated increased spontaneous mutation rate were studied.

3.7. WHOLE GENOME SEQUENCING

WGS approaches were used for in-depth study of the CC274 *P. aeruginosa* collection and for the genotypic characterization of PAO1 aminoglycoside-resistant derivatives (variant calling and genome annotation).

3.7.1. Library preparation and sequencing methodology

P. aeruginosa whole genome DNA sequences were obtained as follows. First, genomic DNA was obtained by using a commercially available extraction kit (High Pure PCR template preparation kit, Roche Diagnostics). DNA purity was assessed with a NanoDrop (ThermoScientifics) using the UV absorbance ratios 260/280nm and 260/230nm; DNA samples with ratio values outside ranges 1.8-2.0 and 2.0-2.2, respectively, were excluded. Then, genomic DNA was quantified using a fluorometric-based method (Quant-iT™ PicoGreen ® dsDNA assay kit, LifeTechnologies) and adjusted to a final concentration of 0.1ng/µl.

Therefore, indexed paired-end libraries were prepared with the Nextera® XT DNA library preparation kit (Illumina Inc, USA) according to the manufacturer's protocol with slight modifications. Briefly, genomic DNA (0.5 ng) was first tagmented and, shortafter, amplified and indexed using a limited-cycle PCR program. Libraries were then cleaned-up and normalized by using either, the manual or the bead-based normalization approaches. For manual normalization a fluorometric-based method was used (Quant-iT™ PicoGreen ® dsDNA assay kit, LifeTechnologies), and the conversion factor 1 ng/µl = 1.5 nM was applied to prepare a 4 nM library. The selection of this conversion factor was based on the results obtained on an Agilent Technology 2100 Bioanalyzer using a High Sensitivity DNA chip. Once normalized, libraries were pooled and appropriately denatured and diluted to result in a 20 pM denatured pooled library that was finally loaded in, either, a MiSeq Reagent Kit v2 or v3 (Illumina inc., USA), and sequenced on an Illumina Miseq ® benchtop sequencer, resulting in 250 bp paired-end reads.

Nextera® XT DNA Library Prep Kit reference guide (#15031942), MiSeq System Denature and Dilute Libraries guide (#15039740) and MiSeq® System User Guide (#15027617) available on Illumina webpage (https://www.illumina.com/index-d.html) can be consulted for further procedures details.

3.7.2. Variant calling

Previously defined and validated protocols were used with slight modifications [Marvig RL *et al*, 2013; Marvig RL *et al*, 2015b]. In short, after mapping obtained 250 bp paired-end reads to the *P. aeruginosa* PAO1 reference genome (GenBank accession number: NC_002516.2) by using Bowtie 2 v2.2.4. (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml), pileup and raw files were generated by using SAMtools v0.1.16

(https://sourceforge.net/projects/samtools/files/samtools/) and PicardTools v1.140 (https://github.com/broadinstitute/picard). Additionally, the Genome Analysis Toolkit (GATK) v3.4-46 (https://www.broadinstitute.org/gatk/) was used for realignment around InDels. Then, from raw files, SNPs were extracted if they met the following criteria: a quality score (Phred-scaled probability of the samples reads being homozygous reference) of at least 50, a root-mean-square (RMS) mapping quality of at least 25 and a coverage depth of at least 3 reads; excluding all ambiguous variants. As well, microInDels were extracted from the totalpileup files applying the following criteria: a quality score of at least 500, an RMS mapping quality of at least 25 and support from at least one-fifth of the covering reads. Pipeline and used scripts are detailed in Annex 4.

For exceptional cases, some positions were indeed manually and individually checked in raw and pileup files without applying any filtering.

3.7.3. De novo assemblies

Sequence reads from each isolate were de novo assembled using Velvet v1.2.10 (https://www.ebi.ac.uk/~zerbino/velvet/) with a k-mer length of 31 and the following parameters: scaffolding = no, ins_length = 500, cov_cutoff = 3, and min_contig_lgth = 500. The script used for this purpose is detailed in Annex 4.

3.7.4. Phylogenetic reconstructions and Beast analysis

Core genome phylogenetic reconstructions were performed using the Parsnp tool from the Harvest Suite package v1.2 with default parameters but forcing the inclusion of all desired genomes (-c) and selecting randomly the reference genome (r!) (http://harvest.readthedocs.io/en/latest/content/parsnp.html).

Bayesian analysis of divergence times was performed using BEAST v2.4.2 (http://beast2.org/). For this purpose, a nexus file including all the curated positions at which at least one of the isolates differed from the reference strain PAO1 was constructed and converted into an.xml file with BEAUTi. Hereafter, BEAST was run with the following user-determined settings; a lognormal relaxed molecular clock model and a general time-reversible substitution model with gamma correction. Divergence times were calculated from a chain length of 50 million steps, sampled every 1,000 steps and discarding the first 5 million steps as a burn-in. Finally, the maximum clade credibility tree was generated using the TreeAnnotator program from the BEAST package and tree parameters were calculated with Tracer v1.6 (http://beast.bio.ed.ac.uk/Tracer). Used scripts are detailed in Annex 4.

Both Phylogenetic reconstructions were displayed using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

3.7.5. Genome annotation: resistome and mutome profiling

SNP and InDels files were annotated by using SnpEff software v4.2 (http://snpeff.sourceforge.net/index.html) with default options.

For the CC274 *P. aeruginosa* collection, annotated SNP and InDels files were then filtered based on an exhaustive literature review that led us to obtain a set of 164 genes known to be related to chromosomal antibiotic resistance in *P. aeruginosa* (see Annex 5). Additionally, the online available tool ResFinder v2.1 (https://cge.cbs.dtu.dk//services/ResFinder/) was used to identify possible horizontally acquired antimicrobial resistance genes. As well, the genetic basis of hypermutation was investigated from WGS data through the analysis of an exhaustive panel of so called mutator genes, thus designated mutome. Genes included within the mutome panel are collected in Annex 5.

4. RESULTS

El alma y el cuerpo

4.1. POPULATION STRUCTURE AND ANTIBIOTIC RESISTANCE OF *Pseudomonas aeruginosa* CYSTIC FIBROSIS RESPIRATORY INFECTIONS

4.1.1. Clonal epidemiology studies

4.1.1.1. Longitudinal analysis of the Balearic Islands collection

A total of 100 *P. aeruginosa* isolates from the Balearic Islands CF collection were studied, including 10 sequential isolates from each of 10 different CF patients covering up an 8-year study period (section 3.2.1.).

Clonal relatedness among isolates was first evaluated by Pulse-field Gel Electrophoresis (PFGE) and the obtained restriction patterns were analysed by using the criteria proposed by *Tenover et al* [Tenover FC *et al*, 1995]. Analysis revealed the presence of 13 different clone types. PFGE clones distribution among the 10 different patients along the 8-year study period is represented in Figure 4.1.
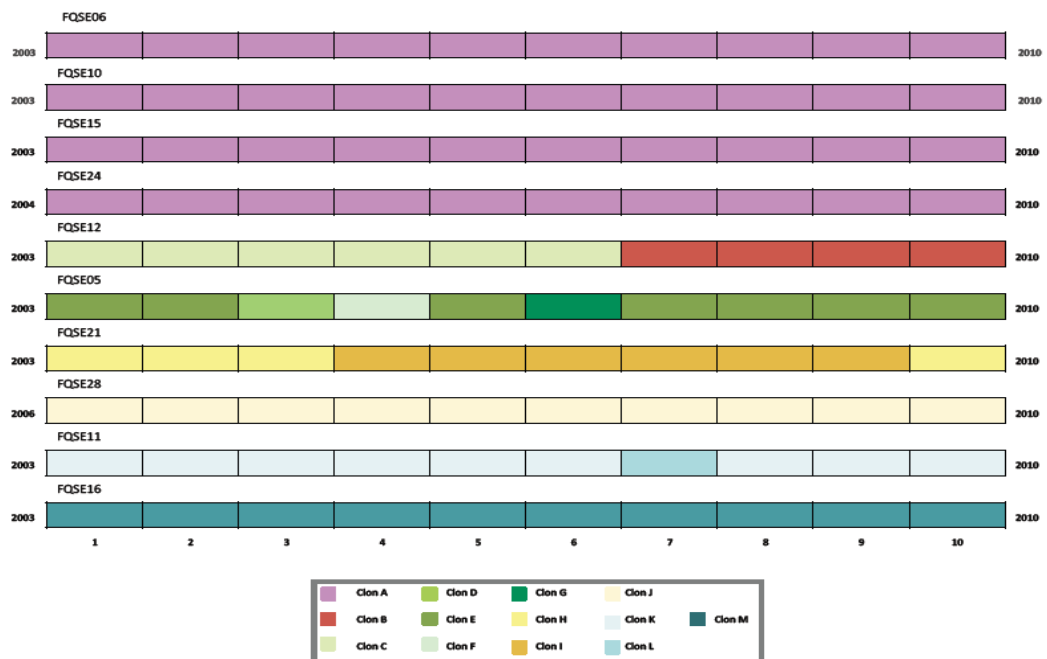
Figure 4.1. PFGE clones distribution. Each bar represents a different patient and each colour represents a different PFGE clone. Isolation years from the first and last isolate included are indicated in the left and right axes, respectively.

As shown, six of the patients showed a single PFGE clone over the whole study period, including those colonized by the designated clone FQSE-A. Conversely, the other four patients showed the coexistence of several clones (2 to 4) or clonal replacements (Figure 4.1.). With the single exception of FQSE-A, all other PFGE clones were detected in unique patients.

First isolate per patient and PFGE clone were further analysed by MLST, yielding 13 different STs not entirely coincident with the clones identified by PFGE (Table 4.1.). Designated FQSE-C and FQSE-D PFGE clones were determined to be the same ST (ST299) and, conversely, the disseminated clone FQSE-A yielded two different ST closely related. Whereas clone FQSE-A isolates from 3 of the 4 colonized patients were identified as ST274, isolates from the fourth patient were identified as a new ST (ST1089). It should also be highlighted the documented superinfection with the LES-1 (ST146) in one of the studied patients (FQSE12, see Figure 4.1. and Table 4.1.).

As highlighted in Table 4.1., up to 8 of the 13 STs encountered had not been previously described (https://pubmlst.org/paeruginosa/), being, indeed, each of them detected in single patients. Of note, just the allelic profile of ST1089 includes new allele sequences, resulting all others from new allele combinations of previously described allele sequences. ST1089 *acsA* and *guaA* non-previously described alleles sequences just differed from those of ST274 by two single point mutations.

Table 4.1. Allelic profiles and associated PFGE clones of the 13 different ST detected.

PFGE clone	ST[a]	Allelic profile[a]						
		acsA	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*
FQSE-A	ST274	23	5	11	7	1	12	7
FQSE-A	**ST1089**	**66**	5	**101**	7	1	12	7
FQSE-B	ST146	6	5	11	3	4	23	1
FQSE-C/D	ST299	17	5	36	3	3	7	3
FQSE-E	**ST1108**	6	3	17	7	3	4	7
FQSE-F	**ST1072**	5	13	25	6	1	7	3
FQSE-G	ST155	28	5	36	3	3	13	7
FQSE-H	**ST1088**	36	27	28	3	4	13	1
FQSE-I	**ST1109**	16	14	3	11	1	15	1
FQSE-J	**ST1071**	5	3	57	6	1	33	42
FQSE-K	**ST701**	29	1	9	13	1	6	23
FQSE-L	ST254	6	5	58	11	3	4	37
FQSE-M	**ST1073**	28	5	36	3	4	10	95

[a]Non-previously described alleles and STs are indicated in bold.

4.1.1.2. Cross-sectional analysis of the Balearic Islands collection

Half of the patients which had had a positive sputum culture for *P. aeruginosa* since 2003 to 2013 were female, being their median age in 2013 of 20.3 years (0-45 years). Most of these patients were chronically colonized by *P. aeruginosa* (n=22) or showed an intermittent infection-colonization pattern (n=12) at the time of the study. Age distribution and associated *P. aeruginosa* infection-colonization patterns have been plotted in Figure 4.2.
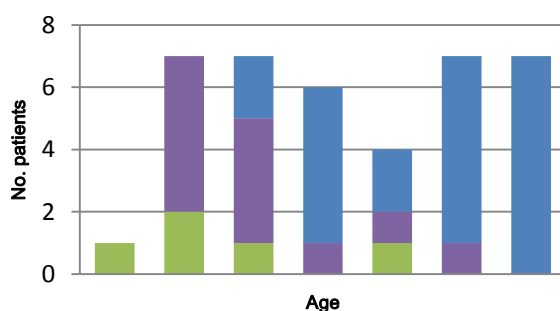
Figure 4.2. Age distribution and infection-colonization pattern of CF patients with a positive sputum culture for *P. aeruginosa* (2003-2013). Colonization patterns are indicated in blue (chronic), purple (intermittent) and green (primocolonization).

Clonal relatedness among isolates was also studied by both, PFGE and MLST. Analysis of PFGE restriction fragments yielded 31 different patterns, collected in Table 4.2.

Table 4.2. PFGE and MLST results summary of the 40 studied isolates.

PFGE Clone	Colonization pattern (No. patients)	ST[a]	Allelic profile[b]						
			acsA	*aroE*	*guaA*	*nuoD*	*mutL*	*ppsA*	*trpE*
FQSE-A	Chronic (4)	ST274	23	5	11	7	1	12	7
	Chronic (1)	**ST1089**	**66**	5	**101**	7	1	12	7
FQSE-C	Chronic (1)	ST299	17	5	36	116	3	7	3
FQSE-F	Chronic (1)	**ST1072**	5	13	25	6	1	7	3
FQSE-I	Chronic (2)	**ST1109**	16	14	3	11	1	15	1
FQSE-K	Chronic (2)	**ST701**	29	1	9	13	1	6	23
FQSE-N	Chronic (1), Intermittent (2)	ST312	5	3	57	6	1	33	47
FQSE-O	Chronic (1), Primocolonization (1)	ST1339	113	5	24	3	1	6	25
FQSE-P	Intermittent(1)	ST277	39	5	9	11	27	5	2
FQSE-Q	Intermittent (1)								
FQSE-R	Intermittent (1)	ST198	11	5	11	11	3	27	7
FQSE-S	Intermittent (1)	ST253	4	4	16	12	1	6	3
FQSE-T	Intermittent (1)	ST279	5	3	57	3	1	33	47
FQSE-U	Chronic (1)	ST285	16	22	6	74	2	41	2
FQSE-V	Intermittent (1)	ST308	13	4	5	5	12	7	15
FQSE-W	Intermittent (1)	ST386	17	5	11	18	4	10	3
FQSE-X	Primocolonization (1)	ST395	6	5	1	1	1	12	1
FQSE-Y	Chronic (1)	ST505	6	20	1	11	4	4	2
FQSE-Z	Intermittent (1)	ST606	23	5	57	30	1	4	3
FQSE-AA	Primocolonization (1)	ST938	15	20	26	13	3	64	2
FQSE-AB	Chronic (1)	ST1527	17	10	129	5	4	112	10
FQSE-AC	Chronic (1)	ST1613	17	5	36	5	4	10	1
FQSE-AD	Primocolonization (1)	ST1637	11	5	3	3	8	1	9
FQSE-AE	Chronic (1)	**ST1837**	28	62	17	3	13	13	7
FQSE-AF	Chronic (1)	**ST1838**	5	1	112	5	1	26	119
FQSE-AG	Intermittent (1)	**ST1839**	17	5	12	9	14	4	7
FQSE-AH	Chronic (1)	**ST1840**	28	5	36	3	2	13	7

[a]Non-previously described alleles and STs are indicated in bold.

Table 4.2. PFGE and MLST results summary of the 40 studied isolates. (Cont.)

PFGE Clone	Colonization pattern (No. patients)	STᵃ	Allelic profileᵇ						
			acsA	*aroE*	*guaA*	*nuoD*	*mutL*	*ppsA*	*trpE*
FQSE-AI	Chronic (1)	**ST1841**	15	5	36	17	27	4	2
FQSE-AJ	Primocolonization (1)	**ST1842**	11	5	30	72	4	4	7
FQSE-AK	Primocolonization (1)	**ST1843**	1	5	17	16	3	4	7
FQSE-AL	Intermittent (1)	**ST2188**	101	84	11	3	4	4	7
FQSE-AM	Intermittent (1)	**ST2189**	11	5	3	3	93	1	9

ᵃNon-previously described alleles and STs are indicated in bold.

A total of five patients (12.5%) showed clone FQSE-A restriction pattern (section 4.1.1.1.), being indeed all of them chronically colonized. Clone FQSE-N and clones FQSE-I, FQSE-K and FQSE-O, were detected in 3 and 2 different CF patients, respectively (Table 4.2.). Within the 3 patients harbouring clone FQSE-N two were siblings, as well as the 2 patients harbouring clone FQSE-I.

When typed by MLST a total of 31 different STs were also found and, with the single exception of clone FQSE-A, the different PFGE patterns were all related with unique sequences types (Table 4.2.). Conversely, the same ST (ST277) was determined for PFGE clones FQSE-P and FQSE-Q.

Of the 31 STs detected, 13 were first reported in this work (41.9%). Nevertheless, just two alleles sequences had not been previously described and, thus, most new STs resulted from new allele combinations (Table 4.2.). Based on their MLST allelic profile, a MST was constructed to infer relatedness among isolates (Figure 4.3.).
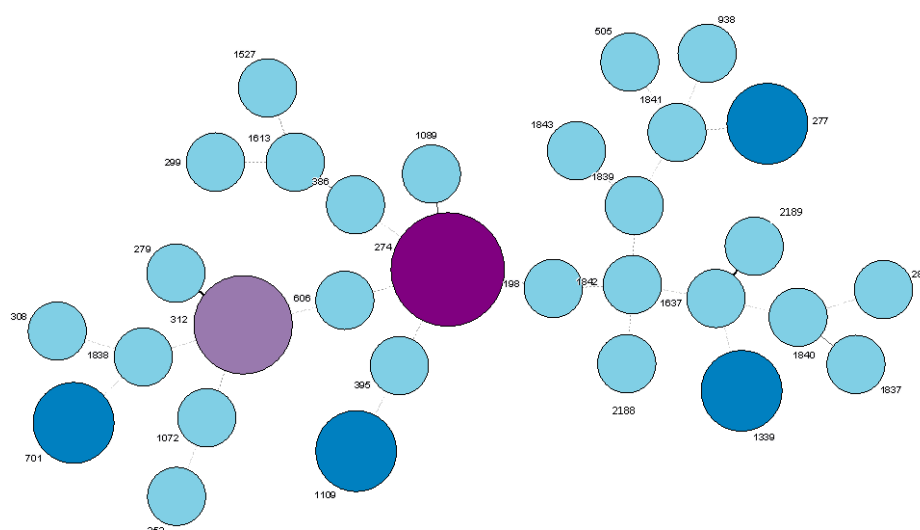
Figure 4.3. MST from the 40 *P. aeruginosa* isolates.

4.1.1.3. Cross-sectional analysis of the Spanish collection

About half of the patients which had had a positive sputum culture for *P. aeruginosa* were female (38/75), being their median age at time of sputum collection of 25.7 (7-51) years. Most of these patients were chronically colonized by *P. aeruginosa* (n=64), whereas just 11 patients showed an intermittent infection-colonization pattern. Primary patients' characteristics are summarized in Table 4.3.

Table 4.3. Primary characteristics of patients colonized by *P. aeruginosa*.

CF Unit	No. patients	Age in years	Colonization pattern (No. patients)	No. Δ508 mutations[a]	
				HT	HM
Paediatric	16	15.4 (7-17)	Chronic (15) Intermittent (1)	3	8
Adult	59	28.5 (18-51)	Chronic (49) Intermittent (10)	28	18

[a]HT: heterozygosis, HM: homozygosis.

Considerable genetic diversity was documented among the *P. aeruginosa* Spanish CF isolates. By PFGE, 70 different restriction patterns were observed among the 79 typed isolates. As well, 72 different STs were detected, each grouping 1 to 3 isolates. Up to 48 (67%) had not been previously described (http://pubmlst.org/paeruginosa/) and most resulted from new allele combinations (83.3%), as just 8 new alleles sequences were defined in this work. The Array Tube genotyping technique enabled the detection of 51 new and 14 previously described Array Tube genotypes, containing one to three isolates each (Table 4.4.).

Table 4.4. Genotyping results obtained from the analysis of the Spanish CF collection.

CF Unit	Isolate[a]	PFGE[b]	Array Tube	ST[c]	MLST allelic profile[c]						
					acsA	*aroE*	*guaA*	*nuoD*	*mutL*	*ppsA*	*trpE*
A	1	1	X298	1748	16	5	30	11	4	13	7
B	2	2	6018	**1886**	7	133	36	63	74	15	2
	3	3	6198	**1887**	11	10	1	61	27	4	7
C	4	3	XX98	**1888**	11	10	1	3	4	4	7
	5	4	8782	**1889**	15	121	36	5	10	15	8
D	6	5	F669	**1890**	5	1	11	13	10	7	23
	7	6	4818	**1891**	17	83	1	61	3	4	2
	8	7	7D98	**1866**	1	5	1	98	1	10	10

[a] Different morphotypes in the same CF patient (from 1 to 4).

[b] Isolates exhibiting similar or identical PFGE patterns have been highlighted in grey.

[c] Non-previously described alleles and STs are indicated in bold.

Table 4.4. Genotyping results obtained from the analysis of the Spanish CF collection. (Cont.)

CF Unit	Isolate[a]	PFGE[b]	Array Tube	ST[c]	MLST allelic profile[c]						
					acsA	*aroE*	*guaA*	*nuoD*	*mutL*	*ppsA*	*trpE*
E	9	8	CC0A	1109	16	14	3	11	1	15	1
	10	9	8C2C	**1867**	6	94	**133**	1	1	12	1
	11	10	2C22	**1868**	23	5	11	98	1	12	7
	12	11	6FA8	**1869**	15	24	5	5	50	4	14
F	13	12	F468	**1870**	17	11	3	13	1	2	4
	14	13	AD80	**1892**	17	5	4	66	4	15	19
G	15	14	ED98	**1893**	101	8	78	72	4	13	7
	16	15	882A	270	22	3	17	5	2	10	7
	17	16	6010	116	28	24	22	18	3	15	7
	18	17	0C2E	395	6	5	1	1	1	12	1
	19	18	0C2A	**1894**	17	**187**	1	3	4	15	3
	20	19	2F80	**1895**	17	6	4	14	4	6	2
	21	20	741C	1228	11	5	5	29	4	4	26
	22	21	0C2A	253	4	4	16	12	1	6	3
	23	22	0C2C	395	6	5	1	1	1	12	1
	24	23	F428	**1896**	5	3	5	3	1	33	**189**
H	25	24	0422	**1897**	7	162	12	3	3	4	185
	26	25	2C18	**1871**	28	22	5	43	3	14	19
	27	26	C40A	**1872**	11	5	1	117	9	4	**190**
	28	27	E022	**1873**	1	5	11	3	2	4	3
	29	28	859A	575	11	5	83	2	4	13	7
	30	29	8428	**1898**	15	5	83	11	4	62	7
	31	30	F419	**1899**	39	5	70	28	4	4	63
I	32	30	B01A	**1900**	39	5	68	28	4	4	63
	33	31	2C20	617	16	101	11	97	4	69	88
J	34	32	B01X	**1901**	16	101	11	13	4	69	88
	35	33	F421	560	5	5	57	13	1	40	3
	36	34	262A	**1902**	11	94	7	98	2	7	33
	37	31	2C20	**1903**	23	5	11	13	1	12	137
	38	35	E42A	**1874**	29	**188**	95	13	8	6	11
K	39	35	E428	**1874**	29	**188**	95	13	8	6	11
	40	36	A998	609	16	22	5	11	4	6	10
	41	35	E428	**1875**	29	**188**	95	127	8	6	11
	42	31	2C20	268	23	5	70	7	1	12	7
	43	37	2F88	**1904**	6	103	11	63	4	15	2

[a] Different morphotypes in the same CF patient (from 1 to 4).

[b] Isolates exhibiting similar or identical PFGE patterns have been highlighted in grey.

[c] Non-previously described alleles and STs are indicated in bold.

Table 4.4. Genotyping results obtained from the analysis of the Spanish CF collection. (Cont.)

CF Unit	Isolate[a]	PFGE[b]	Array Tube	ST[c]	MLST allelic profile[c]						
					acsA	*aroE*	*guaA*	*nuoD*	*mutL*	*ppsA*	*trpE*
L	44	38	2418	**1876**	40	181	11	11	3	4	9
	45	39	E418	564	6	10	5	11	2	15	2
	46	40	2C18	**1905**	6	4	42	5	1	4	26
	47	41	4DA8	**1906**	16	3	1	28	1	55	61
	48	42	2708	644	28	3	94	13	1	4	10
	49	43	7C2C	132	6	20	1	3	4	4	2
	50	44	3E18	**1877**	119	10	83	43	3	6	77
M	51[a1]	45	EC18	**1907**	11	5	11	131	3	53	1
	52[a1]	46	2C2C	**1908**	11	6	19	5	4	15	9
	53	47	6A20	**1909**	17	5	5	4	4	4	**191**
	54	48	6FA8	**1910**	15	5	5	85	8	4	14
N	55[a2]	49	AF90	**1911**	11	3	70	3	1	4	60
	56[a2]	50	F420	1251	35	11	25	6	13	6	84
	57	48	6FA8	**1878**	15	100	66	5	50	4	14
	58	51	2610	**1912**	11	48	11	3	1	15	14
O	59	52	4498	**1913**	11	48	98	5	3	10	85
P	60	53	2810	**1914**	6	14	12	7	1	4	20
	61[a3]	54	E020	**1879**	15	5	11	3	67	4	3
	62[a3]	55	E020	508	15	5	11	3	2	4	3
Q	63	56	8E18	189	26	143	1	3	4	4	10
	64	57	E429	**1880**	13	8	9	97	52	**124**	9
R	65	58	D421	253	4	4	16	12	1	6	3
	66	59	2398	**1881**	22	20	11	48	3	3	7
	67	60	XC10	**1882**	16	5	26	124	4	3	26
	68	59	2398	348	22	20	11	3	3	3	7
S	69	61	DF88	**1883**	17	5	11	97	4	12	56
	70	62	EC10	676	28	5	11	77	3	4	92
	71[a4]	63	E020	508	15	5	11	3	2	4	3
	72[a4]	64	E020	508	15	5	11	3	2	4	3
	73	65	A598	575	11	5	83	2	4	13	7
	74	66	F429	313	47	8	7	6	8	11	40
	75	67	0C48	198	11	5	11	11	3	27	7
	76	68	6E10	569	11	5	11	11	3	6	27
	77	69	0810	**1884**	17	5	12	3	**99**	4	7
T	78	70	EA08	27	6	5	6	7	4	6	7
	79	70	EA08	27	6	5	6	7	4	6	7

[a] Different morphotypes in the same CF patient (from 1 to 4).

[b] Isolates exhibiting similar or identical PFGE patterns have been highlighted in grey.

[c] Non-previously described alleles and STs are indicated in bold.

Results ···

As collected in Table 4.4., identical PFGE band patterns, ST and Array Tube genotypes were detected in *P. aeruginosa* isolates recovered from a pair of siblings (isolates 78 and 79).

The same PFGE band pattern was also observed in unrelated isolates, some of which match with other genotyping methodologies. Isolates from PFGE clone types 3, 30, 35 and 59 were demonstrated to belong to the same CC by MLST (Figure 4.4.) and, with the exception of isolates 31 and 32 (PFGE-30), also to a related Array Tube genotype. The most relevant case concerned a possible intrahospital cross-transmission related to isolates 66 and 68 (PFGE-59), which exhibited an identical PFGE restriction pattern and Array Tube genotype but different MLST, being ascription to different STs due to a single point mutation within *mutL* nucleotide sequence (nt331$_{A \to C}$) that provokes the switch of allele 48 by allele 3, and consequently the assignation of ST1881 instead of ST348. Similarly, isolates 38, 39 and 41 (PFGE-35) were ascribed to STs 1874 and 1875, just differing in their *mutL* sequence by two point mutations, and to Array Tube genotypes E428 and E42A, just differing in the presence or absence of *exoS.* As shown, discordances within *mutL* nucleotide sequences were frequently involved in ascription to different STs. By contrast, isolates from PFGE clone types 31 and 48 yielded a non-related MLST allelic profile but showed the same Array Tube genotype (Table 4.4. and Figure 4.4.).

Conversely, several isolates exhibiting different PFGE restriction patterns were ascribed to the same ST and/or Array Tube genotype (Table 4.4. and Figure 4.4.).

As above described (section 3.3.3.), the Array Tube genotyping method also includes several probes to explore the accessory genome. In this sense, just isolates 66 and 68 (PFGE-59) exhibited an identical repertoire of accessory genes (data not shown). As well, isolates 38, 39 and 41 (PFGE-35) just differ in the presence or absence of *fpvB* gene, coding for the alternative type-I ferripyoverdine receptor (data not shown).
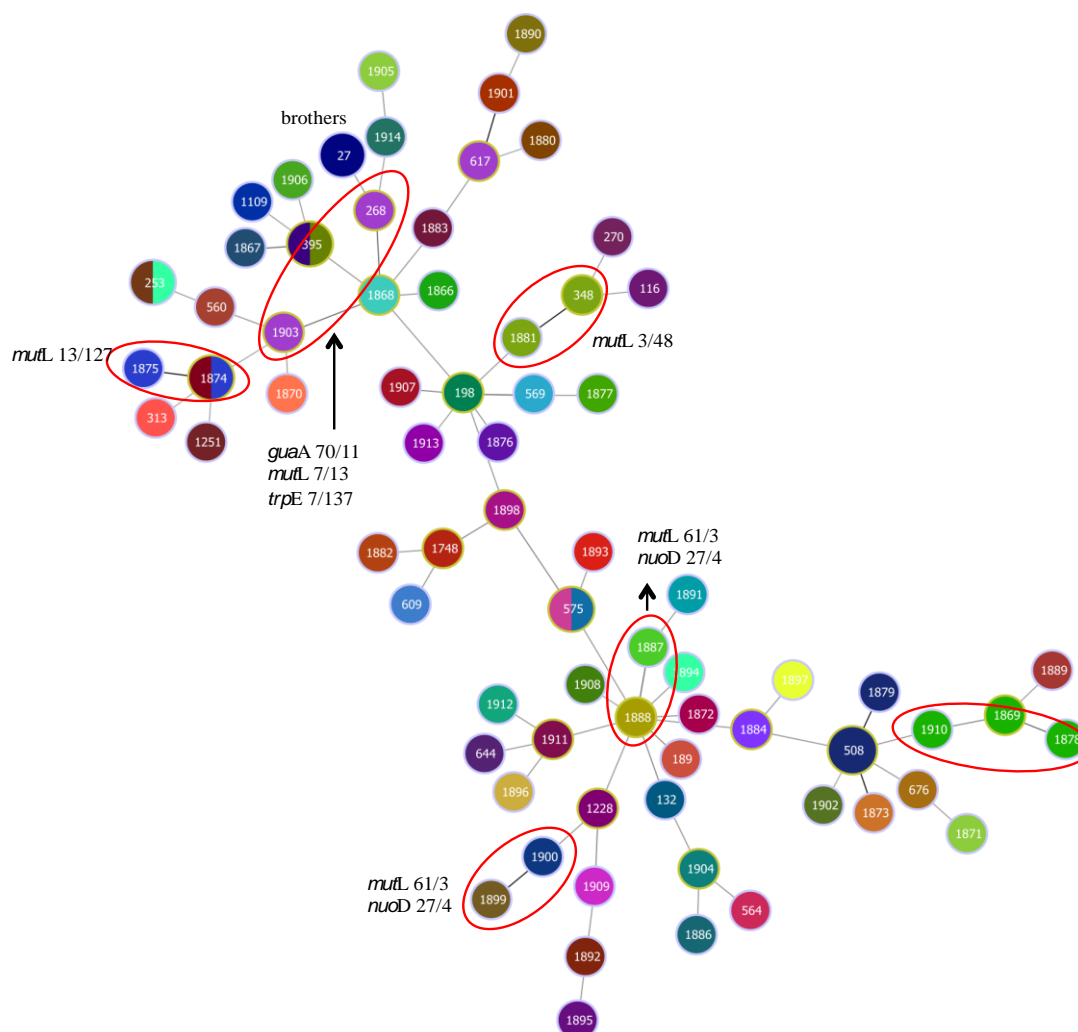
Figure 4.4. MST based on the MLST allelic profiles. Isolates with the same Array Tube genotype are represented with the same colour. As well, isolates ascribed to the same PFGE clone type are indicated with red lines.

Overall, ferripyoverdine receptor genes (*fpvA*) type I, IIa, IIb and III were detected in 23, 22, 6 and 11 isolates respectively, and the alternative type-I ferripyoverdine receptor gene *fpvB* was present only in 45 isolates. Ferripyoverdine receptor genes were not detected in 15 isolates, and all but two were isolates from adult patients. On average, isolates possess 2.3 genome islets and 2.4 genome islands, ranging from 0 to 5. The flagellin glycosylation island was encountered to be the most prevalent one (n=53, 67%). Nevertheless, 2 of these isolates lacked the a-type flagellin and, on the contrary, in 3 isolates expressing the a-type flagellin the flagellin glycosylation island was not detected. PAGI-1 was detected in only 45 isolates (57%) and the genomic islands of the CLC family PAGI-2/3 in 23 isolates (29%). Two isolates harboured both PAPI-1 and PAPI-2, and 43 isolates (54%) harboured only PAPI-2; other pKLC102-like islands were detected in 30 isolates (38%). Statistical differences within the global collection and the different subsets, adult or paediatric population, were not observed (Table 4.5.).

Results

Table 4.5. Prevalence (%) of ferripyoverdine receptor genes, gene islands and genome islets among the Spanish collection.

	fpvA/B	Fla-glyc	PAGI-1/2-3	PAPI-1/2	pKLC102-like	Genome Islets
Global (n=79)	77/57	67	57/29	2/54	38	87
Adult (n=61)	83/59	64	59/26	3/54	36	93
Paediatric (n=18)	72/50	78	50/39	0/56	44	72

The Array Tube genotyping tool also includes probes for detecting T3SS effectors *exoS* and *exoU* genes. An unusual low prevalence, which did not correlate with obtained results by independent specific PCRs (18% vs 81% and 9% vs 10%, respectively), was registered. Coexistence of both genes by specific PCRs was only observed in 3 isolates and for 12 isolates neither *exoS* nor *exoU* was detected.

4.1.2. Antimicrobial resistance

4.1.2.1. Antibiotic susceptibility profiles

Non-susceptibility rates, MIC50 and MIC90 values for the Balearic Islands CF collection (January 2003 to June 2013) are summarized in Table 4.6. Up to 726 *P. aeruginosa* CF isolates were included in the final data analysis.

Colistin was the compound for which a minor percentage of non-susceptibility was registered (5.5%) followed by ceftazidime and meropenem, for which about 20% of the isolates showed *in vitro* resistance. Conversely, aztreonam and ciprofloxacin were the antibiotics exhibiting major resistance rates (44% and 73%, respectively) (Table 4.6.). Overall, the MDR rate was set in 17% and, more worrisome, 1% of the isolates met the PDR criteria.

MIC50 values for all tested compounds but aztreonam were within the susceptibility range, whereas just colistin MIC90 fell within this range (Table 4.6.). Of note, 89% of the isolates exhibited a tobramycin MIC under 64 mg/L, which is the suggested breakpoint for inhaled therapy.

Table 4.6. Non-susceptibility rates, MIC50 and MIC90 obtained for the Balearic Islands collection.

	Antibiotic						
	TZ	IP	MP	CI	TM	CO	AT[a]
% (I+R)[b]	20	33	21	44	28	5	73
MIC50	2	3	0.38	0.5	2	1	3
MIC90	64	32	24	4	96	2	256

a AT was tested just in *P. aeruginosa* isolates obtained in 2012 and 2013 (n=120).

b EUCAST clinical breakpoints version 3.1. was applied (Annex 3).

Results from the antibiotic resistance analysis of the Spanish collection are collected in Table 4.8. Colistin was also the most active compound, and only three isolates (4%) were classified as resistant. Conversely, around the 60% were non-susceptible to both fluoroquinolones tested. Considering co-resistances and excluding aztreonam, 15 isolates

(19%) remained susceptible to all antibiotic compounds and 43 (56%) met the MDR criteria. The XDR rate was set in 16% in the Spanish CF collection.

Antibiotic resistance temporal evolution. When temporal evolution of antibiotic resistance rates was analyzed within the Balearic Islands CF collection, a significant upward trend was registered being of particular concern the increase of the colistin resistance rate, moving from 0% in 2003 to 10% in 2012. As well, the multidrug resistance rate increase from 11% to 37% in the studied period (Figure 4.5.).
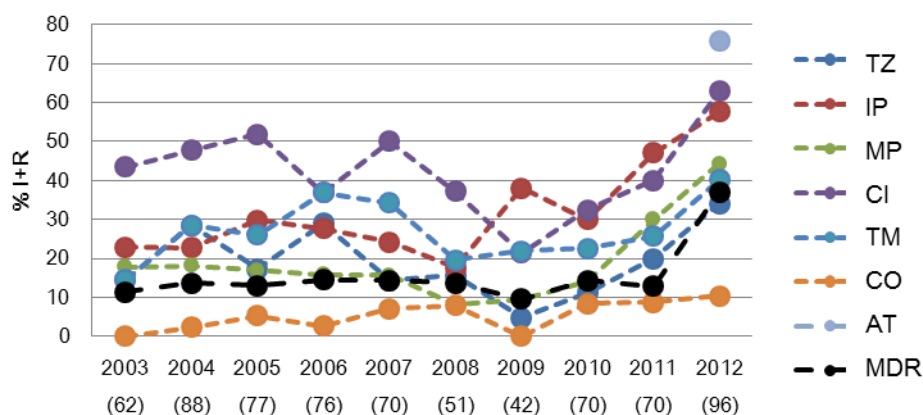
Figure 4.5. Temporal evolution (2003-2012) of antibiotic resistance rates (I+R%) in the Balearic Islands *P. aeruginosa* CF collection. No. of isolates/year is indicated in brackets.

Morphotype and antibiotic susceptibility profiles. Antibiotic resistance depending on colony morphotype was also studied within the Balearic Islands collection, including for this purpose all *P. aeruginosa* isolates obtained from 2003 to 2010. Almost 40% of the isolates showed a mucoid morphotype and about 15% were classified as SCV. Related to patients, up to 61% and 46% did not harbour mucoid or SCV isolate during this period, respectively. In comparison with the entire collection, lower and higher antimicrobial resistance rates for all antimicrobials tested were documented for the mucoid and the SCV subsets, respectively (Table 4.7.).

Table 4.7. Antimicrobial resistance rates (I+R%) determined for the Balearic Islands *P. aeruginosa* CF collection stratified by colonies morphotype.

Morphotype	Antibiotic[a]					
	TZ	IP	MP	CI	TM	CO
Mucoid (n=213)	11	17	9	30	20	2
SCV (n=82)	28	37	18	59	33	8
Global (n=536)	18	26	15	42	26	4

[a] EUCAST clinical breakpoints version 3.1. was applied (Annex 3).

In the Spanish CF collection, the classical CF mucoid morphotype was observed in 17 isolates (21%) and 16 isolates (20%) presented a SCV morphology. Compared to the whole collection, lower and higher antimicrobial resistance rates were also determined for the

mucoid and the SCV subsets, respectively (Table 4.8.). Of note, most SCV (87%) were classified as multi-drug resistant.

Table 4.8. Antimicrobial non-susceptibility rates (I+R%) determined for the whole Spanish *P. aeruginosa* CF collection and stratified by morphotype.

Morphotype	Antibiotic[a]														
	PPT	TZ	PM	AT	IP	MP	CI	LE	TM	GM	AK	CO	FO	MDR	XDR
Mucoid (n=17)	18	23	47	100	18	35	59	59	29	35	41	0	12	18	6
SCV (n=16)	25	50	37	100	44	50	69	69	31	37	37	0	31	87	12
Global (n=79)	18	33	37	100	35	45	59	64	32	37	39	4	19	56	16

[a] EUCAST clinical breakpoints version 6.0. was applied (Annex 3).

Infection-colonization patterns and antibiotic susceptibility profiles. In the analysis of the Balearic Islands collection, isolates obtained from patients chronically colonized exhibited higher resistance rates for all the antibiotics tested. In accordance, MIC90 values for all antibiotics but colistin in the chronically colonized subset fell out the susceptibility range whereas all MIC90 values in the other subset remained within this range. Moreover, all *P. aeruginosa* isolates meeting the MDR criteria had been obtained from respiratory samples of patients chronically colonized. No differences were observed in the MIC50 values for all antibiotics between both subsets, falling all within the susceptibility range (Table 4.9.).

Table 4.9. Non-susceptibility rates, MIC50 and MIC90 obtained for the *P. aeruginosa* CF Balearic Islands collection stratified by patient's colonization pattern.

		Antibiotic					
		TZ	IP	MP	CI	TM	CO
Intermittent and primocolonization (n=47)	% (I+R)[a]	0	4	2	6	4	0
	MIC50	1.5	2	0.25	0.125	1.5	2
	MIC90	4	4	0.5	0.38	3	3
Chronic (n=679)	% (I+R)[a]	22	35	22	47	30	6
	MIC50	2	3	0.38	0.5	2	1
	MIC90	192	32	32	4	256	3

[a] EUCAST clinical breakpoints version 3.1. was applied (Annex 3).

4.1.2.2. Antibiotic resistance mechanisms

Contribution of chromosomically-encoded *P. aeruginosa* efflux pumps, including MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY(-OprM), as well as AmpC overexpression and OprD deficiency to the antibiotic resistance profiles during long-term CRI was evaluated. For this purpose, the 100 CF *P. aeruginosa* isolates chronically colonizing the 10 selected patients of the Balearic Islands were in-depth studied.

The antibiotic resistance profiles were variable within and across patients along the study period; however, a significant trend towards the accumulation of resistance was noted in

individual patients and clones, increasing from an average of resistance to 1.1±1.2 antibiotics in the first isolate of each patient to 2.5±0.85 in the last isolate (paired t test, p = 0.016). Overall, within this subset of 100 CF chronic isolates, the lowest susceptibility rate was observed for aztreonam (60% S) and the highest for meropenem (96% S), whereas resistance rates were highest for cefepime (30% R), tobramycin (30% R) and ciprofloxacin (24% R) and lowest for meropenem (1% R), aztreonam (4% R), and colistin (7% R). As EUCAST considers *P. aeruginosa* intrinsically nonsusceptible to aztreonam (mainly due to the basal expression of MexAB-OprM efflux pump), the percentage of susceptible isolates (60%) documented actually reflected the high number of hypersusceptible isolates falling outside of WT MICs distributions (http://www.eucast.org/mic_distributions/). As well, an important number of these isolates showed hypersusceptibility to meropenem with MICs (<0.06 mg/L) falling outside of WT distributions (Figure 4.6.).
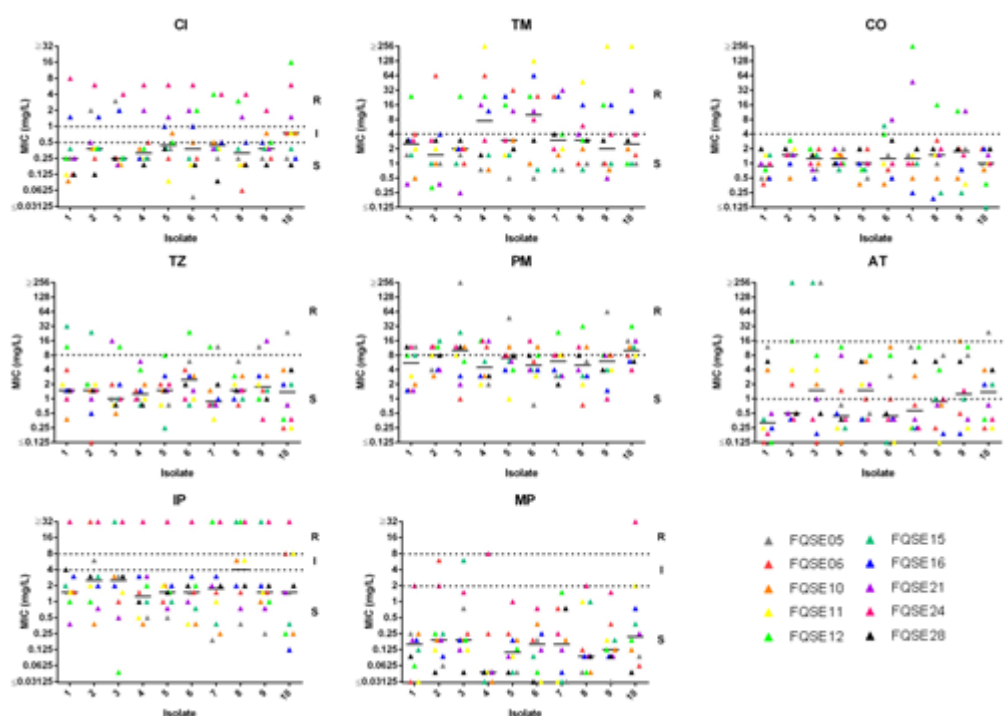
Figure 4.6.. Evolution of minimal inhibitory concentrations (MICs) from the first to the last studied isolate from each patient. Each colour represents a different patient.

Contribution to the documented antibiotic resistance was evaluated within the first and last isolate from each patient and PFGE clone type (Table 4.10.). As for antibiotic resistance, a trend towards accumulation of resistance mechanisms was also noted, moving from 1.4±0.58 in the first to 2.1±0.88 in the last isolates, although differences did not reach statistical significance (p = 0.06).

Table 4.10. MICs and resistance mechanisms detected in first and last isolates from each CF patient and PFGE clone.

Isolate ID	MICs (mg/L)								Resistance mechanisms	*mexZ* mutation[a]
	TZ	PM	AT	IP	MP	CI	TM	CO		
FQSE06-01	0.064	1.5	0.125	1.5	0.016	0.125	4	0.38	*mexY*	S9P
FQSE06-10	1.5	8	0.25	8	0.064	0.75	1.5	2	*mexY*, OprD-	$Nt_{292}\Delta 11$
FQSE10-01	0.38	2	4	1.5	0.25	0.094	0.5	0.5	*mexY*	IS[b]
FQSE10-10	3	12	4	0.25	0.125	0.75	2	0.75	*ampC, mexY*	IS[b]
FQSE15-01	1	8	0.38	2	0.125	0.38	1.5	1.5	*mexY*	A144V
FQSE15-10	1.5	12	2	0.38	0.38	0.38	1	0.016	*mexY*	A194P
FQSE24-01	1	12	0.19	>32	2	8	3	1	*mexY*, OprD-	A194P
FQSE24-10	0.38	4	0.38	>32	>32	6	4	1	*mexY*, OprD-	A194P
FQSE12-01	0.75	8	0.125	1	0.064	0.25	24	0.75	*mexY*	R125P
FQSE12-06	8	8	8	2	0.5	2	24	4	*ampC, mexY*	R125P
FQSE12-07	24	24	12	>32	1.5	4	4	>256	*ampC, mexY, mexF*, OprD-	Q164X
FQSE12-10	1	32	0.38	0.25	0.19	16	1	0.38	*mexY, mexF, mexD*, OprD-	Q164X
FQSE05-01	16	12	12	1.5	0.25	0.25	1.5	0.5	*ampC*	ND[c]
FQSE05-03	2	>256	>256	3	0.75	3	1.5	0.75	*ampC, mexY*	ND[c]
FQSE05-04	1	2	0.75	0.5	0.047	0.25	0.75	2	*mexY, mexF*	W158X
FQSE05-06	0.75	0.75	3	1.5	0.19	0.047	0.5	1.5	-	ND[c]
FQSE05-10	12	16	24	1.5	0.094	0.25	1	1	*ampC, mexY*	V43G
FQSE21-01	0.5	1.5	0.5	0.38	0.19	0.25	0.38	1	*ampC, mexY*	$Nt_{61}\Delta 15$
FQSE21-04	16	16	8	3	0.5	2	16	2	*mexB, mexY*	K131R
FQSE21-09	1.5	8	0.38	0.75	0.094	0.38	0.5	12	*mexY*	$Nt_{61}\Delta 15$
FQSE21-10	8	16	0.75	1.5	0.25	1.5	32	1.5	*mexB, mexY*	K131R
FQSE28-01	1.5	12	6	4	0.094	0.125	3	2	*ampC, mexY*	$Nt_{189}\Delta 12$
FQSE28-10	1	6	4	2	0.047	0.19	3	2	*ampC, mexY*	$Nt_{189}\Delta 12$
FQSE11-01	1	3	0.25	1.5	0.032	0.125	2	1.5	*mexY*	WT
FQSE11-07	1	8	0.125	1	0.023	0.38	2	1.5	*mexY*	$Nt_{279}\Delta 12$
FQSE11-10	2	8	0.25	8	2	0.75	>256	1	*mexY*, OprD-	WT
FQSE16-01	1.5	1.5	0.25	3	0.19	1.5	3	0.5	*mexF, mexD*	WT
FQSE16-10	3	6	2	0.125	0.75	0.25	12	2	*ampC, mexY, mexF*	R125P

[a] PAO1 and PA14 were used as reference WT sequences. Mutations are referred to PAO1 sequence.

[b] 1.2 Kb IS located in *mexX-mexZ* intergenic región (nt -72 respect mexZ coding sequence). Encodes a putative transposase identical to that previously reported in *P. pseudoalcaligenes* CECT 5344 (ref ZP_10763279.1)

[c] ND: not done

The most frequent resistance mechanism was MexXY(-OprM) overexpression, which was documented in all 10 patients. Moreover, this mechanism was already present in most patients (8 of 10) in the early isolates (Table 4.10.). Overexpression of the other efflux pumps was much more infrequent, being MexEF-OprJ overexpression documented in 3 patients, MexCD-OprN in 2 and MexAB-OprM just in one of the patients. As well, AmpC overexpression was evidenced in 6 of the patients and lack of OprD production in the 4 patients colonized by imipenem resistant strains. Although a certain correlation was

documented between AmpC overexpression and ceftazidime resistance and between lack of OprD and imipenem resistance, a correlation between phenotype and genotype was not always evident, particularly concerning efflux pumps overexpression.

In relation to PFGE clone type, isolates 07 and 10 from patient FQSE12 (FQSE-B/ST146/LES-1) were associated with a higher number of resistance mechanisms (Table 4.10.). As shown, the initial MDR isolate from this clone already expressed 4 resistance mechanisms [MexXY(-OprM), MexEF-OprN and AmpC overexpression plus OprD deficiency], as well as the last isolate did [MexXY(-OprM), MexCD-OprJ, MexEF-OprN and OprD deficiency]. Nevertheless, a significant reduction of the MDR profile was documented, likely influenced by the modification of the resistance mechanisms expressed: MexCD-OprJ instead of AmpC overexpression.

Additionally, as almost all early and late isolates overexpressed the efflux-pump MexXY(-OprM), its major regulator *mexZ* was sequenced. Most of the strains showed *mexZ* mutations including deletions, premature stop codons, insertion sequences (IS), or nonsynonymous aminoacid substitutions, thus supporting MexXY overexpression (Table 4.10.).

4.1.3. Prevalence of mutators, mutant frequencies and genetic basis for hypermutation

4.1.3.1. Analysis of the Spanish collection

The prevalence of mutators in the Spanish CF *P. aeruginosa* collection was set at 15.2%. A total of 12 isolates, recovered from 8 adults and 4 children, were classified as mutators ranging their rifampicin mutation frequency from $2x10^{-5}$ to $4.5x10^{-7}$. All these mutators belonged to unrelated genetic lineages (Figure 4.4.), and, of note, most of them were ascribed to new STs.

In 8 of the mutator isolates inactivating mutations within *mutS* (n=7) or *mutL* (n=1) genes were encountered and their implication in the mutator phenotype was confirmed by complementation studies. Three additional mutator isolates showed amino acid substitutions in the MutS and/or MutL proteins, and these aminoacid changes were also demonstrated to be involved in the observed phenotype. The remaining isolate showed WT sequences of *mutS* and *mutL* genes (Table 4.11.).

Table 4.11. Rifampicin mutation frequencies and genetic basis for hypermutation of mutator isolates from the Spanish CF collection.

Isolate ID	ST	Mutation frequency	Detected mutation[a]	Complemented with
6	**1890**	$1.38 \cdot 10^{-6}$	*mutL* (nt644Δ21)	*mutL*
9	1109	$8.26 \cdot 10^{-6}$	*mutL* (nt1120Δ5)	*mutS*
13	**1870**	$5.20 \cdot 10^{-6}$	Not detected	-
16	270	$2.93 \cdot 10^{-7}$	*mutS* (G1290A, E431K)	*mutS*
			mutL (G1872A, G632E)	*mutL*
26	**1871**	$2.50 \cdot 10^{-6}$	*mutL* (T1309C, A437T)	*mutL*
27	**1872**	$4.57 \cdot 10^{-7}$	*mutL* (T647G, V216G)	*mutL*
42	268	$2.47 \cdot 10^{-6}$	*mutS* (nt1600Δ13)	*mutS*
47	**1906**	$1.80 \cdot 10^{-6}$	*mutS* (nt1336Δ2)	*mutS*
49	132	$2.00 \cdot 10^{-5}$	*mutS* (nt399Δ12)	*mutS*
53	**1909**	$3.20 \cdot 10^{-6}$	*mutS* (nt1377Δ1)	*mutS*
55	**1911**	$2.71 \cdot 10^{-6}$	*mutS* (nt2577ins9pb)	*mutS*
60	**1914**	$3.07 \cdot 10^{-6}$	*mutS* (nt1198IS-4-like)	*mutS*

[a] PAO1 and PA14 were used as reference WT sequences. Mutations are referred to PAO1 sequence.

[b] Non-previously described STs are indicated in bold.

4.1.3.2. Analysis of the Balearic Island collection

During CF-CRI, an unusual high prevalence of mutators has been previously demonstrated [Oliver A *et al*, 2000]. Thus, their prevalence within the subset of 100 CF *P. aeruginosa* isolates chronically colonizing patients from the Baleric Islands was additionally evaluated. Up to 29% of the isolates were determined to exhibit a mutator phenotype, showing 6 of the 10 patients at least 1 mutator isolate during the study period (Figure 4.7.). In two of the patients (FQSE16 and FQSE24) all isolates were mutators and in other two, mutators emerged at late stages of colonization (FQSE15 and FQSE21). In one more patient a mutator lineage emerged (FQSE06) but it was not fixed and in other one it was replaced by the multi-drug resistant ST146/LES-1 epidemic strain (FQSE12).
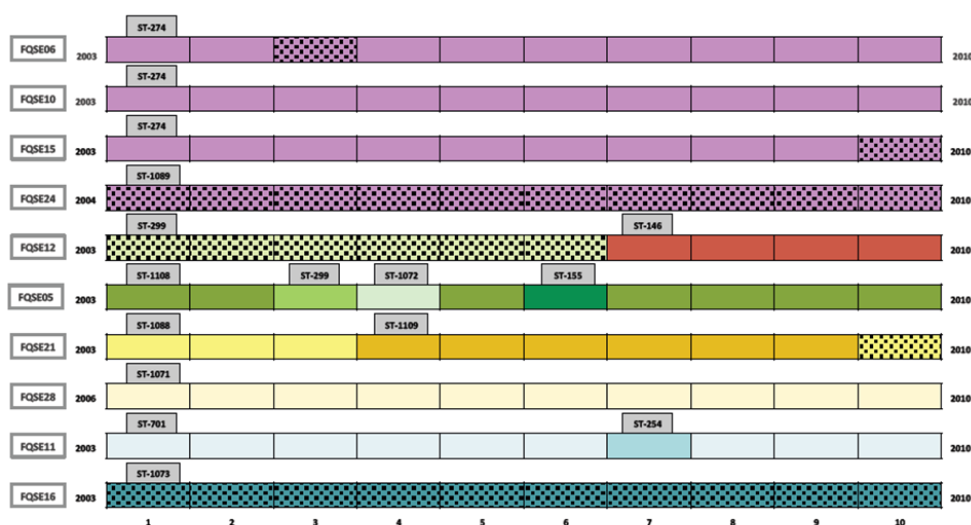
Figure 4.6. Encountered mutator isolates within the 10 sequential isolates from each of the 10 CF patients from the Baleric Islands.
Mutator isolates are marked with a checked pattern.

As shown in Figure 4.6., mutators were detected within 3 of the 4 patients chronically colonized by the widespread clone FQSE-A (ST274/ST1089) being, indeed, all isolates from patient FQSE24 mutators. As initially MLST had been just performed only in the first isolate per PFGE clone type and patient (section 4.1.1.1), and a different ST (ST1089) was detected within this patient we decided to extend the MLST analysis to the last available isolate from each of these patients as well as the two additional sporadic mutator isolates detected in patients FQSE06 and FQSE15. In all cases, the determined ST coincided with that of the first isolate, except for the mutator lineage emerging from one of the patients (FQSE06) that was also identified as ST1089 (Figure 4.6.). So, mutator lineages were detected in 3 of the 4 patients harbouring clone FQSE-A, two belonging to ST1089 and one to ST274. Therefore, available data clearly suggest that ST1089 has recently evolved from ST274 through point mutations linked to the emergence of a mutator lineage. Moreover, when genetic basis of hypermutation was investigated within all these mutator isolates all were demonstrated to be defective in *mutS*. Consequently, MutS encoding gene was sequenced from the three mutator isolates as well as for several representative non-mutator isolates. The three mutator isolates harboured the same inactivating mutation, a 4 bp deletion from nt814; whereas this mutation was absent in the non-mutator isolates.

Hypermutation has been pointed out as a driver of antibiotic resistance. In accordance, a significant trend (p= 0.009) towards resistance to a higher number of antibiotics among mutator isolates (2.28±0.22) than among non-mutator isolates (1.49±0.17) was noted in this subset.

4.2. *Pseudomonas aeruginosa* RESISTOME EVOLUTION IN CYSTIC FIBROSIS CHRONIC RESPIRATORY INFECTIONS

4.2.1. Mutational resistome evolution of the international CC274 cystic fibrosis clone

4.2.1.1. Prevalence and genetic basis for hypermutation

Among the CC274 study collection, nine isolates (31%) were mutators, belonging to six (35%) different patients, residing in both Australia (n=3) and Spain (n=3). Data from sequential isolates were available for the Spanish isolates: one was chronically infected with a persistent mutator lineage (FQSE24), whereas the other two harboured a mixed population of mutator and non-mutator isolates (FQSE06 and FQSE15) (Figure 3.2. and section 4.1.3.).

Sequence variation within an exhaustive panel of so called mutator genes (Annex 5) was analyzed in all mutator and non-mutator isolates. The three Australian mutator isolates showed unique mutations in either *mutL* or *mutS*, whereas all mutator isolates from the three Spanish patients were found to share the same inactivating mutation in *mutS*. Complementation studies with plasmids harbouring WT MMR system genes (*mutS* and *mutL*) were performed in these mutator isolates. As shown in Table 4.12., WT rifampicin resistance mutation frequencies were restored in all mutator isolates upon *mutS* or *mutL* complementation, which correlated in all cases with the presence of specific mutations in these genes. Of note, while mutator phenotypes could be explained in all cases by specific mutations in MMR genes, the contrary was not always true, since one of the non-mutator isolates (FQSE03) showed a missense mutation in *mutS*. Moreover, the presence of polymorphisms in other mutator genes was frequent, but showed no association with mutator phenotypes (Table 4.12.).

Table 4.12. Mutator phenotype and genetic basis for hypermutation in the *P. aeruginosa* CC274 collection.

Isolate ID[a]	ST	Mutation frequency	Complement with	Sequence variation in mutator genes (mutome)[b]									
				ung	*mfd*	*mutS*	*sodB*	*mutT*	*sodM*	*mutL*	*mutM*	*oxyR*	*polA*
AUS034	274	$1.59 \cdot 10^{-6}$	*mutL*					E236D		R631C	D61N L132P		D876E
AUS410	274	$2.04 \cdot 10^{-8}$	-				E25V						D876E
AUS411	274	$6.63 \cdot 10^{-8}$	-					E236D			D61N		D876E
AUS531	274	$1.84 \cdot 10^{-8}$	-					E236D			D61N		D876E
AUS588	274	$2.06 \cdot 10^{-8}$	-				E25V						D876E
AUS601	1043	$1.54 \cdot 10^{-6}$	*mutL*	S13R			E25V			P159S H288Y		F106L H219Y	D876E
AUS603	274	$1.23 \cdot 10^{-8}$	-				E25V						D876E
AUS690	274	$3.71 \cdot 10^{-6}$	*mutS*		Q1123H	C224R T287P		E236D			D61N		D876E
FQRC10	274	$2.22 \cdot 10^{-9}$	-					E236D			D61N		D876E
FQRC15	274	$2.39 \cdot 10^{-8}$	-					E236D			D61N		D876E
FQRC26	274	$9.09 \cdot 10^{-9}$	-					E236D			D61N		D876E
FQSE03	274	$1.11 \cdot 10^{-8}$	-			L374V		E236D			D61N		D876E
FQSE06-0403	274	$3.33 \cdot 10^{-8}$	-					E236D			D61N		D876E
FQSE06-1104	274	$1.04 \cdot 10^{-6}$	*mutS*			Nt814Δ4		E236D			D61N		D876E
FQSE06-0807	274	$5.03 \cdot 10^{-8}$	-					E236D			D61N		D876E
FQSE06-0610	274	$6.50 \cdot 10^{-9}$	-					E236D			D61N		D876E
FQSE10-0503	274	$3.49 \cdot 10^{-9}$	-					E236D			D61N		D876E
FQSE10-0106	274	$2.22 \cdot 10^{-8}$	-					E236D			D61N		D876E
FQSE10-0110	274	$9.80 \cdot 10^{-8}$	-					E236D			D61N		D876E
FQSE10-0111	274	$4.35 \cdot 10^{-8}$	-					E236D			D61N		D876E
FQSE15-0803	274	$9.64 \cdot 10^{-8}$	-					E236D			D61N		D876E
FQSE15-0906	274	$1.18 \cdot 10^{-8}$	-					E236D			D61N		D876E
FQSE15-0310	274	$3.96 \cdot 10^{-8}$	-					E236D			D61N		D876E
FQSE15-1110	1089	$3.12 \cdot 10^{-5}$	*mutS*		A868T	Nt814Δ4		E236D			D61N		D876E
FQSE24-0304	1089	$8.46 \cdot 10^{-6}$	*mutS*			Nt814Δ4		E236D			D61N		D876E
FQSE24-1005	1089	$1.96 \cdot 10^{-5}$	*mutS*			Nt814Δ4		E236D			D61N		D876E
FQSE24-0308	1089	$3.88 \cdot 10^{-6}$	*mutS*			Nt814Δ4		E236D			D61N		D876E
FQSE24-1010	1089	$5.95 \cdot 10^{-6}$	*mutS*			Nt814Δ4		E236D			D61N		D876E
PAMB148	274	$5.00 \cdot 10^{-8}$	-					E236D	L202R		D61N		D876E

[a] Isolates are labelled according to the following format: Patient identification - MMYY isolation code in the case of sequential isolates. Sequential isolates from a same patient are separated by dashed lines.

[b] Sequence variations respect to those of PAO1. No mutations were found in other genes associated with mutator phenotypes, including *pfpI*, *mutY*, *dnaQ*, *PA2583*, *PA2819.1*, *PA2819.2*, *radA* and *uvrD*.

4.2.1.2. PHYLOGENETIC ANALYSIS

PFGE macrorestriction patterns indicated that all isolates were clonally related, including mutators, which were indistinguishable from non-mutators. When an UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram was constructed based on PFGE patterns, all isolates from the Balearic Islands clustered together in the same branch, although patterns from one of the patients (FQSE10) were slightly different. In contrast, Australian isolates were less clonal and clustered in different branches (Figure 4.7.).
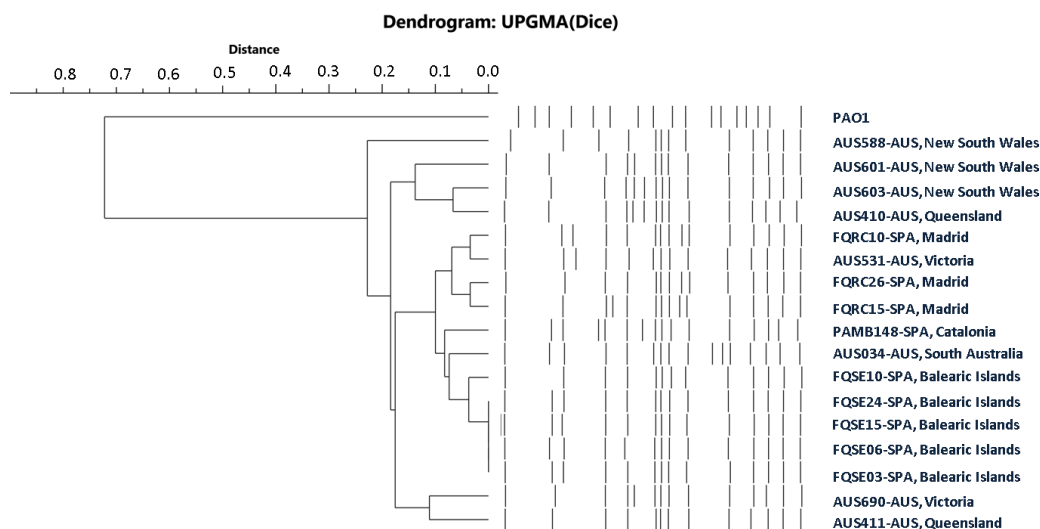
Figure 4.7. UPGMA phylogenetic tree showing the relationship among the CC274 *P. aeruginosa* **collection.** The tree was constructed based on the DNA macrorestriction fragment patterns obtained by pulsed-field gel electrophoresis (PFGE) using SpeI restriction enzyme Isolates are labelled according to the following format: Patient identification - Country (AUS: Australia; SPA: Spain), Region.

Conversely, by MLST, two new and closely ST274-related STs had been detected. As above described, mutators from patients FQSE15 and FQSE24 differed from ST274 by only two point mutations in two of the MLST alleles (*acsA* and *guaA*) leading to ST1089. Nevertheless, the mutator from patient FQSE06, which indeed shared the same inactivating mutation in *mutS*, still belonged to ST274 (section 4.1.1.1.). On the other hand, the Australian mutator AUS601 was also determined to be a new ST (ST1043), but, in this case, just differing from ST274 by two missense mutations in *mutL* allele.

To better understand the evolutionary trajectory, success and international dissemination of CC274, whole-genome based phylogenetic analysis of all 29 isolates were performed. First, to determine the genetic relationship between CC274 isolates and other well-recognized CF epidemic clones, whole-genome sequence reads of all 29 isolates were *de novo* assembled and a phylogenetic tree based on core genome alignment was constructed with default parameters on Parsnp. CC274 was determined to belong to the phylogenetic cluster

containing strain PAO1, as well as other well-known CF epidemic clones such as LESB58, AES-1 and DK2 (Figure 4.8.-A).
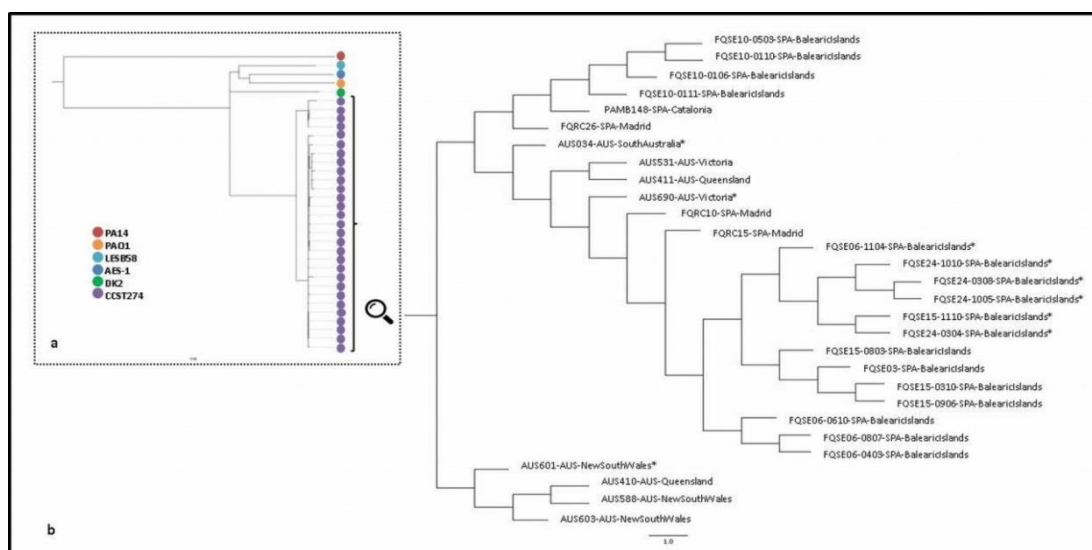
Figure 4.8. Core-genome phylogenetic reconstructions of *P. aeruginosa* CC274 CF clone. (A) Genetic relationship between CC274 and other well-recognized CF epidemic clones. (B) Genetic relationship between the CC274 collection isolates. Both reconstructions were made with Parsnp using default parameters. Isolates are labelled according to the following format: Patient identification - MMYY isolation code in the case of sequential isolates - Country (AUS: Australia; SPA: Spain) - Region. Mutator isolates are identified with an asterisk.

Up to 16,070 common SNP were found by mapping sequence reads for each isolate against *P. aeruginosa* reference PAO1 strain genome. As well, a total of 5,525 high-quality intraclonal SNP were detected, of which 2,294 were unique and thus detected in single isolates. A high degree of intraclonal diversity was observed, with SNP differences between isolates ranging from 20 to 3,256. To elucidate the phylogenetic relationship among isolates two different approaches were used. In both, core-genome and Bayesian time-based analysis, CC274 isolates grouped into two clusters, one including just four Australian isolates and a second major cluster that included all other Australian and Spanish isolates (Figure 4.8-B and figure 4.9.). SNP differences between isolates from the different clusters ranged from 2396 to 3256 and, according to Bayesian time-based analysis, the common ancestor of CC274 was set, approximately, 380 years ago.

As shown, the major cluster further subdivided and, although both phylogenetic reconstructions did not match exactly with each other, both analyses supported that different lineages are currently coexisting with a worldwide distribution, having evolved from a common antecessor set approximately 275 years ago. SNP differences between isolates from Australia and Spain ranged from 114 to 1204, and similar results were obtained when only the Australian (min-max: 230-826) or the Spanish (min-max: 20-839) were compared, supporting no geographical barrier for lineage evolution.

Within the major cluster, all sequential isolates cultured from an individual patient clustered under the same branch with the single exception of all the Spanish isolates that exhibited a mutator phenotype which clustered together, independently of the patient involved and their ST.

Focusing on the sequential isolates, a unidirectional evolution route could not be stablished. Instead, a diversified intrapatient clone evolution that leads to a mix of genetically different sublineages coexisting in the CF respiratory airways was observed. Within a patient, minimum and maximum SNP differences between isolates ranged from 20 to 676, which overlapped with interpatient SNP differences, ranging from 51 to 3256 (51 to 839 for patients from the same hospital).
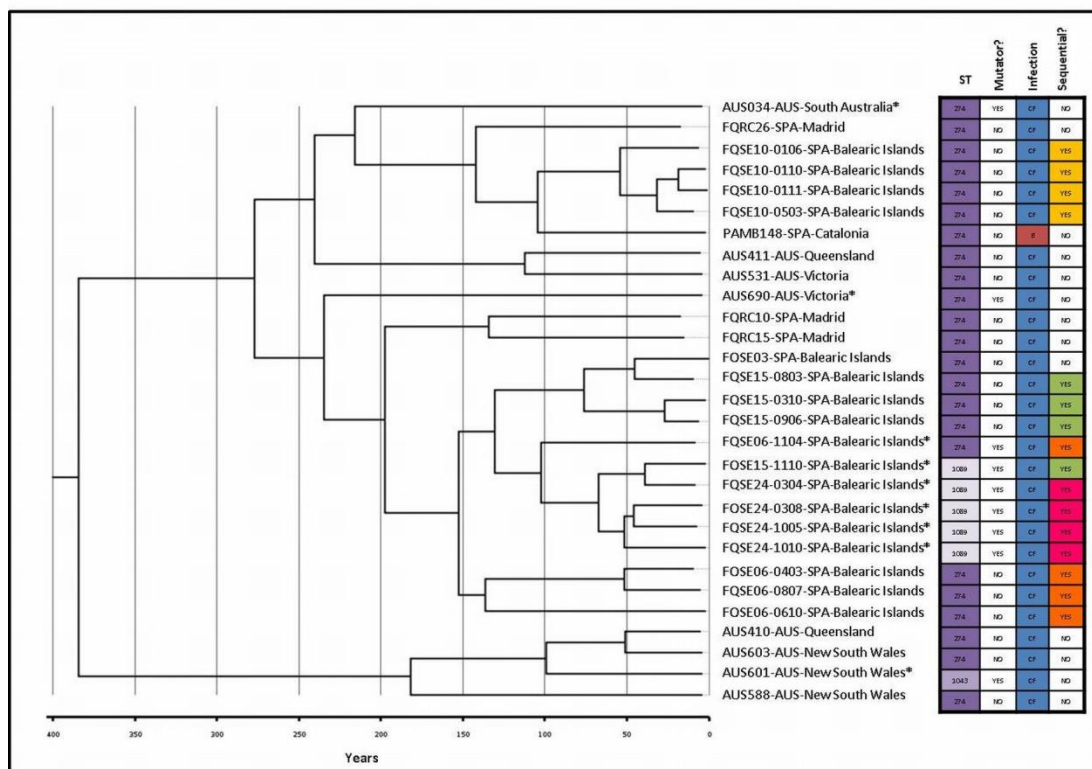
Figure 4.9. Bayesian phylogenetic reconstruction of *P. aeruginosa* CC274 CF clone. The tree was based on 5525 intraclonal variable positions identified by whole-genome sequencing. Divergence times of predicted ancestors and sampling dates can be inferred from the X axis taking into account that time zero corresponds to the most recent isolate (2012). The same labelling of Figure 4.8. was used. Isolates characteristics are summarized at the right board, where: (CF) Cystic Fibrosis CRI, and (B) Bloodstream. Sequential *P. aeruginosa* isolated from a same patient are indicated with the same colour.

4.2.1.3. THE CC274 RESISTOME

MICs determined for a panel of 11 antipseudomonal agents are collected in Table 4.13.

Isolate ID[a]	Antibiotic resistance profile (MIC values)[b]											Hyperexpression?			Main antibiotic resistance mutations encountered[c]
	TZ (≤8)	PM (≤8)	AT (≤1)	PPT (≤16)	TOL/TAZ (≤4)	IP (≤4)	MP (≤2)	TM (≤4)	AK (≤8)	CI (≤0.5)	CO (≤2)	AmpC	MexAB	MexXY	
AUS034*	>256	>256	>256	>256	16	>32	>32	6	>256	1.5	>256	+	-	+	*gyrB* (R441L), *mexR* (R85H), *mexA* (M1X), *mexB* (F178S, M555I), *oprD*(E264X), *phoQ*(E266X), *parR* (M59I), *mexY*(V1000L), *mexZ*(Nt334Δ13), *fusA2*(P329L), PA2489(R12L, A244T), *mexS* (P254Q), *mexT*(L157M), PBP4(W350R), *capD* (I7M, S51G), *gyrA*(T83I), *mexK* (S426G), *mpl* (Nt112ins1, V124G), *fusA1* (V93A, P554L, D588G), *rpoB* (D831G, D964G), *mexW* (A627V, Q771P), PBP3 (P527T, G63S)
AUS410	4	24	1	12	4	>32	>32	64	>256	1	0.38	-	-	+	*gyrB* (S466F), *mexB*(M552T), *oprD* (Nt583Δ1), *lasR* (A50V, D73G), *sucC* (V44G, A384V), *oprF* (Nt574Δ31), *mexY* (V32A), *mexZ* (Q164X), *mexT* (D327Y), *mexE* (F7Y), *mpl* (D168Y), PA2489(A125T, G185S, P260S), *capD*(I7M, S51G), *fusA1*(P618L), *rpoC*(E386K), *mexW*(Q511R), PBP3(G216S), *pagL*(Nt286Δ1), *amgS*(S64L)
AUS411	>256	>256	>256	>256	6	>32	>32	>256	>256	0.38	0.25	-	-	+	*gyrB* (S466F), *mexB* (Q104E, F246C, L376V), *phoQ* (H248P), *lasR* (D73G), *parS* (D381E, T163N), *sucC* (C261G), *mexY* (D201A, G287A), PA2489 (R12L, A244T), *fusA2* (I640L), mexE (V104G), *htpX* (Nt683Δ5), *mexK* (S426G), *capD* (I7M), *fusA1* (K504E), *rpoC* (N690S), *mexW* (A627V,Q771P), PBP3 (Q372P), *pagL* (N159D)
AUS531	3	3	4	12	1	2	0.75	1	6	0.125	1	-	-	-	PA2489 (R12L, A244T), *capD* (I7M, S51G), *mexW* (A627V, Q771P)
AUS588	2	8	3	8	1	1	0.75	1	8	0.125	0.75	-	-	-	PA2489 (A125T, G185S, P260S), *mexE* (F7Y, V276M), *capD* (I7M), *mexW* (Q511R)
AUS601*	>256	>256	>256	1	3	>32	>32	24	>256	16	0.25	-	-	+	*mexB* (M552T), *oprD* (Nt1044ins4), *phoQ* (K234N, T315A), *lasR* (A50V), *sucC* (T102I, A384V), *mexY* (V32A), *mexZ* (Q164X), *fusA2* (S445X), *mexT*(D327Y), *mexE*(F7Y), *ftsK* (A152V), PA2489 (A125T, G185S, P260S), *capD* (S51G), *gyrA* (T83I), *mpl* (G113D), *ampC* (V239A), *fusA1* (P618L), *rpoC* (E386K), *mexW* (Q511R), PBP3 (R504C), *pagL* (E163G), *pmrB* (L31P), *amgR* (E204D)
AUS603	6	8	24	2	1.5	>32	8	1	8	0.25	1.5	+	-	+	*mexB* (M552T), *lasR* (A50V, D73G), *sucC* (V44G, A384V), *mexY*(V32A), *mexZ* (Q164X), *mexT* (D327Y), *mexE* (F7Y), PA2489 (A125T, G185S, P260S), PBP4 (S315G), *opmE* (E204D), *capD* (I7M, Nt1438Δ1), *mpl* (Nt112ins1, Nt1317Δ1), *fusA1* (P618L), *mexW* (Q511R)

[a] Isolates are labelled according to the following format: Patient identification - MMYY isolation code in the case of sequential isolates. Sequential isolates from a same patient are separated by dashed lines. Mutators isolates are identified with and asterisk.

[b] Clinical susceptibility breakpoints established by EUCAST v7.0 for each antibiotic are shown in brackets.

[c] The main antibiotic resistance related mutations documented for each isolate are shown. For this purpose, the full list of mutations in the 164 genes studied was refined to include only those more likely to be involved in the resistance phenotypes, by including: (i) mutations with known effect on resistance according to published evidence (ii) mutations for which our experimental evidence crosslinks resistance phenotypes and genotypes and/or (iii) mutations in genes found to be under high evolutionary pressure.

Table 4.13. Antibiotic susceptibility profiles and main antibiotic resistance related mutations detected among CC274 isolates. (Cont.)

Isolate ID[a]	Antibiotic resistance profile (MIC values)[b]											Hyperexpression?			Main antibiotic resistance mutations encountered[c]
	TZ (≤8)	PM (≤8)	AT (≤1)	PPT (≤16)	TOL/TAZ (≤4)	IP (≤4)	MP (≤2)	TM (≤4)	AK (≤8)	CI (≤0.5)	CO (≤2)	AmpC	MexAB	MexXY	
AUS690*	6	12	0.75	3	6	4	2	24	>256	12	0.125	-	+	+	*gyrB* (Q467R), *mexR* (H133P), *mexB* (Nt712Δ1), *phoP* (T221I), *lasR* (T178I), *parS* (L10P), *oprF* (K250R), *mexY* (G402S, A850T), *mexZ* (Nt529Δ1), PA2489 (R12L, A244T), *fusA2* (L104P, Nt889Δ1), *htpX* (G187D), *capD* (I7M, S51G), *gyrA* (T83A, T325I), *mexK* (G487E), *mexH* (Nt1086ins1), *fusA1* (Y552C, T671I), *rpoC* (E136G, D616G, V808L), *rpoB* (F1046S), *mexW* (A627V, Q771P), *pagL* (P158L), *pmrB* (F124L), *amgS* (R188C), *parE* (P438S)
FQRC10	2	2	4	12	1	1.5	1	1	8	0.094	0.5	-	-	-	PA2489 (R12L, A244T), *capD* (I7M, S51G), *mexH* (D356N), *mexW* (A627V, Q771P)
FQRC15	1	0.75	6	6	1	1.5	1	0.75	8	0.19	1	-	-	-	PA2489 (R12L, A244T), *capD* (I7M), *mexW* (A627V, Q771P)
FQRC26	4	6	24	24	1	0.25	1.5	1	6	1.5	0.38	-	+	-	*mexY* (V875M), *mexT* (R164H), PA2489 (R12L, A244T), *capD* (I7M, S51G), *gyrA* (Q106L), *mexW* (A627V, Q771P)
FQSE03	3	8	0.5	2	1.5	2	0.38	1	6	3	0.25	-	-	+	*mexA* (L338P), *lasR* (P117G), *mexZ* (A144V), PA2489 (R12L, A244T), *capD* (I7M, S51G), *gyrA* (D87N), *mexW* (A627V, Q771P)
FQSE06-0403	0.75	2	0.25	4	0.38	1	0.5	24	16	0.19	0.19	-	-	+	*mexA* (L338P), *lasR* (P117G), *mexY* (G287A), *mexZ* (S9P), PA2489 (R12L, A244T), *mpl* (S257L), *capD* (I7M, S51G), *fusA1* (Y552C, T671I), *mexW* (A627V, Q771P), PBP3 (P215L), *amgR* (A8V)
FQSE06-1104*	0.38	1	0.094	0.38	0.38	6	0.19	1	24	0.75	2	-	-	+	*mexA* (L338P), *lasR* (P117G), *mexZ* (A194P), PA2489 (R12L, A244T), *fusA2* (N236S, N561S), *capD* (I7M, S51G), *gyrA* (D87G), *mexK* (Q585X), *rpoB* (Y583C), *mexW* (A627V, Q771P), *pmrB* (V185I, G221D, R287Q), PBP1A (E161G), *amgR* (A8V)
FQSE06-0807	4	8	0.75	4	2	1.5	0.75	24	>256	0.5	1	-	-	+	*mexA* (L338P), *lasR* (P117G), *mexY* (G287A), *mexZ* (S9P), *mexT* (P270Q), PA2489 (R12L, A244T), *mpl* (S257L), *capD* (I7M, S51G), *fusA1* (N482S, Y552C, T671I), *mexW* (A627V, Q771P), PBP3 (P215L), *amgR* (A8V)
FQSE06-0610	4	24	0.75	8	1.5	1	0.25	1.5	24	0.75	0.19	-	-	+	*mexA* (L338P), *lasR* (P117G), *mexZ* (Nt290Δ11), PA2489 (R12L, A244T), *mexW* (A627V, Q771P), *capD* (I7M, S51G), *amgR* (A8V)

[a] Isolates are labelled according to the following format: Patient identification - MMYY isolation code in the case of sequential isolates. Sequential isolates from a same patient are separated by dashed lines. Mutators isolates are identified with and asterisk.

[b] Clinical susceptibility breakpoints established by EUCAST v7.0 for each antibiotic are shown in brackets.

[c] The main antibiotic resistance related mutations documented for each isolate are shown. For this purpose, the full list of mutations in the 164 genes studied was refined to include only those more likely to be involved in the resistance phenotypes, by including: (i) mutations with known effect on resistance according to published evidence (ii) mutations for which our experimental evidence crosslinks resistance phenotypes and genotypes and/or (iii) mutations in genes found to be under high evolutionary pressure.

Table 4.13. Antibiotic susceptibility profiles and main antibiotic resistance related mutations detected among CC274 isolates. (Cont.)

Isolate ID[a]	Antibiotic resistance profile (MIC values)[b]											Hyperexpression?			Main antibiotic resistance mutations encountered[c]
	TZ (≤8)	PM (≤8)	AT (≤1)	PPT (≤16)	TOL/TAZ (≤4)	IP (≤4)	MP (≤2)	TM (≤4)	AK (≤8)	CI (≤0.5)	CO (≤2)	AmpC	MexAB	MexXY	
FQSE10-0503	1.5	12	4	4	1.5	1	0.25	0.75	8	0.25	0.25	-	-	+	*mexY* (V875M, N1036S), *mexZ* (IS), PA2489 (R12L, A244T), *ftsK* (A38T), *nalD* (Nt459Δ13), *mexW* (A627V, Q771P), *capD* (I7M, S51G)
FQSE10-0106	0.75	3	0.125	0.75	0.5	0.38	0.032	0.75	4	0.38	1.5	-	-	+	*mexB* (L738P), *mexY* (V875M, N1036S), *mexZ* (IS), PA2489 (R12L, A244T), *ftsK* (A38T), *capD* (S51G), *nalD* (Nt396Δ2), *mexW* (A627V, Q771P), *nfxB* (X188ext)
FQSE10-0110	3	8	16	8	2	1	0.125	0.75	4	0.75	0.5	-	+	+	*mexY* (V875M, N1036S), *mexZ* (IS), PA2489 (R12L, A244T), *ftsK* (A38T), *rpoB* (D659E, E904K), *mexW* (A627V, Q771P), *pmrB* (R287Q)
FQSE10-0111	3	16	12	12	8	1.5	1	1	12	0.38	0.38	-	-	+	*mexY* (V875M, N1036S), *mexZ* (IS), PA2489 (R12L, A244T), *ftsK* (A38T, D54Y), *capD* (S51G), *mexW* (A627V, Q771P)
FQSE15-0803	2	12	0.38	4	1.5	6	1	1	12	0.19	0.25	-	-	+	*mexA* (L338P), *lasR* (P117G), *mexZ* (A144V), PA2489 (R12L, A244T), *capD* (I7M, S51G), *pmrB* (E213D), *mexW* (A627V, Q771P), *amgR* (A8V)
FQSE15-0906	0.75	6	0.38	2	1	1	0.047	1.5	12	0.38	0.75	-	-	+	*mexA* (L338P), *lasR* (P117G), *mexZ* (A144V), *mexS* (Nt848Δ2), *mexT* (Nt534Δ17), PA2489 (R12L, A244T), *capD* (I7M, S51G), *mexK* (S426G), *mexW* (A627V, Q771P), *amgR* (A8V)
FQSE15-0310	1	4	1	1	1	12	0.19	1	8	0.38	0.25	-	-	+	*mexA* (L338P), *lasR* (P117G), *mexZ* (A144V), *mexS* (Nt848Δ2), *mexT* (Nt534Δ17), PA2489 (R12L, A244T), *capD* (I7M, S51G), *mexK* (P834S), *mpl* (Nt1266Δ1), *rpoC* (Nt1181Δ3), *mexW* (A627V, Q771P), *amgR* (A8V)
FQSE15-1110*	8	24	6	4	1	>32	>32	1	16	1	0.25	-	-	+	*gyrB* (S466F), *mexA* (N71S, D235G), *mexB* (L376V), *oprD* (V67X), *lasR* (P117G), *mexY* (Y355H), *mexZ* (A194P), galU (P123L), PA2050 (G90R, Q161R), PA2489 (R12L, A244T), *fusA2* (N236S, N561S), *htpX* (A141T), *capD* (I7M, S51G), *fusA1* (K430E), *rpoC* (V693A), *mexW* (A627V, Q771P), *pmrB* (R287Q), PBP1A (E161G), *amgS* (D267N), *amgR* (A8V)

[a] Isolates are labelled according to the following format: Patient identification - MMYY isolation code in the case of sequential isolates. Sequential isolates from a same patient are separated by dashed lines. Mutators isolates are identified with and asterisk.

[b] Clinical susceptibility breakpoints established by EUCAST v7.0 for each antibiotic are shown in brackets.

[c] The main antibiotic resistance related mutations documented for each isolate are shown. For this purpose, the full list of mutations in the 164 genes studied was refined to include only those more likely to be involved in the resistance phenotypes, by including: (i) mutations with known effect on resistance according to published evidence (ii) mutations for which our experimental evidence crosslinks resistance phenotypes and genotypes and/or (iii) mutations in genes found to be under high evolutionary pressure.

Table 4.13. Antibiotic susceptibility profiles and main antibiotic resistance related mutations detected among CC274 isolates. (Cont.)

Isolate ID[a]	Antibiotic resistance profile (MIC values)[b]											Hyperexpression?			Main antibiotic resistance mutations encountered[c]
	TZ (≤8)	PM (≤8)	AT (≤1)	PPT (≤16)	TOL/TAZ (≤4)	IP (≤4)	MP (≤2)	TM (≤4)	AK (≤8)	CI (≤0.5)	CO (≤2)	AmpC	MexAB	MexXY	
FQSE24-0304*	2	24	0.38	8	1	>32	>32	2	24	6	0.38	-	-	+	*gyrB* (S466F), *mexA* (L338P), *oprD* (V67X), *lasR* (P117G), *mexY* (Y355H), *mexZ* (A194P), *galU* (P123L), PA2050 (G90R, Q161R), PA2489 (R12L, A244T), *fusA2* (N236S, N561S), *opmE* (D421G), *capD* (I7M, S51G), *fusA1* (K430E), *rpoC* (V693A), *mexW* (A627V, Q771P), *pmrB* (R287Q), PBP1A (E161G), *amgR* (A8V)
FQSE24-1005*	1	16	0.38	2	1.5	>32	8	3	16	6	1	-	-	+	*gyrB* (S466F), *oprD* (V67X), *lasR* (P117G), *mexY* (Y355H), *mexZ* (A194P), *galU* (P123L), PA2050 (G90R, Q161R), *fusA2* (N236S, N561S), PA2489 (R12L, A244T), *fusA1* (K430E), *rpoC*(V693A), *mexW* (A627V, Q771P), *pmrB* (R287Q), PBP1A (E161G, R407S), *amgR* (A8V)
FQSE24-0308*	1	8	0.25	0.75	1.5	>32	0.25	2	16	4	1	-	-	+	*gyrB* (S466F), *oprD* (V67X), *lasR* (P117G), *mexY* (Y355H), *mexZ* (A194P), *galU* (P123L), PA2050 (G90R, Q161R), *fusA2* (N236S, N561S), PA2489 (R12L, A244T), *capD* (I7M, S51G), *fusA1* (K430E), *rpoC* (V693A), *mexW* (A627V, Q771P), *pmrB* (R287Q), PBP1A (E161G), *amgS* (T92A), *amgR*(A8V)
FQSE24-1010*	1	8	1	1	1	>32	4	4	64	4	0.38	-	-	+	*gyrB* (S466F), *mexA* (L338P), *oprD* (V67X), *lasR* (P117G), *mexY* (Y355H), *mexZ* (A194P), *galU* (P123L), PA2050 (G90R, P97L, Q161R), PA2489 (R12L, A244T), *fusA2* (N236S, N561S), *opmE* (L400P, D421G), *mexH* (V221I), *capD* (I7M, S51G, A165V), *fusA1* (K430E), *rpoC*(V693A), *mexW* (A627V, Q771P), PBP3 (G216S), *pmrB* (R287Q), PBP1A (E161G), *amgS* (A13V), *amgR* (A8V)
PAMB148	>256	64	>256	>256	6	1.5	0.75	1.5	16	0.064	0.5	+	-	-	PA2489 (R12L, A244T), *capD* (I7M, S51G), *mexY* (V875M, N1036S), *mexW* (A627V, Q771P), *ampD* (P41L)
% I + R	13.8	44.8	48.3	13.8	17.2	44.8	27.6	24.1	62.1	48.3	3.4				

[a] Isolates are labelled according to the following format: Patient identification - MMYY isolation code in the case of sequential isolates. Sequential isolates from a same patient are separated by dashed lines. Mutators isolates are identified with and asterisk.

[b] Clinical susceptibility breakpoints established by EUCAST v7.0 for each antibiotic are shown in brackets.

[c] The main antibiotic resistance related mutations documented for each isolate are shown. For this purpose, the full list of mutations in the 164 genes studied was refined to include only those more likely to be involved in the resistance phenotypes, by including: (i) mutations with known effect on resistance according to published evidence (ii) mutations for which our experimental evidence crosslinks resistance phenotypes and genotypes and/or (iii) mutations in genes found to be under high evolutionary pressure.

As shown, resistance rates were lowest for colistin (3.4%), distantly followed by ceftazidime and piperacillin/tazobactam (13.8%). In contrast, resistance to cefepime, aztreonam, imipenem, amikacin and ciprofloxacin was observed in 44.8 to 62% of the isolates. Remarkably, 17.2% of the isolates were resistant to the new combination ceftolozane/tazobactam. As well, antibiotic resistance was shown to be more frequent among mutators, and in Australian isolates in comparison with those from Spain. In fact, all 9 mutator isolates were classified as MDR, as compared to only 3 of 20 non-mutators. Moreover, one of the Australian mutator isolates met the pan-drug resistant (PDR) definition.

The presence of horizontally acquired resistance determinants was explored in the whole-genome sequences using the ResFinder tool. None of the 29 isolates harbored any horizontally acquired genes encoding resistance determinants. The complete list of missense and non-sense mutations encountered in the 164 antibiotic resistance related chromosomal genes investigated (n=164, Annex 5) can be downloaded in the following link https://www.nature.com/articles/s41598-017-05621-5; as well, a summary of these mutations by antibiotic class has been collected in Annex 6. Up to 127 (77.4%) of the 164 studied genes showed non-synonymous mutations in at least one of the isolates studied. Moreover, after discarding non-synonymous mutations present in all isolates (and thus considered intrinsic CC274 polymorphisms), this figure only decreased to 106 (64.6%). The number and distribution of mutations among the 164 antibiotic resistance related genes studied in the CC274 collection has been represented in Figure 4.10. Seventy-three (68.9%) of these genes showed no more than two different mutational events being 44 of them mutated in unique isolates. In contrast, 33 (31.1%) genes appeared to be under high evolutionary pressure showing evidence of at least 3 different mutational events. Particularly noteworthy among them were *mexB* or *mexY*, (coding for efflux pumps proteins), *mexZ* (the main MexXY repressor), *gyrA* (which codes for DNA gyrase subunit A) and *fusA1* (coding for the elongation factor G).

The main antibiotic resistance related mutations documented are listed in Table 4.13. For this purpose, the full list of mutations in the 164 genes studied was refined to include only those more likely to be involved in the resistance phenotypes, by including: (i) mutations with known effect on resistance according to published evidence, (ii) mutations for which our experimental evidence crosslinks resistance phenotypes and genotypes (*e.g.* mutations in genes involved in AmpC, efflux or OprD regulation and β-lactam resistance phenotypes are crosslinked by integrating the analysis of the expression of *ampC*, efflux pumps genes and *oprD*) and/or (iii) mutations in genes found to be under high evolutionary pressure (those with at least 3 different mutational events documented). Overall, the number of mutations was much higher (unpaired T test p<0.0001) in mutator (19.2±3.1) than in non-mutator isolates (6.7±3.1). Nevertheless, some Australian non-mutator isolates (*e.g.* AUS410 or

AUS411) also presented a high number of mutations. Of note, unique mutations detected in specific genes support phylogeny reconstructions (see above Figures 4.8. and 4.9.).
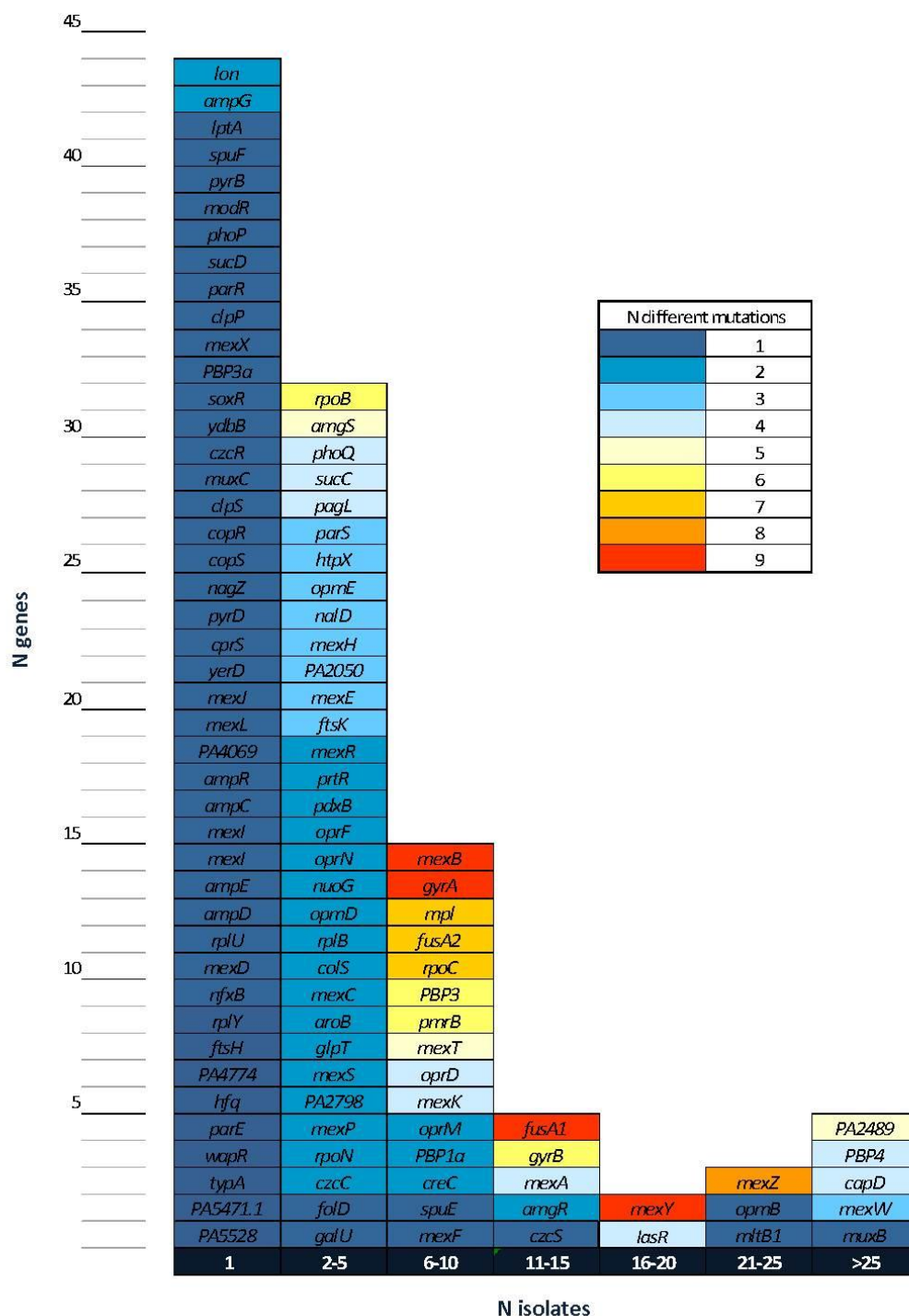
Figure 4.10. Distribution of mutations among the CC274 collection. Mutations encountered among the 164 antibiotic resistance related genes are represented, synonymous and common non-synonymous mutations have been excluded.

To gain insights into the effect on antibiotic resistance profiles of mutations listed in Table 4.13., the median MIC of isolates harboring mutations or not in a specific gene were

compared (Figure 4.11.) Overall, it should be noted that colistin MICs as well as the MICs for the antibiotic combinations piperacillin/tazobactam and ceftolozane/tazobactam were barely affected, whilst carbapenems, aminoglycosides and quinolones MICs are affected by the presence of mutations in many of the selected genes. Apparently, the presence of mutations in some genes such as *capD* (also known as *wbpM*), a gene coding for a protein implicated in O-antigen biosynthesis and previously related with aminoglycoside resistance, or *ftsK,* which codes for a cellular division protein, were not related with an increase in resistance for any antibiotic. Conversely, the presence of mutations in 22 of the genes was shown to produce at least a 2-fold MIC increase for at least 3 different classes of antibiotics. Renowned resistance genes, such as *gyrA*, *gyrB*, *ampD, dacB* (PBP4) or *oprD*, are within this list of 22 genes but, particularly interesting is the presence of not so well-recognized antibiotic resistance related genes such as *fusA1* and *fusA2,* both coding for elongation factor G, or *rpoC,* which codes the β-chain of a DNA-directed RNA polymerase. Mutations in genes coding for two-component regulatory systems, as PhoPQ or ParRS, also require a special mention as mutated isolates showed a strong impact in their MICs for many of the antibiotics tested.

The presence of unique mutations in certain well-known resistance genes, such as *dacB* (PBP4) was observed to increase β-lactam resistance, but mutations within a specific gene did not always correlate or lead to the expected effect on antibiotic resistance (e.g. *pmrB* or *phoP-phoQ* mutated isolates did not exhibit a higher CO MIC). Likewise, several mutations (e.g. *mexZ*, *gyrB* or *oprD*) were associated to extended unexpected antibiotic resistance profiles.

A detailed analysis of the mutational resistome for each class of antibiotics is following provided.

Figure 4.11. MIC-fold change for each antibiotic tested between isolates mutated or not mutated in a specific gene. To evaluate the implication of the presence of mutations in the main genes possibly related with antibiotic resistance the median MIC for both groups were calculated and compared, results are expressed in MIC-fold change. PA2489, *mexW*, *oprF*, *parE* and *nfxB* were excluded since the number of mutated isolates were <3. Some genes were grouped (e.g. *ampD* and *dacB* (PBP4) or *nalD* and *mexR*) according to their well-established effects on resistance (e.g. AmpC or MexAB-OprM overexpression, respectively).

Results

β-lactam resistome. Just three isolates (AUS034, AUS603 and PAMB148) of the CC274 collection were demonstrated to overproduce AmpC. By contrast, at the genomic level, almost all isolates (26/29) contained some variation within *dacB* which codes for the PBP4. Crosslinking phenotypic and genotypic results through *ampC* expression data, suggested that most observed *dacB* allele variations were, in fact, ancestral polymorphisms not involved in antibiotic resistance. AmpC overproduction in the two CF isolates was explained by the presence of specific mutations in *dacB* (S315G or W350R) and by an *ampD* (P41L) mutation in the case of the bloodstream infection isolate PAMB148. Whilst *ampC* overexpression in isolates AUS034 and PAMB148 correlated well with ceftazidime and piperacillin/tazobactam resistance, this was not the case for isolate AUS603 which was documented to be susceptible to these antibiotics. However, unexpected AUS603 β-lactam susceptibility could be explained by the presence of chromosomal mutations whose effects eventually compensate the expected increase in β-lactam resistance. Indeed, this isolate showed an additional non-sense mutation in OprM (Q93X), the OMP of the constitutive MexAB-OprM efflux pump which is well known to play a major role in intrinsic β-lactam resistance. As well, it should also be mentioned that isolate AUS601, exhibiting high-level resistance to ceftazidime, cefepime and aztreonam in the absence of AmpC overproduction, showed an additional mutation (V239A) in AmpC compared to all other CC274 isolates (Table 4.13, Annex 6-β-lactams).

On the other hand, numerous sequence variations were encountered within the essential PBPs coding genes. While some unique mutations were detected in genes coding for PBP1 and PBP3a, the main mutational resistance target among PBPs was found to be PBP3. Up to 7 of 29 isolates presented a mutated PBP3 nucleotide sequence, although β-lactam resistance contribution of each derived *ftsI* (PBP3) allele, if any, depends on the specific point mutation encountered. Missense mutations within the PBP3 (R504C and Q372P) were apparently contributing to resistance in isolates AUS601 and AUS411, since they do not hyperproduce AmpC. Likewise, the P527T mutation of AUS034 likely contributes, together with AmpC overexpression, to the very high-level β-lactam resistance of this isolate, including the new antipseudomonal combination ceftolozane/tazobactam. On the other hand, the P215L and G216S mutations were apparently not linked with phenotypic resistance (Table 4.13. and Annex 6-β-lactams).

Obtained data also demonstrated that the constitutive efflux pump MexAB-OprM is under strong mutational pressure during CF CRI, frequently including inactivating mutations. On the contrary, just 3 isolates showed mutations in regulators leading to MexAB-OprM overexpression (Table 4.13 and Annex 6- β-lactams).

Carbapenem resistome. Imipenem and meropenem resistance correlated in all but two isolates with the presence of non-sense mutations affecting the OMP OprD. High-level meropenem resistance was additionally associated with the presence of PBP3 mutations.

110

Remarkably, all ST1089 mutator isolates shared the same point mutation in *oprD* (V67X) as well as in *galU* (P123L), also related with carbapenem resistance (Table 4.13. and Annex 6-Carbapenems).

MexEF-OprN overexpression was documented just in isolate FQRC26 and MexAB-OprM in 3 isolates; being all susceptible to both carbapenems tested. On the contrary, resistant isolates AUS411 and AUS603 exhibited WT OprD sequences and no efflux pump overexpression was demonstrated (Table 4.13. and Annex 6-Carbapenems).

Aminoglycoside resistome. Among the CC274 collection, a high proportion of the isolates (23/29) were shown to overexpress MexXY and all but one were mutated in *mexZ,* which codes for the mayor MexXY expression regulator. Remarkably, the same point mutation was detected among different and independent isolates. The single MexXY-overproducing isolate showing no mutations in *mexZ,* presented a unique mutation in *parS*, a gene also involved in the modulation of MexXY expression. Nevertheless, MexXY hyperproduction *per se* cannot explain aminoglycoside resistance in the majority of the isolates. Of note, all high-level resistant isolates hyperproduced MexXY and harboured additional mutations in both genes coding for elongation factor G, *fusA1* and *fusA2* (Table 4.13. and Annex 6-Aminoglycosides).

Fluoroquinolone resistome. Obtained data suggest that contribution of efflux pumps overexpression to high-level resistance to fluoroquinolones is very limited, if any. As shown, just isolate FQSE10-0106 (MIC=0.38 mg/L) was demonstrated to hyperproduce MexCD-OprJ due to a non-sense mutation in *nfxB*. On the other hand, our data shows that high-level fluoroquinolone resistance was associated with the presence of missense mutations in *gyrA*, *gyrB* and/or *parC* quinolone resistance-determining regions (QRDRs). Specifically, up to 9 isolates were mutated in *gyrB* QRDR and all but two harbored the same mutation (S466F), 6 showed mutations in *gyrA* QRDR (T83I, T83A, D87N, D87G and Q106L), and just one isolate was mutated in *parE* (P438S). (Table 4.13. and Annex 6-Fluoroquinolones).

Polymyxin resistome. Many isolates were found to be mutated in genes such as *pagL*, *phoQ* or *pmrB,* but with one exception (i.e. isolate AUS034) phenotypic resistance was not observed. For isolate AUS034, a specific non-sense mutation was detected in the two-component sensor PhoQ, as well as two other specific point mutations within *parR* and *colS*. Five additional isolates were shown to harbour mutations in more than one polymyxin-resistance related genes and showed colistin MICs ranging from 0.125 to 2 mg/L. Up to six different and independent mutational events were registered in PmrB sensor and, strikingly, all Spanish mutators shared the same mutation (Table 4.13. and Annex 6-Aminoglycosides).

4.2.2. EVOLUTIONARY DYNAMICS OF *Pseudomonas aeruginosa* AMINOGLYCOSIDE RESISTANCE DEVELOPMENT

When deciphering the aminoglycoside CC274 *P. aeruginosa* resistome, obtained data demonstrated that for high-level resistance development (in absence of aminoglycoside modifying enzymes) the selection of chromosomal mutations that lead to an enhanced membrane impermeability or MexXY-OprM efflux pump overexpression was not enough. Thus, an *in vitro* evolution experiment was performed in an attempt to elucidate the dynamics and chromosomal mutations involved in aminoglycoside resistance development.

As shown in panels A-E of Figure 4.12., *in vitro* resistance development occurred in a stepwise manner, reaching concentrations ranging from 128 to 512 higher than the initial MIC (0.5 µg/mL). The corresponding tobramycin MICs of the purified colonies at day 14 ranged from 64 to 512 µg/mL, whereas those of gentamycin and amikacin were typically 1 or 2 dilutions higher (Annex 7).

Results obtained from whole-genome sequencing experiments are summarized in Figure 4.12. (detailed in Annex 7). Up to 35 different genes were found to be mutated in at least one of the isolates. Mutants from day 14 showed between 3 and 7 mutations and comparison with those from days 1 and 7 evidenced a stepwise acquisition. However, a few mutations documented at these intermediate stages were not fixed in the population and thus were not seen at day 14.

Among the mutated genes, *fusA1* certainly deserves especial attention since non-synonymous mutations within this gene were detected in all 5 replicate experiments. It should also be noted that the time of detection of *fusA1* mutations varied from day 1 to day 14, and that in 3 of them the same amino acid substitution occurred (I61M). Associated aminoglycoside MICs increments were registered (Figure 4.12. and Annex 7).

Another frequently (3 of 5 replicates) mutated gene was *pmrB*. Emergence of *pmrB* mutations at day seven correlated with increased colistin MICs. However, despite the *pmrB* mutations persisted at day 14, colistin resistance disappeared, likely indicating the acquisition of compensatory mutations. One of these isolates showed an additional mutation in *pagL,* involved in lipid A deacylation and polymyxins resistance (31) was documented (Figure 4.12. and Annex 7)*.*
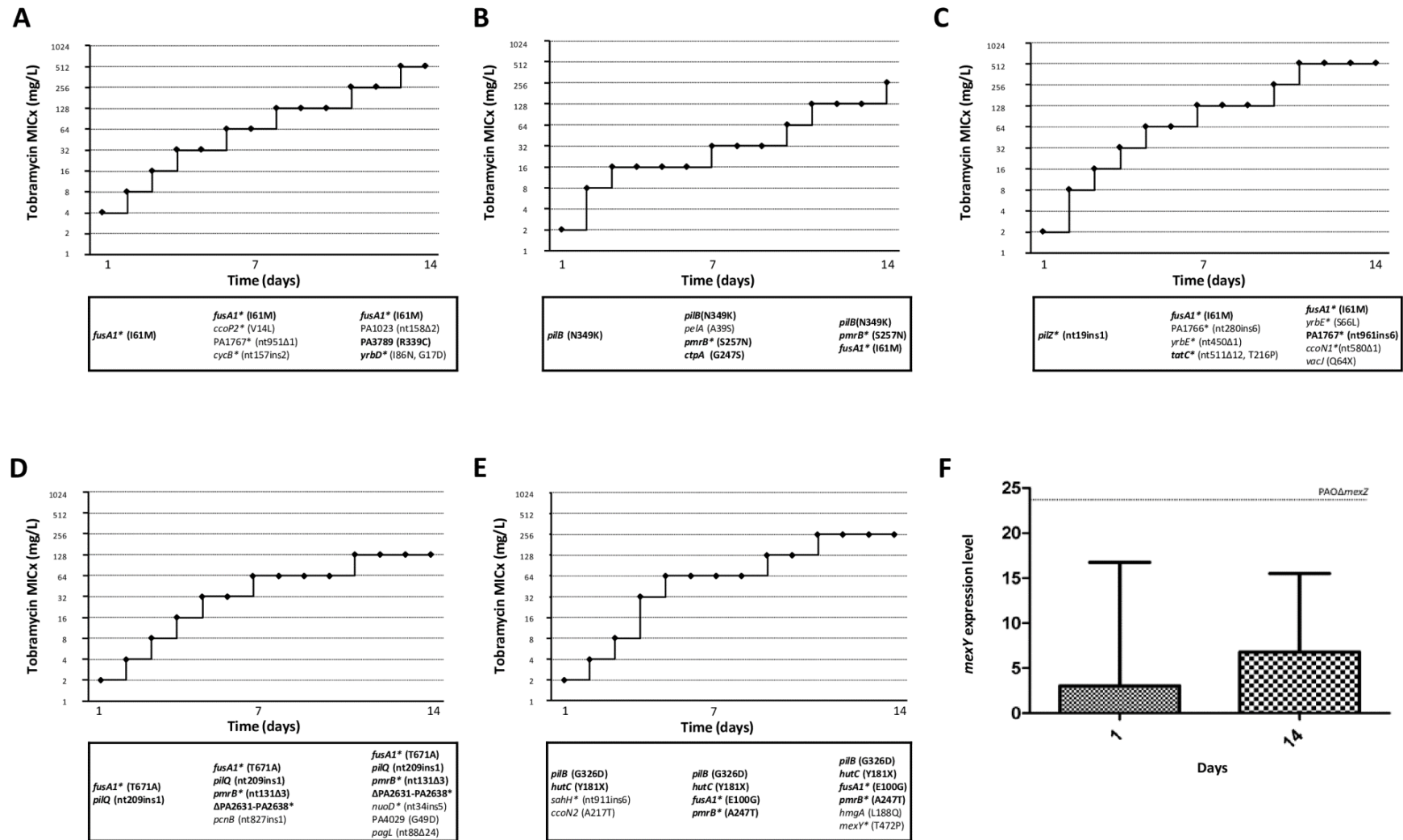
Figure 4.12. Dynamics of resistance development to tobramycin and mutations encountered after 1, 7 and 14 days of tobramycin exposure in the five replicate experiments (A-E). Genetic determinants and specific mutations are highlighted in bold when detected within the two representative colonies studied at each experiment and time point. Genes whose implication in aminoglycoside resistance development has already been demonstrated are indicated with an asterisk. **Median expression level of *mexY* for PAO1-derived resistant mutants after 1 and 14 days of tobramycin exposure (F).**

In relation with other antibiotic classes, a general trend to overtime decreasing MICs particularly for ticarcillin, aztreonam, and ciprofloxacin was noted (Figure 4.13.).

Beyond the mutations actually detected, another relevant aspect to consider are the mutations that were expected but not found in our *in vitro* evolution experiments. No mutations in regulator genes (*mexZ*, PA5471, *parS*) leading to the overexpression of MexXY were seen at any time in any of the 5 replicate experiments. The absence of mutations in these genes was additionally confirmed through Sanger sequencing. Moreover, while *mexY* expression data varied to some extend for the different mutants, values were always below those of a control *mexZ* PAO1 mutant and a statistically significant trend to increased expression at day 14 versus day 1 was not documented (Figure 4.12- panel F).

Among the 3 pairs of isogenic CF *P. aeruginosa* isolates studied, the emergence of *fusA1* mutations was noted in 2 of the 3 tobramycin resistance isolates (Table 4.14. and Annex 7). The resistant isolate not showing *fusA1* mutations was demonstrated to have acquired an exogenous aminoglycoside modifying enzyme (AacA4). As well, in contrast to *in vitro* findings, all 3 CF tobramycin resistant isolates overexpressed *mexY* and showed *mexZ* mutations (Table 4.14. and Annex 7). However, *mexY* overexpression and *mexZ* mutations were also seen in 2 of the 3 susceptible CF isolates.
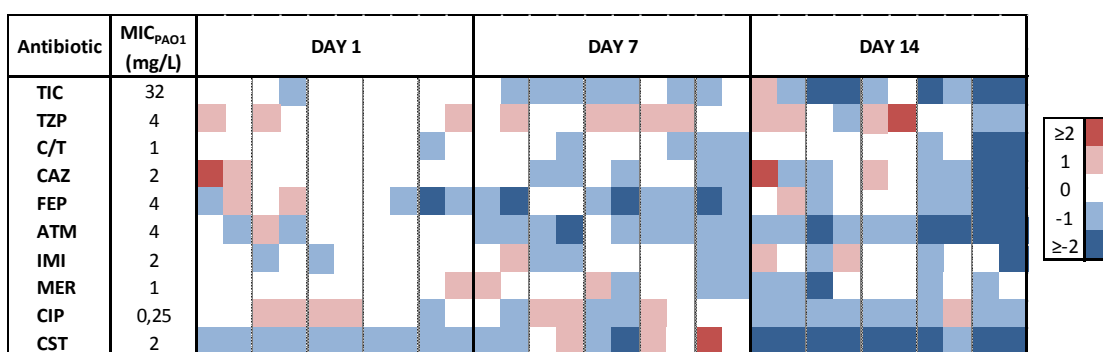
Figure 4.13. MIC-fold changes for each antibiotic tested between the parental strain PAO1 and its derived aminoglycoside resistant mutants. Lower limit for CI and PPT is -1 two-fold dilutions.

Table 4.14. Genomic differences between the three isogenic pairs of tobramycin susceptible-resistant CF isolates.

Locus / Gene[a]	Isolate ID (MIC$_{TOB}$ mg/L)					
	FQSE06-S (1)	FQSE06-R (24)	FQSE11-S (2)	FQSE11-R (>256)	FQSE16-S (4)	FQSE16-R (64)
PA0004/gyrB				R138L		
PA0058/dsbM			C28R,F206L,R212C			
PA0426/mexB			nt772Δ1			Q575R
PA0958/oprD				Q424E,S403A		
PA1430/lasR				R216Q		
PA2018/mexY		G287A				G287S
PA2020/mexZ	nt290Δ11	S9P	L138R	L138R		R125P
PA2492/mexT						G274D,G300D
PA2639/nuoD		G499X				
PA3064/pelA	V446I					
PA3141/capD					nt512ins1	
PA3168/gyrA				Y267N		
PA4020/mpl		S257L		Q248X		
PA4266/fusA1		Y552C,T671I				Y552C
PA4418/PBP3		P215L				
PA4462/rpoN						V473A
PA4568/rplU	I74M					
PA4598/mexD					P721S, L624P	
PA4600/nfxB				E75K		
PA4773/-				A165T		
PA5040/pilQ	E676D,E669D					
Resfinder				AacA4		
mexY overexpression	+	+	+	+	-	+

[a]Genes in which mutations were also detected in the resistance evolution experiment are in bold.

5. DISCUSSION

La levedad y el peso

High genetic diversity among CF *P. aeruginosa* isolates. Cross-sectional studies revealed considerable genetic diversity among *P. aeruginosa* isolates infecting CF patients from both Spain and the Balearic Islands. Up to 65% of the CF patients from the Balearic Islands harbored unique PFGE restriction patterns, percentage that increase to 88.6% in the Spanish cohort (65% and 91.1% unique STs, respectively). Moreover, 42% and 67% of the different STs detected within the Balearic Islands and the Spanish collection have not been previously described, finding which supports the extended idea that most CF patients acquire unique *P. aeruginosa* strains from environmental sources.

Globally, the documented genetic diversity is in accordance with those reported from other CF cohorts in which segregation policies applies. In 2001, *Burns et al.* investigated the genetic background of *P. aeruginosa* isolates infecting a cohort of 40 CF paediatric patients from 3 different hospitals of the United States demonstrating a high degree of genotypic variability. Recently, Kidd *et al.* also investigated this issue in a paediatric cohort from Australia and New Zealand, finally concluding that the environment is the most frequent route for *P. aeruginosa* acquisition among CF children. Likewise, a national observational study across Canada including 1,537 isolates from both adult and paediatric CF patients (n=402) has been conducted by *Middleton and collaborators*. In this work, 403 unique STs were detected and, although 39% of STs were shared, most were only detected among a small number of subjects.

With the exception of clone FQSE-A (CC274), shared STs detected among the Balearic Islands and the Spanish collections were also limited to a small number of patients. Indeed, a direct epidemiological relation could be stablished in the majority of the cases as shared STs were mainly detected infecting pairs of siblings. Transmission of *P. aeruginosa* strains between siblings with CF has already been well documented [Kelly NM *et al*, 1982; Thomassen MJ *et al*, 1985; Grothues D *et al*, 1988; Renders NH *et al*, 1997; Tubbs D *et al*, 2001; Abdul Wahab A *et al*, 2014]. In a study performed in Israel, *Picard et al.* showed that when *P. aeruginosa* was isolated from the first-born sibling, up to 91% of the second siblings were also infected; whereas when the first-born was not positive, only 50% of subsequent siblings were infected. Likewise, they also showed that the age of first isolation was significantly earlier in the second sibling compared to the first-born [Picard E *et al*, 2004], finding that other authors have also reported [Slieker MG *et al*, 2010]. Furthermore, worse clinical outcomes (including lower FEV1, faster decline rate of FEV1, more bacterial airway colonization, increased frequency of lung transplants and a trend towards more hospitalizations) have been found in families with multiple CF patients compared to families with only one CF patient, which may reflect the burden and complexity care of this disease [Lavie M *et al*, 2015].

Scarce representation of *P. aeruginosa* CF epidemic strains. Several European and non-European countries have reported the presence of international epidemic *P. aeruginosa*

Discussion ··

strains infecting a wide number of CF patients. Likewise, high-risk clone ST175 has been documented to be widely distributed in the Spanish nosocomial setting [Cabot G *et al*, 2012; Cabot G *et al*, 2016a; del Barrio-Tofiño E *et al*, 2017].

In the Spanish collection, the C40A AT-genotype was determined in one of the 79 studied isolates. This genotype has been previously described for *P. aeruginosa* Clone C (ST17) [Hilker R *et al*, 2015; Hall AJ *et al*, 2014] but, curiously, this isolate was ascribed to ST1872 which is a double locus variant of ST17, differing in just two point mutations in *mut*L and *trp*E MLST alleles.

More worrisome, when investigating long-term clonal epidemiology of *P. aeruginosa* colonizing the respiratory tract of CF patients from the Balearic Islands, a clonal replacement of a MDR mutator strain by the MDR LES (ST146) was documented in one of the patients, alerting of the first detection of the likely more world-wide concerning CF epidemic clone in Spain. This unusual and awesome characteristic was also reported by *McCallum et al.* in 4 CF patients infected with unique strains after admission for treatment in a CF center [McCallum SJ *et al*, 2001]. In this case, although the epidemiological driver of LES colonization was not specifically investigated, the fact that the patient has family links with a northern European country could help to explain the acquisition of this CF epidemic clone.

As well, clone FQSE-A was detected in 5 unrelated chronically colonized CF patients from the Balearic Islands, clone that was ascribed to the CC274 by MLST. Moreover, in 4 of them, long-term clonal epidemiology was investigated and this strain was demonstrated to persist during the whole 8-years study period. Therefore, results so far suggested that clone FQSE-A is a CF adapted strain: transmissible and persistent. Furthermore, according to the publicly available MLST database (http://pubmlst.org/paeruginosa/), *P. aeruginosa* ST274 has also been detected infecting multiple CF patients from France, Austria and Australia. Thus, our results add further evidence pointing out that ST274 should be added to the growing list of CF epidemic clones.

Discrepant molecular typing results: role of mutators. PFGE and MLST methods are currently considered the gold-standard tecniques for the establishment of epidemiological links. Compared with MLST, PFGE exhibits a higher discriminatory power (or lower stability) and, conversely, MLST results are more reproducible among different laboratories. Thus, PFGE is the preferred technique for studying local epidemiology and to perform outbreaks investigations whereas MLST has been posed as the golden molecular typing tool for global epidemiological studies and for tracking long-term epidemiological relations.

When exploring the Balearic Islands and the Spanish CF *P. aeruginosa* collections, some discrepancies between these molecular typing methods were detected. Not surprising, due to the overall higher discriminatory power of PFGE, several isolates showing different PFGE

patterns were ascribed to the same ST. Conversely, and much more intriguingly, for some isolates showing identical PFGE patterns different STs were determined.

Clone FQSE-A was detected in 5 CF patients from the Balearic Islands. Whereas isolates from 4 of the patients were ascribed to ST274 by MLST, mutator isolates from the fifth patient were ascribed to ST1089. As ST1089 just differs from ST274 by two point mutations in two of the MLST genes each leading to a non-previously described allele, the available data clearly suggest that mutator ST1089 has recently evolved from ST274. Likewise, recently, García-Castillo and collaborators also reported a ST shift within isolates from a chronically colonized CF patient directly linked to the emergence of a mutator phenotype caused by *mutL* mutations [García-Castillo M *et al*, 2012]. As well, within the CC274 collection one of the Australian mutator isolates was ascribed to a new ST which just differs from ST74 by two missense mutations in *mutL* allele, being one of them (H288Y) responsible for the generation of the new ST.

Although not linked with the emergence of stable mutator phenotypes, similar discrepancies were also documented within the Spanish collection. These discrepancies could be explained in terms of an increase prevalence of transient mutator phenotypes (SOS system) during CF CRI, as the CF lungs are known to be a very stressful environment for bacteria in which mutation supply rate is very high. Of note, although not linked to a stable mutator phenotype, the *mutL* gene was frequently involved in the ascription of clonal isolates (PFGE) to different STs.

As well, other authors have also reported that some *P. aeruginosa* strains are not typable by MLST due to the presence of InDel mutations within the *mutL* fragment analyzed [Kidd TJ *et al*, 2011; del Barrio-Tofiño E *et al*, 2017].

All together these results stress the point that *mutL* lacks the neutrality required for an appropriate MLST marker, especially for epidemiological studies involving isolates causing CF CRI in which not only transient mutator phenotypes frequently rise but also MMR deficient mutators are positively selected [Mena A *et al*, 2008] and, therefore, may determine a lower stability of the MLST profiles than expected (leading to discrepant results) both directly (*mutL* inactivating mutations within the gene fragment evaluated in MLST analysis) and indirectly through the increased spontaneous mutagenesis which can facilitate the emergence of novel alleles through point mutations in any of the 7 house-keeping genes evaluated.

Genomic analysis of the phylogeny, within-host evolution and interpatient transmission of the international CC274 CF *P. aeruginosa* clone. CC274 population structure analysis demonstrated the worldwide coexistence of two separated and divergent clonal lineages, but without evident geographical barrier.

Discussion ···

Coexistence of distinct evolved CC274 sublineages within a patient was documented. Similar results have been recently reported by Williams *et al.* concerning the LES [Williams D *et al*, 2015]. In that work, they found that multiple coexisting LES lineages are typically infecting CF patients and that genetic divergence between lineages within patients was greater than interpatient diversity, implying acquisition of diverse genetic populations [Williams D *et al*, 2015]. On the opposite, another study focusing on the LES isolated from patients residing the UK and Canada showed less genetic differences, even when transoceanic isolates were compared [Jeukens J *et al*, 2014]. Likewise, *Yang et al.* also documented a lower genetic divergence in the DK2 epidemic clone [Yang L *et al*, 2011] as well as other previous studies with other relevant and/or persistent CF clones which have also reported divergent results [Feliziani S *et al*, 2014; Marvig RL *et al*, 2013; Cramer N *et al*, 2011]. A possible explanation for these observations could be that different routes for adaptation and survival in the CF lung environment are possible and depend on the specific clonal lineages.

The documented within-host diversity may reflect the coexistence of divergent lineages within the infecting inoculum or the occurrence of several independent transmission events during the course of infection. Based on the substantial phenotypic variation previously observed between samples of the LES taken from patients at successive time points [Mowat E *et al*, 2011; Fothergill JL *et al*, 2010], *Williams et al.* finally pointed out recurrent transmissions as the most suitable driver of rapid population genomic flux in LES infections of the CF airway. To gain more insights, *Williams and collaborators* have recently published a work in which they examined the genetic diversity of chronic *P. aeruginosa* LES infections over 13 months among seven chronically infected CF patients attending the same CF center by genome sequencing, documenting rapid and substantial shifts in the relative abundance of lineages and replacement of dominant lineages likely to represent super-infection by repeated transmissions [Williams D *et al*, 2018]. In the case of CC274, and with the exception of mutators, all isolates from an individual patient clustered together in the same branch which makes the acquisition of a mix of genetically different sublineages a more suitable explanation. Nevertheless, whole-genome sequencing of more longitudinal isolates could help to definitely resolve this issue.

By contrast, and more revealing, both phylogenetic reconstructions and mutational resistome analysis based on WGS data allow us to confirm interpatient transmission of mutators (ST274/ST1089). So, compared with classical molecular typing tools, WGS provides detailed genome fingerprints that might be essential for epidemiological studies in which prevalent and ubiquitous clonal lineages are involved. Indeed, WGS closely clustered isolates from four of the patients from the Balearic Islands, likely indicating interpatient transmission or a common source of colonization, whereas isolates from a fifth patient from the same hospital was distantly related.

Insights into the CF *P. aeruginosa* accessory genome. The pangenome of *P. aeruginosa* consists of two different parts: the conserved core genome (90%) and a combinatorial accesory genome (10%), being the accesory genetic elements esential for surviving under certain selective conditions.

A great example of *P. aeruginosa* adaptability is its ability for producing three different types of pyoverdine and four binding-receptors. The major finding in this variable locus was the absence of the alternative receptor for pyoverdine type I (*fpvB*) in 43% of the isolates from the CF Spanish collection, results that do not correlate with previous studies [Pirnay JP *et al*, 2009; De Vos D *et al*, 2001] in which almost all isolates were demonstrated to harbour it. Recently, *Dingemans et al* also found a significant proportion of CF isolates lacking this alternative receptor (22%) and they hypothesized that this receptor may be relieved from selection because *P. aeruginosa* can utilize multiple iron uptake systems in the CF lung to acquire iron in both its ferric and ferrous forms [Dingemans J *et al*, 2014; Hunter RC *et al*, 2013; Konings AF *et al*, 2013]. An alternative hypothesis for the documented absence may be that loss of *fpvB* can be an advantage for evading the immune system and the action of pyocines [Dingemans *et al*, 2014].

As well, with the exception of the flagellin-glycosylation island, other genomic islands included in the Array Tube genotyping tool were underrepresented when compared with other previous studied collections, which maybe reflects the extraordinary ability of *P. aeruginosa* to explote different paths for adaptation and survival in different environments [Liang X *et al*, 2001; Klockgether J *et al*, 2007; Rakhimova E *et al*, 2009]. The high proportion of isolates harbouring this island clearly suggests that glycosylation may confer some advantages in the CF respiratory tract.

Finally, within its genome, *P. aeruginosa* has a large armamentarium of secreted virulence factors that rely on specialized export systems, including the type III secretion system (T3SS) [Frank, Molecular Microbiology 2007]. In accordance with previously published data for CF respiratory isolates, we encountered that up to 81% and 10% of the isolates possess the ExoS and the ExoU encoding genes, respectively, which reflects a diminished virulence during chronic respiratory infections [Feltman H *et al*, 2001; Pirnay J *et al*, 2009].

Antibiotic resistance trends in CF *P. aeruginosa* isolates. Overall, higher non-susceptibility rates to individual agents were documented for the Spanish (2013-2014) collection in comparison with the Balearic Islands (2003-2012) collection. In both collections, aztreonam and ciprofloxacin were the less active antibiotis and colistin the one for which a minor resistance rate was registered. However, it should be mentioned that EUCAST considers *P. aeruginosa* intrinsically resistant to aztreonam (mainly because of the constitutive expression of MexAB-OprM efflux pump), so, all those isolates showing susceptibility deserve spetial mention (discuss later). High resistance rates to individual agents have also been recently

Discussion

reported by Mustafa *et al.* when studying the antimicrobial susceptibilities of 153 *P. aeruginosa* isolates collected from 2006 to 2012 in 118 CF patients from the United Kingdom, Belgium and Germany [Mustafa MH *et al*, 2016]. Moreover, MDR isolates were also highly prevalent in this Northern European study and within the Spanish collection, finding that compares with the global MDR rate in the Balearic Islands *P. aeruginosa* collection. Of note, a high genetic diversity was documented among the Spanish isolates so maybe the documented higher resistance rates reflect a trend towards increased antibiotic resistance rates as documented in *P. aeruginosa* causing acute infections. Indeed, results from the analysis of antibiotic resistance temporal evolution in the Balearic Islands collection demonstrated a significant upward trend.

Lower non-susceptibility rates were documented for mucoid isolates and, conversely, higher ones were registered for SCV isolates compared with the entire collections. As during CF-CRI an impressive diversification process occur within the infecting population eventually leading to different variants, these results support the importance of perform antibiotic susceptibility testing to at least all different colonies morphotypes encountered within a patient sample.

Non-susceptibility rates values were documented to be higher among chronically colonized CF patients, finding that can be linked to a major antibiotic pressure and, therefore, to an accumulation of resistance mechanisms overtime. Correlation with antibiotics usage was early suggested [Mouton JW *et al*, 1993] but it remains to be demonstrated. In this sense, when we studied long-term CRI, we documented a significant trend towards the accumulation of resistance which was accompanied by a trend towards the accumulation of antibiotic resistance mechanisms.

Mutators as a driver of antibiotic resistance. High proportions of mutator isolates among the CF *P. aeruginosa* population have been demonstrated previously, being frequently associated with antimicrobial resistance [Oliver A, 2010; Montanari S *et al*, 2007; Mena A *et al*, 2008; Ciofu O *et al*, 2005; Marvig RL *et al*, 2013]. Similar rates were documented within the subset of 100 isolates from the Balearic Islands collection and within the CC274 collection, whereas a lower proportion of mutators was found in the Spanish one which maybe reflects earlier stages of chronic colonization.

Defects in the MMR system (*mutS* and *mutL*) were the most frequent cause for hypermutation, which correlate with previous studies [Miller JH, 1996; Oliver A, 2010; Oliver A & Mena A, 2010]. Genetic basis for hypermutation of isolates from the CC274 collection was studied from WGS data (mutome) and, of note, unique missense mutations were encountered in several of the so-called mutator genes in isolates exhibiting a normomutator phenotype, even when located within the MMR system coding-genes.

The obtained antibiotic susceptibility results pointed out mutators as a driver of resistance development in the CF setting, being MDR much more frequent among mutators than in isolates with normal mutation rates. More worrisome, detailed genetic analysis revealed that ST1089 is a *mutS* deficient mutator lineage that have recently evolved from the epidemic strain ST274, which have acquired specific resistance mechanisms and have underwent further interpatient spread.

Altogether these results point out the crutial role of mutators in antibiotic resistance evolution in the CF setting and demonstrate that it can extend beyond intrapatient evolution. Therefore, our results provide evidence of the importance of detecting these hypermutator variants in order to avoid interpatient spread.

Resistome evolution of CF *P. aeruginosa.* Resistome evolution was deeply studied in the CC274 collection by WGS approaches. Whereas horizontally acquired resistance determinants were not encountered, we documented the emergence of mutations in more than 100 genes previously related to antibiotic resistance, which demonstrates the extraordinary capacity of *P. aeruginosa* to develop antibiotic resistance by acquiring chromosomal mutations. While the presence of classical mutational resistance mechanisms was confirmed in several isolates and correlated with resistance phenotypes, our results also provides evidence for a major role of less expected resistance mutations for the majority of antimicrobial classes, including β-lactams, aminoglycosides, fluoroquinolones and polymixins.

β-lactam resistome. The most frequent mutation-driven β-lactam resistance mechanism is likely the overproduction of the chromosomal cephalosporinase AmpC, and it is driven by the selection of mutations in PGN-recycling genes [Juan C *et al*, 2017; Cabot G *et al*, 2011; Moyà B *et al*, 2009]. Among them, the mutational inactivation of *dacB*, encoding the non-essential PBP4, and *ampD*, encoding a *N*-acetyl-muramyl-L-alanine amidase have been found to be the most frequent cause of *ampC* derepression and β-lactam resistance [Juan C *et al*, 2005; Moyà B *et al*, 2009]. The inactivation of PBP4 has also been shown to activate the BlrAB/CreBC regulatory system, further increasing resistance levels [Moyà B *et al*, 2009]. Additionally, specific point mutations leading to a conformation change in the transcriptional regulator AmpR, causing *ampC* upregulation and β-lactam resistance, have been noted among clinical strains. They include the D135N mutation, described in several species besides *P. aeruginosa*, including *Stenotrophomonas maltophilia*, *Citrobacter freundii*, or *Enterobacter cloacae* [Juan C *et al*, 2017] or the R154H mutation, linked to the widespread MDR/XDR ST175 *P. aeruginosa* high-risk clone. Mutation of many other genes, including those encoding other amidases (AmpDh2 and AmpDh3), other PBPs (such as PBP5 and PBP7), lytic transglycosylases (such as SltB1 and MltB), MPL (UDP-*N*-acetylmuramate:Lalanyl-γ-D-glutamyl-meso-diaminopimelate ligase), or NuoN (NADH dehydrogenase I chain N) have been shown to enhance *ampC* expression, either alone or

combined with other mutations, although their impact on β-lactam resistance among clinical strains still needs to be further analyzed [Juan C *et al*, 2017].

Within the CC274 collection, 3 of the isolates overproduced AmpC probably related with the encountered mutations within the PGN recycling genes *dacB* and *ampD*. Moreover, two of them harbored additional inactivating mutations within *mpl,* which may also contribute to AmpC overexpression [Calvopiña K & Avison MB, 2018]. It should be highlighted that one of the isolates (AUS603) did not exhibit phenotypic β-lactam resistance which could be explained in terms of defects in the MexAB-OprM efflux pump system.

Of note, obtained data demonstrated that MexAB-OprM is under strong mutational pressure during CF CRI, including inactivating mutations. This finding correlates with previous investigations that have pointed out that this efflux system is dispensable and, therefore, tends to be lost or inactivated in favour of MexXY-OprM overexpression in CF *P. aeruginosa* subpopulations [Vettoreti L *et al*, 2009]. Results from the CC274 collection support this hypothesis, as while just 3 isolates overexpressed MexAB-OprM, up to 23 overexpressed MexXY. Moreover, many of the isolates showed some degree of hypersusceptibility to aztreonam (substrate of MexAB-OprM) in favour of an increased MIC of cefepime (substrate of MexXY).

Likewise, a susceptibility rate of 60% was documented for aztreonam among the 100 isolates from the 10 chronically colonized patients from the Balearic Islands; percentage that actually reflects the high number of hypersusceptible (MIC ranges 0.125-1 mg/L) isolates falling outside of WT MICs (2-16 mg/L) distributions. As well, in this subset an important number of isolates showed hypersusceptibility to meropenem (substrate of MexAB-OprM) with MICs (<0.06 mg/L) falling outside WT distributions. Although the integrity of MexAB-OprM components was not studied, efflux pumps overexpression was evaluated in the first and last isolate from each patient and clone of this subset and obtained results also support the abovementioned hypothesis.

Nevertheless, results from the *in vitro* experiment under tobramycin pressure and those recently published by *Bolard et al*. [Bolard A *et al*, 2017] suggest that other mechanisms may also be involved in this frequently observed phenotype.

In addition to *ampC* overexpression, recent studies have revealed that β-lactam resistance development, including the novel combinations of β-lactam-β-lactamase inhibitors ceftolozane/tazobactam and ceftazidime/avibactam, may result from mutations leading to the structural modification of AmpC [Cabot G *et al*, 2014; Lahiri SD *et al*, 2014; Fraile-Ribot PA *et al*, 2017; Haidar G *et al*, 2017; MacVane SH *et al*, 2017]. Likewise, recent studies identified diverse AmpC variants associated with high-level cephalosporin resistance, including ceftolozane/tazobactam and ceftazidime/avibactam, in a small proportion (around 1%) of clinical *P. aeruginosa* isolates [Berrazeg M *et al*, 2015]. Currently, over 200

Pseudomonas Derived Cephalosporinase (PDC) variants have been described, including those associated with enhanced ceftolozane/tazobactam and ceftazidime/avibactam resistance. Within the CC274 collection the mutator AUS601, exhibiting high-level resistance to ceftazidime, cefepime and aztreonam in the absence of AmpC overexpression, harbored a mutation within AmpC (V239A) likely contributing to the documented phenotypic resistance. Moreover, in a recent *in vitro* study performed in our group, this specific amino acid substitution was demonstrated to be selected in two of the three β-lactam resistance PAOMS derivatives obtained upon ceftolozane/tazobactam exposure. Of note, contrary to these derivatives, AUS601 remained susceptible to ceftolozane/tazobactam, which illustrates the complexity of mutation-driven resistance within CF isolates.

Besides the chromosomic cephalosporinase AmpC, there is increasing evidence on the role of target modification (essential PBPs) in *P. aeruginosa* β-lactam resistance. Particularly noteworthy are the mutations in *ftsI*, encoding the PBP3, an essential high molecular class B PBP with transpeptidase activity [Chen W *et al*, 2016]. Analysis of the CC274 collection demonstrated that this gene is under strong mutational pressure, as up to 6 different mutations were detected in 7 of the 29 isolates. Although aminoacid substitutions R504C and Q327 are not located in the PBP3 active site, both are very close to two loop regions (residues 332-338 and 526-533) which play an important role in susbstrate recognition [Han S *et al*, 2010]. So, along with the fact that isolates harboring these mutations exhibit β-lactam resistance in the absence of AmpC overexpression, we can conclude that these PBP3 mutations likely contribute to β-lactam resistance. In late years, several other authors have also shown that this PBP is frequently mutated not only among CF *P. aeruginosa* infecting strains [Díaz-Caballero J *et al*, 2015] but also among the so-called high-risk clones [Kos VN *et al*, 2015; Cabot G *et al*, 2016a; del Barrio-Tofiño E *et al*, 2017]. Moreover, PBP3 missense mutations leading to amino acid substitutions in residue 504 (R504C, R504H) have been recently described to occur *in vitro* upon meropenem [Cabot G *et al*, 2016b] and aztreonam [Jorth P *et al*, 2017] exposure as well as among isolates from widespread nosocomial *P. aeruginosa* clones and CF isolates [Cabot G *et al*, 2016a; Kos VN *et al*, 2015; Díaz-Caballero J *et al*, 2015]. Indeed, isolate harboring the Q372P mutation exhibited high-level resistance to carbapenems in the absence of OprD inactivating mutations. As well, mutation P527T may also contribute to β-lactam resistance whereas mutations in residues 215 and 216 apparently not, in agreement with the fact that these residues are not implicated in the formation and stabilization of the inactivating complex β-lactam-PBP3 [Han S *et al*, 2010]. Likewise, several unique mutations were detected in genes coding for PBP1 and PBP3a which role in β-lactam resistance, if any, still needs to be experimentally addressed.

Another apparently relevant mutational β-lactam resistance mechanism is the selection of large (>200 Kb) deletions affecting specific parts of the chromosome upon meropenem exposure [Cabot G *et al*, 2016b]. Although the basis of the conferred resistance phenotype

Discussion ···

still needs to be further clarified, these mutants can be recognized by the characteristic brown pigment (pyomelanine) caused by the deletion of one of the affected genes, *hmgA*, coding for a homogentisate-1,2-dioxygenase. This type of deletion has been documented in both, *in vitro* evolved β-lactam resistant mutants and CF isolates [Cabot G *et al*, 2016b; Hocquet D *et al*, 2016]. However, the deletion of *hmgA* is not responsible of the resistance phenotype, which might be linked to the deletion of another of the affected genes, *galU*, coding for a UDP-glucose pyrophosphorylase required for LPS core synthesis; indeed, analysis of transposon mutant libraries have shown that the inactivation of *galU* increases ceftazidime and meropenem MICs [Alvarez-Ortega C *et al*, 2010; Dötsch A *et al*, 2009]. None of the CC274 isolates showed these large deletions neither exhibited brown pigmentation; however, it should be noted that all ST1089 mutator isolates showed the same missense mutation in galU, likely contributing to their carbapenem resistance.

Apart from these emerging β-lactam resistance mechanisms, phenotypic carbapenem resistance has been clasically linked to the mutational inactivation of the carbapenem porin OprD [Lister PD *et al*, 2009; Castanheira M *et al*, 2014]. Overall, our results, from both the CC274 collection and the subset of 100 isolates from the Balearic Islands, confirm that carbapenem resistance is frequently associated with inactivating mutations within OprD. Additionally, Richardot and collaborators recently reported that some amino acid substitutions within OprD can also confer carbapenem resistance, particularly in the CF setting [Richardot C *et al*, 2015]; nevertheless, no amino acid substitutions were detected within the CC274 isolates. As well, carbapenem resistance may also result from *oprD* repression caused by mutations in the MexEF-OprN efflux pump regulators (*mexS/T*) or the ParRS two-component system [Li XZ *et al*, 2015]; however, our results showed that overexpression of MexEF-OprN is not frequent among CF isolates.

Aminoglycoside resistome. Intravenous antimicrobial combinations including an aminoglycoside are frequently used to manage CF exacerbations. Moreover, in the last decade, tobramycin inhalation has become an important contributor to CF treatment as a means to control CRI as well as a first-line treatment for the eradication of early acquisition of *P. aeruginosa* and several aminoglycoside-based inhaled formulations are currently available [Shteinberg M & Elborn JS, 2015].

Whereas resistance to these agents in acute infections are mainly attributed to the production of aminoglycoside modifying enzymes or 16S rRNA methyltransferases, resistance development in the CRI setting has been linked to the selection of chromosomal mutations leading to enhanced membrane impermeability or MexXY overexpression [Pricket MH *et al*, 2017; Guenard S *et al*, 2014; Poole K, 2015; Vogne C *et al*, 2004]. In accordance, most of the CF clinical studied isolates overexpressed this efflux pump system linked to the presence of mutations within *mexZ*, *amgS* and/or *parRS*; moreover, these mutations occur early and were associated with low-level resistance. Of note, mutations leading to

MexXYoverexpression were not seen in any of the five replicate *in vitro* evolution experiments upon tobramycin pressure. Moreover, while mexY expression data varied to some extent for the different mutants, values were always below those of a control *mexZ* PAO1 mutant, and a statistically significant trend toward increased expression at day 14 versus day 1 was no documented. Thus, these results indicate that mutational overexpression of MexXY is not required for the evolution of high-level tobramycin resistance *in vitro*. Altogether these results may indicate that positive selection of mutations leading to the overexpression of MexXY in CF might be driven by factors beyond exposure to aminoglycosides.

Beyond MexXY overexpression, recent studies have revealed that the aminoglycoside mutational resistome extends far, and that high-level resistance may result from the accumulation of multiple mutations, and the involvement of several novel resistance determinants has been recently documented [El'Garch F *et al*, 2007; Schurek KN *et al*, 2008; Feng Y *et al*, 2016]. WGS data revealed that all high-level resistant CC274 isolates not only overexpressed MexXY but also harbored additional mutations in some of these genes, especially highlighting the presence of mutations in both genes coding for elongation factor G, *fusA1* and *fusA2*. Moreover, *Greipel and collaborators* have also recently reported that these genes are under high evolutionary pressure in the CF environment [Greipel L *et al*, 2016], which can be explained in terms of a wide aminoglycoside use in this setting. As well, several works have associated some specific mutations in FusA1 with aminoglycoside resistance *in vitro* [Feng Y *et al*, 2016] and among clinical, particularly CF, strains [Chung JC *et al*, 2012; Markussen T *et al*, 2014], and, more recently Bolard and collaborators have confirmed the implication of such fusA1 mutations in aminoglycoside resistance through site-directed mutagenesis [Bolard A *et al*, 2017].

In accordance, FusA1 mutations were encountered in all 5 replicates from the *in vitro* evolution experiment associated with a 1- to 3-fold increase in MICs of tobramycin, gentamicin and amikacin, which correlates with *Bolard et al.* observations [Bolard A *et al*, 2017]. As well, resistance development was shown to occur in a stepwise manner, reaching MICs at day 14 close to the maximum tobramycin concentrations achieved through inhaled administration and on the range of the breakpoints suggested for inhaled therapy. Morevoer, 22 of the 35 (63%) different mutated genes have also been related to aminoglycoside resistance development by other authors [Bolard A *et al*, 2017; Yen P & Papin JA, 2017; Feng Y *et al*, 2016; Islam S *et al*, 2009; Schurek KN *et al*, 2009; El'Garch F *et al*, 2007], and thus, our data confirm their relevance in this stepwise process. Mutations within pmrB, traditionally linked with polymyxin resistance development [Moskowitz SM *et al*, 2012; Barrow K & Kwon DH, 2009], rise frequently which alerts from a possible mechanism of co-resistance to two relevant antipseudomonal agents.

Finally, it should be mentioned the fact that although mutational resistance is thought to be the rule in CF CRI, horizontally-acquired resistance always needs to be ruled out.

Fluoroquinolone resistome. Major *P. aeruginosa* RND efflux pumps MexAB-OprM, MexXY, MexCD-OprJ and MexEF-OprN modulate fluoroquinolone resistance. Nevertheless, our results showed that, with the exception of MexXY, efflux pump overexpression is infrequent. The low prevalence of MexCD-OprJ overexpression compares with the fact that hyperproducing mutants tend to emerge after both *in vitro* and *in vivo* fluoroquinolone exposure [Cabot G *et al*, 2016b] and to previous data that have pointed out MexCD-OprJ overexpression as an advantage in the CF setting [Mulet X *et al*, 2011].

So, the fluoroquinolone mutational resistome of CF *P. aeruginosa* generally includes specific missense mutations in DNA gyrase (*gyrA* and/or *gyrB*) and topoisomerase IV (*parC* and/or *parE*) Quinolone Resistance-Determining Regions (QRDRs). Of note, our results revealed that QRDR mutations involved in fluoroquinolone resistance in CF might be more variable.

Polymyxin resistome. As it has been previouskly documented by other authors [Moskowitz SM *et al*, 2012; Gutu AD *et al*, 2013; Miller AK *et al*, 2011; Fernández L *et al*, 2010], the analysis of colistin resistance mechanisms is not always straight forward since the presence of mutations in these two-component regulators is not always associated with clinical colistin resistance, which probably denotes partial complementation between the different regulators. In this sense, *Lee and collaborators* showed that individual two-component systems may not be essential for acquisition of colistin (polymyxin E) resistance in *P. aeruginosa* [Lee JY & Ko KS, 2014]. Nevertheless, it should be highlighted that the isolate exhibiting a premature stop codon in *phoQ* exhibited high-level resistance.

On the whole our results have provided new insights into the evolutionary dynamics and mutation-driven mechanisms of *P. aeruginosa* antibiotic resistance, increasing the current knowledge of the mutational resistome of *P. aeruginosa* , summarized in Table 5.1.

Table 5.1. Main genes and mutations known to be involved in increased antibiotic resistance.

Gene	Resistance mechanisms / Altered target	Antibiotics affected[a]	Type of mutation	Relevant examples	References
gyrA	DNA gyrase	FQ	Gain-of-function	G81D, T83A, T83I, Y86N, D87G, D87N, D87Y, Q106L	Bruchmann S *et al*, 2013 Kos VN *et al*, 2015 Cabot G *et al*, 2016a López-Causapé, C *et al*, 2017 del Barrio-Tofiño E *et al*, 2017
gyrB	DNA gyrase	FQ	Gain-of-function	S466F, S466Y, Q467R, E468D	Bruchmann S *et al*, 2013 Kos VN *et al*, 2015 López-Causapé, C *et al*, 2017 del Barrio-Tofiño E *et al*, 2017
parC	DNA topoisomerase IV	FQ	Gain-of-function	S87L , S87W	Bruchmann S *et al*, 2013 Kos VN *et al*, 2015 Cabot G *et al*, 2016a del Barrio-Tofiño E *et al*, 2017
parE	DNA topoisomerase IV	FQ	Gain-of-function	S457G, S457T, E459D, E459K	Bruchmann S *et al*, 2013 Kos VN *et al*, 2015 López-Causapé, C *et al*, 2017 del Barrio-Tofiño E *et al*, 2017
pmrA	LPS (lipid A)	CO	Gain-of-function	L157Q	Lee JY & Ko KS, 2014
pmrB	LPS (lipid A)	CO	Gain-of-function	L14P, A54V, R79H, R135Q, A247T, A248T, A248V,R259H, M292I, M292T	Barrow K & Kwon DH, 2009 Moskowitz SM *et al*, 2012
phoQ	LPS (lipid A)	CO	Loss-of-function		
parR	LPS (lipid A)	CO	Gain-of-function	M59I, E156K	Muller C *et al*, 2011 Guénard S *et al*, 2014
	OprD downregulation	IP, MP			
	MexEF-OprN hyperproduction	FQ			
	MexXY-OprM hyperproduction	FQ, AMG, PM			
parS	LPS (lipid A)	CO	Gain-of-function	L14Q, V101M, L137P, A138T, A168V Q232E, G361R	Muller C *et al*, 2011 Fournier D *et al*, 2013 Guénard S *et al*, 2014
	OprD downregulation	IP, MP			
	MexEF-OprN hyperproduction	FQ			
	MexXY–OprM hyperproduction	FQ, AMG, PM			

Discussion

Table 5.1. Main genes and mutations known to be involved in increased antibiotic resistance. (Cont.)

Gene	Resistance mechanisms / Altered target	Antibiotics affected[a]	Type of mutation	Relevant examples	References
cprS	LPS (lipid A)	CO	Gain-of-function	R241C	Gutu AD *et al*, 2013
colR	LPS (lipid A)	CO	Gain-of-function	D32N	Gutu AD *et al*, 2013
colS	LPS (lipid A)	CO	Gain-of-function	A106V	Gutu AD *et al*, 2013
mexR	MexAB –OprM hyperproduction	FQ, TZ, PM, PPT, MP, TZ/AVI	Loss-of-function		
nalC	MexAB-OprM hyperproduction	FQ, TZ, PM, PPT, MP, TZ/AVI	Loss-of-function		
nalD	MexAB-OprM hyperproduction	FQ, TZ, PM, PPT, MP, TZ/AVI	Loss-of-function		
nfxB	MexCD-OprJ Hyperproduction	FQ, PM	Loss-of-function		
mexS	MexEF-OprN hyperproduction	FQ	Loss-of-function		
	OprD downregulation	IP, MP			
mexT	MexEF-OprN hyperproduction	FQ	Gain-of-function	G257S, G257T	Juarez P *et al*, 2018
	OprD downregulation	IP, MP			
cmrA	MexEF-OprN hyperproduction	MP, FQ	Gain-of-function	A68V, L89Q, H204L, N214K	Juarez P *et al*, 2017
mvaT	MexEF-OprN hyperproduction	FQ	Loss-of-function		
PA3271	MexEF-OprN hyperproduction	FQ	Loss-of-function		
mexZ	MexXY –OprM hyperproduction	FQ, AMG, PM	Loss-of-function		
PA5471.1	MexXY –OprM hyperproduction	FQ, AMG, PM	Loss-of-function		
amgS	MexXY –OprM hyperproduction	FQ, AMG, PM	Gain of function	V121G, R182C	Lau CH *et al*, 2013
oprD	OprD inactivation	IP, MP	Loss-of-function		
ampC	AmpC structural modification	TZ/AVI, TOL/TAZ	Gain-of-function	T96I, G183D, E247K	Cabot G *et al*, 2014 Fraile-Ribot PA *et al*, 2017
ampD	AmpC hyperproduction	TZ, PM, PPT	Loss-of-function		

Table 5.1. Main genes and mutations known to be involved in increased antibiotic resistance. (Cont.)

Gene	Resistance mechanisms / Altered target	Antibiotics affected[a]	Type of mutation	Relevant examples	References
ampDh2	AmpC hyperproduction	TZ, PM, PPT	Loss-of-function		
ampDh3	AmpC hyperproduction	TZ, PM, PPT	Loss-of-function		
ampR	AmpC hyperproduction	TZ, PM, PPT	Gain-of-function	D135N, G154R	Cabot G *et al*, 2016a Bagge N *et al*, 2002
dacB	AmpC hyperproduction	TZ, PM, PPT	Loss-of-function		
ftsI	Penicillin-binding-protein 3 (PBP3)	TZ, PM, PPT, MP, TZ/AVI, TOL/TAZ	Gain-of-function	R504C, R504H, P527T F533L	Diaz Caballero J *et al*, 2015 Cabot G *et al*, 2016a Cabot G *et al*, 2016b López-Causapé C *et al*, 2017 del Barrio-Tofiño E *et al*, 2017
fusA1	Elongation factor G	AMG	Gain-of-function	V93A, K504E, Y552C, P554L, A555E, N592I, P618L, T671A, T671I	Feng Y *et al*, 2016 López-Causapé C *et al*, 2017 del Barrio-Tofiño E *et al*, 2017 Bolard A *et al*, 2017
glpT	Transporter protein GlpT	FO	Loss-of-function		
rpoB	RNA polymerase β-chain	RI	Gain-of-function	S517F, Q518R, Q518L, D521G, H531Y, H531L, S536F, L538I, S579F, S579Y, N629S, D636Y	Jatsenko T *et al*, 2010

6. CONCLUSIONS

La Gran Marcha

1. The population structure of *P. aeruginosa* isolates infecting CF individuals from the Balearic Islands and Spain is highly diverse, which points out environmental sources as the main route of *P. aeruginosa* acquisition.

2. Epidemic strains were scarce and shared *P. aeruginosa* clones were mainly detected in pairs of siblings involving non-epidemic strains. However, the multidrug resistant Liverpool Epidemic Strain was detected for the first time in Spain.

3. Discrepanices between PFGE and MLST genotyping methods were frequently detected when studying CF isolates mainly due to the lack of neutrality of the *mutL* gene caused by the positive selection of mutator phenotypes. WGS based approaches are a powerful tool that can help in solving this issue.

4. The global trend towards the accumulation of antibiotic resistance during CF-CRI is accompanied by collateral susceptibility to some antibiotics such as aztreonam, which can be explained by the overexpression of MexXY leading to the impairment of MexAB-OprM.

5. Worldwide distributed *P. aeruginosa* CC274 is a well-adapted CF strain, transmissible and persistant and, therefore, it should be added to the list of CF *P. aeruginosa* epidemic clones.

6. Dissemination of evolved mutator lineages, frequently linked to multidrug resistant profiles, between CF patients constitutes a step forward on the spread of antibiotic resistance.

7. Correlation between phenotypes and WGS genotypes of clonal isolates from an epidemic strain allowed us to decipher the *P. aeruginosa* mutational resistome in the CF setting.

8. The β-lactam mutational resistome extends beyond the chromosomic cephalosporinase AmpC. Especial mention deserves gain-of-function mutations within the PBPs, being the PBP3 under high evolutionary pressure in CF isolates.

9. Mutation-driven aminoglycoside resistance development is a stepwise process in which gain-of-function mutations within *fusA1* and loss-of-function mutations within *mexZ* are highly prevalent among CF isolates. The absence of *mexZ* mutations *in vitro* suggests an evolutionary advantage of MexXY overexpression within the respiratory tract of CF patients.

10. Altogether this work demonstrates that clonal epidemiology and antibiotic resistance evolution in the CF setting results from the complex interplay among mutation-driven resistance mechanisms, within host diversification and interpatient transmission of epidemic strains.

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8. ANNEX 1

BACTERIAL CULTURE MEDIA AND LABORATORY REAGENTS

8.1. BACTERIAL CULTURE MEDIA

Brain heart infusion (BHI) broth. The Oxoid (ThermoScientific) commercially available dehydrated media was used (CM1135). As recommended, 37 g were dissolved in 1 litre of distilled water, mixed and sterilized by autoclaving at 121°C for 15 minutes.

Luria-Bertani (LB) broth. 5 g NaCl, 5 g yeast extract and 10 g tryptone were dissolved in 1 litre of distilled water, mixed and sterilized by autoclaving at 121°C for 15 minutes.

Mueller-Hinton broth (MHB). The Oxoid (ThermoScientific) commercially available dehydrated media was used (CM0337). As recommended, 38 g were dissolved in 1 litre of distilled water, mixed and sterilized by autoclaving at 121°C for 15 minutes.

Mueller-Hinton agar (MHA). 15 g of commercially available bacteriological agar per litre of MH broth were added, mixed and sterilized by autoclaving at 121°C for 15 minutes. Once at room temperature, media was plated.

Mueller-Hinton Rifampicin agar (MHA-RI). 1l of room-temperature autoclaved MHA was supplemented with 300 mg of rifampicin and plated.

Mueller-Hinton Gentamycin agar (MHA-GM). 1l of room-temperature autoclaved MHA was supplemented with 50 and 250 mg of gentamycin and plated.

8.2. PULSED-FIELD GEL ELECTROPHORESIS STOCK BUFFERS, AGAROSE AND WORKING SOLUTIONS

1X Tris EDTA (TE) Buffer
1 mM EDTA (pH 7.6)
10 mM Tris-HCl (pH 7.6)
Store at room temperature

5X TBE Stock Buffer/Liter
54 g of Tris base
27.5 g of boric acid
20 ml of 0.5 M EDTA (pH 7.6)
Store at room temperature.
To prepare 0.5X TBE dilute 100 ml 5X TBE with 900 ml of sterile distilled water.

PIV Solution
10 mM Tris-HCl (pH 7.6)
1 M NaCl
Prepare immediately before use.

EC-Lysis Solution
6 mM Tris-HCl (pH 7.6)
1 M NaCl
0.1 M EDTA (pH 7.6)
0.5% Brij®58
0.2% sodium deoxycholate
1% sodium lauroyl sarcosinate
20 µg/mlRNase
100 µg/ml lysozyme
Prepare immediately before use.

ESP Solution
0.5 M EDTA (pH 9.0-9.5)
1% sodium lauroyl sarcosinate
1 mg/ml proteinase K
Prepare immediately before use.

1.6% Low-melt agarose solution
1.6 g low-melt agarose (Bio-Rad)
100 ml 0.5X TBE
Mixed and dissolved well by heating the mixture (60-80 ºC). It can be stored at room temperature and heat before use.

1% Megabase agarose gel
1.5/2 g megabase agarose (Bio-Rad)
150/200 ml 0.5X TBE
Mixed and dissolved well by heating the mixture (60-80 ºC).

8.3. POLYMERASE CHAIN REACTION MASTER MIX

MASTER MIX AmpliTaq Gold™ DNA polymerase (Applied Biosystems™)	
Reagents	**Volume (µl)**
- Buffer II (10X)	10
- DMSO	10
- $MgCl_2$ (25 mM)	6
- dNTPs (10 mM)	2
- Taq polymerase (5 units/µl)	1
- Forward primer 100 µM	1
- Reverse primer 100 µM	1
- PCR grade water	69
- Chromosomal DNA	1
Total reaction volume = 100 µl	

8.4. REAL-TIME QUANTITATIVE REVERSE TRANSCRIPATSE POLYMERASE CHAIN REACTION (QRT-PCR) MASTER MIX

MASTER MIX QuantiTect SYBR green RT-PCR kit (Qiagen)		
Reagents	SMART CYCLER II Instrument (Cepheid)	Eco real-time PCR System (Illumina)
	Volume (µl)	
- RT-PCR master mix (2X)	12.5	5
- Quantitec RT mix	0.25	0.1
- Forward primer	0.1 (100 µM)	0.4 (10 µM)
- Reverse primer	0.1 (100 µM)	0.4 (10 µM)
- RNAse free water	11.3	3.1
- RNA (50ng/µl)	1	1
Total reaction volume	50	10

8.5. ISOLATION OF SMALL AMOUNTS OF OUTER MEMBRANE PROTEINS STOCK BUFFERS

Tris-Mg Buffer
- 10mM Tris-HCl (pH=7.3)
- 5mM $MgCl_2$
Store at 4ºC

Laemmli's sample Buffer
- 0.125 M Tris-HCl (pH=6.8)
- 4% SDS
- 20% Glycerol
- 10% β-mercaptoethanol
Store at -4ºC

8.6. SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) REAGENTS AND BUFFERS

Separating Gel (10%)	
Reagents	Volume
Tris-HCl 1M pH=8.8	1.75 ml
10% ammonium persulfate (*APS*) solution	70 µl
10 % sodium dodecyl sulfate solution	70 µl
TEMED	3 µl
40% polyacrylamide	1.75 ml
Sterile distilled water.	3.36 ml
Total volume = 7 ml	

Stacking Gel (5%)	
Reagents	**Volume**
Tris-HCl 1M pH=6.9	375 µl
10% ammonium persulfate (*APS*) solution	30 µl
10 % sodium dodecyl sulfate solution	30 µl
TEMED	3 µl
40% polyacrylamide	375 µl
Sterile distilled water.	2.2 ml
Total volume = 3 ml	

Running Buffer 1X
3 g Tris_Base
14.4 g glycine
10 ml of 10% sodium dodecyl sulfate solution
Add sterile distilled water up to 1 l and adjust to pH 8.3.
Store at room temperature

8.7. COMPLEMENTATION ASSAYS REAGENTS AND BUFFERS

Sucrose Magnesium Electroporation Buffer (SMEB)		
	Reagents	**Volume**
-	0.5 M HEPES buffer (pH 7)	2 ml
-	1 M MgCl$_2$	1 ml
-	Sucrose	102.7 g
-	Sterile distilled water.	Bring volume to 1000 ml
Autoclave and store at 4ºC until use		

Super Optimal Broth (SOB)		
	Reagents	**Volume**
-	Tryptone	2 g
-	Yeast extract	0.5 g
-	5 M NaCl	0.2 ml
-	1 M KCl	0.25 ml
Add sterile distilled water until 100 ml, adjust to pH 7, autoclave and add…		
-	1M MgCl$_2$	1 ml
-	1 M MgSO$_4$	1 ml

Super Optimal broth with Catabolite repression (SOC)		
	Reagents	**Volume**
-	SOB	20 ml
-	1 M Glucose	0.4 ml

9. ANNEX 2

ARRAY-TUBE GENOTYPING SYSTEM, PROBES AND
PRIMERS INFORMATION

PROBES	REFERENCE	PROBE SEQUENCE	ANTISENSE PRIMER 1	ANTISENSE PRIMER 2
oriC PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	GAAGCCCAGCAATTGCGTGTTTC	AGCCTCGACACCGGTTCTCG	ACCATCTCGTTCATCCCCAGG
oriC non-PAO1	UCBPP-PA14, complete genome, *Lee et al.* 2006	GAAGCCCAGCAACTGCGTGTTTC		
oprL (1) PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	GGTGCTGCAGGGTGTTTCGCCGG	TTCTGAGCCCAGGACTGCTCG	TCGACGCGACGGTTCTGAGCC
oprL (1) non-PAO1	UCBPP-PA14, complete genome, *Lee et al.* 2006	GGTGCTGCAGGGCGTTTCGCCGG		
oprL (2) PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	GTGCTGCAGGGTGTTTCGCCG		
oprL (2) non-PAO1	UCBPP-PA14, complete genome, *Lee et al.* 2006	GCTGCAGGGCGTTTCGCCG		
fliCa (1) PAK	PAK, *Totten and Lory* 1990, flagellin type a2, *Giske et al.* 2006	CAAGATCGCCGCAGCGGTCAAC	AGCTGATGGTATCGCCGTCGC	CTAGTGATCGCACCGGAGCC
fliCa (1) non-PAK	ATCC15691, *Spangenberg et al.* 1998, flagellin type a1, *Giske et al.* 2006	CAAGATCGCCGCTGCGGTCAAC		
fliCa (2) PAK	PAK, *Totten and Lory* 1990, flagellin type a2, *Giske et al.* 2006	CAAGATCGCCGCAGCGGTCAACGAC		
fliCa (2) non-PAK	ATCC15691, *Spangenberg et al.* 1998, flagellin type a1, *Giske et al.* 2006	CAAGATCGCCGCTGCGGTCAACGAC		
alkB2 PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	CCTCGCCCTGTTCCCACCGCTCTGG	TTCCTCGCCGGCATAGTAGGC	
alkB2 non-PAO1	ATCC 15691, *Morales et al.* 2004	CTCGCCCTGTTCCCGCCGCTCTGG		
citS-1 PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	TCGAGCAACTGGCAGAGAAATCCG	GCAGGTAGCAGGTTTCCAGG	AACTGTTCCTTCTGCGCGGCG
citS-1 non-PAO1	UCBPP-PA14, complete genome, *Lee et al.* 2006	CGAGCAACTGGCGGAGAAATCCG		
citS-2 PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	GCGGAAAACTTCCTGCACATGATGTT	TGATCGGCTTGGTCTCGCAGG	GCTGATCGGCTTGGTCTCGC
citS-2 non-PAO1	*Kiewitz and Tummler.* 2000	GCGGAAAACTTCCTCCACATGATGTT		

PROBES	REFERENCE	PROBE SEQUENCE	ANTISENSE PRIMER 1	ANTISENSE PRIMER 2
oprl (1) PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	AGCTCAGCAGACTGCTGACGAGG	GCTGGCTTTTTCCAGCATGCG	TTGCGGCTGGCTTTTTCCAGC
oprl (1) non-PAO1	UCBPP-PA14, complete genome, *Lee et al.* 2006	AGCTCAGCAGACCGCTGACGAG		
oprl (2) PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	GCTCAGCAGACTGCTGACGAGGCTAACG		
oprl (2) non-PAO1	UCBPP-PA14, complete genome, *Lee et al.* 2006	GCTCAGCAGACCGCTGACGAGGCTAAC		
ampC-1 PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	ACGGCCGCCGGGTGACGCC	CGCATCTTGTCCTGGGTCAGG	TCGTCGAGGCGCATCTTGTCC
ampC-1 non-PAO1	*De Champs et al.* 2002, *Kiewitz and Tummler*, 2000	ACGGCCGCCAGGTGACGCCG		
ampC-3 PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	CGACCTACGCGCCGGGCAG	GGCGAGATAGCCGAACAGGC	CACTTGCTGCTCCATGAGCC
ampC-3 non-PAO1	*De Champs et al.* 2002, *Kiewitz and Tummler*. 2000	CGACCTATGCGCCGGGCAGC		
ampC-4 PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	CGTTCGAACGGCTCATGGAGCAG	ACGTCGAGGTGGGTCTGTTCG	GTAGCCTTCGGCATCCAGCG
ampC-4 non-PAO1	*De Champs et al.* 2002, *Kiewitz and Tummler* 2000	CGTTCGAACGACTCATGGAGCAGC		
ampC-5 PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	TGGAGCAGCAAGTGTTCCCGGC	amplified with primers of ampC-4	
ampC-5 non-PAO1	*De Champs et al.* 2002, *Kiewitz and Tummler*. 2000	TGGAGCAGCAACTGTTCCCGGC		
ampC-6 PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	GAACAAGACCGGTTCCACCAACGG	TCGGCATTGGGATAGTTGCGG	
ampC-6 non-PAO1	UCBPP-PA14, complete genome, *Lee et al.* 2006	AACAAGACCGGCTCCACCAACGG		
ampC-7 PAO1	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	CGACCTGGGCCTGGTGATCCT	TTGGGATAGTTGCGGTTGGC	TGGCGTAGGCGATCTTCACCC

PROBES	REFERENCE	PROBE SEQUENCE	ANTISENSE PRIMER 1	ANTISENSE PRIMER 2
fliC a	ATCC15691, *Spangenberg et al.* 1998	GTCGCTGAACGGCACCTACTTCA	CGATCGCGATGTCGACGGTGC	TGCCGATCGCGATGTCGACG
fliC b	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	GCCGACCAACTGAACTCCAACTCG	TGACGTTCTCGCCGGTAGCG	CAGTAGCGGTACCGGTCTGC
exoS	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	CAGCCCAGTCAGGACGCGCA	CAGGGTCGCCAGCTCGCTCGCC	AGGGTCGCCAGCTCGCTCGC
exoU	UCBPP-PA14, complete genome, *Lee et al.* 2006	CGCCAGTTTGAGAACGGAGTCACC	AGTGATCTGCCGCGGCCCTGCC	GTGATCTGCCGCGGCCCTGC
fpvA type I	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	CCTGAATCCGACCATTCGCGAGTC	CGTTCAGGTCGTAGACCGCGC	GCGATACCAACTGTCCTGCGGC
fpvA type IIa	*de Chial et al.* 2003	TCGGACTGTACTCCTACGAAGCAGC	TGCCGAAGGTGAATGGCTTGCC	CCTGATGGTCCGATCCCAGC
fpvA type IIb	*Spencer et al.* 2003	CCAATCCCTATCGCTGGAACCGTACC	GCCGAGGGTCAAGAACCACTGG	TCTTGGCCCAGTCATAGCGGC
fpvA type III	*de Chial et al.* 2003	GCTCGGGACTCGCATTTCGTCC	TAACCCCAAGGCCCATTGGAGG	GCCACCGCCTTCGAATAACCCC
fpvB	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	GCGTTATTGCTCGGTCTCTCCTCG	AATTGCTCGAGGGATGCGGC	GGTCGAAACGGATGCGCAGG
LES	LES400 (personal communication *C. Winstanley*)	TGCATAGGAGTCATGCCGACAGCA	GCCCCGCGTCATTTTCACGTCG	AATGCTCTGGGCAACGAGCC
PA0636	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	GCCAATTGGGTCAGCAAGCAACG	ATGCCATCGTTGAAGGCACCGC	TGCCATCGTTGAAGGCACCG
PA0722	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	CGTGTCGCGAACTCGCATGGC	TCTGGCGGAATCAGGTAGGCC	CTTCCGGGGAGAAACCACCG
PA0728	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	CTGGAGCCTGCGAAAGTGGCTC	AGCCAAGACGGTTGTTCGCGG	TCAATGACGCCGAGTTGGCGC
PA2185	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	ACGAGGGTGATGGCTGGGAATACG	CTCGGACAGGTTCACGCTGG	GCCATTCGCTGCAACACCTCC
PA2221	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	CAGTTGTCGCCAGGTCTGGAGAATCC	TTCCTGGGCCAGAGTTGGACC	AGCTTAAGGCCGTGGCACTCG
PA3835	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	CACATCAATGTCAGCCCACGCCA	CCGGAGAATTCGCGTCCACC	TGCTGACGATGAAGCCCCAGC
fla-island	*Arora et al.* 2001	ACCTGTGTCGCTGGAGGGTATGTT	CCCGTGTTTCCGTAGACCTTGC	

PROBES	REFERENCE	PROBE SEQUENCE	ANTISENSE PRIMER 1	ANTISENSE PRIMER 2
orfA	*Arora et al.* 2001	CGCTGGAGGGTATGTTCCGCAAGG	GTTCCACAGGCGCTGCGGCGC	GTTCCACAGGCGCTGCGGCG
orfI	*Arora et al.* 2001	CCTGGACCTCTCCAAGGTTCGCCT	AAACTGCCCCGCCCCCCATCC	GGAAAAACTGCCCCGCCCCCC
orfJ	*Arora et al.* 2001	GCCATTCCGACGACCAAACAAGGC	ACGCTCGCAGCGCCTCACGCG	GGCCTGGCTGCGAACGCTCGC
PA0980	PAO1-Sequence, *Stover et al.* 2000 (updated 2006)	CGGTATGAAGATGGGTGGTTGGGTCG	ACCTCCAGCACCGACACACC	ATCCGATCCACCTCCAGCACC
XF1753	UCBPP-PA14, complete genome, *Lee et al.* 2006	TGCGAGGACCAGAAACCTTGATGG	GCGCGCGTTCGAGAAACAGG	CGGAGGTTGAAAAGCTGGCCC
acetyltransferase	UCBPP-PA14, complete genome, *Lee et al.* 2006	CGAAGCGTAGGGTCTTCGTAGCC	ACGACGTCACCGTCGAGACCG	ACCGCCTTTCTGGTGAGCTGG
pKL-1	*Klockgether et al.* 2004	CACCATGCAAATGCTCGATGGACTGC	ATCTGAACCGAGGGGATCCGC	CCCGGGAGTCATTGGTCTGG
pKL-3	*Klockgether et al.* 2004	TCTGAACTGCGGCTATCACCTGGA	GACCTACACTCCAACCGCTGG	TTCCCTTGCTGCCGAGAAGC
TB-C47-1	*P. aeruginosa* TB, pKLC102 related gene island integrated in tRNA(Lys) PA4541.1	GCAGGCGTCCAAGTTGGAGCTCTCC	GCCTGTTGGACCCCTTTGACC	TACTCCTGCCTGTTGGACCCC
TB-C47-2	*P. aeruginosa* TB, pKLC102 related gene island integrated in tRNA(Lys) PA4541.1	TCCAACAGGCAGGAGTACAGGGTG	TCTGTCAATCCCCTTTGGGG	AGCCCCTTTCTGTCAATCCCC
PAPI-1 pili chaperone	UCBPP-PA14, complete genome, *Lee et al.* 2006	GGAACACAACGTGGGGCGTGAC	CGCTCAAGCGCTATCCCACC	CGCCATCGGCCTGTACAACG
PAPI-1 luminal binding protein	UCBPP-PA14, complete genome, *Lee et al.* 2006	CCAGTTGGCACCACCATGCTTGC	CGGTAGAGAGCTGGGTTGGC	AACCTGGAGCTAGGGCAGAGC
pKLC conserved hypothetical	*Klockgether et al.* 2004	GCCTGCCTACTTGTTCCCAACGC	CTACCCAGCTTGGGCGTAGC	AAGCGATAGCCGTGCTCCTGC
pKLC adhesin	*Klockgether et al.* 2004	GGCTGTATTGCCCGCCATTCTCC	CCGGCTATATCCGCGGCTACC	ATTGGCGCTGCTGTTTACGCCC
pKLC fatty acid synthase	*Klockgether et al.* 2004	CGACAGACAGAAAGGGTTCTTGCGC	GGTGGCGTCGGGTTTTTCTGC	AGGTCGTAGCGGAAGGTGGTGG
PAGI-2/3-4	*Larbig et al.* 2002	GCGCCTTCTCCTCTTTGCAGATGT	TGTCCCGGCTCAGTTCAACG	GCAACACCTTGGCGTTTGTCC
PAGI-2/3-5	*Larbig et al.* 2002	CAGTATGGTACGGACACGAAGCGC	TCAAGCTCGTTGTGGACCGC	GTTACGACGGCGTGCTGTCGG
PAGI-2/3-6	*Larbig et al.* 2002	CCATGGTCGGAACAGGCACGATATGC	CAACACGCGACTGGCGATCC	TACATCATCCGCAACGGCGGC

PROBES	REFERENCE	PROBE SEQUENCE	ANTISENSE PRIMER 1	ANTISENSE PRIMER 2
C-45	*Larbig et al.* 2002	CGAGGAGTTTCGGACCCGCTTTGA	TCATCCAGCAAGCCATTGCGC	TGGAGTCGCTTTCCGCCATCG
C-46	*Larbig et al.* 2002	CGAAGTCTGAGGTGTGGACCCGC	CGCGGTGCTGGTTGCGCTGC	CGCTGGCAGTTCCGCTGGCC
C-47	*Larbig et al.* 2002	CCACTCGATCATGTTGAGCATCGGCTCC	TATTGACGACCTACCGCGCGCC	CACCAAGAACCCGCTGCTCG
PAGI-2	*Larbig et al.* 2002	GCATCATTGCGCGTCACATCTGGT	ACGCAACGTATTCGGCGACCC	CGCAACGTATTCGGCGACCC
PAGI-2/3-1	*Larbig et al.* 2002	GACCGCAAGCAGAAACGGCATGC	GGTGCTCGACCCAAGCATCG	TCCTTGAGTTCCTTGGCGCGG
PAGI-3-1	*Larbig et al.* 2002	CCCGTTGCTCATAACCCGTTCCTG	GACGAATACCCAGCTGCGTGG	GCAGACGAATACCCAGCTGCG
PAGI-3-8	*Larbig et al.* 2002	GGTTAGTCCCTTCTGCCCGCATCG	ATCGTGGCAGGATGTCCACCG	TAGGCGGGCCTTTTGAAGGTGC
tRNA(Pro)- island 1	*P. aeruginosa* TB, gene island integrated into tRNA(Pro) PA2736.1	GTGTCACGGCCCATGTCTAGCAGC	TCCACGCCGAGGGACGTGCC	GCTCCACGCCGAGGGACGTGCC
tRNA(Pro)- island 2	*P. aeruginosa* TB, gene island integrated into tRNA(Pro) PA2736.1	AGGCCATGGGCTAGCCGGATGC	AGGAGGCCGATGACAACACCC	TGCCGATTCCATGCTCACGCC
PAGI-1	*Liang et al.* 2001	TTCTCGGTGTCGAGGGATTCTCGG	GCATTCGCCACGGAAGGAAGG	GAAGGCATCATGGCATTCGCC

10. ANNEX 3

EUROPEAN COMMITTEE ON ANTIBIOTIC SUSCEPTIBILITY TESTING (EUCAST) CLINICAL BREAKPOINTS FOR *Pseudomonas spp.*

Antibiotic compound[a]	Version 3.1.					Version 6.0.					Version 7.0.				
	MIC breakpoint (mg/L)		Disk content (µg)	Zone diameter breakpoint (mm)		MIC breakpoint (mg/L)		Disk content (µg)	Zone diameter breakpoint (mm)		MIC breakpoint (mg/L)		Disk content (µg)	Zone diameter breakpoint (mm)	
	S≤	R>		S≥	R<	S≤	R>		S≥	R<	S≤	R>		S≥	R<
PPT	16	16	30-6	19	19	16	16	30-6	18	18	16	16	30-6	18	18
TZ	8	8	10	16	16	8	8	10	17	17	8	8	10	17	17
PM	8	8	30	18	18	8	8	30	19	19	8	8	30	19	19
TOL/TAZ	-	-	-	-	-	-	-	-	-	-	4	4	30-10	-	-
IP	4	8	10	20	17	4	8	10	20	17	4	8	10	20	17
MP	2	8	10	24	18	2	8	10	24	18	2	8	10	24	18
AT	1	16	30	50	16	1	16	30	50	16	1	16	30	50	16
CI	0.5	1	5	25	22	0.5	1	5	25	22	0.5	0.5	5	26	26
LE	1	2	5	20	17	1	2	5	20	17	1	1	5	22	22
AK	8	16	30	18	15	8	16	30	18	15	8	16	30	18	15
GM	4	4	10	15	15	4	4	10	15	15	4	4	10	15	15
TM	4	4	10	16	16	4	4	10	16	16	4	4	10	16	16
CO	4	4	-	-	-	4	4	-	-	-	2	2	-	-	-

11. ANNEX 4

USED SCRIPTS IN THE ANALYISIS OF WHOLE-GENOME SEQUENCING DATA

11.1. VARIANT CALLING: PIPELINE

STEP 1: CHECK MiSeq® READS

#!/bin/bash

Use: this script checks all the obtained reads for a sequenced sample (MS). If a single string number is found (no contamination) it will save a new file where the string number would have been replaced with a /1 or /2 depending on the read-file number (forward/1 or reverse/2).

#Input: name of MiSeq sequence file (MS) and if it is "/1" or "/2" (FR)

MS=$1

FR=$2

grep @M ~/$MS |awk '{print $2}' | sort | uniq -c > tmpC

cat tmpC

SEQ_NUM=`cat tmpC | wc -l`

SEQ_ID=`awk '{print $2}' tmpC`

echo $SEQ_ID

if [$SEQ_NUM -eq 1]

then

      echo replace $SEQ_ID of file: $MS with /$FR

      sed "\#@M#s# $SEQ_ID#/$FR#g" ~/$MS > ~/new_$MS

else

      echo error -possible contamination

fi

rm tmpC

STEP 2: PAO1 REFERENCE GENOME MAPPING (SAM FILES)

#!/bin/bash

Use: this script maps all checked reads (forward/reverse) for a sequenced sample (new_MS) to *P. aeruginosa* PAO1 reference genome (previously indexed: "indexed_PAO1_file").

```
~/bowtie2-2.2.6/bowtie2 --phred33 –x ~/indexed_PAO1_file -q -1
~/new_MS_L001_R1_001.fastq -2 ~/new_MS_L001_R2_001.fastq -X 1000 -S
~/MS_mapPAO1.sam 2> ~/output_DO_bowtie2_PAO1_MS.txt
```

STEP 3: GENERATING PILEUP AND RAW FILES FROM SAM FILES

#!/bin/bash

Use: this script will generate the raw and totalpileup files from the sam_file obtained in step 2.

```
        ~/samtools-0.1.16/samtools view -b –S ~/MS_mapPAO1.sam >
~/MS_mapPAO1.bam

        java -jar ~/picard-tools-1.140/picard.jar SortSam INPUT=~/MS_mapPAO1.bam
OUTPUT=~/MS_mapPAO1_sorted.bam SORT_ORDER=coordinate

        java -jar ~/picard-tools-1.140/picard.jar MarkDuplicates
METRICS_FILE=~/MS_metrics.txt

INPUT=~/MS_mapPAO1_sorted.bam OUTPUT=~/MS_mapPAO1_sorted_dedup.bam

        java -jar ~/picard-tools-1.140/picard.jar AddOrReplaceReadGroups
INPUT=~/MS_mapPAO1_sorted_dedup.bam OUTPUT=~/MS_mapPAO1_addrg.bam
LB=XXX PL=Illumina PU=XXX SM=XXX

        java -jar ~/picard-tools-1.140/picard.jar BuildBamIndex
INPUT=~/MS_mapPAO1_addrg.bam

        java -jar ~/GenomeAnalysisTK-3.4-46/GenomeAnalysisTK.jar -T
RealignerTargetCreator -I ~/MS_mapPAO1_addrg.bam -R ~/PAO1complete.fasta -o
/MS_mapPAO1.realigned.intervals

        java -jar ~/GenomeAnalysisTK-3.4-46/GenomeAnalysisTK.jar -T IndelRealigner –I
~/MS_mapPAO1_addrg.bam –R ~/PAO1complete.fasta --maxConsensuses 60 --
maxReadsForConsensuses 240 --maxReadsForRealignment 6000 --targetIntervals
~/MS_mapPAO1.realigned.intervals -o ~/MS_mapPAO1.realigned.bam

        ~/samtools-0.1.16/samtools sort ~/MS_mapPAO1.realigned.bam
/MS_mapPAO1.realigned.sorted
```

~/samtools-0.1.16/samtools pileup -c -f ~/PAO1complete.fasta
~/MS_mapPAO1.realigned.sorted.bam > ~/MS_mapPAO1.realigned.totalpileup

~/samtools-0.1.16/samtools pileup -vc -f ~/PAO1complete.fasta
~/MS_mapPAO1.realigned.sorted.bam > ~/MS_mapPAO1.realigned.raw

NOTE. When adding or replacing groups additional information (XXX) about the sequencing process should be indicated, where: LB: DNA library preparation identifier; PU:platform unit; SM: sample number.

STEP 4: GENERATING SNP AND INDEL FILES

#!/bin/bash

Use: this script will extract SNP and InDel positions from the raw and totalpileup files obtained in step 3.

cat ~/MS_mapPAO1.realigned.raw | awk '$6>=50 && $7>=25 && $8>=3' | awk '$4!="M" && $4!="R" && $4!="W" && $4!="S" && $4!="Y" && $4!="K"' > ~/MS.snps

awk '$3=="*"' ~/MS_mapPAO1.realigned.totalpileup | awk -v var1=500 -v var2=25 '$6>=var1 && $7>=var2' | awk '($9=="*" && $12*5>=$8) || ($10=="*" && $11*5>=$8) || ($9!="*" && $10!="*" && ($12*5>=$8 || $11*5>=$8))' > ~/MS.indels

11.2. DE NOVO ASSEMBLIES

RUNNING VELVET
#!/bin/bash

Use: this script will generate de novo assemblies from checked read sequences files.

sh ~/velvet_1.2.10/contrib/shuffleSequences_fasta/shuffleSequences_fasta.sh
~/new_MS_L001_R1_001.fastq ~/new_MS_L001_R2_001.fastq ~/MS_interleaved.fastq

~/velvet_1.2.10/velveth ~/velvet_MS 31 -shortPaired -fastq ~/MS_interleaved.fastq

~/velvet_1.2.10/velvetg ~/velvet_MS -scaffolding no -ins_length 500 -cov_cutoff 3 -min_contig_lgth 500

mv ~/velvet_MS/contigs.fa ~/MS.500.denovoassembly.fasta

11.3. GENERATING THE NEXUS FILES FOR BEAST ANALYSIS

STEP 1: GENERATING COMMON FILES WITHIN A GROUP OF SAMPLES
#!/bin/bash

#Input: Samples is a file in which all samples included within the group/lineage should be listed in 1 column.

```
SAMPLES=$1

LINEAGE=$2

NO=`cat $SAMPLES | wc -l`

rm tmpS_raw.$LINEAGE

rm tmpS_totalpileup.$LINEAGE

rm tmpS_indels.$LINEAGE

while read line

do

cat ~/"$line"_mapPAO1.realigned.raw | awk '$3!="*"' >> tmpS_raw.$LINEAGE

cat ~/"$line"_mapPAO1.realigned.totalpileup | awk '$8>=3' | awk '$3!="*"' >>
tmpS_totalpileup.$LINEAGE

cat ~/"$line"_mapPAO1.realigned.totalpileup | awk '$3=="*"' >> tmpS_indels.$LINEAGE

done < $SAMPLES

echo "Number of files: $NO"

cat tmpS_totalpileup.$LINEAGE | awk '$8>=3' | cut -f1-3 | sort -nk 2 | uniq -c | awk -v
var1=$NO '$1==var1' | awk '{print $2"\t"$3"\t"$4}' > ~/common_totalpileup_$LINEAGE

cat tmpS_raw.$LINEAGE | cut -f1-4 | awk '$3!=$4' | sort -nk 2 | uniq -c | awk -v var1=$NO
'$1==var1' | awk '{print$2"\t"$3"\t"$4"\t"$5}' > ~/common_raw_$LINEAGE

cat tmpS_indels.$LINEAGE | cut -f1-4 | sort -nk 2 | uniq -c | awk -v var1=$NO '$1==var1' |
awk '{print $2"\t"$3"\t"$4"\t"$5}' > ~/common_indels_$LINEAGE

cat tmpS_totalpileup.$LINEAGE | awk '$4=="M" || $4=="R" || $4=="W" || $4=="S" || $4=="Y"
|| $4=="K"' | cut -f1-3 | sort | uniq > ~/common_ambiguous_$LINEAGE

cat tmpS_totalpileup.$LINEAGE | awk '$5==0' | cut -f1-3 | sort | uniq >
~/common_col5_eq_0_$LINEAGE

rm tmpS_raw.$LINEAGE

rm tmpS_totalpileup.$LINEAGE
```

STEP 2: EXTRACTING COMMON SNP POSITIONS FROM SNP FILES

```
#!/bin/bash

LINEAGE=$1

cat ~MS.snps | fgrep –vf ~/common_raw_$LINEAGE | fgrep -vf
~/common_ambiguous_$LINEAGE | fgrep -vf ~/common_col5_eq_0_$LINEAGE  >
~/MS.$LINEAGE.int.snps

awk 'NR==FNR{c[$1, $2, $3]++;next};c[$1, $2, $3] > 0' ~/common_totalpileup_$LINEAGE
~/MS.$LINEAGE.int.snps > ~/MS.$LINEAGE.snps'
```

STEP 3: GENERATING THE NEXUS FILE

```
#!/bin/bash
```

#Before an input file called SNPS should be prepared (SNPS), containing all positions that will be used for generate the nexus file. As well, a file (ISO) including all isolates (MS) to be used for generate the nexus file should be prepared.

```
ISO=$1

SNPS=$2

N_TAX=`cat $ISO | wc -l`

N_CHAR=`cat $SNPS | wc -l`

while read line

do

        echo "Isolate $line is being processed."

        isolate=`echo $line | awk '{print $1}'`

        fgrep -f $SNPS ~/"$isolate"_mapPAO1.realigned.totalpileup > tmpA

        while read pos

        do

                position=`echo $pos | awk '{print $2}'`

                echo "Position $position is being processed."

                echo "$pos" > tmpB
```

```
fgrep -f tmpB tmpA > tmpC

echo "position $position in $line:"

echo `cat tmpB`

count=`cat tmpC| wc -l`

echo $count

if [ $count -lt 1 ]

then

        echo "Position $position is not present"

        echo $position $line >> missing_positions.txt

        echo "?" >> $isolate.tree

else

        if [ $count -eq 1 ]

        then

                echo "Position $position is found"

                awk '{

                if ($6>=50 || $7>=25 && $8>=3)

                        print $4

                else

                        print "?"

                }' tmpC > $isolate.tmp

                awk '{

                if ($1=="*")

                        print "-"

                else

                        print $0
```

```
                }' $isolate.tmp >> $isolate.tree

           fi

     fi

done<$SNPS

sh ~/transpose.sh $isolate.tree | sed 's/[[:blank:]]//g' > $isolate. trans.tmp

echo "'$isolate'" > name_file_$isolate. tmp

paste name_file_$isolate. tmp $isolate. trans.tmp > alignment.$isolate. tmp
```

done<$ISO

echo "#nexus\n\n[file created from SNP positions found with Bowtie2-GATK-Samtools on `date`]\n\n\

begin data;\n\

\tdimensions ntax = $N_TAX nchar = $N_CHAR;\n\

\tformat datatype = DNA gap = - missing = ?;\n\n\

\tmatrix\n" > head_nex.tmp

echo "\t;\nend;" > tail_nex.tmp

cat head_nex.tmp alignment.*.*.tmp tail_nex.tmp > alignment.nexus.totalpileup

clean

#rm *tmp*

#rm *.*.tree

NOTE. The following script shoud be disposable to run the abovementioned (transpose.sh):

#!/bin/bash

FILE=$1

awk '

{

        for (i=1; i<=NF; i++) {

                a[NR,i] = $i
```

```
                }

        }

        NF>p { p = NF }

        END {

           for(j=1; j<=p; j++) {

                        str=a[1,j]

                           for(i=2; i<=NR; i++){

                                        str=str"\t"a[i,j];

                                   }

                                     print str

                                }

                            }' $FILE
```

12. ANNEX 5

Pseudomonas aeruginosa ANTIBIOTIC-RESISTANCE AND

HYPERMUTATION RELATED CHROMOSOMAL GENES

Table A5.1. Set of 164 genes known to be related with antibiotic resistance in *Pseudomonas aeruginosa* PAO1 reference strain.

LOCUS	GENE	START BP	END BP
PA0004	*gyrB*	4275	6695
PA0005	*lptA*	7018	7791
PA0018	*fmt*	20068	21012
PA0058	*dsbM*	72680	73384
PA0301	*spuE*	339959	341056
PA0302	*spuF*	341111	342265
PA0355	*pfpI*	399493	400032
PA0392	*yggT*	434226	434819
PA0402	*pyrB*	444687	445691
PA0424	*mexR*	471306	471749
PA0425	*mexA*	472024	473175
PA0426	*mexB*	473191	476331
PA0427	*oprM*	476333	477790
PA0463	*creB*	523254	523943
PA0464	*creC*	523943	525367
PA0465	*creD*	525469	526827
PA0486	*yihE*	547432	548406
PA0487	*modR*	548468	549226
PA0610	*prtN*	672777	673091
PA0611	*prtR*	673191	673961
PA0612	*ptrB*	674419	674619
PA0779	*asrA*	845793	848192
PA0807	*ampDh3*	884799	885566
PA0869	PBP6/7	949716	950648
PA0893	*argR*	976410	977399
PA0958	*oprD*	1043983	1045314
PA1178	*oprH*	1277006	1277608
PA1179	*phoP*	1277688	1278365
PA1180	*phoQ*	1278362	1279708
PA1343	*pagP*	1457175	1457633
PA1345	*gshB*	1458707	1460296
PA1375	*pdxB*	1491913	1493055
PA1409	*aphA*	1533238	1534278
PA1430	*lasR*	1558171	1558890
PA1588	*sucC*	1730181	1731347
PA1589	*sucD*	1731347	1732234
PA1777	*oprF*	1921174	1922226
PA1796	*folD*	1946187	1947041
PA1797	-	1948502	1950334
PA1798	*parS*	1950439	1951725
PA1799	*parR*	1951726	1952433
PA1801	*clpP*	1954069	1954710
PA1803	*lon*	1956227	1958623
PA1812	*mltD*	1969635	1971239
PA1886	*polB*	2054911	2057274

PA2006	-	2194058	2195410
PA2018	*mexY*	2208169	2211306
PA2019	*mexX*	2211322	2212512
PA2020	*mexZ*	2212677	2213309
PA2023	*galU*	2215102	2215941
PA2050	-	2244492	2244998
PA2071	*fusA2*	2272460	2274568
PA2227	*vqsM*	2448568	2449545
PA2272	PBP3a	2501720	2503417
PA2273	*soxR*	2503425	2503895
PA2489	-	2805021	2805836
PA2490	*ydbB*	2805917	2806291
PA2491	*mexS*	2806350	2807369
PA2492	*mexT*	2807469	2808512
PA2493	*mexE*	2808743	2809987
PA2494	*mexF*	2810009	2813197
PA2495	*oprN*	2813194	2814612
PA2522	*czcC*	2842019	2843305
PA2523	*czcR*	2843818	2844492
PA2524	*czcS*	2844489	2845907
PA2525	*opmB*	2846283	2847779
PA2526	*muxC*	2847776	2850886
PA2527	*muxB*	2850883	2854014
PA2528	*muxA*	2854011	2855291
PA2615	*ftsK*	2956805	2959240
PA2621	*clpS*	2964607	2964843
PA2642	*nuoG*	2987721	2990438
PA2649	*nuoN*	2996265	2997725
PA2797	-	3154593	3155075
PA2798	-	3155072	3156256
PA2809	*copR*	3162705	3163385
PA2810	*copS*	3163382	3164713
PA2830	*htpX*	3182986	3183861
PA3005	*nagZ*	3365756	3366754
PA3013	*foaB*	3373254	3374429
PA3014	*faoA*	3374460	3376607
PA3047	PBP4	3410264	3411694
PA3050	*pyrD*	3414701	3415729
PA3077	*cprR*	3450838	3451509
PA3078	*cprS*	3451506	3452801
PA3141	*capD*	3524681	3526678
PA3168	*gyrA*	3556427	3559198
PA3521	*opmE*	3938020	3939495
PA3522	*mexQ*	3939492	3942653
PA3523	*mexP*	3942650	3943807
PA3533	*grxD*	3952061	3952387

Table A5.1. Set of 164 genes known to be related with antibiotic resistance in *Pseudomonas aeruginosa* PAO1 reference strain.(Cont.)

LOCUS	GENE	START BP	END BP
PA3574	*nalD*	4006510	4007148
PA3602	*yerD*	4036265	4037875
PA3676	*mexK*	4116188	4119265
PA3677	*mexJ*	4119270	4120373
PA3678	*mexL*	4120469	4121107
PA3719	*armR*	4165719	4165880
PA3721	*nalC*	4166518	4167159
PA3999	PBP5	4478979	4480139
PA4001	*sltB1*	4481230	4482252
PA4003	PBP2	4480139	4485336
PA4020	*mpl*	4498488	4499843
PA4069	-	4546668	4547552
PA4109	*ampR*	4592990	4593880
PA4110	*ampC*	4594029	4595222
PA4119	*aph*	4607578	4608384
PA4205	*mexG*	4705956	4706402
PA4206	*mexH*	4706410	4707522
PA4207	*mexI*	4707535	4710624
PA4208	*opmD*	4710621	4712084
PA4218	*ampP*	4721614	4722858
PA4238	*rpoA*	4754423	4755424
PA4260	*rplB*	4764880	4765701
PA4266	*fusA1*	4769035	4771155
PA4269	*rpoC*	4772279	4776478
PA4270	*rpoB*	4776544	4780617
PA4273	*rplA*	4781985	4782680
PA4315	*mvaT*	4843812	4844186
PA4374	*mexV*	4903466	4904596
PA4375	*mexW*	4904647	4907703
PA4380	*colS*	4910871	4912151
PA4381	*colR*	4912141	4912824
PA4393	*ampG*	4922407	4924191
PA4406	*lpxC*	4938276	4939187
PA4418	PBP3	4952604	4954343
PA4444	*mltB1*	4977869	4978972
PA4462	*rpoN*	4992870	4994363
PA4521	*ampE*	5063941	5064777

LOCUS	GENE	START BP	END BP
PA4522	*ampD*	5064774	5065340
PA4567	*rpmA*	5116032	5116289
PA4568	*rplU*	5116313	5116624
PA4597	*oprJ*	5149633	5151072
PA4598	*mexD*	5151078	5154209
PA4599	*mexC*	5154237	5155400
PA4600	*nfxB*	5155561	5156124
PA4661	*pagL*	5229459	5229980
PA4671	*rplY*	5239466	5240080
PA4700	PBP1b	5277968	5280292
PA4748	*tpiA*	5332746	5333501
PA4751	*ftsH*	5335782	5337701
PA4773	-	5361586	5362068
PA4774	-	5362146	5363195
PA4775	-	5363198	5364058
PA4776	*pmrA*	5364071	5364736
PA4777	*pmrB*	5364760	5366193
PA4878	*brlR*	5473766	5474578
PA4944	*hfq*	5548397	5548645
PA4964	*parC*	5572222	5574486
PA4967	*parE*	5576028	5577917
PA5000	*wapR*	5617534	5618418
PA5038	*aroB*	5674028	5675134
PA5045	PBP1a	5680898	5683366
PA5117	*typA*	5762659	5764476
PA5199	*amgS*	5851239	5852558
PA5200	*amgR*	5852653	5853396
PA5235	*glpT*	5892910	5894256
PA5297	*poxB*	5964859	5966577
PA5332	*crc*	6002121	6002900
PA5366	*pstB*	6033211	6034044
PA5471	*armZ*	6159560	6160699
PA5471.1	-	6160912	6160953
PA5485	*ampDh2*	6176516	6177295
PA5528	-	6219885	6220739
PA5542	-	6234500	6235744

Table A5.2. Set of genes known to be related with hypermutation in *Pseudomonas aeruginosa* PAO1 reference strain.

LOCUS	GENE	START BP	END BP
PA0355	*pfpI*	399493	400032
PA0357	*mutY*	401131	401943
PA0750	*ung*	818003	818698
PA1816	*dnaQ*	1973470	1974210
PA3002	*mfd*	3360875	3364321
PA3620	*mutS*	4054525	4057092
PA4366	*sodB*	4893697	4894278
PA4400	*mutT*	4930748	4931695
PA4468	*sodM*	4997439	4998050
PA4609	*radA*	5167284	5168645
PA4946	*mutL*	5549780	5551681
PA5147	*mutM*	5795954	5797021
PA5344	*oxyR*	6012047	6012979
PA5443	*uvrD*	6131088	6133274
PA5493	*polA*	6183784	6186525

13. ANNEX 6

MAIN MUTATIONS RELATED WITH ANTIBIOTIC RESISTANCE
ENCOUNTERED IN THE CLONAL COMPLEX 274
COLLECTION

Table A6.1. Betalactams. MIC values for betalactams and main mutations related with betalactam resistance encountered in the CC274 collection.

Isolate IDᵃ	ST	MIC values					Hyperexpression?	AmpC and its regulatorsᵇ			
		TZ	PM	AT	PPT	TOL/TAZ		ampR	ampC	ampD	PBP4
AUS034*	274	>256	>256	>256	>256	16	+		T21A, T105A, G391A	R11L, G148A, D183Y	W350R, A394P
AUS411	274	>256	>256	>256	>256	6	-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P
AUS601*	1043	>256	>256	>256	1	3	-		T21A, T105A, G391A, V239A	R11L, G148A, D183Y	
PAMB148	274	>256	64	>256	>256	6	+		T21A, T105A, G391A	R11L, P41L, G148A, D183Y	A394P
FQSE15-1110*	1089	8	24	6	4	1	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
AUS690*	274	6	12	0.75	3	6	-	T275A	T21A, T105A, G391A	R11L, G148A, D183Y	A394P
AUS603	274	6	8	24	2	1.5	+		T21A, T105A, G391A	R11L, G148A, D183Y	S315G
AUS410	274	4	24	1	12	4	-		T21A, T105A, G391A	R11L, G148A, D183Y	
FQSE06-0610	274	4	24	0.75	8	1.5	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
FQSE06-0807	274	4	8	0.75	4	2	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
FQRC26	274	4	6	24	24	1	-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P
FQSE10-0111	274	3	16	12	12	8	-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P
FQSE10-0110	274	3	8	16	8	2	-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P
FQSE03	274	3	8	0.5	2	1.5	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
AUS531	274	3	3	4	12	1	-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P
FQSE24-0304*	1089	2	24	0.38	8	1	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
FQSE15-0803	274	2	12	0.38	4	1.5	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
AUS588	274	2	8	3	8	1	-		T21A, T105A, G391A	R11L, G148A, D183Y	
FQRC10	274	2	2	4	12	1	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
FQSE10-0503	274	1.5	12	4	4	1.5	-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P
FQSE24-1005*	1089	1	16	0.38	2	1.5	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
FQSE24-1010*	1089	1	8	1	1	1	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
FQSE24-0308*	1089	1	8	0.25	0.75	1.5	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
FQSE15-0310	274	1	4	1	1	1	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
FQRC15	274	1	0.75	6	6	1	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
FQSE15-0906	274	0.75	6	0.38	2	1	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
FQSE10-0106	274	0.75	3	0.125	0.75	0.5	-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P
FQSE06-0403	274	0.75	2	0.25	4	0.38	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P
FQSE06-1104*	274	0.38	1	0.094	0.38	0.38	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P

ᵃIsolates have been ordered according to their MIC values and following the subsequent order: TZ, PM, AT, PPT and TOL/TAZ.

ᵇ No mutations were encountered in AmpDh2 and AmpDh3.)

Table A6.1. Betalactams. MIC values for betalactams and main mutations related with betalactam resistance encountered in the CC274 collection. (Cont.)

Isolate IDa	Other penicillin-binding proteinsb					Hyperexpression?	MexAB-OprM and its regulatorsc					
	PBP1A	PBP1B	PBP3	PBP3A	PBP6/7		mexA	mexB	oprM	mexR	nalC	nalD
AUS034*		S25G	G63S, P527T	A104P	S250N	-	M1*	F178S, M555I		R85H	G71E, S209R	
AUS411		S25G	Q372P	A104P	S250N	-		Q104E, F246C, L376V			G71E, S209R	
AUS601*		S25G	R504C	A104P	S250N	-		M552T			G71E, S209R	
PAMB148		S25G		A104P	S250N	-					G71E, S209R	
FQSE15-1110*	E161G	S25G		A104P	S250N	-	N71S, D235G	L376V	E456G		G71E, S209R	
AUS690*		S25G		A104P	S250N	+		$Nt_{712}\Delta1$		H133P	G71E, S209R	
AUS603		S25G		A104P	S250N	-		M552T	Q93*		G71E, S209R	
AUS410		S25G	G216S	A104P	S250N	-		M552T			G71E, S209R	
FQSE06-0610		S25G		A104P	S250N	-	L338P				G71E, S209R	
FQSE06-0807		S25G	P215L	A104P	S250N	-	L338P				G71E, S209R	
FQRC26		S25G		A104P	S250N	+					G71E, S209R	$Nt_{459}\Delta13$
FQSE10-0111		S25G		A104P	S250N	-					G71E, S209R	
FQSE10-0110		S25G		A104P	S250N	+					G71E, S209R	$Nt_{396}\Delta2$
FQSE03		S25G		A104P	S250N	-	L338P				G71E, S209R	
AUS531		S25G		A104P	S250N	-					G71E, S209R	
FQSE24-0304*	E161G	S25G		A95V, A104P	S250N	-	L338P		E456G		G71E, S209R	
FQSE15-0803		S25G		A104P	S250N	-	L338P				G71E, S209R	
AUS588		S25G		A104P	S250N	-					G71E, S209R	
FQRC10		S25G		A104P	S250N	-					G71E, S209R	
FQSE10-0503		S25G		A104P	S250N	-					G71E, S209R	
FQSE24-1005*	E161G, R407S	S25G		A104P	S250N	-			E456G		G71E, S209R	
FQSE24-1010*	E161G	S25G	G216S	A104P	S250N	-	L338P		E456G		G71E, S209R	
FQSE24-0308*	E161G	S25G		A104P	S250N	-			E456G		G71E, S209R	
FQSE15-0310		S25G		A104P	S250N	-	L338P				G71E, S209R	
FQRC15		S25G		A104P	S250N	-					G71E, S209R	
FQSE15-0906		S25G		A104P	S250N	-	L338P				G71E, S209R	
FQSE10-0106		S25G		A104P	S250N	-		L738P			G71E, S209R	
FQSE06-0403		S25G	P215L	A104P	S250N	-	L338P				G71E, S209R	
FQSE06-1104*	E161G	S25G		A104P	S250N	-	L338P				G71E, S209R	

aIsolates have been ordered according to their MIC values and following the subsequent order:TZ, PM, AT,PPT and TOL/TAZ.

bNo mutations were encountered in PBP2 and PBP5.

cNo mutations were encountered in *armR*.

Table A6.1. Betalactams. MIC values for betalactams and main mutations related with betalactam resistance encountered in the CC274 collection. (Cont.)

Isolate ID[a]	Hyperexpre-ssion?	MexXY-OprM and its regulators													
		mexY	*mexX*	*oprM*	*fmt*	*mexZ*	*folD*	*parS*	*parR*	*htpX*	*amgS*	*amgR*	*armZ*	*PA5528*
AUS034*	+	T543A, Q840E,V1000L	K329Q, L331V, D346H, W358R		I181V	Nt$_{334}$Δ13		A82T, H398R	M59I				L88P, D119E	
AUS411	+	D201A, G287A, T543A, Q840E,	E287D, K329Q, L331V, D346H, W358R		I181V			A82T, T163N, D381E, H398R		Nt$_{683}$Δ5			L88P, D119E	
AUS601*	+	V32A, T543A, Q840E,	K329Q, L331V, D346H, W358R		I181V	Q164*		A82T, H398R				E204D	L88P, D119E	
PAMB148	-	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE15-1110*	+	Y355H, T543A, Q840E,	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R		A141T	D267N	A8V	L88P, D119E	
AUS690*	+	G402S, T543A, Q840E, A850T	K329Q, L331V, D346H, W358R		I181V	Nt$_{529}$Δ1		L10P, A82T, H398R		G187D	R188C		L88P, D119E	
AUS603	+	V32A, T543A, Q840E,	K329Q, L331V, D346H, W358R	Q93*	I181V	Q164*		A82T, H398R					L88P, D119E	
AUS410	+	V32A, T543A, Q840E,	K329Q, L331V, D346H, W358R		I181V	Q164*		A82T, H398R			S64L		L88P, D119E	Nt$_{208}$Δ7
FQSE06-0610	+	T543A, Q840E,	K329Q, L331V, D346H, W358R		I181V	A194P		A82T, H398R				A8V	L88P, D119E	
FQSE06-0807	+	G287A, T543A, Q840E,	K329Q, L331V, D346H, W358R		I181V	S9P		A82T, H398R				A8V	L88P, D119E	
FQRC26	-	T543A, Q840E, V875M	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE10-0111	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE10-0110	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE03	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R					L88P, D119E	
AUS531	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE24-0304*	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R				A8V	L88P, D119E	
FQSE15-0803	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R				A8V	L88P, D119E	
AUS588	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQRC10	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE10-0503	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE24-1005*	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R			T92A	A8V	L88P, D119E	
FQSE24-1010*	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R			A13V	A8V	L88P, D119E	
FQSE24-0308*	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R				A8V	L88P, D119E	
FQSE15-0310	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R				A8V	L88P, D119E	
FQRC15	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE15-0906	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R				A8V	L88P, D119E	
FQSE10-0106	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE06-0403	+	G287A, T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	S9P		A82T, H398R				A8V	L88P, D119E	
FQSE06-1104*	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	Nt$_{290}$Δ11		A82T, H398R				A8V	L88P, D119E	

[a]Isolates have been ordered according to their MIC values and following the subsequent order:TZ, PM, AT,PPT and TOL/TAZ.

Table A6.2. Carbapenems. MIC values for carbapenems and main mutations related with carbapenem resistance encountered in the CC274 collection.

Isolate ID[a]	ST	MIC values		OprD	Hyperexpression?	AmpC and its regulators[b]					Other penicillin-binding proteins[c]				
		IP	MP	oprD		ampR	ampC	ampD	PBP4	PBP1A	PBP1B	PBP3	PBP3A	PBP6/7
AUS034*	274	>32	>32	E264*	+		T21A, T105A, G391A	R11L, G148A, D183Y	W350R, A394P		S25G	P527T, G63S	A104P	S250N
AUS410	274	>32	>32	Nt$_{583}$Δ1	-		T21A, T105A, G391A	R11L, G148A, D183Y			S25G	G216S	A104P	S250N
AUS411	274	>32	>32		-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P		S25G	Q372P	A104P	S250N
AUS601*	1043	>32	>32	Nt$_{1044}$ins4	-		T21A, T105A, V239A, G391A	R11L, G148A, D183Y			S25G	R504C	A104P	S250N
FQSE15-1110*	1089	>32	>32	V67*	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P	E161G	S25G		A104P	S250N
FQSE24-0304*	1089	>32	>32	V67*	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P	E161G	S25G		A95V, A104P	S250N
AUS603	274	>32	8		+		T21A, T105A, G391A	R11L, G148A, D183Y	S315G		S25G		A104P	S250N
FQSE24-0308*	1089	>32	8	V67*	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P	E161G	S25G		A104P	S250N
FQSE24-1010*	1089	>32	4	V67*	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P	E161G	S25G	G216S	A104P	S250N
FQSE24-1005*	1089	>32	0.25	V67*	-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P	E161G, R407S	S25G		A104P	S250N
FQSE15-0803	274	12	0.19		-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P		S25G		A104P	S250N
FQSE15-0906	274	6	1		-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P		S25G		A104P	S250N
FQSE06-0610	274	6	0.19		-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P		S25G		A104P	S250N
AUS690*	274	4	2		-	T275A	T21A, T105A, G391A	R11L, G148A, D183Y	A394P		S25G		A104P	S250N
AUS531	274	2	0.75		-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P		S25G		A104P	S250N
FQSE03	274	2	0.38		-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P		S25G		A104P	S250N
FQRC10	274	1.5	1		-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P		S25G		A104P	S250N
FQRC15	274	1.5	1		-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P		S25G		A104P	S250N
FQSE10-0110	274	1.5	1		-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P		S25G		A104P	S250N
FQSE06-0807	274	1.5	0.75		-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P		S25G	P215L	A104P	S250N
PAMB148	274	1.5	0.75		+		T21A, T105A, G391A	R11L, P41L, G148A, D183Y	A394P		S25G		A104P	S250N
AUS588	274	1	0.75		-		T21A, T105A, G391A	R11L, G148A, D183Y			S25G		A104P	S250N
FQSE06-0403	274	1	0.5		-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P		S25G	P215L	A104P	S250N
FQSE06-1104*	274	1	0.25		-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P	E161G	S25G		A104P	S250N
FQSE10-0111	274	1	0.25		-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P		S25G		A104P	S250N
FQSE10-0106	274	1	0.125		-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P		S25G		A104P	S250N
FQSE15-0310	274	1	0.047		-		T21A, T105A, G391A	R11L, G148A, D183Y	A358V, A394P		S25G		A104P	S250N
FQSE10-0503	274	0.38	0.032		-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P		S25G		A104P	S250N
FQRC26	274	0.25	1.5		-		T21A, T105A, G391A	R11L, G148A, D183Y	A394P		S25G		A104P	S250N

[a] Isolates have been ordered according to their MIC values and following the subsequent order: IP and MP

[b] No mutations were encountered in AmpDh2 and AmpDh3.

[c] No mutations were encountered in PBP2 and PBP5.

Table A6.2. Carbapenems. MIC values for carbapenems and main mutations related with carbapenem resistance encountered in the CC274 collection. (Cont.)

Isolate IDª	Hyperexpression?	MexEF-OprN and its regulators						
		mexE	*mexF*	*oprN*	*mexS*	*mexT*	*parS*	*parR*
AUS034*	-				D249N, P254Q	L157M, F172I	A82T, H398R	M59I
AUS410	-	F7Y			D249N	F172I, D327Y	A82T, H398R	
AUS411	-	V104G			D249N	F172I	A82T, T163N, D381E, H398R	
AUS601*	-	F7Y			D249N	F172I, D327Y	A82T, H398R	
FQSE15-1110*	-		Nt$_{1051}$ins9	R127Q	D249N	F172I	A82T, H398R	
FQSE24-0304*	-		Nt$_{1051}$ins9		D249N	F172I	A82T, H398R	
AUS603	-	F7Y			D249N	F172I, D327Y	A82T, H398R	
FQSE24-0308*	-		Nt$_{1051}$ins9		D249N	F172I	A82T, H398R	
FQSE24-1010*	-		Nt$_{1051}$ins9		D249N	F172I	A82T, H398R	
FQSE24-1005*	-		Nt$_{1051}$ins9		D249N	F172I	A82T, H398R	
FQSE15-0803	-			Nt$_{415}$ins2	Nt848Δ2, D249N	Nt534Δ17, F172I	A82T, H398R	
FQSE15-0906	-				D249N	F172I	A82T, H398R	
FQSE06-0610	-		Nt$_{1051}$ins9		D249N	F172I	A82T, H398R	
AUS690*	-				D249N	F172I	L10P, A82T, H398R	
AUS531	-				D249N	F172I	A82T, H398R	
FQSE03	-				D249N	F172I	A82T, H398R	
FQRC10	-				D249N	F172I	A82T, H398R	
FQRC15	-				D249N	F172I	A82T, H398R	
FQSE10-0110	-				D249N	F172I	A82T, H398R	
FQSE06-0807	-				D249N	F172I, P270Q	A82T, H398R	
PAMB148	-				D249N	F172I	A82T, H398R	
AUS588	-	F7Y, V276M			D249N	F172I	A82T, H398R	
FQSE06-0403	-				D249N	F172I	A82T, H398R	
FQSE06-1104*	-				D249N	F172I	A82T, H398R	
FQSE10-0111	-				D249N	F172I	A82T, H398R	
FQSE10-0106	-				D249N	F172I	A82T, H398R	
FQSE15-0310	-				Nt848Δ2, D249N	Nt534Δ17, F172I	A82T, H398R	
FQSE10-0503	-				D249N	F172I	A82T, H398R	
FQRC26	+				D249N	R164H, F172I	A82T, H398R	

ªIsolates have been ordered according to their MIC values and following the subsequent order: IP and MP

[b] No mutations were encountered in *mvaT*.

Table A6.2. Carbapenems. MIC values for carbapenems and main mutations related with carbapenem resistance encountered in the CC274 collection. (Cont.)

Isolate IDa	Hyperexpre ssion?	MexAB-OprM and its regulatorsb					
		mexA	*mexB*	*oprM*	*mexR*	*nalC*	*nalD*
AUS034*	-	M1*	F178S, M555I		R85H	G71E, S209R	
AUS410	-		M552T			G71E, S209R	
AUS411	-		Q104E, F246C, L376V			G71E, S209R	
AUS601*	-		M552T			G71E, S209R	
FQSE15-1110*	-	N71S, D235G	L376V	E456G		G71E, S209R	
FQSE24-0304*	-	L338P		E456G		G71E, S209R	
AUS603	-		M552T	Q93*		G71E, S209R	
FQSE24-0308*	-			E456G		G71E, S209R	
FQSE24-1010*	-	L338P		E456G		G71E, S209R	
FQSE24-1005*	-			E456G		G71E, S209R	
FQSE15-0803	-	L338P				G71E, S209R	
FQSE15-0906	-	L338P				G71E, S209R	
FQSE06-0610	-	L338P				G71E, S209R	
AUS690*	+		Nt$_{712}$Δ1		H133P	G71E, S209R	
AUS531	-					G71E, S209R	
FQSE03	-	L338P				G71E, S209R	
FQRC10	-					G71E, S209R	
FQRC15	-					G71E, S209R	
FQSE10-0110	+					G71E, S209R	Nt$_{396}$Δ2
FQSE06-0807	-	L338P				G71E, S209R	
PAMB148	-					G71E, S209R	
AUS588	-					G71E, S209R	
FQSE06-0403	-	L338P				G71E, S209R	
FQSE06-1104*	-	L338P				G71E, S209R	
FQSE10-0111	-					G71E, S209R	
FQSE10-0106	-			L738P		G71E, S209R	
FQSE15-0310	-	L338P				G71E, S209R	
FQSE10-0503	-					G71E, S209R	
FQRC26	+					G71E, S209R	Nt$_{459}$Δ13

aIsolates have been ordered according to their MIC values and following the subsequent order: IP and MP

b No mutations were encountered in *armR*.

Table A6.2. Carbapenems. MIC values for carbapenems and main mutations related with carbapenem resistance encountered in the CC274 collection. (Cont.)

Isolate ID[a]	Hyperexpression?	MexXY-OprM and its regulators													
		mexY	mexX	oprM	fmt	mexZ	folD	parS	parR	htpX	amgS	amgR	armZ	PA5528
AUS034*	+	T543A, Q840E,V1000L	K329Q, L331V, D346H, W358R		I181V	Nt$_{334}$Δ13		A82T, H398R	M59I				L88P, D119E	
AUS410	+	V32A, T543A, Q840E,	K329Q, L331V, D346H, W358R		I181V	Q164*		A82T, H398R			S64L		L88P, D119E	Nt$_{208}$Δ7
AUS411	+	T543A, Q840E,V1000L	K329Q, L331V, D346H, W358R		I181V	Nt$_{334}$Δ13	A82T, T163N, D381E, H398R	M59I					L88P, D119E	
AUS601*	+	V32A, T543A, Q840E,	K329Q, L331V, D346H, W358R		I181V	Q164*		A82T, H398R				E204D	L88P, D119E	
FQSE15-1110*	+	Y355H, T543A, Q840E,	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R		A141T	D267N	A8V	L88P, D119E	
FQSE24-0304*	+	Y355H, T543A, Q840E,	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R				A8V	L88P, D119E	
AUS603	+	V32A, T543A, Q840E,	K329Q, L331V, D346H, W358R	Q93*	I181V	Q164*		A82T, H398R					L88P, D119E	
FQSE24-0308*	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R				A8V	L88P, D119E	
FQSE24-1010*	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R			A13V	A8V	L88P, D119E	
FQSE24-1005*	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R			T92A	A8V	L88P, D119E	
FQSE15-0803	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R				A8V	L88P, D119E	
FQSE15-0906	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R				A8V	L88P, D119E	
FQSE06-0610	+	T543A, Q840E,	K329Q, L331V, D346H, W358R		I181V	A194P		A82T, H398R				A8V	L88P, D119E	
AUS690*	+	G402S, T543A, Q840E, A850T	K329Q, L331V, D346H, W358R		I181V	Nt$_{529}$Δ1		L10P, A82T, H398R	G187D	R188C			L88P, D119E	
AUS531	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE03	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R					L88P, D119E	
FQRC10	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQRC15	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE10-0110	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE06-0807	+	G287A, T543A, Q840E,	K329Q, L331V, D346H, W358R		I181V	S9P		A82T, H398R				A8V	L88P, D119E	
PAMB148	-	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
AUS588	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE06-0403	+	G287A, T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	S9P		A82T, H398R				A8V	L88P, D119E	
FQSE06-1104*	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	Nt$_{290}$Δ11		A82T, H398R				A8V	L88P, D119E	
FQSE10-0111	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE10-0106	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE15-0310	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R				A8V	L88P, D119E	
FQSE10-0503	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQRC26	-	T543A, Q840E, V875M	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	

[a]Isolates have been ordered according to their MIC values and following the subsequent order: IP and MP

Table A6.3. Aminoglycosides. MIC values for aminoglycosides and main mutations related with aminoglycoside resistance encountered in the CC274 collection.

Isolate ID[a]	ST	TM	AK	Hyperexpression?	mexY	mexX	oprM	fmt	mexZ	folD	parS	parR	htpX	amgS	amgR	armZ	PA5528
AUS411	274	>256	>256	+	T543A, Q840E,V1000L	K329Q, L331V, D346H, W358R		I181V	Nt$_{334}$Δ13		A82T, T163N, D381E, H398R	M59I				L88P, D119E	
AUS410	274	64	>256	+	V32A, T543A, Q840E,	K329Q, L331V, D346H, W358R		I181V	Q164*		A82T, H398R			S64L		L88P, D119E	Nt$_{208}$Δ7
AUS601*	1043	24	>256	+	V32A, T543A, Q840E,	K329Q, L331V, D346H, W358R		I181V	Q164*		A82T, H398R				E204D	L88P, D119E	
AUS690*	274	24	>256	+	G402S, T543A, Q840E, A850T	K329Q, L331V, D346H, W358R		I181V	Nt$_{529}$Δ1		L10P, A82T, H398R		G187D	R188C		L88P, D119E	
FQSE06-0807	274	24	>256	+	G287A, T543A, Q840E,	K329Q, L331V, D346H, W358R		I181V	S9P		A82T, H398R				A8V	L88P, D119E	
FQSE06-0403	274	24	16	+	G287A, T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	S9P		A82T, H398R				A8V	L88P, D119E	
AUS034*	274	6	>256	+	T543A, Q840E,V1000L	K329Q, L331V, D346H, W358R		I181V	Nt$_{334}$Δ13		A82T, H398R	M59I				L88P, D119E	
FQSE24-1010*	1089	4	64	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R			A13V	A8V	L88P, D119E	
FQSE24-0308*	1089	3	16	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R				A8V	L88P, D119E	
FQSE24-0304*	1089	2	24	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R				A8V	L88P, D119E	
FQSE24-1005*	1089	2	16	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R			T92A	A8V	L88P, D119E	
FQSE06-1104*	274	1.5	24	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	Nt$_{290}$Δ11		A82T, H398R				A8V	L88P, D119E	
PAMB148	274	1.5	16	-	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE15-0310	274	1.5	12	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R				A8V	L88P, D119E	
FQSE06-0610	274	1	24	+	T543A, Q840E,	K329Q, L331V, D346H, W358R		I181V	A194V		A82T, H398R				A8V	L88P, D119E	
FQSE15-1110*	1089	1	16	+	Y355H, T543A, Q840E,	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R		A141T	D267N	A8V	L88P, D119E	
FQSE10-0110	274	1	12	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE15-0906	274	1	12	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R				A8V	L88P, D119E	
AUS588	274	1	8	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
AUS603	274	1	8	+	V32A, T543A, Q840E,	K329Q, L331V, D346H, W358R	Q93*	I181V	Q164*		A82T, H398R					L88P, D119E	
FQRC10	274	1	8	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE15-0803	274	1	8	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R				A8V	L88P, D119E	
AUS531	274	1	6	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQRC26	274	1	6	-	T543A, Q840E, V875M	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE03	274	1	6	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R					L88P, D119E	
FQRC15	274	0.75	8	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE10-0111	274	0.75	8	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE10-0503	274	0.75	4	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE10-0106	274	0.75	4	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	

[a]Isolates have been ordered according to their MIC values and following the subsequent order: TM and AK.

Table A6.3. Aminoglycosides. MIC values for aminoglycosides and main mutations related with aminoglycoside resistance encountered in the CC274 collection. (Cont.)

Isolate IDa	Mutationes encountered in other genes related with aminoglycoside resistance						
	galU	*fusA2*	*nuoG*	*capD*	*rplB*	*fusA1*	*rplY*
AUS411		I640L, G695A	T484A	I7M	G138A	K504E	
AUS410		G695A	T484A	I7M, S51G		P618L	
AUS601*		S445*, G695A	T484A	S51G	I144V	P618L	Q81H
AUS690*		L104P, Nt$_{889}$Δ1, G695A	T484A	I7M, S51G		Y552C, T671I	
FQSE06-0807		G695A	T484A	I7M, S51G		N482S, Y552C, T671I	
FQSE06-0403		G695A	T484A	I7M, S51G		Y552C, T671I	
AUS034*		P329L, G695A	T484A	I7M, S51G		V93A, P554L, D588G	
FQSE24-1010*	P123L	N236S, N561S, G695A	T484A	I7M, S51G, A165V		K430E	
FQSE24-0308*	P123L	N236S, N561S, G695A	T484A			K430E	
FQSE24-0304*	P123L	N236S, N561S, G695A	T484A	I7M, S51G		K430E	
FQSE24-1005*	P123L	N236S, N561S, G695A	T484A	I7M, S51G		K430E	
FQSE06-1104*		N236S, N561S, G695A	T484A	I7M, S51G			
PAMB148		G695A	V360A, T484A	I7M, S51G			
FQSE15-0310		G695A	T484A	I7M, S51G			
FQSE06-0610		G695A	T484A	I7M, S51G			
FQSE15-1110*	P123L	N236S, N561S, G695A	T484A, A638T	I7M, S51G		K430E	
FQSE10-0110		G695A	T484A	S51G			
FQSE15-0906		G695A	T484A	I7M, S51G			
AUS588		G695A	T484A	I7M			
AUS603		G695A	T484A	I7M, nt1438Δ1		P618L	
FQRC10		G695A	T484A	I7M, S51G			
FQSE15-0803		G695A	T484A	I7M, S51G			
AUS531		G695A	T484A	I7M, S51G			
FQRC26		G695A	T484A	I7M, S51G			
FQSE03		G695A	T484A	I7M, S51G			
FQRC15		G695A	T484A	I7M			
FQSE10-0111		G695A	T484A				
FQSE10-0503		G695A	T484A	S51G			
FQSE10-0106		G695A	T484A	I7M, S51G			

aIsolates have been ordered according to their MIC values and following the subsequent order: TM and AK.

Table A6.4. Quinolones. MIC values for ciprofloxacin and main mutations related with quinolone resistance encountered in the CC274 collection.

Isolate ID	ST	MIC	QRDRs				Hyperexpression?	MexCD-OprJ and its regulators				
		CI	*gyrB*	*gyrA*	*parC*	*parE*		*mexD*	*mexC*	*oprJ*	*nfxB*	*vqsM*
AUS601*	1043	16	H148N	T83I			-	E257Q, A536S, S845A		D68G, M69V		not present
AUS690*	274	12	H148N, Q467R	T83A		P438S	-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE24-0304*	1089	6	H148N, H148NS466F				-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE24-1005*	1089	6	H148N, S466F				-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE24-0308*	1089	4	H148N, S466F				-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE24-1010*	1089	4	H148N, S466F				-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE03	274	3	H148N	D87N			-	E257Q, A536S, S845A		D68G, M69V		not present
AUS034*	274	1.5	H148N, R441L	T83I			-	L24M, E257Q, A536S, S845A		D68G, M69V		not present
FQRC26	274	1.5	H148N	Q106L			-	E257Q, A536S, S845A		D68G, M69V		not present
AUS410	274	1	H148N, S466F				-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE15-1110*	1089	1	H148N, S466F				-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE06-1104*	274	0.75	H148N	D87G			-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE06-0610	274	0.75	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE10-0110	274	0.75	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE06-0807	274	0.5	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
AUS411	274	0.38	H148N, S466F				-	E257Q, A536S, S845A	E59D	D68G, M69V		not present
FQSE10-0106	274	0.38	H148N				+	E257Q, A536S, S845A		D68G, M69V	*188ext	not present
FQSE10-0111	274	0.38	H148N				-	E257Q, A536S, S845A	F141L	D68G, M69V		not present
FQSE15-0906	274	0.38	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE15-0310	274	0.38	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
AUS603	274	0.25	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE10-0503	274	0.25	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
FQRC15	274	0.19	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE06-0403	274	0.19	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
FQSE15-0803	274	0.19	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
AUS531	274	0.125	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
AUS588	274	0.125	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
FQRC10	274	0.094	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present
PAMB148	274	0.064	H148N				-	E257Q, A536S, S845A		D68G, M69V		not present

[a] Isolates have been ordered according to their MIC value for CI.

Table A6.4. Quinolones. MIC values for ciprofloxacin and main mutations related with quinolone resistance encountered in the CC274 collection.(Cont.)

Isolate ID[a]	Hyperexpression?	MexEF-OprN and its regulators[b]							Hyperexpression?	MexAB-OprM and its regulators[c]					
		mexE	mexF	oprN	mexS	mexT	parS	parR		mexA	mexB	oprM	mexR	nalC	nalD
AUS601*		F7Y			D249N	F172I, D327Y	A82T, H398R				M552T			G71E, S209R	
AUS690*	-				D249N	F172I	L10P, A82T, H398R		-		Nt712Δ1		H133P	G71E, S209R	
FQSE24-0304*	-		Nt1051ins9		D249N	F172I	A82T, H398R	+		L338P		E456G		G71E, S209R	
FQSE24-1005*	-		Nt1051ins9		D249N	F172I	A82T, H398R	-				E456G		G71E, S209R	
FQSE24-0308*	-		Nt1051ins9		D249N	F172I	A82T, H398R	-				E456G		G71E, S209R	
FQSE24-1010*	-		Nt1051ins9		D249N	F172I	A82T, H398R	-		L338P		E456G		G71E, S209R	
FQSE03	-				D249N	F172I	A82T, H398R	-		L338P				G71E, S209R	
AUS034*	-				D249N, P254Q	L157M, F172I	A82T, H398R	M59I	-	M1*	F178S, M555I		R85H	G71E, S209R	
FQRC26	+				D249N	R164H, F172I	A82T, H398R	+						G71E, S209R	Nt459Δ13
AUS410	-	F7Y			D249N	F172I, D327Y	A82T, H398R	-			M552T			G71E, S209R	
FQSE15-1110*	-		Nt1051ins9	R127Q	D249N	F172I	A82T, H398R	M59I	-	N71S, D235G	L376V	E456G		G71E, S209R	
FQSE06-1104*	-				D249N	F172I	A82T, H398R	-		L338P				G71E, S209R	
FQSE06-0610	-		Nt1051ins9		D249N	F172I	A82T, H398R	-		L338P				G71E, S209R	
FQSE10-0110	-				D249N	F172I	A82T, H398R	+						G71E, S209R	Nt396Δ2
FQSE06-0807	-				D249N	F172I, P270Q	A82T, H398R	-		L338P				G71E, S209R	
AUS411	-	V104G			D249N	F172I	A82T, T163N, D381E, H398R	-			Q104E, F246C, L376V			G71E, S209R	
FQSE10-0106	-				D249N	F172I	A82T, H398R	-			L738P			G71E, S209R	
FQSE10-0111	-				D249N	F172I	A82T, H398R	-						G71E, S209R	
FQSE15-0906	-				D249N	F172I	A82T, H398R	-		L338P				G71E, S209R	
FQSE15-0310	-				Nt848Δ2, D249N	Nt534Δ17, F172I	A82T, H398R	-		L338P				G71E, S209R	
AUS603	-	F7Y			D249N	F172I, D327Y	A82T, H398R	-			M552T	Q93*		G71E, S209R	
FQSE10-0503	-				D249N	F172I	A82T, H398R	-						G71E, S209R	
FQRC15	-				D249N	F172I	A82T, H398R	-						G71E, S209R	
FQSE06-0403	-				D249N	F172I	A82T, H398R	-		L338P				G71E, S209R	
FQSE15-0803	-			Nt415ins2	Nt848Δ2, D249N	Nt534Δ17, F172I	A82T, H398R	-		L338P				G71E, S209R	
AUS531	-				D249N	F172I	A82T, H398R	-						G71E, S209R	
AUS588	-	F7Y, V276M			D249N	F172I	A82T, H398R	-						G71E, S209R	
FQRC10	-				D249N	F172I	A82T, H398R	-						G71E, S209R	
PAMB148	-				D249N	F172I	A82T, H398R	-			M552T			G71E, S209R	

[a] Isolates have been ordered according to their MIC value for CI.

[b] No mutations were encountered in *mvaT*, [c] No mutations were encountered in armR.

Table A6.4. Quinolones. MIC values for ciprofloxacin and main mutations related with quinolone resistance encountered in the CC274 collection.(Cont.)

Isolate ID	Hyperexpression?	MexXY-OprM and its regulators												
		mexY	*mexX*	*oprM*	*fmt*	*mexZ*	*folD*	*parS*	*parR*	*htpX*	*amgS*	*amgR*	*armZ*	PA5528
AUS601*	+	V32A, T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	Q164*		A82T, H398R				E204D	L88P, D119E	
AUS690*	+	G402S, T543A, Q840E, A850T	K329Q, L331V, D346H, W358R		I181V	$Nt_{529}\Delta1$		L10P, A82T, H398R		G187D		R188C	L88P, D119E	
FQSE24-0304*	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R				A8V	L88P, D119E	
FQSE24-1005*	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R			T92A	A8V	L88P, D119E	
FQSE24-0308*	+	Y355H, V	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R				A8V	L88P, D119E	
FQSE24-1010*	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R			A13V	A8V	L88P, D119E	
FQSE03	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R					L88P, D119E	
AUS034*	+	T543A, Q840E, V1000L	K329Q, L331V, D346H, W358R		I181V	$Nt_{334}\Delta13$		A82T, H398R	M59I				L88P, D119E	
FQRC26	-	T543A, Q840E, V875M	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
AUS410	+	V32A, T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	Q164*		A82T, H398R			S64L		L88P, D119E	$Nt_{208}\Delta7$
FQSE15-1110*	+	Y355H, T543A, Q840E	K329Q, L331V, D346H, W358R	E456G	I181V	A194P	G182S	A82T, H398R		A141T	D267N	A8V	L88P, D119E	
FQSE06-1104*	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	$Nt_{290}\Delta11$		A82T, H398R				A8V	L88P, D119E	
FQSE06-0610	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A194P		A82T, H398R				A8V	L88P, D119E	
FQSE10-0110	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE06-0807	+	G287A , T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	S9P		A82T, H398R				A8V	L88P, D119E	
AUS411	+	D201A, G287A, T543A, Q840E	E287D, K329Q, L331V, D346H, W358R		I181V			A82T, T163N, D381E, H398R		$Nt_{683}\Delta5$			L88P, D119E	
FQSE10-0106	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE10-0111	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQSE15-0906	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R				A8V	L88P, D119E	
FQSE15-0310	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R				A8V	L88P, D119E	
AUS603	+	V32A, T543A, Q840E	K329Q, L331V, D346H, W358R	Q93*	I181V	Q164*		A82T, H398R					L88P, D119E	
FQSE10-0503	+	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V	IS		A82T, H398R					L88P, D119E	
FQRC15	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQSE06-0403	+	G287A, T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	S9P		A82T, H398R				A8V	L88P, D119E	
FQSE15-0803	+	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V	A144V		A82T, H398R				A8V	L88P, D119E	
AUS531	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
AUS588	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
FQRC10	-	T543A, Q840E	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	
PAMB148	-	T543A, Q840E, V875M, N1036S	K329Q, L331V, D346H, W358R		I181V			A82T, H398R					L88P, D119E	

[a] Isolates have been ordered according to their MIC value for CI.

Table A6.5. Polymyxins. MIC values for colistin and main mutations related with polymyxin resistance encountered in the CC274 collection.

Isolate ID[a]	ST	MIC					Mutationes encountered in other genes related with polymyxins resistance												
		CO	oprH	phoP	phoQ	parS	parR	cprR	cprS	colS	colR	lpxC	pagL	PA4773	PA4774	PA4775	pmrA	pmrB
AUS034*	274	>256		E266*		A82T, H398R	M59I			H415Q						R131Q, A270V	L71R	Y345H
FQSE06-1104*	274	2				A82T, H398R									W274*	R131Q, A270V	L71R	V185I, G221D, R287Q, Y345H
AUS603	274	1.5				A82T, H398R			R56C							R131Q, A270V	L71R	Y345H
FQSE10-0106	274	1.5				A82T, H398R										R131Q, A270V	L71R	Y345H
AUS531	274	1				A82T, H398R										R131Q, A270V	L71R	Y345H
FQRC15	274	1				A82T, H398R										R131Q, A270V	L71R	Y345H
FQSE06-0807	274	1				A82T, H398R										R131Q, A270V	L71R	Y345H
FQSE24-1005*	1089	1				A82T, H398R				E303D						R131Q, A270V	L71R	R287Q, Y345H
FQSE24-0308*	1089	1				A82T, H398R										R131Q, A270V	L71R	R287Q, Y345H
AUS588	274	0.75				A82T, H398R										R131Q, A270V	L71R	Y345H
FQSE15-0906	274	0.75				A82T, H398R										R131Q, A270V	L71R	Y345H
FQRC10	274	0.5				A82T, H398R										R131Q, A270V	L71R	Y345H
FQSE10-0110	274	0.5				A82T, H398R										R131Q, A270V	L71R	Y345H
PAMB148	274	0.5				A82T, H398R										R131Q, A270V	L71R	Y345H
AUS410	274	0.38				A82T, H398R									Nt286Δ1	R131Q, A270V	L71R	Y345H
FQRC26	274	0.38				A82T, H398R										R131Q, A270V	L71R	Y345H
FQSE10-0111	274	0.38				A82T, H398R										R131Q, A270V	L71R	Y345H
FQSE24-0304*	1089	0.38				A82T, H398R										R131Q, A270V	L71R	R287Q, Y345H
FQSE24-1010*	1089	0.38				A82T, H398R										R131Q, A270V	L71R	R287Q, Y345H
AUS411	274	0.25			H248P	A82T, T163N, D381E, H398R									N159D	R131Q, A270V	L71R	Y345H
AUS601*	1043	0.25			K234N, T315A	A82T, H398R									E163G	R131Q, A270V	L71R	L31P, Y345H
FQSE03	274	0.25				A82T, H398R										R131Q, A270V	L71R	Y345H
FQSE10-0503	274	0.25				A82T, H398R										R131Q, A270V	L71R	Y345H
FQSE15-0803	274	0.25				A82T, H398R										R131Q, A270V	L71R	E213D, Y345H
FQSE15-0310	274	0.25				A82T, H398R										R131Q, A270V	L71R	Y345H
FQSE15-1110*	274	0.25				A82T, H398R										R131Q, A270V	L71R	R287Q, Y345H
FQSE06-0403	274	0.19				A82T, H398R										R131Q, A270V	L71R	Y345H
FQSE06-0610	274	0.19				A82T, H398R										R131Q, A270V	L71R	Y345H
AUS690*	274	0.125	T221I		L10P, A82T, H398R										P158L	R131Q, A270V	L71R	F124L, Y345H

[a] Isolates have been ordered according to their MIC value for CO.

14. ANNEX 7

EVOLUTIONARY DYNAMICS OF *Pseudomonas aeruginosa*

AMINOGLYCOSIDE RESISTANCE DEVELOPMENT: EXPANDED RESULTS

Table A7.1. Antibiotic susceptibility profiles and mutations encountered in parental strain PAO1 and its derived aminoglycoside resistant mutants.

ISOLATE			MIC values (mg/L) and susceptibility profiles																							
			TM		GE		AK		TI		PPT		TZ		PM		AT		TOL/TAZ		IP		MP		CI		CO	
			MIC	CLSI	MIC	CLSI	MIC	CLSI	MIC	CLSI	MIC	CLSI	MIC	CLSI	MIC	CLSI	MIC	CLSI	MIC	CLSI	MIC	CLSI	MIC	CLSI	MIC	CLSI	MIC	CLSI
PAO1			0.5	S	1	S	2	S	32	I	4	S	2	S	4	S	4	S	1	S	2	I	1	S	0.25	S	2	S
SERIE	DAY	COLONY																										
1	1	1	4	S	8	I	32	I	32	I	8	S	16	I	2	S	4	S	1	S	2	S	1	S	0.25	S	1	S
1	1	2	4	S	16	R	32	I	32	I	4	S	4	S	8	S	2	S	1	S	2	S	1	S	0.25	S	1	S
2	1	1	2	S	8	I	16	S	32	I	8	S	2	S	4	S	8	S	1	S	1	S	1	S	0.5	S	1	S
2	1	2	2	S	8	I	16	S	16	S	4	S	2	S	8	S	2	S	1	S	2	S	1	S	0.5	S	1	S
3	1	1	2	S	8	I	16	S	32	I	4	S	2	S	4	S	4	S	1	S	1	S	1	S	0.5	S	1	S
3	1	2	2	S	8	I	16	S	32	I	4	S	2	S	4	S	4	S	1	S	2	S	1	S	0.5	S	1	S
4	1	1	2	S	8	I	16	S	32	I	4	S	2	S	4	S	4	S	1	S	2	S	1	S	0.25	S	1	S
4	1	2	2	S	8	I	16	S	32	I	4	S	2	S	2	S	2	S	1	S	2	S	1	S	0.25	S	1	S
5	1	1	1	S	4	S	4	S	32	I	4	S	2	S	1	S	4	S	0.5	S	2	S	1	S	≤0.125	S	1	S
5	1	2	2	S	4	S	4	S	32	I	8	S	2	S	2	S	4	S	1	S	2	S	2	S	0.25	S	1	S
1	7	1	32	R	64	R	256	R	32	I	4	S	2	S	2	S	2	S	1	S	2	S	2	S	0.25	S	1	S
1	7	2	32	R	64	R	128	S	16	S	8	S	2	S	1	S	2	S	1	S	4	I	1	S	≤0.125	S	1	S
2	7	1	16	R	64	R	64	R	16	S	4	S	1	S	4	S	2	S	1	S	1	S	1	S	0.5	S	2	S
2	7	2	16	R	64	R	64	R	16	S	4	S	1	S	4	S	≤1	S	0.5	S	1	S	1	S	0.5	S	4	R
3	7	1	32	R	64	R	128	R	16	S	8	S	2	S	2	S	4	S	1	S	2	S	2	S	≤0.125	S	1	S
3	7	2	64	R	64	R	128	R	16	S	8	S	1	S	1	S	2	S	1	S	2	S	0,5	S	≤0.125	S	0.5	S
4	7	1	32	R	64	R	64	R	32	I	8	S	2	S	2	S	2	S	1	S	2	S	1	S	0.5	S	4	R
4	7	2	16	R	32	R	64	R	16	S	8	S	2	S	2	S	2	S	0.5	S	2	S	1	S	0.25	S	2	S
5	7	1	32	R	64	R	128	R	16	S	4	S	1	S	1	S	2	S	0.5	S	1	S	0,5	S	0.25	S	>4	R
5	7	2	32	R	64	R	128	R	32	I	4	S	1	S	2	S	2	S	0.5	S	1	S	0,5	S	0.25	S	2	S
1	14	1	512	R	1024	R	512	R	64	I	8	S	8	S	4	S	2	S	1	S	4	I	1	S	≤0.125	S	0.25	S
1	14	2	256	R	1024	R	512	R	16	S	8	S	1	S	8	S	2	S	1	S	2	S	0,5	S	≤0.125	S	0.5	S
2	14	1	64	R	256	R	512	R	8	S	4	S	1	S	2	S	≤1	S	1	S	1	S	1	S	≤0.25	S	≤0.125	S	0.5	S
2	14	2	64	R	256	R	512	R	8	S	≤2	S	2	S	4	S	2	S	1	S	4	I	1	S	≤0.125	S	0.25	S
3	14	1	512	R	1024	R	1024	R	16	S	8	S	4	S	4	S	2	S	1	S	2	S	1	S	≤0.125	S	0.25	S
3	14	2	512	R	1024	R	512	R	32	I	16	S	2	S	4	S	2	S	1	S	4	I	1	S	≤0.125	S	0.25	S
4	14	1	64	R	256	R	512	R	8	S	4	S	1	S	2	S	≤1	S	0.5	S	1	S	0,5	S	≤0.125	S	0.5	S
4	14	2	64	R	256	R	512	R	16	S	4	S	1	S	2	S	≤1	S	1	S	2	S	1	S	0.5	S	1	S
5	14	1	128	R	256	R	512	R	8	S	≤2	S	≤0.5	S	1	S	≤1	S	≤0.25	S	2	S	0,5	S	≤0.125	S	0.5	S
5	14	2	128	R	128	R	512	R	8	S	≤2	S	≤0.5	S	1	S	≤1	S	≤0.25	S	≤0.5	S	1	S	≤0.125	S	0.5	S

Table A7.1. Antibiotic susceptibility profiles and mutations encountered in parental strain PAO1 and its derived aminoglycoside resistant mutants. (Cont.)

ISOLATE			ENCOUNTERED MUTATIONS																
			PA0432	PA1023	PA1554	PA1555	PA1557	PA1766	PA1767	PA2009	PA2018	PA2631	PA2632	PA2633	PA2634	PA2635	PA2636	PA2637	PA2638
			sahH	-	ccoN1	ccoP2	ccoN2	-	-	hmgA	mexY	yjcF	-	-	aceA	-	-	nuoA	nuoB
PAO1			WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
SERIE	DAY	COLONY																	
1	1	1																	
1	1	2																	
2	1	1																	
2	1	2																	
3	1	1																	
3	1	2																	
4	1	1																	
4	1	2																	
5	1	1					A217T												
5	1	2	nt910ins6																
1	7	1				V14L													
1	7	2							nt951Δ1										
2	7	1																	
2	7	2																	
3	7	1																	
3	7	2						nt280ins6											
4	7	1										Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ
4	7	2										Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ
5	7	1																	
5	7	2																	
1	14	1																	
1	14	2		nt158Δ2															
2	14	1																	
2	14	2																	
3	14	1							nt961ins6										
3	14	2			nt580Δ1				nt961ins6										
4	14	1										Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ
4	14	2										Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ
5	14	1									T472P								
5	14	2								L188Q									

Table A7.1. Antibiotic susceptibility profiles and mutations encountered in parental strain PAO1 and its derived aminoglycoside resistant mutants. (Cont.)

ISOLATE			ENCOUNTERED MUTATIONS																	
			PA2639	PA2800	PA2960	PA3064	PA3789	PA4029	PA4266	PA4454	PA4455	PA4526	PA4661	PA4727	PA4777	PA5040	PA5070	PA5105	PA5134	PA5300
			nuoD	vacJ	pilZ	pelA	-	-	fusA1	yrbD	yrbE	pilB	pagL	pcnB	pmrB	pilQ	tatC	hutC	ctpA	ctpA
PAO1			WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
SERIE	DAY	COLONY																		
1	1	1							I61M											
1	1	2							I61M											
2	1	1										N349K								
2	1	2										N349K								
3	1	1			nt19ins1															
3	1	2			nt19ins1															
4	1	1							T671A							nt209ins1				
4	1	2							T671A							nt209ins1				
5	1	1										G326D						Y181X		
5	1	2										G326D						Y181X		
1	7	1							I61M											
1	7	2							I61M											
2	7	1			A39S							N349K			S257N					G247S
2	7	2										N349K			S257N					G247S
3	7	1							I61M		nt450Δ1						nt511Δ12			
3	7	2							I61M									T216P		
4	7	1							T671A					nt827ins1	nt131Δ3	nt209ins1				
4	7	2							T671A						nt131Δ3	nt209ins1				
5	7	1							E100G			G326D			A247T			Y181X		
5	7	2							E100G			G326D			A247T			Y181X		
1	14	1					R339C		I61M	I86N										
1	14	2					R339C		I61M	G17D										
2	14	1							I61M			N349K			S257N					
2	14	2							I61M			N349K			S257N					
3	14	1							I61M		S66L									
3	14	2		Q64X					I61M											
4	14	1	nt34ins5						T671A				nt88Δ24		nt131Δ3	nt209ins1				
4	14	2						G49D	T671A						nt131Δ3	nt209ins1				
5	14	1							E100G			G326D			A247T			Y181X		
5	14	2							E100G			G326D			A247T			Y181X		

Table A7.2. MIC$_{TOB}$ values, MexXY overexpression and antibiotic resistance mutations encountered in the clinical isolates. Differences between paired isolates are highlighted in bold.

PATIENT		FQSE06		FQSE11		FQSE16	
Aminoglycoside profile		S	R	S	R	S	R
Sequence Type		274		701		1613	
MIC$_{TOB}$ (mg/L)		1	24	2	>256	4	64
mexY overexpression		+	+	+	+	-	+
AMEs[a]					**aacA4**		
PA0004	*gyrB*	H148N	H148N		**R138L**		
PA0005	*lptA*						
PA0018	*fmt*	I181V	I181V	A308T, L287V, I196V, I181V	A308T, L287V, I196V, I181V	I181V	I181V
PA0058	*dsbM*			**C28R, F206L, R212C**			
PA0301	*spuE*					A301S	A301S
PA0355	*pfpI*			A58T, E57D	A58T, E57D		
PA0392	*yggT*						
PA0402	*pyrB*						
PA0424	*mexR*			V126E	V126E		
PA0425	*mexA*	L338P	L338P			nt472597Δ1	nt472597Δ1
PA0426	*mexB*			**nt772Δ1**			**Q575R**
PA0427	*oprM*						
PA0432	*sahH*					A286T	A286T
PA0463	*creB*	E128G	E128G	A130T	A130T	E128G, A130T	E128G, A130T
PA0464	*creC*	A397V	A397V	G157A	G157A	A397V	A397V
PA0465	*creD*	V394A	V394A	V335I, V394A, F445L, R451K	V335I, V394A, F445L, R451K	V448I	V448I
PA0486	*yihE*	D210E	D210E	D210E	D210E	D258E, D210E	D258E, D210E
PA0487	*modR*			G179E	G179E		
PA0610	*prtN*			S8T, S4T, M1*	S8T, S4T, M1*		
PA0611	*prtR*			T5P, S4N	T5P, S4N		
PA0612	*ptrB*						
PA0779	*asrA*			R778K	R778K		
PA0807	*ampDh3*			A208V	A208V	A219T	A219T
PA0869	PBP6/7	S250N	S250N				
PA0893	*argR*					P42S	P42S
PA0958[a]	*oprD*				**Q424E, S403A**		
PA1023	-	A33V, E209A, E276Q	A33V, E209A, E276Q	A208S	A208S	E209A, E276Q	E209A, E276Q
PA1178	*oprH*						
PA1179	*phoP*						
PA1180	*phoQ*					G370D	G370D

Table A7.2. MIC$_{TOB}$ values, MexXY overexpression and antibiotic resistance mutations encountered in the clinical isolates. (Cont.)

PATIENT		FQSE06		FQSE11		FQSE16	
Aminoglycoside profile		S	R	S	R	S	R
PA1343	*pagP*			F129L	F129L		
PA1345	*gshB*	L17P, D65G, L463R	L17P, D65G, L463R	L17P, L463R, V495I	L17P, L463R, V495I	L463R	L463R
PA1375	*pdxB*	Q150R,179H, R247H,Q365R	Q150R, R179H, R247H, Q365R	H183Y, P192T	H183Y, P192T		
PA1409	*aphA*	L62I, R224L, R297K	L62I, R224L, R297K	L62I, I320V	L62I, I320V	L62I, R297K	L62I, R297K
PA1430	*lasR*	P117G	P117G		R216Q		
PA1554	*ccoN1*						
PA1555	*ccoP2*			V231I	V231I		
PA1557	*ccoN2*						
PA1588	*sucC*						
PA1589	*sucD*						
PA1766	-						
PA1767	-						
PA1777	*oprF*						
PA1796	*folD*					N171S	N171S
PA1797[a]	-						
PA1798	*parS*	A82T, H398R	A82T, H398R	H398R	H398R	H398R	H398R
PA1799	*parR*			S170N, L153R	S170N, L153R		
PA1801	*clpP*	P11S	P11S				
PA1803	*lon*			A499S	A499S		
PA1812	*mltD*						
PA1886	*polB*	D176G	D176G	D176G, R196C	D176G, R196C	D176G, Q664R	D176G, Q664R
PA2006	-			E232Q	E232Q	E220K	E220K
PA2009	*hmgA*			P303H, T226A	P303H, T226A		
PA2018	*mexY*	T543A, Q840E	G287A, T543A, Q840E	Q840E, N709H, G589A, A586T, T543A, I536V	Q840E, N709H, G589A, A586T, T543A, I536V	Q840E, T543A, E152D	Q840E, T543A, G287S, E152D
PA2019	*mexX*	W358R, K329Q, L331V, D346H	W358R, K329Q, L331V, D346H	S382G, W358R, L331V, K329Q, A38P, A30T	S382G, W358R, L331V, K329Q, A38P, A30T	W358R, R351S, K329Q	W358R, R351S, K329Q
PA2020	*mexZ*	nt290Δ11	S9P	L138R	L138R		R125P
PA2023	*galU*						
PA2050	-			C142G	C142G	C142G	C142G

Table A7.2. MIC$_{TOB}$ values, MexXY overexpression and antibiotic resistance mutations encountered in the clinical isolates. (Cont.)

PATIENT		FQSE06		FQSE11		FQSE16	
Aminoglycoside profile		S	R	S	R	S	R
PA2071	*fusA2*	G695A	G695A	S176A, A197D, G695A	S176A, A197D, G695A	S176A, G695A	S176A, G695A
PA2227	*vqsM*	deleted	deleted				
PA2272	PBP3a	A104P	A104P	A104P	A104P	A104P	A104P
PA2273	*soxR*						
PA2489	-	R12L, A244T	R12L, A244T	G185S, A244T	G185S, A244T		
PA2490	*ydbB*	T48S, R104C	T48S, R104C	T48S, R104C	T48S, R104C	T48S, R104C	T48S, R104C
PA2491	*mexS*	D249N	D249N	D249N	D249N	D249N, D201G	D249N, D201G
PA2492	*mexT*	F172I	F172I	F172I	F172I	P60S, F172I	P60S, F172I, **G274D, G300D**
PA2493	*mexE*			S8F	S8F		
PA2494	*mexF*			D230A	D230A		
PA2495	*oprN*			S13P, R363H	S13P, R363H		
PA2522	*czcC*			R225K	R225K	T215A	T215A
PA2523	*czcR*						
PA2524	*czcS*	E44K	E44K	G282S, N353S, G470S	G282S, N353S, G470S	G282S, N353S	G282S, N353S
PA2525	*opmB*	V108A	V108A			S147G, N145S, V108A	S147G, N145S, V108A
PA2526	*muxC*						
PA2527	*muxB*	K646N	K646N			I880V	I880V
PA2528	*muxA*	T261A	T261A	T261A	T261A		
PA2615	*ftsK*	S52P, S287P, G729S	S52P, S287P, G729S	S287P, S52P	S287P, S52P	S52P, S287P, G729S	S52P, S287P, G729S
PA2621	*clpS*						
PA2631	*yjcF*						
PA2632	-	T4A, **P33L**, S127N	T4A, S127N	S74T	S74T		
PA2633	-	S222N, R249H	S222N, R249H		A109T, R249H		A109T, R249H
PA2634	*aceA*	S297A	S297A	S297A	S297A	S297A	S297A
PA2635	-			T410A	T410A		
PA2636	-	T40S, N125D	T40S, N125D	N125D, V178D	N125D, V178D	T180I	T180I
PA2637	*nuoA*						
PA2638	*nuoB*						
PA2639	*nuoD*		G499X				

Table A7.2. MIC$_{TOB}$ values, MexXY overexpression and antibiotic resistance mutations encountered in the clinical isolates. (Cont.)

PATIENT		FQSE06		FQSE11		FQSE16	
Aminoglycoside profile		S	R	S	R	S	R
PA2642	*nuoG*	T484A	T484A	T484A, K585R	T484A, K585R	T484A	T484A
PA2649	*nuoN*					G326D	G326D
PA2797	-						
PA2798	-	G301S	G301S	T317S, G301S, E297D	T317S, G301S, E297D	G301S	G301S
PA2800	*vacJ*			S172G	S172G		
PA2809	*copR*						
PA2810	*copS*			M48L, R320H	M48L, R320H	E359G	E359G
PA2830	*htpX*						
PA2960	*pilZ*					P9S	P9S
PA3005	*nagZ*						
PA3013	*foaB*			I391V, E225Q	I391V, E225Q		
PA3014	*faoA*			V397I	V397I		
PA3047	*PBP4*	A358V, A394P	A358V, A394P				
PA3050	*pyrD*			R96K, K40E	R96K, K40E		
PA3064	*pelA*	V446I, A272T, H141Y, I19T	A272T, H141Y, I19T	V946A, V507A, I377V, T41A	V946A, V507A, I377V, T41A	R862H, V825L, A272T, H141Y, C25R, I19T	R862H, V825L, A272T, H141Y, C25R, I19T
PA3077	*cprR*						
PA3078	*cprS*			T16S, E386D	T16S, E386D		
PA3141	*capD*	I7M, S51G	I7M, S51G	A626V, S51G	A626V, S51G	S486L, nt512ins1	S486L
PA3168	*gyrA*				Y267N	D87N	D87N
PA3521	*opmE*	S175T, A279S, W354R	S175T, A279S, W354R	W354R, S175T	W354R, S175T	W354R, S175T, S46G	W354R, S175T, S46G
PA3522	*mexQ*	I294V, G505D, D602E, G645A, V921M	I294V, G505D, D602E, G645A, V921M	R656K, G505D, V384I, I294V	R656K, G505D, V384I, I294V	R1036H, P428S, V355M	R1036H, P428S, V355M
PA3523	*mexP*	A297E, R366L	A297E, R366L	A297E, R366L	A297E, R366L		
PA3533	*grxD*						
PA3574	*nalD*		A272T, H141Y, C25R, I19T				
PA3602	*yerD*					L21V	L21V
PA3676	*mexK*			K694R, I21L	K694R, I21L		
PA3677	*mexJ*			A314P	A314P		

Table A7.2. MIC$_{TOB}$ values, MexXY overexpression and antibiotic resistance mutations encountered in the clinical isolates. (Cont.)

PATIENT		FQSE06		FQSE11		FQSE16	
Aminoglycoside profile		S	R	S	R	S	R
PA3678	*mexL*					S6P	S6P
PA3719	*armR*			S21T	S21T		
PA3721	*nalC*	G71E, S209R	G71E, S209R	G71E, A145V, S209R	G71E, A145V, S209R	S209R	S209R
PA3789	-	G439S, L195V, E191D, V71A	G439S, L195V, E191D, V71A	L205F, L195V, E191D, V71A	L205F, L195V, E191D, V71A		
PA3999	PBP5						
PA4001	*sltB1*						
PA4003	PBP2						
PA4020	*mpl*	M297V	**S257L,** M297V	S306N, M297V	S306N, M297V, **Q248***	M297V	M297V
PA4029	-			A218T	A218T		
PA4069	-	T234I	T234I				
PA4109	*ampR*			M288R, G283E	M288R, G283E		
PA4110	*ampC*	T21A, T105A, G391A	T21A, T105A, G391A	R79Q, T105A, V205L, V356I, G391A	R79Q, T105A, V205L, V356I, G391A	T105A	T105A
PA4119	*aph*	A42V	A42V				
PA4205	*mexG*						
PA4206	*mexH*			P218T, D302E	P218T, D302E	A123T	A123T
PA4207	*mexI*			A782E	A782E	A491T, A782E	A491T, A782E
PA4208	*opmD*			S112N, A270G	S112N, A270G	S112N, A243E	S112N, A243E
PA4218	*ampP*	M87I, T172A	M87I, T172A	L98F, M87I, L74F	L98F, M87I, L74F		
PA4238	*rpoA*						
PA4260	*rplB*						
PA4266	*fusA1*		**Y552C,** **T671I**			T456A, K187R	**Y552C,** T456A, K187R
PA4269	*rpoC*					R905C, N606S	R905C, N606S
PA4270	*rpoB*	V51I	V51I	V51I	V51I	G158D, V51I	G158D, V51I
PA4273	*rplA*						
PA4315	*mvaT*						
PA4374	*mexV*	A229G, Q321R	A229G, Q321R	A229G, Q321R	A229G, Q321R	Q321R	Q321R
PA4375	*mexW*	A627V, Q771P	A627V, Q771P	Q511R, G758S	Q511R, G758S	Q511R	Q511R
PA4380	*colS*						

Table A7.2. MIC$_{TOB}$ values, MexXY overexpression and antibiotic resistance mutations encountered in the clinical isolates. (Cont.)

PATIENT		FQSE06		FQSE11		FQSE16	
Aminoglycoside profile		S	R	S	R	S	R
PA4381	*colR*					D32G	D32G
PA4393	*ampG*	V81I, A583T	V81I, A583T	A583T	A583T	A583T	A583T
PA4406	*lpxC*						
PA4418	PBP3		P215L			Q568R	Q568R
PA4444	*mltB1*	H64R, P106Q	H64R, P106Q	A29T, H64R, T139A	A29T, H64R, T139A	H64R, T139A, V147I, S267N	H64R, T139A, V147I, S267N
PA4454	*yrbD*						
PA4455	*yrbE*						
PA4462	*rpoN*					W305R	W305R, **V473A**
PA4521	*ampE*	S33G, S69P	S33G, S69P	L190F, E129D, S69P, S33G	L190F, E129D, S69P, S33G	S69P, S33G	S69P, S33G
PA4522	*ampD*	R11L, G148A, D183Y	R11L, G148A, D183Y	G148A	G148A	V90A	V90A
PA4526	pilB	R470K, K482Q	R470K, K482Q			I211T, T266N	I211T, T266N
PA4567	*rpmA*						
PA4568	*rplU*	**I74M**					
PA4597	*oprJ*	D68G, M69V	D68G, M69V			M69V	M69V
PA4598	*mexD*	E257Q, A536S, S845A	E257Q, A536S, S845A	K1031R, S845A, E257Q	K1031R, S845A, E257Q	**P721S, L624P,** E257Q	E257Q
PA4599	*mexC*			A378T, S330A, H310R, R76Q, P47S	A378T, S330A, H310R, R76Q, P47S		
PA4600	*nfxB*			R21H, D56G	R21H, **E75K,** D56G	A170T, Ter188Rext*	A170T, Ter188Rext*
PA4661	*pagL*			T55S	T55S		
PA4671	*rplY*			A123S	A123S		
PA4700	PBP1b	S25G	S25G	L353Q, S25G	L353Q, S25G	L353Q, S25G	L353Q, S25G
PA4727	pcnB						
PA4748	*tpiA*						
PA4751	*ftsH*						
PA4773	-			C161S, V217I, T271A	C161S, **A165T,** V217I, T271A		
PA4774	-						
PA4775	-	R131Q, A270V	R131Q, A270V	G31S, G152D, E153D	G31S, G152D, E153D		

Table A7.2. MIC$_{TOB}$ values, MexXY overexpression and antibiotic resistance mutations encountered in the clinical isolates. (Cont.)

PATIENT		FQSE06		FQSE11		FQSE16	
Aminoglycoside profile		S	R	S	R	S	R
PA4776	*pmrA*	L71R	L71R			L71R	L71R
PA4777	*pmrB*	Y345H	Y345H	A4T, V15I, G68S, Y345H	A4T, V15I, G68S, Y345H	S2P, A4T, Y345H	S2P, A4T, Y345H
PA4878	*brlR*	T111L	T111L	Q42R, P61Q	Q42R, P61Q		
PA4944	*hfq*					D9A	D9A
PA4964	*parC*					Q405R	Q405R
PA4967	*parE*			D533E	D533E		
PA5000	*wapR*	T85A	T85A	T85A, R78K	T85A, R78K	T85A	T85A
PA5038	*aroB*	V85A, A200E, I292T	V85A, A200E, I292T	V85A	V85A	A200E, V85A	A200E, V85A
PA5040	*pilQ*	T690S, **E676D, E669D,** T266M, A106T	T690S, T266M, A106T	K139R, A106T	K139R, A106T	L523I, S505N, S481R, A135T, A128T	L523I, S505N, S481R, A135T, A128T
PA5045	PBP1a			5682740insCGC	5682740insCGC	A368T, S631I, 5682740ins CGC	A368T, S631I, 5682740insCG C
PA5070	*tatC*	D195E	D195E	D195E	D195E	D195E	D195E
PA5105	*hutC*						
PA5117	*typA*						
PA5134	*ctpA*	D65N	D65N				
PA5199	*amgS*			I260V	I260V		
PA5200	*amgR*	A8V	A8V				
PA5235	*glpT*					A439T	A439T
PA5297	*poxB*			V19I, V193I, I303V, E506D	V19I, V193I, I303V, E506D		
PA5300	*cycB*						
PA5332	*crc*						
PA5366	*pstB*						
PA5471	*armZ*	L88P, D119E	L88P, D119E	V243A, I237V, D119E, S112N, L88P, C40R	V243A, I237V, D119E, S112N, L88P, C40R	D119E, L88P	D119E, L88P
PA5471.1	-						
PA5485	*ampDh2*			P116S	P116S		
PA5528	-			6220278insACG	6220278insACG		
PA5542	-	I106V	I106V	I106V	I106V	I106V, T68S	I106V, T68S

15. Publications derived from this thesis

Clonal Dissemination, Emergence of Mutator Lineages and Antibiotic Resistance Evolution in *Pseudomonas aeruginosa* Cystic Fibrosis Chronic Lung Infection

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Abstract

Chronic respiratory infection by *Pseudomonas aeruginosa* is a major cause of mortality in cystic fibrosis (CF). We investigated the interplay between three key microbiological aspects of these infections: the occurrence of transmissible and persistent strains, the emergence of variants with enhanced mutation rates (mutators) and the evolution of antibiotic resistance. For this purpose, 10 sequential isolates, covering up to an 8-year period, from each of 10 CF patients were studied. As anticipated, resistance significantly accumulated overtime, and occurred more frequently among mutator variants detected in 6 of the patients. Nevertheless, highest resistance was documented for the nonmutator CF epidemic strain LES-1 (ST-146) detected for the first time in Spain. A correlation between resistance profiles and resistance mechanisms evaluated [efflux pump (*mexB, mexD, mexF*, and *mexY*) and *ampC* overexpression and OprD production] was not always obvious and hypersusceptibility to certain antibiotics (such as aztreonam or meropenem) was frequently observed. The analysis of whole genome macrorestriction fragments through Pulsed-Field Gel Electrophoresis (PFGE) revealed that a single genotype (clone FQSE-A) produced persistent infections in 4 of the patients. Multilocus Sequence typing (MLST) identified clone FQSE-A as the CF epidemic clone ST-274, but striking discrepancies between PFGE and MLST profiles were evidenced. While PFGE macrorestriction patterns remained stable, a new sequence type (ST-1089) was detected in two of the patients, differing from ST-274 by only two point mutations in two of the genes, each leading to a nonpreviously described allele. Moreover, detailed genetic analyses revealed that the new ST-1089 is a *mutS* deficient mutator lineage that evolved from the epidemic strain ST-274, acquired specific resistance mechanisms, and underwent further interpatient spread. Thus, presented results provide the first evidence of interpatient dissemination of mutator lineages and denote their potential for unexpected short-term sequence type evolution, illustrating the complexity of *P. aeruginosa* population biology in CF.

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Introduction

Chronic respiratory infection by *Pseudomonas aeruginosa* is a major driver of morbidity and mortality in cystic fibrosis patients [1,2]. Traditionally, initial colonization is considered to be produced by unique strains acquired from environmental sources that undergo an extensive adaptation within the patient's lungs, leading to life-long persistent infections with little patient to patient transmission [3]. The establishment of the characteristic biofilm structures and the acquisition of a plethora of adaptive mutations (leading to enhanced antimicrobial and host defenses resistance, specific metabolic adaptation and an adapted virulence), are the main responsible for the persistence of these infections despite extensive antimicrobial therapy [4–9].

One of the hallmarks of *P. aeruginosa* chronic respiratory infections, in contrast to acute processes, is the high prevalence of hypermutable (or mutator) strains [10–13]. These variants are found in 30 to 60% of CF patients and show up to 1000-fold increased spontaneous mutation rates caused by defective DNA repair pathways. Among them, the mismatch repair (MMR) system is the one most frequently affected, due to mutations in *mutS* or *mutL* genes [14–16]. Indeed, mutator variants are positively selected during the establishment of chronic infections, linked to the acquisition of mutations related to antibiotic resistance, biofilm growth, metabolic adaptation, or acute virulence attenuation [10,16–20].

Additionally, there is growing evidence suggesting that adaptation to the CF lung environment may escape from the scale of the individual patients [21]. Indeed, the existence of concerning epidemic strains, such as the Liverpool Epidemic Strain (LES-1), capable of infecting hundreds of CF patients in different geographical locations, has been well documented for over two

decades [22,23]. Mutator variants have also been detected in a small proportion of isolates from patients infected by the LES-1 epidemic strain [24,25], but interpatient spread of mutator variants has never been demonstrated. Moreover, recent whole genome sequence analyses have revealed that the origination of CF adapted epidemic strains may result from a limited number of specific mutations with pleiotropic effects [26].

Although whole genome sequencing and microarray analysis will soon take the lead, the current gold standard for typing *P. aeruginosa* strains with the purpose of investigating patient to patient transmission is still the analysis of whole genome macrorestriction fragments through Pulsed-Field Gel Electrophoresis (PFGE) [27]. However, the instability of PFGE profiles, mainly consequence of frequent genome rearrangements, makes this procedure unsuitable for long-term and global epidemiology studies [28]. On the other hand, Multilocus Sequence Typing (MLST), based on sequencing of 7 house keeping genes, provides a much more stable genetic signature and is still currently considered the gold standard for global epidemiology and population structure analyses [29].

In this work, we investigated the interplay between the three above described key microbiological aspects of *P. aeruginosa* CF chronic lung infections: the occurrence of transmissible and persistent strains (PFGE-MLST clonal epidemiology), the emergence of variants with enhanced mutation rates (mutators) and the evolution of antibiotic resistance.

Results and Discussion

Long-term Clonal Epidemiology of *P. aeruginosa* in CF: Transmissible and Persistent Strains

A total of 100 *P. aeruginosa* isolates were studied, including 10 sequential isolates from each of 10 CF patients attended at the reference hospital of the Balearic Islands, Spain. Each of the sequential isolates included were separated by at least a 6-month interval, covering up to an 8-year period from 2003 to 2010. PFGE analysis revealed the presence of 13 different clones; one of them (clone FQSE-A) was detected in four patients while the other twelve were detected in single patients. Figure 1 shows the distribution of the different clones among the different patients along the 8-year study period. Six patients, including the 4 colonized by clone FQSE-A, showed a single clone over the study period, while the other 4 showed the coexistence of several (2 to 4) clones or clonal replacements (Figure 1). Therefore, results so far suggested that clone FQSE-A is a CF adapted (transmissible and persistent) strain. The epidemiological setting driving (direct or indirect) interpatient transmission is uncertain, since recommendations on segregation of patients colonized by *P. aeruginosa* from those free of this pathogen are followed in all hospital visits.

Discrepant MLST vs PFGE Results: Role of Mutators

The first isolate per patient and clone were further analyzed by MLST, yielding 13 sequence types (ST) not entirely coincident with the clones identified by PFGE. The allelic profiles and relevant features of the 13 STs identified are shown in Table 1. Not surprisingly due to the overall higher discriminatory power (or lower stability) of PFGE compared to MLST [28–30], two different PFGE clones (FQSE-C and FQSE-D) shared the same ST (ST-299). Much more intriguingly, the disseminated clone FQSE-A yielded two different STs (Table 1, Figure 1). Clone FQSE-A from 3 of the 4 patients was identified as ST-274, previously detected in multiple CF patients from France, Austria and Australia according to the MLST database (http://pubmlst. org/paeruginosa/). Moreover, this clone has been simultaneously detected in several patients from another CF Unit in Madrid [31]

and in a few cases of hospital-acquired infections in recent Spanish multicentre studies [32,33]. Thus, our results add further evidence pointing out that ST-274 should be added to the growing list of CF epidemic clones [22,29]. In contrast, clone FQSE-A from the fourth patient was identified as a new ST (ST-1089) differing from ST-274 by only two point mutations in two of the genes (*acsA* and *guaA*) each leading to a nonpreviously described allele (the only two novel alleles found in the complete collection). Additionally, in contrast to ST-274, ST-1089 showed a mutator phenotype (Table 1). Therefore, the available data clearly suggested that ST-1089 has recently evolved from the CF epidemic clone ST-274 through point mutations linked to the emergence of a mutator lineage. As expected, due to the mutational spectra of DNA MMR deficient strains [12], both mutations were G to A transversions. Moreover, both mutations were apparently not neutral, since they lead to nonsynonymous substitutions in the acetyl-coenzyme A synthetase (G435D) and the GMP synthase (G312S), which are key metabolic enzymes. Whether these mutations were positively selected because the play a role in the intense metabolic adaptation to the CF chronic lung infection setting [18] remains to be explored.

Consistently with our findings, while mutators series appeared to be no more variable in their MLST haplotypes than nonmutator series in a previous study, the only novel alleles found were also from patients with mutator strains [34]. Moreover, a recent study has reported discrepant MLST vs PFGE results directly linked to the emergence of a mutator phenotype caused by *mutL* mutations [35], stressing the point that this gene lacks the neutrality required for an appropriate MLST marker, since MMR deficient mutators (*mutS* and *mutL*) are positively selected in CF chronic infection [15]. Likewise, a recent work has reported a strain that was not typable by MLST due to the presence of a deletion in the *mutL* fragment analyzed [30]. All together, these results indicate that frequent mutator variants from chronic infection may determine a lower stability of the MLST profiles than expected (leading to discrepant results compared to PFGE) both directly (*mutL* inactivating mutations within the gene fragment evaluated in MLST analysis) and indirectly through the increased spontaneous mutagenesis (facilitating the emergence of novel alleles through point mutations in any of the 7 genes evaluated in MLST analysis).

Regarding the other STs detected, as expected from the frequent acquisition of unique clones by CF patients from environmental sources [3,36], 8 of the 13 MLST clones detected corresponded to nonpreviously described STs, each found in single patients (Table 1). Nevertheless, in addition to the above described clone FQSE-A/ST-274 CF epidemic strain, clonal replacement (of a MDR mutator strain) by the MDR LES-1 epidemic strain ST-146 [29,37] was documented in one of the patients, alerting of the first detection of the likely more world-wide concerning CF epidemic clone in Spain. Although the epidemiological driver of LES-1 colonization was not specifically investigated, the fact that the patient has family links with a northern European country could help to explain the acquisition of this clone not previously detected in Spain.

Evidence for Interpatient Spread of a Mutator Lineage (ST-1089) Evolved from a CF Epidemic Strain (ST-274)

Since ST-274 and ST-1089 show the same PFGE pattern and MLST was initially performed only in the first isolate per patient and clone, MLST analysis was extended to the last available isolate from each patient colonized by clone FQSE-A as well as the two additional sporadic mutator isolates detected in two of the patients colonized by this clone (Figure 1). In all cases, the MLST

Figure 1. Schematic representation of the 10 sequential isolates from each of the 10 CF patients in the time frame of the study period. Each different clone is represented by a different colour. Resistance profiles and presence mutator phenotypes are indicated for each isolate. Results of MLST analysis are also provided for specific isolates.
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Table 1. Allelic profiles and relevant features of the different ST detected.

Clone	Sequence type	Allelic profile[a]							Relevant features
		acsA	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*	
FQSE-A	ST-274	23	5	11	7	1	12	7	Detected in CF patients in Australia, Austria and France
FQSE-A	ST-1089	66	5	101	7	1	12	7	New Sequence Type, DLV of ST-274 (Mutator)
FQSE-B	ST-146	6	5	11	3	4	23	1	MDR Liverpool Epidemic Strain (LES-1)
FQSE-C/D	ST-299	17	5	36	3	3	7	3	Detected in CF patients in Australia
FQSE-E	ST-1108	6	3	17	7	3	4	7	New Sequence Type
FQSE-F	ST-1072	5	13	25	6	1	7	3	New Sequence Type
FQSE-G	ST-155	28	5	36	3	3	13	7	Detected in CF patients in Australia, Canada and France
FQSE-H	ST-1088	36	27	28	3	4	13	1	New Secuence Type
FQSE-I	ST-1109	16	14	3	11	1	15	1	New Sequence Type
FQSE-J	ST-1071	5	3	57	6	1	33	42	New Sqcuence Type
FQSE-K	ST-701	29	1	9	13	1	6	23	New Sequence Type
FQSE-L	ST-254	6	5	58	11	3	4	37	Detected in CF patients in Australia, Canada
FQSE-M	ST-1073	28	5	36	3	4	10	95	New Sequence Type (mutator)

[a]Nonpreviously described alleles are shown in bold.
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profiles coincided with that of the first isolate, except for the mutator lineage emerging from one of the patients colonized by ST-274 that was also identified as ST-1089 (Figure 1). Thus, the mutator lineage ST-1089 was detected from the first to the last isolate analyzed in one of the patients and only in the last isolate (the only one with mutator phenotype) from a second one. Nevertheless, extended analysis of available isolates of this patient from 2010 to 2012 confirmed the persistence of the ST-1089 mutator lineage. On the other hand, the sporadic mutator lineage detected in the third patient was still ST-274. Therefore, mutator lineages were detected in 3 of the 4 patients with clone FQSE-A, two belonging to ST-1089 and one to ST-274. In order to evaluate the genetic basis of hypermutation, complementation studies with plasmids harboring wild-type MMR genes (*mutS* and *mutL*) were performed in mutator isolates from these three patients. In all cases the isolates were found to be defective in *mutS*. Thus, *mutS* was sequenced from the three mutator isolates and representative nonmutator isolates. Surprisingly, the three mutator isolates had the same inactivating mutation in *mutS* (4 bp deletion from nt 814), obviously absent in the nonmutator isolates. This specific mutation has not been previously noted in dozens of mutator variants sequenced so far [13,14,16,38,39], and is not reasonable to believe that it might have emerged independently in three different occasions. Therefore, these results provide evidence for the first time of interpatient spread of mutators. Moreover, they demonstrate the interpatient spread of a mutator lineage (ST-1089) evolved from a CF epidemic strain (ST-274).

Interplay between Clonal Lineages, Mutator Phenotypes, Antimicrobial Susceptibility and Resistance Mechanisms

The overall susceptibility data for the collection of 100 isolates to 8 antipseudomonal agents is summarized in Table 2. Lowest susceptibility was observed for aztreonam (60% S) and highest for meropenem (96% S). However, resistance rates were highest for cefepime (30% R), tobramycin (30% R) and ciprofloxacin (24% R) and lowest for meropenem (1% R), aztreonam (4% R), and colistin (7% R). A significant trend towards increased MICs overtime to individual antibiotics was not noted, although all colistin resistant isolates occurred in the second half of the study (Figure 2). The low percentages of Aztreonam resistance might be of particular interest, considering its recent introduction for the treatment of CF chronic lung infection as inhaled therapy [40]. It should also be noted that EUCAST considers *P. aeruginosa* intrinsically nonsusceptible to this antibiotic (mainly due to the basal expression of MexAB-OprM efflux pump and pharmacokinetic/ pharmacodynamic issues) (http://www.eucast.org/ clinical_breakpoints/). Therefore, the percentage (60%) of susceptible isolates documented actually reflects the high number of hypersusceptible (MIC ranges 0.125–1 mg/L) isolates falling outside of wild-type MICs (2–16 mg/L) distributions (http:// www.eucast.org/mic_distributions/). In addition of aztreonam, an important number of isolates showed hypersusceptibility to meropenem with MICs (<0.06 mg/L) falling outside of wild-type distributions.

Resistance profiles (I+R) to the 8 antipseudomonal agents and mutator phenotypes for the 100 isolates are also indicated in Figure 1. Although the resistance profiles were variable within and across patients along the study period, a significant trend towards the accumulation of resistances was noted, increasing from an average of resistance to 1.1 ± 1.2 antibiotics in the first isolate of each patient to 2.5 ± 0.85 in the last isolate (paired t test, p = 0.016).Consistently with previous data [10,11,15], 29% of the isolates showed mutator phenotypes and 6 of the 10 patients showed at least 1 mutator isolate. In two patients all isolates were

mutators and in other two, mutators emerged at late stages of colonization. In one more patient a mutator lineage emerged but it was not fixed and in other one it was replaced by the LES-1 epidemic strain (Figure 1). As described above, mutator variants were detected in 3 of the 4 patients colonized by epidemic clon A (ST-274/ST-1089). Mutator variants have also been previously detected in a small proportion of isolates of the LES-1 epidemic strain, but interpatient spread was not previously evidenced [24,25]. In agreement with previous findings [10,11,19,20,41] a significant trend (p = 0.009) towards resistance to a higher number of antibiotics among mutator isolates (2.28 ± 0.22) than among nonmutator isolates (1.49 ± 0.17) was also noted, although not all mutator isolates were resistant and some nonmutator isolates, particularly noteworthy the LES-1 epidemic strain ST-146, were resistant to multiple antibiotics (Figure 1).

Resistance mechanisms [efflux pump (*mexB*, *mexD*, *mexF*, and *mexY*) and *ampC* overexpression and OprD production] were evaluated in the first and last isolate from each patient and clone and results are shown in Table 3. A trend towards accumulation of resistance mechanisms was noted, from 1.4 ± 0.58 in the first to 2.1 ± 0.88 in the last isolates, although the differences did not reach statistical significance (p = 0.06). The most frequent mechanism was *mexY* overexpression, documented in all 10 patients. Moreover, this mechanism was present in most patients (8 of 10) already in the early isolates (Table 3). The overexpression of the other efflux pumps was far more infrequent; *mexF* was documented in 3 patients, *mexD* in 2 and *mexB* only in one. AmpC overexpression was evidenced in 6 of the patients and lack of OprD production in the 4 patients colonized by imipenem resistant strains. Although a certain correlation was documented between *ampC* overexpression and ceftazidime resistance and lack of OprD and imipenem resistance, as previously observed [42,43], a correlation between phenotype and genotype was not always evident, particularly concerning efflux pumps overexpression. Previously described efflux unbalance in CF [44] and antagonistic interactions between certain resistance mechanisms [45] could explain these discrepancies. Particularly, the previously documented frequent inactivation of the constitutive MexAB efflux system could well be responsible of the frequently observed aztreonam and meropenem hypersusceptibily (Table 3, Figure 2). The clone associated with a higher number of resistance mechanisms was ST-146/LES-1 previously associated with MDR phenotypes [43]. The initial MDR isolate from this clone already expressed 4 resistance mechanisms (MexY, MexF, MexD and OprD). The last isolate also expressed 4 resistance mechanisms (MexY, MexD, MexF, and OprD), but showed a significant reduction of the MDR profile, likely influenced by the modification of the mechanisms expressed (MexD instead of AmpC). Although not particularly associated with a high number of resistance mechanisms, all early and late isolates from the epidemic clone FQSE-A (ST-274/ST-1089) overexpressed *mexY*. Thus, *mexZ* was sequenced in all strains that overexpressed *mexY*, in order to determine the underlying genetic mechanism of resistance and to use *mexZ* mutations as epidemiological marker. As expected [46] most of the strains overexpressing *mexY* showed *mexZ* mutations, including deletions, premature stop codons, insertion sequences (IS), or nonsynonymous substitutions (Table 3). Remarkably, clone FQSE-A (ST-274/ST-1089) isolates from the different patients showed different *mexZ* mutations, denoting that interpatient spread precedes resistance development, except for the ST-274/ST-1089 *mutS* deficient mutator lineages (Table 3). Indeed, the ST-274/ST-1089 *mutS* deficient isolates showed the same *mexZ* mutation, demonstrating that the interpatient spread of the mutator lineages occurred after the acquisition of the resistance mechanism.

Figure 2. Evolution of minimal inhibitory concentrations (MICs) from the first to the last studied isolate from each patient. Each color represents a different patient. CI, ciprofloxacin; TM, tobramycin; CO, colistin; TZ, ceftazidime; PM, cefepime; AT, aztreonam; IP, impenem; MP, meropenem.
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In summary, the presented results provide evidence for the interpatient spread of the CF epidemic strain ST-274. Much more importantly, they strongly suggest that ST-274 bacterial populations spreading among different patients were not a single genotype, but rather included a *mutS* deficient subpopulation that

had already evolved into the new mutator lineage ST-1089 and had acquired specific resistance mutations. In other words, presented results provide the first evidence of interpatient dissemination of mutator lineages and denote their potential for unexpected short-term sequence type evolution (leading to MLST vs PFGE discrepancies), illustrating the complexity of *P. aeruginosa* population biology in CF.

Materials and Methods

Ethics Statement

This study was approved by the Research Committee of Hospital Son Espases. All clinical isolates used were obtained from a preexisting collection recovered over years from routine cultures and the study does not include patient's data.

Clinical Isolates and Susceptibility Testing

The collection studied included 10 sequential *P. aeruginosa* isolates from each of 10 CF patients attended at hospital Son Espases, reference hospital of the Balearic Islands, Spain. Each of the sequential isolates included were separated by at least a 6-month interval, covering up to an 8-year period from 2003 to 2010. The first available isolate and the last available isolate (when the project was initiated) from each of the patients was always included in the studied collection. PAO1 strain was used as

Table 2. Antimicrobial susceptibility of the studied *P. aeruginosa* isolates.

Antibiotic	No. of isolates (n = 100)			No. of patients (n = 10)	
	S[a]	I[a]	R[a]	I+R[a]	R[a]
Ceftazidime	89	NA[b]	11	4	4
Cefepime	70	NA	30	8	8
Imipenem	79	5	16	7	4
Meropenem	96	3	1	3	1
Aztreonam	60	36	4	10	2
Ciprofloxacin	69	7	24	8	5
Tobramycin	70	NA	30	6	6
Colistin	93	NA	7	3	3

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Table 3. Antimicrobial susceptibility results and resistant mechanisms detected in first and last studied isolates from each patient and clone.

Patient-Isolate	PFGE Clone	ST	Mutator (Y/N)	MIC (mg/L)								Resistance mechanisms	mexZ mutations[a]
				TZ	PM	AT	IP	MP	CI	TO	CO		
FQSE06-0403	FQSE-A	274	N	0.064	1.5	0.125	1.5	0.016	0.125	4	0.38	mexY	S9P
FQSE06-1104	FQSE-A	274	Y	0.094	1	0.094	1	0.25	0.25	1	2	mexY	A194P
FQSE06-0610	FQSE-A	274	N	1.5	8	0.25	8	0.064	0.75	1.5	2	mexY, OprD-	Nt$_{292}$Δ11
FQSE10-0503	FQSE-A	274	N	0.38	2	4	1.5	0.25	0.094	0.5	0.5	mexY	IS [b]
FQSE10-0111	FQSE-A	274	N	3	12	4	0.25	0.125	0.75	2	0.75	ampC, mexY	IS
FQSE15-0803	FQSE-A	274	N	1	8	0.38	2	0.125	0.38	1.5	1.5	mexY	A144V
FQSE15-0110	FQSE-A	1089	Y	1.5	12	2	0.38	0.38	0.38	1	0.016	mexY	A194P
FQSE24-0304	FQSE-A	1089	Y	1	12	0.19	>32	2	8	3	1	mexY,OprD-	A194P
FQSE24-1010	FQSE-A	1089	Y	0.38	4	0.38	>32	>32	6	4	1	mexY,OprD-	A194P
FQSE12-0603	FQSE-C	299	N	0.75	8	0.125	1	0.064	0.25	24	0.75	mexY	R125P
FQSE12-1206	FQSE-C	299	N	8	8	8	2	0.5	2	24	4	ampC, mexY	R125P
FQSE12-1007	FQSE-B	146	N	24	24	12	>32	1.5	4	4	>256	ampC, mexY, mexF, OprD-	Q164X
FQSE12-1110	FQSE-B	146	N	1	32	0.38	0.25	0.19	16	1	0.38	mexY, mexF, mexD,OprD-	Q164X
FQSE05-0403	FQSE-E	1108	N	16	12	12	1.5	0.25	0.25	1.5	0.5	ampC	WT
FQSE05-0704	FQSE-D	299	N	2	>256	>256	3	0.75	3	1.5	0.75	ampC, mexY	ND
FQSE05-0305	FQSE-F	1072	N	1	2	0.75	0.5	0.047	0.25	0.75	2	mexY, mexF	W185X
FQSE05-0807	FQSE-G	155	N	0.75	0.75	3	1.5	0.19	0.047	0.5	1.5	–	ND
FQSE05-0111	FQSE-E	1108	N	12	16	24	1.5	0.094	0.25	1	1	ampC, mexY	V43G
FQSE21-1003	FQSE-H	1088	N	0.5	1.5	0.5	0.38	0.19	0.25	0.38	1	ampC, mexY	Nt$_{61}$Δ15
FQSE21-0505	FQSE-I	1109	N	16	16	8	3	0.5	2	16	2	mexB, mexY	K131R
FQSE21-0410	FQSE-H	1088	N	1.5	8	0.38	0.75	0.094	0.38	0.5	12	mexY	Nt$_{61}$Δ15
FQSE21-1110	FQSE-I	1109	Y	8	16	0.75	1.5	0.25	1.5	32	1.5	mexB, mexY	K131R
FQSE28-1006	FQSE-J	1071	N	1.5	12	6	4	0.094	0.125	3	2	ampC, mexY	Nt$_{189}$Δ12
FQSE28-1110	FQSE-J	1071	N	1	6	4	2	0.047	0.19	3	2	ampC, mexY	Nt$_{189}$Δ12
FQSE11-0603	FQSE-K	701	N	1	3	0.25	1.5	0.032	0.125	2	1.5	mexY	WT
FQSE11-0608	FQSE-L	254	N	1	8	0.125	1	0.023	0.38	2	1.5	mexY	Nt$_{279}$Δ12
FQSE11-1010	FQSE-K	701	N	2	8	0.25	8	2	0.75	>256	1	mexY, OprD-	WT
FQSE16-0803	FQSE-M	1073	Y	1.5	1.5	0.25	3	0.19	1.5	3	0.5	mexF, mexD	WT
FQSE16-0910	FQSE-M	1073	Y	3	6	2	0.125	0.75	0.25	12	2	ampC, mexY, mexF	R125P

[a]PA01 and PA14 were used as reference wild-types sequences (www.pseudomonas.com).
[b]1.2 Kb IS located in mexX-mexZ intergenic region (nt -72 respect mexZ coding sequence). Encodes a putative transposase identical to that previously reported in Pseudomonas pseudoalcaligenes CECT 5344 (ref ZP_10763279.1).
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reference. The antibiotic susceptibility profiles (ceftazidime, cefepime, aztreonam, imipenem, meropenem, ciprofloxacin, tobramycin and colistin) were determined by Etest, using EUCAST breakpoints (http://www.eucast.org/).

Molecular Typing

Clonal relatedness was evaluated in all isolates by PFGE. For this purpose, bacterial DNA embedded in agarose plugs prepared as described previously [47] was digested with SpeI. DNA separation was then performed in a contour-clamped homogeneous-electric-field DRIII apparatus (Bio-Rad, La Jolla, CA) under the following conditions: 6 V/cm^2 for 26 h with pulse times of 5 to 40 s. DNA macrorestriction patterns were interpreted according to the criteria established by Tenover et al. [48]. Representative isolates from each clone and patient were further analyzed by MLST using available protocols and databases (http://pubmlst.org/paeruginosa/).

Characterization of Resistance Mechanisms

The levels of expression of *ampC*, *mexB*, mexD, *mexF* and *mexY* were determined by Real-time reverse transcription (RT)-PCR according to previously described protocols [49,50]. Briefly, strains were grown in 10 ml of LB broth at 37°C and 180 rpm to the late log phase (optical density at 600 nm [OD$_{600}$] of 1) and collected by centrifugation. Total RNA was isolated by using the RNeasy minikit (Qiagen), dissolved in water, and treated with 2 U of Turbo DNase (Ambion) for 30 min at 37°C to remove contaminating DNA. The reaction was stopped by the addition of 5 µl of DNase inactivation reagent to the mixture. A 50-ng sample of purified RNA was then used for one-step reverse transcription and real-time PCR amplification using the Quanti Tect SYBR green RT-PCR kit (Qiagen) with a SmartCycler II instrument (Cepheid). Previously described primers [49,50] were used for the amplification of *ampC*, *mexB*, *mexD*, *mexF*, *mexY*, and *rpsL* (used as a reference to normalize the relative amount of mRNA). Strains were considered positive for *ampC*, *mexD*, *mexF* or *mexY* overexpression when the corresponding mRNA level was at least 10-fold higher than that of PAO1, negative if lower than 5-fold, and borderline if between 5- and 10-fold. Strains were considered positive for *mexB* overexpression when the corresponding mRNA level was at least 3-fold higher than that of PAO1, negative if lower than 2-fold and borderline if between 2- and 3-fold. All PCRs were performed in duplicate. Mean values (± standard deviations) of mRNA levels obtained in three independent duplicate experiments were considered. Previously characterized strains overexpressing these

mechanisms were used as controls [50]. Additionally, the gene encoding the transcriptional regulator of MexXY-OprM efflux pump, *mexZ*, was fully sequenced in representative isolates, from each patient and clone, showing *mexY* overexpression [32]. After duplicate PCR amplification, sequencing reactions were performed with the Big Dye Terminator kit (PE Applied Biosystems, Foster City, CA), and sequences were analyzed on an ABI Prism 3100 DNA sequencer (PE Applied Biosystems). The resulting sequences were then compared with that of wild-type PAO1 and those available at GenBank. Finally, outer membrane protein (OMP) profiles were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue following previously described protocols [45]. Obtained OprD profiles were compared with those of PAO1 and its OprD-deficient mutant.

Mutant Frequencies and Genetic Basis of Hypermutation

Rifampicin (300 mg/L) resistance mutant frequencies were determined in all strains following previously established procedures [10]. To explore the genetic basis for the mutator phenotypes, complementation studies were performed as described previously [15]. Briefly, plasmid pUCPMSharbouring PAO1 wild-type *mutS*, plasmid pUCPML harbouring PAO1 wild-type *mutL*, and plasmid pUCP24, a control cloning vector, were electroporated into the mutator isolates. Complementation was demonstrated by reversion of the increased rifampicin resistance mutant frequencies in two independent transformant colonies for each strain. Previously described primers [15] were used for the amplification and sequencing of *mutS* or *mutL* genes according to the results of complementation experiments.

Statistical Analysis

The Graph Pad Prism 5 software was used for graphical representation and statistical analysis. Quantitative variables were compared using the Mann-Whitney U-test or the Student's *t* test as appropriate. Categorical variables were compared using the χ^2 test. A *p* value of less than 0.05 was considered statistically significant.

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The problems of antibiotic resistance in cystic fibrosis and solutions

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Chronic respiratory infection is the main cause of morbidity and mortality in cystic fibrosis (CF) patients. One of the hallmarks of these infections, led by the opportunistic pathogen *Pseudomonas aeruginosa,* is their long-term (lifelong) persistence despite intensive antimicrobial therapy. Antimicrobial resistance in CF is indeed a multifactorial problem, which includes physiological changes, represented by the transition from the planktonic to the biofilm mode of growth and the acquisition of multiple (antibiotic resistance) adaptive mutations catalyzed by frequent mutator phenotypes. Emerging multidrug-resistant CF pathogens, transmissible epidemic strains and transferable genetic elements (such as those encoding class B carbapenemases) also significantly contribute to this concerning scenario. Strategies directed to combat biofilm growth, prevent the emergence of mutational resistance, promote the development of novel antimicrobial agents against multidrug-resistant strains and implement strict infection control measures are thus needed.

Cystic fibrosis (CF) is the most prevalent autosomal recessive hereditary disease affecting Caucasian populations. Approximately 70,000 people are affected worldwide, but the estimated incidence varies considerably from country to country, within countries and with ethnic background. White populations from Europe, Canada and the USA account for the highest estimated incidences, among whom the disease occurs in 1 in 2500–5000 newborns [1].

CF is caused by mutations disrupting the function of the CF transmembrane conductance regulator (*CFTR*) gene, which encodes a chloride channel that is expressed on the apical surface of many epithelial and blood cells. Since the discovery of the *CFTR* gene, over 1950 different variations have been identified. The most prevalent mutation worldwide is the three-base pair deletion F508del which accounts for approximately two-thirds of all *CFTR* mutations.

The clinical spectrum of CF disease is wide, and depends not only on the *CFTR* genotype but also on other genetic and environmental factors [2]. When CF disease was first recognized in 1938 by Dorothy Hansine Andersen, malnutrition was the leading cause of death among CF patients. The introduction of pancreatic enzyme replacement therapy prompted pulmonary insufficiency to be the first cause of CF morbidity and mortality. Nowadays, approximately 80% of CF-related deaths are associated with chronic lung infection [1].

The respiratory tract of CF children is apparently normal by the time of birth, but soon after, it becomes inflamed and infected. The mechanisms underlying the early acquisition of infection and the establishment of chronic respiratory infection (CRI) are complex. So, several hypotheses have been proposed over the years. Recently, it has been demonstrated that an impaired mucociliary transport is a primary defect in CF, which favors bacterial trapping and persistence in CF lungs [3].

Microorganisms infecting and/or colonizing the CF airway, as well as its frequency vary with CF patients' age. During the initial years, viral pathogens or species such as *Mycoplasma pneumoniae* or *Chlamydophila pneumoniae* are usually involved. Shortly

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	AXC	CTX	CAZ	TZP	IMP	MER	LVX	AMG	COL	SXT	MIN
P. aeruginosa	red	yellow	yellow	yellow	yellow	yellow	yellow	yellow	green	red	red
S. maltophilia	red	red	red	red	red	red	yellow	red	yellow	green	green
A. xylososidans	red	red	yellow	yellow	yellow	yellow	yellow	red	red	yellow	yellow
B. cepacia complex	red	red	red	red	red	red	red	red	red	red	red
Pandoraea spp	red	red	red	yellow	yellow	red	red	red	yellow	red	green
Ralstonia spp	red	red	red	yellow	yellow	red	yellow	yellow	red	red	red
Cupriavidus respiraculi	red	red	red	green	red	green	red	green	red	green	green
Inquilinus limosus	red	red	red	green	green	green	red	red	red	red	yellow

Figure 1. Antimicrobial susceptibility profiles of most frequent non-fermenting Gram-negative rods isolated from cystic fibrosis patients.
Color codes for susceptibility profiles: green: resistance not described or infrequent; yellow: frequently acquired resistance; red: intrinsic resistance or very frequently acquired resistance.
AMG: Aminoglycosides; AXC: Amoxicillin–clavulanic acid; CAZ: Ceftazidime; CTX: Cefotaxime; COL: Colistin; IMP: Imipenem; LVX: Levofloxacin; MER: Meropenem; MIN: Minocycline; SXT: Sulfamethoxazole–trimethoprim; TZP: Piperacillin–tazobactam.

after, common respiratory pediatric pathogens like *Haemophilus influenzae* or *Streptococcus pneumoniae* become predominant; but they are soon replaced by *Staphylococcus aureus* and then by *Pseudomonas aeruginosa*. Eventually, and mainly as a consequence of wide antibiotic use and pulmonary function decline, opportunistic pathogens such as *Achromobacter* spp., *Stenotrophomonas maltophilia*, *Burkholderia cepacia* complex (Bcc) or other non-fermenting Gram-negative rods (NFGR) may be isolated [4].

Multidrug-resistant pathogens in CF

Current management of CF respiratory tract infection includes wide use of antibiotics. Obviously, this strategy has reduced CF patients' morbidity and increased their life expectancy [4], but it has also led to the collateral damage by causing an increasing prevalence of multidrug-resistant (MDR) bacteria [5,6]. Methicillin-resistant *S. aureus* (MRSA), MDR *P. aeruginosa* and other intrinsically MDR NFGR are of particular concern (FIGURE 1).

S. aureus is the pathogen most frequently involved in early respiratory tract infection in CF pediatric patients and its incidence and prevalence has increased over the last years. In 2012, *S. aureus*, including MRSA isolates, was cultured from the respiratory tract samples of 69.0% of CF patients included in the US CF Foundation Patient Registry versus from 55.9% in 2002. Focusing on MRSA the increase is even more worrisome since in 2002 it was only isolated from 9.2% CF patients rising to 26.5% in 2012 [4]. Outside the US, MRSA prevalence rates are considerably lower ranging from 3 to 11% [7]. The mechanism of resistance to methicillin confers resistance to all β-lactams and it is frequently associated with acquired resistance to unrelated antibiotics, such as quinolones and aminoglycosides.

Short after in the course of CF respiratory tract infection, *P. aeruginosa* and other MDR NFGR became the predominant bacterial species infecting or colonizing CF lungs mainly in response to antibiotic pressure.

P. aeruginosa has been the leading cause of respiratory infection in CF patients for decades but it appears that this scenario may be changing and its prevalence may be decreasing. In the USA, the rate of *P. aeruginosa* infection decreased from 57.8% in 2002 to 49.6% in 2012. Nevertheless, MDR *P. aeruginosa* prevalence is increasing and about 10% isolates exhibit resistance to multiple antibiotics [4].

Early infection by *P. aeruginosa* can be intermittent and usually multiple strains with different antibiotic susceptibility profiles are involved. But, eventually, according to the US CF Foundation Patient Registry, by the age of 25, over 70% CF patients are chronically colonized with this pathogen [4] and a single well-adapted strain predominates. *P. aeruginosa* is intrinsically resistant to several antibiotics and has an enormous capability to develop further resistance. So, frequently, this single well-adapted strain exhibits MDR profiles. The estimation of the clinical impact of MDR *P. aeruginosa* is a subject of growing interest and controversy. Some studies reported a significant lung function decline associated with MDR profiles [8]. On the other hand, a recent large multicenter study suggested that MDR is a marker of more severe disease and more intensive antibiotic therapy, but not a primary driver of FEV_1 decline [9]. However, the very loose definition used in this study hinders the estimation of the impact of truly MDR profiles.

Other MDR NFGR frequently isolated from CF lungs include *Achromobacter* spp., Bcc, *Cupriavidus* species, *Inquilinus limosus*, *Pandoraea* species, *S. maltophilia* and *Ralstonia* species, among others (FIGURE 1) [6]. The isolation rate of these innately MDR bacteria from the CF respiratory tract is increasing, mainly due to the extensive use of antipseudomonal antibiotics.

Physiological resistance in CF chronic lung infection: role of biofilm
Biofilm growth in CF

Biofilms are defined as organized bacterial communities surrounded by an extracellular polymeric matrix. These structures confer resistance against mechanic clearance, the immune system and antibiotics. In fact, the switch from planktonic to biofilm mode of growth is currently recognized as one of the most relevant drivers of chronic infections, thus playing an important

role in CF [10]. Furthermore, the intrinsic properties of biofilms have significant diagnostic and therapeutic consequences [11].

The architecture of biofilms is complex and is highly influenced by the availability of nutrients and oxygen. The biofilm formation involves three stages: attachment, maturation and dispersal [12]. The biofilm development starts with the adherence of planktonic bacteria to a surface with the help of pili and flagella in Gram-negative bacteria [12] or surface proteins in Gram-positive bacteria [13]. Although most biofilm-related infections generally require an attachment to a solid surface, in the case of CF, some studies indicate that the biofilm found in the lung is directly formed on the mucus instead of being in contact with the lung epithelium [14,15].

Attachment is followed by multiplication of bacteria, thus forming microcolonies and the production of the extracellular polymeric matrix. This matrix plays an important role in the biofilm development not only as a protective barrier against host defense, antibiotics, desiccation or reactive oxygen species (ROS), but also by giving cohesion to the structure and acting as a nutrient source [16]. This physiological barrier is composed of a conglomerate of exopolysaccharides, extracellular DNA (eDNA) proteins, surfactants, lipids, bacterial lytic products and host compounds. In *P. aeruginosa*, one of the most extensively studied exopolysaccharides is the alginate, a polymer of uronic and guluronate, due to its importance in the CF lung. Alginate overproduction is a feature of mucoid strains, a phenotype highly adapted and prevalent in chronic infections. Despite initially considered as a residual material from lysed bacterial and host defense cells [16], currently eDNA has been postulated as an integral part of the matrix [17]. Supporting this theory, it has been observed that DNAse acts by dissolving immature biofilms as well as blocking its initial formation [17].

Finally, some bacteria are released from the biofilm matrix in a dispersal stage. Nonsessile bacteria can thus colonize new return to planktonic phase may responds to biological cues like nutrient limitation and growth rate. Such a tangled process is regulated by intra- and extracellular cues that modulate the levels of diffusible signal molecules, second messengers and small RNAs [18]. Quorum sensing (QS) detects these signals as cell density evidence and triggers changes in bacterial gene transcription, including virulence factors and diverse proteins involved in the innate resistance of biofilms to antibiotics and the immune system. *P. aeruginosa* biofilms are able to initiate detachment on their own; this process can be mediated by alginate lyase overexpression [19] or by the up-regulation of motility factors such as the rhamnolipid and type IV pili [20].

It should be noted that these important insights into the biofilm knowledge have been revealed by *in vitro* models, so it cannot be totally extrapolated to the chronic biofilm infection in CF. The most obvious weakness of *in vitro* models is the absence of the involvement of the immune system. A complex interaction between pathogens and host defense mechanisms determines the altered microenvironment and structure of the *in vivo* biofilm. For instance, NO produced by polymorphonuclear cells (PMNs) leads to oxygen depletion and promotes

Figure 2. Schematic representation of the factors contributing to inherent biofilm antimicrobial resistance.
ATB: Antibiotics.

growth and persistence driven by denitrification in biofilms [21]. Besides this, *in vivo* biofilm aggregates seem to be smaller and no mushroom structure has been observed on them [22].

Inherent antimicrobial tolerance of biofilms
One of the most relevant aspects of biofilms is that they determine the persistence of the infection despite long-term antimicrobial treatment. Indeed, it is estimated that biofilms can tolerate up to 100–1000 fold higher concentrations of antibiotics than the planktonic cells [11]. Multiple factors contribute to this inherent biofilm antimicrobial resistance (FIGURE 2).

Antibiotic penetration
The biofilm matrix acts as a primary barrier preventing the entrance of polar and charged antibiotics [23]. Some components of the matrix such as alginate and eDNA show antibiotic chelating activity [24]. Moreover, eDNA also behaves as an antimicrobial shield and contributes to aminoglycoside tolerance [25,26].

Growth rate & nutrient gradients
Internal gradients of biofilms give rise to anaerobic and nutrient-deficient areas, leading to slowing down of the metabolism. The lack of oxygen and the reduced rates of multiplication contribute to the tolerance to fluoroquinolones and aminoglycosides [27]. Moreover, the mucus layers in CF lung are mostly anaerobic, so obligate anaerobes and other pathogens can grow. *P. aeruginosa* can grow in anaerobic or microaerophilic conditions where NO_3^- from PMNs is the final electron acceptor with a lower energetic cost compared to aerobic conditions. Anaerobic biofilms developed in this environment tend to increase alginate production leading to

aminoglycoside tolerance. Furthermore, osmotic stress response may contribute to antibiotic resistance inducing a change in the proportions of porins [10].

Persister phenomenon

Persisters are defined as a dormant phenotypic state of bacteria within biofilms, characterized by a high tolerance to antibiotics. Also, this latent bacterial state behaves as a bumper to host defense and may cause a relapse of infection, being a source of recalcitrant biofilm infection [28].

Induction of resistance mechanisms

Induction of resistance mechanism can significantly differ between biofilm and planktonic growth [29]. Various studies have found a differential expression of several conventional and biofilm-resistance genes in biofilms [30].

Biofilms & mutational resistance

The antibiotic gradient driven by biofilm physiology favors gradual development of mutational resistance during antimicrobial treatment, which is of significance particularly when involving mutator strains which are highly prevalent in CRI [31–33]. Also, endogenous oxidative stress [34] and mutagenic ROS released from PMNs are likely to induce mutability in biofilm cells. In fact, recent findings have shown that mutagenesis is intrinsically increased in biofilms [34,35].

Horizontal gene transfer

Bacterial proximity within a biofilm allows an effective horizontal gene transfer [36]. Moreover, bacterial eDNA may represent a reservoir for the acquisition of exogenous resistance determinants.

Mutational resistance in CF chronic lung infection: role of mutators

Mutational antimicrobial resistance mechanisms in CF pathogens

In addition to intrinsic antibiotic resistance, the antimicrobial susceptibility of bacterial populations can be significantly further compromised by the acquisition of certain chromosomal mutations. This is particularly relevant for the major CF pathogen *P. aeruginosa*, given its extraordinary ability to acquire resistance through mutations that alter the expression and/or function of its chromosomally encoded resistance mechanisms. Although no single mutation can lead to MDR profiles, all antibiotics are prone to being compromised by acquiring mutations that eventually lead to overexpression of efflux pumps, hyperproduction of the chromosomal AmpC cephalosporinase, porin loss or altered antibiotic targets. Table 1 summarizes the most relevant genes involved and the corresponding resistance profiles generated. In addition to these classical resistance mutations, recent whole genome screening mutant libraries reveal a plethora of genes, collectively known as the resistome, which have an impact on antimicrobial susceptibility, including many with central metabolic functions [37].

While it has been shown that mutational resistance can virtually affect all antibiotics, the spontaneous mutation frequencies vary according to the antibiotic agent, the bacterial species and the specific environmental conditions [38]. Moreover, mutational resistance can be significantly enhanced by the presence of mutator phenotypes, which are highly prevalent in CF [39].

Hypermutation and antibiotic resistance in CF

Hypermutable (or mutator) microorganisms are defined as those that have an increased spontaneous mutation rate due to defects in DNA repair or error avoidance systems.

The most frequent cause of hypermutation in natural bacterial populations is the presence of defects on the methyl-directed mismatch repair system. *mutS*, *mutL* and *uvrD* (*mutU*) are the key genes of the methyl-directed mismatch repair system and their inactivation leads to a stable mutator phenotype with an increased rate of mutation from 100- to 1000-fold [40]. Mutations in MutM, MutY and MutT, the three key proteins that compose the GO system, as well as mutations of genes involved in the prevention of oxidative damage produced by ROS, such as *oxyR* and *sodA* (*mutA* and *mutC*) [40] or the recently described *pfpI* [41], also lead to this phenotype.

Under particular circumstances, a transient mutator phenotype can also arise. For instance, bacterial DNA damage induces the SOS response and its error-prone DNA polymerases promote an elevated mutation rate [42] Moreover, some antibiotics can induce a transient mutator phenotype through this mechanism, thus promoting the development of antimicrobial resistance [43–45].

In natural bacterial populations, the presence of the mutator phenotype involves an evolutionary advantage as it can not only enhance mutational resistance but also facilitate bacterial adaptation to new or stressful environments. CRI with *P. aeruginosa* in CF patients represents a major example in nature. Prevalence of mutator *P. aeruginosa* in the CF airways is extremely high, approximately 10–30% of isolates [46], and its presence has been strongly associated with adaptive mechanisms [47] and development of antibiotic resistance [31–33]. The proportion of hypermutable isolates significantly increases during the course of *P. aeruginosa* CRI, as was demonstrated in a 25-year longitudinal study in which the proportion of hypermutable isolates increased from 0% at the onset/early colonization to 65% [48]. Genetic hitch-hiking can explain this observation, which means that mutator alleles reach high frequency by being co-selected with linked beneficial mutations.

Fortunately, CF epidemic strains have not shown an increased prevalence of mutators [49]. Nevertheless, transmission of *P. aeruginosa* hypermutable strains between CF patients has been recently demonstrated [50].

A higher prevalence of mutators in the CF setting has also been noticed for other microorganisms such as *S. pneumoniae*, *H. influenzae*, *S. aureus*, *S. maltophilia* and Bcc [51–56]. Prunier *et al.* found that approximately 14% of CF *S. aureus* isolates were hypermutable in contrast with 1% in non-CF isolates, and that hypermutability was strongly associated with

Table 1. Mutational resistance mechanisms in *Pseudomonas aeruginosa*.

Mutation	Resistance mechanism/altered target	Antibiotics affected							
		TZP	CAZ	CEF	IMP	MER	FQ	AMG	COL
gyrA, gyrB	DNA gyrase						•		
parC, parE	DNA topoisomerase IV						•		
pmrAB	Lipopolysaccharide (lipid A)								•
phoPQ	Lipopolysaccharide (lipid A)								•
parRS	Lipopolysaccharide (lipid A) MexXY-OprM hyperproduction OprD porin downregulation			•	•	•	•	•	•
mexR (nalB)	MexAB-OprM hyperproduction	•	•	•		•	•		
nalC	MexAB-OprM hyperproduction	•	•	•		•	•		
nalD	MexAB-OprM hyperproduction	•	•	•		•	•		
nfxB	MexCD-OprJ hyperproduction			•			•		
mexT	MexEF-OprN hyperproduction OprD porin downregulation				•	•	•		
mexS (nfxC)	MexEF-OprN hyperproduction OprD porin downregulation				•	•	•		
mvaT	MexEF-OprN hyperproduction						•		
mexZ	MexXY-OprM hyperproduction			•			•	•	
PA5471	MexXY-OprM hyperproduction			•			•	•	
ampD	AmpC hyperproduction	•	•	•					
ampD homologues	AmpC hyperproduction	•	•	•					
ampR	AmpC hyperproduction	•	•	•					
dacB	AmpC hyperproduction	•	•	•					
oprD	OprD porin inactivation				•	•			

The bullets indicates which antibiotics are affected by each resistance mechanisms.
AMG: Aminoglycosides; CAZ: Ceftazidime; CEF: Cefepime; COL: Colistin; FQ: Fluoroquinolones; IMP: Imipenem; MER: Meropenem; TZP: Piperacillin–tazobactam.

antibiotic resistance. Similar results were obtained by Román *et al.* for *H. influenzae*. For the emerging CF pathogen *S. maltophilia*, Turrientes *et al.* documented a higher rate of strong mutators in CF patients (17%) versus non-CF patients (3%) and Vidigal *et al.* reported that 31% of CF *S. maltophilia* were strong mutators. Finally, the prevalence of mutators for Bcc isolates has been recently found to be highest among chronically infected CF patients, reaching 40.7% [56].

As evidenced by the unusual high proportion of mutators encountered in chronically colonized CF patients, the CF airway is an ideal environment for mutagenesis. ROS level is increased in CF patients mainly due to an increase in the availability or iron in the CF airways and because the antioxidant mechanisms in CF patients are highly diminished. ROS cause DNA damage and can further increase the inflammatory responses, which eventually lead to the establishment of a vicious cycle of inflammation and hypermutation [57]. The biofilm mode of growth may also increase mutability, as several studies have pointed out [34,35].

Since the first description of hypermutable *P. aeruginosa* strains was made in CF CRI, a strong linkage between mutators and antibiotic resistance has been noticed, as mutators were found to be much more frequently resistant than non-mutators to each of the antipseudomonal agents tested [31]. For instance, the percentage of ceftazidime resistance reached 80% in hypermutable strains in contrast to the 30% observed for non-hypermutable strains and the percentage of fluoroquinolone resistance increased from 5% in non-mutators to 40% in mutators. More recent studies confirmed and extended this observation, establishing a clear link between mutator phenotypes and MDR profiles [33,48,58].

As for *P. aeruginosa*, a strong correlation between hypermutation and mutation-mediated antibiotic resistance has also been observed for *S. aureus* and *H. influenzae* in the CF setting. Prunier *et al.* noted that a high proportion (53%) of the *S. aureus* isolates from CF patients was resistant to erythromycin, and found that more than half of the resistant strains did not contain any acquired macrolide resistance gene but rather

Figure 3. Worldwide distribution of epidemic and transmissible *Pseudomonas aeruginosa* cystic fibrosis strains.
AES: Australian epidemic strain; LES: Liverpool epidemic strain; PES: Prairie epidemic strain.

cepacia from CF centers evidenced that the transmissible strains not only were those of *P. aeruginosa* [67].

Due to the rapid increase of *Burkholderia cenocepacia* infection (formerly Bcc genomovar III) among CF patients and its poor prognosis, attention was focused on this pathogen. An intercontinental epidemic lineage, the ET-12, was soon identified infecting patients from the UK and Ontario in Canada [68]. Since then, numerous transmissible *B. cenocepacia* lineages have been reported worldwide, and evidence of superinfection with epidemic strains in previously colonized patients has been provided [69]. The global epidemiology of *B. cenocepacia* is now better understood thanks to the introduction of Multi Locus Sequence Typing. Analysis of isolates from the epidemic lineage ET-12 has revealed that they belong to at least five different sequence types (STs), with only ST-28 representing the intercontinental ET-12 clone spread in the UK and Canada [70].

P. aeruginosa epidemic and transmissible strains have been recently reviewed by Fothergill *et al.* [71]. The Liverpool Epidemic Strain was first described affecting a unique CF center, but some time later, this strain was detected in other CF centers across the UK and, eventually, it has also been detected to infect CF patients in Canada and Spain [50]. The Liverpool Epidemic Strain isolates develop antibiotic resistance more frequently than the other CF strains, and resistance is more likely to develop over time. A worse prognosis is predicted for patients colonized with the Liverpool Epidemic Strain, and superinfection of patients already colonized with *P. aeruginosa* strains has also been reported; thus, strict patient segregation policies are to be implemented. In Australia, some transmissible strains have also been detected; these are the so-called Australian epidemic strain-1, Australian epidemic strain-2 and a cluster of related strains. Australian epidemic strain-1 exhibits increased antibiotic resistance and increased virulence gene expression during chronic infection. These and other *P. aeruginosa* MDR transmissible strains have been reported worldwide [72,73] are represented in Figure 3.

To date, few studies have investigated the genetic background and transmissibility of MRSA strains in the CF population. In 2006, a heterogeneous glycopeptide-intermediate phenotype of resistance infecting a long cohort of CF patients was detected in France, which alerts that transmissible strains could also exist among MRSA CF strains [74]. Shortly after, a Spanish study demonstrated the presence of a predominant clone among their CF patients, the hospital-acquired MRSA ST228, suggesting either cross-transmission or a common environmental source. This clone, also prevalent among the circulating clones of MRSA in Spain, exhibited MDR, presented

contained mutations in *rrl* (23S rRNA), *rplD* (L4 protein) or *rplV* (L22 protein); indeed, this high prevalence of mutational macrolide resistance was found to be associated with a high prevalence of hypermutable strains [51]. Recently, it has also been published that linezolid-resistant *S. aureus* can emerge through the accumulation of 23S rRNA mutations linked to the acquisition of a mutator phenotype [59]. Similarly, Román *et al.* found a strong correlation between the high prevalence of hypermutable *H. influenzae* strains in CF patients and the high rates of mutational antibiotic resistance [52].

In addition to the clear statistical link obtained between hypermutation and antibiotic resistance from the analysis of collections of clinical CF isolates, several *in vitro* (planktonic and biofilm) and *in vivo* experiments further highlight the strong linkage between hypermutation and antibiotic resistance [60–65].

Transmissible resistance in CF: role of MDR epidemic strains
MDR epidemic strains in CF
For a long time, it was extensively accepted that acquisition of CF pathogens occurs from the environment, so each patient generally harbors his/her own unrelated strain. Although evidence of *P. aeruginosa* cross-infection among CF siblings existed, before the mid-1980s, there was no proof of spread of epidemic strains. But this classical perception changed in 1986 when an outbreak of *P. aeruginosa* resistant to aminoglycosides, carbenicillin, ureidopenicillins, ceftazidime, cefsulodin and imipenem in a CF center in Denmark was published [66]. Shortly after, ribotype analysis of *B.*

SCCmec type 1 and was highly persistent [75]. Nevertheless, an increasing prevalence of community-acquired MRSA among CF patients, including panton valentine leukocydin-positive strains, has been observed [76–78]. Recently, a multicenter Italian survey showed a high prevalence (31.4%) of SCCmecIV. Most of these community-acquired MRSA strains (73%) belonged to known epidemic lineages Globally spread being the ST8-MRSA-IV (genetic signature of the American lineages USA500 and USA300) the most frequent [79].

Transferable resistance determinants in CF isolates

The CF airway hosts a complex microbiome [80] where genetic exchange could occur effectively, thus contributing to the emergence of antibiotic resistance. Most mobile antibiotic resistance genes are encoded on plasmids and transposons, but recent studies suggest that phages may also play an important role in the CF airway environment as the CF virome encodes more antimicrobial resistance sequences than the non-CF virome [81]. Phages also appear to be essential for the adaptation of some successful *S. aureus*, *B. cenocepacia* and *P. aeruginosa* CF strains, which supports this idea [74,82,83].

Among the transferable resistance determinants, extended-spectrum β-lactamases and carbapenemeses are widely distributed worldwide. Although this resistance mechanism seems not to be frequent among CF isolates, several reports have been published recently. In 2006, VEB-1 producing *Achromobacter xylosoxidans* was detected in a CF patient in France [84] and previously, the isolation of three non-characterized extended-spectrum β-lactamases–positive *P. aeruginosa* from CF patients in New Delhi had been reported [85]. Transferable carbapenemases have also been detected among CF isolates, including *P. aeruginosa* producing IMP and VIM metallo-β-lactamases [86,87] and *K. pneumoniae* producing KPC-2 carbapenemase [88].

Current & future antimicrobial therapy strategies to combat resistance

As discussed in previous sections, antibiotic resistance due to the increasing prevalence of MDR pathogens and also due to the physiological, mutational and transmissible mechanisms represents one of the major causes of therapeutic failure in CF patients. Depending on the respiratory infection stage and the microorganisms involved, different therapeutic strategies are chosen. Prophylaxis is still controversial and early eradication is generally attempted with aggressive treatment at the first culture of *P. aeruginosa*, *S. aureus* and MRSA, with the objective of preventing CRI [89].

Classical systemic & inhaled therapy in CF

During CRI, CF patients experience a progressive decline of lung function correlating with strain mucoid conversion and phenotypic diversification, biofilm formation and resistance development. In this stage, the bacterial mass increases which leads to periodic flare-ups of respiratory symptoms known as pulmonary exacerbations. Frequently, exacerbations are associated with a poorer quality of life and an increased mortality. CRI eradication is rarely achieved, especially in *P. aeruginosa* chronically colonized patients, and therefore, the aim of antimicrobial treatment is the reduction of the bacterial load and, thus, the inflammatory response. Success in bacterial reduction is microbiologically defined as a decrease of, at least, two logarithms in bacterial counts on comparing two consecutive cultures.

Traditionally, intravenous antibiotics are used to treat exacerbations. The classical strategy consists of combining two agents from different antimicrobial classes to enhance the treatment effect and prevent the emergence of antimicrobial resistance. For instance, for *P. aeruginosa* treatment, a combination of an aminoglycoside or a fluoroquinolone and an antipseudomonal β-lactam at high doses is typically used. Colistin sulphomethate has also shown efficacy on intravenous administration, alone or in combination [90]; however, it is generally reserved for MDR strains or in cases of therapeutic failure. On the other hand, inhaled therapies are the treatment of choice in suppressive or maintenance therapy during CF CRI, in the absence of exacerbations. Administration of antibiotics by inhalation has demonstrated to be safe and effective due to the high concentrations that reach the infection site (pulmonary epithelia) with a very low systemic effect. Since eradication cannot be achieved, these strategies are based on chronic suppressive therapy (colistin) or administered as a 28-day course (on–off) (tobramycin or aztreonam-lysine [AZLI]). Nebulized administration of sodium colistimethate has demonstrated efficacy for the treatment of *P. aeruginosa* CRI, with the normal dose in adults being 0.5–2 millions of international units, two- or three-times a day, administered without off periods. In the case of tobramycin, the recommended dosage by inhalation is 300 mg twice a day, alternating 4 weeks on–off cycles. Several clinical studies have shown that AZLI is a safe and effective treatment for use in CF patients and recommend the use of an on–off 28-day course of AZLI (75 mg, three-times daily) [89].

Pharmacokinetic & pharmacodynamic approach to antimicrobial therapy in CF

Dosing regimens are based on the changes in the concentration of the antibiotic during the course of treatment (pharmacokinetics [PK]) and on the *in vitro* relationship between antibiotic concentration and the growth or death rate of the targeted bacteria (pharmacodynamics [PD]). These factors comprise the PK/PD indices [91], which are used to estimate the potential efficacy of antibiotic treatment regimens.

Aminoglycoside and fluoroquinolone antibiotics exhibit a concentration-dependent activity. The PK/PD parameters that better predict their activity are Cmax/MIC or area under the curve (AUC_{24h})/MIC. These antibiotics are used at high doses and their prolonged post-antibiotic effect, which is defined as the time that the bacteria need to recover their normal growth, allows using them at long dosage intervals. A Cmax/MIC of ≥10–12 for aminoglycosides predicts their efficacy. In the case of fluoroquinolones, an AUC_{24h}/MIC value >125 is thought to

predict therapeutic success and values above 157 have shown to suppress resistance according to mathematical models [92].

Activity of β-lactam antibiotics is time-dependent; thus, to achieve an optimal therapeutic effect, concentrations should be over MIC for a long period of time. The PK/PD parameter T > MIC is required to be at least 40–50% of the dosage interval of administration; this condition is reached with intravenous perfusion for some antibiotics (piperacillin/tazobactam, ceftazidime, cefepime, meropenem or doripenem) or by inhalation three-times a day in the case of aztreonam.

These PK/PD parameters are based on the general susceptible bacterial population. Regarding the selection of resistant mutants, the concentrations of antibiotic that would prevent the selection of single-step resistant mutants are represented by a parameter known as mutant prevention concentration (MPC), which is defined as the MIC of the least susceptible single-step mutant. Actually, the antimicrobial concentration range extending from the MIC of the general population and the MPC is known as the mutant selection window. Antimicrobial concentrations placed inside this window are expected to select the resistant mutant subpopulations, whereas concentrations above this window are expected to restrict selective enrichment.

In this sense, it would be interesting to consider the MPC/MIC index to define the low or high capacity of antibiotics to select resistant mutants. Similarly, as efficacy should refer not only to obtain an optimal clinical response but also to minimize the selection of resistant subpopulations, PK/PD traditional parameters commented above should be also adapted, for example, using AUC_{24h}/MPC for fluoroquinolones or Cmax/MPC for aminoglycosides instead of AUC_{24h}/MIC or Cmax/MIC, respectively.

Current treatment strategies tend to take into account the knowledge of PK/PD. Administration of antibiotics through inhalation minimizes systemic toxicity while reaching high concentrations in the lung epithelia, generally above the MPC values. For instance, in the case of tobramycin, serum concentration after inhalation is under 1 mg/l, whereas it reaches 1200 mg/l in the sputum. Moreover, administration twice a day helps to take advantage of its post-antibiotic effect. On the contrary, inhaled β-lactams, such as AZLI, need to be administered in concentrations over the MIC for long time intervals (T > MIC ≥40–50% dosage interval), with administration of three-times daily being more favorable. Inhaled formulations have also expanded on fluoroquinolones, such as ciprofloxacin and levofloxacin, which are currently in Phase II and III clinical trials, respectively.

Similarly, the on–off 28-day course is based on reaching high concentrations that reduce the bacterial load for a long term (on) and then let susceptible subpopulations grow (off) at the expense of the resistant mutant subpopulations, without selective pressure. Nevertheless, as evidences show that the beneficial effects diminish during off periods, other strategies, such as combination or alternation of inhaled antibiotics, are now being explored.

Combined & sequential treatments

Combinations of antibiotics are routinely used in the treatment of CF pulmonary infection with the aim of preventing or delaying the onset of resistance. Multiple combination bactericidal testing has been shown to help to choose combinations of antimicrobials with higher levels of in vitro bactericidal activity, especially in P. aeruginosa [93] and Bcc [94]. The impact of multiple combination bactericidal testing on clinical outcome remains, however, controversial and further prospective multicenter studies are required [95,96]. Nevertheless, based on in vitro and in vivo studies, efficient combinations have been identified [97,98]. An interesting combination of inhaled formulations of a 4:1 (w/w) of fosfomycin/tobramycin was recently under research. This combination has shown to be effective in vitro against both Gram-negative and Gram-positive pathogens and has also shown increased activity under anaerobic conditions [99].

Another approach to prevent or delay the onset of resistance may be the use of sequential treatments, for instance, those based on antagonistic resistance mechanisms. Treatment with aminoglycosides often involves the selection of mutants that overexpress the MexXY-OprM efflux pump being frequently related to the inactivation of MexAB-OprM. Taking into account this premise, theoretically, treatment with MexXY-OprM substrates (such as tobramycin) could lead to hypersusceptibility to MexAB-OprM substrates (such as aztreonam). So, sequential treatment with tobramycin followed by aztreonam would entail a clinical benefit by improving the therapeutic efficacy and diminishing the selection of resistant mutants. This was the objective of a recent work [Rojo-Molinero E, Macià MD, Oliver A, unpublished data] where sequential therapies with inhaled antibiotics were found to be superior to individual treatments. Results from this study could support the introduction of sequential regimens with inhaled antibiotics in CF patients' therapy. Furthermore, a double-blind, placebo-controlled multicenter study that used fosfomycin/tobramycin and AZLI suggested that continuous alternating therapy of different inhaled antibiotic therapies could be of benefit in CF patients; unfortunately, this study was lately suspended. Nevertheless, further investigation based on clinical trials is required.

Antimutator strategies

As addressed in the previous sections, hypermutable strains are highly prevalent in CF CRI. Mutation-mediated resistant mechanisms can affect almost all kinds of antipseudomonal antibiotics, including β-lactams, fluoroquinolones and aminoglycosides. However, colistin (as a representative of polymyxins) might apparently be an exception to the strong linkage of mutators with antibiotic resistance in CRI [100], and it is therefore frequently used as last-resource option for the treatment of infections by MDR strains. Similarly, the Phase III antipseudomonal cephalosporin ceftolozane (former CXA-101) is apparently stable to most mutation-driven P. aeruginosa β-lactam resistance mechanisms either in planktonic or biofilm growth [64]. Likewise, in vitro and in vivo studies have shown

that it is possible to suppress resistance due to selection of resistant mutant subpopulations by using appropriate combined regimens [60,61,101].

In addition to methyl-directed mismatch repair–deficient strains, mutator lineages generated by inactivation of the GO system also trigger development of antibiotic resistance, particularly when exposed to conditions promoting oxidative DNA damage as occurs in the CF lungs [102]. Oxidative stress has an important role in the increased mutability of biofilm-growing bacteria, thus contributing to bacterial diversification and development of antibiotic resistance. The addition of antioxidants such as L-proline, N-acetylcysteine, β-carotene and L-cysteine has been shown to decrease the resistance of 5-day-old P. aeruginosa biofilms to tobramycin in vitro [35]. N-acetylcysteine has long been used in patients with CF as a mucolytic agent and also as an anti-inflammatory drug. Although clinical trials are required to evaluate its usage as an enhancer of antibiotic activity on biofilms, combination of N-acetylcysteine and some antibiotics seems promising.

Old & new therapeutic options directed to treat MDR pathogens

The challenge of MDR has driven to a revival of forgotten antimicrobial agents such as fosfomycin and colistin. Fosfomycin seems to be an effective antibiotic that is used intravenously and in combination with other antibiotics to treat resistant bacteria, including MDR P. aeruginosa in CF patients [103]. This antibiotic has a broad-spectrum bactericidal effect by inhibition of the initial step in cell wall synthesis, and also reaches good concentration levels in lungs. Moreover, fosfomycin may have an added benefit, conferring protection against nephrotoxicity [104] and ototoxicity [105]. Colistin shows excellent in vitro activity against Gram-negative bacteria and in vivo efficacy against MDR carbapenemase-producing microorganisms. The disadvantage of colistin is its toxicity, which is reduced by administering colistimethate sodium via inhalation. In spite of the extensive experience with inhaled colistimethate sodium in CF, more studies are needed to explore the effect in combination with intravenous antibiotics to treat multidrug resistance therapy. In conclusion, these old drugs have significant advantages including a low rate of resistance, good activity both in vitro and in vivo against MDR pathogens, known toxicity and lower cost compared to new agents.

Despite the efforts taken to overcome MDR with the available drugs, there is an imperative need for discovering new antimicrobial agents. Presently, there are some antimicrobials active against resistant Gram-negative bacteria in advanced stage of clinical development (Phase II or III). Most of these new agents are β-lactam/β-lactamase inhibitor combination products that act by inhibiting the β-lactamases so that the partner antibiotic can interfere with cell wall synthesis. One of them, ceftolozane/tazobactam, currently in Phase III, has demonstrated an excellent activity against P. aeruginosa. In addition, the development of high-level resistance to ceftolozane/tazobactam is much slower compared to other antibiotics, and appears to occur efficiently only in mutator background [106]. BAL30072 is a

siderophore monosulfactam with an impressive activity against P. aeruginosa and B. cepacia and has shown a powerful synergistic activity in combination with meropenem [107]. Further, some of these new drugs active against resistant Gram-negative rods have also shown in vitro potency versus MRSA, for example, ceftaroline/avibactam and eravacycline, a broad-spectrum fluorocycline. Nevertheless, these achievements will only partial solve the antimicrobial resistance threat, and new drugs with novel mechanisms of action are still needed.

Targeting biofilms

Targeting biofilm arises as an attractive alternative approach to classic therapy since remarkable differences in antimicrobial response have been demonstrated between planktonic and biofilm modes of growth.

Biofilm-guided antibiotic therapy

Antimicrobial susceptibility testing studies performed on biofilm growing bacteria have helped to identify antibiotics that show selective anti-biofilm activity [108]. Such is the case of macrolides against P. aeruginosa. Although according to the standard antimicrobial susceptibility testing, azithromycin has no activity against P. aeruginosa, this macrolide exhibits bactericidal activity on biofilms [63]. Azithromycin inhibits biofilm growth likely due to its interaction with the QS system implicated in the production of alginate and other virulence factors such as rhamnolipid, elastase, protease and chitinase [109]. Despite this good activity on biofilms, resistant mutants are readily selected, particularly for hypermutable strains. The resistance mechanism selected, the overexpression of MexCD-OprJ, also confers resistance to ciprofloxacin or cefepime and, on the contrary, turns the strains hypersusceptible to aminoglycosides. Then, it is important to optimize the selection of appropriate antipseudomonal therapies in patients undergoing azithromycin maintenance treatment. Finally, macrolides have demonstrated synergy with other antibiotics against multidrug-resistant CF pathogens such as B. cepacia, A. xylosoxidans and S. maltophilia [110].

Some studies applying PK/PD models that theoretically predict therapeutic success have been developed using in vitro and in vivo models to mimic the CRI setting and biofilm development. For example, a P. aeruginosa flow cell biofilm model that employed a concentration of 2 g/ml of ciprofloxacin, which correlated with the MPC and provided an AUC_{24h}/MIC ratio of 384 that should predict therapeutic success, was used, which demonstrated, nevertheless, that theoretically optimized PK/PD parameters failed to suppress resistance development on biofilms [65]. The results from this study suggested that the increased antibiotic tolerance driven by the special biofilm physiology and architecture probably raised the effective MPC, favoring gradual mutational resistance development, especially in mutator strains. Actually, the exposure of biofilm-grown cells to sub-inhibitory concentrations of antibiotics may not only fail to eradicate the biofilm but may even promote or enhance biofilm formation. Likewise, results of other PK/PD models of P. aeruginosa biofilm treatment studies showed an alteration of

Figure 4. Schematic representation of the different anti-biofilm strategies proposed.
eDNA: Extracellular DNA.

expected PK/PD antibiotic parameters when acting on biofilms. In the study of Hengzhuang *et al.*, β-lactam antibiotics showed time-dependent killing and ciprofloxacin, colistin and tobramycin showed concentration- or dose-dependent killing being then similar to planktonic growth [111]. However, the concentrations of antibiotics needed were, in all cases, very much higher even in the case of time-dependent killing, where on β-lactamase–overproducing strains, the killing pattern of ceftazidime was changed to concentration-dependent killing for biofilm cells. These results alert us of the complexity of mechanisms taking place on *P. aeruginosa* CRI and the difficulty to predict therapeutic efficacy even applying optimal PK/PD parameters.

Anti-biofilm strategies: new alternatives to classic antimicrobial therapy

In recent years, significant efforts have been made trying to elucidate new therapeutic approaches. Some of the different strategies against biofilms are presented below and in FIGURE 4.

Avoiding biofilm formation

The inhibition of molecules involved in the attachment process seems to be a good approach for this objective. This could be achieved by using specific neutralizing antibodies against flagella, pili, eDNA and exopolysaccharides.

Avoiding biofilm maturation

At this point, the strategies should be directed to weakening of the formed biofilm, mainly targeting the virulence factors, eDNA, QS, small RNAs and iron metabolism. The problem is that during the maturation phase, biofilm loses most of the virulence factors and no drugs have indicated activity in this stage [24].

DNAse

The inhaled recombinant human DNase has shown clinical efficacy in CF patients. This enzyme reduces the viscosity of mucus not only by clearing the DNA released from PMNs but also by dissolving preformed biofilms [17] and facilitating the effects of aminoglycosides [27].

Alginate lyase

The co-administration of inhaled alginate lyase with antibiotics degrades alginate from the extracellular polymeric matrix leading to the elimination of mucoid bacteria from the biofilms [112].

Bacteriophages

Bacteriophage therapy is becoming an attractive co-adjuvant of antibiotics. Phages are able to break through the extracellular

polymeric matrix and reach bacteria within the biofilm, starting a lytic process inside them. The problem is that bacteriophages are species-specific and make the isolation of bacteria necessary to select the suitable phage. Also, bacteria can develop resistance to the lytic activity of phages [113]. Regardless of this, some studies using bacteriophages against *P. aeruginosa* have demonstrated efficacy [114,115].

Iron metabolism
Since iron availability shows up as a critical factor in biofilm formation, chelating agents may be a good option to eliminate the biofilm. The strategy is to take advantage of the iron transport system to introduce inactive metal ions (Sc^{3+}, In^{3+} or Ga^{3+}) or antibiotics conjugated with a siderophore. Several compounds have been shown to interfere with biofilm formation *in vitro*, but most of them have only been effective on abiotic surfaces. Gallium nitrate formulated for inhalation has shown effectiveness in chronic airway infection in animal models [116], although gallium-resistant *P. aeruginosa* mutants have been reported [117]. Also, lactoferrin, a human iron-binding protein, seems to impair biofilm formation in respiratory infections by stimulation of twitching motility [118].

Virulence factors
The development of antibodies against β-lactamase observed in CF [119] supports the development of specific antibodies or substances that bind virulence factors as a good strategy to fight biofilm maturation. The disadvantage of this approach is that only single virulence factors are targeted and they are species- or even strain-specific. Also, there is a risk of inducing immunopathology as a result of increased inflammation owing to an immune complex–mediated reaction.

Quorum sensing inhibitors
Since QS plays a major role in biofilm formation and regulation of the expression of virulence factors, there is an emerging interest in the research of new molecules able to block the QS pathway. The advantage of quorum sensing inhibitors (QSIs) over antibiotics is that the development of resistance is minimized since these molecules target the virulence factor instead of bacterial growth [120]. However, the theoretical benefits of these molecules should be considered with caution since resistance to QSIs has been recently reported in literature [121,122]. Effective QSIs can be found in nature among the secondary metabolites produced by algae, sponges, fungi, food products and higher plants [120]. For example, natural halogenated furanones, solenopsin A, manoalide and its derivate, garlic, patulin and gingseng have shown activity against pathogens implicated in biofilm chronic infections. Nevertheless, activity of these compounds has only been assessed in experimental studies; hence, further clinical studies are needed to establish their efficacy and safety to truly introduce them in the treatment of CF patients.

Driving biofilm dispersal
When the biofilm is mature, some bacteria are released from the biofilm matrix, probably due to the lack of nutrients. Planktonic floating bacteria are more susceptible to antibiotics; taking advantage of this, an adequate antimicrobial treatment with the compounds that promote biofilm disruption could achieve a higher therapeutic success. Studies have investigated the effect of different molecules in the dispersal of biofilms and have obtained promising results. For example, unsaturated fatty acids [123], nitric oxide [124], succinic acid, citrate and compounds that interfere with c-di-GMP levels [125] have shown activity *in vitro*. Curiously, 2-aminoimidazole/triazole may resensitize MDR strains to the effects of conventional antibiotics, apart from its ability to inhibit and disperse biofilms [126].

Infection control in CF
The incorporation of appropriate control measures is one of the most effective available strategies to prevent early infection and transmission of epidemic strains between patients. In the recent past, the use of molecular typing methods has led to an improved understanding of the epidemiology of CF pathogens, highlighting the relevance of epidemic strains among CF patients, described above. In most cases, the initial source of microorganism acquisition is unknown, although the environment seems to be an important reservoir for CF pathogens.

In order to reduce the days of hospitalization and also to improve the quality of life of CF patients, there has been a shift in healthcare delivery from hospitals to ambulatory and home settings. Even though specific measures should be implemented in this new scenario, the lessons learned about transmission of nosocomial pathogens can be applied as infection control strategies for CF.

The American CF Foundation in its last update of infection prevention and control guidelines for CF [127] sets recommendations to prevent transmission, taking into account that all CF patients should be treated as potential transmitters of pathogens.

The most important preventive measure is hand hygiene in healthcare and non-healthcare settings. To prevent droplet transmission, all CF patients, regardless of the respiratory tract culture results, should be separated by at least a distance of 3–6 feet, which has been proposed as the minimal distance to avoid transmission. While the use of mask, gloves and gown is only required for staff in the case of colonization by MDR or epidemic transmissible strains, people with CF should be required to wear a mask in hospitals to reduce the risk of transmission, or even to reduce the acquisition of new pathogens. In particular, CF clinics should schedule and manage patients to avoid contact, minimizing the waiting time in common areas and segregating the patients with MDR or epidemic transmissible strains. In the same way, summer camps and other group activities are not encouraged.

Surfaces and respiratory therapy equipments have to be assiduously cleaned and disinfected to reduce contamination of environmental sources (following institutional policies of control of MDR pathogens). To reduce transmission by medical equipment, the use of single-patient disposable items should be

facilitated. Moreover, it is recommended to clean and disinfect the exam rooms between patients.

Surveillance reports are a valuable tool to document the incidence and prevalence of MDR pathogens, and to review the acquisition of epidemic transmissible strains. The molecular typing of CF pathogens, integrated as a routine in the microbiological diagnosis of respiratory infection, can be used as a marker of success of infection control strategies.

Expert commentary & five-year view

Antimicrobial resistance in CF is a multifactorial problem which includes aspects related to bacterial physiology (development of biofilms), genetic evolution (acquisition of antibiotic resistance mutations linked to mutator phenotypes) and epidemiology (such as the acquisition of infections by MDR pathogens or epidemic strains). Therefore, our present and future strategies should target these key aspects. Among them, the initiatives directed to prevent and/or disrupt biofilms, exemplified by research on QSIs, are particularly encouraging. Likewise, strategies intended to avoid mutational resistance, such as the

optimization of PK/PD parameters, innovative combined and sequential regimens, or the use of 'antimutator' adjuvants, are promising as well. Finally, despite being insufficient, novel compounds currently under clinical development will mitigate our needs for the treatment of MDR strains to some extent, but strict infection control measures will remain a key issue.

Key issues

- The isolation of multidrug resistant (MDR) pathogens from the cystic fibrosis (CF) respiratory tract is increasing, mainly due to the extensive use of antibiotics.
- Biofilm growth is an efficient adaptive strategy for survival and persistence of bacteria in the CF lungs due to its inherent tolerance to the immune system and antibiotics.
- Mutators are highly prevalent in CF chronic respiratory infection and play a major role in resistance development.
- Epidemic and transmissible *Burkholderia cepacia*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* strains have been identified infecting CF patients and frequently show MDR profiles.
- The CF airway hosts a complex microbiome in which genetic exchange can occur contributing to development of resistance.
- Treatments should be based on pharmacokinetic/pharmacodynamic parameters and on the pathogen resistance mechanisms; combined and sequential inhaled antibiotic treatments seem to be a promising alternative.
- Targeting the biofilms and the antimutator strategies arise as innovative approaches to overcome the current lack of effective antimicrobial treatments.
- Strict infection control measures are required to prevent the inter-patient transmission of MDR and epidemic strains.

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SCIENTIFIC REP**O**RTS

Evolution of the *Pseudomonas aeruginosa* mutational resistome in an international Cystic Fibrosis clone

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Emergence of epidemic clones and antibiotic resistance development compromises the management of *Pseudomonas aeruginosa* cystic fibrosis (CF) chronic respiratory infections. Whole genome sequencing (WGS) was used to decipher the phylogeny, interpatient dissemination, WGS mutator genotypes (mutome) and resistome of a widespread clone (CC274), in isolates from two highly-distant countries, Australia and Spain, covering an 18-year period. The coexistence of two divergent CC274 clonal lineages was revealed, but without evident geographical barrier; phylogenetic reconstructions and mutational resistome demonstrated the interpatient transmission of mutators. The extraordinary capacity of *P. aeruginosa* to develop resistance was evidenced by the emergence of mutations in >100 genes related to antibiotic resistance during the evolution of CC274, catalyzed by mutator phenotypes. While the presence of classical mutational resistance mechanisms was confirmed and correlated with resistance phenotypes, results also showed a major role of unexpected mutations. Among them, PBP3 mutations, shaping up β-lactam resistance, were noteworthy. A high selective pressure for *mexZ* mutations was evidenced, but we showed for the first time that high-level aminoglycoside resistance in CF is likely driven by mutations in *fusA1/fusA2*, coding for elongation factor G. Altogether, our results provide valuable information for understanding the evolution of the mutational resistome of CF *P. aeruginosa*.

Pseudomonas aeruginosa chronic respiratory infection (CRI) is the main driver of morbidity and mortality in patients suffering from cystic fibrosis (CF). The CF respiratory tract is a dynamic, heterogeneous, hostile, stressful and very challenging scenario for invading bacteria, but *P. aeruginosa* populations can overcome all these challenges and chronically persist in the CF lungs. Mechanisms underlying early acquisition of *P. aeruginosa* infection and the eventual establishment of CRI are complex and, many factors, related to the patient, the environment and the microorganism, are involved[1–3].

The high versatility and adaptability observed for *P. aeruginosa* can be attributed to its complex and large genome (5–7 Mb), which includes an outstanding intrinsic antibiotic resistance machinery and a large proportion of regulatory genes (>8%). In comparison to other Gram-negative pathogens, *P. aeruginosa* exhibits a basal reduced susceptibility to many antibiotics, attributed to the production of an inducible AmpC cephalosporinase, the constitutive (MexAB-OprM) or inducible (MexXY) expression of efflux pumps, and the reduced permeability of its outer membrane. In addition, *P. aeruginosa* intrinsic resistance can be significantly enhanced by the acquisition of multiple mutations that alter the expression and/or function of diverse chromosomal genes[4–6].

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Figure 1. CC274 *P. aeruginosa* collection. Sampling time from the 29 studied isolates can be inferred from the X axis. Isolates are labelled according to the following format: Patient identification - Country (AUS: Australia; SPA: Spain), Region.

Early infection by *P. aeruginosa* in CF patients can be intermittent and usually different strains with distinct antibiotic susceptibility profiles are involved. However, according to the US CF Foundation Patient Registry, over 70% of CF patients are chronically colonized with *P. aeruginosa* by the age of 25 (Annual Report 2015); moreover, in up to 20% of them the involved strain is multidrug resistant (MDR). Although no single mutation can lead to an MDR profile, during CF CRI, *P. aeruginosa* is exposed to numerous and extended antimicrobial therapies which act as selective forces driving to the acquisition of a plethora of adaptive mutations that eventually lead to an enhanced antimicrobial resistance pattern. Moreover, this genetic adaptation is accelerated by the characteristic high prevalence of hypermutable strains (30–60%)[7–10]. Additionally, there is growing evidence suggesting that adaptation to the CF lungs may escape from the scale of the individual patients. Indeed, another remarkable and challenging issue in the CF setting is the existence of concerning *P. aeruginosa* epidemic strains, such as the Liverpool Epidemic Strain (LES-1), the Denmark Epidemic Strain (DK2) or the Australian Epidemic Strains (AES-1, AES-2 and AES-3), as these successful strains are able of infecting hundreds of CF patients even in different geographical locations and, indeed, in many cases exhibiting a MDR profile[11–13]. Therefore, CF CRI by widespread strains may provide a unique and exceptional opportunity to get insight into long-term evolutionary dynamics of *P. aeruginosa* mutational resistome.

Recent advances in sequencing technologies have made it possible to obtain the whole genome of bacterial pathogens. As mentioned above, *P. aeruginosa* CF CRI represent a unique chance to perform evolutionary studies and, accordingly, several works have been performed in this setting; however, most have focused their attention in pathoadaptive mutations[14]. Moreover, *P. aeruginosa* chronic infections are not limited to CF patients, being also frequently implicated in other chronic underlying diseases such as bronchiectasis and chronic obstructive pulmonary disease (COPD)[15]. Thus, an insight into the resistome evolution during CF CRI could be of great benefit not only for individual patients but also for developing new drugs and new treatment strategies.

In a previous study we detected the presence of a transmissible and persistent *P. aeruginosa* lineage chronically infecting up to 4 of 10 selected chronically infected CF patients attended at the reference hospital of the Balearic Islands, Spain[16]. These isolates belonged to the ST274 clonal complex (CC274), which according to the MLST database, appears to colonize CF patients worldwide (http://pubmlst.org/paeruginosa/). In this work, the whole genome of a collection of CC274 strains was obtained in order to characterize the phylogeny, and the mutational resistome evolution of this widespread clonal complex; the CC274 collection included 29 representative isolates recovered from different regions of two highly distant countries, Australia and Spain, covering an 18-year period (1995–2012) and including sequential isolates from several patients (Fig. 1).

Results and Discussion

Prevalence and genetic basis for hypermutation: CC274 mutome.
Among the CC274 studied collection, nine isolates (31%) were mutators, belonging to six (35%) different patients, residing in both Australia (n = 3) and Spain (n = 3). Data from sequential isolates were available for the Spanish isolates: one was chronically infected with a persistent mutator lineage (FQSE24), whereas the other two harbored a mixed population of mutator and non-mutator isolates (FQSE06 and FQSE15) (Fig. 1).

Isolate ID[a]	ST	Mutator?	Complement with	Sequence variation in mutator genes (mutome)[b]									
				ung	*mfd*	*mutS*	*sodB*	*mutT*	*sodM*	*mutL*	*mutM*	*oxyR*	*polA*
AUS034	274	Yes	*mutL*					E236D		R631C	D61N L132P		D876E
AUS410	274	No	—				E25V						D876E
AUS411	274	No	—					E236D			D61N		D876E
AUS531	274	No	—					E236D			D61N		D876E
AUS588	274	No	—				E25V						D876E
AUS601	1043	Yes	*mutL*	S13R			E25V			P159S H288Y		F106L H219Y	D876E
AUS603	274	No	—				E25V						D876E
AUS690	274	Yes	*mutS*		Q1123H	C224R T287P		E236D			D61N		D876E
FQRC10	274	No	—					E236D			D61N		D876E
FQRC15	274	No	—					E236D			D61N		D876E
FQRC26	274	No	—					E236D			D61N		D876E
FQSE03	274	No	—			L374V		E236D			D61N		D876E
FQSE06-0403	274	No	—					E236D			D61N		D876E
FQSE06-1104	274	Yes	*mutS*			Nt814Δ4		E236D			D61N		D876E
FQSE06-0807	274	No	—					E236D			D61N		D876E
FQSE06-0610	274	No	—					E236D			D61N		D876E
FQSE10-0503	274	No	—					E236D			D61N		D876E
FQSE10-0106	274	No	—					E236D			D61N		D876E
FQSE10-0110	274	No	—					E236D			D61N		D876E
FQSE10-0111	274	No	—					E236D			D61N		D876E
FQSE15-0803	274	No	—					E236D			D61N		D876E
FQSE15-0906	274	No	—					E236D			D61N		D876E
FQSE15-0310	274	No	—					E236D			D61N		D876E
FQSE15-1110	1089	Yes	*mutS*		A868T	Nt814Δ4		E236D			D61N		D876E
FQSE24-0304	1089	Yes	*mutS*			Nt814Δ4		E236D			D61N		D876E
FQSE24-1005	1089	Yes	*mutS*			Nt814Δ4		E236D			D61N		D876E
FQSE24-0308	1089	Yes	*mutS*			Nt814Δ4		E236D			D61N		D876E
FQSE24-1010	1089	Yes	*mutS*			Nt814Δ4		E236D			D61N		D876E
PAMB148	274	No	—					E236D	L202R		D61N		D876E

Table 1. Mutator phenotype and genetic basis of hypermutation in CC274. [a]Isolates are labelled according to the following format: Patient identification - MMYY isolation code in the case of sequential isolates. [b]Sequence variations respect to those of PAO1. No mutations were found in other genes associated with mutator phenotypes, including *pfpI*, *mutY*, *dnaQ*, *PA2583*, *PA2819.1*, *PA2819.2*, *radA* and *uvrD*.

In order to evaluate the genetic basis of hypermutation, complementation studies with plasmids harboring wild-type Mismatch Repair system (MMR) genes (*mutS* and *mutL*) were performed in mutator isolates from these six patients. As shown in Table 1, wild-type rifampicin resistance mutation frequencies were restored in all mutator isolates upon *mutS* or *mutL* complementation, which correlated in all cases with the presence of specific mutations in these genes, documented through whole-genome sequencing. The three Australian mutator isolates showed unique mutations in either *mutL* and *mutS*. Interestingly, all mutator isolates from the three Spanish patients were found to share the same inactivating mutation in *mutS*. On the other hand, while mutator phenotypes could be explained in all cases by specific mutations in MMR genes, the contrary was not always true, since one of the non-mutator isolates showed a missense mutation in *mutS*. Moreover, the presence of polymorphisms in other mutator genes was frequent, but showed no association with mutator phenotypes (Table 1). Overall, the prevalence and genetic basis of hypermutation in CC274 was similar to that previously documented for non-clonal CF populations[9, 10]; this study is however, to our knowledge, the first investigating the genetic basis of hypermutation from whole genome sequence data, through the analysis of the sequence of an exhaustive panel of so called mutator genes, thus designated mutome.

Phylogeny and interpatient dissemination of the international CC274 CF clone. Pulsed Field Gel Electrophoresis (PFGE) macrorestriction patterns indicated that all isolates were clonally related, including mutators, which were indistinguishable from non-mutators. When an UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram was constructed based on PFGE patterns, all isolates from the Balearic Islands clustered together in the same branch, although patterns from one of the patients (FQSE10) were slightly different. In contrast, Australian isolates were less clonal and clustered in different branches (Supplementary Fig. S1).

Conversely, by Multi Locus Sequence Typing (MLST), two new and closely ST274-related sequence types (ST) were detected. Discrepant MLST and PFGE results were linked, directly or indirectly, to the emergence of a mutator phenotype, an event that has already been documented in the CF context[16–18]. Mutators from patients FQSE15 and FQSE24 differed from ST274 by only two point mutations in two of the MLST alleles (*acsA* and *guaA*) leading

Figure 2. Core-genome phylogenetic reconstructions of *P. aeruginosa* CC274 CF clone. (**a**) Genetic relationship between CC274 and other well-recognized CF epidemic clones. (**b**) Genetic relationship between the CC274 collection isolates. Both reconstructions were made with Parsnp using default parameters. Isolates are labelled according to the following format: Patient identification - MMYY isolation code in the case of sequential isolates - Country (AUS: Australia; SPA: Spain) - Region. Mutator isolates are identified with an asterisk.

to ST1089, as previously described[16]. Nevertheless, the mutator from patient FQSE06, which indeed shared the same inactivating mutation in *mutS*, still belonged to ST274 (Table 1). On the other hand, the Australian mutator AUS601 was also determined to be a new ST (ST1043), but, in this case, a direct link of the observed PFGE-MLST discrepancy with its MMR system (MutL) deficiency was suggested, since this isolate showed two missense mutations in *mutL* (Table 1), one of them (H288Y) responsible for the generation of the new ST.

To better understand the evolutionary trajectory, success and international dissemination of CC274, whole-genome based phylogenetic analysis of all 29 isolates were performed. Previous studies have already demonstrated that almost all *P. aeruginosa* strains cluster into two major phylogenetic groups, one including PAO1 and the other PA14[19]. In order to determine the genetic relationship between CC274 isolates and other well-recognized CF epidemic clones, whole-genome sequence reads of all 29 isolates were *de novo* assembled and a phylogenetic tree based on core genome alignment was constructed with default parameters on Parsnp[20]. CC274 was determined to belong to the phylogenetic cluster containing strain PAO1, as well as other well-known CF epidemic clones such as LESB58, AES-1 and DK2 (Fig. 2a).

By mapping sequence reads for each isolate against *P. aeruginosa* reference PAO1 strain genome, up to 16,070 common SNPs were found, as well as a total of 5,525 high-quality intraclonal SNPs, of which 2,294 were unique and thus detected in single isolates. A high degree of intraclonal diversity was observed, with SNP differences between isolates ranging from 20 to 3,256. To elucidate the phylogenetic relationship among isolates two different approaches were used. In both, core-genome and Bayesian time-based analysis, CC274 isolates grouped into two clusters, one including just four Australian isolates and a second major cluster that included all other Australian and Spanish isolates (Fig. 2b and Fig. 3). SNP differences between isolates from the different clusters ranged from 2396 to 3256 and, according to Bayesian time-based analysis, the common ancestor of CC274 was set, approximately, 380 years ago.

The major cluster further subdivided and, although both phylogenetic reconstructions did not match exactly with each other, both analyses supported that different lineages are currently coexisting with a worldwide distribution, having evolved from a common antecessor set approximately 275 years ago. SNP differences between isolates from Australia and Spain ranged from 114 to 1204, and similar results were obtained when only the Australian (min-max: 230–826) or the Spanish (min-max: 20–839) were compared, supporting no geographical barrier for lineage evolution.

Within the major cluster, all sequential isolates cultured from an individual patient clustered under the same branch with the single exception of all the Spanish isolates that exhibited a mutator phenotype which clustered together, independently of the patient involved and their ST. Along with the fact that all these mutators shared the same inactivating mutation in *mutS*, as well as many unique antibiotic resistance mutations (Supplementary Data Set S1), phylogenetic analysis clearly demonstrated that ST1089 mutators evolved from a mutator ST274 isolate and that transmission of mutators among the Spanish CF patients occurred at some time point.

Focusing on the sequential isolates, a unidirectional evolution route could not be stablished. Instead, a diversified intrapatient clone evolution that leads to a mix of genetically different sublineages coexisting in the CF respiratory airways was observed. Within a patient, minimum and maximum SNPs differences between isolates ranged from 20 to 676, which overlapped with interpatient SNPs differences, ranging from 51 to 3256 (51 to 839 for patients from the same hospital). Similar results have been reported recently by Williams *et al.* concerning the

Figure 3. Bayesian phylogenetic reconstruction of *P. aeruginosa* CC274 CF clone. The tree was based on 5525 intraclonal variable positions identified by whole-genome sequencing. Divergence times of predicted ancestors and sampling dates can be inferred from the X axis taking into account that time zero corresponds to the most recent isolate (2012). The same labelling of Fig. 2 was used. Isolates characteristics are summarized at the right board, where: (CF) Cystic Fibrosis CRI and (B) Bloodstream. Sequential *P. aeruginosa* isolated from a same patient are indicated with the same colour.

Liverpool Epidemic Strain, finding that multiple coexisting LES lineages are typically infecting CF patients and that genetic divergence between lineages within patients was greater than interpatient diversity, implying acquisition of diverse genetic populations[21]. However, another study focusing on the LES isolated from patients residing the UK and Canada showed less genetic differences, even when transoceanic isolates were compared[22]. Likewise, Yang *et al.* documented a lower genetic divergence in the DK2 epidemic clone[23]. Moreover, previous studies with other relevant and/or persistent CF clones have also reported divergent results[24–26]. A possible explanation for all these observations could be that different routes for adaptation and survival in the CF lung environment are possible and depend on the specific clonal lineages.

CC274 resistome.   Minimum inhibitory concentrations (MICs) determined for a panel of 11 antipseudomonal agents are shown in Table 2. Resistance rates were lowest for colistin (3.4%), distantly followed by ceftazidime and piperacillin-tazobactam (13.8%). In contrast, resistance to cefepime, aztreonam, imipenem, amikacin and ciprofloxacin was observed in 44.8 to 62% of the isolates. Remarkably, 17.2% of the isolates were resistant to the new combination ceftolozane-tazobactam. As shown, antibiotic resistance was more frequent among mutators, and in Australian isolates in comparison with those from Spain. In fact, all 9 mutator isolates were classified as MDR, as compared to only 3 of 20 non-mutators. Moreover, one of the Australian mutator isolates met the pan-drug resistant (PDR) definition[27].

The presence of horizontally acquired resistance determinants was explored in the whole-genome sequences using the ResFinder tool[28]. None of the 29 isolates harbored any horizontally acquired genes encoding resistance determinants, thus indicating that the observed antibiotic resistance profiles reflected the accumulation of mutations within the chromosomal genes. The complete list of antibiotic resistance related genes investigated (n = 164) as well as all missense and non-sense mutations encountered for each of the isolates studied are reported in the Supplementary Data Set S1. Up to 127 (77.4%) of the 164 studied genes showed non-synonymous mutations in at least one of the isolates studied. Moreover, after discarding non-synonymous mutations present in all isolates (and thus considered intrinsic CC274 polymorphisms), this figure only decreased to 106 (64.6%). Figure 4 shows the number and distribution of mutations among the 164 antibiotic resistance related genes studied in the CC274 collection. Seventy-three (68.9%) of these genes showed no more than two different mutational events being 44 of them mutated in unique isolates. In contrast, 33 (31.1%) genes appeared to be under high evolutionary pressure showing evidence of at least 3 different mutational events. Particularly noteworthy among them were *mexB* or *mexY*, (coding for efflux pumps proteins), *mexZ* (the main MexXY repressor), *gyrA* (which codes for DNA gyrase subunit A) and *fusA1* (coding for the elongation factor G).

The main antibiotic resistance related mutations documented are listed in Table 2 along with the susceptibility profiles for each of the isolates. For this purpose, the full list of mutations in the 164 genes studied (available

Isolate ID[a]	Antibiotic resistance profile (MIC values)[b]											Hyperexpression?			Main antibiotic resistance mutations encountered[c]
	TZ (≤8)	PM (≤8)	AT (≤1)	PPT (≤16)	C/T (≤4)	IP (≤4)	MP (≤2)	TO (≤4)	AK (≤8)	CI (≤0.5)	CO (≤2)	AmpC	MexAB	MexXY	
AUS034*	>256	>256	>256	>256	16	>32	>32	6	>256	1.5	>256	+	−	+	*gyrB* (R441L), *mexR* (R85H), *mexA* (M1*), *mexB* (F178S, M555I), *oprD*(E264*), *phoQ*(E266*), *parR* (M59I), *mexY*(V1000L), *mexZ*(Nt334Δ13), *fusA2*(P329L), PA2489(R12L, A244T), *mexS* (P254Q), *mexT*(L157M), PBP4(W350R), *capD* (I7M, S51G), *gyrA*(T83I), *mexK* (S426G), *mpl* (Nt112ins1, V124G), *fusA1* (V93A, P554L, D588G), *rpoB* (D831G, D964G), *mexW* (A627V, Q771P), PBP3 (P527T, G63S)
AUS410	4	24	1	12	4	>32	>32	64	>256	1	0.38	−	−	+	*gyrB* (S466F), *mexB*(M552T), *oprD* (Nt583Δ1), *lasR* (A50V, D73G), *sucC* (V44G, A384V), *oprF* (Nt574Δ31), *mexY* (V32A), *mexZ* (Q164*), *mexT* (D327Y), *mexE* (F7Y), *mpl* (D168Y), PA2489(A125T, G185S, P260S), *capD*(I7M, S51G), *fusA1*(P618L), *rpoC*(E386K), *mexW*(Q511R), PBP3(G216S), *pagL*(Nt286Δ1), *amgS*(S64L)
AUS411	>256	>256	>256	>256	6	>32	>32	>256	>256	0.38	0.25	−	−	+	*gyrB* (S466F), *mexB* (Q104E, F246C, L376V), *phoQ* (H248P), *lasR* (D73G), *parS* (D381E, T163N), *sucC* (C261G), *mexY* (D201A, G287A), PA2489 (R12L, A244T), *fusA2* (I640L), *mexE* (V104G), *htpX* (Nt683Δ5), *mexK* (S426G), *capD* (I7M), *fusA1* (K504E), *rpoC* (N690S), *mexW* (A627V,Q771P), PBP3 (Q372P), *pagL* (N159D)
AUS531	3	3	4	12	1	2	0.75	1	6	0.125	1	−	−	−	PA2489 (R12L, A244T), *capD* (I7M, S51G), *mexW* (A627V, Q771P)
AUS588	2	8	3	8	1	1	0.75	1	8	0.125	0.75	−	−	−	PA2489 (A125T, G185S, P260S), *mexE* (F7Y, V276M), *capD* (I7M), *mexW* (Q511R)
AUS601*	>256	>256	>256	1	3	>32	>32	24	>256	16	0.25	−	−	+	*mexB* (M552T), *oprD* (Nt1044ins4), *phoQ* (K234N, T315A), *lasR* (A50V), *sucC* (T102I, A384V), *mexY* (V32A), *mexZ* (Q164*), *fusA2* (S445*), *mexT*(D327Y), *mexE*(F7Y), *ftsK* (A152V), PA2489 (A125T, G185S, P260S), *capD* (S51G), *gyrA* (T83I), *mpl* (G113D), *fusA1* (P618L), *rpoC* (E386K), *mexW* (Q511R), PBP3 (R504C), *pagL* (E163G), *pmrB* (L31P), *amgR* (E204D)
AUS603	6	8	24	2	1.5	>32	8	1	8	0.25	1.5	+	−	+	*mexB* (M552T), *lasR* (A50V, D73G), *sucC* (V44G, A384V), *mexY* (V32A), *mexZ* (Q164*), *mexT* (D327Y), *mexE* (F7Y), PA2489 (A125T, G185S, P260S), PBP4 (S315G), *opmE* (E204D), *capD* (I7M, Nt1438Δ1), *mpl* (Nt112ins1, Nt1317Δ1), *fusA1* (P618L), *mexW* (Q511R)
AUS690*	6	12	0.75	3	6	4	2	24	>256	12	0.125	−	+	+	*gyrB* (Q467R), *mexR* (H133P), *mexB* (Nt712Δ1), *phoP* (T221I), *lasR* (T178I), *parS* (L10P), *oprF* (K250R), *mexY* (G402S, A850T), *mexZ* (Nt529Δ1), PA2489 (R12L, A244T), *fusA2* (L104P, Nt889Δ1), *htpX* (G187D), *capD* (I7M, S51G), *gyrA* (T83A, T325I), *mexK* (G487E), *mexH* (Nt1086ins1), *fusA1* (Y552C, T671I), *rpoC* (E136G, D616G, V808L), *rpoB* (F1046S), *mexW* (A627V, Q771P), *pagL* (P158L), *pmrB* (F124L), *amgS* (R188C), *parE* (P438S)
FQRC10	2	2	4	12	1	1.5	1	1	8	0.094	0.5	−	−	−	PA2489 (R12L, A244T), *capD* (I7M, S51G), *mexH* (D356N), *mexW* (A627V, Q771P)
FQRC15	1	0.75	6	6	1	1.5	1	0.75	8	0.19	1	−	−	−	PA2489 (R12L, A244T), *capD* (I7M), *mexW* (A627V, Q771P)
FQRC26	4	6	24	24	1	0.25	1.5	1	6	1.5	0.38	−	+	−	*mexY* (V875M), *mexT* (R164H), PA2489 (R12L, A244T), *capD* (I7M, S51G), *gyrA*(Q106L), *mexW* (A627V, Q771P)
FQSE03	3	8	0.5	2	1.5	2	0.38	1	6	3	0.25	−	−	+	*mexA* (L338P), *lasR* (P117G), *mexZ* (A144V), PA2489 (R12L, A244T), *capD* (I7M, S51G), *gyrA* (D87N), *mexW* (A627V, Q771P)
FQSE06-0403	0.75	2	0.25	4	0.38	1	0.5	24	16	0.19	0.19	−	−	+	*mexA* (L338P), *lasR* (P117G), *mexY* (G287A), *mexZ* (S9P), PA2489 (R12L, A244T), *mpl* (S257L), *capD* (I7M, S51G), *fusA1* (Y552C, T671I), *mexW* (A627V, Q771P), PBP3 (P215L), *amgR* (A8V)
FQSE06-1104*	0.38	1	0.094	0.38	0.38	6	0.19	1	24	0.75	2	−	−	+	*mexA* (L338P), *lasR* (P117G), *mexZ* (A194P), PA2489 (R12L, A244T), *fusA2* (N236S, N561S), *capD* (I7M, S51G), *gyrA* (D87N), *mexK* (Q585*), *rpoB* (Y583C), *mexW* (A627V, Q771P), *pmrB* (V185I, G221D, R287Q), PBP1A (E161G), *amgR* (A8V)
FQSE06-0807	4	8	0.75	4	2	1.5	0.75	24	>256	0.5	1	−	−	+	*mexA* (L338P), *lasR* (P117G), *mexY* (G287A), *mexZ* (S9P), *mexT* (P270Q), PA2489 (R12L, A244T), *mpl* (S257L), *capD* (I7M, S51G), *fusA1* (N482S, Y552C, T671I), *mexW* (A627V, Q771P), PBP3 (P215L), *amgR* (A8V)
FQSE06-0610	4	24	0.75	8	1.5	1	0.25	1.5	24	0.75	0.19	−	−	+	*mexA* (L338P), *lasR* (P117G), *mexZ* (Nt290Δ11), PA2489 (R12L, A244T), *mexW* (A627V, Q771P), *capD* (I7M, S51G), *amgR* (A8V)
FQSE10-0503	1.5	12	4	4	1.5	1	0.25	0.75	8	0.25	0.25	−	−	+	*mexY* (V875M, N1036S), *mexZ* (IS), PA2489 (R12L, A244T), *ftsK* (A38T), *nalD* (Nt459Δ13), *mexW* (A627V, Q771P), *capD* (I7M, S51G)

Continued

Isolate ID[a]	Antibiotic resistance profile (MIC values)[b]											Hyperexpression?			Main antibiotic resistance mutations encountered[c]
	TZ (≤8)	PM (≤8)	AT (≤1)	PPT (≤16)	C/T (≤4)	IP (≤4)	MP (≤2)	TO (≤4)	AK (≤8)	CI (≤0.5)	CO (≤2)	AmpC	MexAB	MexXY	
FQSE10-0106	0.75	3	0.125	0.75	0.5	0.38	0.032	0.75	4	0.38	1.5	–	–	+	*mexB* (L738P), *mexY* (V875M, N1036S), *mexZ* (IS), PA2489 (R12L, A244T), *ftsK*(A38T), *capD* (S51G), *nalD* (Nt396Δ2), *mexW* (A627V, Q771P), *nfxB* (*188ext)
FQSE10-0110	3	8	16	8	2	1	0.125	0.75	4	0.75	0.5	–	+	+	*mexY* (V875M, N1036S), *mexZ* (IS), PA2489 (R12L, A244T), *ftsK* (A38T), *rpoB* (D659E, E904K), *mexW* (A627V, Q771P), *pmrB* (R287Q)
FQSE10-0111	3	16	12	12	8	1.5	1	1	12	0.38	0.38	–	–	+	*mexY* (V875M, N1036S), *mexZ* (IS), PA2489 (R12L, A244T), *ftsK* (A38T, D54Y), *capD* (S51G), *mexW* (A627V, Q771P)
FQSE15-0803	2	12	0.38	4	1.5	6	1	1	12	0.19	0.25	–	–	+	*mexA* (L338P), *lasR* (P117G), *mexZ* (A144V), PA2489 (R12L, A244T), *capD* (I7M, S51G), *pmrB* (E213D), *mexW* (A627V, Q771P), *amgR* (A8V)
FQSE15-0906	0.75	6	0.38	2	1	1	0.047	1.5	12	0.38	0.75	–	–	+	*mexA* (L338P), *lasR* (P117G), *mexZ* (A144V), *mexS* (Nt848Δ2), *mexT* (Nt534Δ17), PA2489 (R12L, A244T), *capD* (I7M, S51G), *mexK* (S426G), *mexW* (A627V, Q771P), *amgR* (A8V)
FQSE15-0310	1	4	1	1	1	12	0.19	1	8	0.38	0.25	–	–	+	*mexA* (L338P), *lasR* (P117G), *mexZ* (A144V), *mexS* (Nt848Δ2), *mexT* (Nt534Δ17), PA2489 (R12L, A244T), *capD* (I7M, S51G), *mexK* (P834S), *mpl* (Nt1266Δ1), *rpoC* (Nt1181Δ3), *mexW* (A627V, Q771P), *amgR* (A8V)
FQSE15-1110*	8	24	6	4	1	>32	>32	1	16	1	0.25	–	–	+	*gyrB* (S466F), *mexA* (N71S, D235G), *mexB* (L376V), *oprD* (V67*), *lasR* (P117G), *mexY* (Y355H), *mexZ* (A194P), *galU* (P123L), PA2050 (G90R, Q161R), PA2489 (R12L, A244T), *fusA2* (N236S, N561S), *htpX* (A141T), *capD* (I7M, S51G), *fusA1* (K430E), *rpoC* (V693A), *mexW* (A627V, Q771P), *pmrB* (R287Q), PBP1A (E161G), *amgS* (D267N), *amgR* (A8V)
FQSE24-0304*	2	24	0.38	8	1	>32	>32	2	24	6	0.38	–	–	+	*gyrB* (S466F), *mexA* (L338P), *oprD* (V67*), *lasR* (P117G), *mexY* (Y355H), *mexZ* (A194P), *galU* (P123L), PA2050 (G90R, Q161R), PA2489 (R12L, A244T), *fusA2* (N236S, N561S), *opmE* (D421G), *capD* (I7M, S51G), *fusA1* (K430E), *rpoC* (V693A), *mexW* (A627V, Q771P), *pmrB* (R287Q), PBP1A (E161G), *amgR* (A8V)
FQSE24-1005*	1	16	0.38	2	1.5	>32	8	3	16	6	1	–	–	+	*gyrB* (S466F), *oprD* (V67*), *lasR* (P117G), *mexY* (Y355H), *mexZ* (A194P), *galU* (P123L), PA2050 (G90R, Q161R), *fusA2* (N236S, N561S), PA2489 (R12L, A244T), *fusA1* (K430E), *rpoC*(V693A), *mexW* (A627V, Q771P), *pmrB* (R287Q), PBP1A (E161G, R407S), *amgR* (A8V)
FQSE24-0308*	1	8	0.25	0.75	1.5	>32	0.25	2	16	4	1	–	–	+	*gyrB* (S466F), *oprD* (V67*), *lasR* (P117G), *mexY* (Y355H), *mexZ* (A194P), *galU* (P123L), PA2050 (G90R, Q161R), *fusA2* (N236S, N561S), PA2489 (R12L, A244T), *capD* (I7M, S51G), *fusA1* (K430E), *rpoC* (V693A), *mexW* (A627V, Q771P), *pmrB* (R287Q), PBP1A (E161G), *amgS* (T92A), *amgR*(A8V)
FQSE24-1010*	1	8	1	1	1	>32	4	4	64	4	0.38	–	–	+	*gyrB* (S466F), *mexA* (L338P), *oprD* (V67*), *lasR* (P117G), *mexY* (Y355H), *mexZ* (A194P), *galU* (P123L), PA2050 (G90R, P97L, Q161R), PA2489 (R12L, A244T), *fusA2* (N236S, N561S), *opmE* (L400P, D421G), *mexH* (V221I), *capD* (I7M, S51G, A165V), *fusA1* (K430E), *rpoC*(V693A), *mexW* (A627V, Q771P), PBP3 (G216S), *pmrB* (R287Q), PBP1A (E161G), *amgS* (A13V), *amgR* (A8V)
PAMB148	>256	64	>256	>256	6	1.5	0.75	1.5	16	0.064	0.5	+	–	–	PA2489 (R12L, A244T), *capD* (I7M, S51G), *mexY* (V875M, N1036S), *mexW* (A627V, Q771P), *ampD* (P41L)
% I + R	13.8	44.8	48.3	13.8	17.2	44.8	27.6	24.1	62.1	48.3	3.4				

Table 2. Antibiotic susceptibility profile and main antibiotic resistance related mutations detected among CC274 isolates. [a]Isolates are labelled according to the following format: Patient identification - MMYY isolation code in the case of sequential isolates. Mutators isolates are identified with and asterisk. [b]Minimal Inhibitory Concentration (MIC) values were determined by grading MIC testing for the following antimicrobial agents: ceftazidime (TZ); cefepime (PM); aztreonam (AT); piperacillin-tazobactam (PPT); cefotolozane-tazobactam (C/T); imipenem (IP); meropenem (MP); tobramycin (TO); amikacin (AK); ciprofloxacin (CI) and colistin (CO). Clinical breakpoints established by EUCAST v7.0 for each antibiotic are shown in brackets. [c]The main antibiotic resistance related mutations documented for each isolate are shown. For this purpose, the full list of mutations in the 164 genes studied (available in Supplementary Data Set S1) was refined to include only those more likely to be involved in the resistance phenotypes, by including: (i) mutations with known effect on resistance according to published evidence (ii) mutations for which our experimental evidence crosslinks resistance phenotypes and genotypes (*e.g.* mutations in genes involved in AmpC, efflux or OprD regulation and β-lactam resistance phenotypes are crosslinked by integrating the analysis of the expression of *ampC*, efflux pumps genes and *oprD* and/or (ii) mutations in genes found to be under high evolutionary pressure (those with at least 3 different mutational events documented).

Figure 4. Distribution of mutations among the CC274 collection. Mutations encountered among the 164 antibiotic resistance related genes are represented, synonymous and common non-synonymous mutations have been excluded.

in Supplementary Data Set S1) was refined to include only those more likely to be involved in the resistance phenotypes, by including: (i) mutations with known effect on resistance according to published evidence, (ii) mutations for which our experimental evidence crosslinks resistance phenotypes and genotypes (*e.g.* mutations in genes involved in AmpC, efflux or OprD regulation and β-lactam resistance phenotypes are crosslinked by integrating the analysis of the expression of *ampC*, efflux pumps genes and *oprD*) and/or (ii) mutations in genes found to be under high evolutionary pressure (those with at least 3 different mutational events documented). As shown in Table 2, overall, the number of mutations was much higher (unpaired T test p < 0.0001) in mutator (19.2 ± 3.1) than in non-mutator isolates (6.7 ± 3.1). This is consistent with the much higher antimicrobial resistance of mutators, documented in this and previous works[8, 29]. However, some Australian (*e.g.* AUS410 or AUS411) non-mutator isolates also presented a high number of mutations, perhaps indicating that under a high antibiotic pressure in long-term CRI, MDR profiles may emerge even in the absence of mutator phenotypes. Unique mutations detected in specific genes support phylogeny reconstructions (see above Fig. 2b and Fig. 3). Moreover, they can be very useful to track interpatient transmission, considering that specific mutations detected in multiple isolates, especially within professional antibiotic resistance genes such as *gyrB*, *oprD*, *mexY*, *creC*, *mexZ* or *fusA2*, are unlikely to have occurred independently in different environments. Moreover, the analysis of these mutations

Figure 5. MIC-fold change for each antibiotic tested between isolates mutated or not mutated in a specific gene. To evaluate the implication of the presence of mutations in the main genes possibly related with antibiotic resistance the median MIC for both groups were calculated and compared, results are expressed in MIC-fold change. PA2489, *mexW*, *oprF*, *parE* and *nfxB* were excluded since the number of mutated isolates were < 3. Some genes were grouped (e.g. *ampD* and *dacB* (PBP4) or *nalD* and *mexR*) according to their well-established effects on resistance (e.g. AmpC or MexAB-OprM overexpression, respectively).

can help to understand the basis for the intrapatient diversification and coexistence of multiple lineages in the CF respiratory tract.

To gain insights into the effect on the antibiotic resistance profiles of mutations listed in Table 2, the median MIC of isolates harboring mutations or not in a specific gene were compared and results are summarized in Fig. 5. Overall, it should be noted that colistin MICs as well as the MICs for the antibiotic combinations piperacillin-tazobactam and ceftolozane-tazobactam were barely affected, whilst carbapenems, aminoglycosides and quinolones MICs are affected by the presence of mutations in many of the selected genes. Apparently, the presence of mutations in some genes such as *capD* (also known as *wbpM*), a gene coding for a protein implicated in O-antigen biosynthesis and previously related with aminoglycoside resistance, or *ftsK*, which codes for a cellular division protein, were not related with an increase in resistance for any antibiotic. Conversely, the presence of mutations in 22 of the genes was shown to produce at least a 2-fold MIC increase for at least 3 different classes of antibiotics. Renowned resistance genes, such as *gyrA*, *gyrB*, *ampD*, *dacB* (PBP4) or *oprD*, are within this list of 22 genes but, particularly interesting is the presence of not so well-recognized antibiotic resistance related genes such as *fusA1* and *fusA2*, both coding for elongation factor G, or *rpoC*, which codes the β-chain of a DNA-directed RNA polymerase. Mutations in genes coding for two-component regulatory systems, as PhoPQ or ParRS, also require a special mention as mutated isolates showed a strong impact in their MICs for many of the antibiotics tested.

The presence of unique mutations in certain well-known antibiotic resistance genes, such as *dacB* (PBP4) was observed to increase β-lactam resistance, but it should be noted that mutations within a specific gene did not always correlate or lead to the expected effect on antibiotic resistance (e.g. *pmrB* or *phoP-phoQ* mutated isolates

did not exhibit a higher colistin MIC). Likewise, several mutations (e.g. *mexZ*, *gyrB* or *oprD*) were associated to extended unexpected antibiotic resistance profiles, perhaps suggesting the co-selection of different resistance mutations during *P. aeruginosa* evolution in CF CRI. A detailed analysis of the mutational resistome for each class of antibiotics is provided below and in the Supplementary Data Set S1.

β-lactam resistome.   Overproduction of the chromosomally encoded cephalosporinase AmpC is the primary pathway for developing resistance to the antipseudomonal β-lactams, and it is driven by the selection of mutations in peptidoglycan-recycling genes (*ampD*, *dacB* and *ampR*)[30, 31]. Just three isolates (AUS034, AUS603 and PAMB148) of the CC274 collection were demonstrated to overproduce AmpC (Table 2). By contrast, at the genomic level, almost all isolates (26/29) contained some variation within *dacB* which codes for the penicillin-binding protein PBP4 (Supplementary Data Set S1-Betalactams). Crosslinking phenotypic and genotypic results through *ampC* expression data, suggested that most observed *dacB* allele variations were, in fact, ancestral polymorphisms not involved in antibiotic resistance. However, AmpC overproduction in the two CF isolates was explained by the presence of specific mutations in *dacB* (S315G or W350R) and by an *ampD* (P41L) mutation in the case of the bloodstream infection isolate PAMB148 (Table 2). Whilst *ampC* overexpression in isolates AUS034 and PAMB148 correlated well with ceftazidime and piperacillin-tazobactam resistance, this was not the case for isolate AUS603 which was documented to be susceptible to these antibiotics. However, unexpected AUS603 β-lactam susceptibility could be explained by the presence of chromosomal mutations whose effects eventually compensate the expected increase in β-lactam resistance. Indeed, this isolate showed a non-sense mutation in OprM (Q93X), the outer membrane protein of the constitutive MexAB efflux pump, which is well known to play a major role in intrinsic β-lactam resistance.

In addition, *P. aeruginosa* may eventually develop β-lactam resistance by acquiring mutations within their macromolecular targets: the essential penicillin-binding proteins (PBPs). While some mutations without apparent effect on resistance were detected in genes coding for PBP1 and PBP3a, the main mutational resistance target among PBPs was found to be PBP3, an essential high molecular class B PBP with transpeptidase activity, in agreement with recent data from CF patients[32] and *in vitro* studies[33]. Indeed, we documented that PBP3 mutations had often occurred (7/29 isolates) among the CC274 collection (Supplementary Data Set S1-Betalactams). Nevertheless, β-lactam resistance contribution of each derived *ftsI* (PBP3) allele, if any, depends on the specific point mutation encountered. Missense mutations within the PBP3 (R504C and Q372P) were apparently the cause of β-lactam resistance in isolates AUS601 and AUS411, since they do not hyperproduce AmpC. Although these mutations are not located in the PBP3 active site, both are very close to two loop regions (residues 332–338 and 526–533) which play an important role in substrate recognition[34]. In fact, PBP3 mutations in residue 504 (R504C, R504H) have been recently described *in vitro*[33] and among isolates from widespread nosocomial *P. aeruginosa* clones[35, 36]. Likewise, the P527T mutation of AUS034 likely contributes, together with the overexpression of AmpC, to the very high-level β-lactam resistance of this isolate, including the new antipseudomonal combination ceftolozane-tazobactam. On the other hand, the P215L and G216S mutations were apparently not linked with phenotypic resistance, in agreement with the fact that residues 215 and 216 are not implicated in the formation and stabilization of the inactivating complex β-lactam-PBP3[34].

Obtained data also demonstrated that the constitutive efflux pump MexAB-OprM is under strong mutational pressure during CF CRI, frequently including inactivating mutations, which correlates with previous investigations that pointed out that this efflux pump is dispensable and, therefore, tends to be lost or inactivated in favor of MexXY-OprM hyperproduction in CF *P. aeruginosa* subpopulations[37]. Our data also support this hypothesis, as just 3 isolates showed mutations in regulators leading to MexAB-OprM overexpression, whereas up to 23 isolates hyperproduced the efflux-pump MexXY-OprM (Supplementary Data Set S1 -Betalactams). Moreover, many of the isolates showed some degree of hypersusceptibiltiy to aztreonam (substrate of MexAB-OprM) in favor of an increased MIC of cefepime (substrate of MexXY-OprM) (Supplementary Data Set S1- Betalactams).

Carbapenem resistome.   Imipenem and meropenem resistance correlated in all but two isolates with the presence of non-sense mutations affecting the outer membrane protein OprD (Table 2, Supplementary Data Set S1-Carbapenems). High-level meropenem resistance was additionally associated with the presence of PBP3 mutations, in agreement with recent *in vitro* studies showing the selection of PBP3 mutations upon meropenem exposure[33]. Remarkably, all ST1089 mutator isolates shared the same point mutation in *oprD* (V67X) as well as in *galU* (P123L), also related with carbapenem resistance, supporting interpatient transmission of this mutator lineage among CF patients attending the reference hospital of the Balearic Islands.

The expression of OprD is known to be modulated by mutations (*mexS* or *mexT*) leading to the overexpression of the efflux pump MexEF-OprN, and meropenem is a well-known substrate for the efflux pump MexAB-OprM. However, carbapenem resistant isolates AUS411 and AUS603 harboring a wild-type *oprD* allele did not overproduce neither of these two efflux pumps. Thus, the observed phenotype could be related with the presence of specific mutations within the genes coding for PBP3 (*ftsI*) and PBP4 (*dacB*), hypothesis that is current being evaluated in our laboratory.

Aminoglycoside resistome.   Intravenous antimicrobial combinations including an aminoglycoside plus a fluroroquinolone or a β-lactam antibiotic are frequently used to manage CF exacerbations. Moreover, in the last decade, tobramycin inhalation has become an important contributor to CF treatment as a means to control chronic infection as well as a first-line treatment for the eradication of early acquisition of *P. aeruginosa* and several aminoglycoside-based inhaled formulations are currently available. Resistance to antipseudomonal aminoglycosides is frequently attributed to the presence of acquired aminoglycoside-modifying enzymes, membrane impermeability or MexXY efflux pump overexpression[38]. Moreover, adaptive resistance, due to MexXY efflux

system overexpression, to this class of antibiotics has been well documented in the CF setting in response to sublethal concentrations[39].

Among the CC274 collection, a high proportion of the isolates (23/29) were shown to overexpress MexXY and all but one were mutated in *mexZ*, which codes for the mayor MexXY expression regulator. In agreement with recent work, which pointed out that mutation of *mexZ* is part of a strongly selected evolutionary pathway[40], several different mutational events were encountered within this regulator. Remarkably, the same point mutation was detected among different and independent isolates, probably indicating interpatient transmission events (Table 2, Supplementary Data Set S1-Aminoglycosides). The single MexXY-overproducing isolate showing no mutations in *mexZ*, presented a unique mutation in *parS*, a gene also involved in the modulation of MexXY expression. Nevertheless, as it has been largely observed in the CF clinical setting[41], MexXY hyperproduction *per se* cannot explain aminoglycoside resistance in the majority of the isolates. In this sense, there is growing evidence that high-level resistance is a stepwise process which arises by the accumulation of several non-enzymatic mechanisms and, moreover, novel genetic resistance determinants have been proposed[42–44]. To our knowledge, no published work has yet investigated the *in vivo* contribution to aminoglycoside resistance of these novel genetic determinants proposed, and many questions remain unresolved. Thus, our work reveals for the first time that all high-level resistant isolates hyperproduced MexXY, but also harbored additional mutations in some of these genes, especially highlighting the presence of mutations in both genes coding for elongation factor G, *fusA1* and *fusA2* (Supplementary Data Set S1 - Aminoglycosides). In fact, *fusA1* and *fusA2* have been recently demonstrated to be under high evolutionary pressure in the CF environment, which can be explained in terms of a wide aminoglycoside use in this setting[45].

Fluoroquinolone resistome.

P. aeruginosa RND (Resistance-Nodulation-Division) efflux pumps MexAB-OprM, MexXY-OprM, MexCD-OprJ and MexEF-OprN are well-known to extrude fluoroquinolones. Nevertheless, our data suggest that the contribution of the overexpression of these efflux pumps to high-level resistance to fluoroquinolones is very limited, if any (Supplementary Data Set S1 -Fluoroquinoles). Concerning MexCD-OprJ overproduction, it has been shown that, although wild type *P. aeruginosa* strains generally do not express this efflux system[46], hyperproducing mutants tend to emerge after both *in vitro* and *in vivo* fluoroquinolone exposure[33]. Moreover, there is some data suggesting that MexCD-OprJ hyperproduction could be an advantage in the CF environment[47]. Among the CC274 collection, however, just 1 isolate (FQSE10-0106), showing aa ciprofloxacin MIC below the resistance breakpoint (MIC = 0.38 mg/L), was demonstrated to hyperproduce MexCD-OprJ due to a non-sense mutation in *nfxB*.

On the other hand, our data shows that high-level fluoroquinolone resistance was associated with the presence of missense mutations in *gyrA*, *gyrB* and/or *parC* quinolone resistance-determining regions (QRDRs). Specifically, up to 9 isolates were mutated in *gyrB* QRDR and all but two harbored the same mutation (S466F), 6 showed mutations in *gyrA* QRDR (T83I, T83A, D87N, D87G and Q106L), and just one isolate was mutated in *parE* (P438S). Mutations in GyrA residues 83 and 87 are well-known to be relevant in the clinical setting and are frequently encountered in fluoroquinolone-resistant *P. aeruginosa*[35, 36], being both residues situated on helix-4. Mutations in residue 106 are in the other hand very infrequent, with only one previous reference of its existence in 1 of 335 quinolone resistant *P. aeruginosa* clinical strains[48].

Polymyxin resistome.

Two component-regulatory systems as well as other genes implicated in lipopolysaccharide biosynthesis have been related with polymyxin resistance[49–51]. Several *in vitro* works have addressed the implication of the two-component regulatory systems in polymyxin resistance development, demonstrating that individual alterations in these systems are generally not sufficient to develop high-level resistance[50, 52–54] and that individual two-component systems may not be essential for acquisition of colistin (polymyxin E) resistance in *P. aeruginosa*[55]. In agreement with these *in vitro* studies, we found that many isolates were mutated in genes such as *pagL*, *phoQ* or *pmrB*, but with one exception (i.e. isolate AUS034) phenotypic resistance was not observed (Table 2, Supplementary Data Set S1-Polymyxins). For isolate AUS034, a specific non-sense mutation was detected in the two-component sensor PhoQ, as well as two other specific point mutations within *parR* and *colS*. Five additional isolates were shown to harbour mutations in more than one polymyxin-resistance related genes and showing colistin MICs from 0.125 to 2 mg/L (Supplementary Data Set S1- Polymyxins). Remarkably, none of the mutations detected in the two-component regulatory systems PmrAB and PhoPQ have been previously described in the clinical setting, reflecting an individual strain adaptation to the CF lung[36, 52, 53, 56]. Six different and independent mutational events were registered in PmrB sensor, being all but two located near the active site (H249). Moreover, Spanish mutators shared the same mutation, again reflecting the interpatient transmission of a CF-adapted mutator lineage.

Considering that colistin is widely used for the management of CF patients, the frequent documentation of mutations in genes such as *phoQ*, *pmrB* or *pagL* suggests a role in polymyxin resistance, tolerance or adaptation *in vivo*, even when phenotypic resistance is not demonstrated *in vitro*. Thus, further *in vivo* and clinical studies should be performed to decipher the impact of these mutations for the therapeutic management of CF patients.

Concluding remarks.

Emergence of international epidemic CF clonal lineages, along with the extraordinary capacity of *P. aeruginosa* to develop resistance to all antibiotic classes, catalyzed by frequent mutator phenotypes, severely compromises the clinical management of *P. aeruginosa* CF CRI. In addition to the assessment of the emerge of mutator phenotypes within an international CF clone, we analyzed for the first time the genetic basis of hypermutation from whole genome sequence data, through the analysis of the sequence of an exhaustive panel of so called mutator genes, thus designated mutome.

CC274 population structure analysis demonstrated the coexistence of two separated and divergent clonal lineages, but without evident geographical barrier. Coexistence of distinct evolved sublineages within a patient

was documented, reflecting coexistence of divergent lineages within the infecting inoculum or alternatively, and less probable, multiple interpatient transmission events. More revealing is the confirmation, by both phylogenetic reconstructions and mutational resistome analysis, of interpatient transmission of mutators. Compared with classical molecular typing tools, WGS provides detailed genome fingerprints that might be essential for epidemiological studies in which prevalent and ubiquitous clonal lineages are involved. Indeed, WGS closely clustered isolates from four of the patients from the Balearic Islands, likely indicating interpatient transmission or a common source of colonization, whereas isolates from a fifth patient from the same hospital was distantly related.

We have documented at whole genome level the extraordinary capacity of *P. aeruginosa* to acquire resistance by mutational events, evidencing the emergence of mutations in over 100 genes related to antibiotic resistance during the evolution of a CF epidemic clone. Moreover, our results confirm that the evolution of *P. aeruginosa* resistome is greatly enhanced when mutator phenotypes are selected. However, the difficulty for correlating genotypic with phenotypic variation (due to random drift mutations among other causes) has been a hallmark of WGS approaches. To minimize this limitation, the full list of mutations in the 164 genes studied was refined to include only those more likely to be involved in the resistance phenotypes. While the presence of classical mutational mechanisms, such as the overexpression of the β-lactamase AmpC, the inactivation of the carbapenem porin OprD, or QRDR mutations, was confirmed in a number of isolates and correlated with the resistance phenotypes, our results also provided evidence for the existence and important role of less expected resistance mutations and their phenotypes. Among them, PBP3 mutations, shaping up β-lactam resistance are particularly noteworthy. Likewise, our work, as previously others, denote the very high selective pressure for *mexZ* mutations, leading to the overexpression of MexXY, associated with aminoglycoside resistance. However, we show for the first time that high-level aminoglycoside resistance in CF is driven by the acquisition of additional mutations, particularly those in *fusA1* or *fusA2*, coding for elongation factor G. Finally, a complex repertoire of mutations in genes related to polymyxin resistance is evidenced, but with limited correlation with *in vitro* phenotypic resistance. Altogether, our results provide valuable information for understanding the evolution and dynamics of the mutational resistome of *P. aeruginosa* CF clones and it is correlation with resistance phenotypes, which might be useful for guiding new diagnostic tools and therapeutic strategies in CRI.

Material and Methods

***P. aeruginosa* CC274 collection and susceptibility testing.** The CC274 collection included 29 isolates: 28 recovered from 18 CF patients from Australia and Spain and 1 blood culture isolate from a Spanish non-CF patient, covering up to an 18-year period from 1995 to 2012. All isolates had been previously classified within the CC274 (sharing at least 5 alleles with ST274) based on MLST using available protocols and databases (http://pubmlst.org/paeruginosa/). All the Australian and 4 CF Spanish isolates were single isolates recovered from patients attending clinical settings located in different geographical areas, being each area represented by at least 2 independent isolates, selected randomly from those available. In addition, we included 4 sequential *P. aeruginosa*, each separated by at least 6-month intervals, from each of 4 CF patients attended at the reference hospital of the Balearic Islands (Son Espases Hospital, Spain)(Fig. 1), thus representing intrapatient clone evolution. These patients were shown to be chronically colonized with this persistent strain in a previous study[16]. *P. aeruginosa* PAO1 strain was used as reference when needed. Minimal inhibitory concentrations (MICs) of ceftazidime, cefepime, aztreonam, piperacillin-tazobactam, ceftolozane-tazobactam, imipenem, meropenem, tobramycin, amikacin, ciprofloxacin and colistin were determined by Etest and classified according EUCAST clinical breakpoints (http://www.eucast.org/).

Molecular typing. Clonal relatedness among isolates was evaluated by PFGE. For this purpose, bacterial DNA embedded in agarose plugs prepared as described previously was digested with SpeI. DNA separation was then performed in a contour-clamped homogeneous-electric-field DRIII apparatus (Bio-Rad, La Jolla, CA) under the following conditions: 6 V/cm^2 for 26 h with pulse times of 5 to 40 s. DNA macrorestriction patterns were analyzed with UPGMA to infer clonal relatedness (CLIQS 1D Pro, Totallab).

Mutant frequencies and genetic basis of hypermutation. Rifampicin (300 mg/L) resistance mutant frequencies were determined in all strains following previously established procedures[9, 10]. To explore the genetic basis for the mutator phenotypes, complementation studies were performed as described previously[9]. Briefly, plasmid pUCPMS harbouring PAO1 wild-type *mutS*, plasmid pUCPML harbouring PAO1 wild-type *mutL*, and plasmid pUCP24, a control cloning vector, were electroporated into the mutator isolates. Complementation was demonstrated by reversion of the increased rifampicin resistance mutant frequencies in two independent transformant colonies for each strain. Additionally, the genetic basis of hypermutation was investigated from whole genome sequence data, through the analysis of an exhaustive panel of so called mutator genes, thus designated mutome. Genes included within the mutome panel, selected according to available informatio[8] were the following: PA0355/*pfpI*, PA0357/*mutY*, PA0750/*ung*, PA1816/*dnaQ*, PA3002/*mfd*, PA3620/*mutS*, PA4366/*sodB*, PA4400/*mutT*, PA4468/*sodM*, PA4609/*radA*, PA4946/*mutL*, PA5147/*mutM*, PA5344/*oxyR*, PA5443/*uvrD* and PA5493/*polA*.

Characterization of resistance mechanisms. The levels of expression of *ampC*, *mexB*, *mexD*, *mexY*, and *mexF* were determined by real-time reverse transcription (RT)-PCR according to previously described protocols[57]. Additionally, for selected isolates, the sequences of resistance genes, such as *oprD* or mexZ was obtained by Sanger sequencing in order to confirm whole-genome sequencing data as needed. Briefly, after duplicate PCR amplification, sequencing reactions were performed with the BigDye Terminator kit (PE Applied Biosystems, Foster City, CA), and sequences were analyzed on an ABI Prism 3100 DNA sequencer (PE Applied Biosystems). The resulting sequences were then compared with that yielded by WGS technology.

Library preparation and whole-genome sequencing.   Genomic DNA was obtained by using a commercially available extraction kit (High Pure PCR template preparation kit; Roche Diagnostics). Indexed paired-end libraries were prepared with Nextera XT DNA library preparation kit (Illumina Inc, USA) and sequenced on an Illumina MiSeq® benchtop sequencer with MiSeq reagent kit v2 (Illumina Inc., USA), resulting in 250 bp paired-end reads.

Variant calling.   Previously defined and validated protocols were used with slight modifications[25, 58]. Briefly, paired-ended reads were aligned to the *P. aeruginosa* PAO1 reference genome (GenBank accession: NC_002516.2) with Bowtie 2 v2.2.4 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml)[59] and, eventually, pileup and raw files were obtained by using SAMtools v0.1.16 (https://sourceforge.net/projects/samtools/files/samtools/)[60] and PicardTools v1.140 (https://github.com/broadinstitute/picard). The Genome Analysis Toolkit (GATK) v3.4-46 (https://www.broadinstitute.org/gatk/) was used for realignment around InDels[61]. Median PAO1 coverage was 95.75% (range: 90.4–97.6%). SNPs were extracted from the raw files if they met the following criteria: a quality score (Phred-scaled probability of the samples reads being homozygous reference) of at least 50, a root-mean-square (RMS) mapping quality of at least 25 and a coverage depth of at least 3 reads; excluding all ambiguous variants. MicroInDels were extracted from the totalpileup files applying the following criteria: a quality score of at least 500, an RMS mapping quality of at least 25 and support from at least one-fifth of the covering reads. Finally, all positions in which at least one of the isolates showed some variation were manually and individually checked in all other isolates without applying any filtering.

***De novo* assembly.**   Sequence reads from each isolate were *de novo* assembled using Velvet v1.2.10 (https://www.ebi.ac.uk/~zerbino/velvet/)[62] with a k-mer length of 31 and the following parameters: scaffolding = no, ins_length = 500, cov_cutoff = 3, and min_contig_lgth = 500. The median size of the *de novo* assembled obtained genomes was 6.1 Mbp, ranging from 5.4 to 6.6 Mbp. MUMmer3 v3.23[63] was used to align the obtained genomes against each other in order to confirm that all belong to the same clone type (genomes differing < 10,000 SNPs).

Phylogenetic reconstructions and BEAST analysis.   Core genome phylogenetic reconstructions were performed using Parsnp from the Harvest Suite package v1.2 with default parameters forcing the inclusion of all genomes and a randomly selected reference genome (flags: -c / -r!) (http://harvest.readthedocs.io/en/latest/content/parsnp.html)[20]. Bayesian analysis of divergence times was performed using BEAST v2.4.2 (http://beast2.org/)[64]. For this purpose, a nexus file including all the curated positions at which at least one of the isolates differed from the reference strain PAO1 was constructed and converted into an.xml file with BEAUTi. BEAST was run with the following user-determined settings; a lognormal relaxed molecular clock model and a general time-reversible substitution model with gamma correction[25]. Divergence times were calculated from a chain length of 50 million steps, sampled every 1,000 steps and discarding the first 5 million steps as a burn-in. The maximum clade credibility tree was generated using the TreeAnnotator program from the BEAST package and tree parameters were calculated with Tracer v1.6 (http://beast.bio.ed.ac.uk/Tracer). Both Phylogenetic reconstructions were displayed using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Profiling of antibiotic resistance genes.   SNPs and InDels for each isolate were annotated by using SnpEff software v4.2 (http://snpeff.sourceforge.net/index.html)[65] with default options. These files were then filtered based on an exhaustive literature review[35] that led us to obtain a set of 164 genes known to be related to chromosomal antibiotic resistance in *P. aeruginosa* (Supplementary Data Set S1). Additionally, we used the online tool ResFinder v2.1 (https://cge.cbs.dtu.dk//services/ResFinder/)[28] to identify possible horizontally acquired antimicrobial resistance genes.

Ethics statement.   The study has been approved by the Research Committee from Son Espases University Hospital. All methods were performed in accordance with the relevant guidelines and regulations. Used isolates derived from frozen stocks of laboratory collections obtained from routine cultures. Patient's information or tissue samples were not used in this study.

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Author Contributions

C.L.-C. conceived the study, performed laboratory experiments and bioinformatics analysis, analyzed results and wrote the manuscript. L.M.S. performed bioinformatics analysis. G.C. performed laboratory experiments and analyzed results. R.R. performed laboratory experiments. A.A.O.-S. contributed materials. H.K.J. contributed analysis tools. J.F. contributed materials. R.C. contributed materials and critically reviewed the manuscript. T.J.K. contributed materials and critically reviewed the manuscript. S.M. contributed analysis tools and critically reviewed the manuscript. A.O. conceived the study, analyzed results and wrote the manuscript.

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Antibiotic resistance and population structure of cystic fibrosis *Pseudomonas aeruginosa* isolates from a Spanish multi-centre study

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ABSTRACT

The first Spanish multi-centre study on the microbiology of cystic fibrosis (CF) was conducted from 2013 to 2014. The study involved 24 CF units from 17 hospitals, and recruited 341 patients. The aim of this study was to characterise *Pseudomonas aeruginosa* isolates, 79 of which were recovered from 75 (22%) patients. The study determined the population structure, antibiotic susceptibility profile and genetic background of the strains. Fifty-five percent of the isolates were multi-drug-resistant, and 16% were extensively-drug-resistant. Defective *mut*S and *mut*L genes were observed in mutator isolates (15.2%). Considerable genetic diversity was observed by pulsed-field gel electrophoresis (70 patterns) and multi-locus sequence typing (72 sequence types). International epidemic clones were not detected. Fifty-one new and 14 previously described array tube (AT) genotypes were detected by AT technology. This study found a genetically unrelated and highly diverse CF *P. aeruginosa* population in Spain, not represented by the epidemic clones widely distributed across Europe, with multiple combinations of virulence factors and high antimicrobial resistance rates (except for colistin).

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1. Introduction

The lower respiratory tract of patients with cystic fibrosis (CF) is usually chronically colonised by a complex microbial ecosystem. This colonisation triggers an inflammatory response that produces respiratory symptoms and acute exacerbations, and influences the patients' clinical course and outcome. *Pseudomonas aeruginosa* is the most relevant micro-organism in this process. During the first stages of the disease, CF *P. aeruginosa* isolates are almost identical to environmental isolates. The evolved disease is characterised by mucoid colonies and/or multi-drug-resistant isolates [1] that result from the particular CF lung environment, a compartmentalised hostile niche for *P. aeruginosa* that forces the bacteria to an ecological adaptation [2], and frequent mutator phenotypes [3].

Previous epidemiological studies on CF *P. aeruginosa* isolates have been performed using different molecular typing tools. For instance, the use of multi-locus sequence typing (MLST) has allowed the identification of international epidemic CF clones, such as the well-known Liverpool epidemic strain or Clone C. Moreover, several hypertransmissible CF *P. aeruginosa* strains have been described [4], the detection of which should alert clinicians to prevent transmission between patients, including siblings [5] and patients from the same or different centres [6]. By using the array tube (AT) multi-marker array, some genotypes have been found to be most abundant in the global *P. aeruginosa* population, particularly AT genotypes 0C2E, 2C22, C40A, D421 and F429 that have been detected in both clinical and environmental isolates [7–13].

In Spain, the genetic background of *P. aeruginosa* isolates obtained from two different CF units has been reported previously [14,15], with ST274 and ST395 identified as endemic clones at each centre. This study, the first Spanish multi-centre study on the microbiology of CF, was conducted from 2013 to 2014, and included a representative patient population from across Spain [16]. The aim of this study was to characterise *P. aeruginosa* isolates to complete the microbiological description of this micro-organism in patients with CF in Spain.

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2. Materials and methods

2.1. Study design

This study involved 24 CF units (12 paediatric and 12 adult) from 17 hospitals [16]. Fifteen consecutive unselected patients per CF unit were recruited, and a single sputum sample from each patient was frozen immediately after collection at –80°C. The frozen samples were sent to Ramón y Cajal University Hospital and, after slow defrosting, were seeded in plates in the appropriate culture medium (see details in [17]). The plates were examined at 24 and 48 h, and the incubation time was extended to 5 days in order to identify potentially slow-growing bacteria. Colonies with compatible *P. aeruginosa* morphology were identified by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (Bruker Daltonics GmbH, Leipzig, Germany) and stored for further studies.

2.2. Antibiotic susceptibility

Antibiotic susceptibility was determined by disk diffusion, except for fosfomycin, for which the agar dilution method was performed [17]. The tested compounds included piperacillin/tazobactam, ceftazidime, cefepime, aztreonam, imipenem, meropenem, colistin, gentamicin, tobramycin, amikacin, ciprofloxacin, levofloxacin and fosfomycin. The European Committee on Antimicrobial Susceptibility Testing clinical breakpoints for systemic infections were applied (www.eucast.org), except for fosfomycin. PAO1 and ATCC 27853 *P. aeruginosa* reference strains were used as controls. Consensus recommendations [18] were used to evaluate the proportion of multi-drug-resistant (MDR, not susceptible to at least three antimicrobial classes), extensively-drug-resistant (XDR, only susceptible to one or two antimicrobial classes) and pandrug-resistant (PDR; not susceptible to any antibiotics) strains, considering the following seven antimicrobial classes: cephalosporins (ceftazidime and/or cefepime), penicillin-β-lactamase inhibitor combinations (piperacillin-tazobactam), monobactams (aztreonam), carbapenems (imipenem and/or meropenem), fluoroquinolones (ciprofloxacin), aminoglycosides (gentamicin, tobramycin, and/or amikacin) and colistin. For percentages of pseudomonas colonisation and antibiotic resistance, 95% confidence intervals (CI) were calculated using the Exact formula.

2.3. Mutant frequencies and genetic basis for hypermutation

Mutant frequencies for rifampicin (300 mg/L) resistance were determined in triplicate for all strains following previously established procedures [3]. To explore the genetic basis of mutator isolates, previously described primers and protocols were employed to amplify and sequence the *mut*S and *mut*L genes [19]. Briefly, plasmid pUCPMS harbouring PAO1 wild-type *mut*S, plasmid pUCPML harbouring PAO1 wild-type *mut*L, and plasmid pUCP24, a control-cloning vector, were electroporated into the mutator isolates. Complementation was demonstrated by reversion of the increased mutant frequencies for rifampicin resistance in two independent transformant colonies for each mutator isolate. Previously described primers and protocols [19] were used for the amplification and sequencing of *mut*S or *mut*L genes according to the results of complementation experiments.

2.4. Population structure

The genetic diversity of the isolates was explored initially by pulsed-field gel electrophoresis (PFGE) with the macrorestriction enzyme *Spe*I [20]. DNA separation was performed using a contour-clamped homogeneous electric field DRIII apparatus (Bio-Rad, La Jolla, CA, USA) under the following conditions: 6 V/cm^2 for 22 h with pulse times of 5–40 s. Finally, DNA macrorestriction

patterns were interpreted according to visual criteria, and after constructing a dendrogram using the Dice coefficient and Phoretix 5.0 software.

All isolates were further genotyped by MLST (http://pubmlst.org/paeruginosa/) using available protocols and databases. MEGA6 software enabled phylogenetic analysis of the MLST alleles and their concatenate sequence. A minimum spanning tree was constructed using the goeBURST algorithm (www.phyloviz.net).

2.5. P. aeruginosa AT genotyping

The Clondiag (Alere Technologies GmbH, Jena, Germany) AT species-specific genotyping system was employed according to the manufacturer's protocol [13]. This species-specific micro-array enables the genotyping of *P. aeruginosa* strains using 13 informative single nucleotide polymorphisms at conserved loci, the *fli*Ca/*fli*Cb multi-allelic locus, and the presence or absence of the *exoS*/*exoU* marker gene. The AT system also includes 38 genetic markers from the accessory genome for defining intraclonal diversity.

2.6. exoS and exoU amplification assays

Polymerase chain reaction (PCR) assays for detecting *exoS* and *exoU* genes were performed using previously described primers and protocols [21], with slight modifications. PCR reactions were performed with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA) in a DNA thermal cycler (Arktic Thermal Cycler; Thermo Fisher, Waltham, MA, USA) under the following conditions: denaturation for 5 min at 94 °C, followed by 35 cycles at 94 °C for 30 s, at 58 °C for 30 s and at 72 °C for 30 s, and a final extension step of 10 min at 72 °C.

3. Results

3.1. Patients, samples and isolates

From a total of 341 respiratory samples (one per patient), 79 *P. aeruginosa* isolates were recovered from 75 patients (four patients presented colonies with two different morphologies). The global colonisation rate was 22% (95% CI 17–26), and was higher in the adult population (32.7%, 95% CI 25–40) than in the paediatric population (9.9%, 95% CI 5–15) ($P < 0.001$). *P. aeruginosa* colonisation status was defined as intermittent (one patient, 6%) or chronic (15 patients, 94%) for the paediatric population; the corresponding numbers were 10 patients (17%) and 49 patients (83%) for the adult population. The primary characteristics of the patients with CF are shown in Table 1. Clinical data from the entire CF population can be found elsewhere [16].

The classical CF mucoid morphotype was observed in 17 (21.5%, 95% CI 13–32) isolates, whereas the others were classified as metallic (23 isolates, 29.2%, 95% CI 19–40) or Enterobacteriaceae-like (23 isolates, 29.2%, 95% CI 19–40). Sixteen isolates (20%, 95% CI 12–30) presented small colony variant (SCV) morphology. Half of the isolates exhibited brown (16 isolates, 20.2%) or green (25 isolates, 31.6%) pigmentation after 48 h of incubation at 37 °C. In 36 (48%, 95% CI 34–57) of the 75 patients, co-existence of *P. aeruginosa* and *Staphylococcus aureus* was detected by classical culture in agar plates, with seven (9.3%) of the isolates resistant to methicillin.

3.2. Antibiotic susceptibility profiles

Overall (intermediate plus resistant) antibiotic resistance rates are shown in Table 2. Colistin was the most active compound, and only three isolates (4%) were classified as resistant to colistin. Considering co-resistances and excluding aztreonam, 15 isolates

Table 1
Primary characteristics of the 341 patients with cystic fibrosis in the Spanish multi-centre study [16].

Sex	Category	No. of patients	Median value (range)			No. (%) of Δ508 mutations	
			Age (years)	Weight (kg)	FEV$_1$	Heterozygosis	Homozygosis
Patients colonised by *Pseudomonas aeruginosa*							
Male	Paediatric	10	15 (11–17)	41 (29–57)	65.2 (38–102)	2 (20.0)	4 (80.0)
	Adult	27	28 (18–51)	65 (47–87)	48.6 (20–106)	11 (40.7)	9 (33.3)
Female	Paediatric	6	16 (7–16)	44 (18–58)	78 (30–107)	1 (16.6)	4 (66.6)
	Adult	32	29 (18–40)	52.7 (39–68)	50.5 (18–87)	17 (50.0)	9 (28.1)
Other patients							
Male	Paediatric	65	11 (2–17)	34 (13–71)	86 (44–185)	27 (41.5)	21 (32.3)
	Adult	59	28 (18–56)	68 (41–89)	66 (20–120)	33 (55.9)	15 (25.4)
Female	Paediatric	80	11 (3–17)	35 (11–61)	75 (29–125)	33 (40.7)	35 (43.2)
	Adult	62	26 (18–48)	51.5 (38–91)	57 (17–108)	32 (51.6)	15 (24.1)

FEV$_1$, forced expiratory volume in 1 s.

(29%) remained susceptible to all antibiotics, 55% were classified as MDR, 16% were classified as XDR, and none corresponded with the PDR phenotype. Isolates displaying a mucoid morphotype were the most susceptible to antibiotics, whereas SCV isolates were the most resistant (Table 2).

3.3. Mutator phenotype

In total, 12 isolates (15.2%) that were recovered from eight adults and four children were considered as mutators (mutation frequency range 2×10^{-5}–4.5×10^{-7}). Eight of these mutator isolates had inactivating mutations in *mut*S ($n = 7$) or *mut*L ($n = 1$) genes. Three additional mutator isolates showed amino acid substitutions in the MutS and/or MutL proteins of uncertain effect, and the remaining isolate showed wild-type sequences of *mut*S and *mut*L genes (Table 3). All mutator isolates belonged to unrelated genetic lineages.

3.4. P. aeruginosa population structure

Considering both overall and individual CF unit data, considerable genetic diversity by PFGE (70 patterns) and MLST [72 sequence types (STs)] was observed among the collection (Table 4 and Fig. 1). Identical PFGE band patterns, STs and AT genotypes were detected in the *P. aeruginosa* isolates recovered from a pair of brothers (strains 78 and 79). Additionally, a similar PFGE pattern was detected in unrelated strains, some of which also matched other typing methodologies. The most relevant case concerned a possible intrahospital cross-transmission related to strains 66 and 68 with identical PFGE band patterns and AT genotypes but different MLST. Ascription to different STs was due to a single A331C *mut*S mutation that provokes the switch of allele 48 by allele 3, and consequently the assignation of ST1881 instead of ST348. Other

groups of strains with similar PFGE band patterns have been detailed in Table 4 and Fig. 2.

The MLST technique was applied to the entire collection (79 isolates), detecting a total of 72 different STs, 49 of which had not been described previously (six new alleles and non-previously described combinations of known alleles). A high genetically diverse background was observed throughout the MLST results, in which only six of the 49 STs grouped more than one isolate. International epidemic clones were not detected (Fig. 1).

A phylogenetic analysis of the concatenated sequences of the MLST alleles was performed with the study isolates and the entire MLST database to identify particular lineages. The results demonstrated a lack of genetic relationship between the study isolates, rejecting cross-transmission between the various CF units and the predominance of particular lineages (Figs 2 and 3).

3.5. AT genotyping: exploring the accessory genome

The AT multi-marker micro-array enabled the detection of 51 new and 14 previously described AT genotypes containing one to three isolates. The five most abundant AT genotypes (0C2E, 2C22, C40A, D421 and F429) in the global *P. aeruginosa* population [7–9] comprise 10.1% of this Spanish CF collection.

Ferripyoverdine receptor genes (*fpvA*) type I, IIa, IIb and III were detected in 23, 22, six and 11 isolates, respectively, and the alternative type I ferripyoverdine receptor gene *fpvB* was present in 45 isolates. Ferripyoverdine receptor genes were not detected in 15 isolates, and all but two were isolated in adults. These results showed that the isolates possess an average of 2.3 genome islets and 2.4 genome islands (ranging from 0 to 5).

The flagellin glycosylation island was the most prevalent (53 isolates, 67.0%). Among this subset of isolates, two lacked the a-type

Table 2
Percentage of antibiotic resistance, including intermediate plus resistant isolates, of the different *Pseudomonas aeruginosa* morphotypes (95% confidence intervals).

Morphotype	Percentage resistant to													% MDR	% XDR
	P/T	CAZ	CEP	AZT	IMI	MER	COL	GEN	TOB	AMK	CIP	LVX	FOS		
Mucoid ($n = 17$)	17.6 (3–43)	23.5 (6–50)	47.0 (22–72)	100	17.6 (3–43)	35.2 (14–61)	0	35.2 (14–61)	29.4 (10–55)	41.1 (18–67)	58.8 (32–81)	58.8 (32–81)	11.7 (1–36)	17.6 (3–43)	5.8 (0–28)
Enterobacteriaceae ($n = 23$)	17.3 (4–38)	30.4 (13–52)	34.7 (16–57)	100	30.4 (13–52)	34.7 (16–57)	0	39.1 (19–61)	39.1 (19–61)	34.7 (16–57)	43.4 (23–65)	56.5 (34–76)	21.7 (7–43)	56.5 (34–76)	21.7 (7–43)
Metallic ($n = 23$)	13.0 (2–33)	30.4 (13–52)	30.4 (13–52)	100	47.8 (26–69)	60.8 (38–80)	8.7 (1–28)	34.7 (16–57)	26.0 (10–48)	43.4 (23–65)	69.5 (47–86)	73.9 (51–89)	13.0 (2–33)	60.8 (38–80)	21.7 (7–43)
SCV ($n = 16$)	25.0 (7–52)	50.0 (24–75)	37.5 (15–64)	100	43.7 (19–70)	50.0 (24–75)	0	37.5 (15–64)	31.2 (11–50)	37.5 (15–64)	68.75 (41–88)	68.75 (41–88)	31.2 (11–50)	87.5 (61–98)	12.5 (1–38)
Total ($n = 79$)	17.7 (10–27)	32.9 (22–44)	36.7 (26–48)	100	35.4 (25–47)	45.5 (34–57)	2.5 (0–8)	36.7 (26–48)	31.6 (21–43)	39.2 (28–50)	59.4 (47–70)	64.5 (52–75)	18.9 (11–29)	55.7 (44–66)	16.4 (9–26)

P/T, piperacillin/tazobactam; CAZ, ceftazidime; CEP, cefepime; AZT, aztreonam; IMI, imipenem; MER, meropenem; COL, colistin; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; CIP, ciprofloxacin; LVX, levofloxacin; FOS, fosfomycin; SCV, small colony variants; MDR, multi-drug-resistant (not susceptible to at least three antimicrobial classes); XDR, extensively-drug-resistant (only susceptible to one or two antimicrobial classes).

Table 3
Mutator isolates detected in the study collection and their molecular basis.

Isolate	ST	Mutation frequency	Complemented with	Detected mutation
49	132	2.00×10^{-5}	*mutS*	$Nt_{399}\Delta12$
42	268	2.47×10^{-6}	*mutS*	$Nt_{1600}\Delta13$
16	270	2.93×10^{-7}	*mutS*	G1290A (E431K)
			mutL	G1872A (G632E)
9	1109	8.26×10^{-6}	*mutS*	$Nt_{1120}\Delta5$
13	1870	5.20×10^{-6}	ND	ND
26	1871	2.50×10^{-6}	*mutL*	T1309C (A437T)
27	1872	4.57×10^{-7}	*mutL*	T647G (V216G)
6	1890	1.38×10^{-6}	*mutL*	$Nt_{644}\Delta21$
47	1906	1.80×10^{-6}	*mutS*	$Nt_{1336}\Delta2$
53	1909	3.20×10^{-6}	*mutS*	$Nt_{1377}\Delta1$
55	1911	2.71×10^{-6}	*mutS*	$Nt_{2577}ins9pb$
60	1914	3.07×10^{-6}	*mutS*	$Nt_{1198}IS$-4-like

ST, sequence type; ND, not detected.

flagellin, and the three isolates expressing the a-type flagellin lacked the flagellin glycosylation island. PAGI-1 was detected in 45 isolates (56.9%), and the genomic islands of the CLC family PAGI-2/3 were found in 23 isolates (29.1%). Two isolates harboured both PAPI-1 and PAPI-2, and 34 (43%) isolates harboured PAPI-2; other pKLC102-like islands were detected in 30 isolates (37.9%). A significant

discrepancy was observed in the prevalence of T3SS effector *exoS* and *exoU* genes detected by this technique (18% and 9% of the isolates, respectively) and by independent specific PCR assays (81% and 10%, respectively). Statistical differences within the overall collection and within the different subsets (mutators, adult population and paediatric population) were not observed.

To investigate potential patient-to-patient and unit-to-unit transmission, the intraclonal diversity of all isolates was analysed, excluding the *exoS* and *exoU* genes as core markers (Table 2 and Fig. 2). This analysis revealed potential patient-to-patient transmission in two cases, including the pair of brothers, and isolates 66 and 68 from unrelated patients. In addition, the same AT genotype was detected in another two patients, each with two morphotypes but with a different repertoire of accessory genes (61, 62, 71 and 72). This finding supports the hypothesis that diversification occurs during adaptation to the CF lung.

4. Discussion

Surveillance and epidemiological studies are useful for detecting and controlling hypertransmissible and hypervirulent strains, and for studying the evolution of antimicrobial resistance trends and the colonisation rates of patients. This study addressed the

Table 4
Results obtained with pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST) and array tube (AT) techniques. Isolates with identical PGFE, MLST or AT genotype are marked in grey.

Centre	Isolate	PFGE	ST	AT genotype	Centre	Isolate	PFGE	ST	AT genotype
A	1	1	1748	X298	L	44	38	1876	2418
B	2	2	1886	6018		45	39	564	E418
	3	3	1887	6198		46	40	1905	2C18
C	4	3	1888	XX98		47	41	1906	4DA8
	5	4	1889	8782		48	42	644	2708
D	6	5	1890	F669		49	43	132	7C2C
	7	6	1891	4818		50	44	1877	3E18
	8	7	1866	7D98	M	51[a1]	45	1907	EC18
E	9	8	1109	CC0A		52[a1]	46	1908	2C2C
	10	9	1867	8C2C		53	47	1909	6A20
	11	10	1868	2C22		54	48	1910	6FA8
	12	11	1869	6FA8	N	55[a2]	49	1911	AF90
F	13	12	1870	F468		56[a2]	50	1251	F420
	14	13	1892	AD80		57	48	1878	6FA8
G	15	14	1893	ED98		58	51	1912	2610
	16	15	270	882A	O	59	52	1913	4498
	17	16	116	6010	P	60	53	1914	2810
	18	17	395	0C2E		61[a3]	54	1879	E020
	19	18	1894	0C2A		62[a3]	55	508	E020
	20	19	1895	2F80	Q	63	56	189	8E18
	21	20	1228	741C		64	57	1880	E429
	22	21	253	0C2A	R	65	58	253	D421
	23	22	395	0C2C		66	59	1881	2398
	24	23	1896	F428		67	60	1882	XC10
H	25	24	1897	0422		68	59	348	2398
	26	25	1871	2C18	S	69	61	1883	DF88
	27	26	1872	C40A		70	62	676	EC10
	28	27	1873	E022		71[a4]	63	508	E020
	29	28	575	859A		72[a4]	64	508	E020
	30	29	1898	8428		73	65	575	A598
	31	30	1899	F419		74	66	313	F429
I	32	30	1900	B01A		75	67	198	0C48
	33	31	617	2C20		76	68	569	6E10
J	34	32	1901	B01X		77	69	1884	0810
	35	33	560	F421	T	78	70	27	EA08
	36	34	1902	262A		79	70	27	EA08
	37	31	1903	2C20					
	38	35	1874	E42A					
K	39	35	1874	E428					
	40	36	609	A998					
	41	35	1875	E428					
	42	31	268	2C20					
	43	37	1904	2F88					

ST, sequence type.
[a] Different morphotypes in the same patient (from one to four).

Fig. 1. Minimum spanning tree combining the detected sequence type (numbers inside the circles) and the virulence determinants (colours). The particular cases of strains sharing pulsed-field gel electrophoresis band patterns (red lines) or pathotype (blue lines) are marked with circles, and the primary features are described.

primary phenotypic and genetic features of CF *P. aeruginosa* isolates recovered in the first microbiological multi-centre study performed in Spain [16].

Although this study had a relatively low representation of the total Spanish population with CF (9–10%) [https://www.ecfs.eu/projects/ecfs-patient-registry/annual-reports], the wide geographical distribution of the involved units (17 tertiary care hospitals) assured adequate sampling. To avoid errors related to the patient selection process, the first 15 consecutive patients who agreed to participate in the study were included, without employing any selective criteria for age, colonisation pattern or lung function. Although

geographical and seasonal differences in the prevalence of *P. aeruginosa* have been reported [22], no geographical differences were observed in this series. However, the seasonal effect may have been underestimated given that recruitment was conducted from March to October 2013, and the coldest period of the year was not included.

The CF patients in this study had low *P. aeruginosa* colonisation rates (22% overall, 32.7% in adults and 9.9% in children). These data, considered as point prevalence colonisation (data from one sputum sample per patient), are consistent with those of other contemporary studies that revealed a currently decreasing and slowing trend in *P. aeruginosa* lung colonisation in patients with CF. This finding could

Fig. 2. Phylogenetic analysis of each multi-locus sequence typing allele, comparing those found in the study collection (distribution is marked with a grey circle).

Fig. 3. Phylogenetic analysis of the concatenate sequences of the seven multi-locus sequence typing (MLST) alleles, comparing the whole MLST database with the study collection. Circles represent the 79 isolates from the present study, and the colour indicates the hospital in which they were isolated.

be related to better clinical management of patients, newborn screening and bacterial ecosystems [23]. Nevertheless, the possible lack of viable bacterial cells at freezing and defrosting has not been evaluated, and therefore this possibility should not be ruled out. Another important factor to consider is the convenience of a single sputum sample as representative of the total *P. aeruginosa* lung population. It has been demonstrated that different genetic lineages can co-exist in the lung compartments, and the entire population is usually underestimated in a single sputum sample [2,24]. Moreover, a recent report demonstrated the co-existence of several lineages along the respiratory tract in a single CF subject, with evidence of spatial separation between the nasopharynx and the lower lung [25].

Finally, the relatively low number of isolates together with their high genetic variability did not allow specific genotypes to be correlated with patients' clinical status. Nevertheless, despite these potential limitations, one of the most important results of this study is the lack of representation of international CF epidemic clones recognised in several European countries [4]. C40A is the AT genotype previously described for Clone C (ST17) [9,26], and was also detected in isolate 27 of the study collection. The epidemic Clone C belongs to ST17, and, curiously, isolate 27 typed as ST1872 is a double locus variant that differs in just two point mutations in *mutL* and *trpE* alleles. On the other hand, ST175 and ST111 are the most frequent *P. aeruginosa* lineages in the Spanish nosocomial setting [27], and neither lineage was present in the CF collection. These data support the hypothesis that CF isolates are usually acquired from the environment and not from hospital sources.

Overall, the mucoid morphotype was the most typical morphotype observed in the patients with CF. The clinical relevance of the SCV morphotype has been highlighted in recent years [28], and in the present study, SCVs were only detected in adults ($n = 16$) (median age 30 years), whereas the mucoid phenotype was present in adults ($n = 13$) and children ($n = 4$) (median ages 29 and 15 years, respectively). Overall, MDR resistance rates were slightly lower than those encountered in a recent study of *P. aeruginosa* isolates

recovered from CF patients in Northern Europe [29]. Moreover, resistance rates to individual agents were also lower than those encountered in Northern Europe.

High proportions of mutator isolates among the CF *P. aeruginosa* population have been demonstrated previously, and are frequently associated with antimicrobial resistance [30]. In this study, a lower proportion of mutators (15%) was found in comparison with another Spanish study (36%) [3], with 55% of these mutator isolates classified as MDR and 16% as XDR. Eight of the 12 mutator isolates were detected in adults, with a clear trend in relation to more evolved stages.

MLST is the reference technique [31], although the particular features of the CF *P. aeruginosa* isolates might limit the use of this technique [19,32]. In the study collection, MLST demonstrated a highly polyclonal structure with 72 different STs, 49 of which are described for the first time. The acquisition and loss of genetic traits between single lineages, combined with the natural exchange of virulence factors between unrelated isolates, was also suggested after the AT genotyping results. Nevertheless, identical AT genotypes (2C18, 2C22, 6FA8, E42A and 2398) were detected in unrelated strains from independent institutions.

P. aeruginosa possesses remarkable adaptability, primarily due to its large and complex genome. The pan-genome of this opportunistic pathogen consists of the conserved core genome (90%) and a combinatorial accessory genome (10%) essential for adaptation and survival in unfavourable conditions. *P. aeruginosa* might therefore have a large armamentarium of secreted virulence factors that rely on specialised export systems, including the type III secretion system (T3SS) [33]. The authors have investigated the presence of ExoS and ExoU (two T3SS effector proteins), and the results are consistent with previously published data for CF respiratory isolates [21,34].

A major example of *P. aeruginosa* adaptability is its ability to produce three different types of pyoverdine and four binding receptors. The major finding in this variable locus was the absence of the alternative receptor for pyoverdine type I (*fpvB*) in 43% of the isolates; this finding does not correlate with a number of previous studies [34] which found that almost all CF isolates harboured this receptor. Nevertheless, Dingemals et al also found that a significant proportion of CF isolates lacked this alternative receptor (22%) [35]. These authors hypothesised that this receptor might not be under selective pressure, because *P. aeruginosa* can use multiple iron uptake systems in the CF lung to acquire iron in both its ferric and ferrous forms [36,37]. As an alternative hypothesis, the authors suggested that the loss of *fpv*B could be an advantage for evading the immune system and the action of pyocines [35].

The precise role of glycosylation in flagellar function remains unclear, and the high proportion of the Spanish CF isolates harbouring the flagellin glycosylation island suggests that it might confer some advantages for persistence or adaptation. In contrast, other genomic islands (with the exception of PAPI-1) were apparently under-represented in the study collection compared with previous studies on CF isolates [7,8].

The intraclonal diversity analysis revealed two possible patient-to-patient transmissions, one of which was explained by the communal living of the two brothers. Clonal dissemination between CF units was not observed, although the paediatric and adult units were located in the same institution. The complete characterisation of the study isolates does not allow sporadic genetic exchange events between different lineages to be ruled out.

5. Conclusion

This study described the genetic and antimicrobial phenotypes of the Spanish CF *P. aeruginosa* isolates recovered from the first multi-centre study on the microbiology of CF performed in Spain.

This study demonstrated a genetically unrelated and highly diverse population with a total colonisation rate of 22% (32.7% in adults and 9.9% in children), moderate prevalence of mutators, and high antimicrobial resistance rates (except for colistin).

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Ethical approval: This project was approved by the hospital ethics committee.

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Insights into the evolution of the mutational resistome of *Pseudomonas aeruginosa* in cystic fibrosis

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> **"**The analysis of WGS mutational resistomes has proven to be useful for understanding the evolutionary dynamics of classical resistance mechanisms and to depict new ones for the majority of antimicrobial classes**"**

Chronic respiratory infection (CRI) by *Pseudomonas aeruginosa* is the main driver of morbidity and mortality in cystic fibrosis (CF) patients [1]. CRI results from an intense adaptation process, where bacterial evolution is tested against host immune responses and years of aggressive antimicrobial treatments [2]. Once established, CRI can seldom be eradicated despite intensive antimicrobial treatments, and therefore our therapeutic goals resignedly move from attempting to cure the infection to minimizing its long-term impact through chronic suppressive therapy [3]. The plasticity of *P. aeruginosa* genome for antimicrobial resistance acquisition, the greatly enhanced mutation supply rate provided by frequent hypermutable variants (mutators) and the highly structured environment determined by the characteristic biofilm growth and the anatomy of the respiratory tract make bacterial evolution and genetic diversification a hallmark of CF CRI [2,4]. While the enhanced evolution of antimicrobial resistance in CF, frequently linked to mutator phenotypes, was noted many years ago [5], it is with the introduction of whole-genome sequencing (WGS) that we are starting to understand its real dimensions [6].

The term resistome was first used to account for the set of primary antibiotic resistance genes that could be eventually transferred to human pathogens [7]. Soon after the concept of intrinsic resistome was introduced to include all chromosomal genes that are involved in intrinsic resistance, and whose presence in strains of a bacterial species is independent of previous antibiotic exposure and is not due to horizontal gene transfer (HGT) [8]. Finally, the term mutational resistome was more recently implemented to account for the set of mutations involved in the modulation of antibiotic resistance levels in the absence of HGT [9]. Recent WGS data obtained from *in vitro* assays on the evolution of antibiotic resistance and clinical isolates, and in particular sequential CF isolates, provide new insights into the evolutionary dynamics and mechanisms of *P. aeruginosa* antibiotic resistance. However, in too many cases, the documented genomic variations fail to provide causative relations in the absence of phenotypic information. The analysis of WGS mutational resistomes has proven to be useful for understanding the evolutionary dynamics of classical resistance mechanisms and to depict new ones for the majority of antimicrobial classes, including β-lactams, aminoglycosides, fluoroquinolones and polymixins.

Regarding β-lactams, the analysis of WGS mutational resistomes has confirmed the major role of classical resistance mutations such as those leading to the overexpression of the chromosomal β-lactamase AmpC (such as DacB [PBP4], AmpD and/or AmpR mutations) or the inactivation of the carbapenem porin OprD. However, the analysis of mutational resistomes of *in vitro* evolved strains and sequential CF isolates have identified other key mutations, such as those occurring in β-lactam targets (essential PBPs), particularly involving mutations in *ftsI* which encodes PBP3, an essential high molecular class B penicillin binding protein (PBP) with transpeptidase

Future Medicine

activity. Indeed, data from CF patients [9,10] as well as from *in vitro* studies [11] have recently demonstrated that PBP3 is under strong mutational pressure, with specific mutations contributing to β-lactam resistance development. Among them are particularly relevant and frequent mutations affecting amino acids R504 or F533, located within the protein domains responsible for the formation and stabilization of the inactivating complex β-lactam–PBP3. Moreover, PBP3 mutations seem to play a role in the emergence of resistance to novel β-lactam–β-lactamase inhibitor combinations, such as ceftolozane/tazobactam [9]. Another relevant mutational β-lactam resistance mechanism is the selection of large (>200 kb) deletions affecting specific parts of the chromosome. Although the basis of the conferred resistance phenotype still needs to be further clarified, these mutants can be recognized by the characteristic brown pigment (pyomelanine) caused by the deletion of one of the affected genes, *hmgA*, coding for a homogentisate-1,2-dioxygenase. This type of deletion has been documented in both, *in vitro* evolved β-lactam-resistant mutants and CF isolates [11,12]. However, the deletion of *hmgA* is not responsible for the resistance phenotype, which may be linked to the deletion of another of the affected genes, *galU*, coding for a UDP-glucose pyrophosphorylase required for lipopolysaccharide core synthesis. Indeed, analysis of transposon mutant libraries has shown that inactivation of galU increases ceftazidime and meropenem minimum inhibitory concentrations [13,14]. Finally, another emerging mutational β-lactam resistance mechanism is the structural modification of AmpC [10,11].

With respect to aminoglycosides, results from analysis of mutational resistomes of CF isolates point to the underlying strong evolutionary pressure of *mexZ* and the relevance of MexXY overexpression for resistance development [15–17]. Moreover, recent *in vitro* studies and findings from CF isolates have revealed that high-level aminoglycoside resistance requires the acquisition of additional mutations; among them, those in FusA1 seem to be particularly frequent and relevant [9,16,18–19]. Likewise, the fluoroquinolone resistome frequently includes mutations in efflux pump regulators, among which *nfxB*, leading to the overexpression of MexCD-OprJ, is particularly noteworthy in the CF setting. However, high-level ciprofloxacin resistance generally involves one or several mutations in the quinolone resistance determining regions of GyrA/B and/or ParC/E [9]. Regarding polymixin resistance, findings from WGS studies of *in vitro* evolved strains and CF isolates have shown that development of high-level colistin resistance requires the acquisition of multiple mutations, including those in the two-component regulators (PmrAB, PhoPQ or ParRS) involved in the addition of 4-amino-4-deoxy-L-arabinose to lipid A from the lipopolysaccharide [9,20]. Finally, in addition to the resistance mechanisms to classical antipseudomonal agents, the CF mutational resistome may also include resistance to other used antibiotics such as the frequent mutations of domain V of 23S rRNA – conferring macrolide resistance [21].

The complexity of the CF isolates resistomes is further enhanced when the intrapatient genetic diversity of CF *P. aeruginosa* populations is introduced. Certainly, to understand the resistance dynamics and evolution, future steps should endeavor to analyze the mutational resistomes in CF at the whole population level, as opposed to the analysis of single isolated colonies. Indeed, full understanding of the evolution of the mutational resistome requires a longitudinal and transversal analysis of *P. aeruginosa* populations in the CF patient. Moreover, recent evidence suggests that interpatient transmission should also be considered when analyzing the evolution of the mutational resistome, especially when introducing mutator lineages of epidemic clones [9].

Beyond addressing a relevant scientific question, the analysis of mutational resistomes would be useful for therapeutic strategy design and monitoring the efficacy of administered antibiotic treatments. Obviously, the evolution of the mutational resistome is a direct consequence of antimicrobial exposure. As such, it is not surprising that exposure to one antibiotic drives evolution of the mutational resistome for that antibiotic. However, the complexity of the actual resistance profile is further increased by the specificity and interactions among different resistance mechanisms. A classic example is cross resistance (or collateral resistance), which implies that exposure to one antibiotic drives also the development of resistance to a different one. Typically this is caused by the developed resistance mechanism (such as efflux pump overexpression) affecting simultaneous different antibiotics. Indeed, potential development of cross resistance is a major issue to consider when using antibiotic combinations [22].

Perhaps less obvious is collateral susceptibility, which implies that exposure to one antibiotic increases the susceptibility to a different one. This might be achieved through two mechanisms. One possible mechanism is that exposure to one antibiotic directly causes increased susceptibility to a different one, for example, mutations in the β-lactamase AmpC increases cephalosporin hydrolysis while reducing that of penicillins or carbapenems [23]. The second possibility is that the development of a resistance mechanism impairs the activity of another existing resistance mechanism, for example, competition between the different efflux pumps, the overexpression of one may impair the expression of another [24]. Indeed, in the CF setting it is very frequent that the overexpression of efflux pump MexXY, involved in aminoglycoside resistance, is linked to the impaired expression of efflux pump MexAB,

involved in the resistance to a broad range of antibiotics including most β-lactams [25]. Thus, the evolution of the mutational resistome for a given antibiotic is not only dependent on the exposure to this antibiotic, but it is also conditioned by the simultaneous or even previous exposures to other antibiotics. An illustrative example is provided in a recent *in vitro* study that demonstrated, for a broad range of antibiotic classes, that the history of exposure and resistance development to a given antibiotic, conditions the dynamics and mechanisms of resistance development when exposed to a second one [18].

Moreover, knowledge of the interactions between resistance mechanisms could be useful in the design of sequential treatments that minimize the risk of resistance development. Such is the case for a recent *in vitro* study showing the effectiveness of aztreonam–tobramycin sequential treatment, based on the antagonism between the resistance mechanisms for each of the antibiotics; overexpression of the efflux pump MexAB (aztreonam) or MexXY (tobramycin) which compete for the same outer membrane channel (OprM) [26].

Finally, it should be noted that a hallmark of *P. aeruginosa* CRI in CF is the biofilm mode of growth. Indeed, biofilm growth, in addition to providing a compact structured environment likely facilitating the evolution of mutational resistance [27], they also add further complexity due to the major differences in the mechanisms involved when compared with conventional planktonic growth that needs to be considered [28]. Likewise, persistence of *P. aeruginosa* in the CF lung despite intensive antimicrobial treatments relays in the acquisition of a vast number of adaptive mutations that extend far beyond classical antibiotic resistance mutations [29].

In summary, the comprehensive analysis of the mutational resistomes of *P. aeruginosa* in CF CRI is expected to become a useful tool for optimizing therapeutic strategies and monitoring the efficacy of administered antibiotic treatments in the near future.

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Evolution of the *Pseudomonas aeruginosa* Aminoglycoside Mutational Resistome *In Vitro* and in the Cystic Fibrosis Setting

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ABSTRACT Inhaled administration of high doses of aminoglycosides is a key maintenance treatment of *Pseudomonas aeruginosa* chronic respiratory infections in cystic fibrosis (CF). We analyzed the dynamics and mechanisms of stepwise high-level tobramycin resistance development *in vitro* and compared the results with those of isogenic pairs of susceptible and resistant clinical isolates. Resistance development correlated with *fusA1* mutations *in vitro* and *in vivo*. *pmrB* mutations, conferring polymyxin resistance, were also frequently selected *in vitro*. In contrast, mutational overexpression of MexXY, a hallmark of aminoglycoside resistance in CF, was not observed in *in vitro* evolution experiments.

KEYWORDS *Pseudomonas aeruginosa*, aminoglycosides, antibiotic resistance, drug resistance mechanisms, mutational resistome, whole-genome sequencing

Pseudomonas aeruginosa is one of the most frequent and severe causes of acute nosocomial infections, as well as the main driver of morbidity and mortality in patients suffering from cystic fibrosis (CF) or other chronic respiratory diseases (1, 2). Compared with other Gram-negative pathogens, *P. aeruginosa* exhibits a basal reduced susceptibility to many antibiotics, and this intrinsic resistance can be much further enhanced by the acquisition of transferable resistance determinants and by the selection of mutations that alter the expression and/or function of diverse chromosomal genes (3–6). This outstanding ability has led to an increasing prevalence of chronic and nosocomial infections produced by multidrug-resistant (MDR) or extensively drug-resistant (XDR) *P. aeruginosa* strains that sharply compromises the selection of appropriate treatments (7, 8). Together with polymyxins, aminoglycosides are often among the few therapeutic options in this scenario (9, 10). Moreover, provision of high local concentrations of tobramycin through inhaled administration has been the basis of the treatment for *P. aeruginosa* chronic respiratory infections in CF patients for many years (11). Whereas resistance to these agents in acute infections is mainly attributed to the production of aminoglycoside-modifying enzymes or 16S rRNA methyltransferases, resistance development in the chronic infection setting has been linked to the selection of chromosomal mutations that lead to enhanced membrane impermeability or MexXY-OprM efflux pump overexpression (12–14). However, high-level resistance development likely requires the accumulation of different resistance mechanisms, and, although recent reports suggest the involvement of additional chromosomal mutations (15–20), there are still important knowledge gaps in this field. Thus, the aim of this work was to analyze the *in vitro* evolution of the aminoglycoside mutational resistome of *P. aeruginosa* and to correlate the documented mutations with those observed *in vivo* during the course of CF chronic respiratory infection.

To determine the dynamics of aminoglycoside resistance development, ~10^6 CFU/ml of exponentially growing *P. aeruginosa* PAO1 reference strain isolates were inoculated into

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FIG 1 (A to E) Dynamics of resistance development to tobramycin and mutations encountered after 1, 7, and 14 days of tobramycin exposure in five replicate experiments. Genetic determinants and specific mutations are boldfaced when detected within the two representative colonies studied at each experiment and time point. Genes whose implications in aminoglycoside resistance development have already been demonstrated are indicated with an asterisk. (F) Median expression level of *mexY* for PAO1-derived resistant mutants after 1 and 14 days of tobramycin exposure.

10-ml Mueller-Hinton tubes containing 0.5×, 1×, 2×, 4×, 8×, 16×, 32×, 64×, 128×, 256×, 512×, and 1,024× MIC (0.5 μg/ml) values and incubated for 24 h at 37°C and 180 rpm. Tubes from the highest antibiotic concentration showing growth were reinoculated (at a 1:1,000 dilution) in fresh medium containing concentrations up to 1,024× MIC and incubated under the same conditions. This evolution experiment was performed for 14 consecutive days in quintuplicate. At days 1, 7, and 14, two colonies from each of the five replicate experiments were purified in antibiotic-free LB agar plates, and MIC values of tobramycin, gentamicin, amikacin, ticarcillin, piperacillin-tazobactam, ceftazidime, cefepime, aztreonam, ceftolozane-tazobactam, imipenem, meropenem, ciprofloxacin, and colistin were determined by broth microdilution and interpreted according to CLSI 2017 clinical breakpoints (21). Whole-genome sequences (WGS) of all the mutants were obtained and analyzed following previously described protocols (9, 22, 23). Likewise, to correlate *in vitro* with *in vivo* findings, three isogenic pairs (confirmed by pulsed-field gel electrophoresis) of tobramycin-susceptible and -resistant isolates obtained from respiratory samples of three different chronically infected CF patients treated with tobramycin at Son Espases University Hospital were also fully sequenced, and variations in 164 genes related to antibiotic resistance were analyzed (22).

As shown in Fig. 1 panels A to E, *in vitro* resistance development occurred in a stepwise manner, reaching concentrations ranging from 128 to 512 higher than the initial MIC (0.5 μg/ml). The corresponding tobramycin MICs of the purified colonies at day 14 ranged from 64 to 512 μg/ml, whereas those of gentamicin and amikacin were typically 1 or 2 dilutions higher (see Data Set S1 in the supplemental material). These concentrations are close to the maximum tobramycin concentrations achieved through inhaled administration and in the range of the breakpoints suggested for inhaled therapy (24).

Results obtained from WGS experiments are summarized in Fig. 1 and detailed in Data Set S1. Up to 35 different genes were found to be mutated in at least one of the

TABLE 1 Genomic differences between the three isogenic pairs of tobramycin-susceptible and -resistant CF isolates

Locus/gene[a]	Isolate ID (MIC$_{TOB}$ mg/liter)					
	FQSE06-S (1)	FQSE06-R (24)	FQSE11-S (2)	FQSE11-R (>256)	FQSE16-S (4)	FQSE16-R (64)
PA0004/*gyrB*				R138L		
PA0058/*dsbM*			C28R, F206L, R212C			
PA0426/*mexB*			nt772Δ1			Q575R
PA0958/*oprD*				Q424E, S403A		
PA1430/*lasR*				R216Q		
PA2018/*mexY*		G287A				G287S
PA2020/*mexZ*	nt290Δ11	S9P	L138R	L138R		R125P
PA2492/*mexT*						G274D, G300D
PA2639/*nuoD*		G499X				
PA3064/*pelA*	V446I					
PA3141/*capD*					nt512ins1	
PA3168/*gyrA*				Y267N		
PA4020/*mpl*		S257L		Q248X		
PA4266/*fusA1*		Y552C, T671I				Y552C
PA4418/PBP3		P215L				
PA4462/*rpoN*						V473A
PA4568/*rplU*	I74 M					
PA4598/*mexD*					P721S, L624P	
PA4600/*nfxB*				E75K		
PA4773/-				A165T		
PA5040/*pilQ*	E676D, E669D					
ResFinder				AacA4		
mexY overexpression	+	+	+	+	−	+

[a]Genes in which mutations were also detected in the resistance evolution experiment are boldfaced.

isolates. Of these, 22 isolates (63%) were already related to aminoglycoside resistance development in previous studies (15–20, 22), so our data confirm their relevance in this stepwise process; mutations in other genes are evidenced for the first time in this work, and their relevance will need to be confirmed in further studies. Mutants from day 14 showed between 3 and 7 mutations, and comparison with those from days 1 and 7 showed a stepwise acquisition. However, a few mutations documented at these intermediate stages were not fixed in the population and thus were not seen at day 14 (Fig. 1).

Among the mutated genes, *fusA1*, which codes for elongation factor G, deserves special attention because nonsynonymous mutations within this gene were detected in all five replicate experiments. Note that the time of detection of *fusA1* mutations varied from day 1 to day 14 and that the same amino acid substitution (I61M) occurred in 3 of them. This novel mechanism was recently confirmed through site-directed mutagenesis by Bolard et al. (15), being associated with a 1- to 3-fold increase in the MICs of tobramycin, gentamicin, and amikacin, which correlates with our observations (Data Set S1). Mutations in *fusA1* were recently noted to be frequent among CF patients (22, 25–27). Moreover, the emergence of *fusA1* mutations was noted in two of the three tobramycin-resistant CF isolates studied in this work (Table 1; see Data Set S2 in the supplemental material). Interestingly, using ResFinder, the resistant isolate not showing *fusA1* mutations was shown to have acquired an exogenous aminoglycoside-modifying enzyme (AacA4). This surprising finding highlights the fact that, although mutational resistance is thought to be the rule in CF chronic infections, horizontally acquired resistance must be ruled out as well.

Another frequently mutated gene (3 of 5 replicates) was, intriguingly, *pmrB*, which codes for the sensor kinase of the two-component regulatory system PmrA-PmrB (28). *pmrB* mutations have traditionally been linked to polymyxin resistance development (29, 30), so this finding alerts from a possible mechanism of coresistance to two relevant antipseudomonal agents (polymyxins and aminoglycosides). Indeed, the emergence of *pmrB* mutations at day 7 correlated with increased colistin MICs (Data Set S1). However, despite the *pmrB* mutations persisting at day 14, colistin resistance disappeared, likely indicating the acquisition of compensatory mutations. In one of these isolates from day

FIG 2 MIC fold changes for each antibiotic tested between parental strain PAO1 and its derived aminoglycoside-resistant mutants. TIC, ticarcillin; TZP, piperacillin-tazobactam; C/T, ceftolozane-tazobactam; CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; IMI, imipenem; MER, meropenem; CIP, ciprofloxacin; CST, colistin. Lower limit for CIP and TZP is −1 2-fold dilutions.

14, an additional mutation in *pagL* involved in lipid A deacylation and polymyxins resistance was documented (Fig. 1; Data Set S1) (31). In relation to other antibiotic classes, we observed a general trend over time toward decreasing MICs, particularly for ticarcillin, aztreonam, and ciprofloxacin (Fig. 2). This phenomenon is globally known as collateral susceptibility (32). It has been largely observed among *P. aeruginosa* clinical isolates (33), and it has traditionally been linked to a mutational or functional loss of the multidrug efflux system MexAB-OprM (34). Moreover, this phenotype has been proposed to result from an efflux imbalance between the MexAB-OprM and MexXY-OprM systems, as both compete for the recruitment of OprM (35). Nevertheless, our results and those recently published by Bolard et al. (15) suggest that other mechanisms may be involved in this frequently observed phenotype.

Beyond the mutations actually detected, another relevant aspect to consider is the mutations that were expected but not found in our *in vitro* evolution experiments. Intriguingly, mutations leading to the overexpression of MexXY (*mexZ*, PA5471, *parS*), which are a hallmark of aminoglycoside resistance development in the CF setting, were not seen at any time in any of the five replicate experiments. The absence of mutations in these genes was additionally confirmed through Sanger sequencing. Moreover, while *mexY* expression data varied to some extent for the different mutants (real-time reverse transcription-PCR [22, 23]), values were always well below those of a control *mexZ* PAO1 mutant, and a statistically significant trend toward increased expression at day 14 versus day 1 was not documented (Fig. 1F). In contrast to *in vitro* findings, all three CF tobramycin-resistant isolates overexpressed *mexY* and showed *mexZ* mutations (Table 1; Data Set S2). However, *mexY* overexpression and *mexZ* mutations were also seen in two of the three susceptible CF isolates. Thus, our results indicate that mutational overexpression of MexXY is not required for the evolution of high-level tobramycin resistance *in vitro*. On the other hand, findings from CF isolates reveal that mutations leading to the overexpression of MexXY are frequent, occur early, and are associated with low-level resistance. These results may indicate that the positive selection of mutations leading to the overexpression of MexXY in CF might be driven by factors beyond exposure to aminoglycosides.

Altogether, this work provides relevant insights into the evolution of the aminoglycoside resistome, balancing understanding of the role of novel (e.g., *fusA1* mutations) and classic (e.g., mutational MexXY overexpression) resistance mechanisms *in vitro* and in CF chronic respiratory infections. However, further studies are needed to decipher the precise role of some of the mutations detected, such as those in PmrB, in the evolution of aminoglycoside resistance in *P. aeruginosa*.

Accession number(s). Sequence files have been deposited in the European Nucleotide Archive under study PRJEB24151 (accession numbers ERS2060747, ERS2060748, ERS2060749, ERS2060750, ERS2077566, and ERS2077567).

SUPPLEMENTAL MATERIAL

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The Versatile Mutational Resistome of *Pseudomonas aeruginosa*

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One of the most striking features of *Pseudomonas aeruginosa* is its outstanding capacity for developing antimicrobial resistance to nearly all available antipseudomonal agents through the selection of chromosomal mutations, leading to the failure of the treatment of severe hospital-acquired or chronic infections. Recent whole-genome sequencing (WGS) data obtained from *in vitro* assays on the evolution of antibiotic resistance, *in vivo* monitoring of antimicrobial resistance development, analysis of sequential cystic fibrosis isolates, and characterization of widespread epidemic high-risk clones have provided new insights into the evolutionary dynamics and mechanisms of *P. aeruginosa* antibiotic resistance, thus motivating this review. Indeed, the analysis of the WGS mutational resistome has proven to be useful for understanding the evolutionary dynamics of classical resistance pathways and to describe new mechanisms for the majority of antipseudomonal classes, including β-lactams, aminoglycosides, fluoroquinolones, or polymyxins. Beyond addressing a relevant scientific question, the analysis of the *P. aeruginosa* mutational resistome is expected to be useful, together with the analysis of the horizontally-acquired resistance determinants, for establishing the antibiotic resistance genotype, which should correlate with the antibiotic resistance phenotype and as such, it should be useful for the design of therapeutic strategies and for monitoring the efficacy of administered antibiotic treatments. However, further experimental research and new bioinformatics tools are still needed to overcome the interpretation limitations imposed by the complex interactions (including those leading to collateral resistance or susceptibility) between the 100s of genes involved in the mutational resistome, as well as the frequent difficulties for differentiating relevant mutations from simple natural polymorphisms.

Keywords: antibiotic resistance, resistome, *Pseudomonas aeruginosa*, multidrug resistance, evolution, resistance development, mutation

INTRODUCTION

Pseudomonas aeruginosa is one of the most frequent and severe causes of hospital-acquired infections, particularly affecting immunocompromised (especially neutropenic) and Intensive Care Unit (ICU) patients. Indeed, *P. aeruginosa* is the first cause of ventilator associated pneumonia (VAP) and burn wound infections, both associated with a very high mortality rate (Vincent, 2003; Bassetti et al., 2012). Likewise, *P. aeruginosa* is the most frequent driver of chronic respiratory infections in cystic fibrosis (CF) patients or other chronic underlying diseases (Döring et al., 2011).

One of the most striking features of *P. aeruginosa* is its outstanding capacity for developing antimicrobial resistance to nearly all available antipseudomonal agents through the selection of chromosomal mutations. Indeed, treatment failure caused by the development of antimicrobial resistance is a too frequent outcome of *P. aeruginosa* infections. The problem of mutation-mediated antibiotic resistance is further amplified in the chronic infection setting, due to the very high prevalence of hypermutable strains, showing greatly enhanced spontaneous mutation rates caused by defective DNA repair or error avoidance systems (Oliver et al., 2000; Maciá et al., 2005).

Beyond the obvious negative impact of resistance development for the treated patient, the accumulation of several of these chromosomal mutations leads to the emergence of multidrug resistant (MDR), extensively drug-resistant (XDR) or even pan-antibiotic-resistant (PDR) strains, which can be responsible for notable epidemics in the hospital setting (Deplano et al., 2005; Suarez et al., 2011). Moreover, recent studies have evidenced the existence of MDR/XDR global clones disseminated in different hospitals worldwide, and for that reason they have been denominated high-risk clones (Woodford et al., 2011; Oliver et al., 2015). Although high-risk clones are frequently associated with transferable antimicrobial resistance determinants, they also typically show a wide range of chromosomal mutations playing a major role in the resistance phenotype (Oliver et al., 2015). Likewise, recent reports have evidenced the interpatient spread of antimicrobial resistance mutations linked to the transmission of epidemic CF strains (López-Causapé et al., 2017).

Along with growing information from mechanistic studies on chromosomal resistance mechanisms and their complex regulatory pathways, involved in adaptive resistance (Lister et al., 2009; Muller et al., 2011; Skiada et al., 2011; Juan et al., 2017), the introduction of whole-genome sequencing (WGS) approaches is shaping up a new dimension for the mutational resistance landscape. The term resistome was first used to account for the set of primary antibiotic resistance genes that could be eventually transferred to human pathogens (D'Costa et al., 2006). Soon after the concept of intrinsic resistome was introduced to include all chromosomal genes that are involved in intrinsic resistance, and whose presence in strains of a bacterial species is independent of previous antibiotic exposure and is not due to horizontal gene transfer (HGT) (Fajardo et al., 2008). Finally, the term mutational resistome was more recently implemented to account for the set of mutations involved in the modulation of antibiotic resistance levels in the absence of HGT (Cabot et al., 2016b; López-Causapé et al., 2017). Recent WGS data obtained from *in vitro* assays on the evolution of antibiotic resistance, *in vivo* monitoring of antimicrobial resistance development, analysis of sequential CF isolates, and characterization of wide spread epidemic high-risk clones provide new insights into the evolutionary dynamics and mechanisms of *P. aeruginosa* antibiotic resistance (Cabot et al., 2016a; Feng et al., 2016; Del Barrio-Tofiño et al., 2017; Jaillard et al., 2017; López-Causapé et al., 2017). Indeed, the analysis of WGS mutational resistomes has proven to be useful for understanding the evolutionary dynamics of classical resistance mechanisms and to depict

new ones for the majority of antimicrobial classes, including β-lactams, aminoglycosides, fluoroquinolones, polymixins and others, as reviewed in the following sections. **Table 1** summarizes the main genes and mutations known to increase resistance levels and therefore shaping up the *P. aeruginosa* mutational resistome.

β-LACTAM MUTATIONAL RESISTOME

The most frequent mutation-driven β-lactam resistance mechanism is likely the overproduction of the chromosomal cephalosporinase AmpC, involving a wide range of genes belonging to complex regulatory pathways of cell-wall recycling (Juan et al., 2017). Among them, the mutational inactivation of *dacB*, encoding the non-essential penicillin-binding protein (PBP) PBP4, and *ampD*, encoding a *N*-acetyl-muramyl-L-alanine amidase have been found to be the most frequent cause of *ampC* derepression and β-lactam resistance (Juan et al., 2005; Moya et al., 2009). The inactivation of PBP4 has also been shown to activate the BlrAB/CreBC regulatory system, further increasing resistance levels (Moya et al., 2009). Additionally, specific point mutations leading to a conformation change in the transcriptional regulator AmpR, causing *ampC* upregulation and β-lactam resistance, have been noted among clinical strains. They include the D135N mutation, described in several species besides *P. aeruginosa*, including *Stenotrophomonas maltophilia*, *Citrobacter freundii,* or *Enterobacter cloacae* (Juan et al., 2017) or the R154H mutation, linked to the widespread MDR/XDR ST175 high-risk clone. Mutation of many other genes, including those encoding other amidases (AmpDh2 and AmpDh3), other PBPs (such as PBP5 and PBP7), lytic transglycosylases (such as SltB1 and mltB), MPL (UDP-*N*-acetylmuramate:Lalanyl-γ-D-glutamyl-meso-diaminopimelate ligase), or NuoN (NADH dehydrogenase I chain N) have been shown to enhance *ampC* expression, either alone or combined with other mutations, although their impact on β-lactam resistance among clinical strains still needs to be further analyzed (Juan et al., 2017).

In addition to *ampC* overexpression, recent studies have revealed that β-lactam resistance development, including the novel combinations of β-lactam-β-lactamase inhibitors ceftolozane/tazobactam and ceftazidime/avibactam, may result from mutations leading to the structural modification of AmpC (Cabot et al., 2014; Lahiri et al., 2014; Fraile-Ribot et al., 2017a; Haidar et al., 2017). Likewise, recent studies identified diverse AmpC variants associated with high-level cephalosporin resistance, including ceftolozane/tazobactam and ceftazidime/avibactam, in a small proportion (around 1%) of clinical *P. aeruginosa* isolates (Berrazeg et al., 2015). Over 200 Pseudomonas Derived Cephalosporinase (PDC) variants have been described so far, including those associated with enhanced ceftolozane/tazobactam and ceftazidime/avibactam resistance (**Table 1**). An update database of PDC variants is maintained in our laboratory and is freely available at https://arpbigidisba.com. Additionally, resistance development to ceftolozane/tazobactam and/or ceftazidime/avibactam may involve mutations leading to the structural modification of narrow spectrum OXA-2 and OXA-10 acquired oxacillinases (Fraile-Ribot et al., 2017a,b).

TABLE 1 | Main genes and mutations known to be involved in increased *P. aeruginosa* antibiotic resistance.

Gene	Resistance mechanisms/ altered target	Antibiotics affected[a]	Type of mutation	Relevant examples of gain-of-function mutations	Reference
gyrA	DNA gyrase	FQ	Gain-of-function	G81D, T83A, T83I, Y86N, D87G, D87N, D87Y, Q106L	Bruchmann et al., 2013; Kos et al., 2015; Cabot et al., 2016b; Del Barrio-Tofiño et al., 2017; López-Causapé et al., 2017
gyrB	DNA gyrase	FQ	Gain-of-function	S466F, S466Y, Q467R, E468D	Bruchmann et al., 2013; Kos et al., 2015; Del Barrio-Tofiño et al., 2017; López-Causapé et al., 2017
parC	DNA topoisomerase IV	FQ	Gain-of-function	S87L, S87W	Bruchmann et al., 2013; Kos et al., 2015; Cabot et al., 2016b; Del Barrio-Tofiño et al., 2017
parE	DNA topoisomerase IV	FQ	Gain-of-function	S457G, S457T, E459D, E459K	Bruchmann et al., 2013; Kos et al., 2015; Del Barrio-Tofiño et al., 2017; López-Causapé et al., 2017
pmrA	Lipopolysaccharide (lipid A)	COL	Gain-of-function	L157Q	Lee and Ko, 2014
pmrB	Lipopolysaccharide (lipid A)	COL	Gain-of-function	L14P, A54V, R79H, R135Q, A247T, A248T, A248V, R259H, M292I, M292T	Barrow and Kwon, 2009; Moskowitz et al., 2012
phoQ	Lipopolysaccharide (lipid A)	COL	Loss-of-function		
parR	Lipopolysaccharide (lipid A)	COL	Gain-of-function	M59I, E156K	Muller et al., 2011; Guénard et al., 2014
	OprD downregulation	IMP, MER			
	MexEF-OprN hyperproduction	FQ			
	MexXY-OprM hyperproduction	FQ, AMG, CEF			
parS	Lipopolysaccharide (lipid A)	COL	Gain-of-function	L14Q, V101M, L137P, A138T, A168V Q232E, G361R	Muller et al., 2011; Fournier et al., 2013; Guénard et al., 2014
	OprD downregulation	IMP, MER			
	MexEF-OprN hyperproduction	FQ			
	MexXY-OprM hyperproduction	FQ, AMG, CEF			
cprS	Lipopolysaccharide (lipid A)	COL	Gain-of-function	R241C	Gutu et al., 2013
colR	Lipopolysaccharide (lipid A)	COL	Gain-of-function	D32N	Gutu et al., 2013
colS	Lipopolysaccharide (lipid A)	COL	Gain-of-function	A106V	Gutu et al., 2013
mexR	MexAB-OprM hyperproduction	FQ, CAZ, CEF, PPT, MER, CAZ/AVI	Loss-of-function		
nalC	MexAB-OprM hyperproduction	FQ, CAZ, CEF, PPT, MER, CAZ/AVI	Loss-of-function		
nalD	MexAB-OprM hyperproduction	FQ, CAZ, CEF, PPT, MER, CAZ/AVI	Loss-of-function		
nfxB	MexCD-OprJ hyperproduction	FQ, CEF	Loss-of-function		
mexS	MexEF-OprN hyperproduction	FQ	Loss-of-function		
	OprD downregulation	IMP, MER			
mexT	MexEF-OprN hyperproduction	FQ	Gain-of-function	G257S, G257A	Juarez et al., 2018
	OprD downregulation	IMP, MER			

(Continued)

TABLE 1 | Continued

Gene	Resistance mechanisms/altered target	Antibiotics affected[a]	Type of mutation	Relevant examples of gain-of-function mutations	Reference
cmrA	MexEF-OprN hyperproduction	MER, FQ	Gain-of-function	A68V, L89Q, H204L, N214K	Juarez et al., 2017
mvaT	MexEF-OprN hyperproduction	FQ	Loss-of-function		
PA3271	MexEF-OprN hyperproduction	FQ	Loss-of-function		
mexZ	MexXY-OprM hyperproduction	FQ, AMG, CEF	Loss-of-function		
PA5471.1	MexXY-OprM hyperproduction	FQ, AMG, CEF	Loss-of-function		
amgS	MexXY-OprM hyperproduction	FQ, AMG, CEF	Gain-of-function	V121G, R182C	Lau et al., 2015
oprD	OprD inactivation	IMP, MER	Loss-of-function		
ampC	AmpC structural modification	CAZ/AVI, C/T	Gain-of-function	T96I, G183D, E247K	Cabot et al., 2014; Fraile-Ribot et al., 2017a
ampD	AmpC hyperproduction	CAZ, CEF, PPT	Loss-of-function		
ampDh2	AmpC hyperproduction	CAZ, CEF, PPT	Loss-of-function		
ampDh3	AmpC hyperproduction	CAZ, CEF, PPT	Loss-of-function		
ampR	AmpC hyperproduction	CAZ, CEF, PPT	Gain-of-function	D135N, G154R	Bagge et al., 2002; Cabot et al., 2016b
dacB	AmpC hyperproduction	CAZ, CEF, PPT	Loss-of-function		
ftsI	Penicillin-binding-protein 3 (PBP3)	CAZ, CEF, PPT, MER, CAZ/AVI, C/T	Gain-of-function	R504C, R504H, P527T, F533L	Diaz Caballero et al., 2015; Cabot et al., 2016a,b; Del Barrio-Tofiño et al., 2017; López-Causapé et al., 2017
fusA1	Elongation factor G	AMG	Gain-of-function	I61M, V93A, E100G, K504E, Y552C, P554L, A555E, N592I, P618L, T671A, T671I	Feng et al., 2016; Bolard et al., 2017; Del Barrio-Tofiño et al., 2017; López-Causapé et al., 2017, 2018
glpT	Transporter protein GlpT	FOS	Loss-of-function		
rpoB	RNA polymerase β-chain	RIF	Gain-of-function	S517F, Q518R, Q518L, D521G, H531Y, H531L, S536F, L538I, S579F, S579Y, N629S, D636Y	Jatsenko et al., 2010

[a]*FQs, fluoroquinolones; COL, colistin; AMGs, aminoglycosides; CAZ, ceftazidime; CEF, cefepime; PPT, piperacillin-tazobactam; IMP, imipenem; MER, meropenem; CAZ/AVI, ceftazidime/avibactam; C/T, ceftolozane/tazobactam; FOS, fosfomycin; RIF, rifampicin.*

Besides β-lactamases, there is increasing evidence on the role of target modification in *P. aeruginosa* β-lactam resistance. Particularly noteworthy are the mutations in *ftsI*, encoding PBP3, an essential high molecular class B PBP with transpeptidase activity (Chen et al., 2016). Indeed, data from CF patients (Diaz Caballero et al., 2015; López-Causapé et al., 2017), epidemic high-risk clones (Cabot et al., 2016b; Del Barrio-Tofiño et al., 2017) as well as from *in vitro* studies (Cabot et al., 2016a) have recently shown that PBP3 is under strong mutational pressure, and that specific mutations in this PBP contribute to β-lactam resistance development. R504C/H and F533L mutations are likely those most commonly reported, and are located within the protein domains implicated in the formation and stabilization of the inactivating complex β-lactam-PBP3 (Han et al., 2010). Moreover, these specific mutations have been documented to emerge *in vivo* during chronic respiratory infection in CF patients (Diaz Caballero et al., 2015; López-Causapé et al., 2017) and upon meropenem (Cabot et al., 2016a) and aztreonam (Jorth

et al., 2017) exposure *in vitro*. However, the precise contribution of PBP3 mutations to β-lactam resistance phenotypes needs to be further investigated using isogenic strains. Likewise, despite unique polymorphisms have also been detected in some clinical strains for other PBPs, their role in β-lactam resistance, if any, still needs to be experimentally addressed.

Other relevant components of the β-lactam mutational resistome are the porins and RND efflux pumps. Mutational inactivation of OprD is well-known to be the primary carbapenem resistance mechanisms in *P. aeruginosa* (Lister et al., 2009; Castanheira et al., 2014). OprD inactivation typically results from indels or nonsense mutations, including the Q142X mutation, characteristic of the widespread ST175 high-risk clone (Cabot et al., 2016b). Additionally, some amino acid substitutions have also been recently associated with OprD-driven resistance, particularly in the CF setting (Richardot et al., 2015). Finally, carbapenem resistance may also result from *oprD* repression caused by mutations in the MexEF-OprN efflux pump regulators

(*mexS/T*) or the ParRS two-component system (Li et al., 2015). Overexpression of MexAB-OprM, caused by mutation of several genes involved in its regulation (*mexR, nalC,* or *nalD*) increases MICs of most β-lactams except imipenem, whereas overexpression of MexXY (*mexZ, parSR, amgS* mutations) is particularly involved in cefepime resistance (Li et al., 2015). Additionally, sequence variations in unique residues are detected in the genes coding for the efflux pump (Del Barrio-Tofiño et al., 2017; López-Causapé et al., 2017); however, their contribution to resistance profiles, if any, still needs to be further explored.

Finally, another potentially relevant mutational β-lactam resistance mechanism is the selection of large (>200 kb) deletions affecting specific parts of the chromosome (Cabot et al., 2016a). Although the basis of the conferred resistance phenotype still needs to be further clarified, these mutants can be recognized by the characteristic brown pigment (pyomelanine) caused by the deletion of one of the affected genes, *hmgA*, coding for a homogentisate-1,2- dioxygenase. This type of deletion has been documented in both, *in vitro* evolved β-lactam-resistant mutants and CF isolates (Cabot et al., 2016a; Hocquet et al., 2016). However, the deletion of *hmgA* is not responsible for the resistance phenotype, which could be linked to the deletion of another of the affected genes, *galU*. This gene codes for a UDP-glucose pyrophosphorylase involved in the synthesis of the lipopolysaccharide (LPS) core. Indeed, analysis of transposon mutant libraries has shown that inactivation of *galU* increases ceftazidime and meropenem MICs (Dötsch et al., 2009; Alvarez-Ortega et al., 2010).

AMINOGLYCOSIDE MUTATIONAL RESISTOME

In the absence of horizontally-acquired aminoglycoside modifying enzymes, resistance to this antibiotic class has been particularly linked to the mutational overexpression of MexXY-OprM. Indeed, mutational overexpression of this pump, mainly caused by *mexZ, amgS,* or *parRS* mutations (**Table 1**), is very frequent among clinical isolates, from both, CF patients and nosocomial infections (Guénard et al., 2014; Prickett et al., 2017). Moreover, recent studies show that the epidemic high-risk clone ST175 overexpresses MexXY due to a specific mutation in *mexZ* (G195E) (Cabot et al., 2016b). However, recent studies have revealed that the aminoglycoside mutational resistome extends far beyond MexXY overexpression, and that high-level resistance may result from the accumulation of multiple mutations, and the involvement of several novel resistance determinants has been recently documented (El'Garch et al., 2007; Schurek et al., 2008; Feng et al., 2016). Among them is particularly noteworthy *fusA1*, coding for the elongation factor G. Indeed, specific FusA1 mutations have been associated with aminoglycoside resistance *in vitro* (Feng et al., 2016; López-Causapé et al., 2018) and among clinical, particularly CF, strains (Chung et al., 2012; Markussen et al., 2014; Greipel et al., 2016; López-Causapé et al., 2017, 2018). Moreover, the implication of *fusA1* mutations in aminoglycoside resistance has been recently confirmed through site-directed mutagenesis (Bolard et al., 2017).

FLUOROQUINOLONE MUTATIONAL RESISTOME

The fluoroquinolone mutational resistome generally includes specific missense mutations in DNA gyrase (*gyrA* and/or *gyrB*) and topoisomerase IV (*parC* and/or *parE*) Quinolone Resistance-Determining Regions (QRDRs) (**Table 1**) (Bruchmann et al., 2013; Kos et al., 2015). High-level fluoroquinolone resistance in *P. aeruginosa* high-risk clones is nearly universal, and typically involves combinations of mutations in GyrA-T83 and ParC-S87 (Del Barrio-Tofiño et al., 2017). QRDR mutations involved in fluoroquinolone resistance in CF might be more variable (López-Causapé et al., 2017). It is also well-known that the mutational overexpression of efflux pumps modulate fluoroquinolone resistance (**Table 1**). While the overexpression of MexAB-OprM and MexXY-OprM is globally more frequent among clinical strains, its contribution to clinical fluoroquinolone resistance is likely more modest (Bruchmann et al., 2013). On the other hand, the mutational overexpression of MexEF-OprN or MexCD-OprJ is associated with high-level (clinical) fluoroquinolone resistance, and although their prevalence is considered low except in the CF chronic infection setting, recent data show that it might be higher than expected (Richardot et al., 2015).

POLYMIXIN MUTATIONAL RESISTOME

Whereas the prevalence of polymyxin (colistin and polymyxin B) resistance is still globally low (<5%), it has increased in the last years because of the frequent use of these last-resource antibiotics for the treatment of MDR/XDR nosocomial and CF strains. Polymyxin resistance results most frequently from the modification of the LPS caused by the addition of a 4-amino-4-deoxy-L-arabinose moiety in the lipid A structure (Olaitan et al., 2014; Jeannot et al., 2017). The involved mutations are frequently located in the PmrAB or PhoPQ two-component regulators, which lead to the activation of the *arnBCADTEF* operon (Barrow and Kwon, 2009). More recent studies have revealed that mutations in the ParRS two-component regulator, not only produce polymyxin resistance due to the activation of the *arnBCADTEF* operon, but also lead to a MDR phenotype determined by the overprexpression of MexXY and the repression of OprD (Muller et al., 2011). Moreover, two additional two-component regulators, ColRS and CprRS, have been recently found to be involved in polymyxin resistance (Gutu et al., 2013). The analysis of colistin resistance mechanisms among clinical strains is not always straight forward, since the presence of mutations in these two-component regulators is not always associated with clinical colistin resistance, probably denoting partial complementation between the different regulators (Moskowitz et al., 2012; Gutu et al., 2013; López-Causapé et al., 2017). Moreover, recent *in vitro* evolution assays have revealed the implication of additional mutations in high level colistin resistance, facilitated by the emergence of mutator (*mutS* deficient) phenotypes (Dößelmann et al., 2017). Particularly noteworthy among them are those occurring in LptD, LpxC, or MigA.

OTHER ANTIBIOTICS

Even if not considered a classical antipseudomonal agent, fosfomycin has emerged in the last decade as a potentially useful antibiotic in urinary tract infections and combined therapy for MDR/XDR *P. aeruginosa* (Michalopoulos et al., 2011). However, fosfomycin resistance spontaneous mutation rates are high and the mechanism involved is typically the mutational inactivation of *glpT*, coding for a glycerol-3-phospate permease required for fosfomycin uptake (Castañeda-García et al., 2009; Rodríguez-Rojas et al., 2010). *glpT* mutations, conferring high-level fosfomycin resistance are also frequently found among MDR/XDR high-risk clones (Del Barrio-Tofiño et al., 2017), and some specific mutations, such as T211P, have been fixed in some widespread lineages as described for ST175 (Cabot et al., 2016b). Another potentially useful antimicrobial for combined therapy against MDR/XDR *P. aeruginosa* is rifampicin (Cai et al., 2017). However, rifampicin resistance may emerge at high frequency due to the selection of specific missense mutations in *rpoB*, coding for the beta subunit of the RNA polymerase (Jatsenko et al., 2010). Another example of newer antibiotic families with antipseudomonal activity are the pacidamycins, uridyl peptide antibiotics, targeting translocase I, an essential enzyme in peptidoglycan biosynthesis (Mistry et al., 2013). Emergence of high-level resistance to this antibiotic class has been shown to involve the selection of mutations in the Opp transporter, a binding protein-dependent ABC transporter used for oligopeptide import. Finally, the *P. aeruginosa* mutational resistome, particularly in the CF setting, may also include resistance to other used antibiotics such as the frequent mutations of domain V of 23S rRNA, conferring macrolide resistance (Mustafa et al., 2017).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The analysis of the *P. aeruginosa* mutational resistome, together with the analysis of the horizontally-acquired resistance determinants, should be useful for establishing the antibiotic resistance genotype, which should correlate with the antibiotic resistance phenotype and as such, it should permit the design of therapeutic strategies and for monitoring the efficacy of administered antibiotic treatments. However, the current applicability of the analysis of the mutational resistome is still limited by the large number of genes involved and the complexity of the resistance phenotypes generated, and, particularly, by the difficulties, in many cases, for differentiating relevant mutations from simple natural polymorphisms. Obviously, the evolution of the mutational resistome is a direct consequence of antimicrobial exposure and as such, it is not surprising that exposure to one antibiotic drives evolution of the mutational resistome for that antibiotic. However, the complexity of the actual resistance profile is further increased by the specificity and interactions among different resistance mechanisms. Indeed, a resistance mutation selected by one antibiotic may have a variable effect among the different agents within the same antibiotic class or family. Likewise, cross resistance (or collateral resistance) implies that exposure to one antibiotic drives also the development of resistance to a different one from the same or other classes. Typically, this is caused by the developed resistance mechanism (such as efflux pump overexpression) affecting simultaneously different antibiotics. Indeed, potential development of cross resistance is a major issue to consider when using antibiotic combinations (Vestergaard et al., 2016). Moreover, cross resistance between antibiotics and antiseptics and other biocides may also occur (Li et al., 2015). Perhaps less obvious is collateral susceptibility, which implies that exposure to one antibiotic increases the susceptibility to a different one (Pál et al., 2015; Imamovic et al., 2017). This might be achieved through two mechanisms. One possible mechanism is that exposure to one antibiotic directly causes increased susceptibility to a different one, for example, mutations in the β-lactamase AmpC increases cephalosporin hydrolysis while reducing that of penicillins or carbapenems (Cabot et al., 2014). The second possibility is that the development of a resistance mechanism impairs the activity of another existing resistance mechanism. An example is competition between the different efflux pumps, since the overexpression of one may impair the expression of another (Mulet et al., 2011). Thus, the evolution of the mutational resistome for a given antibiotic is not only dependent on the exposure to this antibiotic, but it is also conditioned by the simultaneous or even previous exposures to other antibiotics. An illustrative example is provided in a recent *in vitro* study that demonstrated, for a broad range of antibiotic classes, that the history of exposure and resistance development to a given antibiotic, conditions the dynamics and mechanisms of resistance development when exposed to a second one (Yen and Papin, 2017). In summary, the comprehensive analysis of the mutational resistome of *P. aeruginosa* in CF and nosocomial infections is expected to become a useful tool for optimizing therapeutic strategies and monitoring the efficacy of administered antibiotic treatments in the near future.

AUTHOR CONTRIBUTIONS

CL-C and AO wrote the manuscript. CL-C, GC, EdB-T, and AO reviewed the literature.

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¡Quién busque el infinito, que cierre los ojos!

Milan Kundera, 1984