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Faculty of Sciences

Department of Molecular Biology

# Understanding evolution to tackle antibiotic resistance in *Pseudomonas aeruginosa*

Memory presented by

**Pablo Laborda Martínez**

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Thesis Director

**José Luis Martínez Menéndez**

Thesis Co-director

**Sara Hernando Amado**

Thesis Tutor

**María Dolores Irma Marín Palma**

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# *ABSTRACT*

## Abstract

Antibiotic resistance (AR) constitutes a major public health concern, which has been aggravated in recent decades due to the emergence and spread of multidrug-resistant microorganisms, especially Gram-negative bacteria. Among them, *Pseudomonas aeruginosa* stands out; it is an opportunistic pathogen, widely distributed in nature, that frequently infects hospitalized patients and presents low susceptibility to many antimicrobials, as well as an overwhelming capacity to develop AR via mutation, mainly during chronic infections. Hence, novel treatment strategies are needed to deal with the infections caused by this bacterium. Collateral sensitivity, whereby acquiring resistance to one drug increases susceptibility to a second drug, is an evolutionary trade-off that may be exploited for treating bacterial infections by the combination or sequential use of drugs' pairs. This application is only possible if those collateral sensitivity phenotypes are conserved within different genetic contexts, environments and situations; robust collateral sensitivity events were searched for during this thesis. We determined that tobramycin, tigecycline and ceftazidime resistance acquisition in *P. aeruginosa* is associated with a robust fosfomycin collateral sensitivity and ascertained the mechanism responsible for this event. Further, we observed that ciprofloxacin exposure selects distinct mutations in different genetic backgrounds of *P. aeruginosa*, all of them leading to a robust tobramycin and aztreonam collateral sensitivity, and we proposed tobramycin-ciprofloxacin and ciprofloxacin-aztreonam combinations as promising therapies against infections caused by this bacterium. We also determined that media composition and nutrients' availability constrain the pathways towards tobramycin, ceftazidime and ceftazidime-avibactam resistance in *P. aeruginosa*, but fosfomycin collateral sensitivity associated with ceftazidime resistance robustly emerges when *P. aeruginosa* evolves in different media mimicking those that can be encountered during infection. The compensation of fitness costs associated with the acquisition of AR in the absence of selective pressure could cause a decline of AR, which may also be used for designing therapeutic strategies considering those specific antibiotics whose resistance is robustly unstable in absence of selection. In this thesis, we observed that compensatory evolution of fitness costs associated with ceftazidime resistance in *P. aeruginosa* leads to a ceftazidime resistance decline in distinct genetic backgrounds, both in antibiotic-free and in sublethal tobramycin environments. The alternation of ceftazidime with drug restriction periods or the switch back to ceftazidime after a ceftazidime-tobramycin alternation may be feasible therapeutic approaches against *P. aeruginosa* infections. For its part, AR may be transiently induced by some conditions encountered by bacteria during infection, compromising the antibiotic treatments. In this thesis we identified dequalinium chloride, procaine and atropine, which can be present in *P. aeruginosa* site infections, as inducers of the expression of MexCD-OprJ efflux pump encoding genes, hence transiently increasing ciprofloxacin resistance of this bacterium. Finally, by further studying efflux pumps regulation and considering their ancestral function, we determined that the identification of compounds which are both substrates and inducers of efflux pumps of *P. aeruginosa* constitutes an effective strategy for finding molecules that reduce the virulence potential of this pathogen.

Overall, the results of this thesis allow us to propose novel treatment strategies against *P. aeruginosa* infections, based on the identification of novel drugs and on the rational use of the antibiotics that we already have, as well as to better understand AR evolution.

## Resumen

La resistencia a antibióticos (RA) es un problema de salud pública, agravado en las últimas décadas debido a la aparición y propagación de microorganismos multirresistentes, especialmente bacterias Gram-negativas. Entre ellas, destaca *Pseudomonas aeruginosa*, un patógeno oportunista, ampliamente distribuido en la naturaleza, que infecta con frecuencia a pacientes hospitalizados y presenta una baja sensibilidad a muchos antibióticos, así como una abrumadora capacidad para desarrollar RA por mutación, principalmente durante infecciones crónicas. Por tanto, se necesitan nuevas estrategias para tratar las infecciones producidas por esta bacteria. La sensibilidad colateral, por la que la adquisición de resistencia a un fármaco aumenta la sensibilidad a otro, es un *trade-off* evolutivo que puede usarse para tratar infecciones mediante la combinación o el uso secuencial de parejas de antibióticos. Esta aplicación sólo es posible si la sensibilidad colateral se conserva en diferentes contextos genéticos, ambientes y situaciones; durante esta tesis se han buscado eventos de sensibilidad colateral robusta. Determinamos que la adquisición de resistencia a tobramicina, tigeciclina y ceftazidima en *P. aeruginosa* está asociada a una sensibilidad colateral a fosfomicina robusta e identificamos el mecanismo responsable de este fenómeno. Además, observamos que el ciprofloxacino selecciona distintas mutaciones en diferentes contextos genéticos de *P. aeruginosa*, todas ellas produciendo sensibilidad colateral robusta a tobramicina y aztreonam, y siendo las combinaciones de tobramicina-ciprofloxacino y ciprofloxacino-aztreonam terapias prometedoras contra infecciones de esta bacteria. También determinamos que la composición del medio y la disponibilidad de nutrientes constriñen las vías evolutivas hacia la resistencia a tobramicina, ceftazidima y ceftazidima-avibactam en *P. aeruginosa*, pero la sensibilidad colateral a fosfomicina asociada a la resistencia a ceftazidima emerge cuando *P. aeruginosa* evoluciona en diferentes medios que imitan los encontrados durante la infección. La compensación de los costes fisiológicos asociados a la RA en ausencia de presión selectiva podría provocar una disminución de la RA, algo con potencial para diseñar estrategias terapéuticas considerando los antibióticos cuya resistencia es inestable en ausencia de selección. En esta tesis, observamos que la evolución compensatoria de los costes asociados a la resistencia a ceftazidima en *P. aeruginosa* conduce a una disminución de la resistencia en distintos contextos genéticos, tanto sin antibiótico como con concentraciones subletales de tobramicina. El uso de ceftazidima tras periodos de restricción de fármacos o tras el uso alternado de ceftazidima-tobramicina pueden ser enfoques terapéuticos factibles contra las infecciones causadas por *P. aeruginosa*. Por su parte, la RA puede ser inducida transitoriamente por algunas condiciones encontradas por las bacterias durante la infección, comprometiendo los tratamientos antibióticos. En esta tesis identificamos el cloruro de decualinio, la procaína y la atropina, que pueden estar presentes en las infecciones de *P. aeruginosa*, como inductores de la expresión de los genes que codifican la bomba de eflujo MexCD-OprJ, aumentando así transitoriamente la resistencia a ciprofloxacino de esta bacteria. Por último, al profundizar en el estudio de la regulación de las bombas de eflujo y considerar su función ancestral, determinamos que la identificación de compuestos que sean a la vez sustratos e inductores de las bombas de *P. aeruginosa* constituye una estrategia eficaz para encontrar moléculas que reduzcan el potencial de virulencia de este patógeno.

En general, los resultados de esta tesis nos permiten proponer nuevas estrategias de tratamiento de infecciones por *P. aeruginosa*, basadas en la identificación de nuevos fármacos y en el uso racional de los antibióticos que ya tenemos, así como comprender mejor la evolución de la RA.

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## Abbreviations

**3-oxo-C12-HSL**: N-(3-oxododecanoyl)-L-homoserin lactone

**ALE**: Adaptive Laboratory Evolution

**AR**: Antibiotic resistance

**C4-HSL**: N-butanoyl-L-homoserin lactone

**DNA**: Deoxyribonucleic acid

**ESKAPE**: *Enterococcus faecium, Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.

**HGT**: Horizontal gene transfer

**HHQ**: 4-hydroxy-2-heptylquinoline

**HIV**: Human immunodeficiency virus

**Kb**: Kilo base

**LPS**: Lipopolysaccharide

**MDR**. Multidrug-resistant

**MGEs**: Mobile genetic elements

**MIC**: Minimal inhibitory concentration

**PBPs**: Penicillin-binding proteins

**PQS**: *Pseudomonas* Quinolone Signal

**QS**: Quorum Sensing

**RNA**: Ribonucleic acid

**RND**: Resistance-nodulation-cell division

**SCFM**: Synthetic cystic fibrosis sputum

**T3SS**: Type-3-Secretion System

**T6SS**: Type-6-Secretion System

**TOTEM**: Top Ten resistant Microorganisms

# *INTRODUCTION*

# 1. Introduction

## 1.1. The global health challenge of antibiotic resistance

Antibiotics constitute one of the most successful forms of therapy in the history of medicine. Innumerable lives have been saved by avoiding infections through the implementation of hygienic procedures (i.e. hand washing) and infrastructures (i.e. water treatment), and the use of antibiotics has also contributed to this endeavour due to their efficacy in the treatment of infections and in the prevention of those which may occur after surgical interventions or immunosuppressant treatments, among other situations (1).

From the 1950s to the 1970s, the so called "golden era" of antibiotics, many novel antibiotic classes were introduced (2). Regrettably, some bacteria able to grow in presence of those drugs - antibiotic-resistant strains (3)- were reported few years after their introduction, compromising the success of these therapeutic agents (Figure 1). Historically, a kind of "Red Queen" strategy has been applied: novel antimicrobials were developed in order to treat bacterial infections, so when bacteria became resistant to one antibiotic a different one could be chosen for the treatment. However, the present situation of shortage of production of new antibiotics and rapid antibiotic resistance (AR) development and dissemination has led to an increase of infections due to antibiotic-resistant bacteria with limited therapeutic options (4, 5). Hence AR is recognized as one of the 21st century major health challenges by several economic, regulatory and political bodies (6-9).

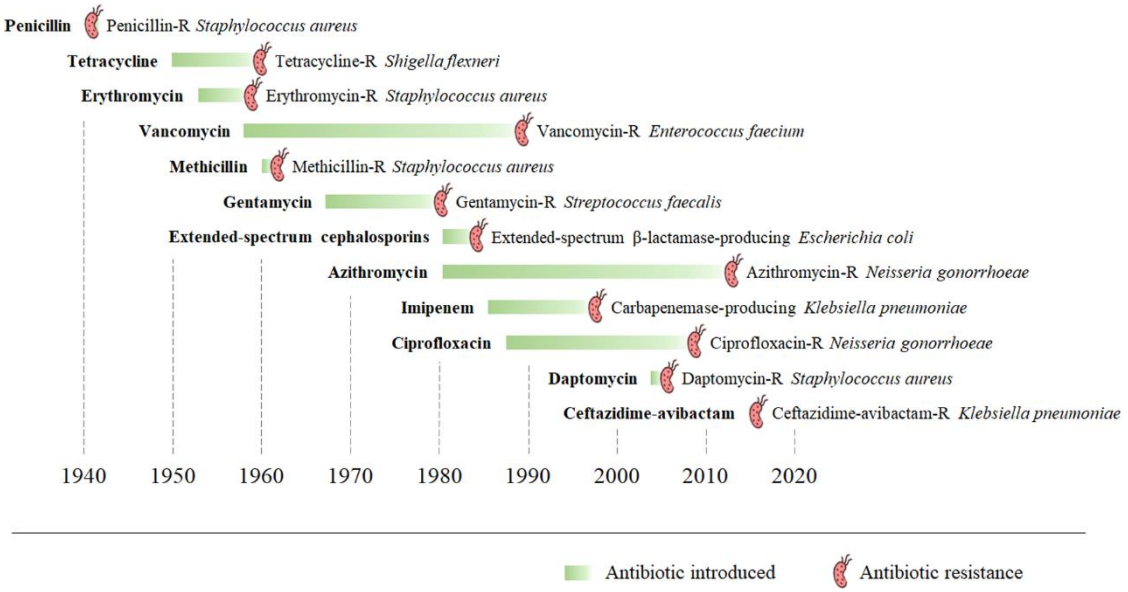


**Figure 1. Timeline of the introduction of antibiotics and the identification of the respective resistance.** The figure shows how rapidly resistance emergence is detected after the development and release of different antibiotics (10-24). Remarkably, methicillin, daptomycin or ceftazidime-avibactam resistance was detected very soon after these drugs were introduced for therapy. We may highlight the case of penicillin, which was widely introduced in 1941, but a bacterial penicillinase was already described one year before (24). Worryingly, while antimicrobial development has slowed down, antibiotic resistance emergence has not.

We may find some social and administrative reasons for the emergence of AR, such as over-prescription of antibiotics, self-medication, noncompliance of treatment plans, overuse of

antimicrobial agents for animal fattening, lax regulations in some countries or a declining interest by pharmaceutical companies in antibacterial research and development (25, 26). In addition, we must also consider the fact that population increment, globalization and lack of efficient sewage treatment in many places, which eventually contain resistant bacteria or resistance genes, may promote dissemination of AR (5, 27). Although these aspects worsen the problem, AR emergence is just a consequence of evolution due to the selection pressure that the use of these drugs, for the treatment of human infections and in animal feeding, sets on susceptible bacteria. Hence, AR is unavoidable even with an optimal use of these drugs.

Even more, an extremely concerning threat to public health is the occurrence of bacterial pathogens resistant to several antimicrobial agents, multidrug-resistant (MDR) bacteria (28, 29). MDR bacteria are associated with high mortality rates: for instance, in Europe they were estimated to be responsible for 33000 deaths during 2015 (30) and 35000 in United States during 2017 (31). These estimations have recently worsened when considering both deaths associated with AR -in patients with previous pathologies- and those directly attributable to antibiotic-resistant bacteria, reaching the concerning levels of 4.95 million and 1.27 million worldwide during 2019, respectively (5). Further, MDR bacteria are continuously transferred among animals, humans and the environment, consequently spreading resistance genes and resistant microorganisms among ecosystems (6, 32, 33), globalizing the problem. Hence, the current approach of analysing and tackling AR is through One Health and Global Health perspectives (6). One Health pivots on the role of geographically connected ecosystems in the emergence and dissemination of antimicrobial resistance and addresses the problem through the implementation of local interventions. For its part, Global Health focuses on the global conditions contributing to the worldwide spread of AR, so deals with it by regulations designed to be integrated at international level. Both of them refer to the interdependence between health of animals, humans, plants and the ecosystems (as wastewater) where bacterial pathogens can be present and, eventually cohabit with antibiotic-resistant microorganisms.

## 1.2. Molecular mechanisms and types of antibiotic resistance

The antibacterial activity of an antibiotic depends on its capacity to bind its target and on the amount of antibiotic that reaches the target. Hence, the capacity to overcome its effect relies on reducing the affinity of the antibiotic/target interaction or the amount of intracellular antibiotic (34). AR may be classified in three types: intrinsic, acquired and transient.

### 1.2.1. Intrinsic resistance

Every bacterial species presents a characteristic level of susceptibility to antibiotics, what is dubbed intrinsic resistance. The intrinsic resistome is thus determined by all the elements contributing to this characteristic susceptibility/resistance that are present independently of previous antibiotic exposure or horizontal gene transfer (HGT) in bacteria (35, 36), which encompasses a wide variety of genes for each species (35, 37-40). Despite the abundance of contributing genes, the most clinically relevant intrinsic resistance determinants in Gram-negative bacteria may be divided in passive and active elements. Those belonging to the first group are a

reduced uptake of the antibiotic and absence of a susceptible target, while the latter group includes antibiotic inactivation and extrusion of the antibiotic (Figure 2) (34).

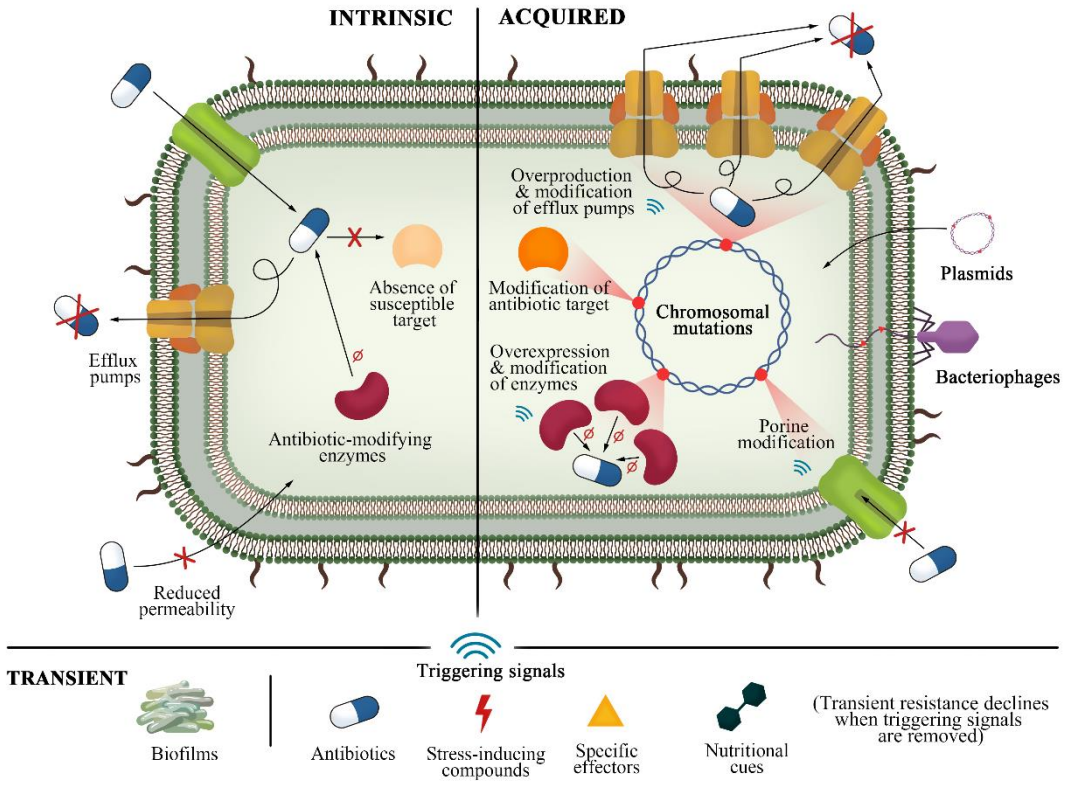


**Figure 2. Scheme including the main intrinsic resistance determinants of Gram-negative bacteria, as well as the best studied acquired and transient antibiotic resistance mechanisms.** Regarding intrinsic antibiotic resistance, the asymmetric bilayer of phospholipids and lipopolysaccharides forming the bacterial membrane prevents the passive diffusion of hydrophobic antibiotics, yet this may be counteracted by the entrance of the antibiotic through membrane porins. Once inside the cell, the antibiotic may produce its toxic effect by reaching its target if the bacterium has an antibiotic-susceptible one. However, some intrinsic elements of the bacterium may reduce its toxic effect by its extrusion outside the cell -multidrug resistance efflux pumps-, or inactivation -antibiotic-modifying enzymes-. In addition, bacteria can also acquire antibiotic resistance. The entrance of antibiotics through membrane porins may be avoided by loss-of-function mutations in their encoding genes, as well as by mutations modifying their pore sizes or the expression of their encoding genes. Mutations in local regulators of efflux pumps, leading to their overproduction and increased antibiotic extrusion once it has entered the cell, is another well-studied acquired resistance mechanism. Incorporation of mobile genetic elements, like plasmids, containing antibiotic resistance genes, such as those encoding antibiotic-modifying enzymes, is a commonly reported mechanism of acquiring antibiotic resistance. Antibiotic-target binding avoidance through mutations in the antibiotic target encoding gene can also lead to acquired antibiotic resistance. Finally, transient antibiotic resistance may be accomplished in certain situations. Environmental signals, such as stress-inducing compounds, the presence of antimicrobial molecules, changes in growth conditions or appearance of specific effectors, may lead to transiently antibiotic-resistant bacteria. When the triggering signal ceases, resistant bacteria recover the susceptible phenotype.

The ability to resist the action of an antibiotic may be due to passive inherent structural and functional features of the bacterium, hence not all antibiotics have the same effect on every bacterial species. First, in order to produce its toxic effect, an antibiotic has to enter the cell in a sufficient amount. In Gram-negative bacteria, the entrance may be prevented by the structure of their membrane, formed by an asymmetric bilayer of phospholipids and lipopolysaccharides (LPS) (41). This barrier decelerates the passive diffusion of hydrophobic compounds among

which we find some classes of antibiotics, for example macrolides, like vancomycin, that is not effective against Gram-negative bacteria for this reason (42). Hence, for some types of antibiotics, the principal route of entry inside the cell is through membrane porins, being determinant in the antibiotic susceptibility of the microorganism the number and type of porins it possesses. For instance, the most abundant porin of *Pseudomonas aeruginosa*, OprF, has a low permeability to the entrance of antibiotics, being this feature one of the major intrinsic AR determinants of this bacterium (43, 44).

Once the antibiotic enters into the cell, it still needs to bind a susceptible target in order to produce a toxic effect. Therefore, the absence of a target or the presence of an antibiotic-resistant allele is the simplest intrinsic AR mechanism. For example, the antistaphylococcal drug daptomycin is not active against Gram-negative bacteria since they have low proportion of anionic phospholipids in their cytoplasmic membrane, reducing the insertion of daptomycin in the membrane which is required for its toxicity (45). Another example relates to fosfomycin, an antibiotic that inhibits the cell wall peptidoglycan synthesis through the binding to the cysteine 11S of the active site of MurA, which catalyses the initial step of the peptidoglycan synthesis (46). Therefore, bacterial species with a cysteine in the active site of MurA, as *Escherichia coli* or *P. aeruginosa*, are susceptible to fosfomycin, while others harbouring different MurA variants, such as *Chlamydia* or *Borrelia burgdorferi*, are intrinsically resistant to this antibiotic (47, 48).

Apart from the main intrinsic AR mechanisms described above, the intrinsic resistome is comprised by a high amount of genes among which we find basic elements of the bacterial physiology (35) such as lipid biosynthesis (49) or carbon catabolism regulation (50). Hence, passive intrinsic resistance is not a specific response to the presence of antibiotics, rather a characteristic derived from the bacterial physiology, metabolism or cell structure (51).

Besides, some active bacterial elements can decrease the amount of active antibiotic, preventing the drug to reach its intracellular target. Bacterial multidrug efflux pumps extrude a wide variety of antibiotics, therefore being one of the most relevant contributors to the intrinsic resistance of Gram-negative bacteria (52). The basal level of expression of some of these systems in some microorganisms is high enough to contribute to their intrinsic resistance, such as AcrAB-TolC from *E. coli* (53), CmeABC from *Campylobacter jejuni* (54), MexAB-OprM from *P. aeruginosa* (55) or SmeYZ from *Stenotrophomonas maltophilia* (56).

The inactivation of the antibiotic is another way to reduce its toxic effect once inside the cell. This may be achieved by the modification of its structure -impeding its binding to the cellular target- through the addition of chemical groups, or by enzymatic hydrolysis. The first possibility includes transferases, such as aminoglycoside acetyltransferases, nucleotidyltransferases, macrolide kinases (57) or the glutathione-S-transferase FosA, which confers intrinsic fosfomycin resistance to *P. aeruginosa* (58). Among the enzymes with hydrolytic capacity we find β-lactamases, able to cleave the β-lactam ring of β-lactam antibiotics (59), like AmpC of *P.aeruginosa* (60).

Some bacteria with environmental origin, where antibiotic concentration is normally low, present an intrinsic low susceptibility to different antibiotics due to the presence of efflux pumps or antibiotic-inactivating enzymes, such as *P. aeruginosa* or *S. maltophilia* (61-63). This fact

suggests that the original physiological function of the active elements that determine intrinsic AR was not to provide bacteria with resistance to the antibiotics currently used in clinics, and that this is a new function developed as a result of their application (35, 61); this constitutes a case of exaptation, by which the acquisition of a new function is not the consequence of a genetic change but of a change of habitat (64).

### 1.2.2. Acquired resistance

Although some bacterial species can be intrinsically resistant to a drug, the actual problem hinges on the fact that AR may also be acquired after incorporating resistance genes from other bacteria of the same genus or species, or after the acquisition of DNA mutations (65) (Figure 2). Genetic mutations leading to acquired AR may be found in elements affecting the membrane permeability, such as membrane porins or transporter encoding genes, reducing the antibiotic entrance into the cell. These AR mutations may produce a loss of the porin or transporter functionality; for example, loss-of-function mutations in *glpT*, encoding the fosfomycin transporter, constitute a common cause of fosfomycin resistance in *P. aeruginosa* (58, 66). Also, the expression level of the gene encoding the transport element may be modified; e.g. mutations in the promoter of the OmpF porin encoding gene in *Enterobacteriaceae*, or in the gene encoding its negative regulator *ompR*, leads to ertapenem-resistant isolates due to a reduced production of the porin (67, 68). Finally, a reduction of the size of the porin's channel can also be involved in AR; *E. coli* isolates from an antibiotic-treated patient were found to present mutations in the constriction zone of OmpC porin encoding gene, resulting in a reduced cefotaxime and other β-lactams passage and, consequently, diminished bacterial susceptibility to them (69).

As mentioned before, efflux pumps may extrude antibiotics outside the cell. Therefore, the occurrence of mutations leading to an increased expression of an efflux pump encoding gene or to an amino acid change in the efflux pump that increases the efficiency of extrusion of antibiotics outside the cell, will produce acquired AR to its substrates (70). For instance, *P. aeruginosa*'s MexAB-OprM encoding genes are overexpressed through mutations in its local negative regulator encoding gene *mexR* (71). Further, although less frequently described, a mutation in the transporter protein of an efflux pump encoding gene may increase the efficiency or alter the specificity of extrusion of antibiotics. For example, a mutation in *mexY*, encoding the transporter protein of *P. aeruginosa*'s MexXY efflux pump, was found to increase aminoglycosides resistance (72). Similarly, mutations in AcrAB-TolC efflux pump encoding genes in *E. coli* and *Salmonella enterica* (73), or in SmeH encoding gene in *S. maltophilia* (74), increase ciprofloxacin and β-lactams resistance, respectively.

The antibiotic inactivation by modifying enzymes may be enhanced by their overproduction. This may occur after mutations in regulators of their expression, such as AmpR, the positive regulator of the expression of the gene encoding the AmpC cephalosporinase of *P. aeruginosa* (75). In addition, mutations in antibiotic-modifying enzymes encoding genes can broader their substrate spectrum. For example, AmpC does not hydrolyse fourth generation cephalosporins, such as cefepime or cefpirome, but novel AmpC variants resulting from the acquisition of genetic

variations are able to do it, as detected *in vitro* (76, 77) and in *Enterobacter aerogenes* and *Serratia marcescens* clinical isolates (78, 79).

The structure of antibiotic targets may be modified by mutations in their encoding genes, reducing the affinity of the antibiotic to the target and preventing its binding. Some examples of this situation are modification of penicillin-binding proteins (PBPs) leading to β-lactams resistance, such as the *P. aeruginosa* PBP3 encoding gene *ftsI* (80) or RpoB alterations causing reduced affinity for rifampicin (81). An increased expression of the antibiotic target may also increase AR, as described for MurA, the target of fosfomycin, in *E. coli* (82).

Acquired AR may also be originated by mutations in genes besides these above described to be involved in canonical mechanisms of resistance. Namely, mutations in genes encoding transcriptional regulators, motility-related proteins, virulence determinants or enzymes involved in different bacterial processes have been described to be associated with a reduction of susceptibility to antibiotics (83-87).

The incorporation of resistance genes constitutes the other major path for acquiring AR. It may occur through transformation or HGT, with these genes being part of cassettes contained in integrons (88) or in mobile genetic elements (MGEs) such as plasmids (89), bacteriophages (88), transposons (90) or even DNA from dead bacterial cells (65). Among the most worrisome cases, we find plasmids carrying extended-spectrum β-lactamases in several *Enterobacteriaceae* species (91) or genomic islands of *S. enterica* containing an integron that harbours several resistance genes (92). In the case of *P. aeruginosa*, the acquisition of rifampicin-, fluoroquinolone-, chloramphenicol-, β-lactam- and aminoglycoside-modifying enzymes has been described, being the most frequent the two latter (93-97). Specially concerning in this bacterium is the acquisition of integrons simultaneously containing genes encoding aminoglycoside-modifying enzymes, β-lactamases and other resistance determinants (96, 98-100). Although this way of acquiring resistance is an ubiquitous source of dissemination and emergence of antimicrobial resistance in many bacteria, during this PhD we have focused on the acquisition of AR through mutation.

### 1.2.3. Transient resistance

Transient AR, also named adaptive or phenotypic resistance, occurs when an environmental signal or cellular condition triggers a temporary alteration in the bacterial physiology, which increases the bacterial ability to survive an antibiotic (70). Transient resistance does not involve genetic changes, and therefore is not inheritable, in contrast to intrinsic and acquired resistance, and it reverts when induction ceases (101, 102) (Figure 2). An example of this type of resistance is persistence, by which a bacterial subpopulation develops a quiescent and refractory behaviour towards the action of an antibiotic (103). The fraction of persisters can increase under low-nutrient conditions or in presence of Quorum Sensing (QS) signalling molecules, as it has been described in *P. aeruginosa* (104, 105). Besides, non-inherited AR can also occur during specific growth conditions, such as biofilms (106). Biofilms form complex structures in which the diffusion of compounds is difficult (106), they contain elements reducing the activity of antimicrobials like glycerophosphorylated β-(1,3)-glucans which sequester aminoglycosides (107), and subpopulations of persisters that can be found in some parts of the biofilm (108). Further,

induction of a resistance phenotype by specific signals and/or conditions must not be disregarded, since they may be found during an antibiotic treatment of bacterial infections, compromising its efficacy (109). Some examples of those inducing conditions are temperature, oxidative or nitrosative stress, antibiotics themselves, other drugs used during the treatment or compounds produced by the host (cationic peptides, bile salts or fatty acids, among others) (101, 109-113).

Transient AR has not been as widely studied as intrinsic and acquired resistance, maybe due to the fact that it is a temporary phenotype, thus making difficult to track it using classical susceptibility tests. As a consequence, our knowledge is limited to few microorganisms and/or inducer conditions, mostly derived from the study of antibiotics as inducers of their associated resistance determinants. Nevertheless, we may find some examples with diverse mechanisms in the basis of this non-inheritable phenotype. Among those examples, we encounter several cases of induction of the expression of efflux pumps encoding genes, which may lead to multidrug resistance. For instance, disinfectants are able to transiently reduce susceptibility to antibiotics of *P. aeruginosa* by inducing the expression of the operon encoding the MexCD-OprJ efflux pump (114, 115). Also, the expression of the genes encoding the efflux pumps AcrAB of *E. coli* and CmeABC of *C. jejuni* is induced by bile salts from the intestinal tract, which these bacteria are able to colonize, increasing resistance to several antibiotics (110, 116).

The presence of inducible β-lactamases in many Gram-negative bacteria, such as AmpC in *Enterobacteriaceae*, whose expression is indirectly enhanced by the action of some β-lactam antibiotics, may compromise a therapeutic outcome (75). Besides, the modulation of the expression of genes encoding porins, by which antibiotics diffuse, as a response to environmental stresses (117), or the temperature- and nutrient limitation-dependent expression of outer membrane vesicles (102), which are able to bind cationic peptides and antibiotics (118), has been reported. Another example is the finding that magnesium limitation enhances resistance to positively charged antimicrobials, such as polymyxin and cationic antimicrobial peptides, in *P. aeruginosa* and *S. enterica*. This occurs through a transient reduction of the net negative charge of the cell surface due to an up-regulation of a LPS modification operon (119-122).

## 1.3. Functions and regulation of RND efflux pumps

Bacterial multidrug efflux pumps are particularly relevant elements in AR, since they may contribute to intrinsic, acquired and transient resistance. This contribution is due to their capacity to extrude a wide variety of compounds outside the cell, among which we find antibiotics (123). Six families of bacterial efflux pumps have been currently identified: the major facilitator superfamily (124), the small multidrug resistance family (125), the ATP-binding cassette family (126), the multidrug and toxin extrusion family (127), the proteobacterial antimicrobial compound efflux family (128, 129) and the resistance-nodulation-cell division (RND) superfamily (130, 131). The latter is specific to Gram-negative bacteria and the AcrAB-TolC system of *E. coli* has been studied as a model of the structure and mechanism of action of pumps belonging to this family (130). RND efflux pumps constitute a structure across the two membranes of Gram-negative bacteria formed by an inner cell membrane transporter powered by the proton motive force to efflux its substrates, an outer membrane channel that reaches the

outside surface of the cell and a periplasmic adaptor protein that connects both membranes (131, 132).

Apart from their role as AR determinants, which has been widely studied, RND efflux pumps are also able to extrude diverse compounds such as organic pollutants, heavy metals and bacterial metabolites, among others (Figure 3), being involved in cellular detoxification of exogenous and endogenous compounds (123). Further, RND efflux pumps have been described to mediate cell-to-cell communication through the extrusion of signalling molecules. For instance, MexAB-OprM, MexGHI, MexEF-OprN and MexCD-OprJ efflux pumps of *P. aeruginosa* can extrude QS signal molecules or their precursors (133-137).

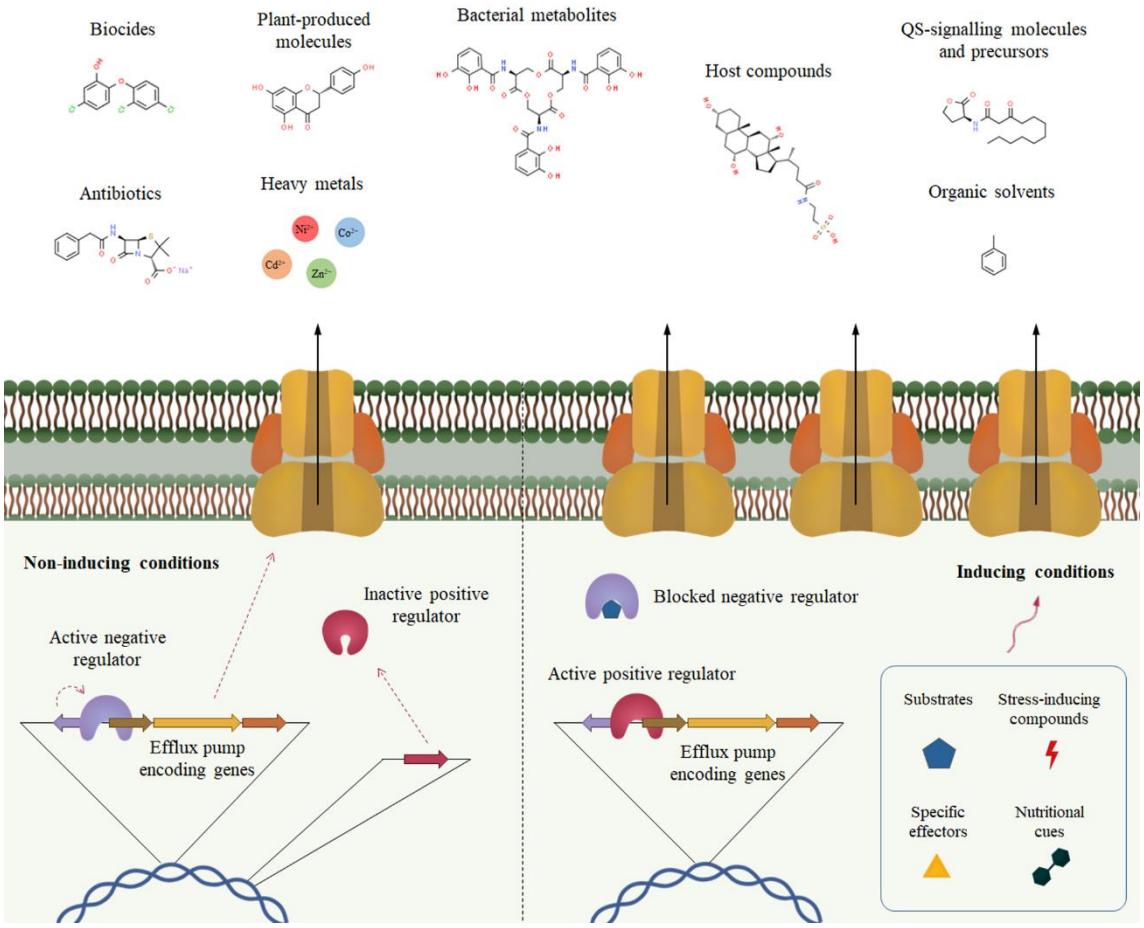


**Figure 3. Role and regulation of bacterial efflux pumps.** Efflux pumps are able to extrude several molecules among which we find biocides, antibiotics, heavy metals, plant-produced compounds, bacterial metabolites, host compounds, QS-signalling molecules and organic solvents, therefore having different roles in bacterial physiology. The expression of efflux pumps encoding genes is tightly regulated by principal regulators, usually encoded upstream the efflux pump encoding genes, and secondary regulators, sometimes involved in general stress-response networks. Further, the expression of the genes coding for efflux pumps may also be affected by several other factors, such as the presence of their substrates -i.e. plant-produced compounds, antibiotics or biocides-, specific effectors, nutritional cues or different stresses.

Importantly, RND efflux pumps also extrude innate bacterial host defence molecules, being necessary in some cases for colonization and propagation during infection. As an example, MexAB-OprM is necessary for *P. aeruginosa* to invade epithelial cells (138); some *Vibrio cholerae* RND efflux pumps are required for intestinal colonization (139) or AcrAB-TolC of *E.*

*coli* is needed for extruding bile salts and antimicrobial peptides from the host facilitating the intestinal colonization (116). This situation is not specific of animal hosts, it has also been described that efflux pumps of some environmental microorganisms have a role in plant colonization. The flavonoid-responsive efflux pump MexAB-OprM of *Pseudomonas syringae* is needed for its efficient colonization of tomato plants (140, 141), the IfeABR efflux pump of *Agrobacterium tumefaciens* extrudes the isoflavonoid coumastrol from root exudates, being this molecule able to reduce the virulence of this bacterium (142), or AcrAB system of *Erwinia amylovora* which is able to extrude the flavonoids naringenin and phloretin (143). Further, SmeDEF efflux pump, the principal determinant of resistance to quinolones of the opportunistic pathogen *S. maltophilia*, is needed for the colonization of plants by this bacterium (144).

Since RND efflux pumps extrude a wide range of substrates, being therefore involved in diverse physiological, communication and infectiveness-related functions, its expression is tightly regulated. It is controlled by one or more local or global regulators, transcription or post-transcription factors (145) or small proteins (146), and it is influenced by some environmental conditions, such as the presence of certain compounds or stress signals, as detailed in the transient resistance section. For example, the expression of the genes encoding the efflux pumps AcrAB-TolC, MexAB-OprM, IfeABR or AcrAB, involved in extruding host-produced anti-virulence compounds for maintaining the infective capacity of *E. coli*, *P. syringae*, *A. tumefaciens* and *E. amylovora*, respectively, is induced when some host-produced compounds -the natural efflux pump's substrates- are present, increasing the efficiency of their extrusion outside the cell (116, 140-143). Hence, knowing the factors that upregulate the expression of RND efflux pumps encoding genes is of relevance, since it may lead to the identification of molecules that can compromise the treatment of an infection by transiently increasing AR, or for better understanding the role of these elements in bacterial physiology or virulence.

## 1.4. Evolution towards acquisition of antibiotic resistance

As above said, AR can be acquired through several mechanisms; in other words, bacteria may follow different evolutionary routes towards the acquisition of AR. The prediction and understanding of the factors that may drive and constrain this evolutionary process have a huge clinical relevance, since this knowledge will be essential for minimizing the AR rise (147). For the prediction of AR evolution to be accomplished, firstly, we will need to know which mutations and/or resistance genes are most likely selected in the presence of a drug (147, 148).

To study mutational acquisition of AR, Adaptive Laboratory Evolution (ALE) experiments in presence of antibiotics is a valuable and frequently used method. ALE experiments were introduced by Richard Lenski at the end of the 1980s (149). Given the rapid bacterial growth rate and big size of their populations, this methodology paved the way for the study of bacterial evolution in presence of a selective pressure, such as that exerted by the presence of an antibiotic (150-153). The combination of ALE assays with bacterial genome sequencing techniques has allowed a certain prediction of AR evolution.

This technique consists of exposing a given bacterial population to antimicrobial concentrations that are high enough to partially inhibit its growth, while being sufficiently permissive to allow

the selection of spontaneous mutants (150). ALE experiments might be adapted for different purposes (Figure 4). Firstly, the concentration of antibiotic can be increased over the serial passages of the experiment, which will be prolonged for the necessary time to give rise to hundreds or thousands of generations, favouring the selection of the best-adapted bacteria (154). These ALE experiments with gradual antibiotic dose increase (Figure 4A) can be used for ascertaining all possible mutations -and the order of their acquisition- for becoming resistant to a drug. Secondly, short-term ALE experiments, with no drug increment, are closer to clinics, where after the appearance of resistant bacteria, the treatment is rapidly redirected to the use of a different antibiotic. Since being a short period of time, few genetic changes are selected, and this type of experiment can lead to the identification of conserved trade-offs of evolution (Figure 4B), such as collateral sensitivity. This would lead us to know which drug could be alternated or combined with the first one in a sequential or combinatorial evolution-based antibacterial strategy. Finally, ALE assays can also be useful for analysing the compensation of fitness costs imposed by resistance mutations by ALE experiments in the absence of drugs (Figure 4C). After the evolution of the bacterial populations, the level of AR is determined, as well as those mutations associated with the phenotype of interest by Whole-Genome Sequencing (Figure 4).

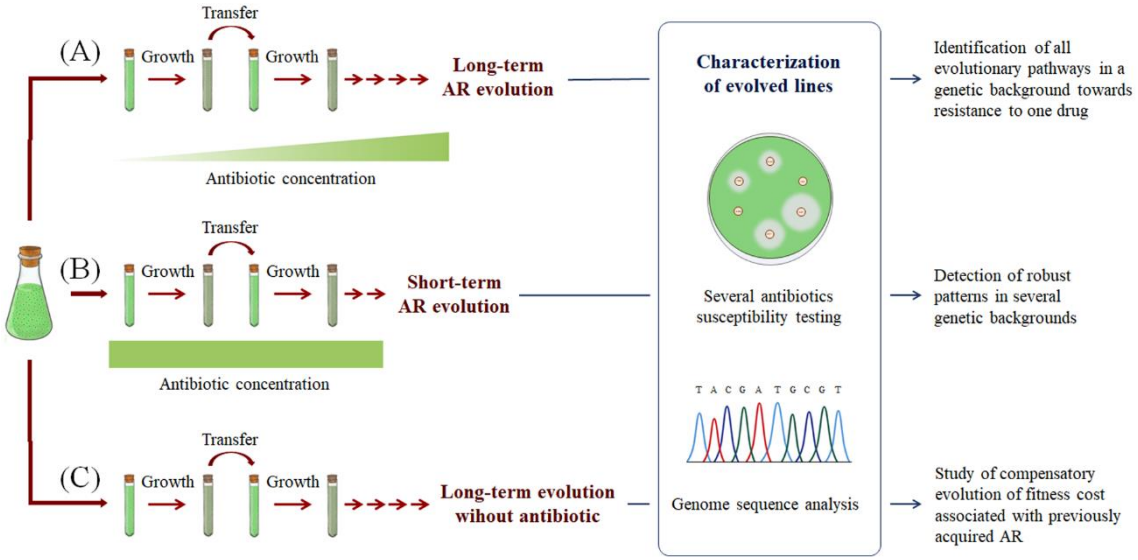


**Figure 4. Diagram of Adaptive Laboratory Evolution experiments for studying antibiotic resistance evolution.** An initial bacterial population is grown with antibiotic as selection pressure. Serial passages into fresh media with antibiotic are performed, up to a determined number of generations. Antibiotic concentration may be increased every few generations (A) or maintained during a short period of time (B), depending on the aim of the study. The experiment may also be performed without antibiotic for a long period of time (C) in order to study compensatory evolution. Finally, the antibiotic susceptibility of the evolved bacterial population is measured by antibiotic susceptibility tests and the genetic causes of resistance are ascertained by Whole-Genome Sequencing.

The large number of genes that could contribute to the acquisition of resistance by mutation in a bacterial population might suggest that evolution of AR is stochastic and unpredictable (87). Nevertheless, there are some factors that constrain this evolution, making some evolutionary pathways more likely to be selected for in a specific genetic background of a species when an antibiotic is applied, existing space for a certain conservation and predictability. As later on detailed, some of these determinants are mutation load, clonal interference, population

bottlenecks, compensatory evolution, fitness cost and resistance level of each mutation, epistasis and the effect of the genetic background (148, 155-164).

Besides, the acquisition of resistance usually involves alterations in bacterial elements that often have several functions in the cellular physiology, leading to collateral effects. These trade-offs of the evolution of AR should be deeply studied, since their exploitation could allow a more efficient use of antibiotics.

### 1.4.1. Consequences of acquiring antibiotic resistance

The acquisition of resistance to a given antibiotic has repercussions on the bacterial physiology, depending on the elements modified. Among these consequences, the most prominent cases are fitness cost, cross-resistance and collateral sensitivity.

#### 1.4.1.1. Fitness cost

Fitness cost is a loss of competitiveness that bacteria may suffer when acquiring AR due to the metabolic alterations it causes (165, 166). The consequences of such cost will result in a reduction in growth rate compared to a wild-type, susceptible, strain, especially when the genetic changes leading to AR involve essential elements for the bacterial physiology (167). Thus, fitness cost is a key parameter determining the evolution and persistence of AR.

When resistance is acquired through the incorporation of resistance determinants via HGT, fitness cost may be the result of the physiological demands involved in replication, transcription and translation of the acquired genes (167). For instance, plasmids might sequestrate the replication machinery and alter the transcriptional profile of the bacteria, or lead to the production of foreign proteins that alter the cellular networks of the host (168, 169).

In those cases in which the cause of AR acquisition are mutations in either genes encoding antibiotic targets or transporters, or genes encoding global regulators, fitness costs could arise from the malfunction of such elements of importance in bacterial physiology (165, 170). Also, genetic modifications leading to overexpression of AR determinants, such as efflux pumps or antibiotic-modifying enzymes, could lead to a metabolic burden in antibiotic-free environments due to a constant, non-physiological, overproduction of these systems (51).

Since antibiotic-resistant bacteria might have a physiological burden, a reduction in the use of the selective antibiotic would suppose an advantage for the fitter susceptible wild-type strain, being the resistant strain outcompeted (167, 171). Therefore, once the existence of the fitness cost associated with AR acquisition was realised, the establishment of drug restriction periods was proposed (172). Nevertheless, such strategy was not always as successful as expected (173), and several cases in which resistance happened to be neutral (174) or even beneficial (175, 176) regarding bacterial fitness were detected. For example, some fluoroquinolone resistance mutations in *C. jejuni* lead to improved fitness (177), although in this case the beneficial effect was dependent on the strain containing the change, pointing to the relevance of epistasis and genetic background in bacterial fitness (see below).

When resistance acquisition is neither neutral nor beneficial, the fitness cost can be compensated, and in some cases, this compensation could lead to a decline of drug resistance. This can occur through a genetic reversion by restoration of the wild-type allele, which is a rare event (167, 178), or through the acquisition of secondary mutations that compensate the fitness cost (179, 180). The compensation can be accomplished by replacing the affected function, reducing the need for that function, or by restoring the activity of the affected protein (181, 182). Compensatory evolution has been described *in vitro* and *in vivo*, and, interestingly, the compensatory mutations selected depend on the bacterial habitat (179, 181, 183).

Although less common, the acquisition of secondary AR mutations able to compensate the cost associated with the primary ones has also been described. For instance, fitness costs derived from streptomycin resistance mutations in *E. coli* and *P. aeruginosa*, can be compensated with mutations in *rpsL* leading to rifampicin resistance (175, 184, 185). This genetic event leads to MDR bacteria with greater fitness than the mutants resistant to just one drug. Further, quinolone-resistant *E. coli* mutants were described to compensate the fitness costs of their mutations by the acquisition of an additional quinolone resistance mutation, leading to strains with both higher fitness and resistance (186).

Compensation of fitness cost may occur through gene amplifications too, as those found in actinonin-resistant mutants of *S. enterica* (187). Besides, bacteria can also compensate these costs without genetic modifications, through a metabolic rewiring that causes variations in the expression of genes that may be involved in other cellular processes. This situation has been described for *P. aeruginosa* mutants overexpressing multidrug efflux pumps (188) and in capreomycin-resistant mycobacteria (147).

#### 1.4.1.1. Cross-resistance and collateral sensitivity

The acquisition of AR, besides eventually being accompanied by a fitness cost, is also frequently associated with changes of susceptibility to other antimicrobials. When acquiring resistance to one antimicrobial, the increase of resistance or susceptibility to other antibiotics might occur, phenomena that have been known since the 1950s as cross-resistance and collateral sensitivity, respectively (189).

Regarding cross-resistance, one of the major contributors to its emergence are mutations leading to the overproduction of multidrug efflux pumps, given the diverse array of substrates they are able to extrude (190). Besides, cross-resistance among antibiotics belonging to the same structural family can arise from mutations in the target or the transporter they share (191). In addition, compensatory (192) and resistance mutations producing an alteration of the expression of genes encoding global stress response proteins (193, 194), can lead to cross-resistance between different antimicrobials.

While cross-resistance constitutes a challenge for the treatment of bacterial infections, collateral sensitivity could be exploited for the elimination of resistant bacteria. This could be done through the use of antibiotics for which bacteria have acquired collateral sensitivity, by combination therapy (195, 196) or the alternation of antimicrobials' pairs (197, 198). Nevertheless, the molecular mechanisms underlying collateral sensitivity phenomena are less understood than those

of cross-resistance, and are usually attributed to pleotropic consequences of resistance mutations (160, 197) and to the altered function of the mutated genes (155). A well-known molecular mechanism responsible for collateral sensitivity, is the one linking resistance to aminoglycosides and collateral sensitivity to β-lactams in *E. coli*. The entry of aminoglycosides into the bacterial cell requires a functional proton motive force and aminoglycosides resistance may occur due to a mutation which reduces it. The function of AcrAB-TolC is also dependent on the proton motive force, and consequently, its reduction leads to a decreased extrusion of β-lactams outside the cell (199, 200).

For the applicability in clinics of collateral sensitivity, besides better understanding the molecular mechanisms responsible for them, the robustness of these phenomena is needed. The durability and evolutionary conservation of collateral sensitivity patterns in different conditions and between different bacterial species or strains of the same species, which may coexist during an infection, are factors to be taken into account (201). Namely, the finding of conserved collateral sensitivity events in different genetic contexts, such as pre-existing antibiotic-resistant mutants - a situation closer to clinics than the more frequently studied situation using single model laboratory strains-, or in different conditions encountered during infection, would be a significant boost to the implementation of evolution-based treatments for bacterial infections.

### 1.4.2. Factors that determine the evolution of antibiotic resistance

As above said, bacteria can acquire AR through several different mutations. However, the number and type of resistance mechanisms actually selected is limited, hence the evolutionary trajectories that bacteria may follow to acquire AR are restricted. This restriction is imposed by several factors (Figure 5), four of them playing a principal role: the level of resistance conferred by each resistance mechanism, the rate at which resistance mutations arise in a bacterial population, the impact of each mutation in relative fitness, and the intensity of selective pressure. These four central determinants are influenced by each other and by other factors.

The mutation load depends on mutation rate and the population size which, for its part, is determined by population bottlenecks. Namely, the inoculum size of a bacterial population starting an infection can produce a population bottleneck eliminating certain genotypes (202). At the other end, the level of resistance achieved is dependent on the type of AR determinant and the environmental conditions which may produce a variation in the resistance determinants' expression level. For instance, anaerobic conditions and acidic pH in urine render clinical isolates of *E. coli* more susceptible to fosfomycin (203). Moreover, *Listeria monocytogenes* rearranges its virulence potential during infection conditions, causing an epistatic suppression of fosfomycin resistance through an increase of the antibiotic influx (204).

The strength and type of selection pressure on a bacterial population has an influence on relative fitness (192), mutation frequency (205), the level of resistance achieved (206) and cross-resistance or collateral sensitivity (192). It has been described that sublethal antibiotic concentrations, as those found in the clinic (in tissues with limited drug accessibility or as a consequence of incomplete treatments) (207) and in natural environments (such as rivers, lakes, sewage, etc.) (61, 208), select different evolutionary trajectories towards AR from those selected

in presence of higher antibiotic doses (209). Nevertheless, it is worth mentioning that high-level, clinically relevant, antibiotic-resistant mutants were selected under both conditions.

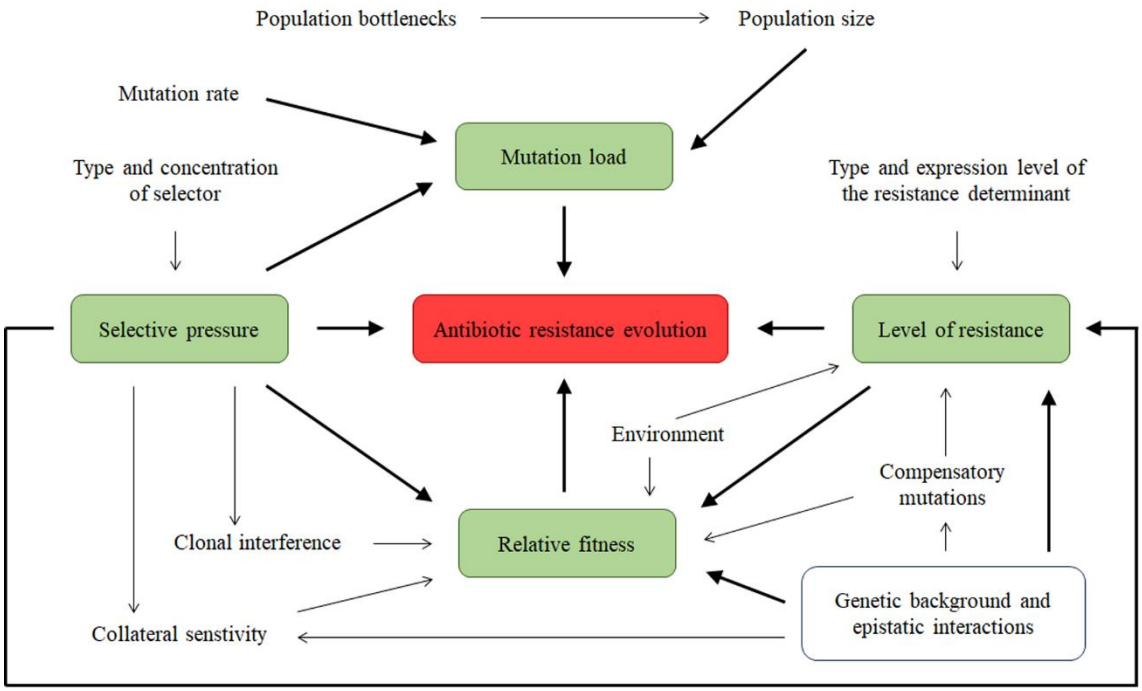


**Figure 5. Conceptual scheme bringing together the factors that constrain the evolution of antibiotic resistance and showing the interplay among them.** Four factors have a central effect on the evolution of antibiotic resistance: the relative fitness of the resistant mutant, the level of resistance conferred by the resistance mechanism, the strength of selection pressure, and the frequency of mutation. Genetic background and epistatic interactions have a major effect on antibiotic resistance evolution: resistance level, as well as the potential genetic modifications to compensate it, fitness cost and collateral sensitivity associated with a specific mutation, are strongly dependent on genetic background. The central factors are also influenced by other aspects, such as the emergence of compensatory mutations or the population size.

As above stated, the imposition of a fitness cost in absence of selective pressure is one of the consequences of acquiring AR. The relative fitness is a key determinant of the evolutionary success of a resistant mutant in the bacterial population within the host or in other environments (148). The competition between mutants with selectable phenotype (clonal interference) will ultimately depend on their fitness. Therefore, the acquisition of mutations to restore the reduced fitness (compensatory evolution) as well as the influence of the genetic context in the fitness cost caused by the acquired mutations (epistasis) will have a major role in AR evolution (156-159). Finally, collateral sensitivity phenomena affect both fitness and resistance level to a second drug, limiting the spectrum of possible resistant mutants that could emerge in the presence of such a second drug (155, 160).

Epistasis has a relevant role in AR evolution, not just by influencing the fitness but also the AR level of a mutant. It is defined as the phenomenon whereby different genes interact in the expression of a phenotypic trait. The effect of a mutation in a particular gene is influenced by the genetic context (180), hence the level of resistance to the selective antibiotic, collateral sensitivity and relative fitness associated with a specific resistance mutation may differ depending on the presence of other mutations (210, 211). If the acquired phenotype (i.e. resistance or fitness modification) is higher than that of the individual mutants, it is called positive epistasis; while if

it is lower than that of the individual mutants, it is called negative epistasis (180). Therefore, the possibility of selection of new mutations during evolution depends on the presence of preceding ones, a concept known as historical contingency which determines the order in which mutations are acquired (212, 213). Each acquired mutation is contingent on prior ones and might constrain the following steps during evolution. Hence, the gradual accumulation of mutations in different genes constitutes a barrier that canalizes the potentially selectable evolutionary trajectories towards AR (156, 180, 214). However, the fact that the most likely evolutionary routes towards resistance may change depending on the genetic context might hinder the prediction of both evolution towards AR and its associated trade-offs, as collateral sensitivity. Because of that, the clinical exploitation of collateral sensitivity requires to identify robust patterns associated with the use of an antibiotic in bacteria presenting different genetic backgrounds.

During this thesis, we have studied an evolutionary process (AR evolution) by following several bacterial generations in a short and observable period of time, in the presence of a known and defined environment and selective pressure. These experimental settings have also provided valuable information to better understand evolution in general terms.

## 1.5. *Pseudomonas aeruginosa*

*P. aeruginosa* is a rod-shaped facultative anaerobe Gram-negative bacterium with high metabolic versatility, which allows this microorganism to colonize a wide range of different habitats and hosts (215). Among these habitats we find soil (216, 217), crude oil (218) or different aquatic systems, such as freshwater and seawater environments (219-222) or wastewater (223), being the most frequent ones those environments closely associated with human activity (224). Considering that a host is another specific environment that bacteria may colonize (225), it is understandable why *P. aeruginosa* is also able to cause infections in a wide range of hosts comprising animals, such as nematodes (226), insects (227), fishes (228) or mammals -including humans (229)-, plants (230, 231) or even the amoeba *Dictyostelium discoideum* (232).

As a human pathogen, *P. aeruginosa* stands out for its capacity to produce nosocomial infections, as well as chronic infections in patients with cystic fibrosis (233) and chronic obstructive pulmonary disease (234), or acute infection in patients with HIV (235), cancer (236) and burn injuries (237), among others. As it tends to infect patients with basal pathologies or immunocompromised, the mortality produced by *P. aeruginosa* infections is particularly concerning in Intensive Care Units (238). One characteristic of *P. aeruginosa* that complicates the treatment of the infections it produces is its ability to form biofilms, which is especially worrisome in lung infections, prosthetic joints and catheter-associated urinary tract infections (239-241). Further, due to the low susceptibility of this bacterium to a wide range of antibiotics (36, 39, 242, 243) and its overwhelming capacity to acquire AR, *P. aeruginosa* is considered one of the pathogens most difficult to eradicate. It acquires AR by mutations, particularly frequent during chronic infections (244), or by the incorporation of resistance genes (245).

For all these reasons, *P. aeruginosa* is included in the groups of bacteria with high clinical relevance and high-risk concerning AR, dubbed ESKAPE pathogens and Top Ten resistant Microorganisms (TOTEM), as stated by the World Health Organisation (246-248).

### 1.5.1. Antibiotic resistance mechanisms in *P. aeruginosa*

*P. aeruginosa* has a very low membrane permeability (specifically, it is an 8% of that of *E. coli*), which hampers the passage of several compounds, being an important contributor to its low susceptibility to different drugs (249, 250). This feature may be explained by the fact that OprF, the most abundant porin in this bacterium (43), is known to have a low efficiency for antibiotic influx (44). Even more, it folds into two different conformers -closed and open channel- and more than 95% of this protein in *P. aeruginosa* is encountered in the closed conformer (251). Consequently, several antibiotics have to cross the outer membrane through specific channels, being not rare the acquisition of AR through mutation of the genes encoding those transporters. An example of such case is the acquisition of fosfomycin resistance by loss-of-function mutations of the gene encoding its transporter inside the cell, GlpT, which is the most common mechanism for acquiring fosfomycin resistance in *P. aeruginosa* (66).

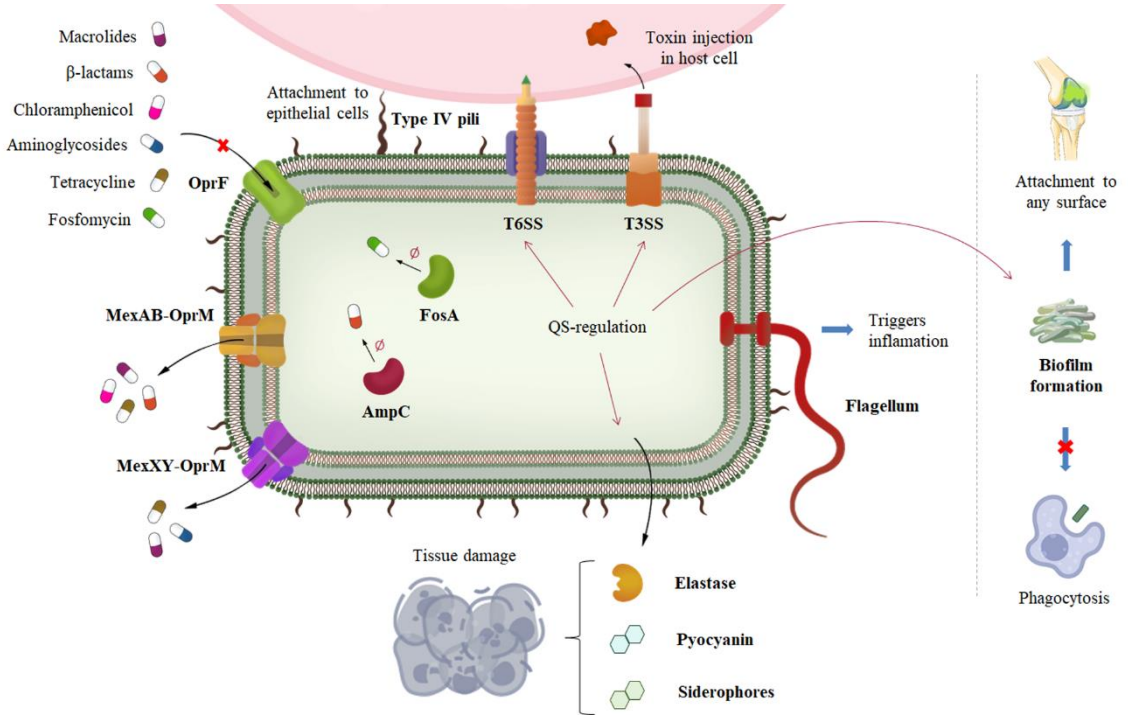


**Figure 6. Scheme representing the main mechanisms of intrinsic antibiotic resistance and virulence of *P. aeruginosa*.** The major determinants of intrinsic antibiotic resistance of *P. aeruginosa* are its low membrane permeability -caused by a closed conformation status of most of its OprF porins-, the presence of a diverse group of antibiotic-modifying enzymes -such as the β-lactamase AmpC or the fosfomycin-modifying enzyme FosA- and a set of efflux pumps able to extrude a wide range of different antibiotics -among which MexAB-OprM and MexXY-OprM are remarkable-. For its part, biofilm formation impedes phagocytosis and allows the attachment of bacteria to catheters, prosthesis or lungs; Type-6-Secretion System (T6SS) and Type-3-Secretion System (T3SS) inject different toxins directly inside the human cells; pyocyanin, elatase and siderophores have a damaging effect for the host tissues; flagellum is needed for bacterial motility and, therefore, for host tissue colonization. Importantly, Quorum Sensing (QS) has a major role in the regulation of several *P. aeruginosa* virulence factors, such as T6SS, T3SS, biofilm formation or the production of elastase, pyocyanin and siderophores.

In the basis of the low intrinsic susceptibility to antibiotics of this bacterium is also the production of antibiotic-inactivating enzymes, among which we may highlight the β-lactamase AmpC (60) or the fosfomycin-inactivating enzyme FosA (58) (Figure 6). Besides, increased resistance may

be acquired by mutations in the regulators of the expression of the genes encoding antibiotic-modifying enzymes, such as the positive regulator of *ampC*, AmpR (252).

In addition to the low membrane permeability and the expression of antibiotic-inactivating enzymes, major contributors to the high intrinsic AR of *P. aeruginosa* are RND efflux pumps, able to extrude a wide range of antibiotics (253, 254) (Figure 6). Among the 12 RND efflux systems described so far in *P. aeruginosa* (255), 4 stand out for their clinical relevance: MexAB-OprM (55), MexCD-OprJ (256), MexEF-OprN (257) and MexXY-OprM (258). Among them, MexAB-OprM contributes to intrinsic resistance to several drugs, such as β-lactams, macrolides, quinolones, chloramphenicol or tetracyclines, due to its sufficiently high basal levels of expression (259). However, mutations in the negative regulators of the genes encoding these RND efflux systems, producing their overexpression, also contribute to acquired resistance to the antibiotics that the pumps are able to extrude (Table 1). For instance, overexpression of *mexAB-oprM* through mutations in the gene encoding its local regulator MexR (71, 260), or in genes encoding secondary regulators such as NalC (261) or NalD (262), has been described in clinical situations (262-264). Also mutations in the gene encoding NfxB, the negative regulator of *mexCD-oprJ* (265, 266), MexS, which modulates the expression of *mexEF-oprN* (267) or MexZ, the local negative regulator of the *mexXY* system (264), among other mutations (Table 1), have been described to contribute to acquired resistance in clinics (263, 264, 268-272). Indeed, mutations in *mexZ* are among the most prevalent ones encountered in *P. aeruginosa* clinical strains (273).

Besides, the transient increased production of these RND efflux systems in the presence of specific triggering signals may also transiently reduce susceptibility to the antibiotics they extrude (123, 253). The expression of *mexAB-oprM* is induced by oxidative stress (274), triclosan and pentachlorophenol (275). It has also been observed that the presence of human antimicrobial peptides as LL-37 (276), as well as molecules that produce membrane damage (114) such as some disinfectants (115), induce the expression of *mexCD-oprJ*. The expression of *mexEF-oprN* is induced by nitrosative stress (277) and by contact with human airway epithelial cells (278), and it has also been described that oxidative stress or the presence of ribosome inhibitors, such as aminoglycosides or tetracyclines, induce the expression of *mexXY* (279). Since some of the described inducers/inducing conditions may be found during the course of an infection (e.g. oxidative or nitrosative stress or compounds produced by host cells), and bacteria can be in contact with inducer molecules used in clinical practice, like disinfectants or some antibiotics, this transient AR may have more relevance in a clinical context than currently thought.

Besides these three major mechanisms of AR in *P. aeruginosa*, several others have been described. For instance, the biocide triclosan is effective against Gram-positive and many Gram-negative bacteria, but not against *P. aeruginosa* due to the presence of an insensitive allele of *fabI*, encoding the target of triclosan (280). This opportunistic pathogen can also acquire resistance by the acquisition of mutations in genes encoding the antibiotic target (e.g. mutations in *gyrA*/*gyrB* which confer resistance to quinolones (281) or in PBP3 causing resistance to β-lactam antibiotics (80)). Finally, the acquisition of AR genes located in plasmids or integrons

through HGT (93, 282) or its transient resistance state, as a result of the bacterial organisation in a biofilm (102, 283), may also be of relevance in *P. aeruginosa*.

**Table 1. RND efflux pumps of *P. aeruginosa* with clinical relevance.**

| Efflux pump | Substrates | Regulators | Inducers | References |
|---|---|---|---|---|
| MexAB-OprM | β-lactams (except for imipenem), cephalosporins, macrolides, quinolones, chloramphenicol, tetracyclines, novobiocin, sulphonamides and trimethoprim | MexR, NalC, NalD, CpxR, MexT, RocA2, BrlR, AmpR, | Oxidative stress, triclosan and pentachlorophenol | (71, 253, 259-262, 274, 275, 284-289) |
| MexCD-OprJ | Quinolones, cefepime, cefpirome, meropenem, penicillin, macrolides, chloramphenicol, tetracyclines, novobiocin, trimethoprim | NfxB, EsrC | IL-37 and membrane damaging compounds such as disinfectants | (114, 115, 253, 265, 276, 289, 290) |
| MexEF-OprN | Chloramphenicol, quinolones, tetracyclines, trimethoprim | MexS, MexT, MvaT, AmpR, MxtR | Nitrosative stress and contact with human airway epithelial cells | (253, 270, 277, 278, 288, 291-293) |
| MexXY-OprM | Aminoglycosides, tetracyclines, macrolides, quinolones, cefepime, cefpirome, meropenem, penicillin | MexZ, ParRS, AmgRS | Oxidative stress and ribosome inhibitors | (253, 259, 263, 264, 279, 289, 294, 295) |

### 1.5.2. Virulence factors of *P. aeruginosa*

Among the systems which play an essential role in *P. aeruginosa* virulence, we find its previously mentioned capacity to form biofilms, which offers protection against host defence elements, as macrophages, or enhances its capacity to adhere to surfaces, making more difficult its eradication, being therefore involved in the establishment of chronic infections (296) (Figure 6). Also relevant for this type of infections is the production of siderophores, involved in iron uptake, like pyoverdine (297, 298).

Besides, this bacterium also possesses several factors involved in acute infections, like secretion systems, particularly the Type-3-Secretion System (T3SS) that allows the injection of toxins into the host cell (299, 300) (Figure 6). T3SS has been associated with recalcitrant infections (301) and increased pathogenesis in animal model infections (302-304), as well as increased mortality in human infections (305-308). Type-6-Secretion System (T6SS) is another secretion apparatus

which has been associated with virulence (309-312) and biofilm formation (313). Further, the flagella and type IV pili are elements necessary to colonize and invade the site of infection (314-316), since they contribute to different forms of motility and adhesion to surfaces (317-319) (Figure 6). Also, *P. aeruginosa* produces proteases such as elastase or protease IV, as well as a phospholipase, whose activity damages host tissues (297), and toxins, as pyocyanin, a green pigment that may induce the production of reactive oxygen species in the host (320) (Figure 6). Another pigment generated by *P. aeruginosa* is pyomelanin, a brown-staining molecule that is frequently detected (up to 13%) in clinical isolates from CF patients (321). The most widely accepted hypothesis about its role hinges on the resistance it may confer to macrophage-generated oxidative stress, thus favouring persistence in chronic lung infections (322).

Finally, the QS system plays a principal role in the virulence of *P. aeruginosa*. It is an intercellular communication system that controls the bacterial physiology through the production of autoinducer molecules in a cell density-dependent manner (323, 324). The *P. aeruginosa* QS response depends mainly on three highly interconnected regulatory systems, dubbed Las, Rhl and Pqs. The respective regulatory proteins, LasR, RhlR and PqsR, are responsible for the regulation of the expression of more than 300 genes (325). These regulators also control the synthesis of autoinducer molecules N-(3-oxododecanoyl)-L-homoserin lactone (3-oxo-C12-HSL), N-butanoyl-L-homoserin lactone (C4-HSL) and *Pseudomonas* Quinolone Signal or its immediate precursor 4-hydroxy-2-heptylquinoline (PQS and HHQ respectively). These autoinducer molecules bind to their respective regulator, causing an active form that exerts its regulatory function (326-329). Among the genes controlled by QS we find several virulence determinants encoding genes, as those related to the production of elastase, protease IV, phospholipase, pyocyanin and pyoverdine, as well as the expression of genes controlling the biofilm formation, T3SS and T6SS (330-336) (Figure 6).

### 1.5.3. Therapeutic strategies against *P. aeruginosa* infections

The infections caused by *P. aeruginosa* are currently treated with cephalosporins -especially ceftazidime- sometimes in combination with β-lactam inhibitors (337), aminoglycosides, especially tobramycin, which is often administered aerosolized (338, 339), fosfomycin, polymyxins, carbapenems, aztreonam and fluoroquinolones, especially ciprofloxacin (337, 340). However, *P. aeruginosa* infections are increasingly difficult to treat, due to its low susceptibility to several antibiotics and to its capacity of acquiring resistance to those antibiotics to which it is intrinsically susceptible, consequently, new therapeutic strategies against *P. aeruginosa* are needed (341). For this endeavour, the finding of novel types of antimicrobials or virulence inhibitors, as well as the improvement of the efficacy of available antibiotics, would be a significant step-forward in addressing this health problem.

Regarding the first approach, we may highlight the discovery of anti-virulence compounds. Virulence determinants contribute to the bacterial capacity to invade a host and to produce damage during an infection, therefore they may be targets of new compounds able to reduce the infectiveness of this bacterium, either used alone or in combination with antibiotics (342-344). Supporting this possibility, it has been reported a reduction of morbidity and an improvement of

antibacterial immune responses, *in vitro* and in animal models, when using compounds that reduce virulence by blocking QS (345). For this reason, during this PhD we have searched for novel anti-virulence compounds, specifically by taking into account the already known role of efflux pumps of plant-colonizing bacteria -including species of the *Pseudomonas* genus- in detoxifying the plant-produced anti-virulence compounds (140-143).

Besides, studying the evolution towards AR can lead to the understanding of the Achilles' heel of such evolutionary process (201), which could make a difference in the design of anti-infection therapies using the antibiotics that we already have. Some evolutionary trade-offs previously mentioned, such as collateral sensitivity or fitness costs, constitute a burden for antibiotic-resistant bacteria, that could be exploited in combination (195, 196) and sequential therapies (197, 198), or in treatments based in the alternation of the use of an antibiotic with drug restriction periods (167, 171), respectively. However, for these evolutionary trade-offs to be implementable in clinics, we need to understand the conditions in which they arise, as well as for them to be robust within different genetic contexts, environments and situations. That is why finding robust evolution trade-offs could pave the way for the development of evolution-based treatment strategies. As part of the present PhD, we have addressed this issue by looking for robust (appearing in different genetic backgrounds or environments with different nutrients' availability), clinically exploitable, trade-offs of AR evolution in *P. aeruginosa*. Further, we have more closely examined the extent to which nutrients' availability and genetic background constrain the progressive acquisition of resistance, collateral sensitivity or fitness costs. By doing so, we have defined the potential and possible limitations of sequential or combinatory use of determined antibiotics, and drug-restriction periods, for dealing with *P. aeruginosa* infections. Given that AR acquisition is one of the few evolutionary processes that can be monitored and experimentally studied in detail, besides the importance of our findings for the AR field, they have allowed us to propose conclusions with relevance for understanding evolution in general.

In addition, understanding how the environment during the infection can modify the bacterial response to an antibiotic may allow to detect conditions in which an antibacterial drug could be ineffective. That is why, during this PhD, we have also focused on the study of transient AR caused by molecules and signals able to increase the production of *P. aeruginosa*'s efflux pumps. This approach has provided information about some situations encountered in clinics that could be compromising treatments against this opportunistic pathogen.

# *OBJECTIVES*

## 2. Objectives

2.1. Study of the molecular mechanism responsible for fosfomycin collateral sensitivity robustness arising after exposure of *P. aeruginosa* to tigecycline, tobramycin or ceftazidime.

2.2. Analysis of the causes and consequences of evolution of ciprofloxacin, tobramycin and aztreonam resistance in different *P. aeruginosa* genetic backgrounds.

2.3. Analysis of the effect of media composition and nutrients' availability in the phenotypic and genotypic evolutionary trajectories of *P. aeruginosa* in presence of tobramycin, ceftazidime and ceftazidime-avibactam, and their associated trade-offs.

2.4. Study of compensatory evolution of fitness costs associated with ceftazidime resistance in different genetic backgrounds of *P. aeruginosa*, in absence of drugs or in presence of sublethal tobramycin concentrations.

2.5. Screening for compounds inducing the expression of MexAB-OprM and MexCD-OprJ efflux pumps encoding genes and analysis of their transient effect on antibiotic resistance.

2.6. Screening for inhibitors of *P. aeruginosa* virulence by searching for natural-like compounds that are inducers and substrates of MexAB-OprM and MexCD-OprJ efflux pumps.

# *RESULTS*

# 3. Results

The results of this thesis are supported by their publication in the following scientific articles:

I. Convergent phenotypic evolution towards fosfomycin collateral sensitivity of *Pseudomonas aeruginosa* antibiotic-resistant mutants

II. Mutational background influences *P. aeruginosa* ciprofloxacin resistance evolution but preserves collateral sensitivity robustness

III. Evolution of habitat-dependent antibiotic resistance in *Pseudomonas aeruginosa*

IV. Rapid decline of ceftazidime resistance in antibiotic-free and sublethal environments is contingent on genetic background

V. Novel inducers of the expression of multidrug efflux pumps that trigger *Pseudomonas aeruginosa* transient antibiotic resistance

VI. Discovery of inhibitors of *Pseudomonas aeruginosa* virulence through the search for natural-like compounds with a dual role as inducers and substrates of efflux pumps

## Article I

**Convergent phenotypic evolution towards fosfomycin collateral sensitivity of *Pseudomonas aeruginosa* antibiotic-resistant mutants**

**Laborda P, Martínez JL, Hernando-Amado S.**

***Microbial Biotechnology*. 2022 Feb;15(2):613-629. doi: 10.1111/1751-7915.13817.**

Novel strategies to fight infections are currently needed, due to the rise of AR and the reduced development of novel antibiotics. Collateral sensitivity, the event by which the acquisition of resistance to an antibiotic leads to increased susceptibility to another, is an evolutionary trade-off with potential clinical application. Indeed, the identification of pairs of drugs presenting collateral sensitivity between them, could allow the design of evolution-based strategies using the antibiotics that we already have, based on the alternation or combination of drugs. However, the emergence of collateral sensitivity is not always conserved and the mechanism underlying these evolutionary trade-offs is not always well understood, precluding the exploitation of this phenomenon. Hence identifying robust collateral sensitivity patterns, as well as studying the molecular mechanisms responsible for these phenotypes, may provide valuable information for the clinical exploitation of collateral sensitivity.

In this work, we describe that different *P. aeruginosa* antibiotic-resistant mutants selected in presence of antibiotics belonging to diverse structural families -tobramycin, tigecycline and ceftazidime- converge in a robust collateral sensitivity to fosfomycin. The underlying mechanism of the collateral sensitivity observed consisted on parallel genes' expression profiles shared by different resistant clones selected by tobramycin, tigecycline or ceftazidime and caused by different AR mutations. Namely, they all had a reduced expression of the genes encoding the peptidoglycan-recycling pathway, which preserves the peptidoglycan synthesis in situations where its *de novo* synthesis is blocked -i.e. in presence of fosfomycin-, and a reduced expression of *fosA*, encoding a fosfomycin-inactivating enzyme.

These results exemplify a phenotypic convergence towards a non-adaptive phenotype when applying different selective forces, suggesting that some phenotypes may have been caused by side selection driven by unrelated selective forces along evolution. Further, this work points to the possibility of using fosfomycin, alternated or combined with other drugs, for treating *P. aeruginosa* infections.

**Specific contributions**:

*Experimental work*: Laborda P. and Hernando-Amado S. contributed to experimental work. I performed most of the experimental work of this article, including RNA and DNA extraction, Whole-Genome Sequencing analysis, transcriptome analysis, gene expression measurements, antibiotic resistance measurement, construction of overexpression mutants, measurement of intracellular fosfomycin, growth curves measurement and determination of resistant mutants frequency, as well as the interpretation of the results.

*Manuscript writing*: All the authors contributed to the writing and correction of the manuscript, being the first version of the manuscript elaborated by me.

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# Convergent phenotypic evolution towards fosfomycin collateral sensitivity of *Pseudomonas aeruginosa* antibiotic-resistant mutants

**Pablo Laborda, José L. Martínez* and Sara Hernando-Amado***

*Centro Nacional de Biotecnología, CSIC, Madrid, 28049, Spain.*

## Summary

**The rise of antibiotic resistance and the reduced amount of novel antibiotics support the need of developing novel strategies to fight infections, based on improving the use of the antibiotics we already have. Collateral sensitivity is an evolutionary trade-off associated with the acquisition of antibiotic resistance that can be exploited to tackle this relevant health problem. However, different works have shown that patterns of collateral sensitivity are not always conserved, thus precluding the exploitation of this evolutionary trade-off to fight infections. In this work, we identify a robust pattern of collateral sensitivity to fosfomycin in *Pseudomonas aeruginosa* antibiotic-resistant mutants, selected by antibiotics belonging to different structural families. We characterize the underlying mechanism of the collateral sensitivity observed, which is a reduced expression of the genes encoding the peptidoglycan-recycling pathway, which preserves the peptidoglycan synthesis in situations where its *de novo* synthesis is blocked, and a reduced expression of *fosA*, encoding a fosfomycin-inactivating enzyme. We propose that the identification of robust collateral sensitivity patterns, as well as the understanding of the molecular mechanisms behind these phenotypes, would provide valuable information to design evolution-based strategies to treat bacterial infections.**

## Introduction

Infections due to multidrug-resistant (MDR) bacteria, with limited therapeutic options, constitute an increasing concern for human health. Among them, *Pseudomonas aeruginosa* infections entail a clinical problem because of the low susceptibility of this microorganism to several antibiotics, its disruptive virulence mechanisms and its capacity to produce nosocomial infections, as well as chronic infections in patients with cystic fibrosis (CF) or chronic obstructive pulmonary disease (Martinez-Solano *et al*., 2008; Tummler *et al*., 2014; Talwalkar and Murray, 2016). The problem is aggravated by the increasing prevalence of infections due to *P. aeruginosa* antibiotic-resistant strains. In a situation where the amount of novel antibiotics introduced for therapy is low, novel therapeutic strategies are needed. Those based on the knowledge of the evolution of antibiotic resistance (AR) are particularly interesting to specifically tackle this relevant health problem.

One promising therapeutic strategy for specifically counteract AR would be the exploitation of trade-offs of AR evolution, such as collateral sensitivity. This phenomenon, firstly described in the fifties (Szybalski and Bryson, 1952), implies that the acquisition of AR to a given antimicrobial may lead to increased susceptibility to a second drug (Pal *et al*., 2015; Podnecky *et al*., 2018; Nichol *et al*., 2019). Some works have explored the possibility of exploiting collateral sensitivity in the aim of implementing therapeutic strategies that could reduce the chances for selecting AR (Pal *et al*., 2015; Baym *et al*., 2016), such as alternation of drug pairs (Imamovic and Sommer, 2013; Kim *et al*., 2014; Imamovic *et al*., 2018) or combinatory therapy (Munck *et al*., 2014; Barbosa *et al*., 2018; Jahn *et al*., 2021). Nevertheless, these approaches still have some drawbacks. As it could be expected, collateral sensitivity depends on the antibiotic used for selection and on the mechanisms involved in the acquisition of resistance (Imamovic and Sommer, 2013; Lazar *et al*., 2013; Barbosa *et al*., 2017; Podnecky *et al*., 2018; Nichol *et al*., 2019; Kavanaugh *et al*., 2020). In addition, different works have shown that the genetic background may determine not only the evolutionary pathways towards AR but also the collateral sensitivity patterns acquired, indicating that historical contingency

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For correspondence. *E-mail jlmtnez@cnb.csic.es; **E-mail shernando@cnb.csic.es; Tel. 34 915854542; Fax 34 915854506.
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modulates AR evolution and the associated trade-offs (Baquero, 2013; Jochumsen *et al*., 2016; Hernando-Amado *et al*., 2019; Nichol *et al*., 2019). This implicates that, unless robust phenotypic patterns are found (Imamovic *et al*., 2018), the exploitation of collateral sensitivity for tackling AR may be compromised. In addition, the fact that the molecular mechanisms responsible for collateral sensitivity are still scarcely understood further compromises the possibility of taking advantage of this evolutionary trade-off to manage bacterial infections (Pal *et al*., 2015).

In previous works, we observed that all the *P. aeruginosa* PA14 populations that were submitted to adaptive laboratory evolution (ALE) in the presence of different antibiotics, namely ceftazidime, tobramycin or tigecycline (Sanz-Garcia et al., 2018a,2018b), presented collateral sensitivity to fosfomycin. This result is remarkable given that it provides a case of phenotypic convergence associated with the acquisition of resistance to antibiotics from different structural families, which suggests that fosfomycin could be alternated or used simultaneously with any of the three mentioned drugs.

Fosfomycin is an antibiotic that inhibits cell wall synthesis by targeting the enzyme MurA (UDP-N-acetylglucosamine 1-carboxyvinyltransferase), which catalyses the initial step of the peptidoglycan biosynthesis of the bacterial cell wall (Kahan *et al*., 1974). The major mechanism of fosfomycin resistance described for *P. aeruginosa* is the loss-of-function of *glpT* (Castaneda-Garcia *et al*., 2009), which encodes the fosfomycin transporter of this bacterium (Winkler, 1973). In addition, an increased expression of the gene encoding the fosfomycin target enzyme MurA, as well as the acquisition of mutations on it, has been described to increase fosfomycin resistance in other species, such as *Mycobacterium tuberculosis, Borrelia burgdorferi* or *Chlamydia* sp. (Venkateswaran and Wu, 1972; De Smet *et al*., 1999; McCoy *et al*., 2003; Jiang *et al*., 2011; Couce *et al*., 2012). Besides that, the expression of *fosA,* which encodes a fosfomycin-inactivating enzyme (De Groote *et al*., 2011; Silver, 2017), together with the activity of an alternative peptidoglycan-recycling pathway (Borisova *et al*., 2014; Fumeaux and Bernhardt, 2017; Hamou-Segarra *et al*., 2017), able to bypass MurA in the conversion of cell wall turnover products (Gisin *et al*., 2013), also contributes to the intrinsic resistance to fosfomycin of *P. aeruginosa* (Figure 1). Regarding the latter, the deletion of genes belonging to the peptidoglycan-recycling pathway has shown to increase susceptibility to fosfomycin in this bacterial species (Borisova *et al*., 2014).

Fosfomycin, alone or in combination with other antibiotics, as colistin, gentamicin, tigecycline, aztreonam, ceftolozane/tazobactam or piperacillin/tazobactam, is a valuable alternative for the treatment of difficult-to-treat infections produced by Gram-negative bacteria, such as carbapenemase-producing *Enterobacteriaceae* or MDR *P. aeruginosa* (Falagas *et al*., 2008a; Michalopoulos *et al*., 2010; Michalopoulos *et al*., 2011; Pontikis *et al*., 2014; Cuba *et al*., 2020). In addition, the co-administration of fosfomycin with ceftazidime-avibactam or tobramycin has been described to be synergistic against MDR *P. aeruginosa* strains (Papp-Wallace *et al*., 2019), as well as against CF *P. aeruginosa* biofilms (Diez-Aguilar *et al*., 2018). Furthermore, collateral sensitivity to fosfomycin is associated with the acquisition of resistance during ALE experiments in the presence of ceftazidime (alone or in combination with avibactam) or tobramycin (Sanz-Garcia et al., 2018a,2018b), a feature that has not been analysed in detail yet.

As above stated, it is quite uncommon to find conserved collateral sensitivity patterns (Barbosa *et al*., 2017), even when replicated populations from a single genetic background are treated with the same drug, which limits the exploitation of this evolutionary trade-off, although some particular examples have been described so far (Hernando-Amado *et al*., 2020; Roemhild *et al*., 2020). For instance, we recently described that tobramycin collateral sensitivity associated with ceftazidime resistance acquisition was conserved in different genetic backgrounds. In this study, resistance acquisition was mediated by a conserved mechanism consisting in chromosomal deletions including several genes among which we found the aminoglycoside extruding efflux pump encoding genes *mexXY* (Hernando-Amado *et al*., 2020). This robust collateral sensitivity phenotype was then the consequence of the parallel evolution towards ceftazidime resistance that, besides leading to such resistance, rendered the associated loss of a tobramycin intrinsic resistance determinant in all analysed lineages. Further, we observed that ceftazidime resistance acquisition also leaded to a conserved collateral sensitivity to fosfomycin, although the molecular mechanism implicated in this phenotype was not analysed in this previous work. In the present work, we found that, besides the mentioned collateral sensitivity to fosfomycin associated with the use of ceftazidime, this phenotype also emerges upon the selection of tobramycin or tigecycline-resistant mutants. Common patterns of collateral sensitivity of mutants selected in presence of different antibiotics have been rarely reported, so that we analyse in the current work the molecular mechanism behind this phenotype. We have found that this is the result of parallel expression profiles in the mutants resistant to each of the mentioned drugs. Therefore, we suggest that

 

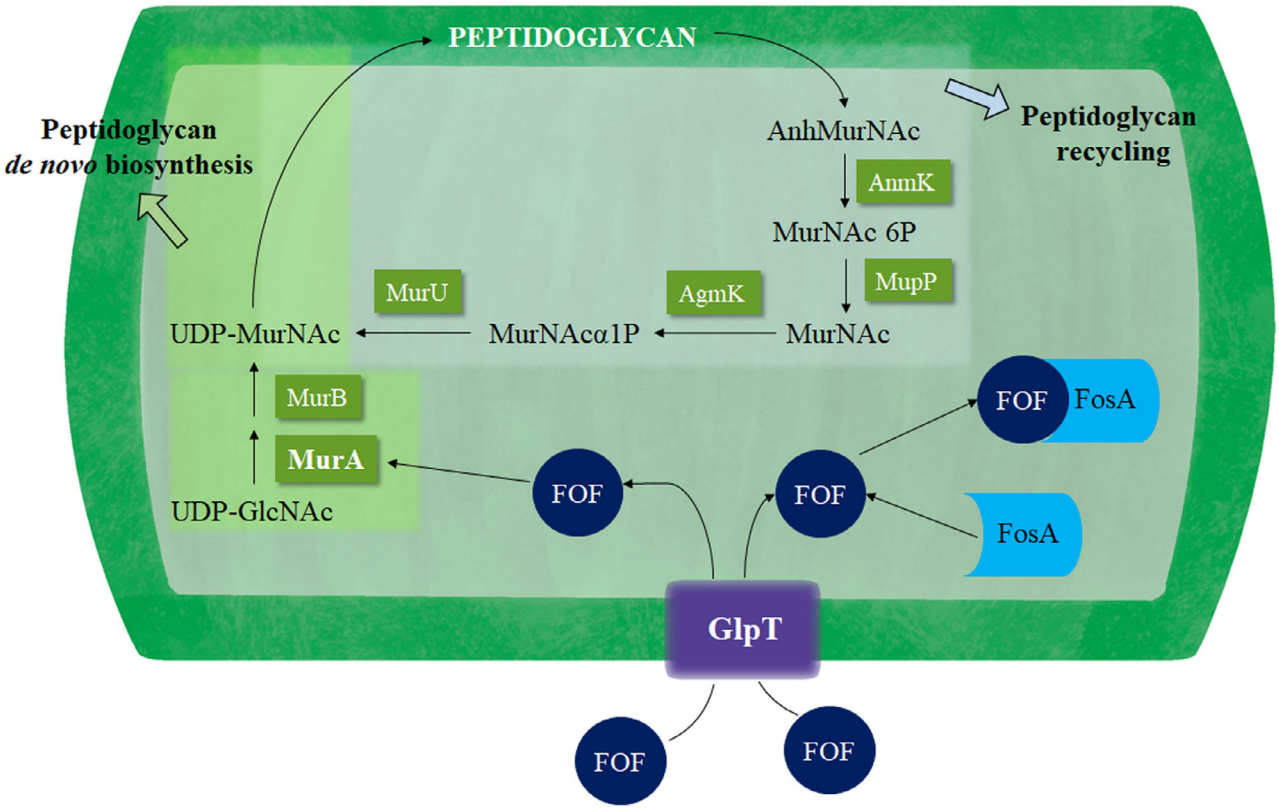


**Fig. 1.** Schematic representation of the *Pseudomonas aeruginosa* fosfomycin resistome. Fosfomycin (FOF) enters the cell through GlpT transporter (Winkler, 1973) and, once inside the cell, fosfomycin could be inactivated by FosA. A reduced expression of *fosA* increases the intracellular amount of active fosfomycin. Active fosfomycin blocks the *de novo* peptidoglycan synthesis by the inhibition of MurA (Kahan *et al.*, 1974). In this situation, the synthesis of the peptidoglycan may be maintained by the peptidoglycan-recycling pathway, whose loss-of-function leads to hyper-susceptibility to fosfomycin (Borisova *et al.*, 2014). Data shown in this work propose that a reduced expression of both, genes belonging to the peptidoglycan-recycling pathway and *fosA*, is at the molecular basis of collateral sensitivity to fosfomycin observed in different antibiotic-resistant mutants.

fosfomycin, either alone or in combination with these antimicrobials, could be used to treat *P. aeruginosa* infections.

## Results

### *Experimental evolution of* P. aeruginosa *in presence of different antibiotics leads to convergent collateral sensitivity to fosfomycin*

The evolutionary pathways of *P. aeruginosa* PA14 in the presence of tobramycin, tigecycline or ceftazidime were previously determined after 30 days of ALE assays (Sanz-Garcia et al., 2018a,2018b). Interestingly, all the resistant populations to tobramycin (four replicates), tigecycline (four replicates) or ceftazidime (four replicates) presented collateral sensitivity to fosfomycin, while control populations (evolved in the absence of antibiotics) did not show this phenotype (Sanz-Garcia et al., 2018a,2018b). To determine if collateral sensitivity to fosfomycin of these populations was acquired at the initial step of the ALE experiments, the Minimal Inhibitory Concentration (MIC) of fosfomycin was determined, at day 5 of evolution, in a representative population resistant to either tigecycline, tobramycin or ceftazidime (hereafter dubbed as PpTgc5d, PpTob5d and PpCaz5d, respectively; see Table 1) (Table 2). At fifth day of evolution, fosfomycin MIC decreased from 32 $\mu$g ml$^{-1}$ in the PA14 wild-type strain to 4 $\mu$g ml$^{-1}$ in PpTgc5d and PpTob5d populations and to 2 $\mu$g ml$^{-1}$ in PpCaz5d population (Table 2). In addition, no changes in fosfomycin MIC were observed at 5 days of ALE in the control populations (PpControlTgc5d-Tob5d and PpControlCaz5d; see Table 1), indicating that collateral sensitivity to fosfomycin was early selected by the three antibiotics (Table 2). The MIC of the antibiotic used as selective pressure in each evolved population was also measured to determine the level of resistance acquired on the fifth day of each ALE, compared to the control populations (Table 2). In addition, since there could be heterogeneity in fosfomycin susceptibility within each of the three populations tested, ten individual clones from each population were isolated and their fosfomycin susceptibility was

 

**Table 1.** Bacterial strains and plasmids used in this work.

| Bacterial strains | Description | Reference/origin |
|---|---|---|
| *Escherichia coli* | | |
| DH5α | Host strain used for the maintenance of cloning plasmids | Laboratory collection |
| OmniMAX™ | Fosfomycin susceptible strain used for the estimation of the intracellular content of fosfomycin of the studied antibiotic-resistant mutants | Invitrogen |
| *Pseudomonas aeruginosa* | | |
| PA14 | Wild-type strain of *P. aeruginosa* | Laboratory collection |
| PpTgc5d | Population evolved 5 days in tigecycline | Sanz-Garcia *et al.* (2018b) |
| PpTob5d | Population evolved 5 days in tobramycin | |
| PpControlTgc5d-Tob5d | Control population evolved 5 days in the absence of antibiotics | |
| PpCaz5d | Population evolved 5 days in ceftazidime | Sanz-Garcia *et al.* (2018a) |
| PpControlCaz5d | Control population evolved 5 days in the absence of antibiotics | |
| Tgc5d | Isolated clone from PpTgc5d | This study |
| Tob5d | Isolated clone from PpTob5d | This study |
| Caz5d | Isolated clone from PpCaz5d | This study |
| PLM011 | Wild-type strain of PA14 carrying pPLM001 vector | This study |
| PLM021 | Tgc5d clone carrying pPLM001 vector | This study |
| PLM031 | Tob5d clone carrying pPLM001vector | This study |
| PLM041 | Caz5d clone carrying pPLM001vector | This study |
| PLM010 | Wild-type strain of PA14 carrying pSEVA234 vector | This study |
| PLM020 | Tgc5d clone carrying pSEVA234 vector | This study |
| PLM030 | Tob5d clone carrying pSEVA234 vector | This study |
| PLM040 | Caz5d clone carrying pSEVA234 vector | This study |
| *fosA*:Tn | *P. aeruginosa* PA14 transposon insertion mutant in *fosA* | Liberati *et al.* (2006) |
| *agmK*:Tn | *P. aeruginosa* PA14 transposon insertion mutant in *agmK* | Liberati *et al.* (2006) |
| *murU*:Tn | *P. aeruginosa* PA14 transposon insertion mutant in *murU* | Liberati *et al.* (2006) |
| *glpT*:Tn | *P. aeruginosa* PA14 transposon insertion mutant in *glpT* | Liberati *et al.* (2006) |
| *orfN** | *P. aeruginosa* PA14 mutant in *orfN* | Hernando-Amado *et al.* (2019) |
| Plasmids | | |
| pGEM-T Easy | Commercial plasmid used for cloning optimization of PCR products. $Amp^R$ | Promega |
| pSEVA234 | Plasmid used for overexpression of genes with a strong promoter controlled by IPTG. $Km^R$ | Donated by Victor de Lorenzo's laboratory<br>GenBank: KC847292.2 |
| pPLM001 | Plasmid pSEVA234 used for overexpression of *fosA* gene with a strong promoter controlled by IPTG. $Km^R$ | This study |

**Table 2.** MIC values (μg $ml^{-1}$) of *P. aeruginosa* populations and strains used in this study.

| | TGC | TOB | CAZ | FOF |
|---|---|---|---|---|
| PA14 | 2 | 0.75 | 0.75 | 32 |
| PpControlTgc5d-Tob5d | 2 | 0.75 | 0.75 | 32 |
| PpControlCaz5d | 2 | 0.75 | 0.75 | 32 |
| PpTgc5d | **32** | 0.75 | 1 | 4 |
| PpTob5d | 48 | **4** | 1.5 | 4 |
| PpCaz5d | 2 | 0.38 | **8** | 2 |
| Tgc5d | **32** | 0.75 | 1 | 4 |
| Tob5d | 48 | **4** | 1.5 | 4 |
| Caz5d | 2 | 0.38 | **8** | 2 |
| *orfN** | 12 | 3 | 3 | 8 |

CAZ, ceftazidime; FOF, fosfomycin; TGC, tigecycline; TOB, tobramycin.
MIC values of the antibiotics used as selective pressure during ALE experiments are highlighted in bold.

measured by disc diffusion. No differences were observed, neither among the clones within a population, nor with respect to the populations to which they belong. Therefore, a representative clone from each population (hereafter dubbed as Tgc5d, Tob5d and Caz5d; see Table 1) was chosen. To ascertain if the fitness of Tgc5d, Tob5d and Caz5d mutants could be affected respect to the wild-type strain, growth curves in LB were performed. Growth defects (growth rate and/or final optical density) were not detected in the resistant mutants. Further, a growth increase was observed in Tob5d mutant (Figure 2).

### *Whole-genome sequencing of the Tgc5d, Tob5d and Caz5d mutants*

In order to elucidate the genetic causes associated with collateral sensitivity to fosfomycin of the studied clones, their genomes were sequenced. The genetic variations found in these clones are described in Table 3. The Tgc5d clone contains two different genetic variations in the gene coding for NfxB, the negative regulator of the expression of *mexCD-oprJ* (Poole *et al.*, 1996; Purssell and Poole, 2013), which encodes an efflux pump that extrudes quinolones, β-lactams and chloramphenicol (De

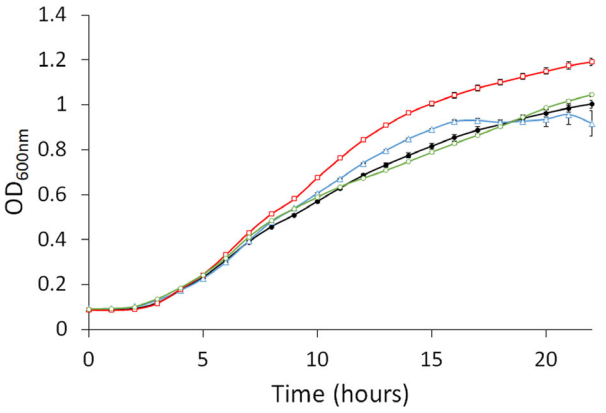


**Fig. 2.** Fitness effects of the genetic events acquired in Tgc5d, Tob5d and Caz5d mutants. Growth curves in LB medium were recorded during 20 h for Tgc5d, Tob5d and Caz5d mutants and the wild-type PA14. No growth defect was observed in Tgc5d (open triangles) and Caz5d (open circles) mutants and an increased fitness was observed in Tob5d (open squares), regarding the wild-type strain PA14 (filled circles). Error bars indicate standard deviations of six technical replicates.

**Table 3.** Genetic changes detected in the Tgc5d, Tob5d and Caz5d clones.

| Gene | Genetic change | Localization[a] | Aa change | Clone |
|---|---|---|---|---|
| *orfN* | Del G | 139 | Val50fs | Tgc5d, Tob5d |
| | Ins G | 138-139 | Val50fs | Caz5d |
| *nfxB* | Del GGAGGC | 231-236 | Glu78Ala79del | Tgc5d |
| Ins GGAGGC | | 394-395 | Lys132delinsArg ArgGln | |
| *fusA* | G→C | 1783 | Ala595Pro | Tob5d |
| – | Del 3499932 | – | 299,658 kb | Caz5d 3200274- |

**a**. Nucleotide location of the mutations, referred to the gene in which they are located, and the amino acid changes associated. Location of the large chromosomal deletion refers to the nucleotide position in *P. aeruginosa* UCBPP-PA14 reference chromosome (NC_008463.1). fs: frameshift. Del: deletion. Ins: insertion.

Kievit *et al*., 2001). The Tob5d clone presents a mutation in *fusA*, which encodes an elongation factor associated with aminoglycosides resistance (Wang *et al*., 2015; Feng *et al*., 2016; Bolard *et al*., 2018). A large chromosomal deletion of around 300 kb was detected in the Caz5d clone, containing the gene *galU*, whose inactivation increases ceftazidime resistance (Alvarez-Ortega *et al*., 2010; Sanz-Garcia et al., 2018a), which was previously described to be robustly acquired upon ceftazidime selective pressure in several genetic backgrounds (Hernando-Amado *et al*., 2020). Genetic alterations detected in the studied clones, such as mutations in *fusA* or *nfxB*, or the detected large deletions, have been described to be encountered in clinical strains (Jalal and Wretlind, 1998; Mayer-Hamblett *et al*., 2014; Bolard *et al*., 2018), a feature supporting that our ALE-derived results have clinical implications.

Importantly, a genetic change in *orfN*, which encodes a putative glycosyl transferase needed for the glycosylation of type A flagellins (Schirm *et al*., 2004), was shared by the three resistant clones. While this gene may be contributing to AR in the three clones, since *orfN* mutations have been selected during ALE experiments in presence of ciprofloxacin, ceftazidime, tobramycin, aztreonam or tigecycline (Wong *et al*., 2012; Jorth et al., 2017; Sanz-Garcia et al., 2018a,2018b; Hernando-Amado *et al*., 2019), its role in susceptibility to fosfomycin is unknown. Therefore, susceptibility to fosfomycin of an *orfN* mutant (*orfN**) (Hernando-Amado *et al*., 2019) was determined. As shown in Table 2, the MIC of *orfN** (8 $\mu$g ml$^{-1}$) was lower than that of the wild-type strain (32 $\mu$g ml$^{-1}$). However, this reduction did not reach the levels of those observed in the hyper-susceptible populations (up to 2 $\mu$g ml$^{-1}$), indicating that the observed collateral sensitivity was not just due to the mutation in *orfN*. However, no other mutations that could explain collateral sensitivity to fosfomycin observed in the three mutants were found. Neither in *fosA*, which encodes a fosfomycin-inactivating enzyme (Silver, 2017), nor in *anmK, mupP, agmK* and *murU*, which encode the enzymes of the peptidoglycan-recycling pathway (Borisova *et al*., 2014; Fumeaux and Bernhardt, 2017) or *nagZ,* the gene upstream this pathway (Borisova *et al*., 2014). As might be expected, given that the mutants present an increased susceptibility to fosfomycin, no mutations were detected in the gene encoding GlpT, the only fosfomycin transporter described so far in *P. aeruginosa* (Winkler, 1973; Castaneda-Garcia *et al*., 2009), neither in *murA*, that encodes the fosfomycin target (Silver, 2017).

### *Collateral sensitivity to fosfomycin and transcriptomic profile of the Tgc5d, Tob5d and Caz5d mutants*

The genetic changes detected by whole-genome sequencing (WGS) in Tgc5d, Tob5d and Caz5d mutants did not show a direct correlation with their collateral sensitivity to fosfomycin, since they do not present mutations in any of the elements that have been described to play a role in intrinsic resistance to fosfomycin. To ascertain if this phenotype might be due to common changes in the level of expression of genes encoding intrinsic fosfomycin resistance determinants (Figure 1) in the three mutants, a whole transcriptomic analysis was performed for each mutant (see Data S1). We specifically focused on genes with a fold change $>$ 1.5 or lower than 0.75, respect to the wild-type strain. Several of the genes that

 

**Table 4.** RNA-seq expression level of genes encoding fosfomycin resistance determinants in the Tgc5d, Tob5d and Caz5d clones.

| Gene | Synonym | Product | Tgc5d | Tob5d | Caz5d |
|---|---|---|---|---|---|
| *nagZ* | PA14_25195 | β-hexosaminidase | 0.75 | **0.74** | 0.90 |
| *anmK* | PA14_08520 | anhydro-N-acetylmuramic acid kinase | 0.78 | **0.67** | 1.10 |
| *mupP* | PA14_23210 | phosphoglycolate phosphatase | **0.71** | 0.77 | **0.59** |
| *agmK* | PA14_07780 | hypothetical protein | **0.63** | **0.69** | **0.52** |
| *murU* | PA14_07790 | nucleotidyl transferase | **0.71** | **0.70** | **0.45** |
| *fosA* | PA14_49780 | fosfomycin resistance protein | **0.67** | **0.67** | **0.58** |
| *murA* | PA14_57810 | UDP-N-acetylglucosamine 1-carboxyvinyltransferase | 0.81 | 0.80 | 1.07 |
| *glpT* | PA14_69130 | sn-glycerol-3-phosphate transporter | **0.48** | **0.41** | 1.08 |

Fold changes respect to the wild-type strain PA14. Values below the threshold are highlighted in bold.

are known to be associated with intrinsic resistance of *P. aeruginosa* to fosfomycin presented a reduced expression in the studied mutants (Table 4; Data S1). Among them, *fosA* and the genes encoding the peptidoglycan-recycling pathway (*agmK, murU*, *anmK*, *nagZ* and *mupP*) can be highlighted. In addition, despite the observed hyper-susceptibility to fosfomycin of the three mutants, the expression of *glpT,* that encodes the only known fosfomycin transporter in *P. aeruginos*a, was reduced in Tgc5d and Tob5d, but not in Caz5d (Table 4; Data S1).

The expression of the mentioned genes was analysed by quantitative reverse transcription PCR (qRT-PCR) (Figure 3), thus validating the RNA-seq analysis. A significant reduction of the expression of *fosA*, up to the half in Tgc5d or Tob5d and a quarter in Caz5d mutant, and of some of the genes encoding the peptidoglycan-recycling pathway enzymes, was observed (Figure 3). It

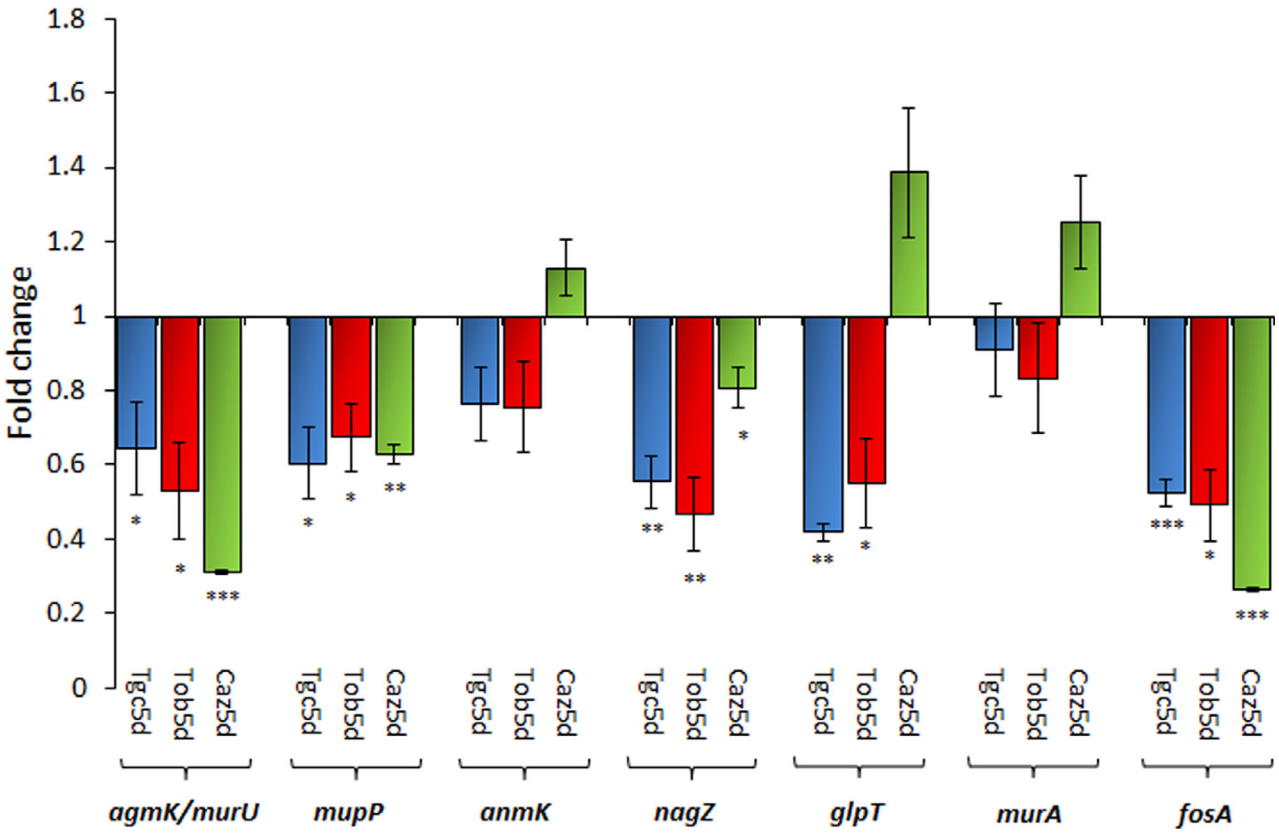


**Fig. 3.** Expression level of genes encoding intrinsic fosfomycin resistance determinants in Tgc5d, Tob5d and Caz5d mutants. Fold changes of Tgc5d (blue), Tob5d (red) and Caz5d (green) were estimated regarding the expression of the PA14 wild-type strain by qRT-PCR. Error bars indicate standard deviations of the results from three biological replicates. Statistically significant differences regarding PA14 were calculated with *t*-test for paired samples assuming equal variances: $*P < 0.05$; $**P < 0.005$; $***P < 0.0005$.

 

is known that both, the inactivation of genes encoding the peptidoglycan-recycling pathway (Borisova *et al*., 2014), as well as the inhibition of FosA (Ito *et al*., 2017), increase *P. aeruginosa* susceptibility to fosfomycin. Hence, the reduced expression of these genes may be the reason for the fosfomycin collateral sensitivity of the analysed antibiotic-resistant mutants.

To determine if the expression changes detected in *fosA* and the genes that encode the peptidoglycan-recycling pathway could be caused by the *orfN** mutation present in the three hyper-susceptible mutants, a transcriptomic analysis was performed for *orfN** (Data S1). Although this mutant presents a reduced MIC to fosfomycin compared to the wild-type, no alteration in the expression of the genes encoding the enzymes of the peptidoglycan-recycling pathway and a slight reduction of *fosA* expression (just in the 0.75 threshold) was detected (Data S1). The reduction of *fosA* expression may explain the increased fosfomycin susceptibility of the *orfN** mutant, as well as part of the collateral sensitivity to fosfomycin observed in the three mutants. However, since this phenotype is weaker in *orfN** than in the analysed resistant mutants (Table 2) and, in addition, *orfN** does not present alterations in the expression of genes encoding the peptidoglycan-recycling pathway, we hypothesize that the parallel expression profiles observed in the three mutants might result from an unknown epistatic interaction between *orfN** and the AR mutations present in the hyper-susceptible mutants. In fact, epistatic interactions in which *orfN** participates have been previously described (Hernando-Amado *et al*., 2019).

Regarding the expression of genes that might contribute to the AR phenotype of the Tgc5d, Tob5d and Caz5d mutants, the RNA-seq analysis showed that the expression of *mexCD-oprJ* in the Tgc5d clone is increased by 500-fold respect to the wild-type strain (Data S1), possibly contributing to tigecycline resistance (Sanz-Garcia *et al*., 2018b). This may be explained by the genetic modifications present in *nfxB* (Table 3), which encodes the local *mexCD-oprJ* repressor (Purssell and Poole, 2013). The genes *clpB, hslV* and *ibpA* are overexpressed in the Tob5d mutant (Data S1). Since *P. aeruginosa* knockout mutants in these genes are tobramycin hyper-susceptible (Wu *et al*., 2015), their overexpression may be contributing also to tobramycin resistance in the Tob5d mutant. In agreement with the reduced susceptibility to ceftazidime of clone Caz5d, the β-lactamase encoding gene *ampC* is overexpressed by more than 100-fold respect to the wild-type strain (Data S1). In addition, as expected, no expression of *galU,* included in the large chromosomal deletion of this mutant, was detected, which may also be contributing to ceftazidime resistance (Alvarez-Ortega *et al*., 2010).

Finally, common expression changes of genes that encode catabolic-related enzymes were observed for every fosfomycin hyper-susceptible mutant (Data S1). Regarding the carbohydrate metabolism, we observed an increased expression of genes encoding enzymes of the glycolysis pathway (*gapA* and *glk*), the phosphate pentose pathway (*zwf* and *pgl*), the Entner Doudoroff pathway (*edd*), the Krebs cycle (*acnA* and *gltA*) and the glyoxylate cycle (*glcB* and *PA14_30050*), as well as of the glucose transporters PA14_22980, PA14_22990 and PA14_23000, yet a decreased expression of lactate dehydrogenase encoding genes (*lldA* and *lldD*). Moreover, we also found increased expression of some genes that encode amino acid and fatty acid degradation enzymes, such as the *bkd* operon (*lpdV*, *bkdB*, *bkdA1* and *bkdA2*), which controls the assimilation of branched amino acids (Corona *et al*., 2018), or *fadE,* that encodes an enzyme that catalyses one reaction in the β-oxidation pathway (Zarzycki-Siek *et al*., 2013). As far as we know, a correlation between an increased expression of the genes encoding for catabolic-related enzymes, which are differentially expressed in the Tgc5d-, Tob5d- and Caz5d-resistant mutants, and collateral sensitivity to fosfomycin, has not been described.

### *The reduced expression of fosA and the peptidoglycan-recycling pathway genes are jointly responsible for collateral sensitivity to fosfomycin of Tgc5d, Tob5d and Caz5d mutants*

The transcriptomic analysis of the Tgc5d, Tob5d and Caz5d mutants showed a reduced expression of *fosA* and of the genes encoding the peptidoglycan-recycling pathway that could explain their collateral sensitivity to fosfomycin. However, we also observed that two of the mutants (Tgc5d and Tob5d) presented a reduced expression of *glpT*, which could render an impaired accumulation of intracellular fosfomycin. To find out if the altered expression of these genes could modify the amount of active fosfomycin in these mutants, the intracellular concentration of this antibiotic was measured and compared with that of the wild-type strain. Insertion mutants in the genes *glpT* and *fosA,* from a non-redundant transposon insertion library of *P. aeruginosa* PA14 (Liberati *et al*., 2006), were used as controls for low and high amount of intracellular fosfomycin, respectively. As shown in Figure 4, the intracellular concentration of fosfomycin in the mutants Tgc5d, Tob5d and Caz5d, after incubation with this antibiotic (see Experimental procedures), was threefold the one of the wild-type strain, but lower than in the *fosA* insertion mutant, whose intracellular concentration of fosfomycin was eightfold the one of the wild-type strain. In addition, the intracellular accumulation of fosfomycin in the *orfN** mutant was also

 

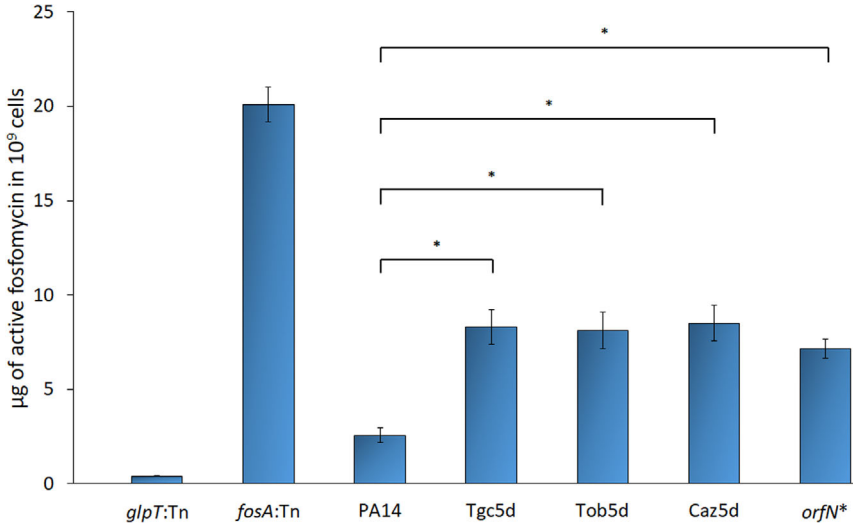


**Fig. 4.** Active intracellular fosfomycin accumulation in Tgc5d, Tob5d, Caz5d and *orfN** mutants. The amount of active intracellular fosfomycin is represented as μg of active fosfomycin in $10^9$ cells, after 1 h of incubation with 2 mg $ml^{-1}$ of the antibiotic. The resulting values were estimated regarding the hallo produced in an *E. coli* OmniMAX™ seeded plate, by a disc soaked with the intracellular content. *P. aeruginosa* PA14 *glpT* and *fosA* transposon insertion (Tn) mutants were used as controls of low and high amount of intracellular fosfomycin, respectively. Error bars indicate standard deviations of the results from three biological replicates. Statistically significant differences were calculated with *t*-test for paired samples assuming equal variances: $*P < 0.05$.

measured, since it presents a reduced expression of *fosA* compared to the wild-type strain. Similar levels to those ones measured in Tgc5d, Tob5d and Caz5d mutants were detected for *orfN** (Figure 4), indicating that the mutation in this gene may be, at least in part, responsible for the reduced expression of *fosA* and the augmented fosfomycin accumulation in these mutants. Nevertheless, the MIC to fosfomycin of *orfN** is higher than those ones of Tgc5d, Tob5d and Caz5d mutants (Table 2). As stated above, these results support that besides the increased accumulation of intracellular fosfomycin, the reduced expression of the peptidoglycan-recycling pathway genes is contributing to fosfomycin hyper-susceptibility of the studied mutants.

In order to estimate the contribution of FosA and of the peptidoglycan-recycling enzymes to the intrinsic resistance to fosfomycin of *P. aeruginosa*, the fosfomycin MIC of the *fosA, agmK* and *murU* insertion mutants, obtained from the mentioned non-redundant transposon insertion library of *P. aeruginosa* PA14 (Liberati *et al.*, 2006), was determined and compared with the ones of the Tgc5d, Tob5d and Caz5d mutants (Table 5). The transposon insertion mutants in either *agmK* or *murU* presented a fosfomycin MIC of 8 μg $ml^{-1}$ (a quarter of the one of the wild-type parental strain), while the insertion mutant in *fosA* presented a fosfomycin MIC of 2 μg $ml^{-1}$ (Table 5). Fosfomycin MICs for Tgc5d and Tob5d were close to the one of the *fosA* insertion mutant, being exactly the same in the case of Caz5d (Table 5), while the intracellular concentration of

**Table 5.** Fosfomycin MIC values (μg $ml^{-1}$) in presence or absence of an inhibitor of FosA of transposon insertion mutants in genes encoding intrinsic fosfomycin resistance determinants and of Tgc5d, Tob5d and Caz5d mutants.

| | PA14 | *fosA*: Tn | *agmK*: Tn | *murU*: Tn | Tgc5d | Tob5d | Caz5d |
|---|---|---|---|---|---|---|---|
| MHA | 32 | 2 | 8 | 8 | 4 | 4 | 2 |
| PPF | 8 | 2 | 1.5 | 2 | 2 | 2 | 1 |

PPF, FosA inhibitor phosphonoformate (500 μM).

fosfomycin in the mutants Tgc5d, Tob5d and Caz5d was much lower than in the *fosA* insertion mutant (Figure 4). These results suggest that collateral sensitivity to fosfomycin may not be just a result of the observed reduced expression of *fosA*. Indeed, if the increased intracellular concentration of fosfomycin was the only element involved in the collateral sensitivity of the studied mutants, they should present fosfomycin MICs higher than the *fosA* mutant. Consequently, other factors, such as the mentioned reduced expression of the genes belonging to the peptidoglycan-recycling pathway, must be contributing to collateral sensitivity to fosfomycin of the studied mutants.

To further analyse the different contribution of the peptidoglycan-recycling pathway and of FosA to *P. aeruginosa* fosfomycin susceptibility, the fosfomycin MICs of the *fosA, agmK* and *murU* insertion mutants, as well as of the Tgc5d, Tob5d and Caz5d mutants, were determined in presence of phosphonoformate (Table 5), an

 

inhibitor of FosA (Ito *et al*., 2017). As expected, this FosA inhibitor does not affect the fosfomycin MIC of the *fosA* insertion mutant (Table 5). However, its presence reduces fosfomycin MICs in the *agmK* and *murU* insertion mutants to levels close to those of the *fosA* insertion mutant (Table 5). Importantly, fosfomycin MIC of the Tgc5d, Tob5d and Caz5d mutants was even lower in the presence of the inhibitor of FosA, up to 1.5 μg $ml^{-1}$, while inhibition of FosA in the wild-type strain reduced the MIC just to 8 μg $ml^{-1}$ (Table 5). These results indicate that intrinsic fosfomycin resistance in *P. aeruginosa* depends on the activity of both, FosA and the peptidoglycan-recycling pathway and that the observed reduced expression of these genes in the mutants Tgc5d, Tob5d and Caz5d is jointly contributing to their collateral sensitivity to fosfomycin.

Finally, *fosA* was overexpressed in Tgc5d, Tob5d and Caz5d mutants and the wild-type strain and the intracellular concentration of fosfomycin was measured in all of them. As shown in Figure 5, *fosA* overexpression reduces the accumulation of fosfomycin in the studied mutants, as well as in the wild-type strain. Consistent with these findings, fosfomycin MIC increased above the limit of detection by E-test (> 1024 μg $ml^{-1}$) in the mutants and in the wild-type strain when *fosA is overexpressed*. This result further supports that the collateral sensitivity to fosfomycin observed in the mutants is, at least in part, a consequence of a reduced *fosA* expression. However, the fact that the inhibition of FosA leads to higher susceptibility in the mutants than in the wild-type strain (Table 5), supports that the reduced activity of the peptidoglycan-recycling pathway in the mutants, which impedes the alternative preservation of the cell wall in situations where MurA does not work, is also contributing to the convergent phenotype of the three analysed mutants.

### *The frequency of emergence of fosfomycin-resistant mutants is lower in Tgc5d, Tob5d and Caz5d mutants than in the wild-type strain*

One of the main drawbacks traditionally argued against the clinical use of fosfomycin is the high frequency of resistant mutants that are selected by this drug *in vitro*, particularly in the case of *P. aeruginosa* (Rodriguez-Rojas *et al*., 2010). However, acquisition of resistance to fosfomycin is rarely observed *in vivo* during therapy of urinary tract infections (Silver, 2017), which allows the use of fosfomycin for treating, among others, infections caused by MDR pathogens (Falagas *et al*., 2008b; Falagas *et al*., 2009; Falagas *et al*., 2010). In addition, it is known that the 'apparent mutation frequency' for one

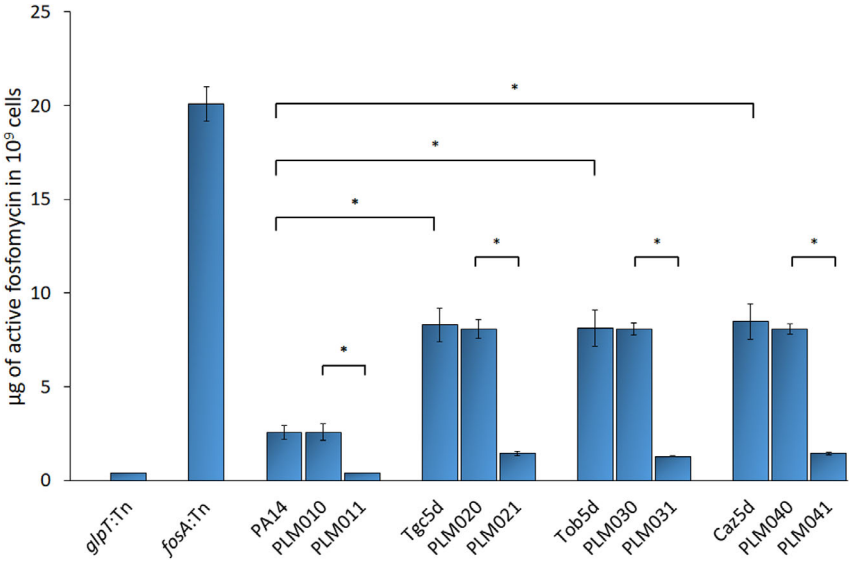


**Fig. 5.** Effect of *fosA* overexpression on the amount of active intracellular fosfomycin in Tgc5d, Tob5d and Caz5d mutants. The amount of active intracellular fosfomycin is represented as μg of active fosfomycin in $10^9$ cells, after 1 h of incubation with 2 mg $ml^{-1}$ of the antibiotic. In the case of bacteria carrying pSEVA234 derived plasmids, overexpressing or not *fosA*, incubation with the antibiotic was performed after 90 min of induction with 1 mM IPTG (see Experimental procedures). PLM011, PLM021, PLM031 and PLM041 are PA14, Tgc5d, Tob5d and Caz5d clones overexpressing *fosA,* respectively. PLM010, PLM020, PLM030 and PLM040 are PA14, Tgc5d, Tob5d and Caz5d clones containing a pSEVA234 empty vector, respectively, and were used as controls. The resulting values were estimated regarding the hallo produced in an *E. coli* OmniMAX™ seeded plate, by a disc soaked with the intracellular content. *P. aeruginosa* PA14 *glpT* and *fosA* transposon insertion (Tn) mutants were used as controls of low and high amount of intracellular fosfomycin, respectively. Error bars indicate standard deviations of the results from three biological replicates. Statistically significant differences were calculated with *t*-test for paired samples assuming equal variances: $*P < 0.05$.

 

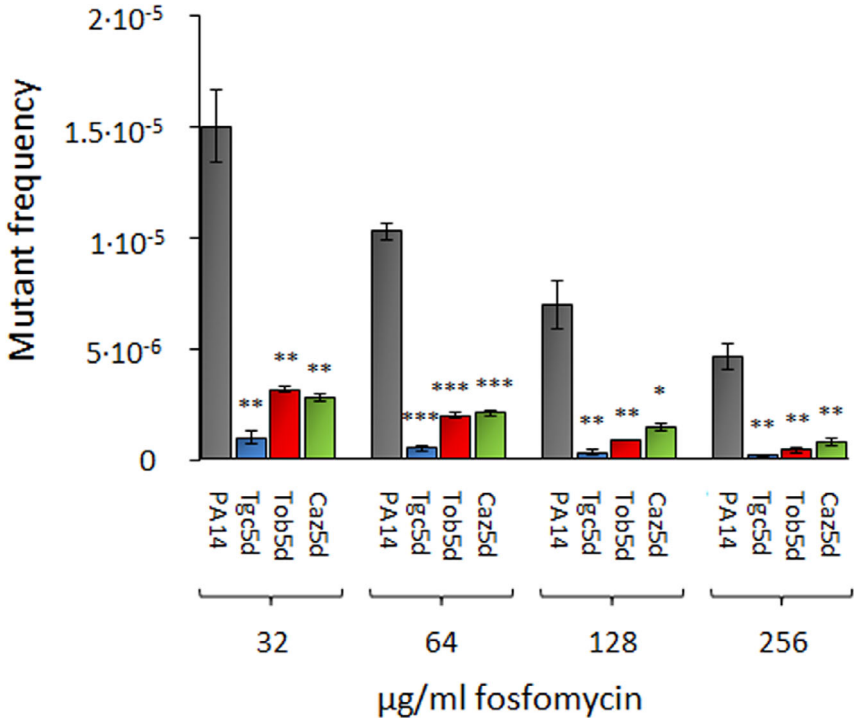


**Fig. 6.** Fosfomycin-resistant mutant frequency of wild-type *P. aeruginosa* PA14 and Tgc5d-, Tob5d- and Caz5d-resistant mutants. The frequency of fosfomycin-resistant mutants in the wild-type PA14 (grey) and the mutants Tgc5d (blue), Tob5d (red) and Caz5d (green) was estimated in the presence of different concentrations of fosfomycin. In the four genomic backgrounds analysed, it resulted to be inversely proportional to the concentration used for selection. As shown, the observed mutant frequency was lower in all the analysed mutants than in the wild-type strain. Error bars indicate standard deviations of the results from three biological replicates. Statistically significant differences regarding PA14 were calculated with *t*-test for paired samples assuming equal variances: $*P < 0.05$; $**P < 0.005$; $***P < 0.0005$.

antibiotic depends on the concentration of the antibiotic used for selection (Garcia-Leon *et al.*, 2014), a feature described in the case of fosfomycin (Demir and Buyukguclu, 2017; Falagas *et al.*, 2019). To ascertain if the studied resistant mutants, presenting collateral sensitivity to fosfomycin, may also present a decreased fosfomycin resistance mutant frequency (at least *in vitro*), the emergence of fosfomycin-resistant mutants at different concentrations of fosfomycin (32, 64, 128 and 256 $\mu$g ml$^{-1}$) was measured in the three mutants and the wild-type strain. As shown in Figure 6, the fosfomycin-mutant frequency decreased in the three mutants compared with the wild-type strain in a concentration-dependent way. Indeed, for the highest concentration analysed, fosfomycin-mutant frequency was reduced by up to 19.6-, 9.4- and 5.5-fold in Tgc5d, Tob5d and Caz5d, respectively.

## Discussion

The analysis of collateral sensitivity networks has been proposed as a good approach for implementing strategies in the use of anticancer (Pluchino *et al.*, 2012) and antimicrobial (Imamovic *et al.*, 2018; Podnecky *et al.*, 2018) compounds, which would reduce the burden of resistance (Lazar *et al.*, 2013; Hancock, 2014; Baym *et al.*, 2016). Nevertheless, with few exceptions (Imamovic *et al.*, 2018), collateral sensitivity depends on the antibiotic used for selection and the resistance mechanism involved. Further, in most cases so far studied, collateral sensitivity is not conserved across different genetic backgrounds (Lazar *et al.*, 2013; Lazar *et al.*, 2014; Barbosa *et al.*, 2017; Podnecky *et al.*, 2018) and the molecular bases of collateral sensitivity remain obscure (Pal *et al.*, 2015). This stochasticity in the collateral sensitivity networks preclude a general use of this evolutionary trade-off, since it can be exploited just when robust collateral sensitivity patterns are found.

We have recently identified a robust collateral sensitivity pattern to tobramycin in *P. aeruginosa* mutants selected in presence of ceftazidime (Hernando-Amado *et al.*, 2020). Despite the original strains submitted to evolution presented a different genetic background, the genetic event leading to ceftazidime resistance were the same; the deletion of a chromosomal region that, among several genes, contains the intrinsic tobramycin resistance determinant *mexXY* (Hernando-Amado *et al.*, 2020). Collateral sensitivity in this case was a consequence of parallel evolution of different bacterial lineages submitted to the same selective pressure.

 

In the current work, the situation is more complex, since the different bacterial lineages are submitted to different selective pressures. Despite this situation, we identified a convergent, robust phenotype of collateral sensitivity displayed by different antibiotic-resistant mutants of *P. aeruginosa* previously selected *in vitro* along ALE experiments in the presence of different antibiotics: tobramycin, tigecycline or ceftazidime. Differing to the situation analysed in (Hernando-Amado *et al*., 2020), this is an example of phenotypic convergence as a response to different selective pressures. Moreover, we identified the underlying molecular mechanisms of collateral sensitivity to fosfomycin in the three antibiotic-resistant mutants: a reduced expression of the genes encoding the peptidoglycan-recycling pathway, that preserves the peptidoglycan synthesis in situations where its *de novo* synthesis is blocked, and a reduced expression of *fosA*.

The WGS analysis of the resistant clones showed that Tgc5d, Tob5d and Caz5d do not present mutations in any of the elements that have been described to play a role in intrinsic resistance to fosfomycin, but all of them acquired a mutation in *orfN*. The analysis of Tgc5d, Tob5d, Caz5d and *orfN** transcriptomes showed a reduced expression of *fosA* that could be responsible for the reduced amount of intracellular fosfomycin detected in all of them. However, Tgc5d, Tob5d and Caz5d mutants also presented a reduced expression of the genes encoding the peptidoglycan-recycling pathway and, therefore, an increased susceptibility to fosfomycin compared with *orfN**. We hypothesize that the rewiring of the transcriptome caused by the combination of *orfN** and the AR mutations present in Tgc5d, Tob5d and Caz5d mutants is responsible for the parallel expression profiles of the three mutants. Although it is generally assumed that the effect of loss-of-function mutations in the global transcriptome may reflect the loss of specific molecular functions associated with the disrupted gene, it has recently been described that rewiring of the transcriptome upon deleterious gene inactivation is frequently non-specific and mimics stereotypic responses to external environmental changes (Kovács *et al*., 2021). Additional experiments will be required to address these questions in the framework of the acquisition of AR and the associated collateral sensitivity patterns.

Although common expression profiles were detected for the three studied mutants regarding *fosA* and peptidoglycan-recycling enzymes encoding genes, this was not a general feature for their overall transcriptomes. Indeed, many differences in expression of other genes unrelated to fosfomycin susceptibility were detected. Among them, it seems that the genes for pyochelin biosynthesis and uptake are quite overexpressed in Tgc5d, Tob5d, but not in Caz5d, whereas QS dependent on PQS is downregulated in the latter. This indicates that the observed robust collateral sensitivity pattern is not a result of a common global physiological changes, but rather of the parallel expression changes in genes involved in fosfomycin resistance. This is a feature that may be expected having into consideration that mutations in a global regulator, an elongation factor or the loss of more than 250 genes occurs in Tgc5d, Tob5d or Caz5d mutants, respectively, and it is difficult that the effect of these disparate genetic alterations in bacterial physiology is overall the same. Actually, mutations in *nfxB* or *fusA* have been described to produce global changes in the transcriptome and proteome of *P. aeruginosa* (Stickland *et al*., 2010; Maunders *et al*., 2020). It is possible that the different transcriptomic changes observed in Tgc5d, Tob5d or Caz5d may alter their virulence, however, this possibility is beyond the purpose of the current work. Nevertheless, the observation that, despite the global transcriptomes of Tgc5d, Tob5d or Caz5d differ, common changes in the expression of fosfomycin intrinsic-resistant genes are found, reinforces the importance of having detected a convergent collateral sensitivity to fosfomycin associated with the downregulation of the same genes after bacterial adaptation to different drugs.

Besides collateral sensitivity to fosfomycin, we also found that fosfomycin-mutant frequency is highly reduced in the studied mutants compared with the wild-type PA14. Previous work has shown that fosfomycin-resistant mutants of *P. aeruginosa* emerge at high frequency *in vitro* when model, antibiotic susceptible strains, are analysed (Rodriguez-Rojas *et al*., 2010), although they are not so frequently detected *in vivo*, where antibiotic selective pressure is high (Silver, 2017). Despite previous concerns, this situation and the early selection of this phenotype (at fifth day of ALE experiments) allow the use of this antibiotic, alone or in combination, for treating different types of infections, including those caused by MDR pathogens (Falagas *et al*., 2008b; Falagas *et al*., 2009; Falagas *et al*., 2010). Since we found that the studied resistant mutants present a robust collateral sensitivity to fosfomycin, together with a reduced fosfomycin-mutant frequency, it is tempting to speculate that these evolutionary trade-offs might contribute to the success of fosfomycin for inhibiting MDR *P. aeruginosa* strains. Altogether, the observations here reported and previously published information (Falagas *et al*., 2008a; Falagas *et al*., 2009; MacLeod *et al*., 2012; Díez-Aguilar *et al*., 2015; Keepers *et al*., 2017; Diez-Aguilar *et al*., 2018; Gopichand *et al*., 2019; Papp-Wallace *et al*., 2019; Hernando-Amado *et al*., 2020) point to fosfomycin as a valuable antibiotic to be introduced in sequential or combinatory therapies against *P. aeruginosa* infections. According to the *in vitro* data here

 

discussed and the results previously obtained (Hernando-Amado *et al.*, 2020), an alternation or combination of the antibiotics fosfomycin-tobramycin-ceftazidime could be particularly promising.

In agreement with previous statements (Imamovic *et al.*, 2018; Hernando-Amado *et al.*, 2020; Roemhild *et al.*, 2020), we propose that robust phenotypes of collateral sensitivity should be identified and exploited for treating bacterial infections. Particularly relevant will be finding phenotypic convergent patterns (Imamovic *et al.*, 2018) as those described in the current study. However, the feasibility of rational broad-spectrum collateral sensitivity-based strategies will require a deeper knowledge of the molecular mechanisms that increase antibiotic susceptibility upon resistance acquisition to certain antibiotics. In this regard, here we describe that a reduced expression of both *fosA* and the genes encoding the peptidoglycan-recycling pathway are responsible for collateral sensitivity to fosfomycin in different antibiotic-resistant mutants. Another limitation of collateral sensitivity to be clinically applied is that resistance to antibiotics frequently occurs by the acquisition of AR genes mediated by plasmids. Nevertheless, a recent work has identified a case of collateral sensitivity robustness associated with the acquisition of the clinically important carbapenem resistance conjugative plasmid pOXA-48 (Herencias *et al.*, 2021), which may open the possibility of exploiting the said trade-off even in the case of acquired genes. In summary, although extensive work is still required until evolution-based anti-infection approaches could be applied in clinics, we think that the results here presented and those already published provide enough evidence to support that the time has come to translate evolutionary knowledge into medical breakthrough to tackle AR.

## Experimental procedures

### *Culture conditions and determination of susceptibility to antibiotics*

Unless stated otherwise, all strains were grown in Lysogeny Broth (LB) (Lennox, Pronadisa, Torrejón de Ardoz, Spain) at 250 rpm or in LB agar (1.5% agar), at 37°C. The *E. coli* strains carrying the pSEVA234-derived plasmid or pGEM-T Easy Vector (Promega, Madison, WI, USA) were grown in LB with 50 $\mu g\ ml^{-1}$ of kanamycin or 100 $\mu g\ ml^{-1}$ of ampicillin, respectively. Strains and plasmids used in this work are included in Table 1.

Antibiotic susceptibility was determined in Mueller Hinton Agar (MHA, Pronadisa, Torrejón de Ardoz, Spain) at 37°C using MIC Test Strips (Liofilchem®, Roseto degli Abruzzi, Italy) following supplier's instructions or by disc diffusion. MHA plates were supplemented with 500 $\mu M$ of phosphonoformate (PPF) when needed.

### *Whole-genome sequencing and bioinformatics analysis*

The genomic DNA of each isolated clone was extracted using the Gnome® DNA kit (MP Biomedicals, Solon, OH, USA). The DNA quality analysis and the whole-genome sequencing (WGS) were performed by Novogene Bioinformatics Technology (Tianjin, China). Libraries constructed were pair-end ($2 \times 350$) and sequenced with an Illumina PE150 system. The average number of reads per sample represents a coverage $> 200x$. WGS data were analysed by using the CLC Genomics Workbench 12.0 software (Qiagen, Düsseldorf, Germany) by trimming genomic information and aligning the reads against the GenBank *P. aeruginosa* UCBPP-PA14 reference chromosome (NC_008463.1).

### *RNA preparation, RNA-sequencing and qRT-PCR*

Overnight cultures of *P. aeruginosa* PA14, Tgc5d, Tob5d, Caz5d and *orfN** were used to inoculate 20 ml of LB medium to a final $OD_{600nm}$ of 0.01 and were grown until exponential phase ($OD_{600nm} = 0.6$). Afterwards, centrifugation of 10 ml at 7000 rpm and 4°C, for 20 minutes, was performed.

The RNA extraction from the collected cells was performed as previously described in (Blanco *et al.*, 2017). DNA was removed by treatment with Turbo DNA-free (Ambion), and the absence of DNA was checked by PCR using primers *rpsL_Fw* and *rpsL_Rv* (Table 6). From 10 $\mu g$ of RNA, cDNA was obtained by using the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA).

RNAs obtained from three independent cultures of each strain were pooled, and RNA-sequencing (RNA-seq) was performed at the Next Generation Sequencing Service of the Centre for Research in Agricultural Genomics by using paired end format lectures ($2 \times 75bp$) in an Ion PGM™ Sequencer. Rockhopper (McClure *et al.*, 2013) was used for analysing the results. Those genes with a level of expression below 15 Reads Per Kilobase Million (RPKM) in the wild-type strain and in the mutants were discarded for further analysis. The fold change threshold for the genes up- or downregulated in the mutants compared with the wild-type strain was settled at 1.5 and 0.75, respectively. RNA-seq data included in this work are deposited in GEO database with accession GSE153006.

qRT-PCR was performed in an ABI Prism 7500 Real-time system (Applied Biosystems), using Power SYBR green PCR master mix (Applied Biosystems). Primers at 400 nM (Table 6) and 50 ng of cDNA were used in each reaction. A first denaturation step of 10 min at 95°C was followed by amplification and quantification with 40 cycles of 15 s at 95°C and 1 min at 60°C. Primers

 

**Table 6.** Primers used in this work.

| Gene | Primer fw (5'-3') | Primer rv (5'-3') | Description |
|---|---|---|---|
| *glpT* | GCAGATCTTCGCCGGTATCT | TTGGAACGGTCCGAGACCAG | qRT-PCR |
| *murA* | CATTTCCGGCGCGAAGAACT | ATGCTGCTGGCGTCGACTTCGA | qRT-PCR |
| *agmK/murU* | AGCTGAATCGCTGGTTGGAC | AACGGTCGGCAGTCTTCCTG | qRT-PCR |
| *anmK* | CAACGTGCTGATGGACGCCT | AGCCAGGACAGGTTGAAGCG | qRT-PCR |
| *nagZ* | AGGTGGGCGGGCTGATCATCTT | ATTGGGGTTGTCGGCGATCG | qRT-PCR |
| *mupP* | GCCGGACTTCATCGCCATCA | AATGCTCCTGGTAGCGGTCGAG | qRT-PCR |
| *fosA* | ACCAGGGCGCCTATCTCGAA | CGCTGCGGTTCTGCTTCCAT | qRT-PCR |
| *rplU* | CGCAGTGATTGTTACCGGTG | AGGCCTGAATGCCGGTGATC | qRT-PCR |
| *rpsL* | GCAAGCGCATGGTCGACAAGA | CGCTGTGCTCTTGCAGGTTGTG | Check DNA contamination in RNA samples |
| OE_*fosA* | CCTAGGTCACACAGGAAACAGATGCTTACCGGTCTCAATCA | GGATCCCTAGTCGGCGAAACGCATTC | Amplify *fosA* by PCR |

*rplU_Fw* and *rplU_Rv* were used to quantify the expression of the housekeeping gene *rplU* (Table 6). Primer3 Input software was used to design the primers; their specificity was tested by BLAST alignment against *P. aeruginosa* genome from Pseudomonas Genome Database (http://www.pseudomonas.com/); and their efficiency was analysed by RT-PCR using serial dilutions of cDNA. Differences in the relative amounts of mRNA were determined according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001; Pfaffl, 2001). In all cases, the values of relative mRNA expression were determined as the average of three independent biological replicates.

### *Construction of* fosA *overexpressing mutants*

The gene *fosA* was amplified using OE_*fosA_Fw* and OE_*fosA_Rv* primers (Table 6). The oligonucleotides contain the restriction enzyme target sequences of AvrII and BamHI, respectively, to allow the cloning of the amplicon into the plasmid pSEVA234 (Table 1), previously digested using these restriction enzymes. The PCR product was purified from an agarose gel, by using a DNA purification kit (GE Healthcare, Chicago, IL, USA) and, afterwards, cloned into the pGEM-T Easy vector, following supplier's instructions. *E. coli* DH5α competent cells were then transformed with these plasmids, which were afterwards purified using the QIAprep Spin miniprep kit 250 (Qiagen) and digested with AvrII and BamHI. The resulting fragments were purified from an agarose gel, and the plasmid pPLM001 (pSEVA234*fosA*) (Table 1) was obtained through a ligation reaction by the T4 DNA ligase (New England BioLabs, Ipswich, MA, USA), transformation of *E. coli* DH5α competent cells and purification using the QIAprep Spin miniprep kit 250 (Qiagen). The preparation of competent *P. aeruginosa* PA14, Tgc5d, Tob5d and Caz5d cells was performed as previously described (Irani and Rowe, 1997) and the plasmids pPLM001 (pSEVA234*fosA* overexpressing plasmid) or pSEVA234 (control plasmid) were then introduced by transformation. The resulting strains were PLM011 (PA14 overexpressing *fosA*) and PLM010 (PA14 control), PLM021 (Tgc5d overexpressing *fosA*) and PLM020 (Tgc5d control), PLM031 (Tob5d overexpressing *fosA*) and PLM030 (Tob5d control), PLM041 (Caz5d overexpressing *fosA*) and PLM040 (Caz5d control) (Table 1).

### *Measurement of intracellular fosfomycin*

The wild-type strain PA14, and the resistant mutants Tgc5d, Tob5d and Caz5d were grown in 20 ml of LB medium until exponential phase ($OD_{600nm}$ = 0.6). The cultures were centrifuged and each of the pellets suspended in 1 ml of LB. Cells were incubated with 2 mg $ml^{-1}$ of fosfomycin for 60 min at 37°C and then washed three times with 1 ml of a buffer (10 mM Tris pH 7.3, 0.5 mM $MgCl_2$ and 150 mM NaCl) by centrifugation at 7000 rpm during 10 min. In the case of the mutants carrying either pPLM001 or pSEVA234, cells were grown during 90 mintes more with 1 mM of isopropyl-β-D-1-thiogalactopyranoside (IPTG) after reaching the exponential phase, before the incubation with fosfomycin. After the last wash, pellets were suspended in 60 μl of 0.85% NaCl. A 10 μl aliquot of each culture was serially diluted and plated on LB agar to estimate number of colony-forming units (CFUs) after the incubation with fosfomycin. Cells from the remaining 50 μl suspension were broken at 100°C for 5 min and centrifuged at 13 200 rpm for 10 min. Then, paper disks (9 mm, Macherey-Nagel, Düren, Germany) were soaked with 40 μl of each supernatant and plated on LB agar plates previously seeded with *Escherichia coli* OmniMAX™ (Invitrogen, Waltham, MA, USA). The concentration of fosfomycin was quantified by measuring the diameter of the inhibition halos and by extrapolating these values to those from a standard curve of fosfomycin, obtained by measuring the hallo diameter produced by disks containing known concentrations of fosfomycin, adjusted to a

 

logarithmic equation. Intracellular fosfomycin concentration was normalized to the number of CFUs of each strain after the incubation with the antibiotic and is presented as the amount (μg) of fosfomycin per $10^9$ cells.

As a control of cell death after the 100°C treatment, 10 μl of each culture was plated on LB agar plates and growth checked after incubation during 20 h at 37°C. No growth was detected in any case. As a control of fosfomycin stability after the 100°C treatment, 40 μl of fosfomycin at 50 mg $ml^{-1}$, treated or not at 100°C during 5 min, was soaked on disks and deposited on LB agar plates previously seeded with *Escherichia coli* Omni-MAX™ and incubated at 37°C for 20 h.

### *Growth curves measurement*

The growth of P. aeruginosa was analysed by measuring the absorbance $OD_{600nm}$ of bacterial cultures. 10 μl of overnight bacterial cultures was added to 140 μl of LB in 96-well microtiter plates (Nunc™, Rochester, NY, USA), at a final $OD_{600nm}$ of 0.01. Measures were made every 10 min during 20 h, in a Tecan Infinite 200 plate reader (Tecan, Männedorf, Switzerland) at 37°C. Six technical replicates were used to estimate the average value of absorbance for each strain.

### *Determination of fosfomycin resistance mutant frequency*

The fosfomycin resistance mutant frequency was determined by plating $10^8$ cells of each of the studied strains in LB plates containing different fosfomycin concentrations, from 32 to 256 μg $ml^{-1}$. Control plates without antibiotic were also seeded with sequential dilutions of the cultures. After 24 h at 37°C, the colonies were counted in order to determine the mutant frequency of each resistant mutant at each concentration and calculated as the ratio between colonies in presence and absence of fosfomycin. The values were determined as the average of three independent replicates for each strain and condition.

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## Conflict of interest

The authors declare that they have no conflict of interest.

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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Data S1**. Whole transcriptomatic analysis.

 

## Article II

**Mutational background influences *P. aeruginosa* ciprofloxacin resistance evolution but preserves collateral sensitivity robustness**

**Hernando-Amado S, Laborda P, Valverde JR, Martínez JL.**

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Epistatic interactions and the effect of the genetic background have a major role in determining the most likely evolutionary route to be followed by bacteria when they are exposed to an antibiotic. Hence, they may have a huge influence not only in AR evolution but also in its associated trade-offs, as collateral sensitivity. Therefore, the feasibility of using information about collateral sensitivity for the design of evolution-based treatments of bacterial infections, relies on the conservation of this phenotype among strains presenting different genetic backgrounds, an issue rarely addressed in the available publications. Particularly relevant is studying collateral sensitivity robustness of mutants that acquired resistance in previous antibiotic treatments when they are challenged with a new drug, something that commonly occurs in clinical settings.

In this work, we addressed the identification of robust collateral sensitivity patterns in a set of *P. aeruginosa* antibiotic-resistant mutants when they were exposed to ciprofloxacin, aztreonam and tobramycin, antimicrobials frequently used to treat *P. aeruginosa* infections. Ciprofloxacin selected mutations in *mexS*, *nfxB* and *gyrAB*, the most commonly mutated genes reported to contribute to ciprofloxacin resistance in clinical strains. Each mutated gene was more prevalently fixed in some genetic backgrounds, but all of them led to a robust aztreonam and tobramycin collateral sensitivity. This observation constitutes an example of a convergent phenotype - collateral sensitivity to tobramycin and aztreonam- not providing a direct adaptation to the selective force and not caused by parallel evolution but by the acquisition of different genetic modifications, a situation that has not been widely studied in general evolution. In addition, populations evolved in presence of tobramycin and aztreonam did not present collateral sensitivity to ciprofloxacin, showing that collateral sensitivity was not reciprocal, hence supporting that the order in which drugs are applied for treatment really matters.

Further, we analysed whether the identified collateral sensitivity event could be exploited to drive *P. aeruginosa* antibiotic-resistant mutants to extinction. We observed that the ciprofloxacin-aztreonam alternation is more efficient than the alternation of ciprofloxacin with tobramycin, and that the combinations ciprofloxacin-aztreonam and ciprofloxacin-tobramycin are the most promising strategies to treat *P. aeruginosa* infections.

**Specific contributions**:

*Experimental work*: Laborda P. and Hernando-Amado S. contributed to experimental work. I performed some of the antibiotic resistance measurements and the drug pairs synergy assessments, as well as contributed to the interpretation of the results.

*Manuscript writing*: All the authors contributed to the writing and correction of the manuscript.

PNAS RESEARCH ARTICLE | APPLIED BIOLOGICAL SCIENCES

# Mutational background influences *P. aeruginosa* ciprofloxacin resistance evolution but preserves collateral sensitivity robustness

Sara Hernando-Amado[a,1], Pablo Laborda[a,b], José Ramón Valverde[a], and José Luis Martínez[a,1]

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**Collateral sensitivity is an evolutionary trade-off whereby acquisition of the adaptive phenotype of resistance to an antibiotic leads to the nonadaptive increased susceptibility to another. The feasibility of harnessing such a trade-off to design evolutionary-based approaches for treating bacterial infections has been studied using model strains. However, clinical application of collateral sensitivity requires its conservation among strains presenting different mutational backgrounds. Particularly relevant is studying collateral sensitivity robustness of already-antibiotic-resistant mutants when challenged with a new antimicrobial, a common situation in clinics that has hardly been addressed. We submitted a set of diverse *Pseudomonas aeruginosa* antibiotic-resistant mutants to short-term evolution in the presence of different antimicrobials. Ciprofloxacin selects different clinically relevant resistance mutations in the preexisting resistant mutants, which gave rise to the same, robust, collateral sensitivity to aztreonam and tobramycin. We then experimentally determined that alternation of ciprofloxacin with aztreonam is more efficient than ciprofloxacin–tobramycin alternation in driving the extinction of the analyzed antibiotic-resistant mutants. Also, we show that the combinations ciprofloxacin–aztreonam or ciprofloxacin–tobramycin are the most effective strategies for eliminating the tested *P. aeruginosa* antibiotic-resistant mutants. These findings support that the identification of conserved collateral sensitivity patterns may guide the design of evolution-based strategies to treat bacterial infections, including those due to antibiotic-resistant mutants. Besides, this is an example of phenotypic convergence in the absence of parallel evolution that, beyond the antibiotic-resistance field, could facilitate the understanding of evolution processes, where the selective forces giving rise to new, not clearly adaptive phenotypes remain unclear.**

collateral sensitivity | antibiotic resistance | convergent evolution | *Pseudomonas aeruginosa* | phenotypic convergence

## Significance

Bacterial adaptation to the presence of an antibiotic often involves evolutionary trade-offs, such as increased susceptibility to other drugs (collateral sensitivity). Its exploitation to design improved therapeutic strategies is only feasible if collateral sensitivity is robust, reproducible, and emerges in resistant mutants; these issues are rarely addressed in available publications. We describe a robust collateral sensitivity phenotype that emerges in different antibiotic-resistance mutational backgrounds, due to different genetic events, and propose therapeutic strategies effective for treating infections caused by *Pseudomonas aeruginosa* antibiotic-resistant mutants. Since conserved collateral sensitivity phenotypes do not confer adaptation to the presence of antibiotics, our results are also relevant for understanding convergent evolution processes in which the force selecting the emerging phenotype remains unclear.

Besides its relevance for human health, antibiotic resistance (AR) is one of the few evolutionary processes that can be experimentally addressed. Consequently, the study of the evolution processes involved in the acquisition of resistance is relevant not just in the AR field, but also in that of evolution in general. Since AR is the result of bacterial evolution, evolution-based approaches aiming to find the Achilles' heel associated with AR acquisition could be useful to tackle this relevant health problem (1). In this regard, evolutionary therapeutic strategies aiming to improve the efficacy of available antibiotics and to reduce the probability of selection of resistance are particularly interesting (2). One of the most promising trade-offs associated with the acquisition of resistance that could be exploited for implementing such novel therapeutic approaches is collateral sensitivity (CS), by which the acquisition of resistance to one drug renders an increased susceptibility to another (3).

Several studies, based on the alternation (4–7) or the combination of pairs of drugs (8–10), have tried to exploit CS (11, 12). However, for this exploitation to occur, a conserved CS phenotype must emerge when different strains become resistant to a particular drug. Unfortunately, although a few cases of robust CS have been described in model strains (5, 13, 14), this is not a common trait. Indeed, CS phenotypes are rarely conserved when different strains are compared; they differ not just among different isolates of the same species (15, 16), but also when replicated populations of the same strain and evolving in the presence of the same antibiotic are compared (17–20). While in the first case, epistasis and pleiotropy may shape the fitness effects associated with AR acquisition, restricting the type of mutations that can be selected in each genomic background (15, 21–23) and, therefore, the associated CS; in the second situation, genetic drift and population bottlenecks (24) might be responsible for the observed

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[1]To whom correspondence may be addressed. Email: jlmtnez@cnb.csic.es or shernando@cnb.csic.es.

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lack of conservation. This lack of conservation when different strains and replicated populations that present the same genetic background are analyzed would be a major drawback for implementing therapies based on the exploitation of CS networks since the emergence of this phenotype will be unpredictable if it is different for each isolate.

As mentioned above, most works in the field have focused on the evolution of model antibiotic-susceptible strains, despite the fact that antibiotic-resistant mutants are frequently encountered in clinics. When an isolate is resistant to one antibiotic, a different one is used for treating the infection that it produces—a common situation in clinics—with a possibility that resistance to this second drug could be selected. If this novel resistance is associated with a robust CS phenotype, this information may lead to prioritization of the use of a third antimicrobial, to which the newly selected resistant strain becomes hypersusceptible. Therefore, the identification of robust CS patterns, conserved in different antibiotic-resistant mutants, is a prerequisite for using this evolutionary trade-off for tackling AR. In the current work, we intend to fill this gap by analyzing the robustness of the CS phenotypes that may emerge in different *Pseudomonas aeruginosa* antibiotic-resistant mutants, previously identified in adaptive laboratory evolution (ALE) experiments of *P. aeruginosa* PA14 (13, 21), when they are submitted to short-term ALE experiments in the presence of antimicrobials belonging to different structural and functional categories.

The acquisition of robust CS patterns in different genomic backgrounds—understood as isolates with different genomes—and genetic mutational backgrounds—here understood as strains with the same genome, but presenting genetic changes that might impact AR evolution (21)—can be considered an example of convergent evolution, understood as the acquisition of the same phenotype by different organisms when confronted with similar selection pressures (25). Particularly relevant is the fact that any conserved phenotype emerging in different organisms, from bacteria to humans, as a result of convergent evolution is generally considered to be selected because it improves the adaptation of the evolved organism to the applied selective force (26, 27). Opposite to this situation, CS is not adaptive to the presence of antibiotics; it is a trade-off associated with the selection of the primary adaptive phenotype, which is resistance to the selective antimicrobial. One of the possible reasons behind convergent evolution is parallel evolution, defined as a situation in which the same genetic event responsible for the convergent phenotype (in our case, CS) is selected, irrespective of the genetic background (28). However, although one example of parallel evolution leading to robust CS has been recently reported (29), the genetic events behind CS robustness are not always conserved (30, 31).

*P. aeruginosa* is a relevant opportunistic pathogen with high prevalence at hospitals and the main cause of chronic infections in cystic fibrosis (CF) patients. It is included in the critical priority list of antibiotic-resistant pathogens (32), as well as in the group of ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter spp.*) that comprises six of the organisms for which the development of novel antibiotics/therapeutic options is an urgent need (33). The identification of robust CS networks associated with the use of drugs commonly used to treat infections caused by *P. aeruginosa*, such as fluoroquinolones, may then be useful.

The fluoroquinolone ciprofloxacin is extensively used to treat a wide range of infections caused by *P. aeruginosa* (34, 35). The two principal mechanisms of acquisition of ciprofloxacin resistance in this bacterium include mutations in the ciprofloxacin target-encoding genes *gyrAB* (encoding DNA gyrase) and *parCE* (encoding DNA topoisomerase IV) (36–40) and the acquisition of mutations leading to overexpression of *mexEF–oprN*, *mexA-B–oprM*, *mexXY*, and *mexCD-oprJ* (34, 38, 41–43), which encode efflux pumps able to extrude ciprofloxacin. Antibiotic-resistant *P. aeruginosa* clinical isolates often contain multiple ciprofloxacin-resistance mutations in *gyrA*, *gyrB*, *mexA*, *mexB*, *nfxB*, *mexZ*, or *mexS* (5, 43, 44). Importantly, ciprofloxacin-resistant clinical isolates of *P. aeruginosa* presenting mutations in *gyrA*, *gyrB*, *mexB*, and *nfxB* seem to display CS to aminoglycosides (5), which also form part of usual therapies against *P. aeruginosa* (45). Although the authors suggested that *nfxB* mutations might be responsible for the observed CS to aminoglycosides, an example of parallel evolution leading to phenotypic convergence, it remains to be firmly established to what extent different ciprofloxacin-resistance mutations, individually or jointly acquired in different mutational backgrounds, as preexisting antibiotic-resistant mutants, may be shaping the robustness of CS to aminoglycosides.

In a previous work, we determined that different antibiotic-resistant mutants of *P. aeruginosa* PA14 submitted to ALE in the presence of ceftazidime displayed a robust pattern of CS to the aminoglycoside tobramycin. This phenotype is associated with the deletion of large chromosomal regions—also found in isolates from CF patients (46)—containing *mexXY*, which encodes an intrinsic aminoglycosides-resistance efflux pump (29), hence being an example of parallel evolution. In this work, we go one step further and analyze the robustness of CS associated with short-term ALE assays performed by using preexisting antibiotic-resistant mutants in the presence of ciprofloxacin, tobramycin, or aztreonam, antibiotics widely used for treating *P. aeruginosa* infections. We found that the different preexisting resistant mutants that evolved under ciprofloxacin challenge present conserved, robust CS to tobramycin and aztreonam. However, unlike the situation in which ceftazidime was the selective force (29), the genetic events selected by ciprofloxacin were different depending on the AR mutational background. The conservation of an emerging CS phenotype in these different antibiotic-resistant mutants provides an example of phenotypic convergence in the absence of parallel evolution that can be exploited to tackle AR. Indeed, based on this information, we rationally designed and experimentally validated in vitro that evolution-based approaches can drive extinction of preexisting antibiotic-resistant mutants of *P. aeruginosa*, supporting the potential of exploiting CS convergence for tackling *P. aeruginosa* infections, including those due to antibiotic-resistant mutants.

## Results

**Evolution of Resistance and CS Associated with Short-Term ALE in the Presence of Tobramycin, Aztreonam, or Ciprofloxacin.** In order to find out robust CS phenotypes, ALE experiments in the presence of antibiotics should be performed, using different antibiotics and in different genetic backgrounds. Indeed, it has been described that the loss-of-function of a single gene, not directly related with AR, modifies the evolutionary trajectories followed by *P. aeruginosa* PA14 in the presence of antibiotics and their patterns of CS (21), a feature that might compromise CS exploitation. Further, to have a situation closer to that found at clinics, strains to be tested should include antibiotic-resistant mutants, not only a model susceptible strain—an experimental approach that has been rarely addressed. Consequently, we analyzed the evolutionary conservation of CS associated with the short-term

use of three different antibiotics—tobramycin, aztreonam, and ciprofloxacin—in antibiotic-resistant *P. aeruginosa* mutants presenting different AR mutational backgrounds. To such goal, we used a set of well-defined antibiotic-resistant mutants (29), derived from *P. aeruginosa* PA14 and containing single (*nfxB*, *parR*, *orfN*, and *mexZ*) and multiple (MDR6 and MDR12) mutations in genes encoding both, regulatory and nonregulatory proteins (Table 1). These different types of mutations were specifically chosen because, besides the fact that they are regularly found in clinical isolates, it has been previously described that AR mutations may lead to either robust or variable CS patterns in different genomic backgrounds, depending on whether they produce "target" or "regulatory" alterations, respectively (16).

Four biological replicates of each single (*nfxB177*, *parR87*, *mexZ43*, and *orfN50*) and multiple antibiotic-resistant mutant (MDR6 and MDR12) strain and the wild-type PA14 strain were submitted to ALE in the presence of tobramycin, aztreonam, or ciprofloxacin, or in the absence of antibiotics (control populations) for 3 d (112 populations in total). As expected, a decrease in the susceptibility to each of the three drugs—tobramycin, aztreonam, and ciprofloxacin—was observed in all the populations evolved in their presence, respectively (Fig. 1 and *SI Appendix*, Tables S1–S3). Statistical analysis was performed by using both nonparametric tests on nontransformed data and parametric tests of $\log_2$(FC), where FC is fold change, as described in *Materials and Methods*. Nonparametric tests were used to assert significance in the differences between each combination of strain treatment with its respective control populations grown in the absence of any drug (*SI Appendix*, Table S4). Since statistically significant changes may be detected with a magnitude not associated with a relevant change in resistance, in the present study, we have settled for a threshold (above or below 2- or 0.5-fold, respectively) as the indicator of the potential biological significance. Differences surpassing this threshold were statistically validated by using $\log_2$(FC)-based parametric tests. In all cases, both types of statistical analyses (parametric and nonparametric) were consistent in detecting significant differences ($P < 0.05$).

In the populations submitted to ALE in the presence of tobramycin, the tobramycin minimal inhibitory concentration (MIC) increased up to 4-fold in PA14, 16-fold in *parR87*, 8-fold in *orfN50*, 8-fold in *nfxB177*, 12-fold in *mexZ43*, 5.3-fold in MDR6, and 3-fold in MDR12 ($P < 0.05$ in all cases and using all approaches). Under aztreonam exposure, aztreonam MIC increased up to 8-fold in PA14; 2.7-fold in *parR87*; 4-fold in *orfN50* and MDR12; 24-fold in *nfxB177*; 12-fold in *mexZ43*; and 12-fold in MDR6 ($P < 0.005$ in all cases). Finally, ALE challenge with ciprofloxacin increased the MIC to this drug up to 46.9-fold in PA14, 128-fold in *parR87*, 15.8-fold in *orfN50*, 16-fold in *nfxB177*, 48-fold in *mexZ43*, 24-fold in MDR6, and 10.5-fold in MDR12 ($P < 0.005$ in all cases). It is worth noting that the greatest increase of AR occurred in *parR87* in the presence of tobramycin and ciprofloxacin and *nfxB177* in the presence of aztreonam, indicating that some mutational backgrounds are more prone to evolve toward higher levels of resistance than others. The question remains whether these differences are due to the acquisition of different AR mutations or whether the same AR mutations have a different effect depending on the mutational background where they are acquired. Control populations that evolved in the absence of antibiotics generally did not present variations in their susceptibility to antibiotics with respect to the MIC of parental strains, and, in the few cases in which these changes occurred, they were minor, not statistically significant, changes (*SI Appendix*, Table S4). This feature supports that the changes in MICs (above or below 2- or 0.5-fold, respectively) observed in populations challenged with antibiotics were due to antibiotic selection, not to a nonspecific adaptation to the growth medium.

In order to ascertain the evolutionary conservation of CS associated with the short-term use of tobramycin, aztreonam, or ciprofloxacin in the different mutational backgrounds, MICs to antibiotics from different structural families were determined for each of the final populations and their parental strains, making a total of 714 MICs (*SI Appendix*, Tables S1–S4). Short-term ALE in the presence of tobramycin or aztreonam resulted in cross-resistance to ceftazidime, ciprofloxacin, and aztreonam, at least in some of the analyzed mutational backgrounds ($P < 0.05$). The tested populations did not present a clear pattern of CS to other drugs, except for the case of fosfomycin (Fig. 1 and *SI Appendix*, Tables S1 and S2). In particular, CS to fosfomycin was observed in replicate populations from PA14 and four out of six mutational backgrounds (*orfN50*, *nfxB177*, *mexZ43*, and MDR12) submitted to short-term ALE in the presence of tobramycin ($P < 0.05$). The possibility of alternating or combining these two antibiotics has been proposed (13, 29, 31, 47, 48) and hence will not be discussed here. Interestingly, no cross-resistance to other drugs was observed in populations submitted to short-term ALE in the presence of ciprofloxacin, while CS to all the analyzed drugs was observed in replicate populations from at least three different mutational backgrounds. It is worth noting the robustness of CS toward two antibiotics (tobramycin and aztreonam), which was detected in replicate populations from PA14 and from six and five out of six mutational backgrounds analyzed, respectively (Fig. 1 and *SI Appendix*, Table S3). In particular, tobramycin MIC was reduced up to 2-fold in *nfxB177*, *mexZ43*, and MDR12; 2.6-fold in PA14; 3-fold in *parR87* and MDR6; and 6-fold in *orfN50* ($P < 0.05$ in all cases except in *mexZ43*). In the case of aztreonam, MIC was reduced up to 2-fold in PA14, *nfxB177*, and MDR6; 3-fold in *parR87* and MDR12; and 6-fold in *orfN50* ($P < 0.05$ in all cases except in MDR12). It is relevant to highlight that the observed CS was not reciprocal; while populations evolved in the presence of

**Table 1. Original genetic events of the *parR87*, *orfN50*, *nfxB177*, *mexZ43*, MDR6, and MDR12 mutational backgrounds**

| Mutational background | Gene | Type of genetic modification | Amino acid change |
|---|---|---|---|
| *parR87* | *parR* | SNP | Glu87Lys |
| *orfN50* | *orfN* | Deletion | Val50fs |
| *nfxB177* | *nfxB* | SNP | Phe177Ser |
| *mexZ43* | *mexZ* | SNP | Val43Gly |
| MDR6 | *mexC* | SNP | Thr267Ala |
| | *pmrB* | SNP | Leu87Gln |
| | *frr* | SNP | Ile98Ser |
| | *phoQ* | SNP | Val260Gly |
| MDR12 | *fusA* | SNP | Tyr552Cys |
| | *fusA* | SNP | Tyr683Cys |
| | *orfN* | Deletion | Val50fs |
| | *pmrB* | SNP | Met46Ile |
| | *mexZ* | SNP | Val43Gly |
| | *gabP* | SNP | Ser267Phe |
| | *ptsP* | SNP | Leu537Pro |
| | *nuoC* | SNP | Gln184* |

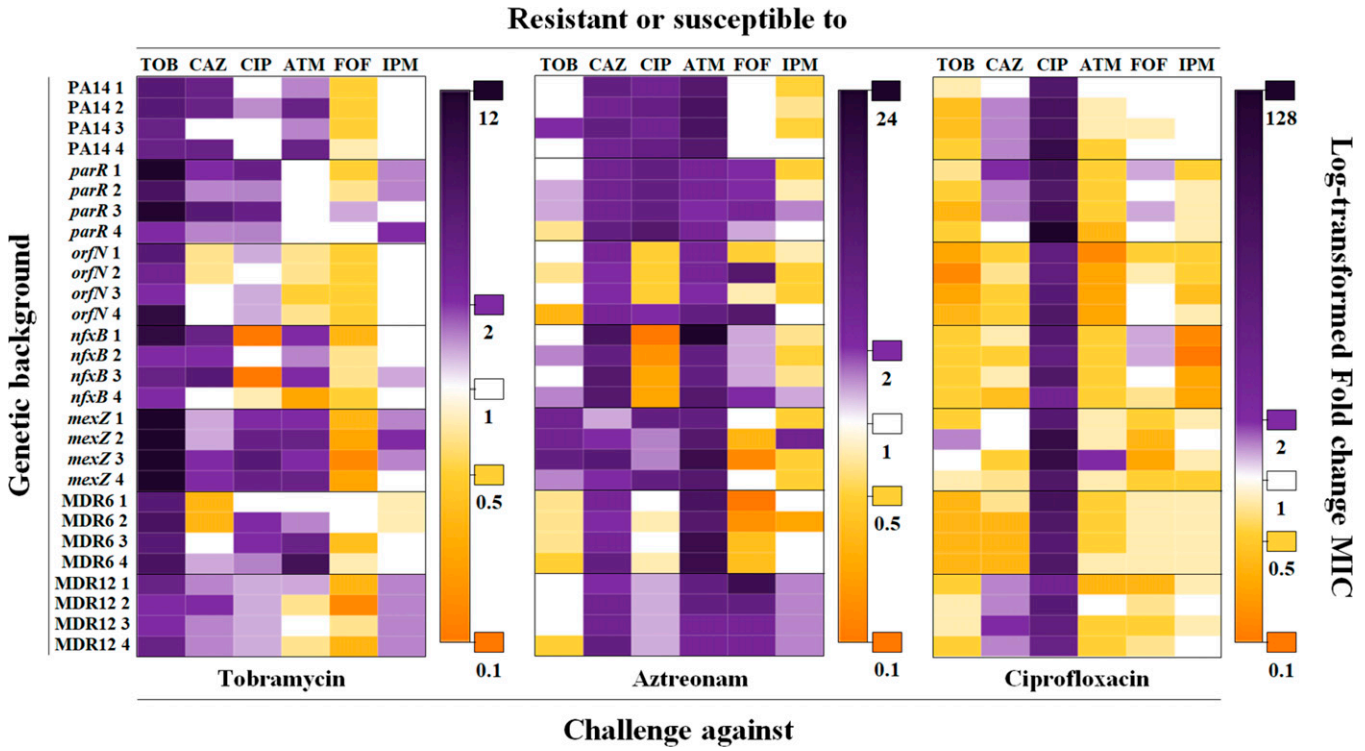


**Fig. 1.** Diagram showing robustness of CS to tobramycin and aztreonam in PA14 and in different mutational backgrounds submitted to short-term ALE on ciprofloxacin. Cross-resistance and CS to antibiotics from different structural families were analyzed in PA14 and the mutational backgrounds *parR87*, *orfN50*, *nfxB177*, *mexZ43*, MDR6, and MDR12 (four replicate populations for each) submitted to ALE in the presence of tobramycin, aztreonam, or ciprofloxacin for 3 d. Intensity of the color is proportional to the log-transformed FC regarding the MIC of the respective parental strain. Since control populations evolved in the absence of antibiotics may present, on rare occasions, subtle changes (below or above 2- or 0.5-fold, respectively) in their susceptibility to antibiotics with respect to the MIC of parental strains, changes in MICs above or below 2- or 0.5-fold, respectively, were considered physiologically relevant to classify a population as "resistant" (purple) or "susceptible" (orange). MIC values (μg/mL) of populations evolved in the presence of tobramycin, aztreonam, or ciprofloxacin are included in *SI Appendix*, Tables S1–S3, respectively. MIC values (μg/mL) of control populations evolved in the absence of drugs are included in *SI Appendix*, Table S4. ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; FOF, fosfomycin; IPM, imipenem; TOB, tobramycin.

ciprofloxacin presented a robust CS to aztreonam and tobramycin, populations challenged with either aztreonam or tobramycin did not present an equivalent CS to ciprofloxacin. Further, while ciprofloxacin-resistant populations did not present cross-resistance to other antibiotics, cross-resistance was frequently observed in aztreonam- and tobramycin-resistant populations. This means that the order in which antibiotics are used might be fundamental for implementing evolution-based strategies to deal with infections and AR.

**Evolutionary Strategies Based on Robustness of CS to Drive Extinction of Preexisting *P. aeruginosa*-Resistant Mutants.** CS to aminoglycosides has been described in clinical strains of *P. aeruginosa* from CF patients treated with ciprofloxacin (5), and CS to tobramycin and aztreonam has been observed in PAO1 during ALE experiments in the presence of ciprofloxacin (5). Here, we have observed that ciprofloxacin rapidly selects mutants presenting CS to tobramycin and aztreonam in the *P. aeruginosa* PA14 genomic background. Since this strain presents genomic and physiological differences with PAO1 (49, 50), the conservation of the same CS pattern between them supports its robustness among strains presenting different genomic backgrounds. In addition, we found that the same robust CS phenotype was found when different mutants, already presenting AR mutations, were submitted to ciprofloxacin-selective pressure. Altogether, these findings support the robustness of the observed CS in different strains, including former antibiotic-resistant mutants. Therefore, we tested the possibility of alternating ciprofloxacin with these antibiotics. This strategy consisted of two stages: a first step on ciprofloxacin, which would drive evolution toward CS to tobramycin and aztreonam; and a second step on tobramycin or aztreonam, which would drive extinction of tobramycin–aztreonam-susceptible cells (Fig. 2*A*). Between treatments, cells were stocked in glycerol. This two-step design of the experiment allowed us to decouple CS (the aim of this work) from potential hysteresis situations (51) that might compromise the interpretation of the results.

We started with the 28 ciprofloxacin-resistant populations (*SI Appendix*, Table S3) belonging to PA14 and to six different mutational backgrounds (*parR87*, *orfN50*, *nfxB177*, *mexZ43*, MDR6, and MDR12), four replicate populations of each, previously submitted to short-term ALE on ciprofloxacin, and 28 control populations (previously unchallenged with ciprofloxacin). As mentioned above, these ciprofloxacin-resistant populations became resistant to ciprofloxacin, being all MICs above the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoint (0.5 μg/mL) (*SI Appendix*, Table S3), and presented CS to aztreonam and tobramycin, being MICs below the EUCAST clinical breakpoint (16 and 2 μg/mL, respectively), with the exception of tobramycin ones in MDR12 (*SI Appendix*, Table S3). At this point, we focused on the switch from ciprofloxacin to tobramycin or aztreonam (Fig. 2*A*). Even though we had observed conservation of CS to tobramycin and aztreonam after evolution in the presence of ciprofloxacin within the analyzed set of mutational backgrounds (Fig. 1 and *SI Appendix*, Table S3), a critical point would be determining if the proposed strategies could be effective to eliminate cell viability in most of them. Hence, we switched the selective pressure from ciprofloxacin to tobramycin (28 ciprofloxacin-resistant populations and 28 control

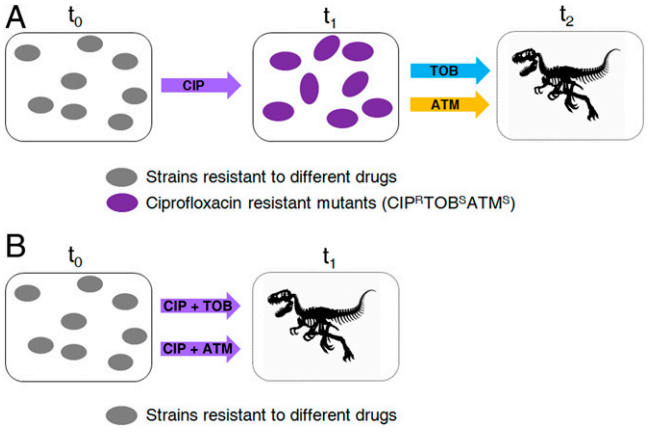


**Fig. 2.** General model illustrating evolution of antibiotic-resistant mutants of *P. aeruginosa* submitted to the alternation of ciprofloxacin with tobramycin or aztreonam or the combination of ciprofloxacin with tobramycin or aztreonam. (*A*) Evolution starts when different antibiotic-resistant mutants are treated with ciprofloxacin at time 0 ($t_0$). Then, there is evolution toward ciprofloxacin resistance and CS to tobramycin and aztreonam (purple cells), rendering ciprofloxacin ineffective ($t_1$). Subsequently, treatment is switched to tobramycin (TOB) or aztreonam (AZT) that may result in the elimination of cells susceptible to tobramycin and aztreonam ($t_2$). (*B*) Evolution starts when different antibiotic-resistant mutants are treated with a ciprofloxacin–tobramycin or a ciprofloxacin–aztreonam combination at time 0 ($t_0$). Since ciprofloxacin-resistance acquisition leads to CS to tobramycin and aztreonam, it may be expected that drug combinations result in a reduced rate of adaptation or the elimination of cells ($t_1$).

populations) or aztreonam (28 ciprofloxacin-resistant populations and 28 control populations) (*Materials and Methods*). As shown in Fig. 3*A*, 11 out of 28 ciprofloxacin-resistant populations submitted to short-term ALE in the presence of aztreonam became extinct, whereas this only occurred in 4 out of 28 ciprofloxacin-resistant populations submitted to short-term ALE on tobramycin. In both cases, the extinction differences were statistically significant ($P < 0.05$). These results suggest that exploiting the aztreonam CS associated with the use of ciprofloxacin by switching selective pressure from ciprofloxacin to aztreonam, although a feasible approach, could be ineffective in driving extinction of the model strain PA14 and of some mutational backgrounds, such as MDR12, at least at the concentrations tested.

It has been suggested that CS may not only improve treatment when drugs are applied sequentially, but it may also serve to optimize combinatory therapy, as it has been described for the combinations ciprofloxacin–aminoglycosides and ciprofloxacin–β-lactams (8). Thus, we decided to analyze the antibiotic-combination efficacy of the ciprofloxacin–tobramycin and ciprofloxacin–aztreonam pairs of drugs in our set of preexisting antibiotic-resistant mutants. We submitted the populations belonging to PA14 and six different mutational backgrounds (*parR87*, *orfN50*, *nfxB177*, *mexZ43*, MDR6, and MDR12), four replicate populations of each, to the drug combinations ciprofloxacin–tobramycin (28 populations) or ciprofloxacin–aztreonam (28 populations) at the concentrations used for previous ALE assays in the presence of ciprofloxacin, aztreonam, or tobramycin (Fig. 1 and *SI Appendix*, Tables S1–S3) (*Materials and Methods*). As shown in Fig. 3*B*, 23 and 25 out of 28 populations submitted to short-term ALE in the presence of either ciprofloxacin–tobramycin or ciprofloxacin–aztreonam became extinct. In both cases, the extinction differences were statistically significant ($P < 0.05$). To further analyze if the high efficiency of these combinatory therapies could be influenced by a synergistic antibiotic interaction between the antibiotics used, we performed 14 checkerboard analyses (*Materials and Methods*) for PA14 and the six mutational backgrounds here analyzed and the two pairs of drugs tested. We did not observe synergy (neither antagonism) between ciprofloxacin and tobramycin or between ciprofloxacin and aztreonam in any of the genetic backgrounds analyzed (fraction inhibitory concentration [FIC] index $\geq 0.5$ and $\leq 4$ in all cases) (*SI Appendix*, Table S5). This observation, in addition to the above-mentioned results (Fig. 3*B*), reinforces that CS to tobramycin and aztreonam associated with the use of ciprofloxacin optimizes the efficiency of the ciprofloxacin–tobramycin and ciprofloxacin–aztreonam pairs of drugs. These results suggest that exploiting the CS associated with the use of ciprofloxacin is a possibility that should be considered, particularly in the case of preexisting antibiotic-resistant mutants, a feature in agreement with previous data indicating that quinolone-resistant subpopulations isolated from CF patients may be eradicated using aminoglycoside and β-lactam drugs (5).

**Genetic Variations Associated with both Ciprofloxacin Resistance and CS in *P. aeruginosa* PA14 and in Different Antibiotic-Resistant Mutants.** To gain insights into the genetic causes of the acquisition of ciprofloxacin resistance and the robust CS pattern observed in the populations submitted to short-term ALE in the presence of ciprofloxacin, the 28 populations independently evolved during 3 d and their parental strains (PA14, *parR87*, *orfN50*, *nfxB177*, *mexZ43*, MDR6, and MDR12) were subjected to whole-genome sequencing. Control populations evolved in the absence of drugs were not whole-genome-sequenced since they did not present any relevant change in the MICs of the tested antibiotics. The genome of the 28 ciprofloxacin-resistant populations was compared to the ones of their respective parental strains in order to determine newly genetic events acquired during evolution. A total of 50 genetic events were identified (Table 2): 8 in PA14, 8 in *parR87*, 5 in *orfN50*, 4 in *nfxB177*, 11 in *mexZ43*, 9 in MDR6, and 5 in MDR12. All detected gene variants were located just within five different genes, and their acquisition was dependent on the genetic background (Table 2): *mexS* variants were acquired in PA14, *parR87*, *mexZ43*, and MDR6; *nfxB* variants were acquired in *parR87*, *orfN50*, *mexZ43*, and MDR12; *gyrAB* variants were acquired in *nfxB177* and MDR12; and an *orfN* variant was acquired in *mexZ43*.

Despite *orfN* mutations having been described in ALE assays in the presence of ciprofloxacin (52), only a single replicate of *mexZ43* presented a mutation in this gene. Instead, the most prevalent mutations were found in *gyrAB*, encoding the quinolones' target (36–40), in *mexS*, encoding a regulator of the expression of *mexEF–oprN*, and in *nfxB*, encoding a regulator of the expression of *mexCD–oprJ*, which may lead to overexpression of these efflux pumps (34, 38, 41–43) (Table 2 and Fig. 4). Importantly, *P. aeruginosa* isolates from chronically infected CF patients treated with ciprofloxacin frequently present mutations in these genes (5, 43, 44). In particular, we identified 15 different variants of *mexS*, 5 different variants of *nfxB*, 3 different *gyrA* variants, and a single *gyrB* variant. Notably, variant NfxBHis21Pro was acquired in three out of the four mutational backgrounds presenting mutations in this gene, suggesting an important phenotypic impact, and variants NfxBThr39Pro, GyrAThr83Ile, GyrAAsp87Gly, and GyrBSer466Phe have already been described as clinically relevant (34, 36, 40, 43), further supporting our experimental approach.

Variants in which an isoleucine replaces the threonine at position 83 of GyrA are among the most frequent alterations associated with ciprofloxacin resistance in *P. aeruginosa* occurring in clinical and in vitro-selected resistant mutants (34, 36, 38–40, 53–55). The second most frequent variant in GyrA is

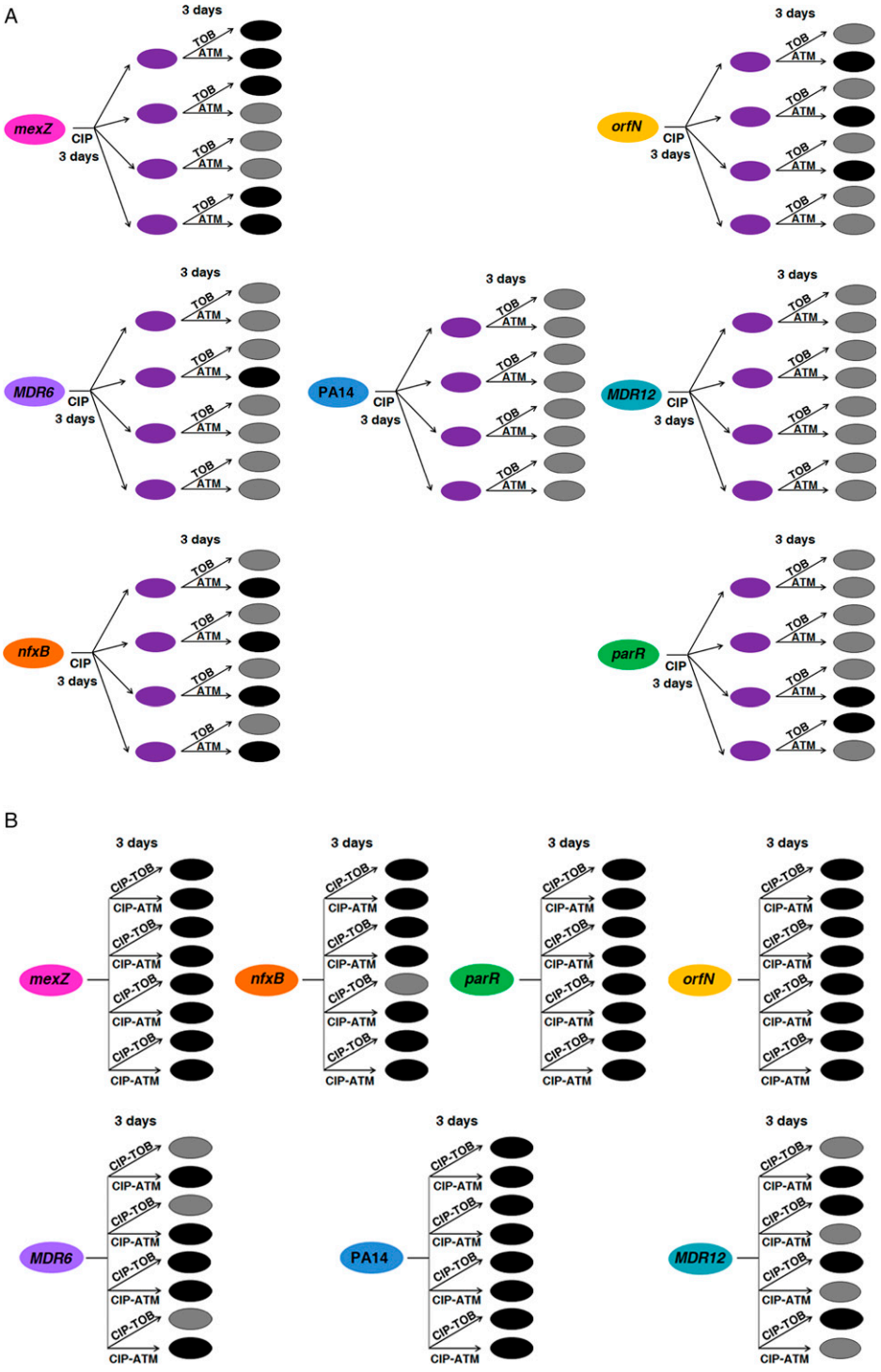


**Fig. 3.** Diagram showing the efficacy of the alternation of ciprofloxacin with tobramycin or aztreonam and the combination of ciprofloxacin with tobramycin or aztreonam for driving extinction of *P. aeruginosa* PA14 and different antibiotic-resistant mutants. (*A*) Short-term evolution of PA14 and six mutational backgrounds (*nfxB177*, *parR87*, *mexZ43*, *orfN50*, MDR6, or MDR12), four replicate populations of each parental strain, was performed during 6 d: 3 d in the presence of ciprofloxacin (CIP) or the absence of antibiotic (control populations), leading to ciprofloxacin-resistant populations (purple cells), and 3 d in the presence of tobramycin (TOB) or aztreonam (AZT). CS to tobramycin and aztreonam was observed in 21 and 19 out of 28 populations after a first step on ciprofloxacin (*SI Appendix*, Table S3). Populations that were extinct at the end of the experiment are represented in black, while surviving populations are colored in gray. Most of the populations (24 out of 28) submitted to short-term ALE in the presence of tobramycin grew after 3 d. However, short-term ALE in the presence of aztreonam led to extinction of 11 out of 28 populations. This evolutionary strategy was efficient in driving extinction of ciprofloxacin-resistant mutants belonging to *nfxB177*, *parR87*, *mexZ43*, *orfN50*, and MDR6, but ineffective in driving extinction of PA14 and MDR12. (*B*) Short-term evolution of PA14 and six mutational backgrounds (*nfxB177*, *parR87*, *mexZ43*, *orfN50*, MDR6, or MDR12), four replicate populations of each parental strain, was performed during 3 d in the presence of the ciprofloxacin–tobramycin (CIP-TOB) or the ciprofloxacin–aztreonam (CIP-ATM) combination. Growth of the 84 control populations was confirmed in the three drugs independently used at the concentrations present in the drugs combinations. A total of 25 out of 28 populations submitted to short-term ALE in the presence of the ciprofloxacin–aztreonam combination were extinct, while it occurred in 23 out of 28 populations submitted to short-term ALE in the presence of the ciprofloxacin–tobramycin combination. These results indicate that CS may not only improve treatment when drugs are applied sequentially, but it may also serve to optimize combinatory therapy.

**Table 2. Newly acquired genetic events after ciprofloxacin ALE by replicates of PA14 and of *parR87*, *orfN50*, *nfxB177*, *mexZ43*, MDR6, and MDR12 mutational backgrounds**

| Mutational background | Gene | Position | Genetic change | Nucleotide location* | Amino acid change | Replicate | Coverage,%† |
|---|---|---|---|---|---|---|---|
| PA14 | *mexS* | 2820352 | SNP | 379T > G | Tyr127Asp | 1, 2, 3, 4 | 43, 28, 23, 50 |
| | *mexS* | 2820074 | SNP | 101G > C | Arg34Pro | 1, 2, 3 | 20, 10, 14 |
| | *mexS* | 2820118 | SNP | 145C > T | Gln49* | 3 | 17 |
| *parR87* | *mexS* | 2820695 | SNP | 722T > A | Val241Glu | 1, 2 | 17 |
| | *mexS* | 2820327 | Deletion | 355delA | Thr119fs | 2, 3, 4 | 86, 10, 23 |
| | *mexS* | 2820695 | SNP | 722T > G | Val241Gly | 2 | 23 |
| | *nfxB* | 5428144 | SNP | 115A > C | Thr39Pro | 1, 3 | 39, 18 |
| | *nfxB* | 5428448 | Deletion | 421delG | Ala141fs | 4 | 80 |
| *orfN50* | *nfxB* | 5428228 | SNP | 199C > T | Gln67* | 1, 2, 4 | 16, 37, 87 |
| | *nfxB* | 5428148 | SNP | 119T > G | Leu40Arg | 2 | 49 |
| | *nfxB* | 5428091 | SNP | 62A > C | His21Pro | 3 | 100 |
| *nfxB177* | *gyrB* | 5671 | SNP | 1397C > T | Ser466Phe | 1, 2 | 99, 95 |
| | *gyrA* | 2015001 | SNP | 248C > T | Thr83Ile | 3 | 84 |
| | *gyrA* | 2015289 | SNP | 536C > T | Ala179Val | 4 | 93 |
| *mexZ43* | *mexS* | 2820463 | SNP | 490C > T | Gln164* | 1 | 36 |
| | *mexS* | 2820619 | SNP | 646A > C | Asn216His | 2, 3 | 10, 31 |
| | *mexS* | 2820782 | SNP | 809T > G | Leu270Arg | 2, 4 | 10, 23 |
| | *mexS* | 2820380 | SNP | 407G > T | Gly136Val | 2, 3, 4 | 10, 11, 21 |
| | *mexS* | 2820020 | SNP | 47T > C | Leu16Pro | 4 | 15 |
| | *orfN* | 2040286 | Insertion | 148insG | Val50fs | 2 | 79 |
| | *nfxB* | 5428091 | SNP | 62A > C | His21Pro | 2 | 26 |
| MDR6 | *mexS* | 2820797 | SNP | 824G > C | Gly275Ala | 1, 2, 3, 4 | 38, 28, 11, 36 |
| | *mexS* | 2820239 | SNP | 266T > C | Phe89Ser | 1, 2, 4 | 30, 29, 28 |
| | *mexS* | 2820529 | SNP | 556C > T | Leu186Phe | 3 | 22 |
| | *mexS* | 2820758 | SNP | 785A > T | His262Leu | 3 | 20 |
| MDR12 | *gyrA* | 2015013 | SNP | 260A > G | Asp87Gly | 1, 2, 3, 4 | 18, 100, 100, 100 |
| | *nfxB* | 5428091 | SNP | 62A > C | His21Pro | 1 | 31 |

Del, deletion; fs, frameshift; ins, insertion.
*Nucleotide location of the mutations referred to the specific gene in which they are located and their associated amino acid changes.
†The coverage indicates the percentage of reads of each mutant allele in the total number of reads, corresponding to the same region of the genome, within each population at the end of the ALE assay.

the replacement of aspartate at position 87 with asparagine, glycine, or tyrosine residues (34, 36, 38–40, 43, 55). It is important to notice that, while new mutations were acquired in *nfxB*, a gene encoding a regulator of *mexCD–oprJ* expression, in *parR87, orfN50*, *mexZ43*, and MDR12, target mutations in *gyrAB* were the only ones acquired in *nfxB177*, and, with the exception of MDR12, no mutations were found in genes encoding the quinolone targets in the other mutational backgrounds (Table 2 and Fig. 4). It is remarkable noting that MDR12 originally presented a mutation in *mexZ*, which encodes the negative regulator of the expression of the *mexXY* efflux pump encoding genes (56). Altogether, these results suggest that mutations in regulators of the expression of efflux pumps may precede target mutations in the acquisition of quinolones' resistance, something that has already been reported in clinical strains of patients treated with ciprofloxacin, in which mutations in *nfxB* were generally acquired early in the lineage evolution and were less persistent than mutations in *gyrAB* (57). According to what is found in clinical strains presenting mutations in both target-encoding genes and genes encoding negative regulators of efflux pumps (40, 58), the ciprofloxacin resistance level of the ciprofloxacin-resistant *nfxB177* populations is higher than the ones of ciprofloxacin-resistant populations selected from other mutational backgrounds (*SI Appendix*, Table S3).

In this work, we describe a conserved CS to tobramycin in the populations submitted to short-term ALE on ciprofloxacin (Fig. 1). This phenotypic convergence toward CS to aminoglycosides has been described in ciprofloxacin-resistant clinical isolates of *P. aeruginosa* presenting mutations in *gyrA*, *gyrB*, *mexB*, and *nfxB* (5). In agreement with the findings of this work, our results indicate that mutations in *nfxB* are clearly associated with CS to tobramycin. This is easily deduced from the ciprofloxacin-resistant *orfN50* populations, which only acquired mutations in *nfxB* and whose tobramycin MIC was reduced up to sixfold, with respect to the parental strain *orfN50*. To further analyze these genotype–phenotype relationships, individual clones, with each one presenting single mutations in either *nfxB*, *mexS*, *gyrA*, or *gyrB*, were isolated from the ciprofloxacin-resistant populations as described in *Materials and Methods*, and their susceptibility to antibiotics was determined. In particular, a NfxBHis21Pro ciprofloxacin-resistant mutant was isolated from an *orfN50* population (replicate 3). Confirming our hypothesis, the mutant presents a reduction of tobramycin MIC, from 3 to 0.75 μg/mL. Moreover, a ciprofloxacin-resistant mutant containing the single-mutation NfxBAla141fs was isolated from a *parR87* population (replicate 4), presenting a reduction of tobramycin MIC, from 1.5 to 0.5 μg/mL These results indicate that *nfxB* mutations cause CS to tobramycin (at least the ones selected in *orfN50* and *parR87*). Further, mutations in *mexS* have been associated with an increased susceptibility to aminoglycosides and β-lactams (59). Taking into consideration those observations, mutations in *mexS* are

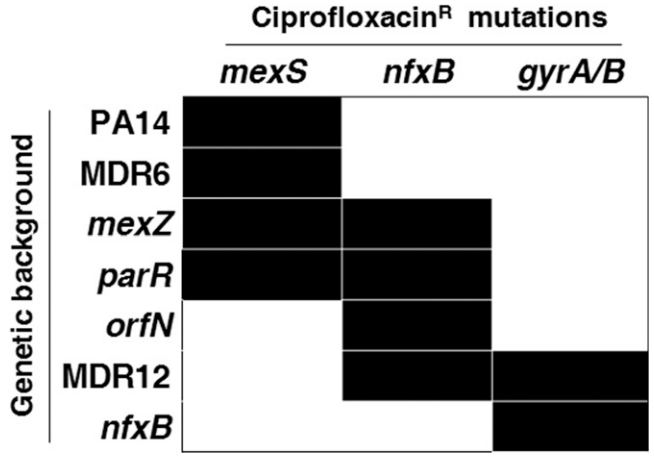


**Fig. 4.** Diagram showing genetic causes of ciprofloxacin-resistance acquisition and mutational background dependence. Classical mutations regularly found in clinical isolates from patients treated with ciprofloxacin, within *gyrAB*, *nfxB*, and *mexS*, were acquired (black boxes) during ALE in the presence of ciprofloxacin for 3 d in 28 populations belonging to PA14 and 6 different mutational backgrounds (*parR87*, *orfN50*, *nfxB177*, *mexZ43*, MDR6, and MDR12). We identified 15, 5, 3, and 1 different variants of *mexS*, *nfxB*, *gyrA*, and *gyrB*, respectively. As observed, early steps of ciprofloxacin-resistance evolution are dependent on the mutational background (Table 2).

likely responsible for the CS to tobramycin observed in PA14 and MDR6. This conclusion is further supported by the fact that the ciprofloxacin-resistant populations from these mutational backgrounds only acquired mutations in *mexS* and showed a 2.6-fold and 3-fold reduction of tobramycin MIC, respectively. To further analyze this possibility, a ciprofloxacin-resistant mutant containing the single-mutation MexSThr119fs was isolated from the *parR87* replicate population 4. The mutant presents a reduction of tobramycin MIC, from 1.5 to 0.75, confirming the role of the *mexS* mutation in tobramycin CS. In the case of ciprofloxacin-resistant populations derived from *nfxB177,* which only acquired mutations in *gyrAB*, their tobramycin MICs were reduced in twofold, with respect to the parental strain, indicating that mutations in *gyrAB* may as well be responsible for tobramycin CS in this mutational background. Two different ciprofloxacin-resistant mutants containing the single-mutations GyrAThr83Ile or GyrBSer466Phe were isolated from two different *nfxB177* populations (replicate 3 and 1, respectively), presenting a reduction of tobramycin MIC from 1 to 0.5. This denotes that phenotypic convergence toward CS to aminoglycosides in clinical isolates from patients treated with ciprofloxacin may be associated with the selection of mutations in different genes that lead to an increased efflux of antibiotics or to alterations in drug targets, although the strength of SC to aminoglycosides associated with *nfxB* mutations is considerably higher than that of mutations in the other genes. Besides tobramycin CS, we observed that CS to aztreonam is also associated with the acquisition of resistance to ciprofloxacin in the different mutational backgrounds tested. In this regard, the contribution of *mexS*, *nfxB*, *gyrA*, or *gyrB* mutations in CS to aztreonam is observed in the mutants MexSThr119fs, NfxBAla141fs, NfxBHis21Pro, GyrAThr83Ile, and GyrBSer466Phe isolated from ciprofloxacin-resistant populations derived from *parR87*, *orfN50*, and *nfxB177*. The NfxBHis21Pro ciprofloxacin-resistant mutant isolated from the *orfN50* population presented a reduction of aztreonam MIC from 6 to 1.5 μg/mL, and the NfxBAla141fs ciprofloxacin-resistant mutant isolated from the *parR87* population presented a reduction of aztreonam MIC from 3 to 0.5 μg/mL, indicating that *nfxB* mutations cause CS to aztreonam (at least the ones of *orfN50* and *parR87*). The ciprofloxacin-resistant mutant isolated from the *parR87* population containing MexSThr119fs as a single mutation presented a reduction of aztreonam MIC from 3 to 1 μg/mL, indicating that in this mutational background—at least—this mutation leads to CS to this antibiotic. Finally, the GyrAThr83Ile and GyrBSer466Phe ciprofloxacin-resistant mutants isolated from two different *nfxB177* populations presented a reduction of aztreonam MIC from 2 to 1 μg/mL, indicating that *gyrA* and *gyrB* mutations cause CS to aztreonam (at least the ones selected in *nfxB177*), but that the strength of this phenotype is considerably lower than the ones associated with *mexS* and *nfxB* mutations.

It is worth emphasizing that the mutations that have been selected during our ALE experiments in the presence of ciprofloxacin are the most frequently found in patients infected by *P. aeruginosa* and treated with ciprofloxacin (34, 36, 38–40, 53–55, 57) and that mutations in *nfxB*, the ones that present a higher CS to tobramycin and aztreonam, are generally early acquired during ciprofloxacin treatment (57), supporting the potential impact of the results reported in the present work, regarding robustness of CS in clinical settings.

## Discussion

Phenotypic convergence is a common process in evolution that may be the consequence of parallel evolution: The same genetic events are selected when organisms confront the same selective force (60). In this regard, we have recently described that parallel evolution underlies the acquisition of a robust pattern of CS to certain antibiotics, associated with the short-term ALE in the presence of ceftazidime in different mutational backgrounds of *P. aeruginosa* (29). However, besides being the result of parallel evolution, phenotypic convergence can also emerge, even when different genetic events, giving up to the same phenotype, are selected during evolution (61). In this case, it is usually argued that the emergent, conserved phenotype is selected because it is adaptive to the selective pressure, despite different genetic solutions being taken to deal with the challenge. However, it has been discussed that, on occasion, the observed convergent phenotypes do not provide direct adaptation to the selective force (62), and CS, a phenotype that is maladaptive to the presence of antibiotics, is an example of this situation. We have identified a robust, convergent CS pattern in different antibiotic-resistant mutants, associated with the use of ciprofloxacin, which is not caused by parallel evolution. In particular, we observed the selection of mutations in different genes driven by the same selective force (ciprofloxacin) in different mutational backgrounds, leading to a functionally equivalent change in susceptibility to second drugs (tobramycin and aztreonam). The current study describes a robust pattern of CS associated with the use of a specific drug that is not caused by parallel evolution in different genetic backgrounds, including preexisting antibiotic-resistant mutants.

Parallel evolution leading to the emergence of a conserved maladaptive phenotype was proposed by Haldane (63), in order to explain the high prevalence of some inherited diseases. The hypothesis, which was later experimentally validated (64), is that the selective force behind the unexpectedly high prevalence of those inherited diseases was infection; the selected mutations protect from infection, and the inherited disease was just the associated, maladaptive, trade-off. However, when parallel evolution is not the cause of convergent evolution, phenotypic convergence is usually explained as a mechanism of adaptation

to the selective force (26, 27); a maladaptive, convergent phenotype is not expected to be selected. Differing from this situation, CS is maladaptive to antibiotic challenge; it is an evolutionary trade-off, whose emergence is hardly inferred, even when the selective force underlying evolution is known. Our results hence provide an example of phenotypic convergence (CS) unlinked to parallel evolution and not providing direct adaptation to the antibiotic-selective force. Besides the relevance concerning AR, our findings have consequences for understanding processes of phenotypic convergence without a clear link between selection and adaptation (62).

As stated above, the exploitation of the information concerning CS requires this phenotype to be conserved among different strains; otherwise, the phenotype will be unpredictable and with no use in clinics. Despite efforts in the field, few cases of robust CS have been reported, and, with the exception of one recent study from our laboratory (29), none of them has been performed using well-defined antibiotic-resistant mutants as a starting point. We believe that this aspect is relevant because infections by antibiotic-resistant organisms are common, and they need to be treated with another different drug, which can lead to the selection of additional resistance phenotypes. Knowing if the novel resistance concurs with a robust CS phenotype, shared in different resistant mutants, is needed to implement evolution-based strategies to fight multidrug resistance. Herein, we found a robust CS phenotype to aztreonam and tobramycin, associated with the acquisition of ciprofloxacin resistance by different *P. aeruginosa* mutants, formerly resistant to antibiotics, which constitutes an important step forward in this endeavor.

The exploitation of in vitro-generated knowledge regarding AR mutations requires that these mutations are also present in clinical isolates. Notably, mutations in the same genes reported in the present article to be involved in ciprofloxacin resistance, such as mutations in target-encoding genes *gyrAB* or in efflux pumps' regulators encoding genes *mexS* and *nfxB*, have been ascertained in *P. aeruginosa* clinical isolates from patients treated with ciprofloxacin (5, 43, 44). Further, variants of clinical relevance, such as NfxBThr39Pro, GyrAThr83Ile, GyrAAsp87Gly, or GyrBSer466Phe (34, 36, 40, 43), were acquired in the populations submitted to short-term ALE in the presence of ciprofloxacin during this study, indicating that our results are not only close to reality, in terms of clinical relevance, but that these clinically relevant mutations might be associated with CS to tobramycin and aztreonam in clinical isolates, a feature that has not been addressed yet. This led us to consider the alternation of ciprofloxacin with tobramycin or aztreonam and the use of a ciprofloxacin–tobramycin or a ciprofloxacin–aztreonam combination. We observed that 25 out of 28 populations (belonging to PA14 and 6 different preexisting antibiotic-resistant mutants) challenged with the ciprofloxacin–aztreonam combination and 23 out of 28 populations challenged with the ciprofloxacin–tobramycin combination were extinct after 3 d of short-term ALE. This is in agreement with a previous work performed with the PA14 model strain of *P. aeruginosa* that pointed out the efficacy of the combination of drug pairs containing ciprofloxacin and aminoglycosides or β-lactams (8). In addition, we observed that 11 out of 28 populations were also extinct after the alternation of ciprofloxacin with aztreonam. Therefore, our results support that it might be possible to exploit CS associated with ciprofloxacin-resistance acquisition to design evolution-based approaches to tackle *P. aeruginosa* infections containing preexisting antibiotic-resistant mutants. In this regard, it is worth mentioning that blind—not evolutionary-based—clinical trials of antibiotic combinations for fighting *P. aeruginosa* infections are regularly performed (65), while rational evolutionary-based information, as the results presented here, supporting these trials are generally absent. However, we are aware that testing the proposed treatments in different infection models, as well as confirming the robustness of CS in clinical strains of *P. aeruginosa*, presenting different genomic backgrounds, not just different mutational backgrounds, would also be needed for the translation of the results into clinical practice. Besides, the fact that the acquisition of quinolone resistance, when due to overexpression of MDR efflux pumps, can be associated with cross-resistance to other drugs (66) and that, on occasion, growing in biofilms may hide the CS phenotype (67) should also be taken into consideration.

We conclude that robust CS patterns may result not just from parallel evolution, as we recently described (29), but also upon the selection of different genetic mechanisms, as we describe here. We therefore propose that the search for robust CS patterns in different genetic backgrounds (including antibiotic-resistant isolates) of a species could pave the way for the design of new evolutionary strategies to promote the extinction of bacteria causing infections. The finding of these robust CS networks is particularly relevant in the case of already-resistant mutants challenged with another antibiotic, a situation rarely explored until now, which is explored here and that is rather common and highly relevant in clinical settings.

## Materials and Methods

**Growth Conditions and Antibiotic-Susceptibility Assays.** Bacteria were grown in glass tubes in Luria–Bertani (LB) broth at 37 °C with shaking at 250 rpm. MICs of ceftazidime, aztreonam, imipenem, tobramycin, ciprofloxacin, and fosfomycin were determined at 37 °C in Mueller Hinton (MH) agar using E-test strips (MIC Test Strip, Liofilchem).

**Short-Term ALE Experiments in the Presence of Ciprofloxacin, Tobramycin, or Aztreonam.** Four single mutants, two multiple mutants, and PA14 were subjected to short-term ALE–four replicates of each–in the presence of ciprofloxacin, tobramycin, or aztreonam or the absence of antibiotic (control populations), resulting in a total of 112 independent bacterial populations. Cultures were grown at 37 °C and 250 rpm for 3 d in independent glass tubes to avoid cross-contamination. Every day, the cultures were diluted (1/100), adding 10 μL of bacteria in 1 mL of fresh LB containing the concentration of antibiotic (close to MIC) that hinders the growth of *P. aeruginosa* PA14 and each mutational background under these culture conditions (ciprofloxacin: 0.1 μg/mL for MDR6; 0.2 μg/mL for MDR12; 0.3 μg/mL for PA14, *orfN50*, *nfxB177*, and *mexZ43*; and 0.4 μg/mL for *parR87*; tobramycin: 0.75 μg/mL for PA14 and *nfxB177*; 1.5 μg/mL for MDR6 and *mexZ43*; 2 μg/mL for *orfN50*; 2.5 μg/mL for *parR87*; and 12 μg/mL for MDR12; aztreonam: 1 μg/mL for MDR12; 4 μg/mL for PA14, *nfxB177*, and MDR6; 6 μg/mL for *parR87*; 7 μg/mL for *orfN50*; and 8 μg/mL for *mexZ43*) or without antibiotics (control populations). During the 3 d, the concentration of ciprofloxacin, tobramycin, or aztreonam was maintained. Every replicate population was preserved at −80 °C at the end of the experimental evolution. In addition, the MIC of the antibiotic used for selection and of antibiotics from other structural families was determined at 37 °C in MH agar using E-test strips.

**Alternation of Ciprofloxacin with Tobramycin or Aztreonam Using Short-Term ALE Experiments.** Short-term ALE experiments in the presence of tobramycin or aztreonam were performed for 3 d at 37 °C and 250 rpm using the 28 populations previously challenged with ciprofloxacin for 3 d and belonging to PA14 and six different mutational backgrounds (*parR87*, *orfN50*, *nfxB177*, *mexZ43*, MDR6, and MDR12). The 28 ciprofloxacin-resistant populations and the 28 control populations (not challenged with ciprofloxacin) were grown from glycerol stocks, and every day, during 3 d, the cultures were diluted (1/100) in fresh LB containing tobramycin or aztreonam at the concentration that hinders–but allows–the growth of each *P. aeruginosa* mutational background under these culture conditions, which are described in *Short-Term ALE Experiments in the Presence of Ciprofloxacin, Tobramycin, or Aztreonam*. Extinction of the populations

was determined by measuring the absorbance $OD_{600nm}$ of 100 μL of bacterial cultures the last day of ALE in a 96-well microtiter plate (NUNC) in a Tecan Infinite 200 plate reader and by plating out final cultures on LB to look for viable cells.

**Combination of Ciprofloxacin with Tobramycin or Aztreonam Using Short-Term ALE Experiments.** Four replicate populations from PA14 and six different mutational backgrounds (*parR87*, *orfN50*, *nfxB177*, *mexZ43*, MDR6, and MDR12) were grown from glycerol stocks. Every day, during 3 d, the cultures were diluted (1/100) in fresh LB medium containing a combination of ciprofloxacin–tobramycin (28 populations), ciprofloxacin–aztreonam (28 populations), or each single drug (84 control populations). Each antibiotic was added at the concentration that hinders–but allows–the growth of each *P. aeruginosa* mutational background under these culture conditions, which are described in *Short-Term ALE Experiments in the Presence of Ciprofloxacin, Tobramycin, or Aztreonam*. Extinction of the populations was determined by measuring the absorbance $OD_{600nm}$ of 100 μL of bacterial cultures the last day of ALE in a 96-well microtiter plate (NUNC) in a Tecan Infinite 200 plate reader and by plating out final cultures on LB to look for viable cells.

**Whole-Genome Sequencing and Analysis of Genetic Changes.** The genomic DNA of each ciprofloxacin-resistant population and parental strain was extracted with the Gnome DNA kit (MP Biomedicals). The assay of DNA quality, libraries' construction, and whole-genome sequencing was performed by Macrogen. Pair-end libraries (2 × 150) were constructed with Truseq DNA PCR-free and sequenced by using an Illumina NovaSeq6000 system. Coverage was greater than 300x for all samples. Genome sequence, gene coordinates, and annotations were obtained from the nucleotide database GenBank. The quality of Illumina short reads was verified by using FASTQC (68). RNA-STAR was used to align reads against *P. aeruginosa* genome UCBPP-PA14 (NC_008463.1) (69). Optical and PCR duplicates were detected by using the MarkDuplicates (Picard) function of The Genome Analysis Toolkit (70). SAMtools was used to index alignment files in BAM format (71). Single-nucleotide polymorphism (SNPs) and small insertions and deletions (INDELs) were detected by using freebayes (72). The impact of SNPs and INDELs was evaluated by using SnpEff (73), and annotated results were saved in the VCF format. Genetic variants were detected by using SNPer viewer (74) and the IGV browser (75).

**Isolation of Ciprofloxacin-Resistant Mutants.** Clones presenting either of the single mutations NfxBAla141fs, NfxBHis21Pro, MexSThr119fs, GyrAThr83Ile, or GyrBSer466Phe were isolated from the ciprofloxacin-resistant populations *parR87.4*, *orfN50.3*, *parR87.4*, *nfxB177.3*, or *nfxB177.1*, respectively. The presence or absence of mutations in the quinolone-resistance-determining regions of *gyrA* and *gyrB* in *mexS* or in *nfxB* were searched by PCR in each individual clone by using the oligonucleotides described in *SI Appendix*, Table S5 and Sanger sequencing. Four pairs of primers, which amplify 378 bp of *gyrA*, 511 bp of *gyrB*, 627 bp of *nfxB*, and 1,076 bp of *mexS*, were used (*SI Appendix*, Table S6). After PCR, the amplicons were purified by using the QIAquick PCR purification kit (QIAGEN) and Sanger sequenced at Macrogen.

**Synergy Assessed by Checkerboard Analysis.** Standard checkerboard broth microdilution assays were performed in PA14 and in six different mutational backgrounds (*parR87*, *orfN50*, *nfxB177*, *mexZ43*, MDR6, and MDR12) using 10 serially diluted concentrations of ciprofloxacin, seven of aztreonam or tobramycin, and a no-drug control, a total of 14 96-U-well plates. First, 90 μL of MH medium with ciprofloxacin (0.025 to 12.8 μg/mL), aztreonam (1 to 64 μg/mL), or tobramycin (0.125 to 8 μg/mL) was added to each well of two different 96-U-well plates for the analysis of each mutational background, with the exception of MDR12, for which tobramycin concentrations ranged between 4 and 256 μg/mL Ten microliters of cells were inoculated into each well to a final $OD_{600}$ of 0.01. Bacteria were grown at 37 °C for 48 h without sacking. The FIC of ciprofloxacin, aztreonam, and tobramycin was calculated as the MIC of the combination of ciprofloxacin with each of the other drugs divided by the MIC of each of the drugs alone. The FIC index was calculated as the addition of the FICs of both drugs. An FIC index value of $<0.5$ was considered to indicate synergy, and an FIC index of $>4$ was considered to indicate antagonism (76).

**Statistical Analysis.** Bidirectional nonparametric tests on the raw data and parametric bidirectional and unidirectional tests on $log_2$-transformed data were performed.

Data were first subjected to Sapiro–Wilk and Levene's tests to assert normality and homocedasticity. Subsequently, each combination of strain and treatment was compared against its respective control. Nonparametric Kruskal–Wallis, Dwass–Steel–Critchlow–Fligner, and Mann–Whitney *U* tests were applied to the untransformed data to assert significance of the difference. Additionally, FC was calculated, and the significance of the FCs observed was asserted by using ANOVA and bidirectional *t* tests on $log_2$-transformed data ($log_2$ FC). The significance of the variation in the number of extinct populations in alternated and combined evolution experiments was estimated by using a likelihood-ratio test. All statistical analyses were performed by using the R package (https://www.R-project.org/).

**Data Availability.** All study data are included in the article and/or supporting information.

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Author affiliations: [a]Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, 28049 Madrid, Spain; and [b]Programa de Doctorado en Biociencias Moleculares, Universidad Autónoma de Madrid, 28049 Madrid, Spain

Author contributions: S.H.-A. and J.L.M. designed research; S.H.-A. and P.L. performed research; S.H.-A. and J.R.V. analyzed data; and S.H.-A., P.L., J.R.V., and J.L.M. wrote the paper.

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## Article III

**Evolution of habitat-dependent antibiotic resistance in *Pseudomonas aeruginosa***

**Laborda P, Martínez JL, Hernando-Amado S.**

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AR evolution can also be constrained by the nutrients' availability of bacteria when acquiring resistance, since AR and metabolism are closely interlinked -i.e. AR acquisition might change bacterial metabolism and metabolic dysregulation can affect antibiotic susceptibility-. Hence, the evolutionary routes towards AR may vary in different body locations, something that has been barely addressed, since experimental evolution assays are usually performed in regular laboratory growing media. Importantly, *P. aeruginosa* is a metabolically versatile pathogen, able to cause infections in different and changing in-patient environments. During this work, we studied the evolution of *P. aeruginosa* towards resistance to tobramycin, ceftazidime and ceftazidime-avibactam, three drugs frequently used to treat infections caused by this pathogen, in rich laboratory medium, urine and synthetic cystic fibrosis sputum (SCFM). By doing so, we studied the extent to which media composition and nutrients' availability constrains AR evolution and its associated trade-offs.

We found that tobramycin, ceftazidime and ceftazidime-avibactam resistance evolution is dependent on the medium in which the selection pressure is applied. Resistance level to the selective antibiotic and to other drugs -cross-resistance and collateral sensitivity- differed depending on whether the populations had evolved in urine, SCFM or rich laboratory media. The reason for this was that some of the mutations acquired were specific of each medium. We observed that fitness cost and AR associated with particular mutations were distinct in each medium, being some genetic modifications the optimal "solution" to the selective force exerted by the antibiotic in the respective medium. This indicates that the selection of resistance mutations is environment- and metabolic state-dependent.

Since AR evolutionary trade-offs change in different environments, the design of evolution-based treatment strategies to tackle *P. aeruginosa* infections should take into account the restrictions imposed in each particular infection in different body locations. Indeed, using media closer to what is found in clinics for prediction AR evolution, might favour the implementation of such evolution-based anti-infection procedures in clinical practice.

**Specific contributions**:

*Experimental work*: Laborda P. and Hernando-Amado S. contributed to experimental work. I performed most of the experimental work of this article, including antibiotic susceptibility assays, ALE experiments, Whole-Genome Sequencing analysis, relative fitness determination, RNA extraction and gene expression measurements, as well as the interpretation of the results.

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RESEARCH ARTICLE

# Evolution of Habitat-Dependent Antibiotic Resistance in *Pseudomonas aeruginosa*

**Pablo Laborda,[a,b] José Luis Martínez,[a] Sara Hernando-Amado[a]**

[a]Centro Nacional de Biotecnología, CSIC, Madrid, Spain
[b]Programa de Doctorado en Biociencias Moleculares, Universidad Autónoma de Madrid, Madrid, Spain

**ABSTRACT** *Pseudomonas aeruginosa* is an opportunistic human pathogen that usually causes difficult-to-treat infections due to its low intrinsic antibiotic susceptibility and outstanding capacity for becoming resistant to antibiotics. In addition, it has a remarkable metabolic versatility, being able to grow in different habitats, from natural niches to different and changing inpatient environments. Study of the environmental conditions that shape genetic and phenotypic changes of *P. aeruginosa* toward antibiotic resistance supposes a novelty, since experimental evolution assays are usually performed with well-defined antibiotics in regular laboratory growth media. Therefore, in this work we address the extent to which the nutrients' availability may constrain the evolution of antibiotic resistance. We determined that *P. aeruginosa* genetic trajectories toward resistance to tobramycin, ceftazidime, and ceftazidime-avibactam are different when evolving in laboratory rich medium, urine, or synthetic sputum. Furthermore, our study, linking genotype with phenotype, showed a clear impact of each analyzed environment on both the fitness and resistance level associated with particular resistance mutations. This indicates that the phenotype associated with specific resistance mutations is variable and dependent on the bacterial metabolic state in each particular habitat. Our results support that the design of evolution-based strategies to tackle *P. aeruginosa* infections should be based on robust patterns of evolution identified within each particular infection and body location.

**IMPORTANCE** Predicting evolution toward antibiotic resistance (AR) and its associated trade-offs, such as collateral sensitivity, is important to design evolution-based strategies to tackle AR. However, the effect of nutrients' availability on such evolution, particularly those that can be found under *in vivo* infection conditions, has been barely addressed. We analyzed the evolutionary patterns of *P. aeruginosa* in the presence of antibiotics in different media, including urine and synthetic sputum, whose compositions are similar to the ones in infections, finding that AR evolution differs, depending on growth conditions. Furthermore, the representative mutants isolated under each condition tested render different AR levels and fitness costs, depending on nutrients' availability, supporting the idea that environmental constraints shape the phenotypes associated with specific AR mutations. Consequently, the selection of AR mutations that render similar phenotypes is environment dependent. The analysis of evolution patterns toward AR requires studying growth conditions mimicking those that bacteria face during *in vivo* evolution.

**KEYWORDS** experimental evolution, *Pseudomonas aeruginosa*, antibiotic resistance, evolution constraints

*Pseudomonas aeruginosa* is a nosocomial opportunistic pathogen (1, 2), producing infections in immunocompromised patients and in people with underlying diseases (3–8). This bacterium is able to grow in several body locations, being one of the main causative agents of chronic infections in the lungs of cystic fibrosis (CF) or chronic

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Address correspondence to José Luis Martínez, jlmtnez@cnb.csic.es, or Sara Hernando-Amado, shernando@cnb.csic.

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obstructive pulmonary disease (COPD) patients (3) and also being a major cause of urinary tract infections (UTIs) (9, 10). In addition to its metabolic versatility, *P. aeruginosa* presents a characteristic low susceptibility to a large variety of antibiotics (11–14), and it has a high capacity to acquire further resistance to antibiotics, something that frequently occurs by the acquisition of mutations in patients under treatment (15–17).

The problem of antibiotic resistance (AR) traditionally has been tackled by introducing novel antibiotics into the market, in a sort of a "Red Queen" strategy. However, this strategy is currently insufficient, and approaches to improve the use of the antibiotics we already have and to reduce the emergence of AR are needed (18). For such conservative interventions focused on the rational design of efficient evolution-based treatments to manage bacterial infections, knowledge of the evolutionary trajectories that bacteria can follow to acquire AR and their associated trade-offs is needed. In fact, adaptive laboratory evolution (ALE) studies have shown that the evolutionary landscapes that bacterial populations submitted to a specific selective pressure follow are limited (19–23), supporting that mutation-driven evolution may be constrained and, hence, may be predictable to some extent. However, reproducibility of such evolution is contingent on several factors, which include resistance level and the impact of each mutation on bacterial fitness, mutation rate, the strength of selection pressure, population bottlenecks, clonal interference, cross-selection, compensatory evolution, collateral sensitivity, and epistasis (24–34), and as we discuss here, it may also be contingent on environmental conditions (i.e., nutrient composition of colonized habitats). That is why the study of constraints of evolution of AR and its associated trade-offs is of relevance in order to rationally design novel strategies to eradicate populations of bacterial pathogens, *P. aeruginosa* included (35–38).

It has been described in previous studies that the evolution of AR may result in changes of bacterial metabolism and of growth dynamics (39), which might be exploited to tackle AR (40). It is also known that dysregulation of metabolism and infective conditions may affect susceptibility to antibiotics and expression of resistance determinants (41–43). In fact, bacterial metabolism has an effect on the efficacy of certain antibiotics (44–46). Furthermore, it has been determined that changes in metabolism may constrain AR evolution (47). Overall, these studies show that AR and bacterial metabolism are closely interrelated (48). Nevertheless, deeper studies are still required to understand the functional constraints imposed by the environment on the evolution of AR. This is a critical issue since fitness costs associated with resistance acquisition can be metabolically compensated for (49–51), and selection of mutations that compensate for fitness costs depends on the bacterial habitat (38, 52). Therefore, environmental conditions determine the resistant mutants that will be established within a population (47, 52). This has special relevance for metabolically versatile bacteria, such as *P. aeruginosa*, a pathogen capable of causing infections in distinct body locations, each one presenting different nutrients' availability and physicochemical composition. In fact, when *P. aeruginosa* migrates from an environment with limited amount of nutrients to the lungs of a host presenting CF, changes in nutrients' availability lead to major metabolic modifications that result in increased resistance to oxidative stress and a reduction of cell growth, decreasing effectiveness of antibiotics (53).

In order to know the extent to which the evolutionary trajectories toward AR of *P. aeruginosa* are affected as a function of the type of infection, we performed ALE experiments mimicking the nutritional compositions present in different body locations. The experiments were performed in the presence of antibiotics commonly used to treat *P. aeruginosa* infections (tobramycin, ceftazidime and the combination of ceftazidime with the $\beta$-lactamase inhibitor avibactam) (54–56) in urine and synthetic sputum, and the results were compared with those obtained in previous ALE assays in the presence of these antibiotics in rich laboratory medium (20, 57). Our results show that both genotypic and phenotypic evolutionary trajectories in the presence of the analyzed antibiotics are contingent on growth conditions.

## RESULTS AND DISCUSSION

**Impact of growth conditions in *P. aeruginosa* stepwise evolution toward tobramycin, ceftazidime, and ceftazidime-avibactam resistance.** The aim of the work was to determine the effect of environments similar to those encountered by *P. aeruginosa* during infections on the evolution of resistance to tobramycin, ceftazidime, and the combination ceftazidime-avibactam. For such a goal, we compared the evolution of AR during 30 days of ALE experiments (four replicates for each environment) in urine and synthetic cystic fibrosis sputum medium (SCFM) with the ones previously obtained in rich laboratory medium (20, 57). The MIC for the antibiotic used as selective agent was measured every 5 days of the ALE, before doubling the concentration of the selective antibiotic. The MICs increased over the evolutionary process in every replicate population, showing stepwise evolutionary trajectories in either urine, SCFM, or rich laboratory medium (Fig. 1; see Tables S1 and S2 in the supplemental material). The evolution of tobramycin resistance showed similar resistance levels after 30 days of evolution in the different media and in the different replicate populations, while in the presence of ceftazidime or its combination with avibactam, differences were observed among populations evolved in rich medium, SCFM, or urine. In particular, the increase of MICs was lower in populations evolved in urine than in rich laboratory medium or SCFM (Fig. 1; Table S2). This indicates that phenotypic evolution of ceftazidime and ceftazidime-avibactam resistance is contingent on environmental conditions.

**Cross-resistance and collateral sensitivity associated with the acquisition of tobramycin, ceftazidime, and ceftazidime-avibactam resistance in *P. aeruginosa* are dependent on the environment.** In order to determine the potential cross-resistance and collateral sensitivity patterns associated with the evolution of tobramycin, ceftazidime, and ceftazidime-avibactam resistance in urine, SCFM, or rich laboratory medium, MICs of a set of antibiotics representative of different structural families were determined for the final evolved populations in urine and SCFM and compared with those previously described in rich laboratory medium (20, 57) (Fig. 2; Table S3).

In every replicate population that evolved in the presence of tobramycin, cross-resistance to quinolones, aminoglycosides, or tigecycline was observed, independently of the growth medium used for the experiment (Fig. 2). Nevertheless, collateral sensitivity patterns were dependent on the medium, since a remarkable collateral sensitivity to fosfomycin was only observed in populations that evolved in rich laboratory medium,

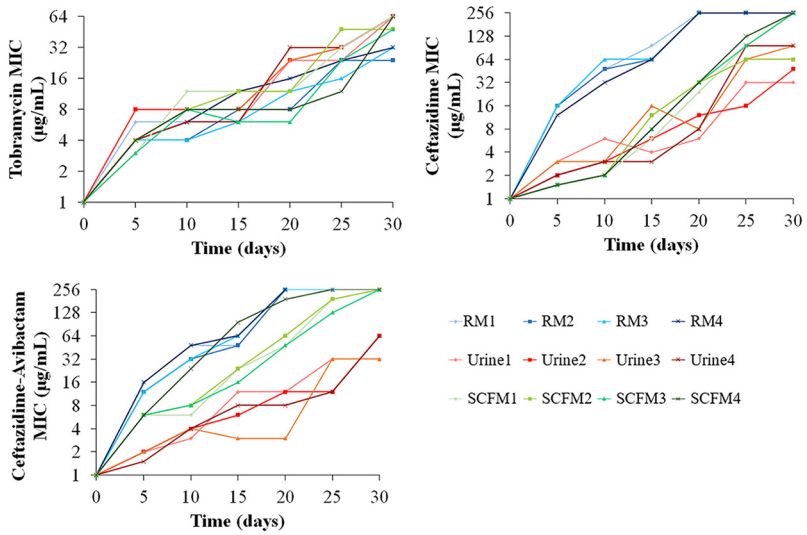


**FIG 1** Evolution of *P. aeruginosa* toward tobramycin, ceftazidime, or ceftazidime-avibactam resistance in rich laboratory medium, urine, or SCFM. MICs for the antibiotic used as selective agent were determined every 5 days in populations that evolved 30 days in the presence of tobramycin, ceftazidime, or ceftazidime-avibactam in rich laboratory medium (RM), urine, or SCFM. Raw data for each evolved replicate are included in Tables S1 and S2.

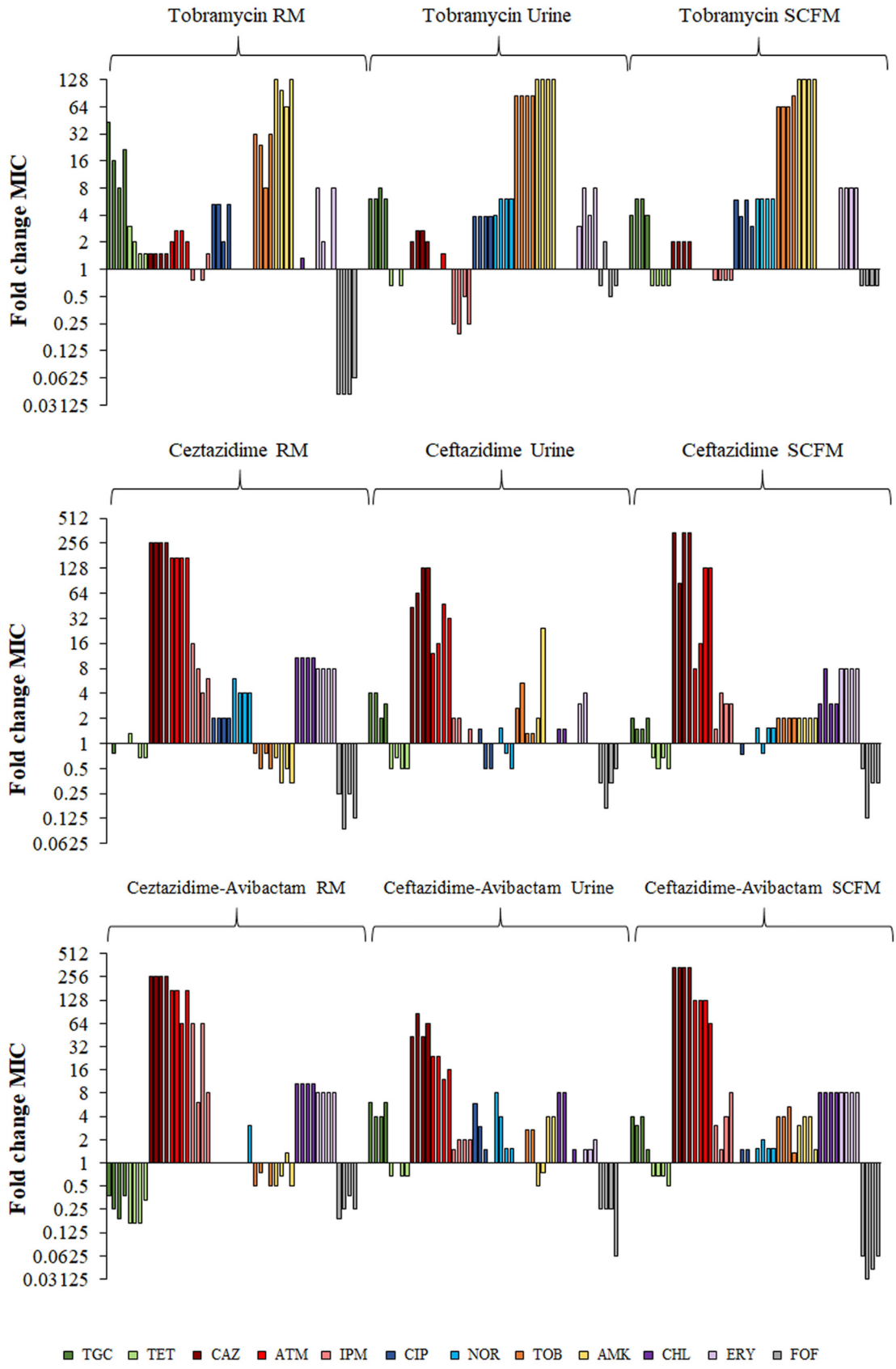


**FIG 2** Susceptibility to antibiotics of *Pseudomonas aeruginosa* populations resulting from evolution in the presence of tobramycin, ceftazidime, and ceftazidime-avibactam in rich laboratory medium (RM), urine, or SCFM. Fold change of MICs of the populations

(Continued on next page)

and an important collateral sensitivity to imipenem was detected only in urine-evolved populations (Fig. 2).

Cross-resistance to $\beta$-lactam antibiotics was observed in every ceftazidime- and ceftazidime-avibactam-evolved population. Increased erythromycin and chloramphenicol resistance was also observed in all replicates evolved in rich medium and in SCFM, but was seen in only half of the urine-evolved populations. In addition, populations that evolved in the presence of ceftazidime or ceftazidime-avibactam, in either rich medium, urine, or sputum, presented a robust pattern of collateral sensitivity to fosfomycin. Remarkably, the populations that evolved in the presence of ceftazidime-avibactam in SCFM had a stronger increase in fosfomycin susceptibility than the populations that evolved in other environments. Collateral sensitivity to aminoglycosides was observed only in the populations that evolved in ceftazidime or ceftazidime-avibactam in rich medium, and tigecycline and tetracycline collateral sensitivity was observed only in populations that evolved in the presence of ceftazidime-avibactam in rich medium (Fig. 2).

Overall, these results indicate that the cross-resistance and collateral sensitivity associated with tobramycin, ceftazidime, and ceftazidime-avibactam resistance in *P. aeruginosa* are contingent on the environment (nutritional composition of growth medium) in which resistance is acquired, although some patterns (i.e., collateral sensitivity to fosfomycin associated with the use of ceftazidime or ceftazidime-avibactam) may be conserved in different environments.

**Genetic basis of resistance to tobramycin, ceftazidime, and ceftazidime-avibactam in different ecosystems.** Once we determined that phenotypic AR evolution is contingent on growth conditions, we analyzed the genetic causes of such evolution. In order to identify the genetic modifications responsible for AR in the populations that evolved in urine or SCFM in the presence of tobramycin, ceftazidime, or ceftazidime-avibactam, their genomes, as well as those of control populations grown in the absence of antibiotics, were sequenced after 30 days of evolution. All detected genetic variations are described in Table S4. A search of the mutated genes in the *Pseudomonas* Genome Database (58) showed that orthologs of all of them are present in different *P. aeruginosa* isolates, supporting that our findings can be generalized to other strains besides PA14. While, in most cases, the acquired mutations have a negative effect on the activity of the encoded protein—like the ones in the transcription-negative regulators DacB, DacC, MexR, NalC, and NalD, in targets of antibiotics or proteins related to its function, like FtsI, FtsL, FtsB, MurF, AnmK, and Mpl, in proteins that influence the antibiotics' passage through the membrane, like NuoD or OrfN, or in enzymes involved in general stress responses, like ClpS, ClpA, FusA, and PmrB—the effect is likely positive when the mutation occurs in genes encoding intrinsic resistance determinants, like the subunits of multidrug efflux pumps MexY, MexB, or PA14-45890 or their positive regulators, like AmpR. A Boolean analysis of common and specific genetic modifications acquired in populations evolved in the presence of tobramycin, ceftazidime and ceftazidime-avibactam in rich laboratory medium, urine or SCFM is presented in Fig. 3.

To ascertain if our results reflect *in vivo* evolution toward AR, the identified mutations were searched in the genome of *P. aeruginosa* clinical isolates using the database BACTOME (59) and by regular bibliographic search. Reinforcing the reliability of our work, several of these genetic changes and most of the mutated genes, although presenting different genetic variations, had been already found in clinical *P. aeruginosa* isolates, as well as in previous ALE experiments using different *P. aeruginosa* strains, being related to AR to the analyzed antibiotic (Tables S5 and S6, respectively). Notably, mutations in genes encoding the regulators of the quorum sensing system MvfR (60) and LasR (61) were prevalent in control populations grown in the absence of antibiotics.

**FIG 2** Legend (Continued)
that evolved in the presence of tobramycin (top), ceftazidime (middle), and ceftazidime-avibactam (bottom) in different media was calculated relative to the MIC values of the PA14 parental strain. Fold change MIC values for each antibiotic are represented as bars with the same color and ordered from replicates 1 to 4. Raw data for each evolved replicate population and antibiotic are included in Table S3. TGC, tigecycline; TET, tetracycline; CAZ, ceftazidime; ATM, aztreonam; IPM, imipenem; CIP, ciprofloxacin; NOR, norfloxacin; TOB, tobramycin; AMK, amikacin; CHL, chloramphenicol; ERY, erythromycin; FOF, fosfomycin.

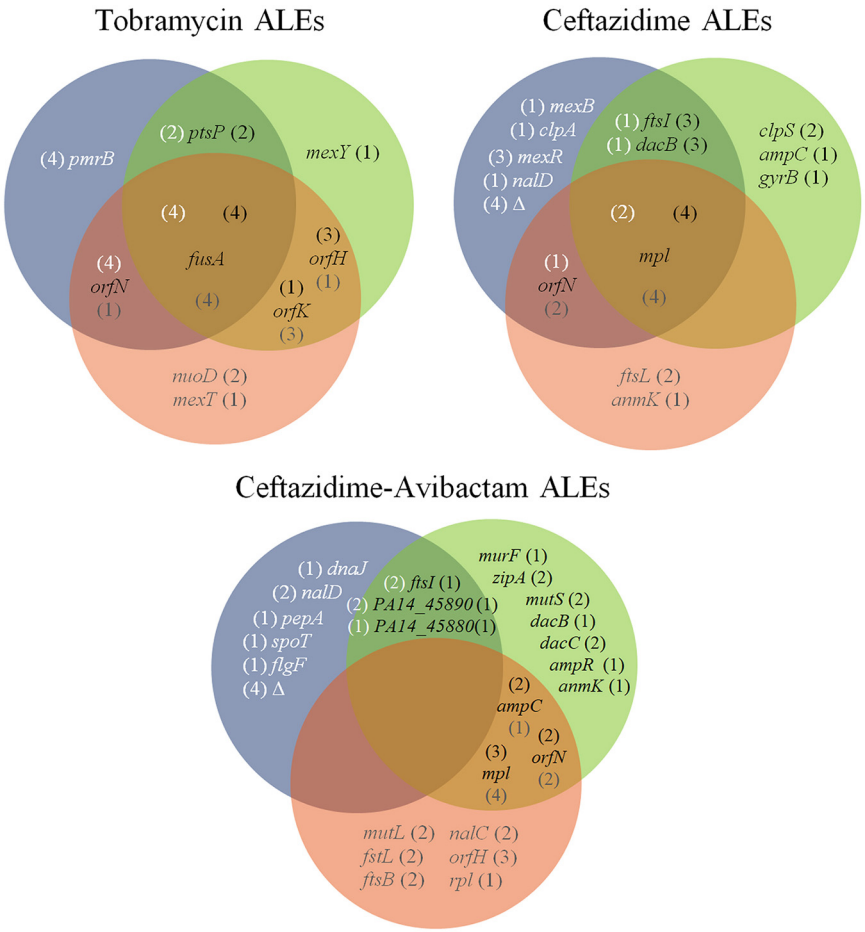


**FIG 3** Venn diagram of common and specific genetic modifications in populations evolved in the presence of tobramycin, ceftazidime, and ceftazidime-avibactam in rich laboratory medium, urine, or SCFM. Mutated genes known to be related to AR acquired in rich laboratory medium (20, 57) (blue circles), SCFM (green circles), and urine (red circles) are represented. The numbers in parentheses indicate the number of replicates with mutations in the respective gene in evolutions performed in rich laboratory medium, SCFM, or urine. All detected genetic modifications identified and detailed related information are included in Table S4.

*lasR* mutants are frequently selected in infected CF patients—more recently it has been described to be selected in a variety of habitats—and this mutation is suggested to be selected as an adaptation to the growing conditions (62–65). However, genetic variations in these genes were absent in ALE assays in the presence of antibiotics (Table S4). This fits with previous results from our laboratory in which mutations in *lasR* were acquired only in control populations. The reason for the absence of mutations in this gene after ALE in the presence of tobramycin or tigecycline was that AR and *lasR* mutations are reciprocally contingent; selection of AR mutations impedes the secondary selection of *lasR*-defective mutants (33). Our results support that not only tigecycline or tobramycin resistance mutations, but also mutations acquired in the presence of ceftazidime or ceftazidime-avibactam, restrict the selection of mutations in *lasR*.

**Effect of the environment on the genetic modifications selected during tobramycin ALEs.** Mutations in *fusA*, encoding an elongation factor (66), were acquired in all replicates of every environment, supporting the importance of mutations within this gene in the acquisition of tobramycin resistance (20), independently of the environment. Indeed, four different genetic variations identified in this work in *fusA* have been described as being associated with acquisition of aminoglycoside resistance, not only in experimental studies (67) but also in clinical strains (59, 68, 69) (Table S5). Mutations in different genes of the *orfKHLN* operon were identified in all replicate populations in

every environment. In particular, mutations in *orfN* were acquired in all replicates grown in rich medium, whereas *orfK* and *orfH* mutations were the most prevalent in urine and SCFM, respectively (Table S4). The *orfKHLN* operon encodes the lipopolysaccharide (LPS) O-antigen biosynthesis enzymes (67, 70–72), and it has been proposed that such mutations might reduce drug binding or uptake by alteration of outer membrane (67). The genomic variations detected in *orfN* had been previously described to be involved in tobramycin resistance in experimental studies (33, 67, 73) (Table S5) as well as in collateral sensitivity to fosfomycin in tobramycin-resistant clones obtained after tobramycin ALE in rich medium (36). This is consistent with the fact that only the tobramycin-resistant populations selected in rich medium present a robust collateral sensitivity to fosfomycin (Fig. 2).

Environment-specific mutations were also selected by tobramycin. Mutations in *ptsP*, encoding a phosphoenolpyruvate phosphotransferase, which previously had been related to tobramycin resistance (20, 74), were detected in both rich laboratory medium and in SCFM. More specific was the mutation of *mexY*, encoding a subunit of MexXY efflux pump—an intrinsic aminoglycoside resistance determinant (75)—selected only in SCFM. Interestingly, the His908Leu amino acid change in MexY had been detected previously in tobramycin-resistant clinical isolates (59) (Table S5). Modifications in *nuoD*, which encodes the NADH-quinone oxidoreductase subunit C/D, whose mutations block tobramycin uptake through a disruption of the proton motive force (33, 74), were specifically selected in urine. In addition, a mutation in *mexT*, encoding a regulator of the expression of the genes coding for the MexEF-OprN efflux pump (76) and whose mutations may lead to tobramycin resistance in clinical strains (77), was also selected in this medium. Finally, mutations in *pmrB*, encoding a protein belonging to a two-component regulatory system known to have a role in resistance to polymyxins, fluoroquinolones, $\beta$-lactams, aminoglycosides (78), and, specifically, resistance to tobramycin in both experimental (67, 77) and clinical (79) studies, were specifically selected in rich medium.

**Effect of the environment on the genetic modifications selected during ceftazidime ALEs.** The mutations acquired in the presence of ceftazidime were more diverse than the ones selected in the presence of tobramycin, particularly in rich laboratory medium and SCFM. The only gene commonly mutated after ALE in the three different media was *mpl*, which encodes a protein involved in peptidoglycan muropeptide recycling (80). Genetic variations acquired in this gene, leading to Met38fs, Val384Gly, Tyr35Ser, or Val124Gly changes, have also been found in clinical isolates of *P. aeruginosa* (59, 79) (Table S5). The genetic variation in *orfN* that was selected in the presence of tobramycin or ceftazidime-avibactam (see below) (Fig. 3) was also acquired in ceftazidime, in both urine and rich laboratory medium, supporting the relevant role of this gene in AR evolution of *P. aeruginosa*. Mutations in *dacB*, encoding a regulator of the expression of the $\beta$-lactamase-encoding gene *ampC* (81), and in *ftsI*, which encodes PBP3 (the target of several $\beta$-lactam antibiotics) (82), were selected in rich laboratory medium and in SCFM. Those genetic variations leading to a truncated DacB (Gln372*) and to amino acid variations in FtsI (Arg504His and Ala482Val) had been detected previously in clinical isolates (59, 82–84) (Table S5).

Other mutations selected in the presence of ceftazidime were contingent on each specific environment. In SCFM, specific mutations were found in *clpS*, encoding an intracellular protease involved in $\beta$-lactam resistance among other physiological processes (85), and in *ampC*, which encodes an intrinsic $\beta$-lactamase (86). The Val239Gly amino acid variation in AmpC has been detected in ceftazidime-resistant clinical isolates (59, 79) (Table S5).

Populations evolved in urine presented specific mutations in *anmK*, encoding an enzyme of the peptidoglycan recycling pathway, disruption of which was previously associated with ceftazidime resistance and collateral sensitivity to fosfomycin (87, 88). Interestingly, the genetic variation leading to the Gly232Asp amino acid change has also been detected in ceftazidime-resistant clinical isolates (59) (Table S5). Genetic variations in *ftsL*, encoding a protein needed for FtsI functionality, and leading to a

Gly59Asp amino acid change, have been previously described in clinical strains (59) (Table S5). The reduced number of ceftazidime resistance mutations acquired in urine may be responsible for the lower ceftazidime resistance level acquired in these populations than the in other environments analyzed (Fig. 1). Ceftazidime ALE in rich medium led to the acquisition of environment-specific mutations. Some of them may lead to the overexpression of genes encoding MexAB-OprM, an efflux pump that extrudes $\beta$-lactams, such as the mutations in *mexR* or *nalD*, encoding its regulators (89, 90). The loss of large chromosomal regions containing *galU* and *mexXY*, previously described to be involved in $\beta$-lactam resistance and in intrinsic aminoglycosides resistance, respectively (80), were also specifically selected in populations evolved in rich medium. This explains collateral sensitivity of these populations to aminoglycosides (35, 57), which is absent in urine- and SCFM-evolved populations (Fig. 2).

In agreement with our previous findings showing that fosfomycin collateral sensitivity of rich medium-evolved populations may be associated with mutations in *orfN* (36), we found that fosfomycin collateral sensitivity was conserved in the ceftazidime-evolved populations (Fig. 2).

**Effect of the environment on the genetic modifications selected during ceftazidime-avibactam ALEs.** Evolution in the presence of ceftazidime-avibactam in SCFM and urine led to the selection of genomic variations in *mutS and mutL*, respectively, in some of the evolved populations (Fig. 3). These genes encode components of the mismatch repair system, and their inactivation renders hypermutator phenotypes (91). Since these populations presented a huge number of genetic modifications, only those known to be related to AR are discussed here, in order to simplify such discussion. No common mutations were selected in the three environments. However, in urine and SCFM, common mutations were acquired. Among them, mutations in *mpl*, *ampC*, and *orfN*, also selected in ceftazidime-evolved populations, were selected. Mutations in *mpl* leading to Met38fs and Tyr35Ser were previously detected in *P. aeruginosa* clinical isolates (59, 79) (Table S5). Evolution in SCFM and rich laboratory medium selected mutations in *ftsI*, which were also selected after ceftazidime ALE, and in *PA14_45890*, encoding an efflux pump involved in *P. aeruginosa* intrinsic resistance to carbapenems (92, 93) and acquired resistance to ceftazidime-avibactam (57). Finally, growth in SCFM and rich laboratory medium selected mutations in *PA14_45880*, encoding a two-component response regulator. It has been proposed that the two-component system encoded by *PA14_45880-PA14_45870* may regulate the expression of the mentioned *PA14_45890* efflux pump-encoding gene (57).

Among the environment-specific acquired mutations, SCFM ALE selected mutations in *dacB*, *dacC*, *ampR*, *murF*, *zipA*, and *anmK*. Mutations in *dacB*, *dacC*, and *ampR*, which encode regulators of the expression of *ampC* (94), may increase *ampC* expression (95). In addition, the *dacB* genetic variation leading to Trp350Arg was previously detected in ceftazidime-resistant clinical isolates (79) (Table S5). Mutations in *murF*, encoding an enzyme involved in peptidoglycan synthesis, have been described to be involved in *Staphylococcus aureus* $\beta$-lactam resistance (96), and mutations in *zipA*, encoding a cell division protein, are involved in *Acinetobacter baumannii* $\beta$-lactam resistance (97). As mentioned, AnmK participates in the peptidoglycan recycling pathway (98), and its loss of function is associated with $\beta$-lactam resistance and collateral sensitivity to fosfomycin (87, 88). However, the mutation in *anmK* was acquired in SCFM and not in urine, as occurred in ceftazidime ALE.

In the case of urine, specific mutations were acquired in *nalC*, encoding a repressor of the expression of the genes encoding the MexAB-OprM efflux pump (99), in *ftsB* and *ftsL*, encoding division proteins from the FtsB/FtsL complex needed for the proper functioning of FtsI (100), and in the transcriptional regulator-encoding gene *rpl*, which produces a transcriptional regulator that regulates the expression of the *dad* operon (101), which controls intracellular D-alanine levels and peptidoglycan synthesis (102).

Apart from genetic modifications in *pepA*, *spoT*, *dnaJ*, and *flgF*, which have been previously related to $\beta$-lactam resistance (57), ceftazidime-avibactam rich medium-evolved populations also acquired mutations in *nalD*, encoding a transcriptional regulator of MexAB-OprM efflux pump, and large chromosomal deletions, as those previously

mentioned in populations evolved in ceftazidime and rich medium (57), which lead to collateral sensitivity to aminoglycosides (Fig. 2).

Despite the fact that no common genes were mutated in all the environments, it is relevant noting that different mutations might be functionally equivalent and produce similar effects on molecular mechanisms associated with $\beta$-lactam resistance. For instance, mutations in *ftsI* or functionally related genes, such as *ftsL* or *ftsB*, were acquired in every medium. Notably, mutations leading to Arg504His in FtsI and to Gly59Asp in FtsL were previously detected in ceftazidime-resistant clinical isolates of *P. aeruginosa* (59, 82, 83) (Table S5). Furthermore, mutations in genes encoding the regulators of MexAB-OprM efflux pump, such as *nalD* and *nalC*, were selected in urine and in rich medium, respectively, and mutations in the gene encoding the predicted efflux pump, *PA14_45890*, or the genes encoding its likely regulator, *PA14_45880-PA14_45870*, were selected in SCFM and rich medium, respectively.

Finally, as it occurred in the ceftazidime evolved populations, fosfomycin collateral sensitivity was acquired independently of the medium used (Fig. 2). As mentioned, this phenotype may be associated with genetic variations in *orfN*, *anmK* (36, 87), or genes related to peptidoglycan synthesis, which is blocked by fosfomycin, such as *murF* and *rplI*.

**Differential fitness cost and levels of resistance are the basis of the differential prevalence of mutations in each environment.** To further understand why specific mutations were acquired in each growth medium, clones from populations evolved in the presence of tobramycin, ceftazidime, or ceftazidime-avibactam were isolated, their mutations were ascertained by Sanger sequencing and those with a representative set of mutations of each ALE were selected for further analysis (Table 1). The isolated representative clones from tobramycin ALE experiments in rich medium, urine, and SCFM were dubbed TobRM, TobU, and TobS, respectively, representative clones of ceftazidime ALE experiments in rich medium, urine, and SCFM were referred to as CazRM, CazU, and CazS, respectively, and representative clones of ceftazidime-avibactam ALE experiments in rich medium, urine, and SCFM were referred to as Caz-AviRM, Caz-AviU, and Caz-AviS, respectively (Table 1).

The fitness in rich laboratory medium, urine, and SCFM of each representative clone as well as their susceptibility to the respective antibiotic of selection in each medium were measured. Notably, higher MICs do not imply higher fitness costs in the absence of antibiotics (Fig. 4). Differences in MICs were observed for each representative clone in the different media in which they were determined. This finding supports that AR acquisition and the associated trade-offs, as fitness costs or collateral sensitivity, may depend on the place of infection and its nutritional composition, a feature that deserves to be studied in greater depth. Among the three tobramycin representative clones, TobU was that one with highest relative fitness in urine. However, it presented the lowest fitness in rich medium and in SCFM (Fig. 4). This supports that differential fitness costs are based on the selection of the specific set of mutations present in TobU, which was prevalently selected in urine and not selected in SCFM or rich medium. For their part, TobS and TobRM had very similar relative fitness in SCFM (Fig. 4). Nevertheless, the set of mutations acquired in SCFM ALE produces a higher tobramycin resistance level without a relevant fitness cost in SCFM (Fig. 4), therefore being the optimal evolutionary solution for tobramycin selective pressure in SCFM. TobRM had higher tobramycin resistance level and relative fitness than TobS in rich medium (Fig. 4), explaining the selection of the TobRM group of genetic modifications in rich medium (Fig. 4).

Regarding representative clones of ceftazidime ALEs, CazRM presented the highest ceftazidime resistance level in every medium and CazU the lowest (Fig. 4). CazRM, probably due to the loss of a great number of genes (Table 1), presented very low relative fitness in SCFM and urine, but slightly higher fitness in rich medium (Fig. 4). This may indicate that, although the loss of those genomic regions leads to a great level of ceftazidime resistance, its selection is costly, with rich medium the growth medium in which the fitness cost is lower and, hence the medium in which these deletions might be selected (Fig. 4). However, it is worth mentioning that, although these large deletions are not selected *in vitro* in SCFM or urine, clinical *P. aeruginosa* isolates presenting large

**TABLE 1** Genomic variations identified in representative clones of tobramycin and ceftazidime ALEs in urine, SCFM and rich medium

| Clone | Gene | Genetic event | Amino acid change[a] |
|---|---|---|---|
| TobU | *fusA* | 2011A→G | Thr671Ala |
| | *orfK* | 355G→A | Glu119Lys |
| | *nuoD* | 183_184insC | Lys63fs |
| TobS | *ptsP* | 1135dupG | Ala379fs |
| | *fusA* | 2038C→T | Arg680Cys |
| | *orfH* | 286C→T | Arg96* |
| TobRM | *ptsP* | 2156delG | Glu677fs |
| | *fusA* | 1634G→A | Gly545Asp |
| | *orfN* | 148delG | Val50fs |
| | *pmrB* | 853G→C | Val285Leu |
| CazU | *mpl* | 353C→G | Thr118Ser |
| | *ftsL* | 176G→A | Gly59Asp |
| CazS | *mpl* | 706A→C | Thr236Pro |
| | *dacB* | 326G→A | Gly109Asp |
| | *clpS* | 248A→C | Gln83Pro |
| | *ftsI* | 1510C→T | Arg504Cys |
| CazRM | *mpl* | 416T→G | Val139Gly |
| | *dacB* | 343C→T | Gly115Ser |
| | *nalD* | 32G→T | Thr11Asn |
| | *pitA* | 367A→C | Thr123Pro |
| | del299,648 bp | del3200274–3499932 | |
| Caz-AviU | *mpl* | 742C→T | Gln248* |
| | *ftsL* | 176G→A | Gly59Asp |
| Caz-AviS | *anmK* | 197G→A | Trp66* |
| | *mpl* | 111delC | Met38fs |
| | *mpl* | 104A→C | Tyr35Ser |
| Caz-AviRM | *PA14_45890* | 1001G→A | Ser334Leu |
| | *ftsI* | 1567C→T | Val523Met |
| | *ftsI* | 1511C→T | Arg504His |
| | *clpA* | 1634A→G | Tyr545Cys |
| | del220701 bp | del3288650–3509351 | |

[a]An asterisk indicates the mutation led to a stop codon.

deletions are not infrequent (103). CazU was the representative clone with the lowest number of mutations (Table 1) and the lowest level of ceftazidime resistance (Fig. 4). Nevertheless, the ceftazidime resistance level of this clone was higher in urine than in rich medium or SCFM and led to the lowest fitness cost (Fig. 4), explaining the selection of this set of mutations in this medium. Finally, CazS achieved great levels of ceftazidime resistance with a set of mutations that produced the lowest fitness cost in SCFM among the three representative clones (Fig. 4), being the optimal ceftazidime resistance mutational solution in SCFM.

Regarding representative clones of Caz-Avi ALEs, some similarities were observed with that of Caz representative clones. Caz-AviRM presents a large deletion and high resistance levels but very reduced fitness in urine and SCFM, explaining why those deletions are selected only in rich medium. Caz-AviU clones, although presenting the lowest resistance levels, had the lowest fitness cost in urine, being selected in this medium. Finally, Caz-AviS mutations were selected because they produce high levels of resistance and the lowest fitness cost in SCFM.

**Robustness of collateral sensitivity to fosfomycin.** A robust collateral sensitivity to fosfomycin was observed in all the populations that evolved in the presence of ceftazidime or ceftazidime-avibactam in different media (rich medium, SCFM, and urine),

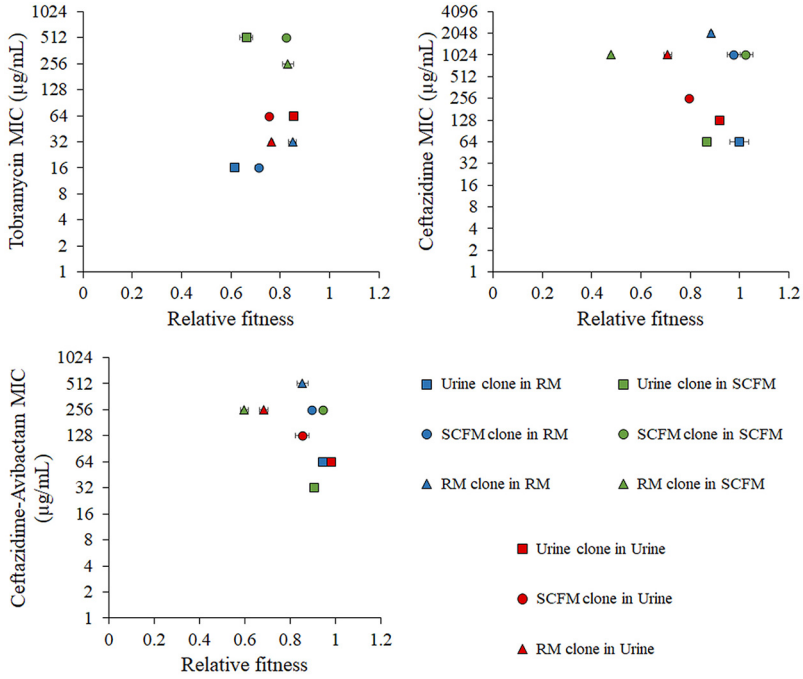


**FIG 4** MICs and relative fitness of tobramycin, ceftazidime, and ceftazidime-avibactam ALE representative clones in rich laboratory medium, urine, and SCFM. Growth curves of representative clones of tobramycin, ceftazidime and ceftazidime-avibactam ALEs in urine, SCFM, and rich laboratory medium were recorded in rich laboratory medium, urine, and SCFM. The fitness of each strain was measured as the area under the growth curve. The relative fitness of each clone was calculated with respect to the fitness of PA14 wild-type strain in the same medium. The values shown represent the mean from three replicates. For their part, MICs of tobramycin, ceftazidime and ceftazidime-avibactam clones to the antibiotic in which they were selected were also measured in each medium. The relative fitness of each clone with respect to its MIC values is represented.

but not in the ones that evolved in the presence of tobramycin (Fig. 2). In the last case, collateral sensitivity to fosfomycin was only observed in populations evolved in rich medium. In order to delve into the molecular causes of collateral sensitivity to fosfomycin observed in these populations, the MIC to fosfomycin in all the representative ceftazidime, ceftazidime-avibactam, and tobramycin clones was analyzed. All the ceftazidime and ceftazidime-avibactam representative clones presented increased susceptibility to fosfomycin, but only the tobramycin clone isolated from ALE in rich medium did so, confirming that the ceftazidime and ceftazidime-avibactam ALEs, but not the tobramycin ALE, led to a robust collateral sensitivity to fosfomycin (Table 2).

**TABLE 2** Fosfomycin MICs of tobramycin, ceftazidime, and ceftazidime-avibactam representative clones at the end of ALE assays in urine, SCFM, or rich laboratory medium and of the PA14 wild-type strain

| Strain or clone | Fosfomycin MIC ($\mu$g/mL) |
|---|---|
| Strain | |
| PA14 (wild type) | 32 |
| Clones | |
| TobRM | 8 |
| TobU | 48 |
| TobS | 32 |
| CazRM | 6 |
| CazU | 16 |
| CazS | 12 |
| Caz-AviRM | 6 |
| Caz-AviU | 12 |
| Caz-AviS | 1.5 |

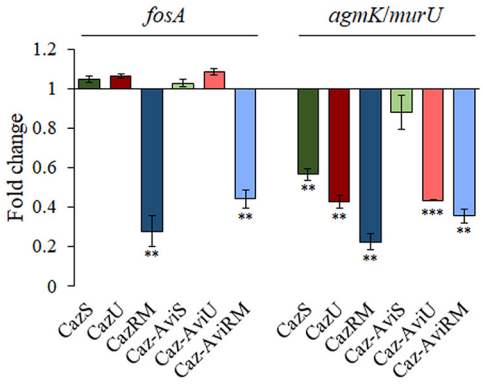


**FIG 5** Expression level of genes encoding fosfomycin resistance determinants in the CazS, CazU, CazRM, Caz-AviS, Caz-AviU, and Caz-AviRM clones. Fold changes in expression of CazS, CazU, CazRM, Caz-AviS, Caz-AviU, and Caz-AviRM were calculated relative to the expression of the PA14 wild-type strain and measured by qRT-PCR. Error bars indicate standard deviations from three biological replicates. Statistically significant differences from PA14 were calculated by *t* test for paired samples, assuming equal variances: *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

Collateral sensitivity to fosfomycin in clones from ceftazidime ALEs in rich medium was previously described to be caused by a reduced expression of *fosA*, encoding a fosfomycin-inactivating enzyme, and of genes encoding enzymes from the peptidoglycan recycling pathway (36). Hence, the expression level of such intrinsic fosfomycin resistance determinant-encoding genes was measured in the ceftazidime and ceftazidime-avibactam clones, which presented a robust collateral sensitivity to fosfomycin with respect to the wild type, independent of the medium in which they evolved (rich medium, SCFM, and urine). In agreement with previous information (36), CazRM and Caz-AviRM presented reduced expression levels of both *fosA* and *agmK/murU* (encoding the last enzymes of the peptidoglycan recycling pathway) (87) (Fig. 5). For their part, CazS, CazU, and Caz-AviU had reduced expression of *agmK/murU* (Fig. 5), which may be related to the genetic variations that these clones acquired in genes encoding proteins associated with synthesis of the cell wall (i.e., in *mpl*, *dacB*, *ftsL*, or *ftsI*). CazRM and Caz-AviRM clones have lower fosfomycin MICs than CazS, CazU, or Caz-AviU clones, possibly because the last ones present impaired expression of the peptidoglycan recycling-encoding genes, but not of *fosA* (Table 2 and Fig. 5). Finally, the Caz-AviS clone acquired a genetic variation leading to a truncated AnmK protein (Table S4), belonging to the peptidoglycan recycling pathway. Although no expression changes in *fosA* were detected in this clone (Fig. 5), it is the clone most susceptible to fosfomycin, confirming that a loss-of-function mutation in *anmK*, whose inactivation has been associated previously with both an increase of ceftazidime resistance and fosfomycin susceptibility (87, 88), has an important impact in this phenotype, as previously described (36).

These results indicate that the robust collateral sensitivity to fosfomycin associated with the acquisition of different ceftazidime resistance mutations in ceftazidime- and ceftazidime-avibactam-evolved populations in SCFM, urine, and rich laboratory medium is caused by reduced activity of the peptidoglycan recycling pathway, either by reduced expression of the genes encoding these enzymes or directly by acquisition of variations in the genes encoding these enzymes.

**Concluding remarks.** Although it has been established that several factors may constrain the evolution of AR (24–34), the extent to which nutritional composition of the colonized environment may modify the evolutionary process in the presence of a specific drug has not been deeply studied, despite the fact that the evolution process largely depends on the habitat where it takes place. In the case of bacterial infections, this can be particularly relevant, since the physicochemical composition, including nutrients' availability, largely varies in different body locations, a feature that might

influence evolution toward AR. Indeed, our results show that the genetic variations acquired by *P. aeruginosa* in the presence of tobramycin, ceftazidime, or ceftazidime-avibactam are different, depending on whether they are acquired in urine, synthetic sputum, or laboratory rich medium. The reason behind this may be that different mutational patterns lead to different levels of resistance and fitness depending on the composition of growing medium. This indicates that fitness costs associated with specific resistance mutations are not just a nonspecific burden that equally occurs in any ecosystem, but rather fitness costs are habitat dependent. In other words, the phenotype associated with specific resistance mutations is not something rigid and immovable, but it is variable and dependent on the metabolic state of bacteria growing in each particular habitat. This means that different resistance mutations could be selected in different infected locations and that, furthermore, the contribution of these mutations to AR is specific to each environment. It is noteworthy that most of the mutations identified during this work have been previously detected, exactly the same or in the same genes, in antibiotic-resistant clinical isolates of *P. aeruginosa* (Tables S5 and S6, respectively), supporting the clinical relevance of our results.

The fact that the habitat may restrict the possible selected mutants in the presence of a specific antibiotic has relevant implications for the design of evolution-based approaches to tackle *P. aeruginosa* infections. Indeed, collateral sensitivity has been explored as a mean to rationally design therapeutic strategies against bacterial infections, but its exploitation largely depends on the robustness of this phenotype in different genetic backgrounds, such as preexisting antibiotic-resistant mutants (35, 37). However, robustness of collateral sensitivity not only implies phenotypic conservation in bacteria presenting different genetic backgrounds evolved in the presence of the same drug, but also refers to the conservation of the phenotype observed when bacteria acquire resistance in different locations within the infected patient, each one presenting a specific nutritional composition. Importantly, we show that collateral sensitivity to fosfomycin associated with the acquisition of ceftazidime and ceftazidime-avibactam resistance is conserved in urine, synthetic sputum, and rich laboratory medium. It is important to emphasize that the latter is the conventionally used medium to experimentally predict patterns of AR and collateral sensitivity, possibly limiting the translation of evolution experiments in clinical settings. This work supports that the evolution of AR in *P. aeruginosa* is habitat dependent, and therefore, the design of evolutionary strategies to tackle infections should be based on robust patterns of evolution identified within each particular patient environment.

## MATERIALS AND METHODS

**Media and growth conditions.** Overnight cultures were obtained by growing bacteria in lysogeny broth (LB) (Lennox; Pronadisa) at 37°C and shaking at 250 rpm. For the ALE (see below), urine and synthetic cystic fibrosis sputum medium (SCFM) were used. The urine used in this work was obtained by pooling urine samples from four healthy volunteers who had not received antibiotic treatment during the previous year. Urine was then filtered through 0.2-$\mu$m-pore-size filters (Whatman) and stored at −20°C until use.

Fresh SCFM was prepared each day for the ALE as described in reference 104, where the concentrations of the components of SCFM were based on the average concentrations of CF sputum samples.

**Antibiotic susceptibility assays.** The concentration of antibiotic (ceftazidime, ceftazidime-avibactam, or tobramycin) used for selection in the ALEs was that one that hinders the growth of *P. aeruginosa* PA14 parental strain. It was determined in glass tubes in each different medium used for the ALEs at 37°C and 250 rpm.

Susceptibility of evolved populations to different drugs, namely, tigecycline, tetracycline, aztreonam, ceftazidime, imipenem, ciprofloxacin, norfloxacin, tobramycin, amikacin, chloramphenicol, erythromycin, and fosfomycin, was determined by MIC test strips (Liofilchem) in Mueller-Hinton agar (MHA) (Pronadisa) at 37°C following the supplier's instructions.

MICs of representative clones to ceftazidime or tobramycin in MHA, urine, or SCFM were determined by the broth microdilution method. For that, 96-well plates with round bottoms (Thermo Scientific Nunc) were used. Bacteria were inoculated at an initial optical density at 600 nm ($OD_{600}$) of 0.01, and the concentration of antibiotic in which there was no bacterial growth was determined after incubation at 37°C without shaking for 48 h.

**Adaptive laboratory evolution experiments.** ALE assays were performed as previously described (20, 57), but using SCFM and urine as growth media. Thirty-two bacterial populations of *P. aeruginosa* PA14 were grown in parallel in urine or SCFM at 37°C with shaking at 250 rpm for 30 days. Four replicates were grown in the presence of either tobramycin, ceftazidime, or the combination ceftazidime-avibactam in SCFM or urine. In addition, eight controls were grown without antibiotic for each medium (4 populations in SCFM and 4 populations in urine). Initial concentrations of antibiotic were determined as previously specified, with 0.5 $\mu$g/mL of ceftazidime and 0.5 $\mu$g/mL of tobramycin in urine, whereas 3 $\mu$g/mL and 2.5 $\mu$g/mL of ceftazidime and tobramycin, respectively, were used in SCFM. The avibactam concentration was constantly maintained at 4 $\mu$g/mL, as used in clinical tests (105). Each day, the cultures were diluted 1/250 in fresh medium. Every 5 days, the antibiotic concentration was doubled up to 32 times at 30 days. Samples were preserved at −80°C for further research, and MICs for the antibiotic of selection were determined.

**Whole-genome sequencing and bioinformatics analysis.** Genomic DNA of each of the 30-day-evolved populations was extracted by using the Gnome DNA kit (MP Biomedicals, Santa Ana, CA, USA). The DNA quality check and sequencing were performed by Macrogen. Paired-end libraries (2 × 150 bp) were obtained by using the Truseq DNA PCR-free system and sequenced with an Illumina NovaSeq 6000 instrument. The average number of reads per sample represents a coverage of greater than 300×.

Genome sequence, gene coordinates, and annotations were obtained from GenBank. FASTQC was used to verify Illumina short-read quality (106). The alignment of reads against *Pseudomonas aeruginosa* genome UCBPP-PA14 (GenBank accession no. NC_008463.1) was performed with RNA-STAR (107). The MarkDuplicates (Picard) function of the Genome Analysis Toolkit was used to detect optical and PCR duplicates (108). For indexing of alignment files in BAM format, SAMtools was used (109). Freebayes was used to detect single nucleotide polymorphisms (SNPs) and small insertions and deletions (indels) (110). The impact of indels and SNPs was evaluated with SnpEff (111), and annotated results were saved in VCF format. By using the SNPer viewer (https://bioinfogp.cnb.csic.es/tools/snper) and the IGV browser, genetic variants were detected (112).

The presence of mutations detected in the genome sequencing analysis (see Table S4 in the supplemental material) in representative clones of the treatments was verified by PCR and by Sanger sequencing using the primers shown in Table S7. DNA fragments were purified with the QiAquick PCR purification kit (Qiagen) and Sanger sequenced at Macrogen.

**Relative fitness determination.** Bacteria were inoculated at initial $OD_{600}$ of 0.01 in each well of 96-well microtiter plates with delta surface (Thermo Scientific Nunc) previously filled with 100 $\mu$L of LB, urine, or SCFM. Growth curves were obtained by measuring the $OD_{600}$ every 10 min for 48 h at 37°C in a Spark 10M plate reader (Tecan). Triplicates of each condition were performed. The area under the growth curve was considered an estimation of fitness of each bacterial strain in each medium, and relative fitness was calculated with regard to the area under the growth curve of PA14 wild-type strain in the respective medium.

**RNA extraction and qRT-PCR.** Overnight bacterial cultures of the wild-type strain PA14 and of the representative clones CazS, CazU, CazRM, Caz-AviS, Caz-AviU, and Caz-AviRM were inoculated in 20 mL of LB at an $OD_{600}$ of 0.01 and incubated at 37°C and 250 rpm until the exponential phase of growth was reached ($OD_{600}$ of 0.6). Then, the RNA extraction was done as previously described (113), and cDNA was obtained from 10 $\mu$g of RNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems).

Quantitative real-time PCR (qRT-PCR) was performed in an ABI Prism 7300 real-time system (Applied Biosystems). Power SYBR green PCR master mix (Applied Biosystems) and 50 ng of cDNA were used in each reaction, which consisted of a denaturation step (95°C for 10 min) followed by 40 cycles of 95°C for 15 s and 1 min at 60°C for amplification and quantification. Primers amplifying a specific fragment of *fosA* (ACCAGGGCGCCTATCTCGAA; CGCTGCGGTTCTGCTTCCAT), *agmK* (AGCTGAATCGCTGGTTGGAC; AAC GGTCGGCAGTCTTCCTG), or the housekeeping gene *rplU* (CGCAGTGATTGTTACCGGTG; AGGCCTGAATGC CGGTGATC) (36) were used at 400 nM. The threshold cycle ($2^{-\Delta\Delta CT}$) method (114) was used to analyze differences in the relative amounts of mRNA of three independent biological replicates, each containing three technical replicates.

**Data availability.** Whole-genome sequencing data of this work can be found at NCBI with the accession number PRJNA810193.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.4 MB.

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## Article IV

**Rapid decline of ceftazidime resistance in antibiotic-free and sublethal environments is contingent on genetic background**

**Hernando-Amado S, Laborda P, Valverde JR, Martínez JL.**

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Fitness cost in absence of antibiotics is another AR evolutionary trade-off with potential to be exploited to address bacterial infections, since compensatory evolution of fitness cost could result in a decline of drug resistance. However, decline of resistance depends on the antibiotic used and the resistance mechanism selected. Hence it is not a general trait to be exploited by means of blind, not evidence-based, antibiotic restriction periods, something that has probably compromised the success of this strategy for tackling AR. Therefore, identifying specific antibiotics for which the most frequent resistance mechanisms are not preserved when removing the selection pressure is needed for the application of this kind of approaches.

In this article, we have studied compensatory evolution of mutants, presenting different genetic backgrounds, which previously acquired ceftazidime resistance. This was addressed both in antibiotic-free medium and in presence of sublethal concentrations of tobramycin -an antibiotic for which the resistant mutants presented collateral sensitivity- which can be present in certain clinical situations, such as incomplete treatments or limited drug accessibility. We observed that ceftazidime resistance decline occurs within 450 generations both in absence of drugs and in presence of sublethal tobramycin concentrations, and that the frequency and degree of AR decline is contingent on genetic background. Ceftazidime resistance decline was not caused by reversion of the original mutations but by newly acquired secondary mutations in the genes that were originally mutated in a previous antibiotic exposure. These genetic modifications also caused an improved fitness and a decline of cross-resistance to other β-lactam drugs. Consequently, the alternation of ceftazidime with drug restriction periods may be feasible. Even more, ceftazidime could be also used after the previously suggested ceftazidime-tobramycin alternation to treat tobramycin-resistant bacteria arising from sublethal tobramycin concentrations.

Importantly, tobramycin collateral sensitivity was preserved after the aforementioned evolution in absence of drugs. The stability of collateral sensitivity after drug restriction periods has not been previously explored and it may be relevant for the implementation of such antibiotic use policies. Even more, these results show that, when the selection pressure ceases, non-adaptive phenotypes may be preserved. This observation suggests that stable non-adaptive phenotypes might have more relevance in evolution than traditionally considered.

**Specific contributions**:

*Experimental work*: Laborda P. and Hernando-Amado S. contributed to experimental work. I performed some antibiotic resistance measurements and the β-lactamase activity determinations, as well as contributed to the interpretation of the results.

*Manuscript writing*: All the authors contributed to the writing and correction of the manuscript.

# Rapid Decline of Ceftazidime Resistance in Antibiotic-Free and Sublethal Environments Is Contingent on Genetic Background

Sara Hernando-Amado,* Pablo Laborda, José Ramón Valverde, and José Luis Martínez*

Centro Nacional de Biotecnología, CSIC, Madrid, Spain

***Corresponding authors:** E-mails: shernando@cnb.csic.es; jlmtnez@cnb.csic.es.

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## Abstract

**Trade-offs of antibiotic resistance evolution, such as fitness cost and collateral sensitivity (CS), could be exploited to drive evolution toward antibiotic susceptibility. Decline of resistance may occur when resistance to other drug leads to CS to the first one and when compensatory mutations, or genetic reversion of the original ones, reduce fitness cost. Here we describe the impact of antibiotic-free and sublethal environments on declining ceftazidime resistance in different *Pseudomonas aeruginosa* resistant mutants. We determined that decline of ceftazidime resistance occurs within 450 generations, which is caused by newly acquired mutations and not by reversion of the original ones, and that the original CS of these mutants is preserved. In addition, we observed that the frequency and degree of this decline is contingent on genetic background. Our results are relevant to implement evolution-based therapeutic approaches, as well as to redefine global policies of antibiotic use, such as drug cycling.**

***Key words:* *Pseudomonas aeruginosa*, decline of antibiotic resistance, collateral sensitivity, fitness cost, compensatory evolution.**

## Introduction

The effectiveness of antibiotics is currently compromised by the emergence and spread of antibiotic resistance (AR). This evolutionary process presents two sequential steps: emergence and fixation. Although emergence is the consequence of selection, fixation of AR mainly depends on the fitness costs associated with resistance and on the chances of compensating these costs by acquiring secondary mutations.

It has been generally accepted that mutational acquisition of AR may impose a functional/metabolic burden, enhancing the probability of the resistant mutants to be outcompeted by a wild-type susceptible strain in antibiotic-free environments (Andersson and Levin 1999; Bjorkman and Andersson 2000; Martinez and Baquero 2000; Andersson 2006; Baquero et al. 2009; Balasubramanian et al. 2012; Tian et al. 2016). However, some AR mutations are cost-free (Wasels et al. 2015) or may even produce an increased fitness in antibiotic-free environments (Skurnik et al. 2013; Durão et al. 2015; Maharjan and Ferenci 2017). Besides, mutations that compensate fitness costs are frequently selected more likely than genetic reversion by restoration of the wild-type allele (Levin et al. 2000). This means that simply restricting the use of antibiotics would be insufficient to solve the AR problem (Schrag et al. 1997; Enne et al. 2001; Andersson and Hughes 2010; Brolund et al. 2010).

Despite the above, a rapid decline of AR has been observed using adaptive laboratory evolution (ALE) assays on antibiotic-free medium (Dunai et al. 2019). Nevertheless, resistance loss associated with compensatory evolution was observed to be drug specific. In addition, it is possible that AR decline may depend on both, the initial fitness cost and the original genetic background in which those fitness costs are compensated, because epistatic interactions, in addition to restricting AR evolution (Trindade et al. 2009; Ward et al. 2009; Salverda et al. 2011; Kryazhimskiy et al. 2014; Vogwill et al. 2016; Knopp and Andersson 2018; Hernando-Amado et al. 2019), may also affect compensatory evolution, something that has not been studied so far. Further, it is also possible that AR decline may depend on the environment, as fixation of AR does. It is known that high-cost AR genetic variations are preserved or rapidly outcompeted depending on whether or not antibiotics are present in the environment. Because of that, most studies in the field have focused on the study of AR evolution on antibiotic-free environments or inhibitory (hereafter dubbed lethal, to simplify) concentrations of antibiotics. However, subinhibitory (hereafter dubbed sublethal, to simplify) concentrations can also select antibiotic resistant mutants (Sanz-García et al. 2021) and they can be present in certain clinical situations (e.g., incomplete treatments or limited drug accessibility) (Baquero and Negri 1997). Therefore, it is possible that sublethal concentrations of antibiotics may be relevant during the evolutionary compensation of fitness costs, a feature that has not been addressed yet.

In a previous work, we determined to what extent the presence of pre-existing AR mutations may affect the robustness of ceftazidime resistance evolution in *Pseudomonas aeruginosa*. For this purpose, we submitted different







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*P. aeruginosa* resistant mutants, presenting either single mutations, NfxB or ParR, or multiple resistance mutations, MDR6, to short-term evolution in the presence of ceftazidime (Hernando-Amado et al. 2020). The evolved populations presented large chromosomal deletions leading to increased ceftazidime minimal inhibitory concentration (MIC) and reduced tobramycin MIC (Hernando-Amado et al. 2020), antibiotics that form part of usual therapies against *P. aeruginosa* (Cheer et al. 2003). Therefore, we proposed that this robust phenotypic convergence toward collateral sensitivity (CS) (Szybalski and Bryson 1952; Pal et al. 2015), by which acquisition of resistance to a drug (i.e., ceftazidime) increases susceptibility to another (i.e., tobramycin), could be exploited to rationally counteract *P. aeruginosa* infections, alternating ceftazidime with tobramycin. However, the stability of ceftazidime resistance and CS to tobramycin once ceftazidime is removed, where compensatory evolution of fitness costs may occur, was not determined. Furthermore, we did not study the evolution of tobramycin resistance in the presence of tobramycin sublethal concentrations and if it could lead to CS to ceftazidime, a possibility supported by previous findings showing the existence of reciprocal CS between aminoglycosides and $\beta$-lactams (Barbosa et al. 2019).

In this work, we analyze the possible decline of ceftazidime resistance during 8 weeks of ALE experiments in two different environments: 1) antibiotic-free medium (e.g., drug restriction periods) and 2) sublethal tobramycin concentrations (e.g., concentrations resulting from tobramycin and ceftazidime alternation in sequential therapies). Our hypothesis was that fitness cost of ceftazidime resistant mutants could drive compensatory evolution in nonselective environments (antibiotic-free or tobramycin sublethal concentrations that do not select resistance) and a decline of ceftazidime resistance (see "nonselective environment" in conceptual fig. 1). In particular, we raised the question of the extent to which both, genetic background and initial fitness costs could shape compensatory evolution and the decline of resistance, if it occurred. Besides, we analyzed if sublethal tobramycin concentrations able to select resistance in each genetic background could also lead to CS to ceftazidime (see "selective environment 2" in conceptual fig. 1). Finally, we studied the stability of CS to tobramycin of the analyzed ceftazidime resistant mutants in antibiotic-free environments, because compensatory evolution may not only cause a decline of ceftazidime resistance but also variations in trade-offs associated with this phenotype, as it is CS to tobramycin.

## Results

### Characterization of NfxB-CAZ, ParR-CAZ, and MDR6-CAZ Ceftazidime Resistant Mutants

A clone from each of the three different ceftazidime resistant/tobramycin hyper-susceptible populations (NfxB, ParR, and MDR6) previously obtained (Hernando-Amado et al. 2020), was isolated (hereafter dubbed NfxB-CAZ, ParR-CAZ, or MDR6-CAZ, to simplify). The three clones were subjected to whole-genome sequencing. Consistent with previous information concerning the genotype of the populations containing these mutants, we identified three genetic events in NfxB-CAZ, including a large chromosomal deletion and two single-nucleotide polymorphisms (SNPs), three genetic events in ParR-CAZ, including a large chromosomal deletion and two SNPs, and seven genetic events in MDR6-CAZ, including a large chromosomal deletion, and six SNPs (supplementary table 1, Supplementary Material online). Given that the three ceftazidime resistant clones lacked large genomic regions, it was sensible to hypothesize that they could present high fitness costs when grown in the absence of antibiotics. In addition, it is important to notice that MDR6-CAZ and ParR-CAZ mutants presented a SNP in *rpoB*, that encodes the subunit $\beta$ of the RNA polymerase (RNAP) (Villain-Guillot et al. 2007), and in *ampR*, which encodes a LysR-type transcriptional regulator (LTTR) that regulates the expression of the $\beta$-lactamase AmpC (Kong et al. 2005; Balcewich et al. 2010), respectively (supplementary table 1, Supplementary Material online), respectively. Mutations in the genes encoding these two proteins are known to reduce bacterial fitness (Severinov et al. 1993; Qi et al. 2014; Perez-Gallego et al. 2016; Balbontin et al. 2021). We, therefore, compared the fitness of NfxB-CAZ, ParR-CAZ, or MDR6-CAZ mutants, before and after short-term evolution on ceftazidime, in antibiotic-free medium (fig. 2). For that, we estimated fitness of each mutant and parental strain as the area under the growth curve recorded in antibiotic-free medium and we calculated fitness costs of each ceftazidime resistant mutants respect to its parental strain (see Materials and Methods). The three ceftazidime resistant mutants presented a reduction of fitness with respect to their parental strains, from 20% in the case of NfxB-CAZ or ParR-CAZ, up to 40% in the case of MDR6-CAZ. Given the strength of the fitness costs, it was worth thinking that these ceftazidime resistant mutants might acquire compensatory mutations, which would reduce the burden associated with ceftazidime resistance, in antibiotic-free, nonselective environments. In addition, it might be possible that some of the newly acquired compensatory mutations could produce a reversion or decline of resistance. Since these ceftazidime resistant mutants presented CS to tobramycin (supplementary table 2, Supplementary Material online), being possible to alternate ceftazidime with tobramycin (Hernando-Amado et al. 2020), it was also of interest to know the range of tobramycin sublethal concentrations that could select for tobramycin resistance, eventually altering the pattern of compensatory evolution.

### Evolution of Ceftazidime and Tobramycin Resistance on Antibiotic-Free and Sublethal Tobramycin Environments

To analyze changes in ceftazidime and tobramycin susceptibility upon evolution in antibiotic-free and sublethal tobramycin environments, we performed an 8-week ALE assay with the ceftazidime resistant mutants NfxB-CAZ, ParR-CAZ, and MDR6-CAZ, using four replicates of each genetic background, in four different environments: antibiotic-free (12 populations), 1/4 of tobramycin MIC (12 populations), 1/8 of tobramycin MIC (12 populations), and 1/16 of

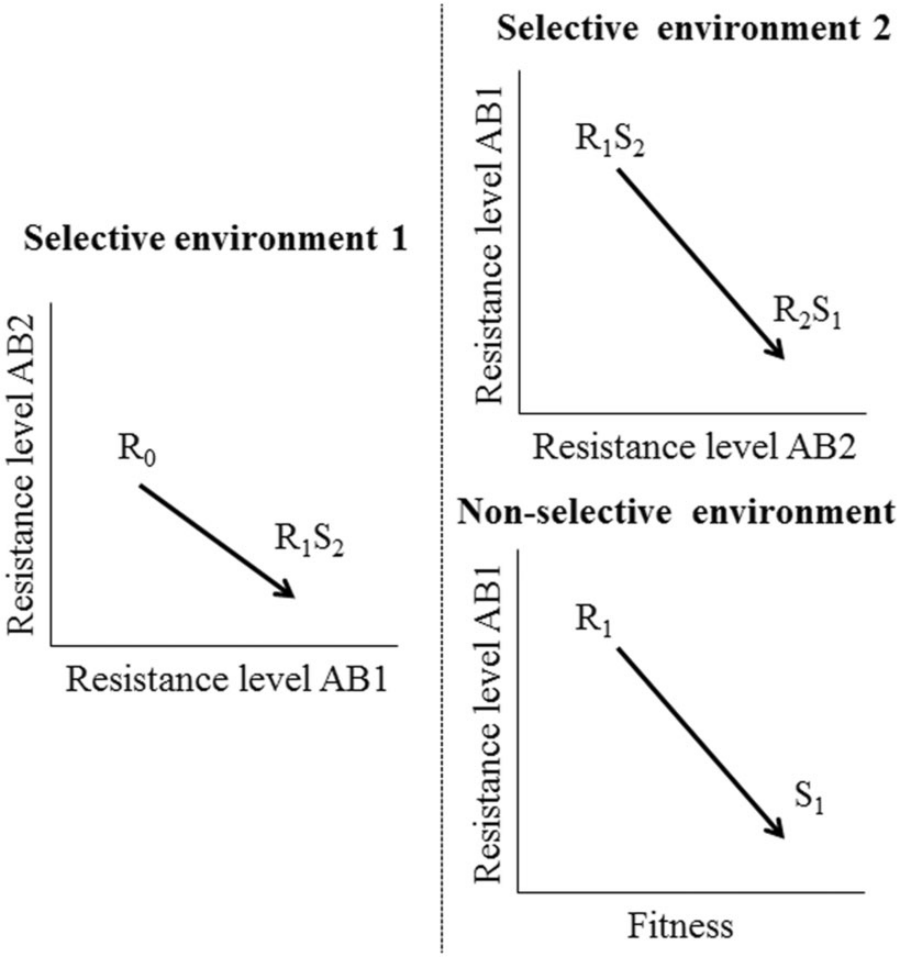


FIG. 1. Conceptual figure representing phenotypic reversion of drug resistance. Departing from an initial AR phenotype ($R_0$), exposure to a first antibiotic (AB1) may select resistance to that first drug ($R_1$) and CS ($S_2$) to a second antibiotic (AB2) (selective environment 1). After that first step, which leads to an organism resistant to the first drug and susceptible to the second antibiotic ($R_1S_2$), reversion of resistance acquired may occur in sublethal concentrations of AB2 that select resistance to this antibiotic and CS to AB1 ($R_2S_1$) (selective environment 2). This decline of resistance may also occur in antibiotic-free environments or sublethal antibiotic concentrations that do not select resistance, when fitness costs of resistance to the first drug are compensated (nonselective environment), hence moving from low fitness and resistance to AB1 ($R_1$) to a high fitness related susceptible phenotype ($S_1$).

tobramycin MIC (12 populations). This resulted in a total of 48 independently evolved populations. The populations were propagated for 56 days, approximately 450 generations, by diluting 1% of the saturated cultures into fresh medium every day. The tobramycin and ceftazidime MICs for each population were determined every 7 days during the 56 days of the experiment (supplementary tables 2 and 3, Supplementary Material online, respectively).

Statistical analysis of the data, using Multi- and One-Way ANOVA with Welch correction followed by Tukey HSD multiple comparisons, revealed the existence of significant differences when considering each factor or combination thereof ($P < 0.005$ in all cases). Notably, a decline of ceftazidime resistance was observed, within the 56 days of ALE, in 35 out of 48 populations (72%) from both, nonlethal and tobramycin sublethal environments (supplementary table 3, Supplementary Material online and fig. 3C). Decline of ceftazidime MIC was observed in 9 out of 12 populations evolved in antibiotic-free environment, 9 out of 12 populations evolved in 1/4 of tobramycin MIC, 7 out of 12 populations evolved in 1/8 of tobramycin MIC, and 10 out of 12 populations evolved in 1/16 of tobramycin MIC (supplementary table 3, Supplementary Material online). However, the degree of resistance decline varied, depending on the genetic background, being up to 128-fold in ParR-CAZ, up to 4-fold in NfxB-CAZ, and up to 3-fold in MDR6-CAZ. Because ANOVA showed that differences depending on genetic background and time of evolution were significant ($P < 0.001$), we carried an endpoint analysis, as described in Materials and Methods. This revealed significant reductions of ceftazidime MIC in NfxB-CAZ populations in all the growth conditions ($P < 0.01$), in MDR6-CAZ populations in all conditions except in 1/8 of tobramycin MIC, and in ParR-CAZ populations in 1/16 of tobramycin MIC ($P < 0.05$).

Our initial hypothesis was that a decline of ceftazidime resistance in antibiotic-free conditions might be possible, due to compensatory evolution of fitness costs associated with ceftazidime resistance, and that the presence of

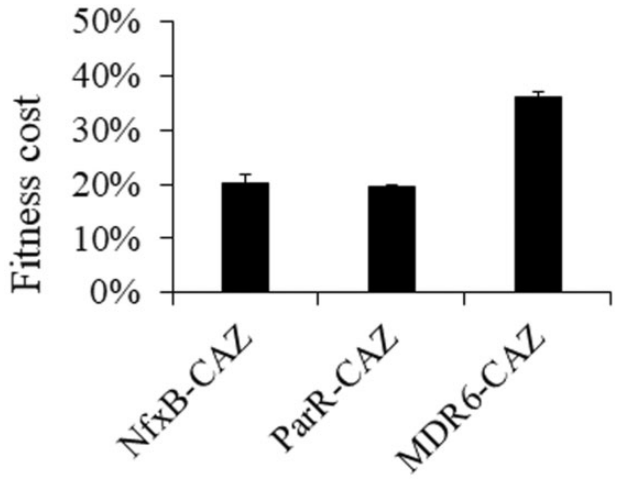


FIG. 2. Ceftazidime-resistant mutants present a high fitness cost in antibiotic-free medium. Growth curves of the resistant mutants, before and after ceftazidime evolution, were recorded in antibiotic-free medium (LB). The fitness ($W$) of each strain was measured as the area under the growth curve. Fitness cost of each ceftazidime resistant mutants respect to its parental strain was calculated, using the equation: $1 - (W_{mutant}/W_{parental\ strain})$ and was expressed as percentage. Acquisition of resistance to ceftazidime is associated with fitness cost in antibiotic-free medium. Error bars indicate standard deviations from three different replicates.

concentrations of tobramycin unable to select tobramycin resistance mutations would not impede this AR decline (see conceptual fig. 1). In addition, our hypothesis was that the newly selected tobramycin resistance mutations, acquired in the presence of selective concentrations of tobramycin, could also present CS to ceftazidime (see conceptual fig. 1), because reciprocal CS between aminoglycosides and $\beta$-lactams has been previously described (Barbosa et al. 2019). In these two situations, ceftazidime resistance could decline, something clinically relevant, allowing a switch back to ceftazidime after drug restriction periods or alternation of ceftazidime with tobramycin.

End-point analysis (comparing MIC levels at $t = 0$ and $t = 56$ days) revealed significant differences ($P < 0.05$) in the three parental, antibiotic resistant, strains when treated with 1/4 and 1/8 of tobramycin MIC but not with the lower sublethal concentration (1/16 of tobramycin MIC) or without antibiotic. In the absence of drugs, compensatory evolution led to the decrease of ceftazidime resistance, but CS to tobramycin was preserved (see supplementary table 2, Supplementary Material online). For its part, a progressive increase in the level of resistance to the antibiotic of selection was observed for the two higher sublethal tobramycin concentrations (1/4 and 1/8 of tobramycin MIC) for the three analyzed genetic backgrounds, suggesting either accumulation of mutations along the evolution or the displacement of low-level resistant mutants by higher-level ones. In all the genetic backgrounds (and all replicate populations) analyzed, 1/4 and 1/8 of tobramycin MIC resulted in a tobramycin resistance increase at the end of the ALE experiments, although the resistance levels acquired were significantly lower ($P < 0.001$, using Tukey HSD) in the presence of 1/8 of tobramycin MIC than in the presence of 1/4 of tobramycin MIC (supplementary table 2, Supplementary Material online and fig. 3A). This is in agreement with previous data from our laboratory that described, in a PA14 wild-type strain, a threshold of the sublethal selective window higher than 1/10 of tobramycin MIC (Sanz-Garcia et al. 2020). In fact, only the higher sublethal tobramycin concentration (1/4 of tobramycin MIC) was able to select mutants presenting a tobramycin MIC above the EUCAST clinical breakpoint (2 μg/ml) (fig. 3A). However, it is important to highlight that, even in these cases, the MICs reached by the mutants were not high; well below those described when the PA14 wild-type strain was submitted to tobramycin sublethal selection (Sanz-Garcia et al. 2018b). In addition, a very subtle, not statistically significant, increase was observed in the case of antibiotic-free medium or the lower tobramycin concentration tested (1/16 of tobramycin MIC) (supplementary table 2, Supplementary Material online). No significant increase of resistance was observed in 1/16 of tobramycin MIC in any of the NfxB-CAZ replicate populations, nor in 2 out of 4 ParR-CAZ replicate populations. However, a detectable increase was observed in 2 out of 4 ParR-CAZ replicate populations and MDR6-CAZ replicate populations. ANOVA showed that the level of tobramycin resistance significantly depends on genetic background ($P < 0.001$) and time of evolution ($P < 0.01$) for all treatments. Significant differences ($P < 0.01$) could be found among genetic backgrounds and treatments in subsequent Tukey's test, indicating that the probability to select resistance at a particular sublethal tobramycin concentration depends on the genetic background (fig. 3A). These results support the influence of the genetic background on determining the sublethal selective concentration that selects resistance to tobramycin, something that had not been previously described.

Altogether, these results support that the alternation of ceftazidime with tobramycin for treating *P. aeruginosa* infections, that we previously suggested (Hernando-Amado et al. 2020), could be followed, if required, by a switch back to ceftazidime. Further, these results also suggest the possibility of alternating ceftazidime with periods of drug restriction, allowing a rapid decline of resistance, preserving tobramycin CS, and possibly avoiding the selection of resistance to other antibiotics, a feature that we study below.

## Evolution of Cross-Resistance and Collateral Sensitivity on Antibiotic-Free and Sublethal Tobramycin Environments

The ceftazidime resistant mutants NfxB-CAZ, ParR-CAZ, and MDR6-CAZ obtained after short-term evolution on ceftazidime presented CS, not only to tobramycin, but also to fosfomycin and tetracycline, and cross-resistance to aztreonam and imipenem (Hernando-Amado et al. 2020). To ascertain if the populations obtained after ALE on both, antibiotic-free or tobramycin sublethal environments, besides presenting a decline of ceftazidime MIC, could also present variations in the level of resistance to other antibiotics belonging to different structural families (fosfomycin, tetracycline, ciprofloxacin,

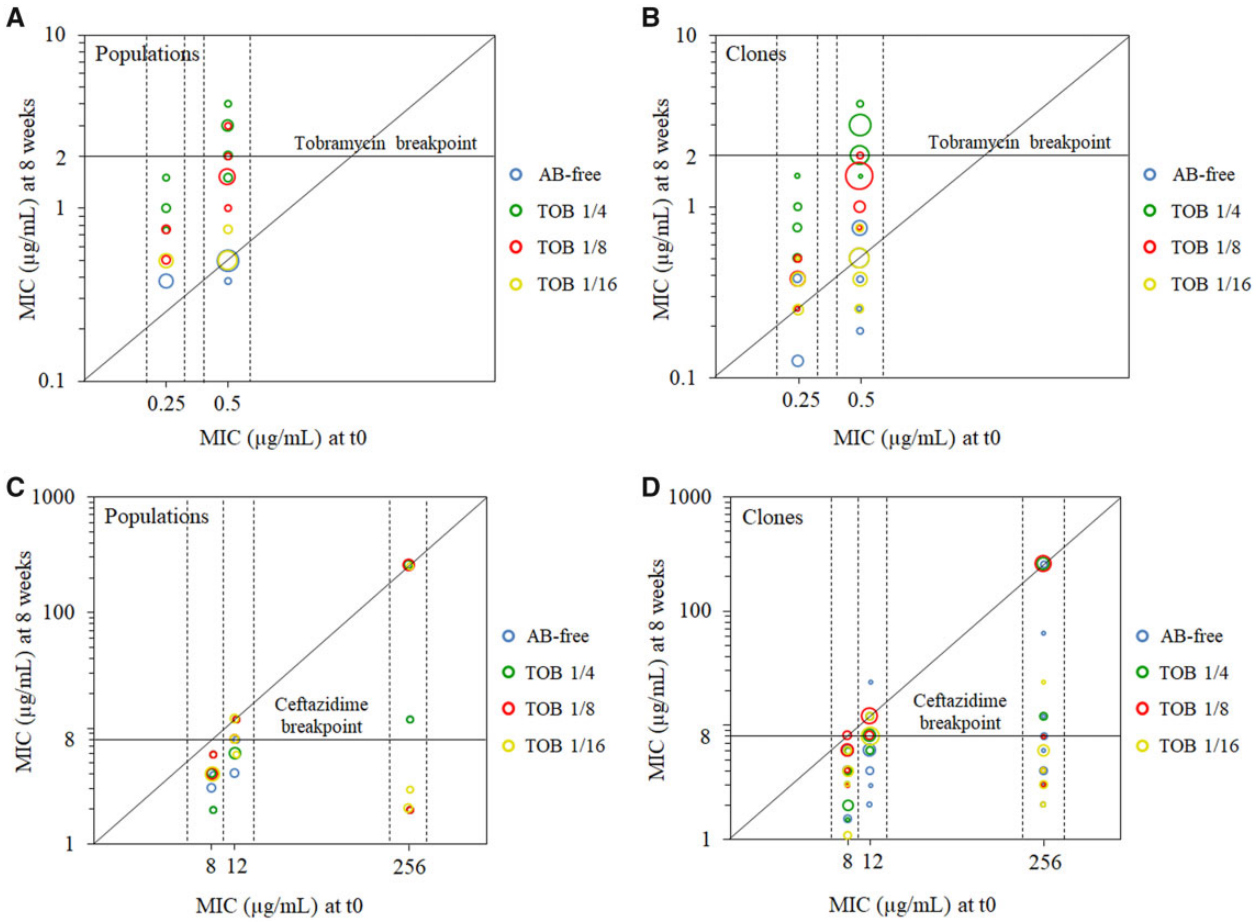


FIG. 3. Impact of antibiotic-free and tobramycin sublethal environments on AR level. (*A* and *B*) The figure shows the MICs (µg/ml) to tobramycin (log10-scale) of each starting genetic background at time zero ($t_0$) and the corresponding MIC (µg/ml) acquired in (*A*) the populations or (*B*) their isolated clones, after 8 weeks of ALE. Each of the points represents a population (in *A*) or clone (in *B*); and the colors indicate different treatments: AB free, 1/4 of tobramycin MIC, 1/8 of tobramycin MIC, and 1/16 of tobramycin MIC. The size of the points is proportional to the number of clones/populations within the same treatment presenting the same value. Raw MIC values appear in supplementary tables 2, 3, and 5, Supplementary Material online. Points above the diagonal line indicate cases with an increase of tobramycin resistance level after resistance evolution. Points above the horizontal line indicate cases with a tobramycin MIC above the clinical breakpoint (2 µg/ml). 1/4 of tobramycin MIC was able to select mutants presenting a MIC above the clinical breakpoint (4 out of 12 populations and 18 out of 48 clones), whereas 1/8 of MIC did in just 1 out of 12 population and none of the 48 clones (see supplementary tables 2 and 5, Supplementary Material online). (*C* and *D*) The figures show MIC (µg/ml) to ceftazidime (log10-scale) of each starting genetic background at time zero ($t_0$) and the corresponding MIC (µg/ml) acquired in the populations (*C*) or their isolated clones (*D*) after 8 weeks of ALE. Each of the points represents a population (in *C*) or clone (in *D*); and the colors indicate different treatments: AB free, 1/4 of tobramycin MIC, 1/8 of tobramycin MIC, and 1/16 of tobramycin MIC. The size of the points is proportional to the number of clones/populations within the same treatment presenting the same value. Raw MIC values appear in supplementary tables 2, 3, and 5, Supplementary Material online. Points below the diagonal line indicate cases with a decrease of ceftazidime resistance level after compensatory or tobramycin resistance evolution. Points below the horizontal line indicate cases with a ceftazidime MIC below the clinical breakpoint (8 µg/ml). For mixed populations, MIC value of the susceptible subpopulation was represented (see supplementary table 3, Supplementary Material online). Most of the populations (35 out of 48) and clones (145 out of 192) showed a decline of resistance after 8 weeks of ALE on both, antibiotic-free or tobramycin sublethal environments. Importantly, only 25% of the clones presented a ceftazidime MIC above the clinical breakpoint. 75.5% of clones presented a reduction of ceftazidime MIC, being the degree of decline dependent on the genetic background: up to 128-fold in ParR-CAZ, 10.7-fold in NfxB-CAZ, and 6-fold in MDR6-CAZ.

chloramphenicol, aztreonam, and imipenem), MICs for these antibiotics were determined for the 48 populations and analyzed using ANOVA followed by post-hoc tests (Tukey for all vs. all and Dunnett with Hochberg correction for many to one). MIC changes were evaluated using EUCAST values, proportion tests and log-transformed confidence intervals, as described in Materials and Methods. An important, statistically significant, decrease of resistance to aztreonam ($P < 0.01$) and imipenem ($P < 0.001$) was observed, whereas the phenotypes of CS were maintained and no further change of susceptibility to other antibiotics was detected (supplementary table 4, Supplementary Material online). The case of imipenem is particularly remarkable, because decline of MIC was observed in 41 out of 48 populations (85%) from both, nonlethal and sublethal environments (supplementary table 4, Supplementary Material online) with 31 populations

having values below the EUCAST breakpoint ($P < 0.001$, test for equality of proportions). Namely, imipenem MIC was reduced in 10 out of 12 populations evolved in antibiotic-free environment, 10 out of 12 populations evolved in 1/4 of tobramycin MIC, 11 out of 12 populations evolved in 1/8 of tobramycin MIC, and 10 out of 12 populations evolved in 1/16 of tobramycin MIC (supplementary table 4, Supplementary Material online). Altogether, our data support that compensatory evolution allows not only the decrease of resistance to ceftazidime but also of cross-resistance to other $\beta$-lactams while preserving CS to tobramycin, a trade-off originally associated with ceftazidime resistance evolution.

## Decline of Ceftazidime Resistance in Environments without Antibiotics or Containing Sublethal Concentrations of Tobramycin

As mentioned above, a reduction of ceftazidime MIC was observed in 72% of the populations evolved in environments without antibiotics (nonselective environments) or containing sublethal tobramycin concentrations (selective environments) (supplementary table 3, Supplementary Material online; fig. 3C). However, occasionally, subpopulations still resistant to ceftazidime were observed within the susceptible ones (supplementary table 3, Supplementary Material online). Therefore, we selected for further analysis four random clones from each of the 48 populations, hereafter dubbed as ParR, NfxB, or MDR6, followed by the number of the population from which they proceed and a letter (i.e., ParR 1a, a clone isolated from population ParR-CAZ 1). Ceftazidime and tobramycin MICs were measured in the 192 isolated clones (supplementary table 5, Supplementary Material online and fig. 3B and D) and analyzed checking EUCAST values and using ANOVA followed by post-hoc tests. Interestingly, we observed a ceftazidime MIC decrease in 76% of the clones and only 25% of all the clones presented ceftazidime MICs above the EUCAST clinical breakpoint (8 μg/ml) (fig. 3D and supplementary table 5, Supplementary Material online), depending on genetic background and selective environment ($P < 0.001$, Multi-Way ANOVA). It is important to highlight that, although we observed in most of these clones a decrease of ceftazidime MIC, it was not possible to know the degree of decrease for others, because their ceftazidime MICs were above the detection limit of the E-test strips (supplementary table 5, Supplementary Material online).

We then analyzed the relevance of both, the selective environment and the genetic background, in the decrease of ceftazidime MIC observed in that 76% of the clones. Nonselective environments (antibiotic-free and 1/16 of tobramycin MIC) were the ones in which a higher number of clones showed a decline of ceftazidime MIC (44 out of 48 clones), followed by the 1/4 of tobramycin MIC (36 out of 48 clones) and 1/8 of tobramycin MIC (21 out of 48 clones) selective environments (supplementary table 5, Supplementary Material online and fig. 4A). MIC changes were evaluated using $\log_2$ fold change with ANOVA followed by the corresponding post-hoc tests as described in Materials and Methods. The reduction of ceftazidime MICs observed within each environment was dependent on the genetic background, statistically ascertained both when considering number of changes and fold change (minimal support $P < 0.005$). In particular, the NfxB-CAZ genetic background presented the highest number of MIC declines (59 out of 64 clones), followed by MDR6-CAZ (44 out of 64 clones) and ParR-CAZ (42 out of 64 clones) (fig. 4A). Moreover, the genetic background also determined the degree of resistance decline ($P < 0.001$), being up to 128-fold in ParR ($\mu = 39.7$), up to 11-fold in NfxB ($\mu = 2.65$) and up to 6-fold in MDR6 ($\mu = 1.7$) clones (fig. 4B). These data indicate that epistasis and the existence of different genetic backgrounds does not only constrain AR evolution (Hernando-Amado et al. 2019), but also compensatory evolution, something that had not been previously reported.

## Relative Fitness of ParR-CAZ-, NfxB-CAZ-, and MDR6-CAZ-Derived Clones

We observed that decline of ceftazidime resistance can occur not only in antibiotic-free environments, where compensatory mutations may be a reasonable cause, but also in environments containing tobramycin, where tobramycin resistance mutations, which might also produce additional fitness costs that may influence the selection of said compensatory mutations, are expected to be acquired. Therefore, we compared fitness of the 48 ParR-CAZ-, NfxB-CAZ-, and MDR6-CAZ-derived clones with respect to their parental strains (ParR-CAZ, NfxB-CAZ, or MDR6-CAZ), by estimating the area under the growth curve recorded in antibiotic-free medium (fig. 5 and supplementary table 6, Supplementary Material online). Fitness of the selected clones evolved in antibiotic-free, 1/4 of tobramycin MIC, 1/8 of tobramycin MIC, and 1/16 of tobramycin MIC was improved in 67%, 67%, 75%, and 33% of them, respectively. After ANOVA, relative changes in fitness were tested with single-sample $t$-tests for $\mu = 1$ (no change). An important fitness improvement was observed, as expected, in antibiotic-free medium ($P = 0.001$), where compensatory but no resistance mutations are expected. However, a high number of the clones evolved in 1/4 and 1/8 of tobramycin MIC environments, which may present mutations responsible for the increased tobramycin resistance, also showed an improvement of fitness ($P = 0.0036$ and $P = 0.0033$, respectively, fig. 5), suggesting that evolution toward improved fitness is uncoupled from tobramycin resistance evolution. ANOVA and Tukey also revealed major and significant differences in relative fitness across clones belonging to different genetic backgrounds ($P < 0.001$, fig. 5A). Namely, all the clones selected from MDR6-CAZ, the genetic background that originally showed the higher initial fitness cost (fig. 2), 50% of the clones belonging to ParR-CAZ and 31% of the clones belonging to NfxB-CAZ, showed an improved fitness after 8 weeks of ALE. This suggests that fitness improvement is influenced by the set of mutations initially present in parental strains. Although a further analysis would still be necessary, these results also suggest that the higher fitness cost associated with acquisition of resistance in a mutant, the greater the mutational space for compensatory evolution to occur, a

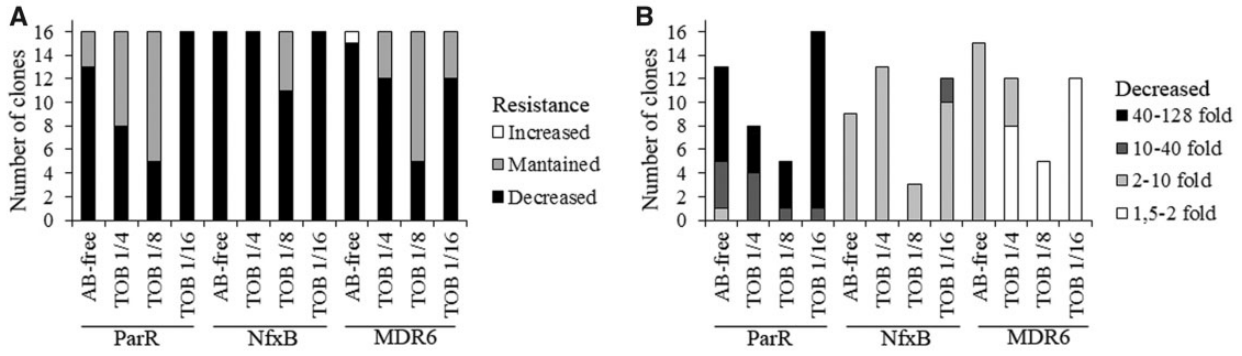


FIG. 4. Decline of ceftazidime resistance in antibiotic-free and tobramycin sublethal environments depending on the genetic background. (*A*) The figure shows the number of clones that present decreased, maintained, or increased resistance to ceftazidime after ALE on antibiotic-free or tobramycin sublethal environments. Nonselective environments (antibiotic free and tobramycin 1/16) showed the higher number of ceftazidime declines (44 out of 48 clones, $P < 0.05$), followed by 1/4 of tobramycin selective environment (36 out of 48 clones) and 1/8 of tobramycin selective environment (21 out of 48 clones) (see supplementary table 5, Supplementary Material online). In addition, the number of MIC declines within each treatment was dependent on the genetic background, being NfxB the genetic background presenting a higher percent of declines (59 out of 64 clones, $P < 0.001$), followed by MDR6 (44 out of 64 clones, $P < 0.001$) and ParR (42 out of 64 clones, $P < 0.001$). (*B*) The figure shows the degree of ceftazidime decline depending on the genetic background. The higher decline of ceftazidime MIC was observed in ParR (up to 128-fold), followed by NfxB (up to 11-fold) and MDR6 (up to 6-fold).

question previously raised (Dunai et al. 2019) but that had not been analyzed until now.

We analyzed the possibility that resistance loss may be associated with an increase in fitness. We found both, an increase of relative fitness and a ceftazidime resistance decline in 58%, 60%, 43%, and 33% of the clones evolved in antibiotic-free, 1/4 of tobramycin MIC, 1/8 of tobramycin MIC, and 1/16 of tobramycin MIC, respectively; relative fitness was also improved in all clones that presented a maintenance of resistance (fig. 5*B* and supplementary table 6, Supplementary Material online). This indicates that the decline in the original ceftazidime resistance level may be, at least partly, associated with compensatory evolution of fitness costs, although a correlation between the level of fitness compensation and the level of ceftazidime resistance decline was not firmly established. It is worth noting here that tobramycin selects tobramycin resistant mutants, which may reduce the overall *P. aeruginosa* fitness, hence counteracting the fitness improvements associated with the decline of ceftazidime resistance.

## Decline of Ceftazidime Resistance Is Not Associated with Molecular Reversion of Resistance

ParR-CAZ-, NfxB-CAZ-, and MDR6-CAZ-derived clones were subjected to whole-genome sequencing, to gain insights into the underlying molecular causes of decline of ceftazidime resistance. Their genomes were compared with the ones of their parental strains, to determine new genetic events acquired during evolution. Some of the clones became mutators and, to facilitate the comparative analysis between replicates (within the same genetic background or across different genetic backgrounds), only those genes that also presented mutations in nonmutator clones were considered for this analysis. In the case of the clones evolved in antibiotic-free medium, a total of 59 genetic events were identified (supplementary table 7, Supplementary Material online), 15 in ParR-CAZ, 18 in NfxB-CAZ, and 26 in MDR6-CAZ genetic backgrounds. In the case of the clones evolved in 1/4 of tobramycin MIC, a total of 62 genetic events were identified (supplementary table 8, Supplementary Material online), 24 in ParR-CAZ, 18 in NfxB-CAZ, and 20 in MDR6-CAZ genetic backgrounds. In the case of the clones evolved in 1/8 of tobramycin MIC, a total of 46 genetic events were identified (supplementary table 9, Supplementary Material online), 22 in ParR-CAZ, 17 in NfxB-CAZ, and 7 in MDR6-CAZ genetic backgrounds. Finally, in the case of the clones evolved in 1/16 of tobramycin MIC, a total of 53 genetic events were identified (supplementary table 10, Supplementary Material online), 18 in ParR-CAZ, 24 in NfxB-CAZ, and 11 in MDR6-CAZ genetic backgrounds. The genome of the 48 clones was initially screened to determine whether molecular reversion of resistance (Durao et al. 2018) could account for the observed decline of ceftazidime resistance, although it is expected that phenotypic reversion, by the acquisition of new genetic variations, could be more likely than genetic reversion by the restoration of the mutated allele to a wild-type one (Levin et al. 2000; Dunai et al. 2019). The three mutants here analyzed present large deletions, a genetic event that cannot be restored, as well as mutations in different genes. None of the original mutations present in each of the parental genetic backgrounds reverted back to the wild-type sequence, so that compensatory evolution, and not genetic reversion, may be the cause of the observed decline of ceftazidime resistance.

## Genetic Variations Associated with the Acquisition of Tobramycin Resistance in Sublethal Tobramycin Environments

The genomes of 36 clones from the populations independently evolved during 8 weeks in different tobramycin sublethal concentrations, and presenting increased tobramycin resistance, were analyzed to gain insights into the underlying molecular causes of the acquisition of said resistance. It is relevant to remind that the original ceftazidime resistant mutants were tobramycin hyper-susceptible because they

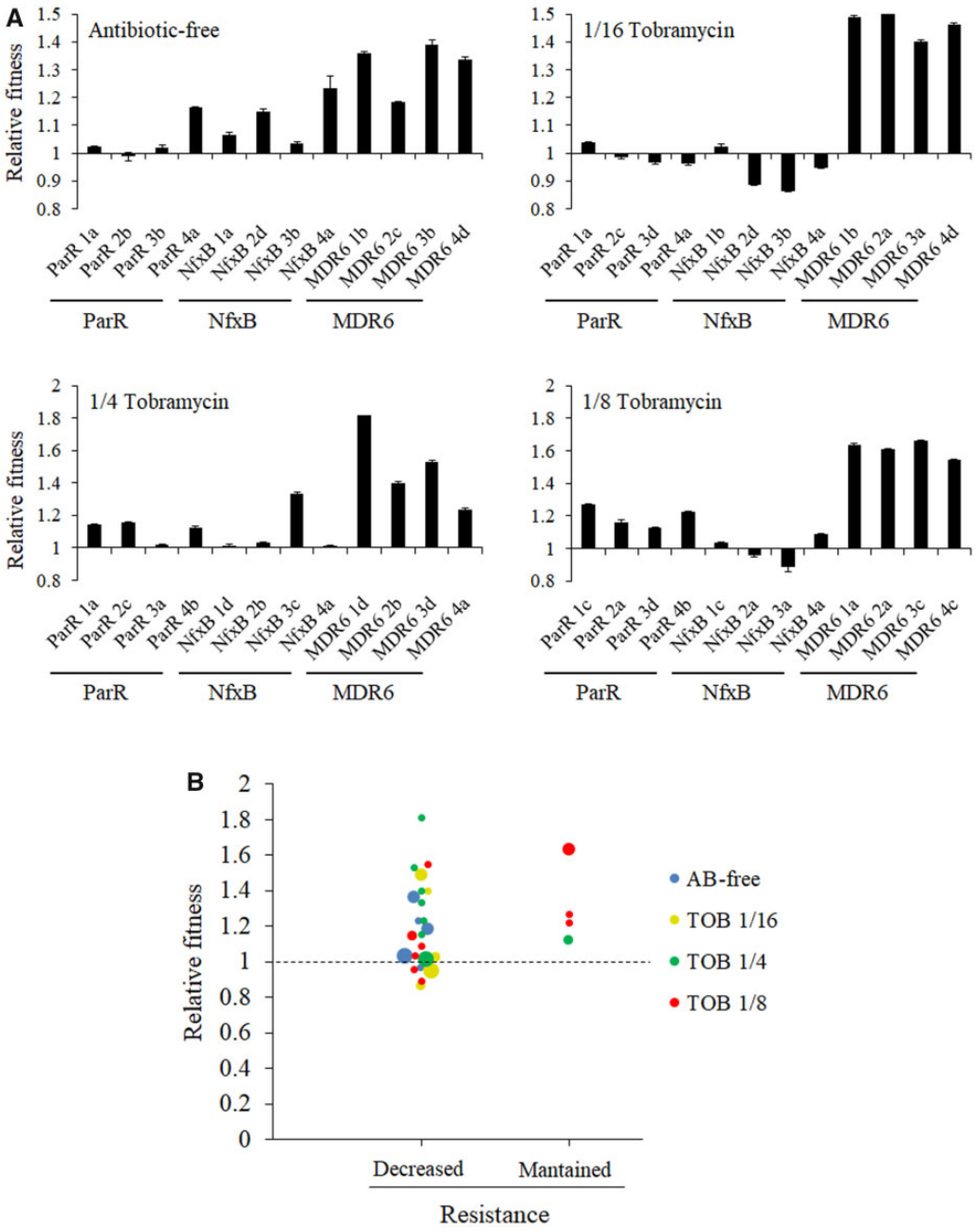


FIG. 5. Impact of fitness recovery on AR decline. (*A*) Relative fitness of clones evolved 8 weeks in antibiotic-free and tobramycin sublethal environments. Growth curves of the resistant mutants, before and after antibiotic-free or tobramycin evolution, were recorded in antibiotic-free medium (LB). The fitness of each clone was measured as the area under the growth curve. Relative fitness of the clones was calculated respect to their parental strains. Relative fitness was increased (relative fitness value $\geq$1.1) in 67%, 33%, 67%, and 75% of clones isolated from populations evolved in antibiotic-free medium, 1/16 of tobramycin MIC, 1/4 of tobramycin MIC, and 1/8 of tobramycin MIC, respectively (see supplementary table 6, Supplementary Material online). The analysis also showed differences in fitness improvement within each genetic background. All the clones belonging to MDR6-CAZ presented an increased fitness followed by ParR-CAZ (50%) and NfxB-CAZ (31%). The mean of values from three replicates are represented. (*B*) Relationship between fitness and ceftazidime resistance decrease of clones evolved during 8 weeks in antibiotic-free and tobramycin sublethal environments. As shown, ceftazidime resistance decreases as a function of relative fitness in antibiotic-free and tobramycin sublethal environments. Each of the points represents a clone and the colors indicate different treatments. Relative fitness was increased (fitness value $\geq$1.1) in 58%, 33%, 60%, and 43% of clones that showed a resistance decrease after evolution in antibiotic-free, 1/16 of tobramycin MIC, 1/4 of tobramycin MIC, and 1/8 of tobramycin MIC, respectively (see supplementary table 6, Supplementary Material online). Importantly, relative fitness was also improved in all the clones presenting resistance maintenance. The size of the points is proportional to the number of clones within the same treatment presenting the same value.

lack the aminoglycosides efflux pump *mexXY* (Hernando-Amado et al. 2020); their evolutionary trajectories toward tobramycin resistance could be different than those of the wild-type strain (Sanz-Garcia et al. 2018b). As mentioned above, a total of 62 genetic events were identified in the clones isolated from populations evolved in 1/4 of

tobramycin MIC (supplementary table 8, Supplementary Material online), 46 genetic events were identified in the clones isolated from populations evolved in 1/8 of tobramycin MIC (supplementary table 9, Supplementary Material online), and a total of 53 genetic events were identified in the clones isolated from populations evolved in 1/16 of tobramycin MIC (supplementary table 10, Supplementary Material online), although some of these variations were most likely compensatory genetic events acquired during evolution (supplementary table 11, Supplementary Material online). Variations in a limited number of genes, 6 in total, were commonly acquired in different sublethal tobramycin environments and genetic backgrounds (supplementary table 12, Supplementary Material online). Among them, genetic variations in *fusA*, encoding an elongation factor whose mutations are clearly associated with acquisition of aminoglycoside resistance in clinical and nonclinical strains (Feng et al. 2016; Bolard et al. 2018; Ibacache-Quiroga et al. 2018; Lopez-Causape et al. 2018; Sanz-Garcia et al. 2018b), were commonly selected in the three genetic backgrounds analyzed. In particular, variations in *fusA* were detected in all ParR-CAZ- and NfxB-CAZ-derived clones and some MDR6-CAZ-derived clones (MDR6 1d and 4a clones), isolated from populations evolved in 1/4 of tobramycin MIC (supplementary table 8, Supplementary Material online), and in all NfxB-CAZ-derived clones isolated from populations evolved in 1/8 of tobramycin MIC (supplementary table 9, Supplementary Material online). In addition, mutations in *ptsP*, encoding a phosphoenolpyruvate phosphotransferase, whose mutation is associated with acquisition of tobramycin resistance (Schurek et al. 2008; Sanz-Garcia et al. 2018b), were acquired in ParR-CAZ and MDR6-CAZ genetic backgrounds, whereas they were not selected in the NfxB-CAZ genetic background. In particular, mutations in this gene were acquired in clones ParR 1a, 3a, and 4b and in clone MDR6 3d, isolated from populations evolved in 1/4 of tobramycin MIC (supplementary table 8, Supplementary Material online) and in all ParR-CAZ-derived clones, isolated from populations evolved in 1/8 of tobramycin MIC (supplementary table 9, Supplementary Material online). The fact that all the ParR-CAZ-derived clones, and no NfxB-CAZ-derived clones, isolated from populations evolved in 1/8 of the MIC of tobramycin present mutations in *ptsP*, and that all NfxB-CAZ-derived clones, and no ParR-CAZ-derived clones, isolated from populations evolved in 1/8 of the MIC of tobramycin present mutations in *fusA*, illustrates to which extent the genetic background can constrain the evolution of AR (Hernando-Amado et al. 2019; Hernando-Amado et al. 2020; Card et al. 2021).

## Intragenic Compensatory Mutations in the AmpR Regulator and the RpoB Subunit of the RNA Polymerase

The genome of the 12 clones evolved during 8 weeks in antibiotic-free medium was analyzed to identify all possible compensatory genetic events acquired during evolution (supplementary table 7, Supplementary Material online). Afterwards, genetic variations in these genes were searched within those identified in the clones evolved during 8 weeks in sublethal tobramycin environments (supplementary tables 8–10, Supplementary Material online) to distinguish between compensatory mutations and genetic events leading to tobramycin resistance. Variations in a limited number of genes, 9 in total, were commonly acquired in both, antibiotic-free and sublethal tobramycin environments (supplementary table 11, Supplementary Material online). Among them, some mutations were located in genes that were originally mutated in the parental genetic backgrounds ParR-CAZ and MDR6-CAZ (supplementary table 13, Supplementary Material online), supporting that intragenic compensation might be the basis for alleviating the fitness costs associated with acquisition of ceftazidime resistance in these mutants. Indeed, in ParR-CAZ-derived clones we observed intragenic mutations in *ampR*, which encodes an LTTR that regulates the expression of the $\beta$-lactamase AmpC (Kong et al. 2005; Alvarez-Ortega et al. 2010; Balcewich et al. 2010; Gifford et al. 2018; Sanz-Garcia et al. 2018a), and in MDR6-CAZ-derived clones, in *rpoB*, which encodes the subunit $\beta$ of the RNAP (Villain-Guillot et al. 2007), whose mutation frequently confers rifampicin resistance (Severinov et al. 1993; Qi et al. 2014) (supplementary table 13, Supplementary Material online).

The ParR-CAZ genetic background, originally presented an amino acid variation in AmpR (Asp135Asn) (supplementary table 1, Supplementary Material online), and displayed intragenic mutations in *ampR* after 8 weeks of evolution in both, antibiotic-free medium and tobramycin sublethal concentrations. Clone ParR 1a, isolated from a population evolved in antibiotic-free medium, presented a Pro164Leu amino acid variation, clone ParR 2c, isolated from a population evolved in 1/4 of tobramycin MIC, presented an His39Tyr amino acid variation, and clones ParR 1a, 3d, and 4a, isolated from populations evolved in 1/16 of tobramycin MIC sublethal concentration, presented two distinct Glu274fs and Leu110Pro amino acid modifications, respectively (supplementary table 13, Supplementary Material online). In addition, clone ParR 3 b isolated from a population evolved in antibiotic-free medium presented a Phe19fs amino acid variation in AmpC. AmpR is a transcriptional regulator that controls the expression of *ampC* (Balcewich et al. 2010) and, therefore, the level of resistance to $\beta$-lactam antibiotics. The ParR-CAZ parental genetic background, that presents an amino acid variation in AmpR (Asp135Asn) (supplementary table 1, Supplementary Material online), is highly resistant to ceftazidime (MIC $>$ 256 $\mu$g/ml; supplementary table 3, Supplementary Material online) showing an increased $\beta$-lactamase activity (up to 55-fold) with respect to its ParR parental strain (fig. 6A). In addition, the ParR-CAZ mutant presents a high fitness cost that could be partly associated with the mutation present in *ampR* (Perez-Gallego et al. 2016). We hypothesize that the intragenic mutations in *ampR* acquired after 8 weeks of ALE in antibiotic-free and sublethal tobramycin concentrations could modify AmpR activity, reducing the amount of AmpC and, therefore, $\beta$-lactamase activity. Actually, a strong decline of ceftazidime resistance (up to 128-fold) was observed in ParR-CAZ-derived clones. In particular, clone ParR 1a, isolated from populations evolved in antibiotic-free

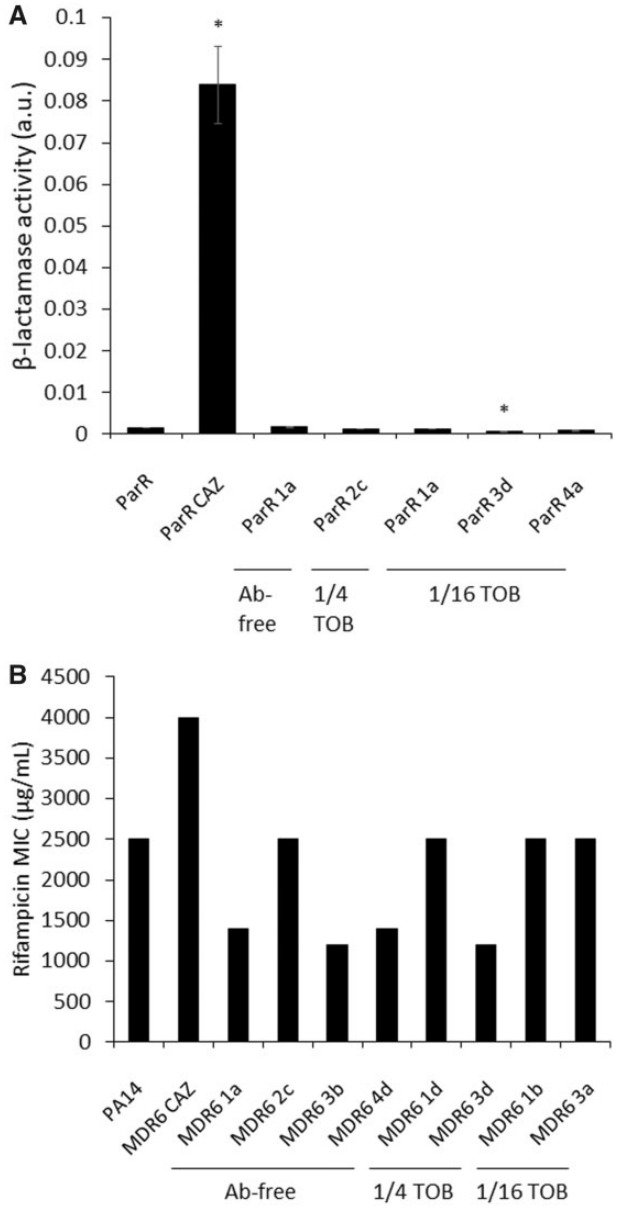


FIG. 6. Reversion of phenotypes due to intragenic secondary mutations in *ampR* and *rpoB*. (*A*) Figure shows $\beta$-lactamase activity of protein extracts from ParR, ParR-CAZ, and Par-CAZ-derived clones: ParR 1a, isolated from a population evolved in antibiotic-free medium, ParR 2c isolated from a population evolved in 1/4 of tobramycin, or ParR 1a, 3d, and 4a isolated from populations evolved in 1/16 of tobramycin MIC sublethal concentration. $\beta$-Lactamase activity was increased up to 55-fold in ParR-CAZ mutant respect to its parental strain, whereas a reversion of this phenotype was achieved after 56 days of ALE in antibiotic-free and tobramycin sublethal environments and the acquisition of secondary mutations in *ampR*. Even a significantly reduced $\beta$-lactamase activity respect to the original ParR parental strain was measured for one of them. Error bars indicate standard deviations of the results from three independent biological replicates. Statistically significant differences regarding ParR were calculated with *t*-test for paired samples assuming equal variances: $^*P < 0.05$. (*B*) Rifampicin MIC ($\mu$g/ml) of PA14 wild-type strain, MDR6-CAZ parental strain, MDR6-CAZ-derived clones: MDR6 1b, 2c, 3b, and 4d isolated from populations evolved in antibiotic-free medium, clones MDR6 1d and 3d isolated from populations evolved in 1/4 of tobramycin MIC sublethal concentration, and clones MDR6 1b and 3a isolated from populations evolved in 1/16 of tobramycin MIC sublethal concentration are represented. As shown, MDR6-CAZ parental strain has an increased rifampicin resistance respect to PA14 wild-type strain, whereas a reversion of this phenotype was achieved after 56 days of ALE in antibiotic-free and tobramycin sublethal environments and the acquisition of secondary mutations in *rpoB*.

medium, ParR 2c isolated from a population evolved in 1/4 of tobramycin sublethal concentration, or ParR 1a, 3d, and 4a, isolated from populations evolved in 1/16 of tobramycin MIC sublethal concentration, presented a reduced ceftazidime MIC of 128-fold, 32-fold, or 64-, 85-, and 43-fold, respectively, compared with the ParR-CAZ parental strain (supplementary table 5, Supplementary Material online). Confirming this hypothesis, a reduced $\beta$-lactamase activity (up to 140-fold) was observed in these compensated, evolved clones (fig. 6A), indicating that the intragenic mutations acquired in *ampR* (supplementary table 13, Supplementary Material online) are the main cause of ceftazidime resistance decline in ParR-CAZ genetic background.

In order to interpret the effect of *ampR* mutations, the AmpR structures of the wild-type, ceftazidime resistant and ceftazidime compensated mutants were modeled. Structure modeling of the different AmpR mutants in a DNA-bound tetramer complex formed by two dimers composed of a compact and extended form of AmpR each and bound to DNA, sheds new light on the observed resistance phenotype (fig. 7A). The Asp135Asn mutation, present in the mutant ParR-CAZ is located in a region involved in DNA binding, where this new amino acid likely stabilizes the AmpR binding to the intergenic region that contains the *ampC* and *ampR* promoters, leading to constitutive expression of *ampC*. For its part, the various reversal phenotypes can be explained by changes that hamper AmpR function: Phe19fs results in a truncated, defective protein, His39Tyr lies in a DNA-binding helix likely reducing affinity for the promoter and hence expression of *ampC*; Leu110Pro would affect one of the helices in the intermonomer interface destabilizing packing and formation of the active complex; Pro164Leu is located at the hinge between the two domains that line the effector-binding pocket, likely rendering AmpR unresponsive to its activators, and Glu274 frame shifts would alter the C-terminal domain, impairing the interaction with RNAP and therefore transcription of genes regulated by AmpR (Balcewich et al. 2010).

The MDR6-CAZ genetic background, originally presenting an amino acid variation in RpoB (Asp203Glu) (supplementary table 1, Supplementary Material online), suffered intragenic mutations in *rpoB* after 8 weeks of evolution in both, antibiotic-free and sublethal tobramycin environments. Clones MDR6 1b, 2c, 3b, and 4d, isolated from populations evolved in antibiotic-free medium, presented an Arg184His, Arg184Cys, Ser186Phe, and Val474Glu amino acid variation, respectively (supplementary table 13, Supplementary Material online). Clones MDR6 1d and 3d, isolated from populations evolved in 1/4 of tobramycin MIC sublethal concentration, showed a Ser186Phe amino acid modification, and clones MDR6 1b and 3a, isolated from populations evolved in 1/16 of tobramycin MIC sublethal concentration, presented a

Val471Glu and Arg184Cys amino acid modification, respectively (supplementary table 13, Supplementary Material online). It is well-known that *rpoB* mutants present pleiotropic effects, including rifampicin resistance (an antibiotic that targets RpoB), reduced transcriptional efficiency, altered expression of essential genes, or DNA breaks (Severinov et al. 1993; Qi et al. 2014). Strikingly, the ceftazidime resistant MDR6-CAZ parental strain (supplementary table 5, Supplementary Material online), presenting an Asp203Glu variation in RpoB, displays a high fitness cost in antibiotic-free medium (fig. 2) and, in addition to resistance to ceftazidime, it also presents resistance to rifampicin (MIC $>$ 4 mg/ml; fig. 6*B*), supporting that RpoB function is affected in this mutant. We hypothesize that the intragenic mutations acquired in *rpoB* after 8 weeks of ALE in antibiotic-free and sublethal tobramycin concentrations could be mutations that compensate fitness through restoring RpoB functionality. Indeed, secondary mutations in *rpoB* have been described to restore the defective RNAP activity caused by mutations in this gene in *P. aeruginosa* (Hall et al. 2010; Hall and MacLean 2011). All the mentioned MDR6-CAZ-derived clones presented an increased fitness, measured as growth in antibiotic-free medium (fig. 5*A*). In addition, rifampicin and ceftazidime MIC of the mentioned clones were reduced up to 3.3- and 6-fold, respectively (fig. 6*B* and supplementary table 5, Supplementary Material online). The reduction of rifampicin MIC suggests that the secondary *rpoB* mutation allows RpoB to recover functionality close to that of the wild-type.

The effect of the *rpoB* intragenic mutations in the activity of the RNAP can be explained by carefully analyzing the predicted models of RpoB substituted into a hybrid *Escherichia coli* RNAP complex with DNA bubbles and nascent RNAs (fig. 7*B*). Asp203 is followed by two arginines and is spatially close to Ser186. These amino acids interact with the beginning of the transcription bubble (Zhang et al. 2012) in all reported complexes, likely helping to open the DNA bubble in a zipper-like fashion, and contributing to reorient nucleotide bases in the nontranscribed (NT) DNA strand away from the transcribed (T) strand. Asp203Glu introduces a large side chain and a negative charge that force a reorientation of the arginines and a slight displacement of the NT strand. We propose that, at such an early point in the bubble, the small displacement of the NT strand also has a small effect in the nearby T strand; this would not be enough to affect interactions with the active site and transcription, but as the distortion is transmitted through the T strand, it would allow the nascent RNA to grow beyond the third nucleotide stage. In addition, Asp203Glu would affect the "scrunching" of the DNA bubble, pushing it in the cavity toward a channel open between Arg184 and Val471-Val474, whose interaction with the sixth, seventh, and eighth nucleotides of the bubble's NT strand might modify its tension, possibly affecting the progression of transcription. The question remains how these structural changes are associated with resistance to ceftazidime of this mutant. The fact that *rpoB* mutations, besides rifampicin resistance, can select pleiotropic mutants presenting resistance to other drugs, as quinolones and ampicillin (Pietsch et al. 2017), suggests that transcriptional changes associated with RpoB malfunctioning might be the underlying molecular cause of ceftazidime resistance in these mutants. Concerning the effect of compensatory mutations on RpoB functionality, Ser186Phe introduces a large side chain close to Asp203Glu that would rearrange the stacking of the first bases in the NT strand correcting the changes of the latter amino acid change. The effect of Arg184His and Arg184Cys could be explained by the reduction in side chain size, which would leave more space and relieve the tension on the NT strand. Finally, the effect of Val471Glu and Val474Glu might be explained by the introduction of a repulsive charge and larger side chain pushing the NT strand toward Arg184 at the opposite wall, hampering progression of the NT strand through the channel between them and Arg184 and constraining release of the tension in the DNA bubble. Thus, the seemingly most plausible explanation is that the intragenic mutations in *rpoB* would compensate the distortion introduced by the amino acid change Asp203Glu, restoring the orientation of the T strand. We hypothesized that these structural changes may be responsible for restoring RpoB functionality, hence allowing the recovery of fitness and the decline of ceftazidime resistance in the analyzed clones presenting these compensatory mutations.

The above-mentioned intragenic modifications acquired in *ampR* and *rpoB*, both in antibiotic-free and in sublethal tobramycin environments but specifically in ParR-CAZ and MDR6-CAZ genetic backgrounds, respectively, which originally presented mutations in these genes, shows the importance of genetic background in constraining compensatory evolution.

## Discussion

Recent years have seen a growing interest in the development of evolutionary approaches to tackle AR from a conservative point of view, improving the use of available antibiotics (Laxminarayan 2014). In this sense, numerous studies have proposed to exploit some evolutionary trade-offs associated with the acquisition of AR, as CS (Imamovic and Sommer 2013; Kim et al. 2014; Pal et al. 2015; Baym et al. 2016; Barbosa et al. 2018; Imamovic et al. 2018; Barbosa et al. 2019; Dunai et al. 2019; Hernando-Amado et al. 2020) or the possible decline of AR in antibiotic-free environments (Dunai et al. 2019).

The evolutionary forces that modulate the emergence of a specific AR mutation have been studied in detail; mutation rate and mutational input, clonal interference, cross-selection, level of resistance, fitness under selection, epistatic interactions, and contingency are factors that constrain the evolutionary trajectories leading to AR (Weinreich 2005; de Visser and Krug 2014; Szamecz et al. 2014; Maddamsetti et al. 2015; Gifford et al. 2016; Hughes and Andersson 2017; Imamovic et al. 2018; Nichol et al. 2019; Rosenkilde et al. 2019). Fixation of such mutations requires that they persist, even when selective pressure is suspended. For this stability to happen, fitness costs associated with the acquisition of AR should be low or easily compensated by the acquisition of secondary mutations that do not reduce AR to the selective antibiotic

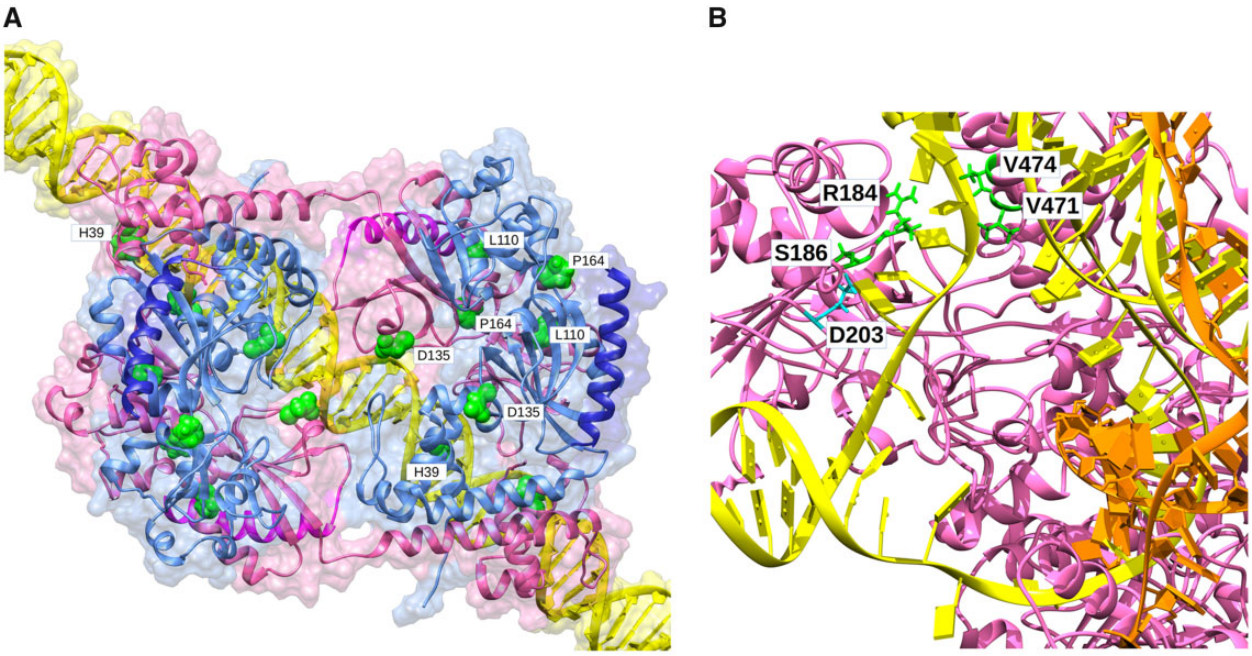


FIG. 7. Modeling of the effect of AR and compensatory mutations in the structures of AmpR and RpoB. (*A*) Proposed model of the AmpR tetramer bound to the putative promoter of *ampC* of *P. aeruginosa* PA14. Mutated amino acids are shown as balls for better identification. Only amino acids in one pair of monomers have been labeled. The C-terminal regions affected by 274 frame shifts are highlighted. D135N and H39Y would affect promoter recognition, F19fs truncates the protein, L110P would destabilize the complex, P164L would destabilize the effector binding pocket, and G274fs would affect interaction with RNAP. (*B*) Proposed model of RpoB bound to a DNA bubble and a nascent RNA molecule. Mutated amino acid D203, whose mutation causes the resistant phenotype (possibly through a change in promoter specificity), and amino acids with reversion mutations are shown as sticks. D203E would affect the interaction with the open DNA bubble, pushing the nontranscribed (NT) strand toward the channel between R184 and V471-V474. Reversion mutants S186F, R184C, V471E, and V474E would correct stacking, tensions, and "scrunching" distortions in the NT strand.

(Bjorkman, Nagaev et al. 2000; Nagaev et al. 2001; Marcusson et al. 2009). Although some detailed studies on the effect of fitness costs in the stability of AR have been published, most works still focus on the emergence of AR. Information on the degree to which compensatory evolution is contingent on genetic background, on initial fitness costs and/or on the environmental conditions and how these factors impact the stability or reversion of AR, as well as their associated trade-offs (i.e., CS), is almost absent in the field.

It is debatable whether reversion of resistance upon antibiotic therapy discontinuation is a common situation (Schrag et al. 1997; Enne et al. 2001; Andersson and Hughes 2010; Brolund et al. 2010), because it has been shown that eliminating the use of an antibiotic in a geographical area does not revert resistance (Sundqvist et al. 2010). Actually, decline of resistance is not a black-and-white issue, but rather depends on different factors. An important one is the type of antibiotic used to treat an infection (Seppala et al. 1997; Enne et al. 2001; Gottesman et al. 2009; Andersson and Hughes 2010; Dunai et al. 2019), and hence, the selected resistance mechanisms, because the fitness cost associated with acquiring resistance depends on the cellular functions affected (Baquero et al. 2009; Melnyk et al. 2015). Consequently, the identification of antibiotics for which resistance could be unstable in absence of selection is of utmost relevance for the design of evolution-based approaches to tackle AR. Here, we have identified compensatory mutations responsible for the decline of ceftazidime resistance that, importantly, do not affect pre-existing CS to tobramycin.

CS has been explored as a trade-off potentially exploitable to tackle AR, besides fitness costs associated with the AR acquisition. For that, CS must be robust and emerge in different genetic backgrounds, being particularly relevant the case of pre-existing antibiotic resistant mutants (Hernando-Amado et al. 2020), because infections caused by *P. aeruginosa* in CF patients can contain different strains with distinct antibiotic susceptibility profiles (Lopez-Causape et al. 2017). In addition, CS must be stable. The stability of CS, which had not been explored, is particularly relevant for the implementation of antibiotic use policies, such as evolutionary-based therapies alternating two different antibiotics, using the antibiotic for which there is CS. Here, we have observed that compensatory evolution of fitness costs associated with ceftazidime resistance in antibiotic-free environments does not affect pre-existing CS to tobramycin.

We observed a robust decline of ceftazidime resistance, within 450 generations, in 92% of the clones isolated from populations evolved during 8 weeks in antibiotic-free medium as well as in populations evolved in sublethal concentrations of tobramycin. These results agree with a clinical study at a Shanghai hospital, in which the restriction of ceftazidime consumption resulted in a significant decrease of ceftazidime resistance levels in *P. aeruginosa* (Guo et al. 2015). In addition, we found that the degree and frequency

of ceftazidime resistance decline is dependent on the genetic background, showing ParR-CAZ-derived clones the highest decrease and NfxB-CAZ-derived clones the most frequent decline. As far as we know, this is the first work describing that decline of AR is contingent on genetic background and that it can occur in environments containing sublethal antibiotic concentrations. Further, it is important noticing that, in addition, we observed that compensatory evolution in antibiotic-free medium allows the recovery of *P. aeruginosa* susceptibility to drugs to which these mutants presented cross-resistance (other $\beta$-lactams).

Not only the type but the strength of selection (i.e., lethal or sublethal antibiotic concentrations) can restrict the type of AR mutations acquired (Sanz-Garcia et al. 2020) and their associated fitness costs. In this work, we answer the question of the extent to which sublethal concentrations of tobramycin that can be found in certain clinical situations (Baquero and Negri 1997) may determine, beyond the evolution of tobramycin resistance either the evolution of CS to ceftazidime or compensatory evolution (and a possible decline of ceftazidime resistance), allowing a switch back to this drug. Sublethal tobramycin concentrations (1/4 and 1/8 of tobramycin MIC) selected tobramycin resistant mutants in the three analyzed genetic backgrounds, which is in agreement with the described sublethal selective window for tobramycin in *P. aeruginosa* PA14 (Sanz-Garcia et al. 2020). However, tobramycin resistance was not only selected in the presence of 1/4 and 1/8 of tobramycin MIC but also in the presence of 1/16 of tobramycin MIC in the MDR6-CAZ and some subpopulations of the ParR-CAZ genetic backgrounds. This indicates that the probability to select resistance at a particular sublethal tobramycin concentration depends on the starting genetic background, a feature that had not been previously explored. In addition, we observed that ceftazidime resistance declines in these sublethal tobramycin concentrations due to compensatory evolution.

From a genetic point of view, reversion or decline of AR can result from a molecular (Durao et al. 2018) or phenotypic reversion of resistance (Levin et al. 2000; Dunai et al. 2019), by the restoration of the mutated allele to a wild-type one or the acquisition of new genetic variations, respectively. In this work, after whole-genome sequencing of 48 clones presenting a decline of ceftazidime resistance, the first possibility was discarded, because none of the original mutations present in each of the parental genetic backgrounds reverted back to the wild-type sequence. In fact, intragenic compensatory mutations in *ampR* and *rpoB*, specifically acquired in ParR-CAZ- and MDR6-CAZ-derived clones, respectively, seem to be responsible for the decline of ceftazidime resistance observed in these mutants.

Besides their relevance in the AR field, our results provide general information for the theory of evolution. The evolution of *P. aeruginosa* in the presence of ceftazidime leads to three phenotypes of interest from the clinical perspective: ceftazidime resistance is adaptive to the selective pressure whereas their associated trade-offs, fitness costs and CS to tobramycin, are not. The evolutionary process is usually considered to increase complexity by the cumulatively acquisition of adaptive genetic changes, giving evolution a direction, an arrow of time (Ekstig 2015). Our results support that this arrow only operates if the adaptive phenotype (i.e., ceftazidime resistance) is preserved -fixed- when selection ends; otherwise, processes of short-sighted evolution could be expected (Levin and Bull 1994; Martinez 2013). Although here we found that resistance to ceftazidime declines in absence of selection, and hence it is not fixed, we also observed that its evolutionary trade-off, CS to tobramycin, remains. This implies that stable, not necessarily adaptive phenotypes might have a relevance in evolution that is currently underestimated.

Antibiotic cycling has been usually unsuccessful in reducing AR burden (Schuetz and Beardmore 2018; van Duijn et al. 2018). One of the underlying reasons could be that, when implemented, these programs have been mainly based on blind assays, under the assumption that any AR mechanism would present a fitness cost and supposing that AR would be lost under exposure to the subsequent antibiotic (Nichol et al. 2018). However, for decline of AR to occur, the adaptation to a specific drug must cause the emergence of conserved trade-offs, such as high fitness costs in antibiotic-free environments or CS to a second drug. Further, compensatory evolution of fitness costs does not unequivocally imply a decline of AR, because it may depend on the antibiotic and the resistance mutations involved (Seppala et al. 1997; Enne et al. 2001; Gottesman et al. 2009; Andersson and Hughes 2010; Dunai et al. 2019) and on other factors, as the ones here described: genetic background, initial fitness costs and strength of selection.

Here we propose that the alternation of ceftazidime with tobramycin for the treatment of *P. aeruginosa* infections, even those due to pre-existing antibiotic resistant mutants, could be followed by a switch back to ceftazidime, to drive tobramycin resistant mutants that could result from sublethal tobramycin concentrations present in certain tissues or situations to extinction. In addition, we suggest that it might be possible to alternate the use of ceftazidime with periods of drug restriction, allowing a rapid decrease of ceftazidime resistance, preserving tobramycin CS (see conceptual fig. 8). However, it is important to emphasize that our results should not be generalized to other antibiotics or specific situations. Specific ALE assays, as those done here, are necessary to find drugs for which periods of drug restriction or drug-cycling policies could be effective.

## Materials and Methods

### Growth Conditions and Antibiotic Susceptibility Assays

Unless stated otherwise, bacteria were grown in Luria Bertani (LB) Broth at 37 °C and shaking of 250 rpm, in glass tubes. Antibiotic susceptibility was determined for ceftazidime, tobramycin, aztreonam, imipenem, tetracycline, fosfomycin, ciprofloxacin, and chloramphenicol at 37 °C, in Mueller Hinton (MH) agar, using Test strips (MIC Test Strip, Liofilchem). Rifampicin susceptibility (above the limit of detection of E-test strips) was determined by broth microdilution method in 96-well plates with round bottoms (Thermo

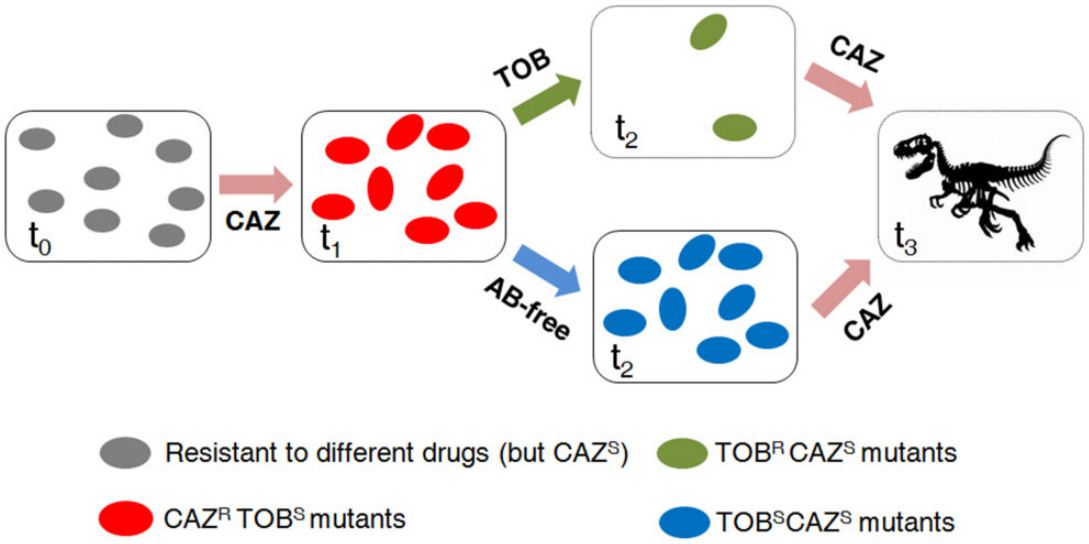


**Fig. 8.** Conceptual figure illustrating two different evolutionary approaches to drive different antibiotic resistant mutants of *P. aeruginosa* to extinction. Different genetic backgrounds of *P. aeruginosa* ($t_0$) show a robust evolution toward CS to tobramycin after short-term evolution in the presence of ceftazidime ($t_1$) (Hernando-Amado et al. 2020). Furthermore, these $CAZ^RTOB^S$ mutants also have a high fitness cost in antibiotic-free environments. Therefore, it is possible to alternate the use of ceftazidime with periods of drug restriction, allowing the compensation of fitness costs and the decline of ceftazidime resistance ($t_2$), or with tobramycin (Hernando-Amado et al. 2020), driving the populations to extinction ($t_2$). In the second case, the hypothetical presence of sublethal tobramycin concentrations in certain situations, although selecting an increase of tobramycin resistance, would also lead to a decline of ceftazidime resistance in these populations. Therefore, a switch back to ceftazidime in either of the two strategies could finally result in the extinction of the resistant populations ($t_3$).

Scientific Nunc) filled with MH supplemented with growing concentrations of antibiotic and bacteria at initial $OD_{600\ nm}$ of 0.01, after incubation at 37 °C for 24 h.

## Adaptive Laboratory Evolution Experiments

Three ceftazidime resistant mutants of *P. aeruginosa* PA14—four replicates each—were subjected to 8 weeks of ALE in presence or absence of sublethal concentrations of tobramycin (1/4, 1/8, and 1/16 of MIC of each linage), resulting in a total of 48 independent bacterial populations (12 grown in presence of 1/4 of tobramycin MIC, 12 in 1/8 of tobramycin MIC, 12 in 1/16 of tobramycin MIC, and 12 grown without antibiotic). Cultures were grown at 37 °C and 250 rpm for 56 days. Every day, the cultures were diluted (1/100), adding 10 µl of bacteria in 1 ml of fresh LB, either containing or lacking tobramycin. Each tobramycin concentration was maintained during ALE. Every replicate population was preserved, at the end of the experimental evolution, at −80 °C. In addition, the MIC of the antibiotic used for selection in populations (tobramycin) and of the one to which the mutants were initially resistant (ceftazidime), was determined at 37 °C in MH agar, using E-test strips.

## Growth Measurements

Growth curves were obtained inoculating overnight cultures to a final $OD_{600\ nm}$ of 0.01 in LB, by triplicate, in a 96-well microtiter plate (Nunclon Delta Surface) and by measuring the $OD_{600\ nm}$ of the bacterial cultures every 10 min during 20 h at 37 °C using a Spark 10M Plate Reader (Tecan). Fitness (W) was measured as the area under the growth curve recorded in antibiotic-free medium. Fitness cost of each ceftazidime resistant mutants respect to its parental strain was calculated as stated (Dunai et al. 2019), using the equation: $1 - (W_{mutant}/W_{parental\ strain})$ and was expressed as percentage.

## Whole-Genome Sequencing and Analysis

The genomic DNA of each isolated clone was extracted using the Gnome DNA kit (MP Biomedicals). Whole-genome sequencing (WGS) and DNA quality was performed by Macrogen. Pair-end libraries (2 × 150) were constructed with Truseq DNA PCR free and sequenced using an Illumina NovaSeq6000 system. Coverage was greater than 300× for all samples. Genome sequence, gene coordinates, and annotations were obtained from GenBank nucleotide database. The quality of Illumina short reads was verified using FASTQC (Wingett and Andrews 2018). Reads were aligned against *P. aeruginosa* genome UCBPP-PA14 (NC_008463.1) using RNA-STAR (Dobin et al. 2013). Optical and PCR duplicates were marked using MarkDuplicates (Picard) function of The Genome Analysis Toolkit (McKenna et al. 2010). Alignment files in BAM format were indexed using SAMtools (Li et al. 2009). SNPs and small insertions/deletions (INDELs) were detected using freebayes (Garrison and Marth 2012). Only primary alignments were considered as effective reads. Impact of detected SNP and INDEL was evaluated using SnpEff (Cingolani et al. 2012) and annotated results were saved in VCF format. Genomic regions with no coverage (large chromosomal deletions) were identified using BEDTools (Quinlan and Hall 2010). Relevant genetic variants were detected with the help of SNPer viewer

(https://bioinfogp.cnb.csic.es/tools/snper) and IGV browser (Thorvaldsdottir et al. 2013).

### $\beta$-Lactamase Activity Determination

Cells from overnight 20 ml cultures were harvested by centrifugation at 7,000 rpm for 10 min and then suspended in 500 $\mu$l of 0.1 M $Na_2HPO_4$ (pH 7.4) buffer. Crude protein extracts were obtained after sonication at 0.7 Hz of the suspended cells and subsequent centrifugation for 15 min at 13,000 rpm. Bradford protein assay with bovine serum albumin was performed for determining the protein content of each extract. Nitrocefin (Oxoid) was added to an equal amount of protein from each extract at a final concentration of 500 $\mu$g/ml, with $Na_2HPO_4$ 0.1M (pH 7.4) as the test buffer. The $\beta$-lactamase activity was quantified by measuring $OD_{486\ nm}$ every 2 min during 2 h at 37 °C in an Infinite M200 plate reader (Tecan).

### Statistical Analysis

R package AMR (Berends et al. 2021) was used to check all MIC values against EUCAST for classification of susceptibility. Collected MIC data were analyzed with R ((Version 4.0), retrieved from https://cran.r-project.org) using whole data sets and subsets by treatment, genetic background and their combinations: Multi- and One-Way ANOVA tests applying Welch's correction for unequal variances, were used to test each and all variables and their combinations. Successful ANOVA was followed by post-hoc Tukey's HSD all versus all tests. As Tukey may be too demanding for many of the desired comparisons, MIC changes over time were subject to longitudinal analysis using the parental strain values as time-zero reference: endpoint analyses relied on paired *t*-tests, and time series analyses used Dunnett's test with Hochberg correction (Hochberg 1988), which was also used for other comparisons of multiple groups against a single reference.

Changes in susceptibility to various antibiotics were asserted with ANOVA and a two-sample test for equality of proportions of resistant and susceptible populations. The significance of $\log_2$ fold changes was tested using ANOVA, Welch *F*-Test and post-hoc Tukey HSD tests. Significance of relative changes in fitness was checked, after ANOVA, using single-sample *t*-tests with $\mu = 1$ (no change). Kruskal–Wallis and Wilcoxon tests were used for count data. Comparisons between punctual MIC reference values of representative clones were calculated on a spreadsheet determining the 95% confidence interval of the mean of the log-transformed replicated experimental data and testing the log-transformed reference for inclusion in the computed interval (or the reference value in the back-transformed interval; Turnidge et al. 2006).

### Molecular Modeling

Models of wild-type and mutants of AmpR were obtained with I-TASSER (Yang et al. 2015) and Modeller (Webb and Sali 2016) using the corresponding sequences for *P. aeruginosa* UCBPP-PA14. Separate models for the extended and compact conformations of wild-type and mutant variants of AmpR were obtained using different combinations of reference structures (PDB: 5MMH, 4WKM, 3FZV, 3T1B). The structures were inspected to choose representative models using UCSF Chimera (Pettersen et al. 2004). A tetramer complex composed of two homodimers of AmpR, each with one subunit in extended and one in compact conformation, attached to the PA14 *ampC-ampR* intergenic region DNA was built using as reference the structures of *V. cholera* AphB variant N100E and BenM/BenA from *Acinetobacter* sp. ADP1 (PDB: 3T1B, 4IHT).

Models of wild-type and mutants of RpoB were obtained using I-TASSER, Modeller, and IntFold (McGuffin et al. 2018). I-TASSER and IntFold output were used to further identify potential interacting molecules and their conformation. The predicted structures were inspected with UCSF Chimera and substituted in over 80 of the available structures of the full RNAP complex from *E. coli* at different stages of the transcription process and bound to various $\sigma$ and transcription-related factors, to elucidate the potential role of mutants. Hybrid models of *P. aeruginosa* RpoB with molecules (DNA, RNA, antibiotic) transferred from *E. coli* structures were further minimized with the AMBER force field using UCSF Chimera. The minimized proposed hybrid model of the *P. aeruginosa* Asp203Glu mutant bound to an open DNA loop and a nascent RNA molecule was subjected to a Molecular Dynamics simulation using NAMD to verify the stability of the proposed model.

## Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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## Author Contributions

S.H.-A. participated in the design of the study and performing experimental work. P.L. participated performing experimental work. J.R.V. participated performing the statistical and computational modeling analyses. J.L.M. participated in the design of the study. All authors participated during writing of the manuscript and approved the submitted version.

## Data Availability

The data undelying this article are available in the article and its oline supplementary material.

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## Article V

**Novel inducers of the expression of multidrug efflux pumps that trigger *Pseudomonas aeruginosa* transient antibiotic resistance**

**Laborda P, Alcalde-Rico M, Blanco P, Martínez JL, Hernando-Amado S.**

***Antimicrobial Agents and Chemotherapy*. 2019 Oct 22;63(11):e01095-19. doi: 10.1128/AAC.01095-19.**

MDR efflux pumps are elements capable of extruding different compounds outside the cell, hence presenting several roles in bacterial physiology. Due to their antibiotic extrusion capacity, efflux pumps are AR determinants, and because of their varied and relevant functions, the expression of their encoding genes is tightly regulated. Therefore, bacteria may encounter situations in which the expression of MDR efflux pumps is increased, consequently increasing AR. If these conditions occur during an infection, the emergence of such non-inheritable AR increase may compromise an antibiotic treatment.

During this work, we have studied the expression of the genes encoding the clinically relevant efflux pumps of *P. aeruginosa* MexCD-OprJ and MexAB-OprM, in presence of different compounds. For that, we developed biosensor strains in which the expression of the *luxCDABE* operon was controlled by the promoter of the expression of the efflux pump encoding genes and we use them for a high-throughput analysis of 240 compounds. Several putative inducers of the expression of such genes were identified, and we focused on those compounds that could be found in clinical settings. Namely, dequalinium chloride -a disinfectant-, procaine and atropine -anaesthetic agents-, were found as inducers of *mexCD-oprJ*. Consequently, the presence of the studied compounds led to a reduced ciprofloxacin -a substrate of MexCD-OprJ- susceptibility. The induction was transient since, when removing the compounds, the expression of *mexCD-oprJ* returned to wild-type levels, and the "memory of induction" was dependent on the inducer compound, being larger with dequalinium chloride than with atropine or procaine.

These observations point to dequalinium chloride, procaine and atropine as compounds that, if found by *P. aeruginosa* when causing an infection, could threaten its treatment. Also, this study reinforces the need to study situations in which transient AR may arise, a phenotype difficult to detect using classical susceptibility tests.

**Specific contributions**:

*Experimental work*: Laborda P., Hernando-Amado S., Blanco P. and Alcalde-Rico M. contributed to experimental work. I performed most of the experimental work of this article, including the screening of potential inducers of efflux pumps encoding genes, analysis of luminescence measurements, antibiotic susceptibility determination, RNA extraction and gene expression measurements, determination of mutations by Sanger sequencing and checkerboard analysis, as well as the interpretation of the results.

*Manuscript writing*: All the authors contributed to the writing and correction of the manuscript, being the first version of the manuscript elaborated by me.

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# Novel Inducers of the Expression of Multidrug Efflux Pumps That Trigger *Pseudomonas aeruginosa* Transient Antibiotic Resistance

**Pablo Laborda,[a] Manuel Alcalde-Rico,[a,b,c] Paula Blanco,[a] José Luis Martínez,[a] Sara Hernando-Amado[a]**

[a]Centro Nacional de Biotecnología, CSIC, Madrid, Spain
[b]Instituto de Biología, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile
[c]Millennium Nucleus for Collaborative Research on Bacterial Resistance (MICROB-R), Valparaíso, Chile

**ABSTRACT** The study of the acquisition of antibiotic resistance (AR) has mainly focused on inherited processes, namely, mutations and acquisition of AR genes. However, inducible, noninheritable AR has received less attention, and most information in this field derives from the study of antibiotics as inducers of their associated resistance mechanisms. Less is known about nonantibiotic compounds or situations that can induce AR during infection. Multidrug resistance efflux pumps are a category of AR determinants characterized by the tight regulation of their expression. Their contribution to acquired AR relies in their overexpression. Here, we analyzed potential inducers of the expression of the chromosomally encoded *Pseudomonas aeruginosa* clinically relevant efflux pumps, MexCD-OprJ and MexAB-OprM. For this purpose, we developed a set of *luxCDABE*-based *P. aeruginosa* biosensor strains, which allows the high-throughput analysis of compounds able to modify the expression of these efflux pumps. Using these strains, we analyzed a set of 240 compounds present in Biolog phenotype microarrays. Several inducers of the expression of the genes that encode these efflux pumps were found. The study focused in dequalinium chloride, procaine, and atropine, compounds that can be found in clinical settings. Using real-time PCR, we confirmed that these compounds indeed induce the expression of the *mexCD-oprJ* operon. In addition, *P. aeruginosa* presents lower susceptibility to ciprofloxacin (a MexCD-OprJ substrate) when dequalinium chloride, procaine, or atropine are present. This study emphasizes the need to study compounds that can trigger transient AR during antibiotic treatment, a phenotype difficult to discover using classical susceptibility tests.

**KEYWORDS** Biolog, MDR efflux pumps, *Pseudomonas aeruginosa*, transient antibiotic resistance

*Pseudomonas aeruginosa* is included in the groups of bacteria considered to be of high risk concerning antibiotic resistance (AR) (1, 2). These include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa* and *Enterobacter* spp., which altogether have been dubbed ESKAPE pathogens (3) and the TOp TEn resistant Microorganisms (TOTEM), as stated by the World Health Organization (1, 2). This nosocomial pathogen is one of the most prevalent organisms causing infections at hospitals and is the main cause of chronic lung infections in patients with cystic fibrosis and chronic obstructive pulmonary diseases (4, 5). Among the different AR mechanisms of *P. aeruginosa*, multidrug efflux pumps from the resistance-nodulation-cell division (RND) family are relevant elements since they contribute to both intrinsic and acquired resistance (6–10). Among such RND efflux pumps, MexAB-OprM and MexCD-OprJ stand out as significant determinants of multi-

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Address correspondence to José Luis Martínez, jlmtnez@cnb.csic.es, or Sara Hernando-Amado, shernando@cnb.csic.es.

P.L. and M.A-R. contributed equally to this article.

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drug resistance in *P. aeruginosa* (11–13). MexAB-OprM encoding genes are constitutively expressed under regular growing conditions, hence contributing to intrinsic resistance of *P. aeruginosa* to several antibiotics such as quinolones, macrolides, tetracycline, lincomycin, chloramphenicol, novobiocin, and β-lactams (14). In addition, *mexAB-oprM* overexpressing mutants have been isolated from patients (9), so that this overexpression is considered a significant mechanism for acquiring AR under a clinical viewpoint (8). The operon encoding MexCD-OprJ, on the other hand, is expressed at very low levels under regular growing conditions. Thus, it does not seem to have a relevant contribution in *P. aeruginosa* intrinsic resistance (8). Nevertheless, *mexCD-oprJ* overexpression, usually achieved by loss-of-function mutations in the gene that encodes its local repressor, *nfxB*, confers resistance to different antibiotics such as quinolones, tetracyclines, and chloramphenicol (14, 15).

It is worth mentioning that in addition to being AR determinants (16, 17), efflux pumps present other physiological functions important for bacterial behavior, such as modulation of quorum-sensing (QS) signaling (18–20), response to stress situations (21) and to host defenses (22–24), or plant-bacterial interactions (25, 26). Although the basal level of expression of each efflux pump can vary, it is well established that their expression may increase in the presence of some compounds or situations (27). In this regard, knowing which compounds can trigger the expression of the genes that encode efflux pumps and therefore promote a transient reduction in the susceptibility to antibiotics (28, 29), a situation that is not easily detected using common susceptibility methods (8, 29), is a relevant topic.

It is important to highlight that induction of the expression of MDR efflux pumps can be the consequence of the binding of the inducer to the transcriptional regulators of their expression (30, 31) or due to the bacterial response to different injuries (32). In the first case, a structural similarity of potential effectors is expected; in the second case, molecules with different structures, but with similar biological activities, can trigger the expression of MDR efflux pumps.

In the present study, we addressed this issue by analyzing two relevant *P. aeruginosa* efflux pumps, MexAB-OprM and MexCD-OprJ, that present different levels of basal expression. Previous studies have shown that *mexAB-oprM* expression is induced by oxidative stress (33) as well as by triclosan and pentachlorophenol (34), whereas envelope stress, benzalkonium chloride, chlorhexidine, tetraphenylphosphonium chloride, ethidium bromide, rhodamine 6G or antimicrobial human peptides, and interleukin-37 (35–38) are inducers of *mexCD-oprJ* expression. By screening a set of compounds present in Biolog phenotype microarrays, an approach that has been previously validated as a useful strategy to discover novel inducers of the expression of the genes that encode efflux pumps (39, 40), we have detected different molecules that induce the expression of *mexAB-oprM* and *mexCD-oprJ* in *P. aeruginosa*. For further analysis, we focused on molecules that this bacterial pathogen can potentially encounter when producing an infection, such as procaine, atropine, or dequalinium chloride. This study highlights the potential risk associated with the utilization of these compounds in clinical settings, as inducers of transient AR in *P. aeruginosa*, when antibiotic treatments are applied.

## RESULTS AND DISCUSSION

**Construction and validation of reporter strains.** In order to identify potential inducers of the expression of either *mexCD-oprJ* or *mexAB-oprM*, which could trigger *P. aeruginosa* nonheritable resistance to antibiotics, a set of biosensor strains of *P. aeruginosa* PAO1 containing the *mexCD-oprJ* or the *mexAB-oprM* promoter regions controlling the *luxCDABE* operon (PAO1 CTX-*lux*::P*mexCD* and PAO1 CTX-*lux*::P*mexAB*, respectively) and the control strain PAO1 CTX-*lux*, were developed as described in Materials and Methods. The proper functioning of the developed reporter strains was analyzed by measuring the luminescence and optical density at 600 nm ($OD_{600}$) of each strain in the presence of known inducers, benzalkonium chloride at 10 μg/ml for *mexCD-oprJ* (37) and $H_2O_2$ at 0.136 μg/ml for *mexAB-oprM* (33). The luminescence

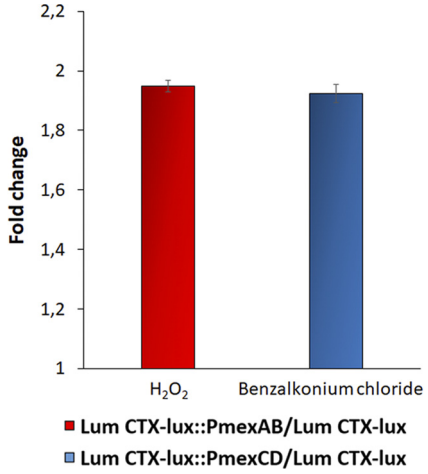


**FIG 1** Effect of known inducers of *mexAB* and *mexCD* expression in luminescence emitted by *mexAB* and *mexCD* bioreporter strains. The luminescence and $OD_{600}$ values for PAO1 CTX-lux::P*mexAB* and PAO1 CTX-lux::P*mexCD* were measured in the presence of 0.136 $\mu$g/ml of $H_2O_2$ and 10 $\mu$g/ml benzalkonium chloride, respectively. Fold changes in the luminescence emitted by strains grown in the presence of the inducers versus strains grown without any inducer are shown. Error bars represent the standard deviations of three independent replicates. As shown, the known inducer compounds increase the production of luminescence of the bioreporter strains.

values were normalized with respect to those obtained from the control strain PAO1 CTX-*lux*, as described in Materials and Methods. The presence of the known inducers produced increases in luminescence emitted by the corresponding biosensor strain of 1.92-fold in the case of benzalkonium chloride and 1.94-fold in the case of $H_2O_2$ compared to that produced in lysogeny broth (LB) medium without inducers (Fig. 1). These results validate the capability of these biosensor strains to detect *mexAB-oprM* and *mexCD-oprJ* inducers.

**Screening for inducers of *mexCD-oprJ* and *mexAB-oprM* expression using Biolog phenotype microarrays.** It has been described that the expression of efflux pumps can be induced by different stressors (32). Consequently, we tested the capability of 240 compounds, several of them with pharmacological interest and present in the PM11C to PM20B bacterial chemical susceptibility arrays of Phenotype MicroArrays (Biolog), for inducing the expression of either *mexCD-oprJ* or *mexAB-oprM*. Among these compounds were antibiotics, heavy metals, antiseptics, fungicides, food preservatives, chelating agents, oxidative stress compounds, amino acids, synthetic organic compounds, and different drugs for human and veterinary use. Four different concentrations of each compound are present in each commercial microarray plate; those in which the growth of the biosensor strain was severely impaired were discarded from the analysis.

The luminescence emitted and the $OD_{600}$ were recorded for each biosensor strain (PAO1 CTX-*lux*::P*mexCD* and PAO1 CTX-*lux*::P*mexAB*), and these values were normalized with those obtained from the control strain (PAO1 CTX-*lux*), as described in Materials and Methods. Luminescence and $OD_{600}$ raw data are shown in the supplemental material. The distribution of normalized values for each promoter was represented in a box plot (Fig. 2), and an induction threshold level was calculated for each reporter strain, as described in Materials and Methods. These threshold values were 1.73 and 1.53 for *mexCD-oprJ* and *mexAB-oprM* promoter, respectively. Compounds that produced a fold change in luminescence that exceeded the calculated threshold were considered potential inducers and are highlighted in Table 1. For *mexCD-oprJ*, 20 putative inducers were detected, while 10 compounds were found to increase luminescence above the threshold in the case of the *mexAB-oprM* biosensor strain.

Among the latter, we found pentachlorophenol, already known to be inducer of *mexAB-oprM* expression (34), as well as compounds that may lead to oxidative stress,

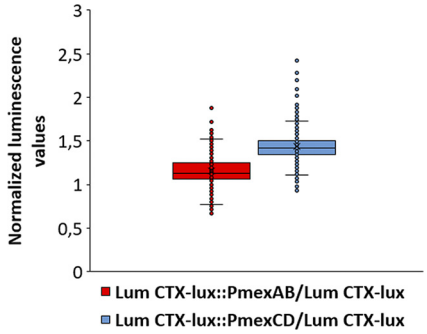


**FIG 2** Effects of different compounds on the expression of either *mexAB-oprM* or *mexCD-oprJ*. The figure shows the normalized luminescence values produced by PAO1 CTX-*lux*::P*mexAB* and PAO1 CTX-*lux*::P*mexCD* in the presence of four different concentrations of 240 compounds from Biolog plates. The outliers of the boxplot represent the conditions under which there was potential overexpression or repression of the genes encoding the studied efflux pumps: values greater than 1.53 for PAO1 CTX-*lux*::P*mexAB* and 1.73 for PAO1 CTX-*lux*::P*mexCD* indicate overexpression, while values less than 0.7 for PAO1 CTX-*lux*::P*mexAB* and 1.1 for PAO1 CTX-*lux*::P*mexCD* indicate repression.

and which is a known inducer condition of the expression of this efflux pump (33), such as flavins or their derivatives (acriflavine, proflavine, or 9-aminoacridine) (41) or iodoacetic acid. These results reinforce the robustness of our experimental approach. Novel inducers of *mexAB-oprM* expression detected during the analysis included antibiotics such as amikacin and azlocillin (14); sanguinarine, a plant-derived compound; ethionamide, used as an antibiotic for treating multidrug-resistant *Mycobacterium tuberculosis* (42); and sodium cyanate, a neurotoxic compound implicated in neurodegenerative disorders in populations subsisting on the cyanogenic plant cassava (43) (Table 1).

Concerning MexCD-OprJ, an efflux pump known to be induced by membrane-damaging agents (35, 36), novel putative inducers of its expression were found. Among them, disinfectants (benthezonium chloride, dequalinium chloride, domiphen bromide, alexidine, and cetylpyridinium chloride), the chelating agent 2,2′-dipyridyl, sodium cyanate, methyltrioctylammonium chloride, iodonitrotetrazolium violet, flavin derivatives (acriflavine, proflavine, and 9-aminoacridine), the anesthetic agent procaine, the antidepressant drug amitriptyline, the antihistaminic agent orphenadrine, the $\beta$-blocker propranolol, the fungicide dodine, and some plant-derived compounds (atropine, harmane, and sanguinarine), were identified (Table 1).

Regarding plant-derived compounds, it is worth mentioning that some of them, such as the flavonoids, play important physiological functions in plants, as well as in plant-bacterial interactions (44), and it is known that the flavonoid-responsive efflux pump MexAB-OprM of *Pseudomonas syringae* is required for the efficient bacterial colonization of tomato plants (45). Since *P. aeruginosa* is widely distributed in different natural habitats, including plants (46), it seems possible that these efflux pumps could have a role in colonizing such habitats (25, 26).

In addition to the detected inducers of *mexCD-oprJ* and *mexAB-oprM* expression, some compounds (Table 2) were found to reduce the expression of these efflux pumps below the lower threshold (1.1 for *mexCD-oprJ* and 0.7 for *mexAB-oprM*) for each of both promoters, calculated as described in Materials and Methods. For *mexCD-oprJ*, these compounds were the antibiotics chloramphenicol, spectinomycin, and spiramycin and the plant-derived compounds nordihydroguaiaretic acid and gallic acid (Table 2). For *mexAB-oprM*, there were antibiotics such as hygromycin and josamycin, chromium chloride, glycine hydroxamate, and protamine sulfate (Table 2). However, these compounds did not seem to be strong inhibitors, since the normalized luminescence values measured in their presence were close to those of the threshold values and were not further studied.

Among the potential inducers, dequalinium chloride, procaine, and atropine, which seemed to induce the expression of *mexCD-oprJ*, were chosen for deeper analysis

**TABLE 1** Potential inducer compounds of *mexCD-oprJ* and *mexAB-oprM* expression detected by the Biolog screening

| Efflux pump induced | Compound[a] | Normalized luminescence value[b] | | | |
|---|---|---|---|---|---|
| | | A | B | C | D |
| *mexCD-oprJ* | Benthezonium chloride | 1.71 | **1.83** | **1.95** | **2.29** |
| | Dequalinium chloride* | **2.09** | **2.21** | **2.28** | **2.2** |
| | 2,2′-Dipyridyl | 1.54 | 1.62 | **1.79** | IG |
| | Acriflavine | 1.57 | 1.65 | 1.66 | **1.91** |
| | Sanguinarine | 1.72 | **1.77** | **1.86** | **1.85** |
| | 9-Aminoacridine | 1.60 | 1.69 | **1.92** | **2.2** |
| | Sodium cyanate | 1.64 | 1.71 | **1.83** | IG |
| | Procaine | 1.57 | 1.64 | **1.93** | IG |
| | Domiphen bromide* | 1.65 | **1.8** | **1.84** | **2.42** |
| | Alexidine* | 1.32 | 1.53 | 1.67 | **1.93** |
| | Cetylpyridinium chloride* | 1.67 | **1.81** | **1.81** | **1.76** |
| | Metyltrioctylammonium chloride | 1.72 | **1.77** | **1.83** | **1.96** |
| | Harmane | 1.59 | 1.71 | **1.93** | **1.87** |
| | Iodonitro tetrazolium violet | 1.46 | 1.57 | **1.79** | **2.19** |
| | Amitriptyline | 1.50 | 1.60 | **1.8** | **1.85** |
| | Orphenadrine | 1.61 | **1.82** | **2.11** | **1.9** |
| | Propanolol | 1.70 | **1.87** | **2.02** | **2.12** |
| | Atropine | **1.78** | **1.83** | **2.09** | IG |
| | Proflavine | 1.49 | 1.53 | **1.76** | **2.02** |
| | Dodine | 1.67 | 1.72 | **1.85** | **1.82** |
| *mexAB-oprM* | Amikacin | 1.42 | 1.50 | **1.72** | IG |
| | Azlocillin | 1.10 | 1.10 | 1.13 | **1.55** |
| | Acriflavine† | 1.31 | 1.42 | 1.45 | **1.65** |
| | Sanguinarine | 1.34 | 1.40 | 1.52 | **1.58** |
| | 9-Aminoacridine† | 1.31 | 1.47 | 1.47 | **1.64** |
| | Sodium cyanate | 1.30 | 1.48 | **1.61** | IG |
| | Ethionamide | 1.12 | 1.23 | **1.75** | **1.55** |
| | Iodoacetate† | 1.26 | 1.34 | **1.75** | IG |
| | Penthachlorophenol[c,*] | 1.14 | 1.16 | 1.39 | **1.9** |
| | Proflavine† | 1.18 | 1.27 | 1.50 | **1.88** |

[a]Compounds marked by an asterisk (*) are disinfectants, a category of compounds known to be inducers of *mexCD-oprJ* (37). Compounds marked by a dagger (†) generate oxidative stress, which is a known inducer condition of *mexAB-oprM* (33).

[b]A, B, C, and D represent normalized luminescence values obtained in the four different concentrations of each compound present in the Biolog phenotype microarray plates; "A" is the normalized value for the lowest compound concentration, and "D" is the normalized value for the highest compound concentration. All normalized luminescence values for each compound that did not impair bacterial growth appear in the table; those above the threshold are highlighted in boldface. IG, impaired growth.

[c]A known inducer of *mexAB-oprM* (34).

because they are used in human therapy and hence *P. aeruginosa* can grow in their presence when causing infections.

**Inducers of expression of *mexCD-oprJ* with relevance in clinical settings.** Dequalinium chloride is used as a disinfectant (47) that belongs to the same group of quaternary amines as benzalkonium chloride and presents some structural similarities to chlorhexidine. Both compounds are known inducers of *mexCD-oprJ* (37) that promote its expression through the induction of an envelope-stress condition (35, 36). Further, several compounds found as inducers in the analysis are similar to them in terms of biological activity (benthezonium chloride, cetylpyridinium chloride, domiphen bromide, or alexidine) and are also used in clinical practice. This finding supports the potential relationship between antibiotics and disinfectants resistance, as well as the possible induction of AR by disinfectants of common use. In particular, the induction of *mexCD-oprJ* by dequalinium chloride could have clinical relevance, since this compound forms part of several antiseptic and disinfection procedures (48), and its use has been considered for the treatment of promyelocytic leukemia (49–52).

Procaine is used as a local anesthetic agent (53) in some minor surgeries or in burn injuries, which involve tissues that *P. aeruginosa* frequently colonizes (54). Therefore, the induction of *mexCD-oprJ* by procaine should be taken into consideration in these

**TABLE 2** Potential inhibitors of *mexAB-oprM* and *mexCD-oprJ* expression detected with Biolog screening

| Efflux pump repressed | Compound | Normalized luminescence value[a] | | | |
|---|---|---|---|---|---|
| | | A | B | C | D |
| *mexCD-oprJ* | Chloramphenicol | 1.34 | 1.23 | 1.26 | **1.09** |
| | Spectinomycin | 1.29 | 1.29 | 1.11 | **1.05** |
| | Spiramycin | 1.14 | **0.98** | **1.08** | **1.09** |
| | Nordihydroguaiaretic acid | 1.38 | 1.38 | 1.21 | **1.06** |
| | Gallic acid | 1.49 | 1.42 | 1.38 | **0.92** |
| *mexAB-oprM* | Protamine sulfate | 1.01 | 0.98 | 0.89 | **0.65** |
| | Chromium chloride | 0.79 | 0.77 | 0.73 | 0.71 |
| | Glycine hydroxamate | 0.73 | **0.70** | **0.69** | **0.67** |
| | Hygromycin | 1.42 | 1.13 | 0.80 | 0.72 |
| | Josamycin | 1.01 | 0.98 | 0.86 | 0.71 |

[a]A, B, C, and D represent normalized luminescence values obtained in the four different concentrations of each compound present in the Biolog phenotype microarray plates; "A" is the normalized value for the lowest compound concentration, and "D" is the normalized value for the highest compound concentration. The normalized luminescence values for each compound that were below the threshold and did not impair bacterial growth appear in the table; those above the threshold are highlighted in boldface.

patients since they are susceptible to *P. aeruginosa* infections. Finally, atropine is considered an essential drug for preoperative medication and sedation by the World Health Organization (42), so it may have an effect on *P. aeruginosa* susceptibility to antibiotics when this microorganism infects surgical patients. It has been reported previously that procaine and atropine have some common pharmacological properties (55), although their structures present few similarities (Fig. 3). Whether their mechanism of induction relies on a response to cellular damage, as happens for some inducers of efflux pumps (32), or whether they are effectors able to bind the regulators of efflux pump expression, as happens for others (30, 31), is a question that remains to be determined.

Since concentrations of each compound in each well of the Biolog phenotype microarrays are not included in the information provided with the plates, the *mexCD-oprJ* reporter strain was grown for 20 h in a range of concentrations in order to select the one at which an increase in luminescence is observed but bacterial growth is not

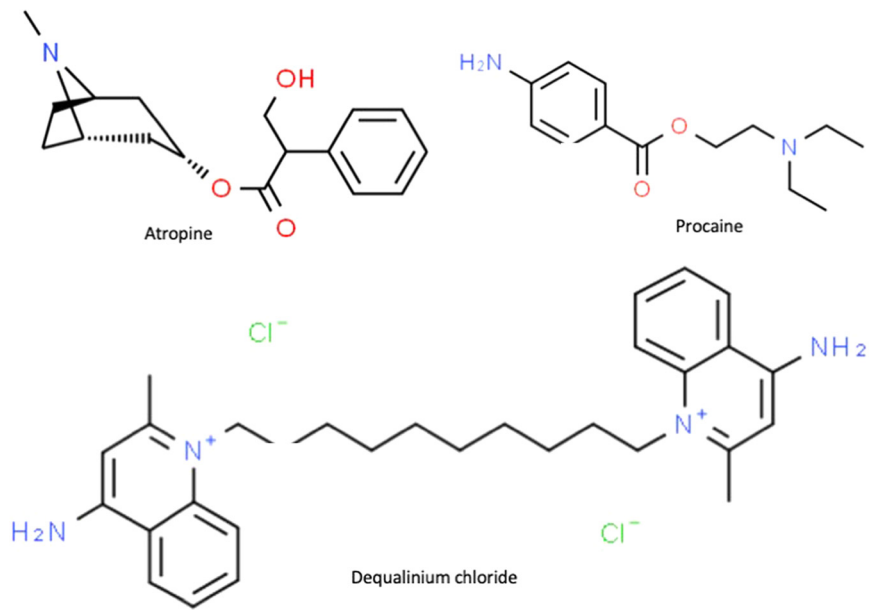


**FIG 3** Structures of inducers of *mexCD-oprJ* expression. The structures of atropine, procaine, and dequalinium chloride were obtained from the ChemSpider database.

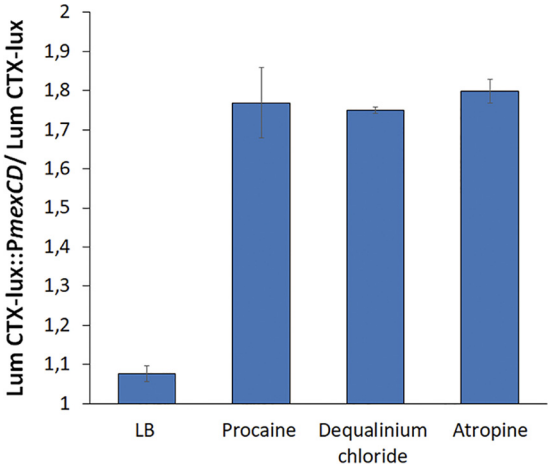


**FIG 4** Effect of dequalinium chloride, procaine, and atropine on the expression of *mexCD-oprJ*. The figure shows the normalized luminescence values produced by reporter strain PAO1 CTX-*lux*::P*mexCD* in the presence of 10 $\mu$g/ml of dequalinium chloride, 2 mg/ml of procaine, and 2 mg/ml of atropine. The luminescence was normalized to that produced by PAO1 CTX-*lux* in the presence of inducer. As shown, the expression of *mexCD-oprJ* is induced by the three tested compounds. Error bars represent the standard deviations of three independent replicates.

compromised. These concentrations are 10 $\mu$g/ml for dequalinium chloride, 2 mg/ml for procaine, and 2 mg/ml for atropine and were the concentrations used for further studies. Luminescence measurements in the presence of inducers at these optimal concentrations were recorded and normalized as previously described. In all cases, the presence of the tested compounds increased the luminescence produced by the biosensor strain (Fig. 4), further supporting that they induce the expression of *mexCD-oprJ*.

**Dequalinium chloride, procaine, and atropine induce *mexCD-oprJ* expression and transient antibiotic resistance.** In order to further confirm the inducing capacity of the compounds found in the screening, the expression level of *mexCD-oprJ* was quantified by real-time PCR in wild-type *P. aeruginosa* PAO1 grown in the presence or absence of the cognate inducer compounds. The strain overexpressing *mexCD-oprJ* (*nfxB**) (Table 3), which differs from the wild-type strain just by a mutation in *nfxB* (20), was used as an efflux pump-overexpressing control strain. The expression of *mexCD-oprJ* increased 54-fold in the presence of dequalinium chloride, 25-fold in the presence of procaine, and 16-fold in the presence of atropine (Fig. 5A). These results confirm that the expression of *mexCD-oprJ* is induced by the compounds selected from the Biolog screening. Although the time for selection is low and bacteria are not growing in the presence of antibiotics, it might be possible that the increased expression of *mexCD-oprJ* could be due to the selection of mutants in the elements that regulate the expression of this efflux pump, namely, its promoter region and its local repressor NfxB (15). To investigate this possibility, *nfxB* and the *nfxB-mexC* intergenic region were amplified by PCR and sequenced in cultures of *P. aeruginosa* after 20 h of induction. None of the mutations was detected, either in the gene coding for the regulator or in the intergenic region between it and the efflux pump-encoding operon. This finding and the results below indicate that the increased expression observed is associated with the presence of the inducers.

An interesting issue was to determine whether or not the induction of *mexCD-oprJ* remains after removing the inducer. To address this possibility, the expression of *mexCD-oprJ* was measured by real-time PCR at different times after removal of the inducers. The expression of *mexCD-oprJ* is reduced to levels close to the untreated cells levels 30 min after the removal of procaine or atropine. However, a 10-fold induction, compared to the strain grown in the absence of inducer, is still observed 30 min after the removal of dequalinium chloride, and a 2-fold induction in *mexCD* expression levels

**TABLE 3** Bacterial strains and plasmids used in this study[a]

| Bacterial strain or plasmid | Description | Reference(s) or origin |
|---|---|---|
| Bacterial strains | | |
| *E. coli* | | |
| One Shot OmniMax | Host strain used to maintain cloning plasmids | Invitrogen |
| S17-1λ*pir* | Conjugative donor strain used to transfer plasmids to *P. aeruginosa* strains by conjugation | 64 |
| *P. aeruginosa* | | |
| PAO1-V | PAO1 strain stored at the laboratory of Victor de Lorenzo | 20, 65 |
| *nfxB** | PAO1 spontaneous mutant in *nfxB* gene that overexpresses *mexCD-oprJ* | 20, 65 |
| *nfxB** Δ*mexD* | Loss of function of MexCD-OprJ system mutant, obtained from *nfxB** by *mexD* deletion by homologous recombination | 20 |
| PAO1 CTX-*lux* | PAO1 strain which contains the mini-CTX-*lux* construction inserted in a neutral zone of the chromosome | This study |
| PAO1 CTX-*lux*::P*mexCD* | PAO1 strain which contains mini-CTX::P*mexCD-lux* construction inserted in a neutral zone of the chromosome | This study |
| PAO1 CTX-*lux*::P*mexAB* | PAO1 strain which contains mini-CTX::P*mexAB-lux* construction inserted in a neutral zone of the chromosome | This study |
| Plasmids | | |
| pGEM-T Easy | Commercial plasmid used for cloning optimization of PCR products; Amp$^r$ | Promega |
| mini-CTX-*lux* | *luxCDABE* operon inserted into the mini-CTX-1 plasmid; Tc$^r$ | 59 |
| mini-CTX::P*mexCD-lux* | Plasmid derived from mini-CTX-*lux* in which the expression of the *luxCDABE* operon is under the control of the *mexCD-oprJ* promoter region of *P. aeruginosa*; Tc$^r$ | This study |
| mini-CTX::P*mexAB-lux* | Plasmid derived from mini-CTX-*lux* in which the expression of the *luxCDABE* operon is under the control of the *mexAB-oprM* promoter region of *P. aeruginosa*; Tc$^r$ | This study |

[a]Tc$^r$, tetracycline resistant; Amp$^r$, ampicillin resistant.

still remains 120 min after removing the inducer (Fig. 5B, C, and D). This indicates that the memory of induction is dependent on the inducer. It has been proposed that *mexCD-oprJ* expression is induced as a response to membrane damage (35, 36). The likely reason behind dequalinium chloride durability of induction over time may then be related to the membrane-damaging effect of this compound, which may remain for a time after removing the compound and can actually cause its inducer capability. The fact that induction is transient further supports that the cause of the increased *mexCD-oprJ* expression is not the selection of mutants overexpressing this efflux pump.

The *P. aeruginosa* PAO1 MIC of ciprofloxacin, one of the MexCD-OprJ substrates, was analyzed in the presence or absence of the aforementioned inducers in order to determine the effect of such induction on the susceptibility of *P. aeruginosa* to this antibiotic. The MIC of ciprofloxacin was higher in the presence of the inducers (Table 4). It might be possible that this increased MIC would be the consequence of antagonism between the inducer and the antibiotic, in which case the activity of the efflux pump should be of not-relevance for the final outcome. To analyze this possibility, the strain that overexpresses *mexCD-oprJ* (*nfxB**) and a *mexD*-defective mutant (*nfxB** Δ*mexD*) (Table 3) were used as controls. Neither when *mexCD-oprJ* is constitutively overexpressed (*nfxB**) nor when the efflux pump is absent (*nfxB** Δ*mexD*) was an antagonism between the inducers and the antibiotic observed; the presence of each inducer did not affect the MIC of ciprofloxacin for either *nfxB** or *nfxB** Δ*mexD* strains. These results indicate that the observed phenotype was caused specifically by *mexCD-oprJ* induction and was not the consequence of nonspecific antagonism between the inducers and the antibiotic. Growth curves of PAO1 in the presence of ciprofloxacin, with or without these inducers, were also analyzed (Fig. 6). The results confirm that atropine, procaine, and dequalinium chloride lead to a transient reduction in *P. aeruginosa* ciprofloxacin susceptibility in the wild-type strain, whereas no change is observed in the *mexD*-defective mutant, further confirming that the phenotype depends on the activity of MexCD-OprJ.

Further, checkerboard analyses were performed as described in Materials and Methods for the *P. aeruginosa* PAO1 wild-type strain and the strain lacking the MexCD-OprJ efflux pump. Inducer compounds showed an antagonistic effect with

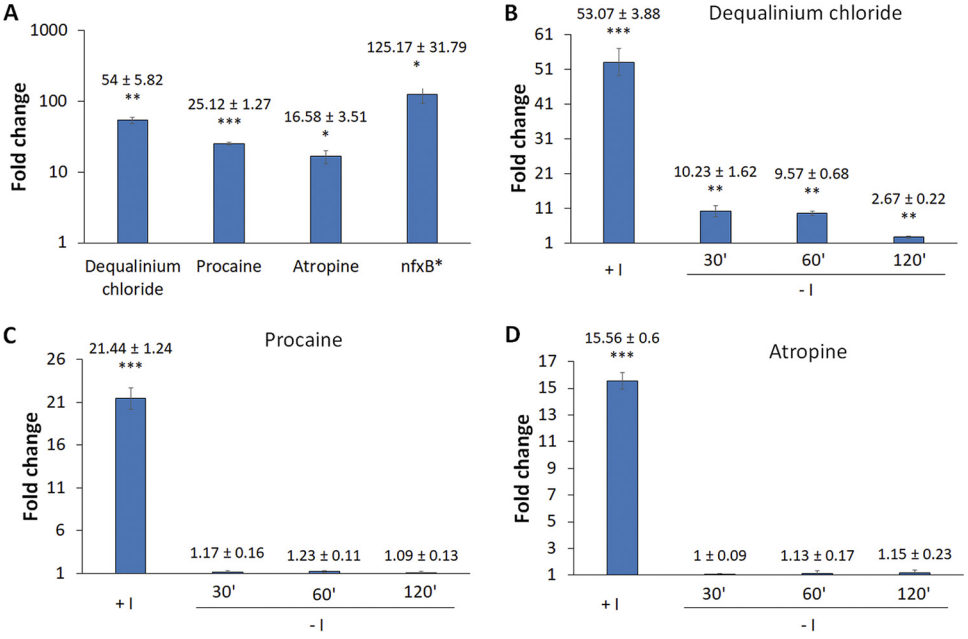


**FIG 5** Analysis of *mexCD-oprJ* expression by real-time PCR in the presence of and after the removal of dequalinium chloride, procaine, or atropine. (A) *mexC* expression after 90 min of incubation with 10 $\mu$g/ml of dequalinium chloride, 2 mg/ml of procaine, or 2 mg/ml of atropine or without inducer. The *nfxB** strain grown in the absence of any inducer was used as a control of overexpression. As shown, the expression of *mexCD-oprJ* is induced by the three tested compounds. (B, C, and D) *mexC* expression 30, 60, and 120 min after the removal of the inducers (–I) and compared to the expression level after 90 min of induction (+I) with 10 $\mu$g/ml of dequalinium chloride (B), 2 mg/ml of procaine (C), and 2 mg/ml of atropine (D). As shown, the overexpression of *mexCD-oprJ* is reduced 30 min after the removal of procaine and atropine to levels similar to those for the untreated bacterial levels; 30 and 60 min after removal of dequalinium chloride, the expression level is reduced to 10 times the untreated bacterial levels and to 2 times the untreated bacterial levels after 120 min. The fold changes were calculated regarding the expression of untreated *P. aeruginosa* PAO1. Each represented value is the average of three biological replicates. Statistically significant differences regarding PAO1 untreated were calculated with a Student *t* test for paired samples assuming equal variances (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$).

ciprofloxacin in the case of PAO1 (FIC index $>$ 2.7 in all cases), while neither an antagonistic nor a synergistic effect (FIC index $\leq$ 2 and $\geq$ 0.5 in all cases) was observed for the strain lacking the efflux pump (Fig. 7). This observation, in addition to the above-mentioned results (Table 4 and Fig. 6), reinforces that dequalinium chloride, procaine, and atropine are molecules able to reduce the effect of ciprofloxacin in *P. aeruginosa* via a *mexCD-oprJ* induction, whereas this antagonistic effect is not observed in the strain lacking the gene encoding the mentioned efflux pump (Fig. 6 and 7).

In order to further confirm the specific induction of *mexCD-oprJ* by these compounds, the MICs of antibiotics which are not substrates of MexCD-OprJ (tobramycin, fosfomycin, and ceftazidime [8]) were also measured in the presence or absence of the identified inducers. The MICs of these antibiotics did not change in the presence of the inducers.

Altogether, these results indicate that the three analyzed compounds are able to transiently increase AR through the induction of the expression of the genes that

**TABLE 4** Ciprofloxacin MICs for *P. aeruginosa* in the presence or absence of inducer compounds

| Strain | Ciprofloxacin MIC ($\mu$g/ml) | | | |
|---|---|---|---|---|
| | No inducers | Dequalinium chloride (10 $\mu$g/ml) | Procaine (2 mg/ml) | Atropine (2 mg/ml) |
| PAO1 | 0.047 | 0.38 | 0.125 | 0.094 |
| *nfxB** | 0.5 | 0.5 | 0.5 | 0.5 |
| *nfxB** Δ*mexD* | 0.047 | 0.047 | 0.047 | 0.047 |

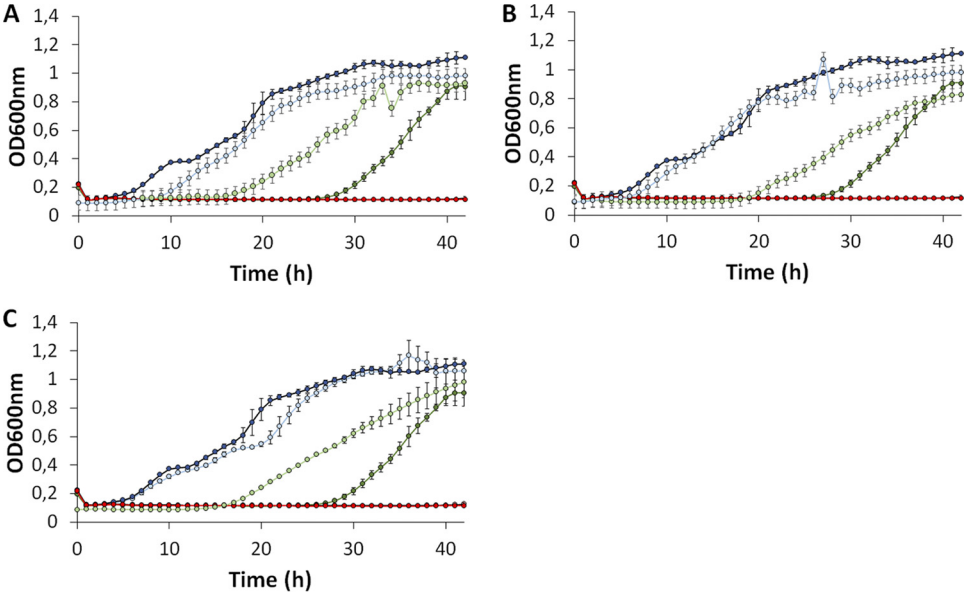


**FIG 6** Effect of inducers of *P. aeruginosa* growth in the presence of ciprofloxacin. The growth curves of *P. aeruginosa* PAO1 (green), *nfxB** (blue), and of *nfxB**Δ*mexD* (red) in LB medium containing 0.25 $\mu$g/ml ciprofloxacin with (light) or without (dark) 10 $\mu$g/ml of dequalinium chloride (A), 2 mg/ml of procaine (B), or 2 mg/ml of atropine (C) are shown. As indicated, wild-type *P. aeruginosa* grows better in the presence of ciprofloxacin when the inducers are added. Each represented $OD_{600}$ value is the average of three biological replicates.

encode MexCD-OprJ. Because of that, the temporal coincidence of any of these compounds with MexCD-OprJ antibiotic substrates for treating patients may be a concern.

**MexCD-OprJ efflux pump extrudes procaine.** It has been found that, on some occasions, inducers of the expression of efflux pumps are also substrates of these AR determinants (22, 31, 56, 57). One example of this situation is the induction of MexXY in *P. aeruginosa* by aminoglycosides, which are substrates of this efflux pump (58). However, in other cases, as in the case of *S. maltophilia* SmeVWX and SmeYZ efflux pumps (40), some of their inducers are not substrates of these efflux pumps.

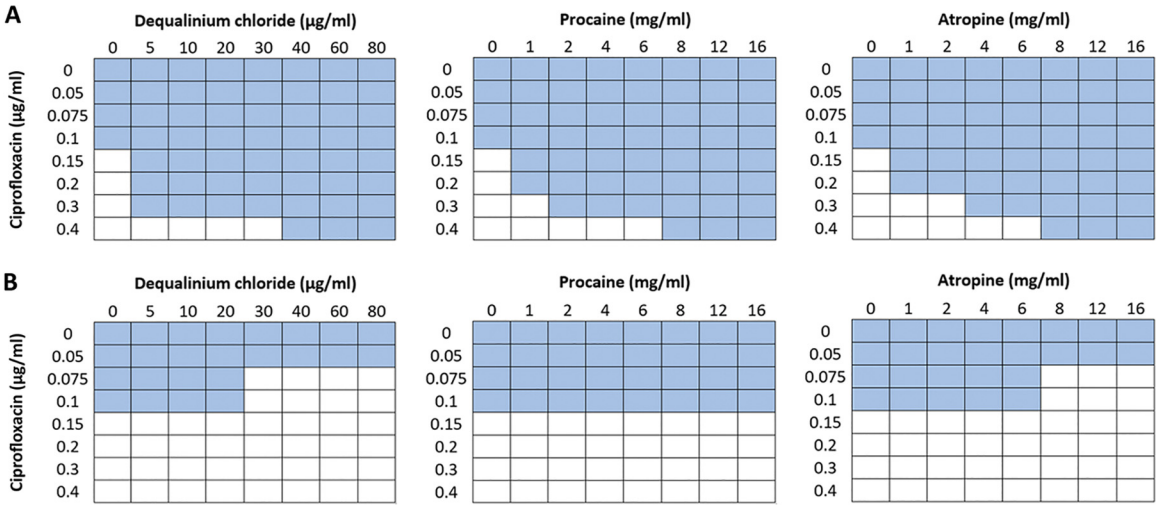


**FIG 7** Checkerboard analysis for ciprofloxacin and *mexCD-oprJ* inducer compounds. Checkerboard analyses were performed with *P. aeruginosa* PAO1 wild-type (A) and *nfxB** Δ*mexD* (B) strains. Wells with bacterial growth are represented in blue, and wells in which there was no growth are represented in white. Inducer compounds showed an antagonistic effect with ciprofloxacin in strain PAO1, but no antagonistic effect was observed for the *mexD*-deficient strain.

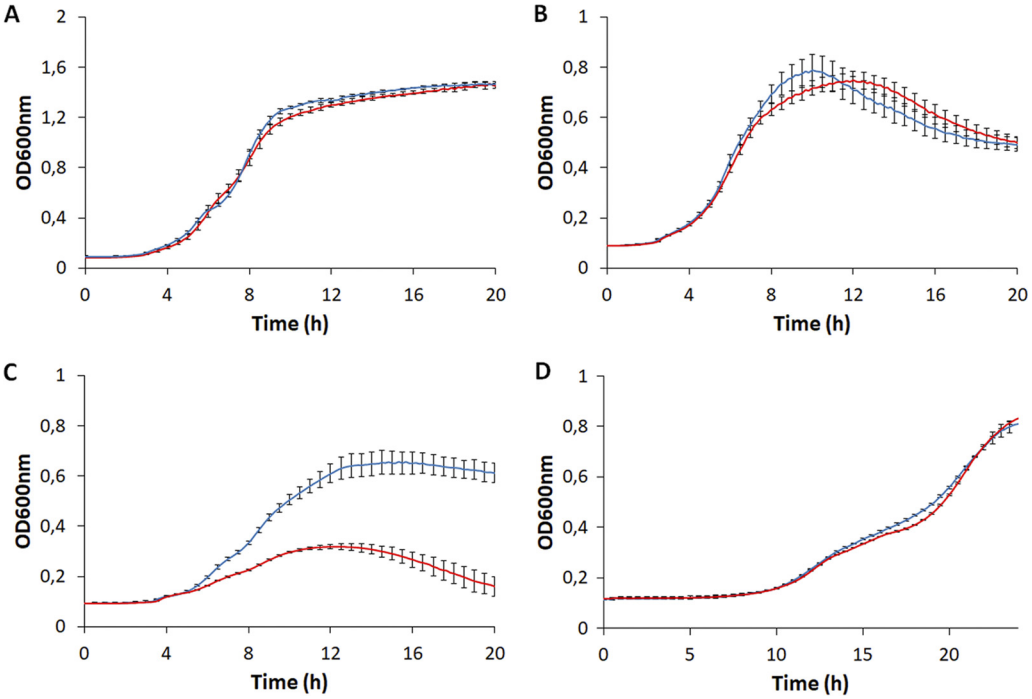


**FIG 8** Effect of MexCD-OprJ on the susceptibility of *P. aeruginosa* to *mexCD-oprJ* inducers. PAO1 wild-type (blue) and *nfxB** Δ*mexD* (red) strains were grown in LB as a control (A) and in the presence of 4 mg/ml of atropine (B), 4 mg/ml of procaine (C), or 20 $\mu$g/ml of dequalinium chloride (D). As shown, the absence of MexCD-OprJ increases *P. aeruginosa* susceptibility to procaine. Each represented $OD_{600}$ value is the average of three biological replicates.

To address the capacity of MexCD-OprJ to extrude its described inducers, the growth of the wild-type *P. aeruginosa* PAO1 and the *mexCD*-deficient *nfxB** Δ*mexD* strain were compared in the presence of a toxic concentration of each inducer (4 mg/ml of atropine, 4 mg/ml of procaine, or 20 $\mu$g/ml of dequalinium chloride). No relevant differences in growth were observed in the presence of dequalinium chloride or atropine in the MexD-deficient strain compared to the wild type. However, growth of the MexD-deficient strain was reduced in the presence of procaine (Fig. 8), indicating that this compound, besides being an inducer, might also be a substrate of the MexCD-OprJ efflux pump.

In summary, the combination of Biolog phenotype microarrays and luminescent biosensor strains has allowed us to describe novel inducers of *P. aeruginosa* efflux pumps, some of which must be carefully taken into consideration in the clinic field, particularly when these compounds and antibiotics are simultaneously applied.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture conditions.** The bacterial strains and plasmids used in this study are listed in Table 3. Unless otherwise stated, all strains were grown in LB (Lennox; Pronadisa) at 37°C and 250 rpm. *Escherichia coli* strains carrying mini-CTX-*lux* ($Tc^r$) or pGEM-T Easy vector-derived plasmids were grown in LB medium with 10 $\mu$g/ml of tetracycline or 100 $\mu$g/ml of ampicillin, respectively.

**Construction of mini-CTX::P*mexAB-lux* and mini-CTX::P*mexCD-lux* reporter plasmids.** To obtain the mini-CTX::P*mexAB-lux* and mini-CTX::P*mexCD-lux* reporter plasmids, a mini-CTX-*lux* ($Tc^r$) plasmid (59) was digested with EcoRI and BamHI (New England BioLabs). The promoter region of *mexAB-oprM* operon was amplified with primers EcoRI_*PmexAB_Fw* and BamHI_*PmexAB_Rv*, while the promoter region of the *mexCD-oprJ* operon was amplified with primers EcoRI_*PmexCD_Fw* and BamHI_*PmexCD_Rv* (Table 5). The products of PCR were purified from an agarose gel by using a DNA purification kit (GE Healthcare) and cloned into the pGEM-T Easy vector according to the supplier's instructions. Afterward, *E. coli* OmniMax competent cells (Invitrogen) were transformed with these plasmids, which were then purified using the QIAprep Spin miniprep kit 250 (Qiagen) and digested with EcoRI and BamHI. The resulting fragments, corresponding to the efflux pump promoters, and the mini-CTX-*lux* ($Tc^r$) plasmid linearized using EcoRI and BamHI were purified from an agarose gel and used to obtain the reporter plasmids, mini-CTX::

**TABLE 5** Primers used in this study

| Primer | Sequence (5′–3′) | Description |
|---|---|---|
| EcoRI_*PmexAB_Fw* | GAATTCTGGTTTGGCCGAGTAAACCT | To amplify the promoter region of *mexAB-oprM* |
| BamHI_*PmexAB_Rv* | GGATCCAGCGTTGTCCTCATGAGCGA | |
| EcoRI_*PmexCD_Fw* | GAATTCCGATGGGTCCCGGTTGGTTT | To amplify the promoter region of *mexCD-oprJ* |
| BamHI_*PmexCD_Rv* | GGATCCGACACACCCGACCGTTGATT | |
| *rpsL_Fw* | CGCAGTGATTGTTACCGGTG | To check DNA contamination from RNA samples |
| *rpsL_Rv* | AGGCCTGAATGCCGGTGATC | |
| *mexC_RTPCR_Fw* | GTGGCGGTATCGAAGTCCT | To amplify *mexC* by real-time PCR |
| *mexC_RTPCR_Rv* | GACCTGCTGTTCCAGATCG | |
| *rplU_RTPCR_Fw* | GCAAGCGCATGGTCGACAAGA | To amplify the housekeeping gene *rplU* by real-time PCR |
| *rplU_RTPCR_Rv* | CGCTGTGCTCTTGCAGGTTGTG | |
| *nfxB_Fw* | CGGCCTCCTGTCGCTCTTCC | To amplify *nfxB* and the intergenic region between this gene and *mexC* |
| *nfxB_Rv* | AGCAACAGCAGGCCAGCCTT | |

P*mexAB-lux* and mini-CTX::P*mexCD-lux*, through a ligation reaction with T4 DNA ligase (New England BioLabs).

**Integration of the reporter plasmids, mini-CTX-*lux*, mini-CTX::P*mexAB-lux* and mini-CTX::P*mexCD-lux* in the chromosome of PAO1 wild-type strain.** The reporter plasmids, mini-CTX::P*mexAB-lux* and mini-CTX::P*mexCD-lux*, in addition to the mini-CTX-*lux*, used as a control plasmid (Table 3), were introduced by transformation into *E. coli* S17-1λ*pir*. Afterward, these constructions were independently inserted into the chromosome of *P. aeruginosa* PAO1 by conjugation by using as the donor strain *E. coli* S17-1λ *pir* harboring each of the plasmids and according to a protocol previously described (60). The *P. aeruginosa* exconjugants carrying the mini-CTX-*lux* (Tc$^r$), mini-CTX::P*mexAB-lux*, or mini-CTX::P*mexCD-lux* reporter constructions integrated into their chromosomes were selected on petri dishes containing *Pseudomonas* isolation agar (Sigma-Aldrich) with 100 μg/ml of tetracycline. The resulting bioreporter strains are PAO1 CTX-*lux*::P*mexAB*, PAO1 CTX-*lux*::P*mexCD*, and PAO1 CTX-*lux* (Table 3).

**Screening of potential inducers of *mexCD-oprJ* and *mexAB-oprM* expression.** The potential ability of 240 compounds to induce *mexAB-oprM* or *mexCD-oprJ* expression was analyzed by using the PAO1 CTX-*lux*::P*mexAB*, PAO1 CTX-*lux*::P*mexCD*, and PAO1 CTX-*lux* strains. For that purpose, these biosensor strains were grown in bacterial chemical susceptibility plates (from PM11C to PM20B) of Phenotype MicroArrays (Biolog), and both the absorbance and the bioluminescence emitted were monitored over time. Then, 100 μl of LB medium was added to each well of 96-well plates, and the plates were incubated for 2 h with agitation at room temperature to dissolve the lyophilized compounds. A volume of 10 μl of cell culture was inoculated into each well to a final $OD_{600}$ of 0.01. Bacteria were grown at 37°C for 20 h, and the $OD_{600}$ and luminescence were measured every 10 min by using a Tecan Infinite 200 plate reader (Tecan). The luminescence and $OD_{600}$ raw data are provided in the supplemental material.

**Normalization of the results.** The luminescence emitted/$OD_{600}$ ratio was calculated for wells in which the growth rate was not impaired, and the resulting values were represented graphically versus time. The area under the curve for each graph was calculated using GraphPad Prism software, giving rise to numeric values that represent the *luxCDABE* expression in PAO1 CTX-*lux*::P*mexCD*, PAO1 CTX-*lux*::P*mexAB*, and PAO1 CTX-*lux* strains when growing in each well. Each numeric value obtained for each tested strain (PAO1 CTX-*lux*::P*mexAB* and PAO1 CTX-*lux*::P*mexCD*) was then normalized by dividing the value corresponding to the same condition for the control strain (PAO1 CTX-*lux*).

The distribution of every normalized luminescence value obtained from each biosensor strain is represented in a box plot in order to determine the threshold from which one value will be considered an indicator of significant induction or repression. This threshold was determined as previously described (40), using the following equations: $Q_3 + 1.5 \times IQR$ for induction or $Q_1 - 1.5 \times IQR$ for repression, where $Q_3$ is the upper quartile, $Q_1$ is the lower quartile, and IQR is the interquartile range for each data set.

**Analyses of potential inducers of *mexCD-oprJ* expression and of *P. aeruginosa* susceptibility to ciprofloxacin.** The growth of *P. aeruginosa* was analyzed by measuring the absorbance (i.e., the $OD_{600}$) of bacterial cultures. First, 10-μl portions of diluted overnight bacterial cultures were added to 140 μl of LB medium with or without 0.25 μg/ml ciprofloxacin, atropine (1 to 8 mg/ml), procaine (1 to 8 mg/ml), or dequalinium chloride (1 to 500 μg/ml) in flat, white, 96-well plates with optical bottoms (Thermo Scientific Nunc) at a final $OD_{600}$ of 0.01. To determine the effect of these potential inducers, the luminescence of either PAO1 CTX-*lux*::P*mexCD* or PAO1 CTX-*lux* reporter strains was measured in the presence or absence of atropine, procaine, or dequalinium chloride. Measurements were taken every 10 min for 20 or 42 h in a plate reader (Tecan Infinite 200) at 37°C. The average of three biological replicates for each strain and condition was used to estimate the absorbance and luminescence values.

**RNA preparation and real-time PCR.** An overnight culture of *P. aeruginosa* PAO1 was used to inoculate Erlenmeyer flasks with 20 ml of LB broth to a final $OD_{600}$ of 0.01. The flasks were incubated at 37°C and 250 rpm until the exponential phase of growth ($OD_{600} = 0.6$) was reached. Then, the optimal concentrations of each tested compound (10 μg/ml of dequalinium chloride, 2 mg/ml of procaine, and 2 mg/ml of atropine) were added to each flask, and cultures were incubated for 90 min with shaking, as previously described (40), to perform the induction assay. Bacterial cultures without any compound or with ethanol, in the case of dequalinium chloride, the solvent of this compound, were used as negative controls, and a *mexCD-oprJ* overexpressing strain (*nfxB**) (Table 3), grown in the absence of inducer, was

used as a control for *mexCD-oprJ* overexpression. When removal of the inducer compounds was necessary, cultures were centrifuged 20 min at 7,000 rpm and then resuspended in the same volume of LB and grown additional for 30, 60, or 120 min. Then, 10 ml of each culture was pelleted by centrifugation at 7,000 rpm and 4°C for 20 min.

The RNA extraction from the collected cells was performed as previously described (61). After a DNase I (Qiagen) treatment, a second treatment with DNase Turbo DNA-free (Ambion) was performed, and the presence of DNA contamination was checked by PCR using the primers *rpsL_Fw* and *rpsL_Rv* (Table 5). By using a high-capacity cDNA reverse transcription kit (Applied Biosystems), cDNA was obtained from 10 $\mu$g of RNA.

Real-time PCR was carried out with a Power SYBR green PCR master mix (Applied Biosystems) in an ABI Prism 7300 real-time system (Applied Biosystems). Portions (50 ng) of cDNA were used in each reaction, except for the wells with no template used as negative controls. A first denaturation step at 95°C for 10 min was followed by 40 cycles at 95°C for 15 s and 60°C for 1 min for amplification and quantification. Primers that amplify specific fragments of *mexC* were used at 400 nM (Table 5). The primers *rplU_RTPCR_Fw* and *rplU_RTPCR_Rv* were used to amplify the housekeeping gene *rplU*. All of the primers used were designed with Primer3 Input software; their specificity was tested by BLAST alignment against *P. aeruginosa* genome from the Pseudomonas Genome Database (62), and their efficiency was analyzed by real-time PCR using serial dilutions of cDNA. Differences in the relative amounts of mRNA were determined according to the $2^{-\Delta\Delta CT}$ method (63). In all cases, the values of relative mRNA expression were determined as the average of three independent biological replicates, each containing three technical replicates.

**Search of genetic modifications in *nfxB*.** To ascertain the absence of putative mutations in *nfxB* or the intergenic region between this gene and *mexC* after the induction assay (see above), this region was amplified by PCR using the primers listed in Table 5. Then, the PCR products were purified using a QIAquick PCR purification kit (Qiagen) and Sanger sequenced at GATC Biotech.

**Determination of the susceptibility to antibiotics of *P. aeruginosa* in the presence of inducers of *mexCD-oprJ* expression.** Ciprofloxacin, fosfomycin, tobramycin, and ceftazidime susceptibility assays were carried out using MIC-test strips (Liofilchem) in Mueller-Hinton agar (Pronadisa) containing either 10 $\mu$g/ml of dequalinium chloride, 2 mg/ml of procaine, 2 mg/ml of atropine, or without any inducer, following the supplier's instructions. Overnight bacterial cultures were normalized to an $OD_{600}$ of 2.5, and a 1:1,000 dilution of each culture was inoculated in the test plates, followed by incubation at 37°C. Growth curves were also recorded using a Tecan Infinite 200 plate reader, as previously described.

**Checkerboard analysis.** Standard checkerboard broth microdilution assays were performed using eight serially diluted concentrations of ciprofloxacin and eight of each inducer compound. First, 140 $\mu$l of LB medium with ciprofloxacin (0.05 to 0.2 $\mu$g/ml), dequalinium chloride (5 to 80 $\mu$g/ml), procaine (1 to 16 mg/ml), or atropine (1 to 16 mg/ml), were added to each well of three different 96-U-well plates. A 10-$\mu$l volume of cell culture was inoculated into each well to a final $OD_{600}$ of 0.01. Bacteria were grown at 37°C for 24 h without sacking. The fraction inhibitory concentration (FIC) of ciprofloxacin, dequalinium chloride, procaine, and atropine was calculated as the MIC of the combination of ciprofloxacin, with each inducer divided by the MIC of each of the drugs alone. The FIC index was calculated as the addition of the FICs of both drugs. An FIC index value of $<0.5$ is considered to indicate synergy, and an FIC index of $>2$ is considered to indicate antagonism.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01095-19.

**SUPPLEMENTAL FILE 1**, XLSX file, 4.8 MB.

## ACKNOWLEDGMENTS

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## Article VI

**Discovery of inhibitors of *Pseudomonas aeruginosa* virulence through the search for natural-like compounds with a dual role as inducers and substrates of efflux pumps**

**Laborda P, Alcalde-Rico M, Chini A, Martínez JL, Hernando-Amado S.**

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Multidrug efflux pumps extrude several molecules outside the bacterial cell: antibiotics, bacterial metabolites, QS signals, biocides, organic solvents, host-produced compounds, heavy metals or plant-produced molecules. Hence, the original function of these ancient elements encoded in all genomes, from bacteria to mammals, may be different from the detoxification of antibiotics during treatment of human infections.

Concerning plant-bacteria interactions, efflux pumps have been described to be involved in facilitating plant colonization by bacteria. This role is mediated by the detoxification of plant-produced anti-virulence compounds. Even more, the expression of efflux pumps encoding genes is induced in presence of such compounds, increasing the cell extrusion capacity, which suggests a coordinated plant-bacteria coevolution.

Since *P. aeruginosa* has an environmental origin, during this work we used the biosensor strains for analysing the expression of *mexCD-oprJ* and *mexAB-oprM* in presence of 1243 compounds from a Natural-Like Product library. Four inducers, two of each studied efflux pump, which were also substrates of the respective pump, were chosen for further analysis. Despite being inducers, these compounds did not trigger transient AR, since they may compete with the antibiotics for their extrusion. Further, the four studied compounds impair swarming motility and exotoxin secretion through the T3SS, consequently reducing the ability of *P. aeruginosa* to kill *Caenorhabditis elegans*. The virulence reduction was caused by a downregulation of genes encoding the flagellum and T3SS in presence of the analysed compounds.

Therefore, departing from a hypothesis based on the original role of a current AR determinant, we determined that new anti-virulence drugs could be found by screening collections of natural libraries searching for compounds which are both inducers and substrates of efflux pumps. This type of compounds, as the ones identified in this thesis, may have clinical application for treating bacterial infections, alone or in combination with antibiotics.

**Specific contributions**:

*Experimental work*: Laborda P., Hernando-Amado S. and Alcalde-Rico M. contributed to experimental work. I performed most of the experimental work of this article, including the screening of potential inducers of efflux pumps encoding genes, analysis of luminescence measurements, antibiotic susceptibility determination, RNA extraction and gene expression measurements, electrophoresis and Western Blot, determination of pyocyanin production, elastase activity, biofilm formation, swarming motility and lethality of *C. elegans*, as well as the interpretation of the results.

*Manuscript writing*: All the authors contributed to the writing and correction of the manuscript, being the first version of the manuscript elaborated by me.

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# Discovery of inhibitors of *Pseudomonas aeruginosa* virulence through the search for natural-like compounds with a dual role as inducers and substrates of efflux pumps

**Pablo Laborda**,[1] **Manuel Alcalde-Rico**,[1,2,3] **Andrea Chini**,[1] **José L. Martínez**[1*] and **Sara Hernando-Amado**[1*]

[1]*Centro Nacional de Biotecnología, CSIC, Darwin 3, Madrid, 28049, Spain.*

[2]*Grupo de Resistencia Antimicrobiana en Bacterias Patógenas y Ambientales (GRABPA), Instituto de Biología, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile.*

[3]*Millennium Nucleus for Collaborative Research on Bacterial Resistance (MICROB-R), Valparaíso, Chile.*

## Summary

**Multidrug efflux pumps are ancient elements encoded in every genome, from bacteria to humans. In bacteria, in addition to antibiotics, efflux pumps extrude a wide range of substrates, including quorum sensing signals, bacterial metabolites, or plant-produced compounds. This indicates that their original functions may differ from their recently acquired role in the extrusion of antibiotics during human infection. Concerning plant-produced compounds, some of them are substrates and inducers of the same efflux pump, suggesting a coordinated plant/bacteria coevolution. Herein we analyse the ability of 1243 compounds from a Natural Product-Like library to induce the expression of *P. aeruginosa mexCD-oprJ* or *mexAB-oprM* efflux pumps' encoding genes. We further characterized natural-like compounds that do not trigger antibiotic resistance in *P. aeruginosa* and that act as virulence inhibitors, choosing those that were not only inducers but substrates of the same efflux pump. Four compounds impair swarming motility, exotoxin secretion through the Type 3 Secretion System (T3SS) and the ability to kill *Caenorhabditis elegans*, which might be explained by the downregulation of genes encoding flagellum and T3SS. Our results emphasize the possibility of discovering new anti-virulence drugs by screening natural or natural-like libraries for compounds that behave as both, inducers and substrates of efflux pumps.**

Received 15 January, 2021; revised 25 March, 2021; accepted 3 April, 2021. *For correspondence. E-mail jlmtnez@cnb.csic.es, shernando@cnb.csic.es; Tel.+34 915854542; Fax: +34915854506.



## Introduction

Besides being a major cause of infections in hospitals, *Pseudomonas aeruginosa* is the main cause of chronic infections in patients with cystic fibrosis and chronic obstructive pulmonary diseases (Martinez-Solano *et al*., 2008; Tummler *et al*., 2014). Poor clinical outcome due to the low susceptibility of *P. aeruginosa* to antibiotics has been observed, placing this bacterium among those considered a high risk concerning antibiotic resistance (Boucher *et al*., 2009; Pendleton *et al*., 2013; Rello *et al*., 2019). In this bacterial species, multidrug efflux pumps are relevant elements contributing to both intrinsic and acquired resistance (Ziha-Zarifi *et al*., 1999; Breidenstein *et al*., 2011; Nikaido, 2011; Garcia-Leon *et al*., 2014a; Hernando-Amado *et al*., 2016). Namely, MexAB-OprM and MexCD-OprJ stand out among such group of antibiotic resistance determinants in *P. aeruginosa* (De Kievit *et al*., 2001; Poole, 2001) being able to extrude and significantly contribute to the resistance to macrolides, quinolones, lincomycin, novobiocin, chloramphenicol and β-lactams or tetracyclines, quinolones and chloramphenicol respectively (Poole *et al*., 1996a; Masuda *et al*., 2000b). *mexAB-oprM* encoding genes are constitutively expressed, contributing to the intrinsic resistance of *P. aeruginosa* (Laborda *et al*., 2019), while MexCD-OprJ has a minor role in intrinsic resistance. MexCD-OprJ overexpression is usually achieved by mutations in *nfxB*, encoding its local repressor (Poole *et al*., 1996b), and leads to acquisition of antibiotic resistance. In addition, both efflux pumps may contribute to inducible, non-inheritable, resistance to antibiotics (Laborda *et al*., 2019).

Although efflux pumps have been extensively studied as relevant elements of both acquired and intrinsic antibiotic resistance (Ziha-Zarifi *et al*., 1999; Breidenstein *et al*., 2011; Nikaido, 2011; Garcia-Leon *et al*., 2014a; Hernando-Amado *et al*., 2016), they also play fundamental roles in bacterial physiology, such as the modulation of quorum sensing (QS) signalling (Evans *et al*., 1998; Olivares *et al*., 2012; Alcalde-Rico *et al*., 2018; Alcalde-Rico *et al*., 2020), response to stress situations (Poole, 2014; Alcalde-Rico *et al*., 2016), or plant–bacteria interactions (Martinez *et al*., 2009; Garcia-Leon *et al*., 2014b; Blanco *et al*., 2016). Regarding the last one, the role of efflux pumps in flavonoids-mediated signalling in bacteria is a good example of Red-Queen adaptive coevolution phenomenon: while plant-produced flavonoids inhibit virulence of *Pseudomonas syringae* (Vargas *et al*., 2011; Vargas *et al*., 2013), efflux pumps of this bacterium, such as MexAB-OprM, are induced by these compounds and are able to extrude them, counteracting their inhibitory effect. In other species, such as *Erwinia amylovora* or *Agrobacterium tumefaciens*, some flavonoids, which are both inducers and substrates of efflux pumps (Palumbo *et al*., 1998; Burse *et al*., 2004), also regulate the capacity of these species to colonize plants. The coordinated plant–bacteria coevolution exemplified in the above-described interactions supports that bacterial efflux pumps may sometimes have a role in plants colonization by the extrusion of plant-derived compounds with putative anti-virulence properties.

The use of anti-virulence drugs has been proposed as an interesting possibility to overcome the problems of antibiotic resistance (Rasko and Sperandio, 2010; Alford *et al*., 2019). In the case of *P. aeruginosa*, most efforts have focused on the inhibition of the QS system (D'Angelo *et al*., 2018; Soukarieh *et al*., 2018). We propose that a better understanding of the ecological conditions and elements that modulate the virulence of this pathogen may help to find inhibitors of virulence. In this regard, it is worth noting that, besides being a high-risk human opportunistic pathogen, *P. aeruginosa* is also a plant pathogen (Azam and Khan, 2019). In agreement with previous findings (Azuama *et al*., 2020; Tahrioui *et al*., 2020), we hypothesized that plant-derived anti-virulence molecules could be found that may be of biomedical interest to counteract *P. aeruginosa* infections.

In this work, based on the evolution of *P. aeruginosa* as a consequence of its interactions with plants, we searched for natural-like molecules that could be efflux pumps' inducers and hence, likely inhibitors of *P. aeruginosa* virulence (Alcalde-Rico *et al*., 2016; Blanco *et al*., 2016), as a result of the ancient adaptation of this bacterium to natural environments. However, we are aware that such inducers could also trigger antibiotic resistance (Hernando-Amado *et al*., 2016), which may compromise their utility. Consequently, we focused on molecules that behave as both, inducers and substrates of *P. aeruginosa* efflux pumps; in particular, of MexAB-OprM or MexCD-OprJ. The hypothesis behind was that these molecules might reduce virulence, while their extrusion might outcompete antibiotics' efflux, hence not increasing antibiotic resistance in *P. aeruginosa*. By the screening of a Natural Product-Like library, we identified four compounds that disrupt bacterial motility, reduce the secretion of the exotoxin ExoS through the Type 3 Secretion System (T3SS) or decrease the capacity to kill the *Caenorhabditis elegans* animal model. Here we describe a proof-of-concept study showing that this approach is a robust strategy to identify novel effectors of MDR efflux pumps, a subset of which present anti-virulence properties against *P. aeruginosa*.

## Results and discussion

### *Screening for inducers of* mexCD-oprJ *and* mexAB-oprM *expression using a Natural Product-Like library*

The effect on the expression of either *mexAB-oprM* or *mexCD-oprJ* of 1243 compounds from a Natural Product-Like library (Supplemental Table S1), including an array of natural-like scaffolds (Boldi, 2004; Fonseca *et al*., 2014), and a collection of 43 natural and synthetic auxins, was tested using the biosensor strains PAO1 CTX-*lux*::P*mexAB* and PAO1 CTX-*lux*::P*mexCD* (Laborda *et al*., 2019) (Supplemental Table S2). The luminescence emitted by each biosensor strain in presence of 100 μM of each compound was normalized with those emitted by the control strain (PAO1 CTX-*lux*), as described in Experimental procedures. Figure 1 shows the distribution of the normalized values for each reporter strain and the induction threshold of the expression of each efflux pump, indicating the outliers of each boxplot.

The induction threshold values of *mexAB-oprM* and *mexCD-oprJ* were 1.60 and 1.91 respectively. Thirty-one and eight inducers of *mexCD-oprJ* and *mexAB-oprM* respectively were detected (Supplemental Table S3). Approximately, one-third of the potential inducers of both efflux pumps were coumarin-like compounds, several *mexCD-oprJ* inducers presented a 1,4-benzodioxan-like structure and an indole-derivative was found to induce *mexAB-oprM* expression. Coumarins and benzodioxans are compounds with bioactive functions that are present in different plants (Pilkington and Barker, 2015; Sarker and Nahar, 2017). Indeed, more than a thousand coumarins have been so far described and some of them have been used as anticoagulants and, more recently, as antioxidants, anti-inflammatory and anti-cancer compounds, highlighting their role as bioactive compounds with therapeutic potential (Aadil and Vikrant, 2020). For their part,

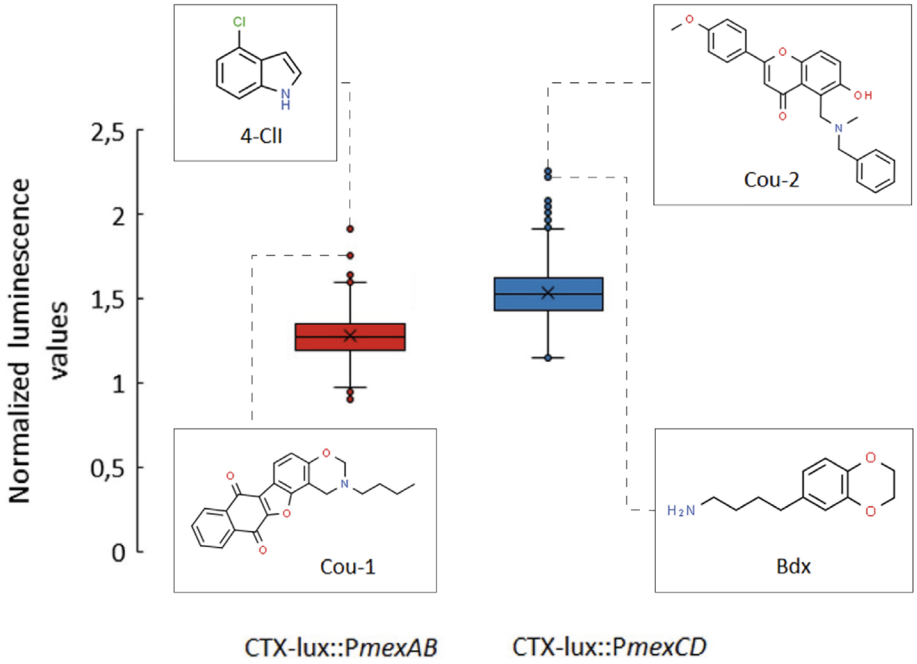


**Fig 1.** Effect of compounds from a Natural Product-Like library on the expression of either *mexAB-oprM* or *mexCD-oprJ*. The normalized luminescence values produced by PAO1 CTX-*lux*::P*mexAB* and PAO1 CTX-*lux*::P*mexCD* in the presence of 1243 different compounds are shown in the figure. Overexpression is represented by the upper outliers of the boxplot: values greater than 1.60 and 1.91 for PAO1 CTX-*lux*::P*mexAB* and PAO1 CTX-*lux*::P*mexCD* respectively. The normalized luminescence value obtained for each compound and each biosensor strain are included in Supplemental Table S1. The chemical structures of 4-Chloroindole: 4-CII, 2-Butyl-2,3-dihydro-1H-naphtol[2′,3′:4,5]furo[2,3-f][1,3] benzoxazine-7,12-dione (Cou-1), 5-([Benzyl(methyl)amino]methyl)-6-hydroxy-2-(4-methoxyphenyl)-4H-chromen-4-one (Cou-2), and 4-(2,3-Dihydro-1,4-benzodioxin-6-yl)-1-butanamine (Bdx), were obtained from ChemSpider database (http://www.chemspider.com).

indoles and indole derivatives present an important role as plant hormones (Leyser, 2018).

Overall, the natural-like molecules identified with capacity to trigger the expression of *mexCD-oprJ* or *mexAB-oprM* in *P. aeruginosa* can be classified into three different groups based on their structure: coumarin-like (Cou) and 1,4-benzodioxan-like (Bdx), and a third heterogeneous group which includes 4-Chloroindole (4-CII). Representative compounds of each group, producing high normalized luminescence values, were selected among all detected inducers for further analysis (Fig. 1). Two compounds with Cou structure were further analysed as potential inducers of *mexAB-oprM* or *mexCD-oprJ* respectively: 2-Butyl-2,3-dihydro-1H-naphtol [2′,3′:4,5]furo[2,3-f][1,3]benzoxazine-7,12-dione (Cou-1) and 5-([Benzyl(methyl)amino]methyl)-6-hydroxy-2-(4-methoxyphenyl)-4H-chromen-4-one (Cou-2). We also selected a Bdx compound, 4-(2,3-Dihydro-1,4-benzodioxin-6-yl)-1-butanamine (Bdx), as a potential inducer of *mexCD-oprJ*, and 4-CII as a possible inducer of *mexAB-oprM*. In order to simplify nomenclature throughout the manuscript, these compounds are named: Cou-1, Cou-2, Bdx and 4-CII.

### *Validation of the inductive capacity of Cou-1, Cou-2, Bdx and 4-CII*

In order to confirm the inductive capacity of Cou-1, Cou-2, Bdx and 4-CII, the normalized luminescence values produced by the reporter strains PAO1 CTX-*lux*::P*mexAB* and PAO1 CTX-*lux*::P*mexCD*, grown at concentrations from 50 to 400 μM of their respective inducer compound, were measured. As shown in Fig. 2, the compounds significantly induce the expression of the tested efflux pumps' encoding genes in a concentration-dependent manner.

To further confirm such induction, the expression level of *mexAB-oprM* and *mexCD-oprJ* was quantified by quantitative reverse transcription PCR (qRT-PCR) in a wild-type *P. aeruginosa* PAO1 strain grown in the absence or presence of 100 μM of the respective cognate inducer compounds. In addition, overexpressing mutants of either *mexAB-oprM* (*mexR**) or *mexCD-oprJ* (*nfxB**) (see Supplemental Table S2) were used as controls of overexpression. The expression of *mexAB-oprM* was significantly increased 2.8- and 2-fold in the presence of 4-CII or Cou-1 respectively, while the expression of *mexCD-oprJ* was significantly increased 7.6- and 11-fold

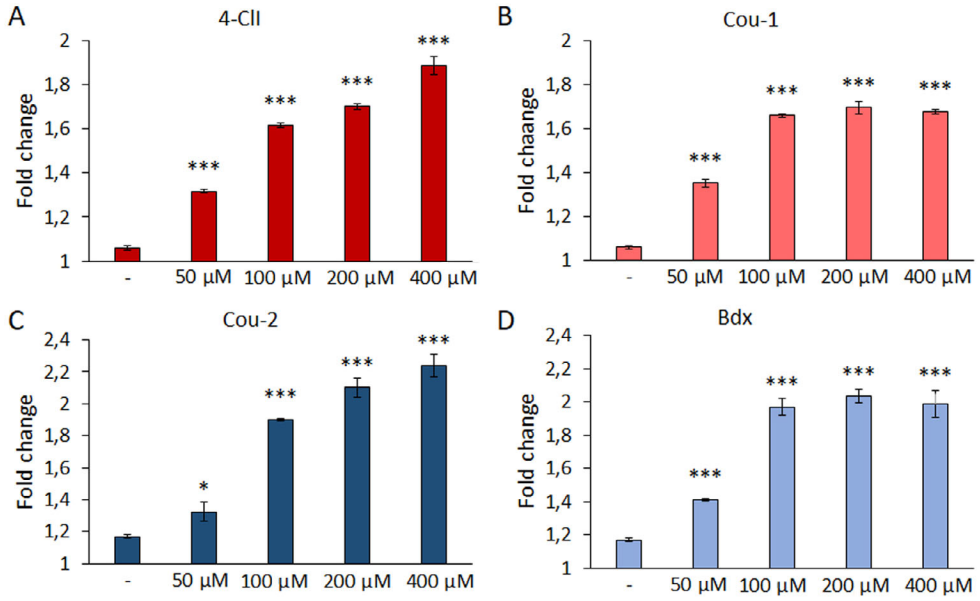


**Fig 2.** Effect of 4-CII or Cou-1 and Cou-2 or Bdx in the expression of *mexAB-oprM* or *mexCD-oprJ* respectively. Normalized luminescence values produced by reporter strains PAO1 CTX-*lux*::P*mexAB* (A and B) and PAO1 CTX-*lux*::P*mexCD* (C and D) grown at concentrations from 50 to 400 µM of 4-CII or Cou-1 and Cou-2 or Bdx respectively, are shown in the figure. The luminescence values were normalized to those produced by the PAO1 CTX-*lux* control strain grown in the same conditions. Error bars represent standard deviations of three independent replicates. Statistically significant differences regarding the biosensor strain with no inducer compound were calculated with *t*-test for paired samples assuming equal variances: $*p < 0.05$; $**p < 0.005$; $***p < 0.0005$.

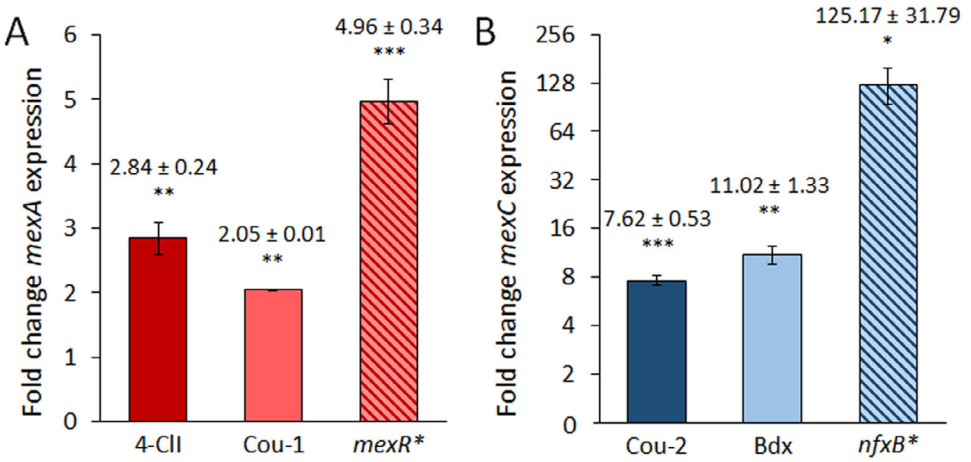


**Fig 3.** Analysis of *mexAB-oprM* and *mexCD-oprJ* expression by qRT-PCR in the presence of natural-like inducers.
A. *mexA* expression was measured after 90 min of incubation with 100 µM of 4-CII or Cou-1.
B. *mexC* expression was measured after 90 min of incubation with 100 µM of Cou-2 or Bdx. The *mexR** and *nfxB** strains (Supplemental Table S2) grown in the absence of any inducer were used as a control of overexpression of *mexA* and *mexC* respectively. Fold changes were calculated regarding the expression in *P. aeruginosa* PAO1 with the compounds' solvent. As shown, expression of *mexAB-oprM* and *mexCD-oprJ* is induced by the tested compounds. Each represented value is the average of three biological replicates. Statistically significant differences regarding PAO1 with no inducer compound were calculated with *t*-test for paired samples assuming equal variances: $*p < 0.05$; $**p < 0.005$; $***p < 0.0005$.

in presence of Cou-2 or Bdx respectively (Fig. 3). These results confirm that the expression of *mexAB-oprM* and of *mexCD-oprJ* is induced by the compounds selected in the screening.

*Cou-1, Cou-2, Bdx and 4-CII produce a minor effect in transient antibiotic resistance mediated by MexAB-OprM and MexCD-OprJ*

We analysed the effect of the induction of the efflux pumps MexAB-OprM and MexCD-OprJ by the identified compounds on the susceptibility of *P. aeruginosa* to antibiotics that are substrates of the studied efflux pumps, such as β-lactams, substrates of MexAB-OprM, and quinolones, tetracycline or chloramphenicol, substrates of both efflux pumps (Masuda *et al*., 2000b). For that, we determined the minimal inhibitory concentration of antibiotics in the presence of 100 μM of Cou-1, Cou-2, Bdx or 4-CII, or the same amount of solvent (DMSO). Regarding the inducers of *mexAB-oprM*, no increase in resistance was observed for the β-lactam ceftazidime, chloramphenicol, tetracycline or the quinolones ciprofloxacin, ofloxacin or levofloxacin, and a minor increase was observed for the β-lactam aztreonam (Table 1). In the case of the inducers of *mexCD-oprJ*, a minor increase of resistance to ciprofloxacin and to ofloxacin was observed while there was no increase of resistance to β-lactams, chloramphenicol or tetracycline (Table 1). No effect on resistance to fosfomycin, imipenem, tobramycin or amikacin which are not substrates of these efflux pumps, was observed (Table 1). These results indicate that the analysed compounds promote a minor transient reduction of *P. aeruginosa* susceptibility to specific antibiotics. This feature differs from the findings concerning previously characterized inducers of the expression of these efflux pumps (Laborda *et al*., 2019), in which a higher transient antibiotic resistance occurred. These differences in the observed phenotypes upon induction might be explained by two reasons: the lower increase of the expression of *mexAB-oprM* and *mexCD-oprJ* in the presence of the natural-like compounds here identified (Fig. 3) compared with the ones previously described (Laborda *et al*., 2019), and/or a possible competition between inducers and antibiotics, both substrates of the pumps, thus making it difficult for the latter to be extruded, an hypothesis that led us to further study this possibility.

*MexAB-OprM or MexCD-OprJ efflux pumps extrude Cou-1 and Cou-2 or Bdx and 4-CII respectively*

Several inducers of the expression of genes encoding efflux pumps have been found to also be substrates of these antibiotic resistance determinants (Rosenberg *et al*., 2003; Hernandez *et al*., 2011). For instance, in *P. aeruginosa*, procaine and some aminoglycosides are inducers and substrates of MexCD-OprJ (Laborda *et al*., 2019) and MexXY-OprM (Masuda *et al*., 2000a) respectively. However, other efflux pump inducers, such as dequalinium chloride in the case of MexCD-OprJ in *P. aeruginosa* (Laborda *et al*., 2019), or selenite and chloramphenicol in the case of SmeVWX and SmeYZ in *Stenotrophomonas maltophilia* (Blanco *et al*., 2018), are not substrates of these efflux pumps.

To address the ability of MexAB-OprM and MexCD-OprJ to extrude the studied inducers, the growth of the wild-type *P. aeruginosa* PAO1, of a mutant defective in MexCD-OprJ (*nfxB** Δ*mexD*) (Alcalde-Rico *et al*., 2018) and of a mutant defective in MexAB-OprM (Δ*mexB*) (see Experimental procedures) (Supplemental Table S2) were compared in the presence of toxic amounts of the compounds able to induce the expression of these two efflux systems: 4-CII (400 μM) or Cou-1 (600 μM) and Cou-2 (600 μM) or Bdx (600 μM) respectively. The growth of both mutants was significantly reduced, compared with the wild-type strain, in the presence of their respective inducers (Fig. 4), indicating that their toxicity is dependent on the presence of each efflux pump. Therefore, 4-CII or

**Table 1.** MIC (μg ml$^{-1}$) values of *P. aeruginosa* PAO1, *nfxB** and *mexR** to different antibiotics, in presence or absence of 100 μM of inducer compounds.

| | PAO1 | | | | | *nfxB** | *mexR** |
|---|---|---|---|---|---|---|---|
| | – | Cou-1 | 4-CII | Cou-2 | Bdx | – | – |
| Ciprofloxacin[a,b] | 0.2 | 0.2 | 0.2 | 0.4 | 0.4 | 3.2 | 1.6 |
| Ofloxacin[a,b] | 0.5 | 0.5 | 0.5 | 1 | 1 | 8 | 4 |
| Levofloxacin[a,b] | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 4 | 2 |
| Aztreonam[b] | 8 | 16 | 8 | 8 | 8 | 4 | 64 |
| Ceftazidime[b] | 8 | 8 | 8 | 8 | 8 | 2 | 32 |
| Imipenem | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Fosfomycin | 120 | 120 | 120 | 120 | 120 | 120 | 120 |
| Chloramphenicol[a,b] | 96 | 96 | 96 | 96 | 96 | 192 | 384 |
| Tobramycin | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Amikacin | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Tetracycline[a,b] | 24 | 24 | 24 | 24 | 24 | 48 | 96 |

[a]Substrate of MexCD-OprJ efflux pump.
[b]Substrate of MexAB-OprM efflux pump.

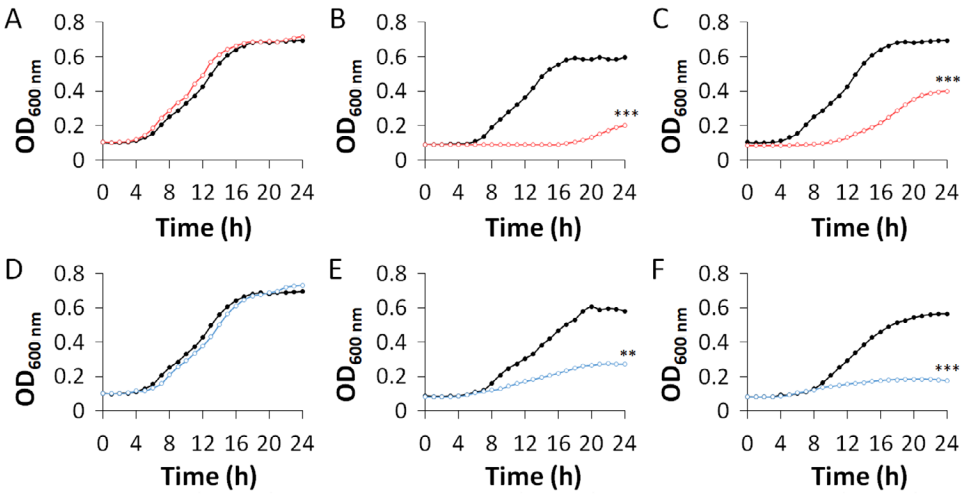


**Fig 4.** Effect of MexAB-OprM and MexCD-OprJ activity on the susceptibility of *P. aeruginosa* to their natural-like inducers. The figure shows growth curves of a deletion mutant of either *mexB* (top of the Figure) or *mexD* (bottom of the figure). Δ*mexB* strain (open circles) and PAO1 wild-type strain (filled circles) were grown in presence of the compounds' solvent as a control (A), in the presence of 400 μM of 4-CII (B) or 600 μM of Cou-1 (C). *nfxB** Δ*mexD* (open circles) and PAO1 strain (filled circles) were grown in presence of the compounds' solvent as a control (D), in the presence of 600 μM of Cou-2 (E) or 600 μM of Bdx (F). Strains lacking MexAB-OprM or MexCD-OprJ presented an increased susceptibility to their own inducers, indicating that these compounds are also substrates of these efflux pumps. Each represented $OD_{600nm}$ value is the average of three biological replicates. Statistically significant differences regarding PAO1 wild-type strain were calculated with ANOVA test: $^{**}p < 0.005$; $^{***}p < 0.0005$.

Cou-1 and Cou-2 or Bdx are also substrates of MexAB-OprM and MexCD-OprJ respectively.

### *Cou-1, Cou-2, Bdx and 4-CII are inhibitors of virulence-related phenotypes in* P. aeruginosa

It has been described that bacterial efflux pumps may play essential roles in bacterial colonization of plant hosts, particularly when they extrude plant-derived compounds whose function is to reduce bacterial colonization or infection (Martinez *et al*., 2009). Therefore, we hypothesized that the extrusion of Cou-1, Cou-2, Bdx and 4-CII by the studied efflux pumps might be a detoxification mechanism of *P. aeruginosa* to tackle a potential plant-driven virulence inhibition carried out by these natural-like compounds. In other words, the natural-like compounds here described, which are both inducers and substrates of the mentioned efflux pumps, could be inhibitors of *P. aeruginosa* virulence. In favour of this possibility is the previous finding that a coumarin impairs *P. aeruginosa* virulence and biofilm formation by affecting QS and T3S (Zhang *et al*., 2018), although the concentrations tested in this previous work were much higher than those used in the present study. To validate our hypothesis, we analysed the effect of the selected compounds on some well-known virulence-related processes of *P. aeruginosa*, such as motility, T3SS-dependent secretion of the exotoxin ExoS, pyocyanin production, elastase activity, biofilm formation and *P. aeruginosa* killing of *C. elegans*. The results showed that these compounds led to a strong reduction of swarming motility (Fig. 5) and of the amount of secreted ExoS protein through the T3SS (Fig. 6) in PAO1 wild-type strain, being the inducers of *mexAB-oprM*, 4-CII and Cou-1, the stronger inhibitors of both phenotypes.

It has been previously described that mutants overexpressing either *mexAB-oprM* or *mexCD-oprJ* have a low production of QS-controlled virulence factors (Sanchez *et al*., 2002;Alcalde-Rico *et al*., 2018; Alcalde-Rico *et al*., 2020), such as pyocyanin, elastase and biofilm formation. However, we observed that these natural-like inducer compounds do not produce a strong effect on the mentioned QS-related phenotypes in the *P. aeruginosa* wild-type strain, at least at the concentrations tested (Fig. 7). Furthermore, swarming motility was also strongly impaired in the presence of the studied compounds in the efflux pumps' overexpressing mutants, while motility of the efflux pumps' overexpressing strains, in the absence of inducers, was slightly affected compared to the one of the wild-type strain (Fig. 5). These results strongly suggest that the inhibitory effect of Cou-1, Cou-2, Bdx and 4-CII over *P. aeruginosa* virulence does not just depend on the impaired QS response known to be associated with the overexpression of these efflux pumps (Alcalde-Rico *et al*., 2018; Alcalde-Rico *et al*., 2020). Overall, these findings reinforce the hypothesis that the observed *mexAB-oprM* and *mexCD-oprJ* induction, and the consequent extrusion of these


**Fig 5.** Effect of 4-CII, Cou-1, Cou-2 and Bdx on swarming motility of *P. aeruginosa* PAO1, *mexR** and *nfxB**. Motility assay of *P. aeruginosa* PAO1, *mexAB-oprM* overexpressing strain (*mexR**) and *mexCD-oprJ* overexpressing strain (*nfxB**) in presence of 100 μM of the natural-like inducers or the same amount of their solvent is shown. Pictures were taken after 17 h of incubation at 37°C. The studied compounds strongly inhibit swarming motility of both, the wild-type strain and efflux pumps' overexpressing strains. The numbers inside each plate represent the areas ($cm^2$) covered by bacteria in each condition were measured with ImageJ software and are the average of three replicates. Statistically significant differences regarding the respective strain with no inducer compound were calculated with *t*-test for paired samples assuming equal variances and all differences were statistically significant: $p < 0.0005$.

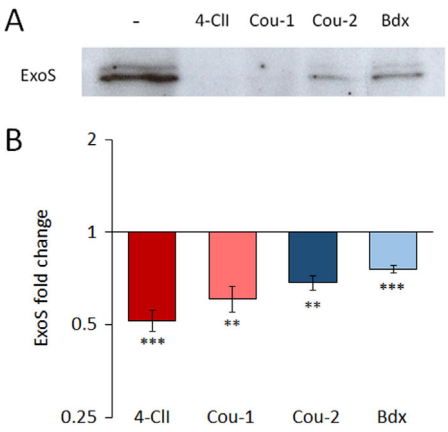


**Fig 6.** Effect of 4-CII, Cou-1, Cou-2 and Bdx on the Type 3 Secretion capacity of *P. aeruginosa*.
A. The amount of ExoS in the supernatant of bacterial cultures grown in presence of 100 μM of 4-CII, Cou-1, Cou-2 or Bdx or the same amount of their solvent was analysed by Western blot, as described in Experimental procedures. As shown, the T3SS-mediated secretion of ExoS is reduced in PAO1 strain in the presence of the four compounds, being 4-CII and Cou-1 the stronger inhibitors of this phenotype.
B. Intensity of the bands was measured with ImageJ software and the fold-change of the value recorded in presence of 4-CII (dark red), Cou-1 (light red), Cou-2 (dark blue) and Bdx (light blue) regarding the wild-type strain was calculated. Each represented value is the average of three biological replicates. Statistically significant differences regarding PAO1 with no inducer compound were calculated with a *t*-test for paired samples assuming equal variances: $^{**}p < 0.005$; $^{***}p < 0.0005$.

natural-like compounds, could be a mechanism to overcome more general inhibitory effects on *P. aeruginosa* virulence.

Finally, in order to determine if these compounds could also impair *P. aeruginosa* virulence in an animal infection model, we performed a *C. elegans* killing assay in the presence of the mentioned compounds or the same amount of their solvent, as a control. As shown in Fig. 8, while an 80% of the population of *C. elegans* were killed by *P. aeruginosa* after 48 h of infection, the presence of 4-CII, Cou-1, Cou-2 or Bdx significantly reduced lethality to 28%, 44%, 34% or 54% respectively.

### *Cou-1, Cou-2, Bdx and 4-CII reduce the expression of* P. aeruginosa *virulence determinants*

Once the virulence-related phenotypes of *P. aeruginosa* affected by the studied natural-like compounds were determined, we measured the expression of genes encoding different virulence determinants by qRT-PCR. In particular, genes coding for proteins related with QS, flagellum T3SS (Veesenmeyer *et al*., 2009)and Type 6 Secretion System (T6SS) (Chen *et al*., 2015). The expression of the genes encoding the master regulators of QS, *lasR* and *rhlR*, and of the genes encoding its effectors, *lasI* and *rhlI* (Veesenmeyer *et al*., 2009), was analysed. The expression of *fliC*, which encodes a

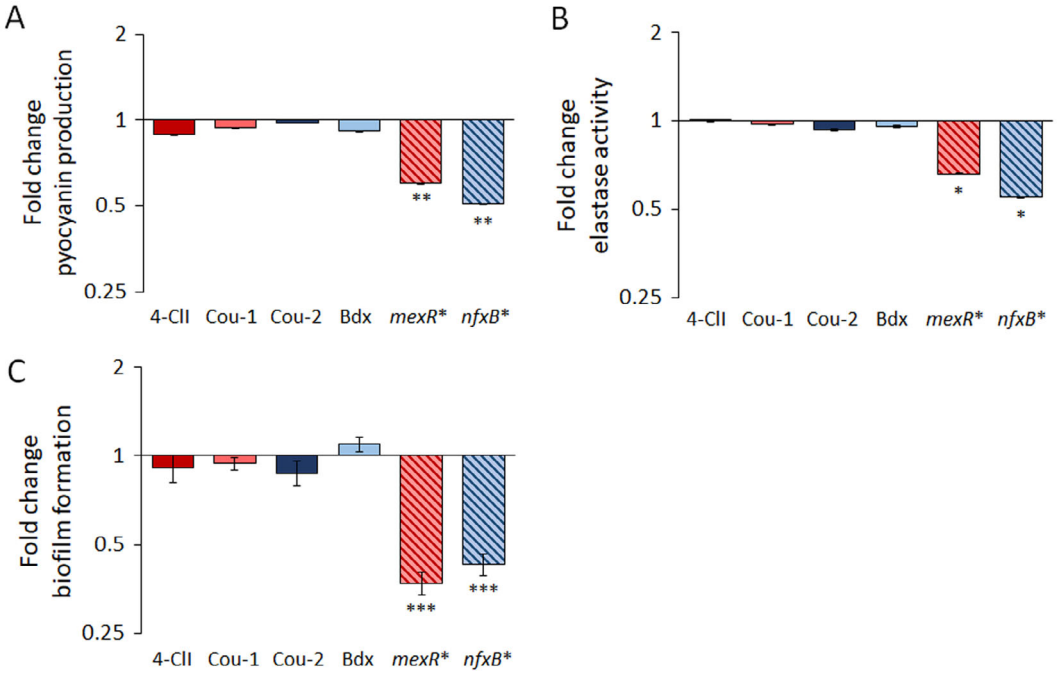


**Fig 7.** Effect of 4-CII, Cou-1, Cou-2 and Bdx on different processes involved in the virulence of *P. aeruginosa*. Fold change of (A) pyocyanin production, (B) elastase activity and (C) biofilm formation in *P. aeruginosa* PAO1 growing in presence of 4-CII (dark red), Cou-1 (light red), Cou-2 (dark blue) and Bdx (light blue), or in *mexAB-oprM* (red stripes) and *mexCD-oprJ* (blue stripes) overexpressing strains growing in presence of their solvent, are represented. The fold changes were calculated regarding the value of the wild-type strain in presence of the solvent. No significant changes in pyocyanin production, elastase activity or biofilm formation were detected in the presence of the studied compounds, while a significantly reduced pyocyanin production, elastase activity and biofilm formation were detected in the efflux pumps' overexpressing strains. Error bars indicate standard deviations of the measured values from three independent replicates for pyocyanin production and elastase activity and from eight independent experiments in the biofilm formation assay. Statistically significant differences regarding PAO1 growth with no inducer compound were calculated with *t*-test for paired samples assuming equal variances: $*p < 0.05$; $**p < 0.005$; $***p < 0.0005$.

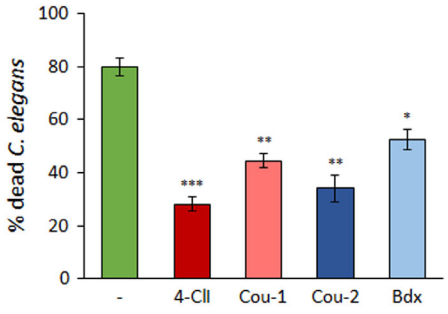


**Fig 8.** Effect of 4-CII, Cou-1, Cou-2 and Bdx on the killing capacity of *P. aeruginosa* in a *C. elegans* model system. Percentage of dead nematodes after 48 h of infection with *P. aeruginosa* PAO1 wild-type strain with 100 μM of 4-CII (dark red), Cou-1 (light red), Cou-2 (dark blue), Bdx (light blue) or the same amount of their solvent (green) is represented. As shown, the presence of the natural-like compounds significantly reduced the percentage of dead nematodes after 2 days of infection with *P. aeruginosa*, from 80% up to 28%, 44%, 34% or 54% in presence of 4-CII, Cou-1, Cou-2 or Bdx respectively. Results are the average of three independent replicates. Statistically significant differences in the survival of nematodes regarding PAO1 with no inducer compound were determined with *t*-test for paired samples assuming equal variances: $*p < 0.05$; $**p < 0.005$; $***p < 0.0005$.

protein that constitutes the flagellar filament, and of *fleQ*, the gene encoding the master regulator of the flagellar assembly (Dasgupta *et al*., 2003), was also measured. Concerning T3SS, the expression of *exsA*, encoding the T3SS master regulator, of *exoS*, encoding a translocated toxin which prevents phagocytosis and produces cytotoxicity, of *pscF*, encoding the protein which forms the needle filament, and of *popB*, encoding a protein which forms the translocon (Yahr and Wolfgang, 2006; Hauser, 2009), was measured. Finally, the expression of *clpV1*, *clpV2* and *clpV3*, as representatives of the expression of each of the three operons encoding proteins of the T6SS in *P. aeruginosa* (Chen *et al*., 2015), was analysed.

As shown in Fig. 9A, expression of *fliC* was reduced in presence of all the compounds, which may explain the strongly affected motility caused by these compounds (Fig. 5). No significant expression change was detected for QS- and T6SS-related genes (Fig. 9B and C), while a reduced expression of *exsA*, *exoS*, *pscF* and *popB* was detected (Fig. 9D). Even more, a significant reduction of

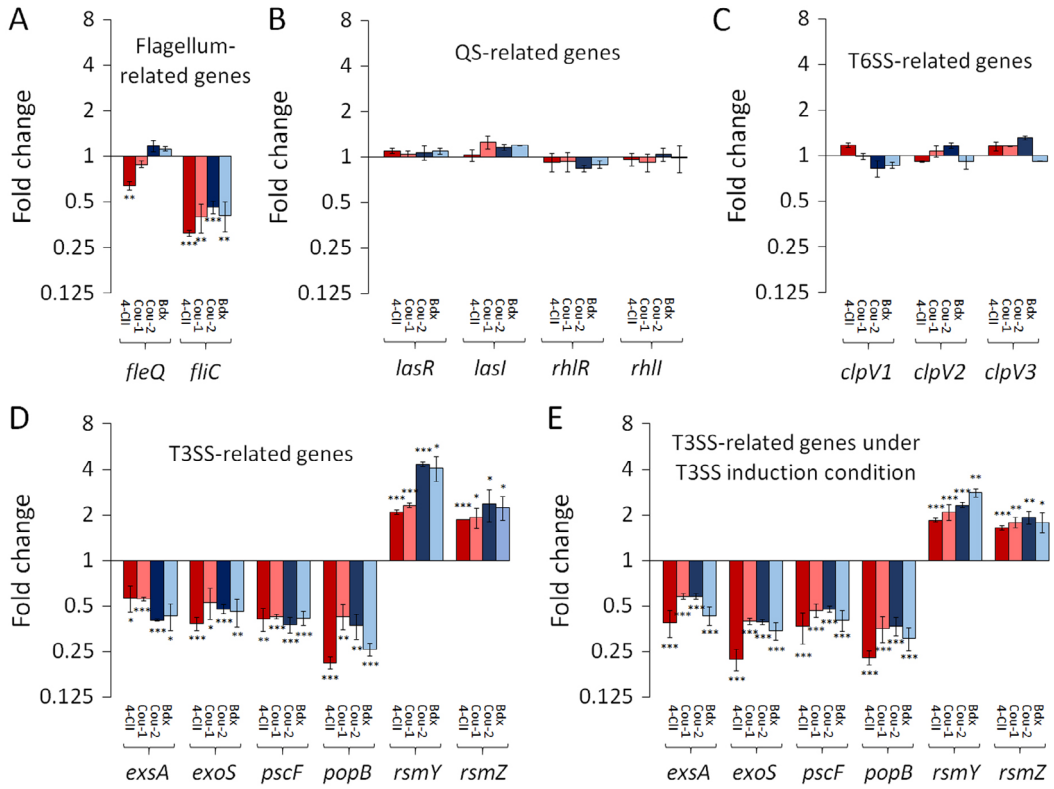


**Fig 9.** Analysis of the expression of virulence-related genes by qRT-PCR in the presence of 4-CII, Cou-1, Cou-2 and Bdx. Expression of (A) flagellum-related genes, (B) QS-related genes, (C) T6SS-related genes, (D) T3SS-related genes and its regulators through the GacS-GacA two-component signal transduction system was measured by qRT-PCR after 90 min of incubation with 100 μM of 4-CII (dark red), Cou-1 (light red), Cou-2 (dark blue), Bdx (light blue) or the same amount of their solvent. (E) T3SS-related genes' expression under T3SS induction conditions (see Experimental procedures) and in presence of 100 μM of 4-CII (dark red), Cou-1 (light red), Cou-2 (dark blue), Bdx (light blue) or the same amount of their solvent, was also measured by qRT-PCR. Fold changes were calculated regarding the expression in *P. aeruginosa* PAO1 grown in the presence of the solvent. As shown, expression of flagellum and T3SS-related genes, under T3SS inducing and non-inducing condition, are reduced by the four tested compounds. Each represented value is the average of three biological replicates. Statistically significant differences regarding PAO1 with no inducer compound were calculated with *t*-test for paired samples assuming equal variances: $*p < 0.05$; $**p < 0.005$; $***p < 0.0005$.

the expression of the T3SS-related genes was also detected under T3SS-inducing conditions (see Experimental procedures) in the presence of the studied compounds (Fig. 9E), which may explain the reduced bacterial secretion of ExoS when growing in presence of Cou-1, Cou-2, Bdx or 4-CII (Fig. 6).

Under inducing conditions, the expression of the T3SS is triggered by ExsA, whose expression is induced by RsmA. For its part, the activity of RsmA is repressed by the small RNAs *rsmY* and *rsmZ*, whose expression is controlled by the GacS-GacA two-component signal transduction system (Yamazaki *et al*., 2012). Besides, this signalling system also controls *Pseudomonas fluorescens* motility (Navazo *et al*., 2009). To further delve into the causes of the decreased expression of T3SS-related genes under inducing conditions, the expression of *rsmY* and *rsmZ* was measured in the presence and absence of the tested compounds. As shown in Fig. 9D, the expression of *rsmY* and *rsmZ* was significantly increased by the studied compounds, suggesting that, as it has been previously described for some plant phenolic compounds (Yamazaki *et al*., 2012), the GacS-GacA two-component signal transduction system might be partly responsible for the observed impaired motility and reduced expression of the T3SS-related genes.

## Conclusions

In this work, taking into consideration the ancient role of efflux pumps as extruding elements of plant-produced anti-virulence compounds, as well as their possible induction mediated by their own substrates, we hypothesized that a chemical screening could identify natural-like anti-virulence compounds acting as inducers and substrates of MexAB-OprM and MexCD-OprJ efflux pumps in *P. aeruginosa*. A potential drawback of this approach might be that these molecules could also increase

antibiotic resistance to, at least, antibiotics expelled by induced efflux pumps (Laborda *et al*., 2019). However, we found, for the identified inducers, that changes in antibiotic susceptibility are minor, if any. This is likely because the inducers analysed seem to be also substrates of the efflux pumps, so they may be competing with the antibiotics extruded by them. Therefore, here we show a solid proof-of-concept for chemical screenings to identify compounds that are both, inducers and substrates of efflux pumps. This approach may help to find anti-virulence compounds that, as observed in this study, will not significantly induce transient antibiotic resistance mediated by efflux pumps.

As a result of this study, we describe four molecules, which significantly reduce *P. aeruginosa* motility, Type 3 Secretion and lethality of *P. aeruginosa* against the animal infection model *C. elegans*, due to the reduction of the expression of genes encoding flagellum components, as well as T3SS-related genes (Fig. 10). Since these processes are of relevance for *P. aeruginosa* virulence and taking into consideration that both, flagellum and T3SS are associated with poor clinical outcomes in acute infections by this bacterium (Montie *et al*., 1982; Feldman *et al*., 1998; Roy-Burman *et al*., 2001; Hauser *et al*., 2002; Shaver and Hauser, 2004; Peña *et al*., 2015), the described compounds should be considered as promising inhibitors of virulence of *P. aeruginosa*. It is relevant to notice that coumarins and their derivatives have been used as anticoagulants for decades (Wittkowsky, 2003) and their use as anti-inflammatory and anti-cancer compounds is under investigation (Kumar *et al*., 2018; Aadil and Vikrant, 2020). This means that their use is safe and would not have problems associated with potential side effects on human health. Additional studies on the structure–activity relationship of these compounds may identify molecules with improved inhibitory capacity of *P. aeruginosa* virulence, without increasing antibiotic resistance. This could allow its use, alone or in combination with antibiotics, to counteract infections caused by these bacteria.

## Experimental procedures

### *Bacterial strains, culture conditions and antibiotic susceptibility determination*

*Pseudomonas aeruginosa* strains used during this work are listed in Supplemental Table S2. All strains were grown at 37°C and 250 rpm in Luria–Bertani (LB) broth (Pronadisa). Antibiotic susceptibility in presence of 100 μM of the studied compounds or the same amount of solvent (DMSO), as a negative control, was determined by broth dilution method.

### *Inactivation of MexAB-OprM efflux system by the partial deletion of* mexB

Upstream (1010 bp) and downstream (917 bp) DNA fragments adjacent to the region of *mexB* to be deleted were amplified by PCR, using FailSafe® DNA polymerase (Epicentre Biotechnologies, Madison, WI) and the primer pairs HindIII_*mexB*_Fw/*mexB*_int_Rv and *mexB*_int-Fw/HindIII:*mexB*-Rev respectively (Supplemental Table S4). DNA products were purified from agarose gel electrophoresis and were used as templates for a new PCR reaction, using the primer pair HindIII_*mexB*_Fw/HindIII_*mexB*_Rev. The DNA product was purified from agarose gel electrophoresis, digested with the restriction enzyme HindIII (New England Biolabs) and ligated, using T4 DNA ligase (Promega), into HindIII-digested and dephosphorylated pEX18Ap plasmid. Then, the resulting ligated plasmids were introduced by transformation into chemically competent *E. coli* S17-1λ *pir* cells (Simon *et al*., 1986). In order to rule out mutations that could be introduced by PCR amplification, the DNA inserted into pEX18Ap plasmid was Sanger sequenced, using the primer pair M13_Fw/M13_Rv (Supplemental Table S4). After that, the partial deletion of *mexB* was carried out in the PAO1 wild-type strain, by homologous recombination, using the *E. coli* S17-1λ *pir* (pEX18Ap-Δ*mexB*) as donor strain (Supplemental Table S2). Conjugation and mutant selection were performed following the methodology previously described (Hoang *et al*., 1998), using 350 μg ml$^{-1}$ carbenicillin and 10% sucrose. Finally, the partial deletion of *mexB* was verified by PCR, using the primer pair *ΔmexB*_comp_Fw/*ΔmexB*_comp_Rv (Supplemental Table S4).

### *Growth and luminescence measurements*

The growth of bacterial cultures ($OD_{600nm}$) and their luminescence were measured in a plate reader (Tecan Infinite 200) every 10 min for 24 h at 37°C. Flat white 96-well plates with optical bottoms (Thermo Scientific Nunc) were inoculated at an $OD_{600nm}$ of 0.01 in 150 μl of the corresponding media. The absorbance and luminescence values were estimated by the average of three biological replicates for each strain and condition.

### *Screening for inducers of the expression of* mexCD-oprJ *and* mexAB-oprM

The capacity of 1243 compounds from a Natural Product-Like library and an indole library (OTAVA Chemicals; (Matilla *et al*., 2018)) to induce *mexAB-oprM* or *mexCD-oprJ* expression was tested using the PAO1 bioreporter strains CTX-*lux*::P*mexAB*, PAO1 CTX-*lux*::P*mexCD* and PAO1 CTX-*lux* (Laborda *et al*., 2019). The strains were inoculated in flat white 96-well plates with optical bottoms (Thermo Scientific Nunc) containing 150 μl of LB broth

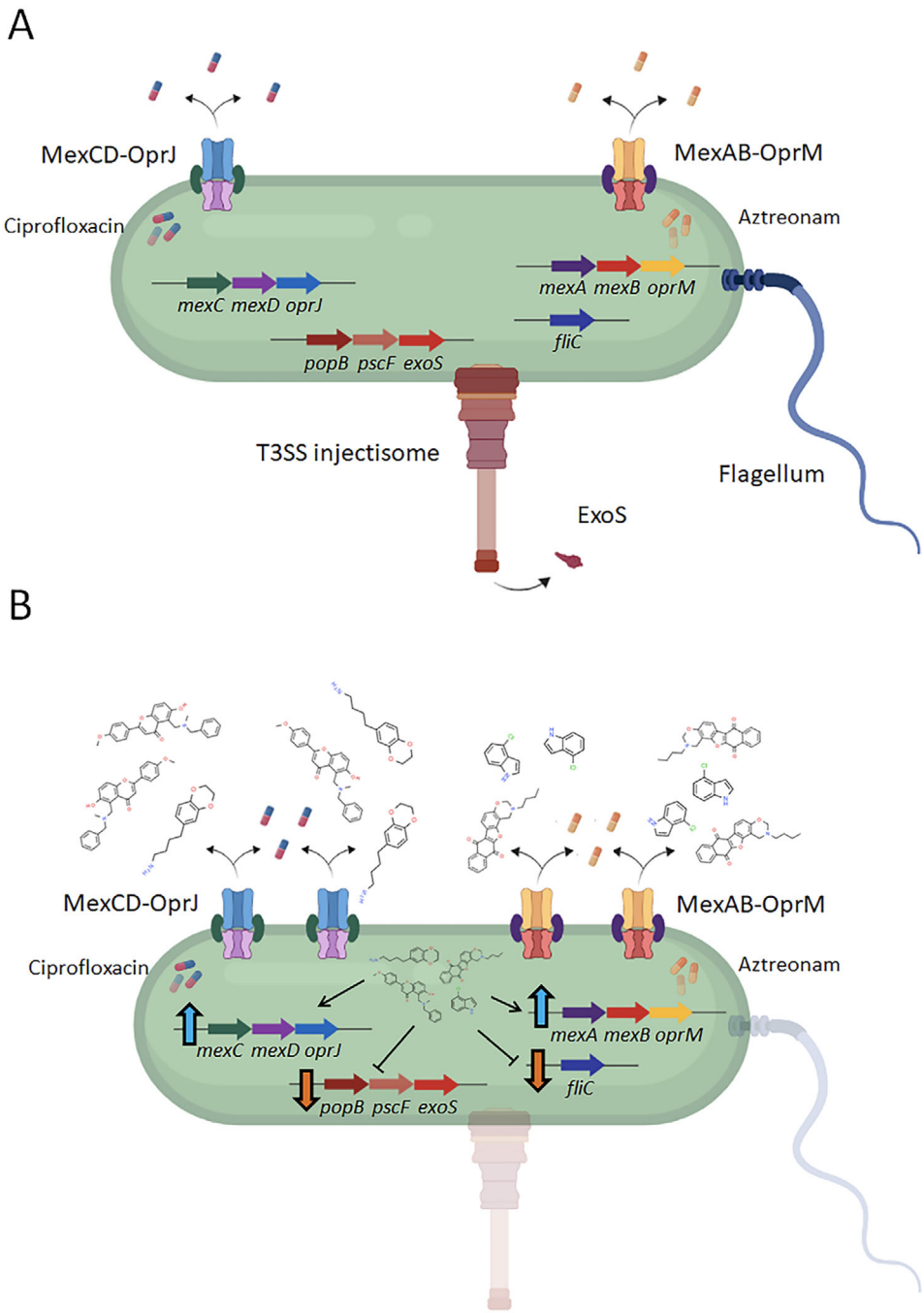


**Fig 10.** Schematic representation of the effect of 4-CII, Cou-1, Cou-2 and Bdx on antibiotic resistance and virulence of *P. aeruginosa*.
A. MexAB-OprM and MexCD-OprJ are *P. aeruginosa* efflux pumps that extrude different antibiotics as aztreonam or ciprofloxacin respectively; besides, elements such as T3SS and flagellum constitute remarkable virulence determinants of this bacterium.
B. 4-CII, Cou-1, Cou-2 and Bdx are inducers of the expression of *mexAB-oprM* and *mexCD-oprJ* efflux pumps' encoding genes and substrates of MexAB-OprM and MexCD-OprJ, which might outcompete antibiotics' efflux. In addition, these natural-like compounds are inhibitors of the expression of T3SS- and flagellum-related genes, leading to a reduction of virulence-related phenotypes, such as swarming motility, Type 3 Secretion and killing capacity to the animal model *C. elegans*.

and each compound at 100 μM or the same volume of solvent, as a negative control. Absorbance and luminescence were monitored during 20 h using a Tecan Infinite 200 plate reader, as described above.

*Normalization of the results from the screening*

The ratio luminescence emitted/$OD_{600nm}$ was determined in each well and represented against time. The area under the curve for each graphic was calculated using the GraphPad Prism software. That gave rise to a collection of numeric values which represent the *luxCDABE* expression in PAO1 CTX-lux::P*mexCD*, PAO1 CTX-lux::P*mexAB* and PAO1 CTX-lux strains (Supplemental Table S2) when growing in presence of each tested compound. Each numeric value from PAO1 CTX-*lux*::P*mexAB* and PAO1 CTX-*lux*::P*mexCD* was normalized dividing it by the one corresponding to the control strain (PAO1 CTX-*lux*) grown in the same condition. The

distribution of normalized luminescence values obtained from each biosensor strain was represented in a boxplot. An upper threshold was calculated using the formula $Q_3 + 1.5 \times$ IQR and a lower threshold with $Q_1 - 1.5 \times$ IQR, being $Q_3$ the upper quartile, $Q_1$ the lower quartile and IQR the interquartile range for each data set. Those values above the upper threshold were considered as compounds with significant capacity to induce the expression of the efflux pump encoding genes, as previously considered (Laborda *et al*., 2019).

### *RNA preparation and qRT-PCR*

*Pseudomonas aeruginosa* PAO1 was inoculated in LB broth to a final $OD_{600nm}$ of 0.01 and grown until exponential phase ($OD_{600nm}$ of 0.6). The induction assay was subsequently performed: each tested compound was added at 100 μM and cultures were incubated for 90 min with shaking, as previously described (Laborda *et al*., 2019). A bacterial culture with the same amount of their solvent was used as negative control. *mexAB-oprM* overexpressing strain (*mexR**) and *mexCD-oprJ* overexpressing strain (*nfxB**) (Supplemental Table S2), grown in the absence of inducer, were used as controls of overexpression. For the analysis of the effect of the tested compounds in the expression of T3SS-related genes under T3SS-inducing conditions, 100 μM of each compound or an equal amount of their solvent were added to LB flasks containing EGTA 5 mM and $MgCl_2$ 20 mM (Linares *et al*., 2005) and cells were grown during 4 h, from $OD_{600nm}$ of 0.1 to early stationary phase. At this point, 10 ml of each culture were pelleted by centrifugation at 7000 rpm and 4°C for 20 min.

RNA extraction was performed as previously described (Blanco *et al*., 2017). After the first treatment with DNase I (Qiagen) and a second one with DNase Turbo DNA-free (Ambion), the absence of DNA contamination was checked by PCR using primers *rpsL_Fw* and *rpsL_Rv* (Supplemental Table S4). cDNA was obtained by using the High-Capacity cDNA reverse transcription kit (Applied Biosystems) from 10 μg of RNA.

qRT-PCR was carried out in an ABI Prism 7300 Real-time system (Applied Biosystems) using Power SYBR green PCR master mix (Applied Biosystems). In each reaction, 50 ng of cDNA were used. The reaction consisted of a denaturation step at 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min for amplification and quantification. Primers *rplU_RTPCR_Fw* and *rplU_RTPCR_Rv* (Supplemental Table S4) were used to amplify the housekeeping gene *rplU*. All the primers (Supplemental Table S4) were designed with Primer3 Input software and their efficiency was analysed by qRT-PCR using serial dilutions of cDNA. Differences in the relative amounts of mRNA were assessed according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001; Pfaffl, 2001). In all cases, the resulting values were determined as the average of three independent biological replicates each one containing three technical replicates.

### *Elastase activity and pyocyanin production*

*Pseudomonas aeruginosa* PAO1 was grown in 10 ml of LB broth, at 37°C during 24 h, with 100 μM of each tested compound or the same amount of their solvent, as negative control. *nfxB** and *mexR** were grown in 10 ml of LB broth with their solvent in the same amount at 37°C during 24 h. Then, 1 ml samples were collected and centrifuged at 7000 rpm for 10 min. The resulting supernatants were filtered by using 0.2-μm-pore-size filters (Whatman) and 100 μl were added to a 96-well microtiter plate (Nunc). Pyocyanin production was determined by measuring $OD_{690nm}$ in a Tecan Infinite 200 plate reader. The elastase assay was performed as previously described (Sanz-Garcia *et al*., 2019): 100 μl of each filtered supernatant were added to 1 ml of Congo red elastin (Sigma-Aldrich) and then incubated for 2 h at 37°C and 250 rpm; after that, the mixtures were centrifuged at 7000 rpm for 10 min, supernatants were filtered and 100 μl were added to a 96-well microtiter plate to determine the elastase activity by measuring $OD_{495nm}$. Three replicates of each tested condition were used to analyse the results.

### *Biofilm formation*

Biofilm formation was analysed as previously described (Sanz-Garcia *et al*., 2019). An overnight bacterial culture of *P. aeruginosa* PAO1 was diluted 100-fold in LB with 100 μM of each tested compound or the same amount of their solvent, as a negative control. An overnight bacterial culture of *nfxB** and *mexR** strains was also diluted 100-fold in LB with the same amount of solvent. 100 μl of cells were added into a 96-well microtiter plate (Falcon 3911 MicroTest III Flexible Assay Plate), previously sterilized with UV light. The inoculated plate was incubated for 48 h at 37°C. After that, 25 μl of crystal violet 0.1% were added to each well and, after 5 min, the excess of dye was thoroughly washed four times with distilled water. The biofilm from the wells was removed with Triton X-100 (0.25%) and 100 μl were added to a 96-well microtiter plate (Nunc) to perform the biofilm quantification by measuring $OD_{570nm}$ in a Tecan Infinite 200 plate reader. The results were estimated by the average of eight biological replicates for each condition.

*Swarming assay*

Petri dishes with 25 ml of a Casamino Acids medium (0.5% Casamino Acids, 0.5% Bacto agar, 0.5% filtered glucose, 3.3 mM $K_2HPO_4$ and 3 mM $MgSO_4$) with 100 μM of each tested compound or the same amount of their solvent as a negative control were used to perform the swarming assay. 4 μl of *P. aeruginosa* PAO1, *nfxB** or *mexR** at $OD_{600nm}$ of 1.0 were placed on the centre of each plate containing each tested compound or the same amount of their solvent, and they were incubated for 17 h at 37°C. The experiment was conducted with three replicates for each condition, a picture was recorded and the area of the swarming motility zone was measured with ImageJ software.

*Electrophoresis and Western Blot*

T3SS-induced cultures of *P. aeruginosa* (10 ml) in the presence of 100 μM of each natural-like compound or the same amount of their solvent were pelleted by centrifugation at 7000 rpm and 4°C for 20 min. The resulting supernatants were filtered by using 0.2-μm-pore-size filters (Whatman) and afterwards concentrated through centrifugation to 1 ml (Amicon Ultra 3K, Millipore). Supernatants were submitted to SDS-PAGE electrophoresis (10% acrylamide) and transferred onto a nitrocellulose membrane. Then, membranes were blocked with a 5% powder milk solution in PBS at 4°C. Anti-ExoS primary antibody (Agrisera), obtained in chicken, was added at 1:5000 to the membrane and incubated during 1 h. After three washes with PBS during 30 min, goat-antichicken secondary antibody conjugated with peroxidase (Bio-Rad) was added at 1:10 000 and further incubated during 1 h. The membrane was then washed with PBS during 1 h and the chemiluminescent HRP substrate-mediated detection was performed (Immobilon Western, Millipore). Chemiluminescence images were saved and relative abundance of proteins determined by using ImageJ software (https://imagej.nih.gov/ij/). Three biological replicates of the experiment were performed.

Caenorhabditis elegans *killing assay*

*Caenorhabditis elegans* killing capacity of *P. aeruginosa* PAO1 was assessed as previously described (Dharmalingam *et al*., 2012). 50 μl from an overnight culture of *P. aeruginosa* PAO1 were inoculated in potato dextrose agar (Sigma-Aldrich) 6-cm-diameter plates with 100 μM of each tested compound or the same amount of their solvent, and incubated for 24 h at 37°C. Forty young adult hermaphrodite *C. elegans* N2 Bristol worms (Brenner, 1974) were then added to each plate. Plates were incubated at 20°C and were examined for living worms after 48 h. The experiment was conducted with three replicates for each condition. A worm was not considered alive when it did not react to contact anymore. *Escherichia coli* OP50, the preferred food source of *C. elegans*, was used as a positive control of survival in each tested compound or their solvent.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Supplemental Table S1.** Normalized luminescence values emitted by *luxCDABE*-based bioreporter strains of *mexCD-oprJ* and *mexAB-oprM* expression in presence of each molecule of a Natural-like library

**Supplemental Table S2.** Bacterial strains used in this work

**Supplemental Table S3.** Potential inducer compounds of *mexCD-oprJ* and *mexAB-oprM* expression detected by the Natural-like library screening

**Supplemental Table S4.** Primers used in this work

# *DISCUSSION*

# 4. Discussion

AR has become a global economic and health problem during the last decades, something worsened by the continuous emergence and dissemination of MDR bacteria (3). Regarding antibiotic-resistant bacteria of concern, *P. aeruginosa* may be highlighted, due to its high intrinsic resistance and its ability to acquire AR mutations and resistance determinants located in MGEs (36, 242, 244, 245). These characteristics allow the selection and spread of isolates resistant to many of currently available antibiotics, including those limited for therapies of critical clinical cases.

During this thesis, we aimed to searching for efficient strategies against infections of *P. aeruginosa*, placing emphasis not only on finding novel drugs but also on the rational use of existing antibiotics. Regarding the first approach, we addressed an evolution-based strategy for the search for compounds with anti-virulence potential. For a more efficient use of the available antibiotics, we focused on the study of the factors that constrain clinically-exploitable trade-offs of AR evolution, trying to identify those robust ones, hence with potential to be applied in clinics where different bacterial strains and conditions occur during an infection. We principally focused on collateral sensitivity, by which acquiring resistance to a drug renders higher susceptibility to a second drug (189), and on fitness costs associated with the acquisition of AR, whose compensation in absence of selective pressure could cause a resistance decline. Further, we studied the impact of different environmental factors in susceptibility of bacteria to antibiotics, in order to detect situations in which antibiotic treatments could be compromised. Besides its relevance within a clinical point of view, some of the obtained results also have importance for better understanding evolution in general terms.

## 4.1. Robust trade-offs of the evolution of antibiotic resistance constitute a promising step-forward for the rational design of antibacterial therapies

Although evolution is usually considered to be stochastic, several factors, such as mutation load, population bottlenecks, clonal interference, fitness cost and resistance level of each mutation, compensatory evolution, cross-selection, epistasis and genetic background, constrain AR evolution (148, 155-164). Therefore, understanding how these factors restrict evolution makes it predictable in some degree. In order to exploit AR evolution trade-offs -i.e. collateral sensitivity or fitness cost-, we need them to be robust and to independently emerge in different situations and genetic backgrounds. Otherwise, these trade-offs would not always occur, compromising the potential strategy of using them in certain situations. However, finding evolutionary events that are conserved is not easy, even when replicates of the same strain evolve in presence of the same antibiotic (194, 199, 346, 347). For this reason, during this PhD we searched for robust trade-offs of *P. aeruginosa* AR evolution to rationally design therapeutic strategies using available antibiotics.

### 4.1.1. Identification of robust collateral sensitivity patterns for the design of evolution-based treatments

We addressed the identification of robust collateral sensitivity patterns at three levels: those arising when distinct antibiotics are applied to a single strain, those that emerge when strains presenting different genetic backgrounds acquire resistance to a specific drug, and those appearing when the same strain is exposed to a single antibiotic in different environments. These different approaches allowed us to answer different evolutionary questions, as detailed below.

Firstly, we described that the selective pressure exerted by antibiotics belonging to different structural families -tobramycin, ceftazidime and tigecycline-, leads to collateral sensitivity to fosfomycin. This provides an example of convergent evolution towards fosfomycin hyper-susceptibility when the same strain acquires different AR mutations. In this regard, common patterns of collateral sensitivity associated with the acquisition of resistance to different antibiotics have been rarely reported (348). Therefore, we analysed the molecular mechanism behind this phenotype.

Fosfomycin-hyper-susceptible clones isolated from populations evolved in presence of tobramycin, ceftazidime or tigecycline did not present mutations in genes previously related to fosfomycin hyper-susceptibility, but presented genetic modifications associated with resistance to the antibiotics used as selective pressure. All of them acquired a mutation in *orfN*, encoding a glycosyltransferase of flagellins (349). Mutations in this gene were later on proposed to affect drug uptake by modification of the outer membrane (350). Besides, the clone selected in presence of tigecycline also acquired a mutation in *nfxB*, the negative regulator of the expression of the genes encoding the MexCD-OprJ efflux pump (256), the clone selected in tobramycin acquired a mutation in *fusA*, encoding an elongation factor whose mutations are related to aminoglycosides resistance (351), and the clone selected in ceftazidime presented a large chromosomal deletion of a region of around 300 kbp containing *galU*, previously associated with ceftazidime resistance (352). The fact that these clones presented mutations in an elongation factor, in a global regulator or lacked almost 250 genes, was possibly responsible for the abundant changes in their transcriptomes, among which we found a common reduction in the expression of *fosA*, encoding a fosfomycin-inactivating enzyme (58), and of the genes encoding the peptidoglycan recycling pathway, that constitutes an alternative pathway to the one for the peptidoglycan synthesis, which is blocked by fosfomycin (353). This parallel reduction in the expression of those genes could be therefore responsible for fosfomycin hyper-susceptibility of the studied mutants. The coincidence of expression profiles was not a general feature for the overall transcriptomes of the studied clones, but many differences were ascertained. This indicates that collateral sensitivity to fosfomycin is not a result of global physiological changes commonly acquired in all of them but to the specific downregulation of intrinsic fosfomycin resistance determinants encoding genes. This observation reinforces the difficulty -in genetic terms- of having found a convergent collateral sensitivity phenotype associated with the selection of mutations conferring resistance to different antibiotics.

A mutant in *orfN*, the commonly mutated gene in the three clones, did not present changes in the expression of the peptidoglycan recycling enzymes encoding genes, but presented a reduced *fosA* expression. This is consistent with the fact that the *orfN** mutant was more susceptible to fosfomycin than the wild-type strain, but not as susceptible as the three evolved clones. Hence,

the parallel expression profiles of the three studied clones are associated with a rewiring of the transcriptome caused by the combination of the mutation in *orfN* with other AR mutations. Indeed, loss-of-function mutations do not always have an effect on the transcriptome that just reflects the loss of the associated disrupted molecular function, but usually cause non-specific changes (354).

The three clones and the *orfN** mutant presented an increased accumulation of active fosfomycin, due to their reduced amount of fosfomycin-inactivating enzyme FosA. This phenotype reverted back by overexpressing *fosA* in the three clones. Nevertheless, as above stated, the minimal inhibitory concentration (MIC) to fosfomycin was higher for the *orfN** mutant than for the three evolved clones, indicating that this phenotype is not just caused by the reduced expression of *fosA*, but the downregulation of the peptidoglycan recycling pathway enzymes encoding genes is also contributing. In agreement with this hypothesis, we found that insertion mutants in *fosA* and in the peptidoglycan recycling enzymes encoding genes are fosfomycin hyper-susceptible. Even more, the presence of a FosA inhibitor caused a further increase of fosfomycin susceptibility of the insertion mutants in the peptidoglycan recycling enzymes encoding genes, indicating that the inhibition of both systems jointly contribute to a fosfomycin susceptibility increase. Further, fosfomycin MIC of the three studied clones was similar to that of the *fosA* insertion mutant, but active fosfomycin accumulation of the *fosA* mutant was much higher than that of the clones. This confirms that both, the reduction of the expression of *fosA* and the peptidoglycan recycling enzymes encoding genes, are contributing to fosfomycin collateral sensitivity.

Concluding, our results provide an example of a convergent, but non-adaptive, phenotype that emerges by the acquisition of different genetic variations when different selective pressures are applied. These findings may have a potential clinical application for therapies that would consist in the alternation of these drugs with fosfomycin or the use of fosfomycin-containing combinations for treating antibiotic-resistant *P. aeruginosa*.

Following with the identification of robust collateral sensitivity patterns, epistasis and genetic background have a major role in the determination of the most probable evolutionary solutions - and their associated trade-offs, such as collateral sensitivity- given a selective pressure. That is why finding robust evolutionary trade-offs in different genetic backgrounds is not a trivial matter, although is needed for the exploitation of such trade-offs in rational anti-infection approaches. Therefore, during this PhD we also addressed the search for conserved collateral sensitivity patterns in a collection of *P. aeruginosa* mutants resistant to different antibiotics, since resistant strains presenting different genetic contexts are usually found causing infections.

The set of *P. aeruginosa* antibiotic-resistant mutants, previously constructed in the lab, which contained single and multiple mutations in genes encoding both regulatory and non-regulatory proteins, was subjected to short-term evolution in presence of tobramycin, aztreonam and ciprofloxacin, three drugs frequently used to treat the infections caused by this bacterium. Collateral sensitivity to aztreonam and tobramycin was associated with acquisition of ciprofloxacin resistance in almost every replicate of each genetic background. The analysis of sequential and combinatory treatment strategies of ciprofloxacin and tobramycin or aztreonam in

the collection of mutants showed that the most promising results arise from the combination strategy. Specifically, 23 and 25 out of 28 populations presenting different genetic backgrounds became extinct after a treatment with ciprofloxacin-tobramycin and ciprofloxacin-aztreonam combinations, respectively. Importantly, a synergistic effect between these pairs of drugs was not ascertained in any genetic context analysed, suggesting that collateral sensitivity associated with the use of ciprofloxacin may be optimising the combinatory effect of the pairs ciprofloxacin-tobramycin and ciprofloxacin-aztreonam.

Populations resulting from ciprofloxacin short-term ALEs acquired mutations in *mexS*, *nfxB* -coding for negative regulators of the efflux pumps encoding genes *mexEF-oprN* and *mexCD-oprJ*, respectively (257, 355-358)- and *gyrAB* -coding for the target of ciprofloxacin (281, 358-361)-. Remarkably, mutations in these genes are the most frequently found in clinical strains isolated from ciprofloxacin-treated patients and some variants acquired in these ALE experiments have been already described as clinically important (281, 357-359, 361-365), reinforcing the clinical relevance of our results. Mutated genes were dependent on the genetic background. For instance, mutations in *gyrAB* were acquired in those genetic backgrounds that already presented mutations in genes encoding negative regulators of efflux pumps. These results suggest that when acquiring resistance to quinolones, mutations in regulators of the expression of efflux pumps may precede mutations in the antibiotic target. In agreement with this hypothesis, it has been described that mutations in *nfxB* are generally early acquired in clinical strains of patients treated with ciprofloxacin, but are less persistent than *gyrAB* mutations (365).

Individual clones presenting a single mutation in either *nfxB*, *gyrA*, *gyrB* or *mexS*, isolated from ciprofloxacin-resistant populations, also presented an increased susceptibility to tobramycin and aztreonam, confirming these phenotypes to be caused by these classical ciprofloxacin resistance-associated mutations in the respective genetic context in which they were acquired. Hence, collateral sensitivity to tobramycin and aztreonam is associated with the selection of mutations in genes encoding regulators of the expression of efflux pumps or quinolones' targets, although mutations in *nfxB* produce the highest susceptibility increase to both drugs.

During this work, we described that the acquisition of ciprofloxacin resistance is associated with tobramycin and aztreonam collateral sensitivity within different genetic backgrounds of *P. aeruginosa*. Nevertheless, this phenotypic convergence is caused by different genetic modifications selected in each genetic background. Importantly, mutated genes ascertained during this work are the most frequently encountered in clinical strains treated with ciprofloxacin. This indicates the potential of our results to be applied in clinics, and that the mutations usually found in clinical isolates might be associated with tobramycin and aztreonam collateral sensitivity. Indeed, ciprofloxacin-resistant clinical strains of *P. aeruginosa* have been previously described to be susceptible to aminoglycosides (161), and, according to our results, this phenotype may be caused by mutations in *gyrA*, *gyrB* and *nfxB*. Further, mutations in *mexS* have been associated with an enhanced susceptibility to β-lactams and aminoglycosides (270). Overall, this work supports the possibility to use the combinations ciprofloxacin-tobramycin and ciprofloxacin-aztreonam to treat *P. aeruginosa* infections caused by different previously resistant mutants; an evolution-based strategy for promoting the extinction of *P. aeruginosa* infections.

Further, during this work we also observed that fosfomycin collateral sensitivity was acquired in most of the genetic backgrounds treated with tobramycin. In addition, a robust collateral sensitivity to this drug was previously observed to be conserved in the mentioned pre-existing antibiotic-resistant mutants evolved in presence of ceftazidime (201). Even more, it has been described that the combination of fosfomycin with either tobramycin or ceftazidime-avibactam is synergistic against MDR *P. aeruginosa* strains and *P. aeruginosa* biofilms (366-368). These observations, together with the previously discussed results from article I, reinforce the potential use of fosfomycin in combination with tobramycin and ceftazidime for treating *P. aeruginosa* infections.

Besides, robustness of collateral sensitivity might also imply the conservation of this phenotype when bacteria evolve in diverse habitats with varying nutritional composition, as different sites within an infected patient. Therefore, we studied the extent to which the nutrients' availability of body locations frequently infected by *P. aeruginosa* have an influence in the evolutionary pathways of this bacterium towards resistance to antimicrobials commonly used for treating such infections and how it affects the robustness of collateral sensitivity. For this goal, we performed ALE experiments in presence of tobramycin, ceftazidime or ceftazidime-avibactam in SCFM or urine, and compared them with those previously performed in rich laboratory media.

Firstly, we observed that tobramycin resistance levels acquired at the end of the evolutions were the same, independently of the medium used, but ceftazidime and ceftazidime-avibactam resistance levels were higher when acquired in rich medium or SCFM than when acquired in urine. Further, collateral sensitivity and cross-resistance to other drugs was dependent on the medium in which populations evolved. Namely, all tobramycin evolved populations acquired resistance to other aminoglycosides, quinolones and tigecycline, but collateral sensitivity to fosfomycin was only observed in populations evolved in rich medium and collateral sensitivity to imipenem only in urine-evolved populations. Populations evolved in presence of ceftazidime or ceftazidime-avibactam acquired cross-resistance to other β-lactams and collateral sensitivity to fosfomycin, independently of the medium in which they evolved, suggesting, again, that the alternation or combination of fosfomycin with ceftazidime (or ceftazidime-avibactam) may be an efficient strategy to treat *P. aeruginosa* infections. Also supporting this possibility, fosfomycin collateral sensitivity associated with ceftazidime resistance acquisition is also robust among different mutational backgrounds, as previously described (201). Finally, collateral sensitivity to tobramycin was specific of ceftazidime and ceftazidime-avibactam rich medium-evolved populations. Therefore, resistance to the selective antibiotic and other antibiotics from different structural families is dependent on the medium in which resistance is acquired.

In agreement with the observed phenotypic differences, genetic evolutionary trajectories towards resistance were also contingent on environmental conditions. Namely, mutations in *fusA* were acquired in all replicate populations submitted to tobramycin selective pressure, independently of the medium of the evolution, while modifications in *nuoD* and *pmrB* were specific of populations that evolved in urine and rich medium, respectively, and mutations in *ptsP* only emerged in populations evolved in SCFM and rich medium. Regarding the *orfKLNH* group of genes, all replicate populations that evolved in rich medium acquired a mutation in *orfN*, *orfH* mutations

were the most prevalent in urine and *orfK* mutations were the most frequent in SCFM. As we described in article I of this PhD, fosfomycin collateral sensitivity of tobramycin-evolved clones in rich medium was partly caused by mutations in *orfN*, which correlates with the fact that only rich medium-evolved populations acquired fosfomycin collateral sensitivity.

Populations that evolved in presence of ceftazidime in every media presented mutations in *mpl*. Nevertheless, deletions of around 300 kbp, mutations in *mexB* as well as in its negative regulators encoding genes *mexR* and *nalD*, were specific of rich medium-evolved populations, a mutation in *anmK* was only acquired in urine and a variation in *ampC* in SCFM. Mutations in *ftsI* and *dacB* were acquired in populations that evolved in SCFM and rich medium, and mutations in *orfN* were acquired in both urine and rich medium.

For their part, populations that evolved in presence of ceftazidime-avibactam also acquired large deletions only in rich medium, and became hyper-mutators in urine and SCFM. Common mutations in all environments were scarce, but some functionally related mechanisms were involved in resistance acquired in different media. For example, mutations in *ftsI*, *ftsB* or *ftsL* were acquired in every medium, while *mpl* or *orfN* mutations were acquired just in urine- and SCFM-evolved populations. Further, mutations in the genes encoding a predicted efflux pump, *PA14_45890*, and its likely regulator, were only observed in SCFM and rich medium, while mutations in genes encoding regulators of *mexAB-oprM*, *nalC* and *nalD*, were selected in rich medium and urine. Finally, some genes related to peptidoglycan synthesis, such as *murF*, *rpl* or *anmK*, presented mutations in urine- and SCFM-evolved populations. It is worth noting that the deletion of large chromosomal genomic regions was only selected in presence of ceftazidime or ceftazidime-avibactam in rich medium. These deletions, besides conferring ceftazidime resistance due to the loss of *galU* (352), cause tobramycin collateral sensitivity due to the loss of *mexXY* -coding for an aminoglycoside-extruding efflux pump (259)-, an event produced in presence of ceftazidime or ceftazidime-avibactam in rich medium but not in urine or SCFM. However, the acquisition of these deletions is frequently observed in clinics (369), suggesting that conditions facilitating the acquisition of such genetic modification may be encountered during infection. Most of the mutations detected during this work were previously observed in clinical isolates of *P. aeruginosa* and are associated with AR, supporting the clinical relevance of these results.

Importantly, fosfomycin collateral sensitivity was acquired within ceftazidime and ceftazidime-avibactam evolved-populations, independently of the medium. Representative clones isolated from populations that evolved in presence of ceftazidime and ceftazidime-avibactam in rich medium, which had a large deletion, presented a reduced expression of *fosA* and the peptidoglycan recycling enzymes encoding genes. For their part, ceftazidime SCFM, ceftazidime urine and ceftazidime-avibactam urine representative clones, had just a reduced expression of the peptidoglycan recycling enzymes encoding genes. This finding justifies that the increase in fosfomycin susceptibility was lower in these last three clones than in the ceftazidime and ceftazidime-avibactam rich medium clones that had a reduced expression of both peptidoglycan recycling encoding genes and *fosA*. Finally, the clone presenting the highest fosfomycin resistance decline, a ceftazidime-avibactam SCFM representative clone, had acquired a mutation in *anmK,* encoding an enzyme which forms part of the peptidoglycan recycling pathway and whose

inactivation was previously associated with both an increase of fosfomycin susceptibility and ceftazidime resistance (353, 370). Then, the robust reduction of the activity of the peptidoglycan recycling pathway, either through a reduction of expression or by inactivating mutations, is in the basis of a robust fosfomycin collateral sensitivity associated with the acquisition of ceftazidime or ceftazidime-avibactam resistance in different nutritional conditions.

Overall, these findings suggest that the translation of evolutionary knowledge into clinical practice requires the analysis of different genetic backgrounds and of different growing conditions, including media closer to what is found in clinics, for the experimental prediction of the evolution of AR. Both, nutrients' availability and the effect of the genetic background have relevant implications for the design of evolution-based approaches to tackle AR, which might be based on robust patterns identified when a specific drug is used in different environments, when using an antibiotic for treating bacteria with diverse genetic backgrounds, or when bacteria are exposed to different drugs.

### 4.1.2. Studying fitness costs associated with antibiotic resistance to counteract such resistance

Another trade-off of AR evolution that might be exploited to tackle AR is fitness cost in the absence of antibiotics associated with the acquisition of AR, since compensatory evolution might lead to AR decline. However, the empirical application of antibiotic restriction periods has not been a successful strategy for combating AR (371, 372). This can be due to the fact that decline of resistance is not a general trait, is infrequent, and depends on the antibiotic used and the resistance mechanism selected -different molecular functions affected will produce different fitness costs- (373, 374). Even more, decline of resistance not only depends on the initial fitness cost but it might also be contingent on the genetic background and the strength of selection. Consequently, blind assays, as those usually implemented, are not enough to manage AR and therefore, the identification of specific antibiotics whose resistance mechanisms are robustly unstable in absence of selection is of utmost importance for successfully applying this kind of approaches.

During this PhD, we studied the compensation of fitness costs associated with ceftazidime resistance acquisition and the possible AR decline after compensatory evolution. Ceftazidime resistance acquisition -in rich medium- is associated with tobramycin collateral sensitivity, due to the deletion of large chromosomal regions containing *mexXY*, which encodes an aminoglycoside-extruding efflux pump, as already discussed. This evolutionary trade-off was described to be robust within different genetic backgrounds (201). Besides, ceftazidime resistance was also associated with a high fitness cost -measured as growth respect to their parental strains- . Herein, the possible compensation of fitness costs of ceftazidime-resistant mutants from previous ALE experiments, presenting different genetic backgrounds, and the possible decline of ceftazidime resistance was studied both in medium without antibiotic and in presence of sublethal concentrations of tobramycin which could be present in certain clinical situations, such as limited drug accessibility or incomplete treatments (207).

Ceftazidime resistance decline was observed in all studied genetic backgrounds after 8 weeks in antibiotic-free medium and in presence of a tobramycin concentration corresponding to 1/4, 1/8 and 1/16 of the respective tobramycin MIC, namely in 92% of clones isolated from populations evolved in antibiotic-free medium and in presence of tobramycin sublethal concentrations. Further, after evolution in absence of drugs, collateral sensitivity to tobramycin was preserved, whereas tobramycin resistance was acquired in presence of 1/4 and 1/8 of tobramycin MIC and with 1/16 of the MIC to tobramycin only in some genetic backgrounds. Importantly, a decrease of resistance to other β-lactams, such as aztreonam and imipenem, was also detected, while susceptibility to other drugs was maintained. Altogether, these results suggest that the alternation of ceftazidime with drug restriction periods could be a feasible approach, since the absence of drugs leads to a decline of ceftazidime resistance, preservation of collateral sensitivity to tobramycin, fosfomycin and tetracycline, and resistance to other antibiotics does not emerge. Even more, the previously suggested ceftazidime-tobramycin alternation (201) could be followed by a switch back to ceftazidime, to drive tobramycin-resistant mutants arising from sublethal antibiotic concentrations to extinction. Remarkably, the stability after periods of ceftazidime restriction of tobramycin collateral sensitivity would also allow a switch to this aminoglycoside. This indicates that the preservation of collateral sensitivity in the absence of drugs may also be relevant for the implementation of AR evolution based-policies.

Fitness was generally improved after the evolution in antibiotic-free medium and in presence of sublethal concentrations of tobramycin in some genetic backgrounds, the latter indicating that tobramycin resistance acquisition and compensatory evolution of fitness cost associated with ceftazidime resistance may be uncoupled. In particular, the clone initially presenting the highest initial fitness cost (MDR6) also presented the highest rate of fitness upgrade after compensatory evolution, suggesting that the higher fitness cost associated with resistance acquisition, the larger the mutational space for compensatory evolution to occur. Then, fitness increase, as well as the ceftazidime resistance decline, was dependent on genetic background, suggesting that epistasis does not only constrain AR evolution but also compensatory evolution.

Original mutations present in the parental genomic backgrounds did not revert to the wild-type sequence during evolution, thus discarding genetic reversion as the cause of ceftazidime resistance decline. Instead, additional mutations in *ampR* and *rpoB* -originally mutated in the ceftazidime-resistant strains- were acquired, indicating that intragenic compensation of fitness costs might be responsible for the observed ceftazidime resistance decline. A mutation in *ampR*, encoding a positive regulator of the expression of the β-lactamase encoding gene *ampC* (375), was originally acquired during the evolution in presence of ceftazidime, producing a β-lactamase activity increase. Afterwards, during compensatory evolution, secondary mutations were acquired in *ampR*, leading to a reduction of β-lactamase activity to the levels of the parental strain. The original mutation in *ampR* is located in a DNA-binding region of the protein, where the new amino acid likely stabilizes the binding to the *ampC* promoter, leading to an increased expression of the β-lactamase encoding gene. Secondary mutations disrupt AmpR from the beginning of its sequence, destabilize a DNA-binding helix reducing affinity for the promoter, impede the formation of the active AmpR dimer by affecting helices of the intermonomer interface, render

AmpR unresponsive to its activators by changing the effector-binding pocket, or impair the interaction of AmpR with the RNA polymerase altering its C-terminal domain. In any case, the activity of AmpR is compromised by these secondary mutations, hence reducing the expression of *ampC*, decreasing β-lactamase activity and being responsible for ceftazidime resistance decline.

Mutations in *rpoB*, encoding the β subunit of the RNA polymerase (376), are known to produce a modified expression of essential genes, DNA breaks and reduced transcription efficiency (377). Besides, mutations in this gene, encoding the target of rifampicin, also increase resistance to this drug (378) and produce as well pleiotropic effects leading to resistance to other drugs, such as quinolones or ampicillin (379). Therefore, the mutation in *rpoB* acquired during ceftazidime evolution, is likely responsible for the high fitness cost, as well as for the ceftazidime and rifampicin resistance of MDR6. Then, secondary intragenic mutations in *rpoB* acquired during evolution in absence of antibiotic might be restoring the defective RNA polymerase activity, as previously described (185, 380). Indeed, those clones with secondary mutations in *rpoB* presented an important fitness improvement, as well as a ceftazidime and rifampicin resistance decrease. The original mutation in *rpoB* introduces a large side chain with negative charge at the beginning of the transcription bubble that might displace the non-transcribed DNA strand, modifying the tension and the progression of the transcription. Secondary mutations lead to smaller side chains close to the original change that may leave more space in the zone relieving the tension of the non-transcribed strand, or to a repulsive charge in the opposite zone of the strand pushing it to its original place. These mutations likely restore the transcription activity of RpoB, possibly by a reorientation of the DNA strand, producing a recovery of fitness and a decline of ceftazidime and rifampicin resistance.

For their part, clones from populations evolved in 1/4 and 1/8 of tobramycin acquired mutations in *fusA* or *ptsP*, previously described to be related to tobramycin resistance (351, 381), depending on the genetic background, illustrating the extent to which epistasis constrains the evolution of AR. Tobramycin resistance was only selected in some genetic backgrounds when evolving in presence of 1/16 of tobramycin, indicating that the probability of acquiring resistance at a particular sublethal concentration is also dependent on genetic background.

Overall, these four articles reinforce the concept that finding robust trade-offs of AR evolution, which are conserved among different genetic backgrounds, selective forces or nutritional conditions, and that are stable when the antibiotic selective pressure is removed, would importantly help in fighting antibiotic-resistant bacteria by exploiting their Achilles' heel.

## 4.2. Conditions encountered during infection can both induce antibiotic resistance and alter the evolutionary pathways towards antibiotic resistance acquisition

Efflux pumps are elements encoded in the bacterial genome capable of extruding several types of antibiotics, thus contributing to multidrug resistance (253). Since they also have relevant roles in bacterial physiology (123), their expression is tightly regulated (145), being not uncommon the existence of specific conditions and compounds whose presence leads to the overproduction of these systems and the consequent increase in resistance to various drugs (111, 112). If bacteria

encountered such inducers during the course of an infection, its treatment would be threatened (102).

Then, during this PhD, we have searched for inducers of the expression of the genes encoding efflux pumps in *P. aeruginosa*, focusing on those with potential clinical relevance. Namely, we studied compounds able to induce the expression of *mexCD-oprJ* and *mexAB-oprM*, as examples of efflux pumps encoding genes with low and high intrinsic, basal, expression, respectively (253, 355). For that, we developed a set of *P. aeruginosa* biosensor strains containing the promoter regions of *mexCD-oprJ* and *mexAB-oprM* controlling the expression of the *luxCDABE* operon. The proper function of the biosensors was confirmed in the presence of membrane damage and oxidative stress, the previously known inducer conditions of *mexCD-oprJ* and *mexAB-oprM*, respectively (115, 274). This technology allowed us to easily determine the expression of the studied efflux pumps encoding genes in presence of 240 compounds present in Biolog phenotype microarrays. We found various inducers of the expression of *mexAB-oprM* and *mexCD-oprJ*, among which we may highlight dequalinium chloride, atropine and procaine. These three compounds, frequently used in clinics, were able to induce the expression of *mexCD-oprJ* and, consequently, enhance ciprofloxacin resistance of *P. aeruginosa*.

Dequalinium chloride was the molecule leading to the highest expression of *mexCD-oprJ* among those detailed during this work. Interestingly, several compounds detected as inducers during the analysis, such as cetylpyridinium chloride, domiphen bromide, benthezonium chloride or alexidine, have a similar biological activity and are also used as disinfectants in clinical practice. These molecules might be inducing *mexCD-oprJ* expression as a final consequence of the response to the envelope stress conditions they produce, similarly to benzalkonium chloride and chlorhexidine, the previously described inducers of *mexCD-oprJ*, which are also disinfectants (114, 115, 290). Alternatively, some of these molecules might induce *mexCD-oprJ* by binding to NfxB and precluding its regulatory capacity, as it has been described for dequalinium chloride in other regulators of the expression of efflux pumps (382).

Then, we studied the stability of transient AR when removing the inducers. The expression levels of the efflux pump encoding genes, returned to those ones of untreated cells after 30 minutes in the cases of atropine and procaine. Nevertheless, when inducing with dequalinium chloride, the expression level was lower but the induction still remained 2 hours after the removal of the inducer. This indicates that the "memory" of induction is dependent on the inducer compound.

It has been described that the expression of the genes encoding efflux pumps may be induced, on some occasions, by their own substrates (116, 383-385). This detoxifying mechanism, that is induced when the substrate is present, has been described for MexXY of *P. aeruginosa,* which is induced by and extrudes aminoglycosides (258). In this work, among the studied inducers, only procaine was detected to be a substrate of MexCD-OprJ, being toxic for an efflux pump-deficient mutant but not for the wild-type strain.

The induction of the expression of the genes encoding the MexCD-OprJ efflux pump by dequalinium chloride (54-fold), procaine (25-fold) and atropine (16-fold) led to an increase of ciprofloxacin resistance. Namely, ciprofloxacin resistance augmented up to 8-fold in the case of

dequalinium chloride, but procaine and atropine led to a similar (around 2-fold) phenotype. The fact that the induction levels caused by procaine were much higher than those caused by atropine but the level of AR was quite similar in the presence of both compounds might be explained by the role of MexCD-OprJ in extruding the first. Our hypothesis is that this efflux pump may be extruding both ciprofloxacin and procaine, hence reducing, to some extent, its capacity to extrude the antibiotic when the anaesthetic is present, despite the inducing capacity of procaine. The implications and possible application of detecting compounds which are both inducers and substrates of an efflux pump are further discussed in the following section.

Dequalinium chloride is a disinfectant, whose use is part of several antiseptic and disinfection procedures (386, 387) and has been considered for the treatment of promyelocytic leukemia (388-391). Procaine is an anaesthetic drug applied for some minor surgeries or burn injuries (392) and atropine is used for preoperative medication and sedation (393). Hence, *P aeruginosa* might get in contact with these compounds when infecting immunocompromised people, burned people, surgical patients or hospitalized patients in general. These situations must be carefully taken into consideration, since infections of *P. aeruginosa* are common in such conditions (236-238, 394) and the use of these drugs might compromise the treatment of such infections.

Besides increasing the level of AR, conditions encountered during infection might also alter the genetic variations acquired towards AR. As previously detailed, during this PhD we studied the evolution towards tobramycin, ceftazidime and ceftazidime-avibactam resistance in media simulating the nutritional composition of different body fluids. We found that mutational acquisition of resistance varied in urine, SCFM or laboratory rich medium, hence modifying the level of resistance to the antibiotic used as selective agent as well as the phenotypes of cross-resistance and collateral sensitivity to other drugs. In addition, we observed that the MIC to the antibiotic of selection and fitness cost of representative clones of each evolution was different in each media, supporting that the phenotype of AR and its consequences in bacterial physiology may depend on the nutritional composition of the place of infection. Further, the group of mutations selected in each medium were usually those ones causing the highest resistance level and/or the lowest fitness cost in the respective medium. For instance, large chromosomal deletions, only selected in rich medium in the presence of ceftazidime or ceftazidime-avibactam, provided a huge AR increase, but their associated fitness costs probably impeded their selection in SCFM or urine, where different genetic variations emerged. This means that fitness costs associated with AR mutations are dependent on the metabolic state of the bacteria in each particular situation, and are not a rigid non-specific burden equally occurring in every habitat. Hence, only some resistance mutations can be selected in different sites of infection since the fitness costs they produce and the level of AR they provide are specific of each environment. This makes evolution of AR highly dependent on the environmental conditions in which selection occurs, something that should be considered in the design of evolution-based strategies to treat infections.

### 4.3. The ancestral role of bacterial efflux pumps provides clues for the identification of anti-virulence compounds

Bacterial efflux pumps extrude not only antibiotics but also heavy metals, organic pollutants, innate host defence molecules or bacterial metabolites, having therefore a role in several aspects of bacterial physiology such as response to stress situations (395, 396), detoxification of damaging compounds (123), modulation of QS signalling (133, 136, 137, 397), colonization and propagation during animal host infection (116, 138, 139, 383, 396) or plant-bacteria interactions (123, 144, 398). Regarding the later, efflux pumps are able to detoxify plant-produced compounds that inhibit bacterial virulence, hence facilitating the colonization of the plant tissues. In addition, the expression of these efflux pumps is induced by such plant-derived substrates, therefore increasing their extrusion and exemplifying a plant-bacteria co-evolutionary phenomenon. This situation has been described in several bacterial species able to infect plants, such as *P. syringae*, *A. tumefaciens* or *E. amylovora,* being plant-produced flavonoids the inducer/substrate compounds (140-143).

Since *P. aeruginosa* is a bacterium with environmental origin, and may act as a plant pathogen, we wondered whether its efflux pumps might also have a role in the response to plant-derived compounds able to reduce the bacterial virulence. For exploring this hypothesis, during this PhD, we studied the expression of *P. aeruginosa mexAB-oprM* and *mexCD-oprJ* when growing in presence of a collection of plant-derived molecules, by using the previously mentioned biosensor strains. One-third of the molecules detected as inducers of both efflux pumps during the screening were coumarin-like compounds; 1,4-benzodioxan-like molecules were frequently found among *mexCD-oprJ* inducers and an indole-derivative induced the expression of *mexAB-oprM*. Both benzodioxans and coumarins are compounds present in several plants, presenting different bioactive functions with pharmacological interest (399, 400). For instance, many coumarins have been applied for different therapies, such as anticoagulant, antioxidants, anti-inflammatory and anti-cancer procedures (401). For its part, indole derivatives fulfil an important role as plant hormones (402).

Then, we observed that two compounds with coumarin-like structure -each one being an inducer of each efflux pump-, a 1,4-bezodioxan-like molecule -an inducer of *mexCD-oprJ*- and 4-chloroindole -a *mexAB-oprM* inducer-, were also substrates of the same efflux pump they induced. This could explain why these inducers produce a minor effect in transient AR. As previously mentioned, if an inducer is also a substrate of the efflux pump, there could be a competition between it and the antibiotic for their extrusion, compensating the transient induction of AR associated with the over-expression of the efflux pump encoding genes with a reduced extrusion capacity of the drug by the over-produced efflux pump.

In line with the initial hypothesis of this work, the fact that these plant-derived inducers are also substrates of the respective efflux pump might constitute a detoxification mechanism of potential anti-virulence compounds. Indeed, we observed a reduction of motility and toxin secretion through the T3SS when adding the studied molecules, which was caused by a reduction of the expression of genes encoding the flagellum and T3SS-related genes. The reduction of these virulence determinants in presence of the compounds led to a decreased lethality of *P. aeruginosa* in a *C. elegans* animal model. Further pointing to these compounds as part of a promising therapy, the activity of T3SS and flagellum is usually associated with poor clinical outcomes in *P.*

*aeruginosa* acute infections (302, 305-307, 314, 316), hence being relevant for the virulence of this bacterium. Therefore, we proposed that these molecules could be used, alone or in combination with antibiotics, to inhibit bacterial virulence, since they produce minor changes, if any, in transient AR.

Hence, taking into consideration the ancient role of efflux pumps, we also defined a strategy for finding *P. aeruginosa* anti-virulence compounds, based on the search for plant-derived molecules which were both inducers and substrates of efflux pumps.

### 4.4. Studying antibiotic resistance to better understand evolution

The acquisition of AR is one of the few evolutionary processes that can be experimentally addressed. Therefore, besides its importance in the AR field, its study may give relevant information about evolution in general terms.

Firstly, we described a case of convergent evolution towards fosfomycin collateral sensitivity associated with the acquisition of resistance to different antibiotics (tobramycin, tigecycline or ceftazidime). Convergent evolution is common when different organisms are confronted with similar selective pressures, hence acquiring equivalent phenotypes that deal with the challenge. In other words, a conserved phenotype emerging in different organisms is generally considered to be selected because of its contribution to adaptation to the same selective force (403, 404). However, the phenotype here described is not selected as a response to similar selective forces and it is not an adaptive phenotype to the challenges to which bacteria were exposed. The selective forces underlying the process -tobramycin, tigecycline or ceftazidime-, are expected to promote the selection of tobramycin, tigecycline or ceftazidime resistance but not a fosfomycin susceptibility alteration. Then, our results support that side selection driven by unrelated selective forces might be in the basis of the higher than expected prevalence of some phenotypes along evolution.

Further, our results also provide an example of convergent evolution towards collateral sensitivity to tobramycin and aztreonam, occurring in different genetic backgrounds when the same selective force -the presence of ciprofloxacin- was applied. Phenotypic convergence may be due to parallel evolution, in which the same genetic event responsible for the convergent phenotype is selected in every genomic background (405). Evolution leading to a conserved maladaptive phenotype is not something new, for instance it was already discussed by Haldane in order to explain the prevalence of some inherited human diseases (406). He proposed that the force driving the selection of sickle cell disease was the infection by *Plasmodium falciparum*; inherited anaemia was just an evolutionary trade-off associated with resistance to this infection, a hypothesis afterwards validated (407). However, convergent evolution may be also due to different genetic events that give rise to the same phenotype (408). In this case, the emerging conserved phenotype is expected to be adaptive to the selective pressure: different genetic solutions can deal with the same selective force. Nevertheless, in this work, we observed that ciprofloxacin selects mutations in different genes in each genetic background that, in addition to producing ciprofloxacin resistance, also lead to susceptibility to tobramycin and aztreonam, being the last phenotype non-adaptive to the selective pressure. Therefore, we report a case of convergence of a phenotype

which does not provide direct adaptation to the selective force and which is not caused by parallel evolution. Our observations demonstrate that deciphering the selective forces that lead to non-adaptive phenotypic convergence associated with different genetic variations constitutes a great challenge.

Furthermore, we also studied compensatory evolution of fitness cost associated with ceftazidime resistance acquisition. In this work, we observed that the presence of ceftazidime leads to an adaptive phenotype -ceftazidime resistance- and two phenotypes which are not adaptive to the selective pressure -tobramycin collateral sensitivity and fitness costs in the absence of drugs-. Evolution's arrow of time is provided by the cumulative acquisition of adaptive genetic changes, increasing complexity along the process (409). This arrow only operates if the adaptive phenotype is fixed and remains after selection ends, because, otherwise, we would expect short-sighted evolution processes (225, 410). In this study, we found that the adaptive phenotype -ceftazidime resistance- and one of its associate trade-offs -fitness costs- decline after removing the selective pressure. However, the other trade-off -tobramycin collateral sensitivity- remains, since it is not associated with mutations in the genes that suffered intragenic compensatory evolution. These results show the relevance that stable non-adaptive phenotypes might have in evolution, something that may have been frequently underscored.

Finally, we studied the role of efflux pumps in the interaction of *P. aeruginosa* with plant-derived compounds. We found several anti-virulence natural-like compounds that are substrates and inducers of the efflux pumps MexCD-OprJ and MexAB-OprM, giving an example of a function of efflux pumps besides the detoxification of antibiotics, possibly selected after co-evolution between plants and bacteria. Further, as previously mentioned, efflux pumps have several roles with importance for bacterial physiology, such as response to stress situations (395, 396), detoxification of damaging compounds (123), modulation of QS signalling (133, 136, 137, 397), colonization and propagation during animal host infection (116, 138, 139, 383, 396) or plant-bacteria interactions (123, 144, 398). Hence, efflux pumps might have a role in an ancient homeostasis, detoxification or signal trafficking, while their role in AR is relevant just in environments with high, selective, antibiotic load. For instance, MexAB-OprM, MexEF-OprN and MexCD-OprJ efflux pumps of *P. aeruginosa* extrude QS signal molecules or their precursors (133-137), and are also AR determinants when this opportunistic pathogen is exposed to high concentrations of antibiotics in clinics (253). Similarly, the efficient colonization of plants by *S. maltophilia* is mediated by the SmeDEF efflux pump, which is also the principal determinant of resistance to quinolones of this opportunistic pathogen (144). The term exaptation defines this kind of processes in which some elements shaped by natural selection to cover a defined function are eventually used to play a different role that becomes the most important one when the environment changes (64). For example, the development of feathers was proposed to fit into this term, since they might be originally selected for thermoregulation and later on used for flight. The original functional role of efflux pumps, as the one studied during this PhD, and their current importance as AR determinants, constitutes an example of exaptation and confirms how relevant this concept is for understanding evolution.

# *CONCLUSIONS*

## 5. Conclusions

I. Tobramycin, tigecycline and ceftazidime resistance acquisition in *P. aeruginosa* is associated with a robust fosfomycin collateral sensitivity. This phenotype is caused by different genetic modifications leading to a parallel downregulation of the expression of the peptidoglycan recycling pathway enzymes encoding genes and the fosfomycin-inactivating enzyme encoding gene *fosA*.

II. Ciprofloxacin exposure leads to the selection of mutations in *mexS*, *nfxB* or *gyrAB* in *P. aeruginosa*. Despite the prevalence of mutations depends on the genetic background, robust tobramycin and aztreonam collateral sensitivity phenotypes emerge in the different genetic backgrounds analysed.

III. The combinations ciprofloxacin-tobramycin and ciprofloxacin-aztreonam constitute promising collateral sensitivity-based therapeutic strategies against *P. aeruginosa* infections caused by populations presenting diverse genetic backgrounds.

IV. Media composition and nutrients' availability constrain the evolutionary pathways towards tobramycin, ceftazidime and ceftazidime-avibactam resistance in *P. aeruginosa*. The level of resistance and fitness costs associated with each AR mutation is dependent on the specific environment where *P. aeruginosa* is present.

V. Fosfomycin collateral sensitivity associated with ceftazidime resistance acquisition robustly emerges when *P. aeruginosa* evolves in different media -mimicking those that can be encountered during infection-, being the downregulation of the peptidoglycan recycling pathway enzymes encoding genes in the basis of such event. This result points to the alternation or combination of fosfomycin and ceftazidime as promising strategies against *P. aeruginosa* causing infections in different body locations.

VI. Compensatory evolution of fitness costs associated with ceftazidime resistance in *P. aeruginosa* leads to ceftazidime resistance decline, both in antibiotic-free and in sublethal tobramycin environments. This is caused by intragenic compensatory mutations.

VII. The alternation of ceftazidime with drug restriction periods and a switch back to ceftazidime may be a feasible approach to tackle AR in *P. aeruginosa*. In addition, it could be possible a switch back to ceftazidime after a ceftazidime-tobramycin alternation.

VIII. Dequalinium chloride, procaine and atropine induce the expression of *mexCD-oprJ* and transiently increase ciprofloxacin resistance of *P. aeruginosa*, which may compromise the treatment of infections caused by this opportunistic pathogen when these drugs are used.

IX. The identification of compounds that are both substrates and inducers of efflux pumps of *P. aeruginosa* constitutes an effective strategy for finding molecules that reduce the virulence potential of this pathogen.

# 5. Conclusiones

I. La adquisición de resistencia a tobramicina, tigeciclina y ceftazidima en *P. aeruginosa* está asociada a una sensibilidad colateral a fosfomicina robusta. Este fenotipo está causado por diferentes modificaciones genéticas que conducen a una disminución paralela de la expresión de los genes que codifican las enzimas de la vía de reciclado del peptidoglicano y del gen que codifica la enzima inactivadora de la fosfomicina, *fosA*.

II. La exposición a ciprofloxacino provoca la selección de mutaciones en *mexS*, *nfxB* o *gyrAB* en *P. aeruginosa*. A pesar de que la prevalencia de las mutaciones depende del fondo genético, emerge un fenotipo robusto de sensibilidad colateral a tobramicina y a aztreonam en los diferentes contextos genéticos analizados.

III. Las combinaciones de ciprofloxacino con tobramicina y de ciprofloxacino con aztreonam constituyen unas estrategias terapéuticas prometedoras, basadas en la sensibilidad colateral, contra las infecciones causadas por poblaciones de *P. aeruginosa* que presentan diversos contextos genéticos.

IV. La composición del medio y la disponibilidad de nutrientes limitan las vías evolutivas hacia la resistencia a tobramicina, ceftazidima y ceftazidima-avibactam en *P. aeruginosa*. El nivel de resistencia y los costes fisiológicos asociados a cada mutación de resistencia a antibióticos dependen del ambiente específico donde se encuentre *P. aeruginosa*.

V. La sensibilidad colateral a fosfomicina asociada a la adquisición de resistencia a ceftazidima emerge de forma robusta cuando *P. aeruginosa* evoluciona en diferentes medios -imitando los que se puede encontrar durante la infección-, siendo la reducción de la expresión de los genes que codifican las enzimas de la vía de reciclado del peptidoglicano la base de dicho evento. Este resultado apunta al uso alternado o la combinación de fosfomicina y ceftazidima como estrategias prometedoras contra *P. aeruginosa* causante de infecciones en diferentes localizaciones corporales.

VI. La evolución compensatoria de los costes fisiológicos asociados a la resistencia a ceftazidima en *P. aeruginosa* conduce a una disminución de la resistencia a ceftazidima, tanto en entornos libres de antibióticos como en presencia de concentraciones subletales de tobramicina. Esto se debe a mutaciones compensatorias intragénicas.

VII. El uso alternado de ceftazidima con periodos de restricción de fármacos puede ser un enfoque factible para abordar la resistencia a los antibióticos en *P. aeruginosa*. Además, sería posible volver a usar ceftazidima después del uso alternado de ceftazidima y tobramicina.

VIII. El cloruro de decualinio, la procaína y la atropina inducen la expresión de *mexCD-oprJ* y aumentan transitoriamente la resistencia a ciprofloxacino de *P. aeruginosa*, lo que puede comprometer el tratamiento de las infecciones causadas por este patógeno oportunista, cuando estos compuestos se utilizan.

IX. La identificación de compuestos que sean a la vez sustratos e inductores de las bombas de eflujo de *P. aeruginosa* constituye una estrategia eficaz para encontrar moléculas que reduzcan el potencial de virulencia de este patógeno.

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# *ANNEXES*

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COMMENTARIES

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# Antimicrobial resistance: A multifaceted problem with multipronged solutions

Teresa Gil-Gil | Pablo Laborda | Fernando Sanz-García | Sara Hernando-Amado | Paula Blanco | José Luis Martínez

Centro Nacional de Biotecnología CSIC, Madrid, Spain

**Correspondence**
José Luis Martínez, Centro Nacional de Biotecnología CSIC, Darwin 3, 28049-Madrid, Spain.
Email: jlmtnez@cnb.csic.es

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## Abstract

Infectious diseases still stand as a major cause of morbidity and mortality, and this problem can be worsened with the current antimicrobial resistance crisis. To tackle this crisis more studies analyzing the causes, routes, and reservoirs where antimicrobial resistance can emerge and expand, together with new antimicrobials and strategies for fighting antimicrobial resistance are needed. In the current special issue of MicrobiologyOpen, a set of articles dealing with the multiple faces of antimicrobial resistance are presented. These articles provide new information for understanding and addressing this problem.

**KEYWORDS**
antibiotic resistance, One Health, Global Health, Infectious diseases

## 1 | COMMENTARY

Antimicrobial resistance (AMR) is a relevant problem for human health as recognized by several independent agencies, governments, and international organizations, including the WHO and the UN among others (Govindaraj Vaithinathan & Vanitha, 2018). The problem is not restricted to human-linked habitats, since different works have shown that other ecosystems, including animals, soil and water bodies contribute to the origin, spread and maintenance of AMR, hence being a One Health problem (Berendonk et al., 2015). Besides, AMR bacteria (AMRB) can expand through different geographical areas, which constitutes a Global Health problem (Hernando-Amado, Coque, Baquero, & Martinez, 2019). Understanding the elements involved in the emergence of resistance as well as the ways of counteracting such resistance, requires then integrated approaches where the ecological and evolutionary aspects of antimicrobial resistance are taken into consideration (Baquero, Alvarez-Ortega, & Martinez, 2009). In the current special issue on Antimicrobial Resistance, MicrobiologyOpen presents a set of articles covering different areas of AMR, from the study of reservoirs and vectors for its dissemination to the analysis of novel antimicrobial compounds with potential therapeutic use.

Besides being a relevant health problem, AMRB and AMR genes (AMRGs) released from human-linked reservoirs constitute nowadays a pollution problem. Thus, knowing where and how they can be inactivated is a subject of interest (Martinez, 2012). This issue has been addressed by analyzing the levels and diversity of AMRGs in sandy beach environments compared with mangroves of South China, these last receiving sewage and aquaculture wastewater from coastal regions (Zhao et al., 2019). Notably, the authors found that the total abundance of AMRGs is lower in mangrove sediments. Despite the authors state that the properties of the sediment and the presence of mobile genetic elements are the most relevant elements in shaping the AMRGs, it is relevant to state that, as described by others (Forsberg et al., 2014), most analyzed genes are intrinsic AMRGs, indicating that the phylogenetic composition of the community should also be relevant. One important element for understanding the spread of AMR is the delimitation of the transmission routes. Wild birds, particularly migratory ones, can contribute to such dissemination, being a potential vector for AMR spread among

geographically distinct areas (Bonnedahl & Jarhult, 2014). The analysis of fecal samples from short- and long-distance migratory wild birds in Switzerland (Zurfluh et al., 2019) showed that 5.8% of the birds contained AMR *Escherichia coli* and several of them harbored clinically-relevant extended-spectrum beta-lactamases as CTX-M-15, CTX-M-55, or CTX-M-65. Since several of these birds are present in both, urbanized and natural environments, they could potentially spread AMRBs within and between these environments.

One of the challenges to face AMR is the lack of quantitative models for analyzing its dissemination. A probabilistic model to estimate cross-contamination and recontamination by methicillin-resistant *Staphylococcus aureus* in retail meat via hands and cookware during a household barbecue is presented in (Plaza-Rodriguez, Kaesbohrer, & Tenhagen, 2019). According to this model, the probability of one ARMB to be transferred from the contaminated raw chicken meat to the final serving is low. The application of this model to other potential transmission routes may help in quantifying the relevance of each one of them. These models can be fueled by the genomic analysis of recent opportunistic pathogens, particularly those with an environmental origin that can spread through human-linked and environmental reservoirs. This is the case of *Elizabethkingia anophelis*, an emerging and frequently misdiagnosed opportunistic pathogen, whose AMR spectra and pathogenesis mechanisms are still unclear. The genome of a clinical isolate presenting resistance to 20 antibiotics was sequenced, and genes encoding different beta-lactamases and efflux pumps, as well as several potential virulence factors, were found (Wang et al., 2019). The comparison of three clinical and two environmental *E. anopheles* strains showed that they present similar AMRGs and virulence determinants, further highlighting the role that natural ecosystems may have in the spread of AMRBs (Martinez, 2008). Even once the genomes of a pathogenic species have been sequenced, and the molecular basis for AMR predicted, fast, reliable, and cheap methods for their detection is needed. One example of this situation is the *Aeromonas* genus, in which the phenotypic identification of species causing human infections is not always easy. Proteomic identification by MALDI-TOF MS, linked to classical susceptibility tests, emerges as a valuable tool, in terms of time-efficiency and low-costs, for solving this issue (Elbehiry et al., 2019). It is important to notice that fast detection and molecular epidemiological surveys of AMR require previous knowledge of the molecular basis of resistance (McArthur & Wright, 2015); and predictive approaches are of utmost relevance for this purpose (Martinez, Baquero, & Andersson, 2007). Two articles in this special issue make use of these methods to predict the emergence of resistance to two antimicrobials produced by lactic bacteria: nisin (Arii, Kawada-Matsuo, Oogai, Noguchi, & Komatsuzawa, 2019), an antibacterial peptide used as a food preservative, and the bacteriocin plantaricin EF (Heeney, Yarov-Yarovoy, & Marco, 2019). Mutations that lead to plantaricin resistance are located in the *corC* gene, which encodes a putative membrane-bound magnesium/cobalt efflux protein, which is supposed to be the bacteriocin target. Thus, the mechanism of action of plantaricin EF could be linked to modifications in the bacterial metal homeostasis. *S. aureus*, on its hand, acquires resistance to nisin as a consequence of the selection of mutations in the two-component system BraRS, which in turn leads to the increased expression of the VraDE efflux pump, also known to be involved in daptomycin and gallidermin resistance (Popella et al., 2016). These results highlight the fact that the acquisition of resistance to one antibiotic can modify the susceptibility to others, either reducing (cross-resistance) or increasing (collateral sensitivity) bacterial susceptibility to them. While the analysis of cross-resistance phenotypes gives clues for avoiding the use of some specific antibiotics when bacteria have acquired resistance to another one, the analysis of the collateral sensitivity networks can help to develop combined or cyclic therapeutic regimes (Podnecky et al., 2018; Sanz-Garcia, Hernando-Amado, & Martinez, 2018). Indeed, *Pseudomonas aeruginosa* mutants selected in the presence of carbapenems presented decreased susceptibility to doripenem, meropenem, and imipenem, while their susceptibility to aminoglycosides, fluoroquinolones, and noncarbapenem β-lactams was higher than that of the parental clinical isolate from which these mutants derive (Harrison, Fowler, Abdalhamid, Selmecki, & Hanson, 2019). While alterations in the porin OprD, the entrance channel for carbapenems, were responsible for the increased resistance to these antibiotics, modifications in the lipopolysaccharide were likely the cause of aminoglycoside hypersensitivity. These evolutionary constraints imposed by collateral sensitivity could be exploited in the clinics to the rational design of treatments.

Most studies on AMR are based on the analysis of resistance to industrially produced antimicrobials used in therapy or for prophylactic purposes. Nevertheless, it is important to recall that the infected organisms can produce a set of antimicrobial compounds and knowing the bacterial mechanisms of resistance to such compounds is of utmost relevance for understanding infection. As shown by Desloges et al., (2019), the capacity of uropathogenic *E. coli* to resist being killed by antimicrobial peptides during urinary tract infections (UTIs) is mediated by the protease OmpT. Noteworthy, the presence of another *ompT*-like gene (*arlC*) causes higher clinical severity in the UTIs. Therefore, the presence of this second AMRG should be taken into consideration for diagnostic and prognostic purposes in *E. coli* urinary infections.

One of the main causes of the increasing problem of AMR is the lack of novel antimicrobials or therapeutic approaches (Bush et al., 2011), a matter that is also explored in the current special issue of MicrobiologyOpen. To that purpose, different strategies could be implemented. The search of naturally-produced compounds is exemplified by the article of Horta et al., (Horta et al., 2019), where the biodiversity of bacteria associated with the red seaweed *Asparagopsis armata*, together with the production of antimicrobial compounds by these microorganisms, is analyzed. Two of the investigated isolates produce potent anti–Gram-positive antimicrobials, suggesting that the microbiota associated with *A. armata* can be a source of compounds with pharmacological interest. Genome mining of antimicrobial producers may also help in developing novel antimicrobials (Desloges et al., 2019). Using this approach, Nakaew et al., (Nakaew, Lumyong, Sloan, & Sungthong, 2019) showed that an antibiotic-producing *Streptomyces* sp. isolate

encodes in its genome, not only biosynthetic pathways of already known antimicrobials but also pathways predicted to be involved in the biosynthesis of novel antibiotics, which, as above stated, are urgently needed. Most antibiotics currently in use are of natural origin or derived from natural antimicrobials. However, purely synthetic antimicrobials as quinolones can also effectively inhibit bacterial pathogen growth. A synthetic approach was followed by Serafim et al., (Serafim et al., 2019) for synthesizing new 1,3-bis(arloxy)propan-2-amines with biological activity against Gram-positive pathogens such as *Streptococcus pyogenes*, *Enterococcus faecalis*, and *S. aureus*. By using docking approaches, the authors suggest that their compounds might bind to the cell division protein FtsZ. Although these predictions are far away to be confirmed, anti-FtsZ antimicrobials should constitute a novel group of antibiotics with clear clinical value (Panda et al., 2016).

It is relevant to remind that the use of antimicrobials is not restricted to the treatment of human infections. They are also used in animal and crop production. Consequently, the use of new anti-infective approaches for avoiding infections associated with these activities would reduce the global antimicrobial load, which will have a positive effect in reducing the AMR burden. One of these approaches can be combination therapy as described by Procopio et al (Procopio et al., 2019). In their study, the authors showed that the lectin CasuL is a bacteriostatic and antibiofilm agent against some Staphylococcal isolates obtained from animals with mastitis. Besides, it presents a synergistic activity when used in combination with antibiotics, supporting the need for conducting new studies to determine the feasibility of this approach in the treatment of caprine and bovine mastitis. A classical way for avoiding infections in crops is the development of transgenic plants with anti-infective properties. Khademi et al., (Khademi, Nazarian-Firouzabadi, Ismaili, & Shirzadian Khorramabad, 2019) developed transgenic tobacco plants which were able to produce dermaseptin B1, an antimicrobial peptide produced by *Phyllomedusa bicolor*. The purified recombinant peptide presented a strong antimicrobial activity, suggesting it can be useful as a novel compound for treating plants infections. Also, the results suggest that transgenic plants expressing dermaseptin B1 might be resistant to infection.

The diversity of the articles published in this special issue of MicrobiologyOpen supports that, as stated in the title, antibiotic resistance is a multifaceted problem that requires multipronged solutions.

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## CONFLICT OF INTEREST

None declared.

## AUTHOR CONTRIBUTIONS

Writing-original draft: TGG, PL, FSG, SHA, PB; Writing-review and editing: TGG, PL, FSG, SHA, PB, JLM; Conceptualization: JLM; Resources: JLM; Supervision: JLM.

## ETHICAL APPROVAL

None required.

## DATA AVAILABILITY STATEMENT

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## ORCID

*José Luis Martínez* https://orcid.org/0000-0001-8813-7607

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# Coming from the Wild: Multidrug Resistant Opportunistic Pathogens Presenting a Primary, Not Human-Linked, Environmental Habitat

**Fernando Sanz-García, Teresa Gil-Gil, Pablo Laborda, Luz E. Ochoa-Sánchez, José L. Martínez * and Sara Hernando-Amado**

Centro Nacional de Biotecnología, CSIC, 28049 Madrid, Spain; fsanz@cnb.csic.es (F.S.-G.); tgil@cnb.csic.es (T.G.-G.); plaborda@cnb.csic.es (P.L.); luzedith.os@gmail.com (L.E.O.-S.); shernando@cnb.csic.es (S.H.-A.)
* Correspondence: jlmtnez@cnb.csic.es

**Abstract:** The use and misuse of antibiotics have made antibiotic-resistant bacteria widespread nowadays, constituting one of the most relevant challenges for human health at present. Among these bacteria, opportunistic pathogens with an environmental, non-clinical, primary habitat stand as an increasing matter of concern at hospitals. These organisms usually present low susceptibility to antibiotics currently used for therapy. They are also proficient in acquiring increased resistance levels, a situation that limits the therapeutic options for treating the infections they cause. In this article, we analyse the most predominant opportunistic pathogens with an environmental origin, focusing on the mechanisms of antibiotic resistance they present. Further, we discuss the functions, beyond antibiotic resistance, that these determinants may have in the natural ecosystems that these bacteria usually colonize. Given the capacity of these organisms for colonizing different habitats, from clinical settings to natural environments, and for infecting different hosts, from plants to humans, deciphering their population structure, their mechanisms of resistance and the role that these mechanisms may play in natural ecosystems is of relevance for understanding the dissemination of antibiotic resistance under a One-Health point of view.

**Keywords:** opportunistic pathogens; MDR; One-Health; intrinsic resistance; environmental bacteria; *Pseudomonas aeruginosa*; *Stenotrophomonas maltophilia*; *Acinetobacter baumannii*; *Burkholderia cepacia*; *Shewanella*; *Aeromonas*



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## 1. Introduction

Bacterial organisms causing human infections can be divided into two categories; those that infect healthy people, and those that mainly infect people with underlying diseases, immunosuppressed or debilitated. While the former are relevant both in the community and in the hospitals, the latter have been dubbed opportunistic pathogens and are primarily a hospital problem [1]. Opportunistic pathogens have historically originated from human commensal bacteria. Indeed, in the seminal paper that led to the search of antibiotic producers in soils, the main reason for such screening was that, despite the soil being a sink for organisms infecting humans, "one hardly thinks of the soil as a source of epidemics" [2]. Nevertheless, in the last decades, an increased prevalence of opportunistic pathogens with an environmental origin, most of them non-fermentative Gram-negative bacteria [3], has been reported [4]. Most of these pathogens present low susceptibility to antibiotics currently used in therapy, suggesting that the enrichment of these pathogens at hospitals can result from the selection pressure exerted by antibiotics used for treating infectious diseases [5]. Actually, one of the risk factors for being infected by these pathogens is previous antibiotic treatment with broad-spectrum antibiotics. As opposed to what Waksman and Woodruff stated in 1940 [2], it is now evident that natural environments encompass an undefined

reservoir of bacterial species, some of which have the potential to infect humans. These infections mainly occur in immunodeficient people and patients with underlying diseases. This fact suggests that, beyond the existence of specific lineages that have evolved towards virulence, the main reason behind infection by this type of opportunistic pathogens is the health status of the infected patient. Indeed, for most of the opportunistic pathogens herein reviewed, there are not clear genomic differences between environmental and clinical isolates. This factor does not mean that epidemic clones are absent, but rather that those clones, more frequently involved in outbreaks at hospitals, are also present in natural ecosystems. Further, the fact that most of their virulence determinants and several antibiotic resistance genes (ARGs) are usually present in their core genomes supports that these elements have evolved to deal with functions other than infecting humans in the natural habitats that these microorganisms colonize. In the present article, we review the most relevant current information on these pathogens, with a particular emphasis on their mechanisms of antibiotic resistance (AR). It is important to notice that, besides being the primary habitat (i.e., the origin) of some opportunistic pathogens, natural ecosystems are the places where all human bacteria, pathogens and commensals end up, along with the ARGs they carry [6–8]. While environmental antibiotic-resistant organisms, such as *Pseudomonas aeruginosa* or *Burkholderia cepacia*, regularly colonize environmental habitats, other pathogens with relevance for the dissemination of resistance, such as *Escherichia coli*, *Enterococcus* or *Klebsiella pneumoniae*, are part of human-linked microbiomes; their finding in a natural ecosystem is considered a sign of anthropogenic pollution [9,10], to the extent that it has been stated that resistant organisms detected in wastewater treatment plants should reflect the overall resistome of the human populations they serve [11–14]. Certainly, upon such pollution, natural ecosystems can be drivers for the evolution and spread of AR in any human pathogen [6,15]; however, in the current review, we focus just on those organisms that present a bona fide, non-clinical, environmental primary habitat, where they have evolved [16,17] before causing human infections.

## 2. *Pseudomonas aeruginosa*

*P. aeruginosa* is a Gram-negative, rod-shaped, non-fermentative, facultative anaerobic bacterium able to colonize a wide range of different habitats due to its high metabolic versatility and broad capacity of adaptation to fluctuating environments [18]. Its presence has been described in soil [19], crude oil [20] or different aquatic systems such as wastewater [21], freshwater and seawater environments [22], being found among the most frequent locations those closely related to human activities [23].

Considering a host as an environment colonizable by bacteria [16], the ability of *P. aeruginosa* to cause infections in a large range of hosts may also be used to exemplify its high adaptability and ubiquitous distribution. *P. aeruginosa* infections have been reported in plants [24], animals -such as insects [25], nematodes [26], fishes [27] or mammals [28], including humans [29]- or even amoebas, such as *Dictyostelium discoideum* [30]. However, despite the wide distribution of *P. aeruginosa*, several studies have indicated that there are no specific clones associated with specific habitats and that environmental and clinical isolates are indistinguishable. In addition, there is a consensus about the non-clonal epidemic nature of *P. aeruginosa* population structure [31].

*P. aeruginosa* is one of the main causes of nosocomial infections, including acute respiratory diseases and bacteraemia [32]. Moreover, it can chronically infect immunocompromised people or patients with underlying diseases, such as chronic obstructive pulmonary disease (COPD) [33], cystic fibrosis (CF) [34], AIDS [35], cancer [36] or those presenting burn or surgical wounds [37], being that these infections are an utmost source of morbidity and mortality in intensive care units (ICUs).

The impact of *P. aeruginosa* on human health cannot be understood without taking into account the vast amount of virulence factors it possesses. Proteases, flagella, secretion systems, biofilm formation (which is particularly worrying when located in catheters, prosthesis or lungs [38–40]) or quorum sensing (QS) (the cell-cell signalling

system that coordinates the expression of most of the said factors [41]), are elements that pave the way for infection and hamper therapies. Further, this microorganism exhibits low susceptibility to a great number of drugs [42], an issue that is dissected below. Overall, these features result in *P. aeruginosa* being subsumed into two bacterial ensembles, namely ESKAPE (acronym of *Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter* spp.) and TOTEM (TOp TEn resistant Microorganisms), which include the currently most relevant multidrug-resistant human pathogens [43,44].

*P. aeruginosa* infections are frequently treated with aminoglycosides, especially tobramycin, as well as with cephalosporines or β-lactam/β-lactamase-inhibitor combinations, such as piperacillin/tazobactam or ceftazidime/avibactam [32]. Besides, fluoroquinolones (ciprofloxacin), polymyxins, fosfomycin, aztreonam and carbapenems are also antibiotics of choice, which usage depends on the characteristics of the infection [45]. Nevertheless, the aforementioned intrinsic resistance of this pathogen to some antibiotics has compelled many to search for novel β-lactam/β-lactamase inhibitors, like imipenem/relebactam [46], or the development of new antimicrobial compounds, such as plazomicin, murepavadin or doripenem [47]. It is also important to remark that non-antibiotic therapies have been delved into, in order to counteract treatment failure when resistance to classical drugs emerges. Among these strategies, anti-virulence compounds, efflux pump inhibitors and permeabilizing membrane compounds (co-administered with antibiotics or on their own) stand out as the most promising alternatives [48–50]. In addition, evolution-based approaches that exploit phenotypic convergence and negative hysteresis phenomena are currently being investigated to fight *P. aeruginosa* and other human pathogen infections [51–54].

The above-stated high intrinsic resistance to antibiotics of *P. aeruginosa* (Figure 1) is due to its low outer membrane permeability [55], the production of antibiotic-modifying enzymes [56], and the large stock of multidrug resistance (MDR) efflux pumps it harbours [47]. Concerning the latter, there are 12 Resistance Nodulation Division (RND) family members that have been ascribed to this bacterium; among them, MexAB-OprM, MexXY-OprM, MexCD-OprJ and MexEF-OprN are of significant interest, given their known role in clinical settings [57]. The first two are the ones that have been shown to contribute to intrinsic resistance, but every system is able to extrude a wide range of antimicrobial agents (Table 1). Regarding antibiotic-modifying enzymes, *P. aeruginosa* can resort to its inherent assortment of β-lactamases, making AmpC the most noteworthy [58], and aminoglycoside-modifying enzymes, namely aminoglycoside acetyltransferases, phosphotransferases and nucleotidyltransferases [59]. Additionally, it is worth emphasizing that its intrinsic resistome does not only consist of classical resistance determinants, but it may encompass basic components of bacterial physiology [60]. For example, Crc is a global regulator of carbon metabolism whose inactivation entails an increased susceptibility to several antimicrobials in *P. aeruginosa* [61]. This situation agrees with the notion that the ancestral, physiological function of intrinsic resistance determinants of pathogens with an environmental origin goes beyond counteracting the activity of antimicrobial agents currently used in therapy. As previously discussed [42], interfering with the effectiveness of antibiotics is a novel functional role of these determinants, promoted by the current antibiotic era.

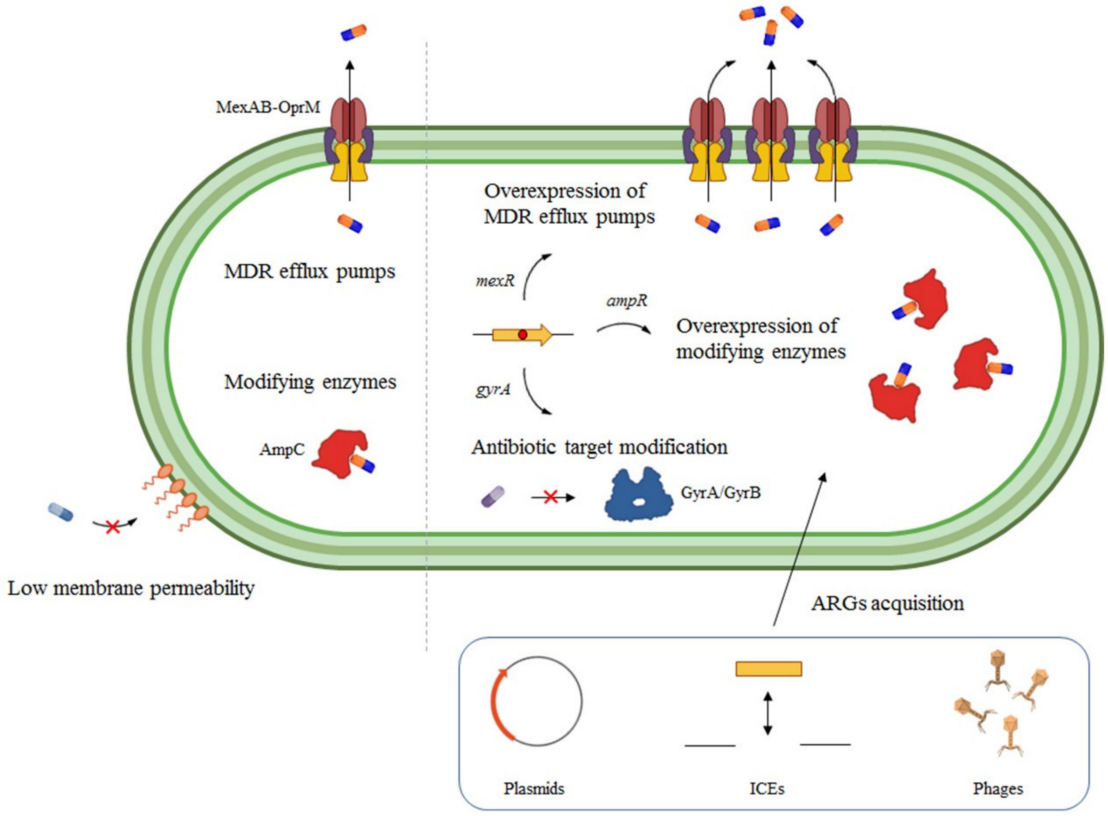


**Figure 1.** Schematic representation of the main elements involved in intrinsic and acquired antibiotic resistance in *Pseudomonas aeruginosa*. *P. aeruginosa* possesses a remarkable intrinsic resistance to antibiotics caused, among other factors, by the production of antibiotic-modifying enzymes (e.g., β-lactamase AmpC), low outer membrane permeability and a great amount of multidrug resistance (MDR) efflux pumps like MexAB-OprM. Antibiotic resistance level may increase by chromosomal mutations in genes encoding negative regulators of the above-described intrinsic resistance determinants, such as genetic modifications within *mexR* or *ampR*, which boost the expression of *mexAB-oprM* and *ampC*, respectively. The modification of the antibiotic target is also a frequent mechanism for acquiring antibiotic resistance in *P. aeruginosa*, as the increased resistance to quinolones by mutations in gyrases encoded by *gyrA* or *gyrB*. Alternatively, this bacterium is also able to acquire novel ARGs, which are located in mobile elements, such as plasmids or integrative and conjugative elements (ICEs). It has been stated that bacteriophages might also be involved in the acquisition of ARGs, but the role of these genetic elements in the spread of resistance in *P. aeruginosa* is not yet fully understood.

In addition to intrinsic AR, increased resistance levels may also be acquired by chromosomal mutations that boost the expression of the above-described determinants (Figure 1), a situation that frequently takes place during chronic infections [62]. For instance, mutations in genes encoding regulators of MDR efflux pumps can lead to the overexpression of the latter and, as a consequence, a more efficient extrusion of drugs. In the case of *mexAB-oprM*, overexpression may be due to mutations in the gene that encodes its local repressor, MexR, or in genes encoding other secondary regulators, NalC or NalD, events that have been reported in vivo [63,64]. Similarly, *mexCD-oprJ* and *mexXY-oprM* overexpression could be driven by spontaneous mutations in genes encoding their repressors, NfxB and MexZ, respectively [63,65]. Concerning β-lactamases, indirect or direct repressors of *ampC* expression, AmpR or AmpD, are commonly found mutated in β-lactam resistant strains of *P. aeruginosa*, presenting an enhanced β-lactamase activity and, consequently, resistance to β-lactams [58].

Besides, mutations can be selected in genes that encode the resistance determinants themselves. In the case of MDR efflux pumps, it has been published that amino acid changes in MexY, a subunit of the MexXY-OprM pump, may optimize antibiotic recognition site and, hence, improve drug efflux [66]. Regarding enzymes, cephalosporinase AmpC variants are

able to extend their substrate spectrum, becoming capable of hydrolyzing carbapenems [67] or recent β-lactam/β-lactamase inhibitor combinations [68].

Beyond modifications in resistance determinants, the mutational resistome of *P. aeruginosa* is still more multifarious. Some examples of the versatile mutational resistome of this opportunistic pathogen are the mutations in genes involved in the peptidoglycan recycling pathway, as *mpl or dacB*, which raise β-lactamase activity [69]; in genes that encode drug targets, as *gyrA* or *gyrB*, which foster quinolone resistance [70]; in genes encoding Penicillin Binding Proteins (PBPs), i.e., PBP3, which cause β-lactams resistance [71]; or even in *loci* that do not seem to be associated with AR, as *pilQ*, a gene that codes for a Type IV pili protein, which can give rise to resistance against various antipseudomonal agents [72].

Alternatively, this microorganism can acquire ARGs through Horizontal Gene Transfer (HGT) [47]. These genes can locate in integrative and conjugative elements (ICEs), plasmids, integrons, transposons or prophages (Figure 1), and they can be transferred by different mechanisms [73]. As expected, there is plethora of examples of ARG acquisition by *P. aeruginosa* through HGT, with ICEs and plasmids being the most usual ARGs carriers [74]. In this sense, an extensive miscellany of β-lactamases, aminoglycoside and fluoroquinolone-modifying enzymes have routinely been detected in these vectors [75–78], either alone or accompanied by large arrays of ARGs [79]. Correspondingly, integrons, which do not transfer independently, but are gene-recruiting elements, may also harbour metallo β-lactamases (MBLs) (i.e., carbapenemases) [80], aminoglycoside-modifying enzymes [81], or both [82], among other resistance mechanisms [83]. Phage particles containing ARGs have been found in the lungs of CF patients suffering chronic infections by *P. aeruginosa* [84]; in addition, a composite phage-like plasmid carrying the β-lactamase-encoding gene *blaKPC-2* has been found in a carbapenem-resistant *P. aeruginosa* isolate [85]. However, a deep understanding of the relevance of prophages in disseminating ARGs in *P. aeruginosa* requires further studies. In conclusion, this pathogen wields an astonishing range of alternatives to achieve AR.

Lastly, from a One-Health perspective, it must be noted that *P. aeruginosa* ubiquity in nature aggravates the problem of AR. This bacterium has a broad sub-lethal selective window to different antibiotics, under which resistant mutants may arise [86,87]. This matter becomes more alarming since substantial concentrations of drugs (i.e., 31 or 61 mg/L of ciprofloxacin or tetracycline, respectively) have been detected in habitats that *P. aeruginosa* can colonize [23,88], besides clinical settings. Further, aquatic ecosystems have been suggested as reservoirs and sources of ARGs, usually carried on plasmids, a situation that may play a critical role in the propagation of antimicrobial resistance among *P. aeruginosa* strains [89].

Besides intrinsic and genetically acquired, stable AR, the resistance phenotype can be acquired transiently, without the need for genetic changes. Transient resistance can be achieved by the bulk of the population, as happens in the case of biofilms [90]. The capacity of *P. aeruginosa* to form a biofilm within a host–which contributes to its ability to inhabit diverse ecological niches- impedes phagocytosis and diminishes the efficiency of antimicrobial treatments, sometimes provoking chronic and persistent infections in host tissues or prosthetic devices [39]. Transient resistance can also be developed just by a bacterial subpopulation, a situation dubbed persistence, which is defined as the ability of a part of the bacterial population to survive under an antimicrobial treatment without acquiring genetic changes conferring resistance [91]. In *P. aeruginosa*, persistence is encountered under nutrient limiting conditions [92], as is the presence of QS signaling molecules [93].

One explanation for the increased antimicrobial resistance of biofilm-growing *P. aeruginosa* is the presence of cells with a slow-growing metabolic state in some parts of the biofilm, which constitute a subpopulation of persisters [94]. Other reasons for an increased transient resistance are a more difficult diffusion of compounds due to the complex structure of the biofilm [90] or the presence of elements that reduce the activity of antimicrobials within the biofilm, such as glycerophosphorylated β-(1, 3)-glucans or cyclic-β-(1, 3)-glucans, which sequester aminoglycoside antibiotics [95]. In addition, changes in the expression levels of ARGs as *mexAB-oprM* and *mexCD-oprJ* during the biofilms state of growth are also of relevance [96,97].

Besides persistence and the formation of recalcitrant biofilm structures, changes in the expression of AR determinants due to specific signals and/or conditions are of relevance for developing transient resistance. Indeed, this scenario may be encountered during infection, thus compromising the efficacy of antipseudomonal treatments [98]. For instance, the presence of the inducible β-lactamase AmpC in *P. aeruginosa*, the expression of which is enhanced by some β-lactam antibiotics, may lead to treatment failure due to transient β-lactam resistance [99]. Changes in the permeability of *P. aeruginosa* associated with magnesium limiting conditions transitorily reduce the negative charge of the cell surface through an up-regulation of an LPS modification operon, driving to enhanced resistance in positively charged antimicrobials, like cationic antimicrobial peptides or polymyxin B [100,101].

The role of MDR efflux pumps regarding *P. aeruginosa* transient AR should also be highlighted. Since these systems are involved in different key processes for bacterial physiology, tight regulatory control over their expression, dependent on environmental conditions, may be expected. Therefore, in some situations or in the presence of specific effectors, a temporary rise of the expression of efflux pump encoding genes is achieved [102]. Some of these inducing conditions may be found in clinical settings, allowing bacteria to resist an antimicrobial treatment through a transitory improved antibiotic extrusion capacity. For instance, expression of MexCD-OprJ efflux pump's encoding genes is induced by molecules that *P. aeruginosa* may run into during an infection. Some of these molecules are disinfectants or anaesthetic agents (e.g., procaine or atropine), which induce quinolone resistance [103], as well as the human host defence peptide LL-37, which increases resistance towards quinolones and aminoglycosides [104].

Further, *mexAB-oprM* expression is induced under oxidative stress conditions [105] and by triclosan or pentachlorophenol [106]. Furthermore, nitrosative stress, chloramphenicol presence and contact with human airway epithelial cells are circumstances that trigger *mexEF-oprN* overexpression [107,108], supporting the idea that the MexEF-OprN efflux pump might contribute to *P. aeruginosa* transient resistance during lung infection. Another *P. aeruginosa*'s efflux pump, MexXY, may also contribute to transient AR in clinical settings since the expression of its encoding genes is induced under oxidative stress conditions or in the presence of antibiotics able to inhibit protein synthesis as aminoglycosides or tetracyclines [109].

In summary, transient resistance must not be neglected during *P. aeruginosa* infections. Different conditions, compounds and modes of growth that may take place during the infection process might transiently increase the resistance of this opportunistic pathogen to several antimicrobial treatments, thus hindering the eradication of the infecting bacterial population. Further, recent work has shown that the early appearance of tolerance mutations facilitates the evolution of AR [110], a feature of particular relevance in the case of chronic *P. aeruginosa* infections [111].

Infections by other *Pseudomonas* species with an environmental origin and biotechnological potential, as *Pseudomonas putida* [112–115], have also been reported, although their prevalence is much lower. Besides intrinsic resistance determinants [116,117], the acquisition of carbapenemases, as KPC-2 [113], constitutes an additional risk for the efficient treatment of infections by these pathogens.

## 3. *Acinetobacter baumannii*

*Acinetobacter* is another non-fermentative Gram-negative bacterial genus, firstly reported as a significant nosocomial pathogen in the late 1970s. This microorganism harbours an entire repertoire of intrinsic resistance determinants, and it easily acquired novel ARGs soon after its detection as a cause of infections [118], becoming nowadays one of the most prevalent resistant pathogens causing problems at hospitals. Unlike other pathogens discussed in the current review, and despite the *Acinetobacter* genus being ubiquitous, the potential primary environmental niches of *Acinetobacter baumannii*, the species causing most problems at hospitals, are still not well established [119]. Hence, more studies are still needed to delimitate the outside-hospitals reservoirs of *A. baumannii* [120]. Notably, it

seems that *A. baumannii* presents the largest pangenome and biochemical versatility within the species of the *Acinetobacter* genus [121,122]. Its open pangenome contains a variety of mobile genetic elements, most notably integrons, transposons and plasmids [123], which may support the capacity of this opportunistic pathogen for acquiring ARGs. Integrons and transposons can be located in genomic islands, some of which have been dubbed islands of resistance due to the presence of multiple ARGs inside them. It has been reported that around 40% of *A. baumannii* pangenome is specific to each strain [121], indicating that gene exchange within this bacterial species has a certain degree of clonal specificity.

With regard to the core genome of *Acinetobacter*, it has been described to contain 950 families of orthologous proteins, including a large number of virulence factors [124] and at least 1590 orthologous proteins that correspond to 44% of the size of the smallest proteome of the species [121].

Due to the importance of hospital outbreaks, the population structure of *A. baumannii* is now well-established [125]. At least six major international clonal lineages (ICL), distributed across continents worldwide, have been described [125]. Three successful clones re-named as "international clones I-III", among which ICLI and ICLII display MDR phenotypes that may be favouring their clonal expansion [126], are included in these lineages. A recent study, based on the analysis of almost 2500 genomes, shows that *A. baumannii* can be divided into two clusters. Notably, the strains of one cluster, which contain a CRISPR/Cas system, rarely harbour plasmids, indicating that CRISPR/Cas elements may modulate the acquisition of novel genes in *A. baumannii* [127].

Concerning human health, *A. baumannii* strains have been isolated primarily from hospitalized patients, and this pathogen is associated with infections of the respiratory tract, bloodstream, wound, skin and soft tissue, urinary tract and central nervous system [128]. Besides humans, *A. baumannii* has been isolated in veterinary medicine, infecting seriously ill animals [129], livestock and wildlife; thereby indicating that this opportunistic pathogen constitutes a One-Health problem [130].

The main limitation regarding the treatment of *A. baumannii* infections is the increasing prevalence of MDR isolates. Intrinsic determinants, such as the OXA-type *Acinetobacter*-derived cephalosporinase [131] or the RND efflux pumps AdeABC [132], AdeFGH and AdeIJK [133], stand out as major determinants of intrinsic AR in this bacterium. While the first efflux pump contributes to acquired resistance when overexpressed, the contribution of the second to this phenotype is less relevant because AdeIJK overexpression is toxic above a given threshold. AdeFGH also confers MDR when overexpressed, while some non-RND efflux systems, such as CraA, AmvA, AbeM and AbeS, have been described to be involved in *A. baumannii* AR too [134].

Notably, it has been shown that one-step AR mutations can be selected in vivo during the treatment of the infected patients [135]. Among them, mutations in the genes encoding the regulators of the expression of MDR efflux pumps lead to their overproduction and to associated cross-resistance to a variety of antimicrobials [136]. In addition, mutations in genes encoding outer membrane proteins, such as OmpA, CarO and OprD, also contribute to AR and modulate virulence of this opportunistic pathogen [137], providing an example of the crosstalk between virulence and AR [138].

Besides intrinsic and mutational acquired resistance, the members of this bacterial species have acquired several β-lactamases and other ARGs by HGT [139]. Among them, and in addition to the intrinsic OXA-type β-lactamase, other OXA derivatives have been found [140], frequently linked to insertion sequences (ISs) located upstream the genes encoding β-lactamases [141]. Indeed, the activity of ISs, capable of modifying the expression of genes involved in resistance when located in the right positions, seems to be also instrumental for the acquisition of the resistance phenotype [142,143]. Despite AR plasmids not being as frequent in *A. baumannii* as in *Enterobacteriaceae* [144], the plasticity of its genome [145] allows the acquisition of ARGs, many of them present in transposons and in integrons, within plasmids and the chromosome. In this regard, it is worth mentioning that more than 130 gene cassettes containing ARGs have been identified in integrons located

in *A. baumannii* genomes [146]. Although several studies have shown the high prevalence of class 1 integrons, which often contain resistance gene cassettes, other studies carried out in Latin American countries, such as Chile, Argentina and Brazil, have also shown a wide distribution of class two integrons in this species [147,148]. It is important to notice that while extended-spectrum β-lactamases (ESBLs) as ESBLs PER-, GES- and VEB-type are the most common *A. baumannii*, TEM- and SHV-type ESBLs, the most prevalent in *Enterobacteriaceae*, are less frequently found in *A. baumannii*, supporting that gene exchange between these two groups of microorganisms is likely low [149], although still possible (see the example of NDM1 below).

Notably, ARGs acquired by *A. baumannii* are frequently clustered, forming part of genomic islands, dubbed AbaRs. These AbaRs [146] present backbones resembling Tn*6019*, Tn*6022* and Tn*6172* transposons [150] and seem to be clone-specific [151]. This feature may mean that, once an AbaR has been acquired, its mobilization to another phylogenomic *A. baumannii* group could be limited. Hence, HGT via plasmids or other mobile genetic elements might be on the basis of the acquisition of resistance by *A. baumannii* [152]. In addition, recent works indicate that ARGs-containing bacteriophages might contribute to AR spread in this microorganism [153,154]. Finally, it has been recently found that this organism can be naturally competent [155,156], opening the possibility that direct transformation could be a relevant mechanism triggering the acquisition of ARGs by this bacterium.

Currently, more studies have shown the importance of other bacterial species within the *Acinetobacter* genus in clinical settings [157]. Along with *A. baumannii*, other species such as *Acinetobacter pittii* and *Acinetobacter nosocomialis* have been frequently isolated in patients [158]. *A. pittii* was isolated in China, and its potential to acquire resistance to carbapenems by a mutation in $bla_{\text{OXA-499}}$ has been observed [159]. Regarding *A. nosocomialis*, the importance of the RND-type efflux pumps, AdeIJK and AdeABC, in its resistance phenotype has been highlighted [160].

Other species of the genus have also been described in natural and clinical environments, such as *Acinetobacter soli*, firstly isolated from a Korean forest [161] and identified in domestic animal lice [162]. Although the first reports indicated that the microorganism came from environmental sources, it has also been found in clinical settings [163]. In China, an MDR isolate of this bacterial species containing the β-lactamase encoding genes ($bla_{\text{OXA-58}}$, $bla_{\text{IMP-1}}$, $bla_{\text{NDM-1}}$ and $bla_{\text{TMB-2}}$) caused the death of a patient under treatment [164]. Notably, it has been suggested that $bla_{\text{NDM-1}}$ is a chimaera constructed in *A. baumannii*; a feature supporting that this species can be the origin as well as a reservoir for the transfer of this relevant carbapenemase of *Enterobacteriaceae* [165]. This has been further reinforced with in vitro data indicating that *Acinetobacter* plasmids could have contributed to the spread of $bla_{\text{NDM-1}}$ in *Enterobacteriaceae* [166]. Also supporting this idea is the characterization of transferable plasmids containing $bla_{\text{NDM-1}}$ in both *A. soli* and *A. pittii* and their mobility between the genus [167]. *A. pittii* isolates containing plasmids belonging to new incompatibility groups, which carry genes encoding OXA-type carbapenemases, and with the ability to transfer them to other species of the genus, have been reported too [168].

The possibility that plasmids carried by *Acinetobacter* spp. might be transferred to other human pathogens, hence contributing to ARGs spread, has been analysed in other studies. For instance, genomic analyses comparing *Acinetobacter* spp. clinical and environmental (water and soil) isolates suggested that Rep_3-type plasmids can be transferred between *Acinetobacter* spp. and bacteria belonging to other genera from different environments [169]. Further, the in silico analysis of 173 plasmids of a wide variety of sizes from 17 countries showed that some plasmid lineages have the capacity to replicate in many bacterial genera, while others only do it within species of the *Acinetobacter* genus [170]. It is important to notice that the number of different plasmid lineages harboured by *A. baumannii* is low, with around one-third of them containing ARGs and that gene flux among different plasmids seems to be mediated by transposons [170].

Reports on *Acinetobacter* spp. carrying ARGs continue to increase. Examples of them are some strains of *Acinetobacter bereziniae*, recently isolated from human clinical

samples [171], carrying $bla_{\text{OXA-type}}$ [172] and MBLs encoding genes, as $bla_{\text{NDM-1}}$; in both cases encoded in plasmids [173]. Another example is *Acinetobacter junii* strains, presenting $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-58}}$, and isolated from hospitals [174].

## 4. *Stenotrophomonas maltophilia*

*Stenotrophomonas maltophilia* is a ubiquitous non-fermentative Gram-negative microorganism described in a variety of environments [175], from natural to anthropogenic niches, such as soil [176], water [177] or sediments [178,179]. Besides its role as an opportunistic pathogen, *S. maltophilia* is also a plant endophyte, and different strains with biotechnological value have been described [180]. This feature makes particularly relevant to distinguish between infective and non-infective *S. maltophilia* strains. However, there is evidence of epidemic *S. maltophilia* lineages [181] and it seems that, as has been described for *P. aeruginosa* [182,183], there are not specific clades evolving towards virulence [184,185]. Instead, the prevalence of *S. maltophilia* infections mainly derives from the underlying health condition of the patient, more than from specific characteristics of the isolate causing such infection. Through different genotyping methodologies, it has been described that the *S. maltophilia species complex* (Smc) contains multiple genospecies [178,186,187]. Four genospecies belong to the *S. maltophilia sensu stricto* species, which is the main cause of infections in humans and the only one that consistently expresses MBLs (Sgn1, Sgn2, Sgn3 and Sgn4) [178]. The Smc displays high genetic, ecological and phenotypic diversity [188,189] as well as heterogeneous resistance and virulence phenotypes [187,190]. In fact, this phenotypic heterogeneity mainly results from problems in species delimitation within the Smc [178,191]. This problem can be aggravated since, based on estimates of genomic mean nucleotide identity values >94%, it has been recently proposed to reclassify *Stenotrophomonas africana*, *Pseudomonas beteli* and *Pseudomonas hibiscicola* as *S. maltophilia* [188,192]; despite these species are not known to be a relevant cause of human infections.

It was in the 1980s when *S. maltophilia* became significantly reported as an emerging pathogen. Nowadays, it has become the third most common cause of nosocomial infections caused by non-fermentative Gram-negative bacilli. Even though it is not considered a highly virulent bacterium, it is one of the leading drug-resistant pathogens of more significant public health concern in hospitals worldwide and is associated with mortality rates between 14 and 69% in patients with bacteraemia. Although it is mainly a nosocomial pathogen, community-acquired infections are an increasing trend [193]. *S. maltophilia* is mostly associated with respiratory infections and acute exacerbations of COPD, followed by bloodstream infections. Less frequently, it causes infections of the skin and soft tissues, biliary and urinary tract, endocarditis, meningitis, intra-abdominal infections and endophthalmitis [194]. The most affected patients are those with previous pathologies (CF, HIV infection or cancer–particularly obstructive lung cancer-), mechanical ventilation, indwelling catheters, corticosteroid or immunosuppressant therapy, together with those hospitalized for prolonged periods or ICU admission and previous broad-spectrum antibiotics therapy [5]. In these patients, *S. maltophilia* infections are associated with high mortality rates [5,175,195]. Infections caused by this microorganism occur in adults and children, and the transmission to susceptible individuals takes place through direct contact with the source. Possible sources are hands of health care professionals, aerosols from CF patients, suction system tubing of dental chair units, contaminated endoscopes or tap water [175].

Treatment of infections caused by *S. maltophilia* is complicated given the intrinsic resistance mechanisms against most antimicrobials that this bacterium presents [196,197]. Trimethoprim/sulfamethoxazole (SXT) is currently the treatment of choice [198]; albeit, combination therapies of SXT plus ciprofloxacin, ceftazidime, tobramycin or tigecycline, which exhibit a greater activity than SXT alone, are also implemented [199,200]. However, the acquisition of resistance to SXT limits its use. Therefore, new therapeutic options are needed to tackle these infections. Ticarcillin/clavulanate or ceftazidime in combination with ciprofloxacin are the agents used in most SXT resistant infections. On the one side, ceftazidime and ticarcillin/clavulanate used to be the most effective β-lactams

against *S. maltophilia*, but the number of resistant isolates is increasing [5]. On the other side, ciprofloxacin or newer fluoroquinolones as levofloxacin are still a helpful alternative, even though the number of resistant isolates is sizable [201]. Ultimately, recent studies have shown that minocycline [202] and colistin, alone or in combination with N-acetylcysteine [203], could be used for treating infections caused by this microorganism.

Genome sequencing of *S. maltophilia* clinical [195] and environmental isolates [204] indicated that several of the elements involved in the characteristic AR phenotype of this bacterial species are shared by strains isolated from different habitats. Therefore, these elements have evolved before the use of antibiotics for human therapy, as described for *P. aeruginosa*. In all these genomes, many genes encoding determinants of resistance to antibiotics (Table 2), such as β-lactams, cephalosporins, macrolides, fluoroquinolones, aminoglycosides or carbapenems, have been found [195,205,206]. These data show that *S. maltophilia* intrinsic resistance has not been acquired upon evolution in the presence of antibiotics currently used in therapy, although increased levels of resistance can be acquired by mutations or by ARGs acquisition through HGT [194,206,207].

Concerning intrinsic resistance, *S. maltophilia* possesses two inducible β-lactamases, L1 and L2. L1 is a broad spectrum (excluding monobactams) MBL [208], while L2 is classified as a class A clavulanic acid-sensitive cephalosporinase [209]. The expression of these enzymes, mostly controlled by the AmpR transcriptional regulator, is directly induced by the antibiotics they provide resistance to [210,211]. AmpR acts as an activator in the presence of inducers, such as β-lactams, but in the absence of them, it is a repressor of *L2* expression [212].

In addition, aminoglycoside-modifying enzymes encoded in the *S. maltophilia* genome confer low susceptibility to several aminoglycosides [213]. Three of these enzymes have been analysed: N-aminoglycoside acetyltransferases AAC(6′)-Iz that contributes to resistance to amikacin, tobramycin, sisomicin and netilmicin [198,214]; AAC(6′)-Iak [198,213] that decreases susceptibility to several aminoglycosides, including arbekacin, kanamycin, neomycin, sisomicin or tobramycin [213]; and the aminoglycoside phosphotransferase APH(3′)-IIc [215], that confers resistance to kanamycin, neomycin, paromycin and butirosin [215].

Another mechanism of intrinsic resistance in this bacterium is the chromosomally-encoded SmQnr protein, which contributes to intrinsic resistance to quinolones [216–218] by protecting DNA gyrase and topoisomerases from fluoroquinolones' activity [219].

Along with the inactivating enzymes, the major contributors to intrinsic resistance to many antimicrobial agents in *S. maltophilia* are the chromosome-encoded MDR efflux pumps. The best-characterized group of pumps is the RND family. Eight of these complexes (SmeABC, SmeDEF, SmeGH, SmeIJK, SmeMN, SmeOP, SmeVWX and SmeYZ) are encoded in the genome of *S. maltophilia*, and the role in AR of seven of them has been studied [220]. Only when the expression level is constitutively significant, as it happens with SmeYZ, SmeDEF, SmeGH, SmeIJK and SmeOP [220–224], do these pumps contribute to intrinsic resistance. The SmeYZ system is involved in intrinsic resistance to aminoglycosides, tetracycline, leucomycin and SXT [220]. SmeDEF overexpression is linked to quinolones, chloramphenicol, tetracycline, tigecycline, macrolides, sulfamethoxazole, trimethoprim and SXT resistance [207,225–227], as well as to resistance to the biocide triclosan [228,229]. SmeGH is involved in intrinsic resistance to β-lactams, quinolones, tetracycline and polymyxin B, as well as to other toxic compounds, such as menadione, tert-butyl hydroperoxide, naringenin and hexachlorophene [221]. For their part, SmeIJK and SmeOP confer resistance to aminoglycosides, tetracycline, ciprofloxacin, levofloxacin, leucomycin or minocycline [222] and nalidixic acid, doxycycline, aminoglycosides or macrolides [223], respectively.

Along with this well-characterized group, other MDR efflux pumps are encoded in the *S. maltophilia* genome. Among them, ATP binding cassette (ABC) efflux pumps such as, SmrA which contributes to fluoroquinolones, tetracycline and doxorubicin resistance [230]; and MacABCsm, which is involved in aminoglycosides, macrolides and polymyxins resistance [231]; and the major facilitator superfamily (MFS)-type efflux pump

EmrCAB, implicated in the extrusion of nalidixic acid, erythromycin and tetrachlorosalicylanilide [232], are of clinical relevance.

This chromosomally-encoded arsenal of resistance elements, together with their low-permeability membranes, are responsible for *S. maltophilia*'s MDR intrinsic phenotype that is independent of the environment in which this bacterium lives [233].

In addition to their contribution to intrinsic resistance, these elements also contribute to acquired resistance when overexpressed or mutated [234]. The overexpression of either β-lactamases or MDR efflux pumps stands as the main cause of the acquisition of resistance in clinical isolates of *S. maltophilia*. In the case of efflux pumps, increased AR is associated with their overexpression, mostly by the acquisition of mutations in their regulators. The selection of these mutations, leading to efflux pumps' overexpression, is particularly problematic since they lead to an MDR phenotype. For example, the most prevalent cause of *S. maltophilia* acquired resistance to quinolones is the overproduction of SmeDEF, mainly by mutations in the gene encoding the negative regulator SmeT [224], and of SmeVWX, by mutations in the gene encoding its SmeRv regulator [225]. Importantly, *S. maltophilia* is the only known bacteria in which high-level resistance to quinolones is only due to the overexpression of MDR efflux pumps, not to mutations in genes encoding quinolones targets [235,236]. Besides, evolution experiments made in the presence of tigecycline have revealed that mutations in *smeT,* leading to SmeDEF overexpression, constitute the first step in *S. maltophilia* tigecycline acquired resistance. In addition, amino acid substitutions in the gene encoding the efflux pump and, thus, changes in its structural elements are also on the basis of acquired resistance. For instance, *smeH* mutations are involved in the acquisition of resistance against ceftazidime, leading to cross-resistance towards other antibiotics, mainly β-lactams [237]. SmeABC, SmeIJK and SmeYZ also contribute to acquired resistance towards aminoglycosides, β-lactams and fluoroquinolones [238] when overexpressed.

Mutations in the antibiotics' target genes are another cause of acquired resistance in this bacterium. Apart from the aforementioned *smeT* mutations, ribosome 30S mutations, the target of tigecycline, are among the mechanisms of tigecycline acquired resistance. Additionally, the inactivation of central carbon metabolism enzymes has also been shown to be responsible for the acquisition of AR by *S. maltophilia.* A group of in vitro selected mutants in which genes encoding the enzymes Eno, GmpA, GapA and Pgk were inactivated has allowed the study of mutation-driven fosfomycin resistance. This study showed that the inactivation of the Embden-Meyerhof-Parnas metabolic pathway is on the basis of this resistance [239].

Finally, not only mutations but also HGT contributes to *S. maltophilia* acquired resistance. Resistance to SXT may occur by the acquisition of the *sul* and *dfrA* genes present in integrons or plasmids [225]. Besides, mobile elements involved in SXT resistance, plasmid-mediated quinolone resistance genes (e.g., *qnrS* [240]), and β-lactamases (e.g., $bla_{CTX\text{-}M\text{-}Gp1}$ [241]), have been found in *S. maltophilia* isolates. Despite the fact that a lysogenic phage containing the dihydrofolate reductase encoding the *folA* gene has been described to contribute to trimethoprim resistance in an *S. maltophilia* isolate [242], the role of these elements in the dissemination of ARGs in this microorganism remains to be studied in detail.

As in other species, different factors, such as medium composition, osmolarity or ionic concentrations, can induce *S. maltophilia* transient AR. Temperature can also modify its antibiotic susceptibility by alterations in the outer membrane LPS conformation. For instance, this bacterium is more susceptible to aminoglycosides at 37 °C than at 30 °C since the binding and/or uptake of the antibiotic is inhibited at a lower temperature [243]. The ability to form biofilms, which reduces antibiotics' susceptibility, is a significant feature of *S. maltophilia*. Environmental factors, such as phosphate or chloride concentrations, temperature and aerobic or anaerobic conditions, can influence the production of biofilms, being enhanced under aerobic conditions [175]. *S. maltophilia* and *P. aeruginosa* can grow together inside dense polymicrobial biofilms in different environments, including the lungs of CF patients. This kind of growth influences their behaviour, including antibiotic

susceptibility [244]. Inside these biofilms, *S. maltophilia* produces a diffusible signal factor that *P. aeruginosa* senses through the two-component sensor BptS, leading to the increased production of proteins implicated in polymyxin and colistin resistance [245].

β-lactamases and MDR efflux pumps also contribute to transient AR since their expression is inducible. The expression of β-lactamases is induced by β-lactams [210], and the MDR efflux pump's expression increases due to the effect of different molecules. On the one side, *smeDEF* expression is induced by plant-derived flavonoids [246] or biocides like triclosan [229]. These molecules can bind the *smeDEF* repressor, SmeT, inducing the expression of this efflux pump and reducing *S. maltophilia* quinolone susceptibility. On the other hand, fluorescence-based analyses have uncovered *smeYZ* and *smeVWX* inducers involved in aminoglycosides and chloramphenicol or quinolone resistance, respectively. Boric acid, erythromycin, chloramphenicol and lincomycin are inducers of *smeYZ* [247], whereas vitamin K3 and its analogues vitamin K2 and plumbagin, as well as iodoacetate, clioquinol and sodium selenite, are *smeVWX* inducers [248]. Finally, the tripartite efflux pump (FuaABC), related to ABC efflux pumps, whose expression is induced by fusaric acid, contributes to transient resistance to this compound [249].

## 5. *Burkholderia cepacia* Complex

The *B. cepacia* complex (Bcc) is a group of closely related non-fermenting Gram-negative bacilli that comprises 22 validated species. The taxonomy of these bacteria is complex and continuously changing [250]. This complex is formed by nine genomovars, namely *B. cepacia* (formerly genomovar I), *Burkholderia multivorans* (II), *Burkholderia cenocepacia* (III), *Burkholderia stabilis* (IV), *Burkholderia vietnamiensis* (V), *Burkholderia dolosa* (VI), *Burkholderia ambifaria* (VII), *Burkholderia anthina* (VIII), *Burkholderia pyrrocinia* (IX) and the group or taxon K (recently split into two species: *Burkholderia contaminans* and *Burkholderia lata*) [250–254].

This complex has a versatile metabolism that allows it to colonize a great variety of niches [251–254]. Moreover, the complex includes species that are important opportunistic human pathogens of CF patients [252,255] or chronic granulomatosis disease, and critical nosocomial pathogens causing bacteraemia or urinary tract infections [256,257]. Although it has been stated that infections by *B. cepacia* could be associated with a fast lung decline and increased mortality of CF patients, dubbed the cepacia syndrome [258], recent works suggest that this statement might not always be true [259]. Further, since Bcc prevalence increases with age, lung deterioration and lung transplantation [260], these underlying conditions might also be contributing to the bad prognosis of Bcc infected patients. Although *B. cenocepacia* has been traditionally the most predominant cause of infections, *B. multivorans* is increasingly being recovered from the lungs of CF patients [261]. In addition, other members of the Bcc complex, as *B. contaminans*, cause infections in CF patients too [262,263]. Besides, outbreaks of healthcare-associated Bcc infections due to the contamination of pharmaceutical products have also been reported [264,265].

In addition to their relevance for human health, species of this complex are also important in agriculture because of their biocontrol and biotechnological properties. Genomovar III has been identified as a commensal of different soil types and the rhizosphere of several cultivated plants, such as maize, wheat and lupin, in natural environments [266,267]. Multilocus sequence typing (MLST) analysis of environmental and clinical isolates showed that at least 20% of the strains causing human infections are also found in nature [268]. Further, soil isolates can produce infections in both plants and animals [269]. Altogether, these findings indicate that natural ecosystems constitute a reservoir of Bcc strains with clinical relevance.

Due to its versatility and relevance in clinic and natural environments and the potential biotechnological application of some strains, the population structure of *B. cenocepacia* (genomovar III) has been studied in detail. It has been found that the population is in linkage disequilibrium and presents a clonal structure, with three major clones displaying variable degrees of recombination distributed worldwide [270].

One of the problems associated with Bcc infections is the low susceptibility to several antibiotics (e.g., carboxypenicillins, first and second generation cephalosporins, tetracycline or tobramycin) that this bacterial group possesses. Particularly relevant is the intrinsic resistance they all have to the last resort antibiotics polymyxins [271]. The main cause of the lack of activity of this drug against Bcc relies on the particular LPS structure of this group of microorganisms. It has been shown that the addition of 4-amino-4-deoxy-l-arabinose (Ara4N) to the lipid A component of the LPS reduces polymyxin susceptibility in different organisms [272,273]. While Ara4N synthesis is usually dispensable in different bacteria, the Ara4N biosynthetic gene cluster seems to be essential for *B. cenocepacia* [274]. This fact supports that the natural incorporation of Ara4N into lipid A is likely a major cause of Bcc polymyxin intrinsic resistance.

Like several other bacteria, Bcc presents in its genome genes encoding different AR determinants, including efflux pumps and inducible class A and class C β-lactamases, as PenB [275] (formerly dubbed PenA [276]) or AmpC [277], respectively, which expression is coregulated [278]. Recent work suggests that β-lactam inhibitors such as relebactam, enmetazobactam, avibactam or vaborbactam, might be useful for increasing the susceptibility to β-lactams of Bcc isolates [279].

Beyond β-lactamases, several efflux pumps are encoded in Bcc genomes. Among these systems, NorM, a member of the multidrug and toxic compound extrusion (MATE) family, has shown to play a role in polymyxin resistance, together with the aforementioned Lipid A modification [280]. Within the identified efflux pumps encoded in Bcc genomes, those present in *B. cenocepacia* stand out as the best studied. Namely, fourteen RND efflux pump-encoding genes have been found in the genome of *B. cenocepacia* [281]. These elements are able to confer resistance to clinically relevant antibiotics as aminoglycosides, chloramphenicol, fluoroquinolones and tetracyclines [281,282]. Among them, at least three are involved in intrinsic AR [283]. Notably, an MFS immunodominant efflux pump, named BcrA and involved in tetracycline and quinolones resistance, has been detected in CF patients infected with Bcc [284], suggesting that BcrA may have a relevant role in Bcc resistance to antibiotics in said patients. Besides, the finding that salicylate, a siderophore produced by *B. cenocepacia,* may induce the expression of an antibiotic efflux pump that confers resistance to chloramphenicol, trimethoprim and ciprofloxacin, suggests that this MDR element can be overexpressed; hence contributing to Bcc transient resistance in environments with low iron availability, such as CF patients' lungs [285].

Besides classical AR determinants, the low susceptibility to antibiotics of Bcc is also due to global mechanisms of response to stressful compounds, such as the production of lipocalins, a family of small proteins capable of binding hydrophobic ligands. It has been shown that a soluble *B. cepacia* lipocalin, produced in the presence of antibiotics, allows the sequestration of such antibiotics, hence contributing to Bcc resistance [286].

Regarding genetic changes leading to acquired resistance, mutation and recombination stand as major players in this process. It has been found that *B. multivorans* diversifies into various clones presenting different phenotypes when causing chronic infections in CF patients; and that some *loci* involved in β-lactams resistance present multiple mutations in recombinogenic regions [287]. Among those, mutations in *ampD,* which encode a transcription factor that coregulates the expression of the two intrinsic β-lactamases AmpC and PenB, stand out among the major causes of resistance to β-lactams in Bcc. Notably, *ampD* is highly prone to acquire AR mutations with an estimated frequency in the range of $10^{-6}$ to $10^{-5}$ [278].

In addition to mutation-driven AR, the acquisition of ARGs through HGT by some Bcc strains has been reported. Amongst them, Type I integrons, containing the sulphonamide resistance gene *sul1* and carrying the aminoglycoside resistance genes *aacA4* or *aacA7*, or *catB3*, encoding a chloramphenicol acetyltransferase, are found [288]. These findings indicate that integrons may participate in the acquisition of resistance to sulfamethoxazole, chloramphenicol and aminoglycosides in Bcc.

The presence of plasmids in Bcc was studied early [289], but comprehensive information on their role in AR is still required. Something similar happens with bacteriophages. The finding of putative ARGs in prophages inserted in the chromosomes of different *B. cenocepacia* strains suggest that these genetic elements might be involved in the spread of resistance among Bcc [290]. However, detailed studies about the contribution of these elements in Bcc AR remain to be established.

## 6. Emerging Opportunistic Pathogens with Environmental Origin

Along with the opportunistic pathogens mentioned above, other environmental bacterial genera, such as *Brevundimonas, Shewanella, Achromobacter, Agrobacterium*, *Aeromonas*, *Erwinia* or *Pantoea*, among others, have been increasingly reported as responsible for emerging infectious diseases [291]. Note that while the most prevalent MDR opportunistic pathogens with a primary environmental habitat are non-fermentative Gram-negative bacteria, some environmental *Enterobacteriaceae* have been reported to cause human infections. Given their taxonomic relationship with highly prevalent human pathogens, as *E. coli* or *K. pneumoniae*, which easily acquire ARGs through HGT, the possibility that these environmental pathogens are a first step in the acquisition of ARGs by human bacterial pathogens [17,89,292,293] must be taken into consideration.

*Brevundimonas* spp. are aerobic Gram-negative bacteria that are not only isolated from soils, submarine sediments and numerous aquatic habitats; but that also cause multiple types of infections, indicating that this genus may be a more widespread pathogen than previously thought [294]. *Brevundimonas diminuta* and *Brevundimonas vesicularis* have been isolated from clinical specimens, including blood, urine and lungs of CF patients [295–299]. The majority of *Brevundimonas* infections have been found in patients with underlying diseases, and many of them are acquired in hospitals [294,300]. Importantly, *Brevundimonas* infections are difficult to treat, as these bacteria can be resistant to different drugs, including fluoroquinolones or β-lactams [301,302]. Resistance to fluoroquinolones may be due to mutations in *gyrA*, *gyrB* and *parC* [301], and resistance to β-lactams to the presence of a VIM-2 MBL [302]. In addition, tetracycline resistance genes have also been detected in environmental isolates of *B. diminuta* [303]. Altogether, these data indicate that these bacteria should be considered as possible causes of nosocomial infections and should be included in prevention programs. Furthermore, their suggested use in bioremediation of contaminated seas and soils [304] should be carefully re-evaluated.

Another microorganism with bioremediation potential is *Shewanella algae*, a marine bacterium [305] that also causes a variety of clinical symptoms in immunocompromised patients [306,307]. It has been suggested that some strains of *S. algae* isolated from clinical samples (skin ulcers and ear infections) [308–311] were mistakenly identified as *Shewanella putrefaciens* [312–314], a very close bacterial species [313]. A recent study has described the presence of β-lactams resistant clones of *S. algae* along the Italian Adriatic coast, containing AmpC and OXA-55-like β-lactamases [315]. Further, these authors have described the possible role of *S. algae* as a reservoir of ARGs, such as *qnrA* and β-lactamase genes (that confer resistance to quinolones and β-lactams, respectively), which could be transferred from the aquatic microbiota of Italian fish farms to bacteria of medical interest [316]. Actually, it has been proposed that *S. algae* are the origin of the quinolone resistance gene *qnrA*, widely distributed among plasmids present in several organisms [317], and different *Shewanella* species (as well as *A. baumannii*, see above) are considered as potential origins of some OXA-type β-lactamases [318]. Besides their contribution as progenitors of mobile ARGs, *Shewanella* can be involved in such mobility too. In fact, a plasmid harbouring several ARGs has been recently identified in *Shewanella xiamenensis* [319]. Although serious infections caused by *Shewanella* have been described [320,321], the rarity of these infections means that treatment guidelines have not been defined yet.

The genus *Achromobacter* is found in soils and aquatic environments, although some isolates can colonize the human intestinal tract, becoming opportunistic pathogens in immunosuppressed patients [322]. These bacteria can cause bacteraemia, meningitis and

urinary tract infections [323–325]. Moreover, *Achromobacter* genus-belonging bacteria have also been isolated from CF patients [326–328]. *Achromobacter xylosoxidans* is the predominantly reported species among CF clinical isolates [322], but other *Achromobacter* species have also been isolated from these patients, such as *Achromobacter ruhlandiiand* [329]. They are intrinsically resistant to several drugs [328] due to the presence in their genomes of genes encoding RND MDR efflux pumps that extrude cephalosporins, aztreonam, carbapenems, quinolones, chloramphenicol, tetracyclines and erythromycin [330,331], as well as to the activity of β-lactamases [332,333]. Moreover, they are becoming increasingly resistant to carbapenems [328]. Furthermore, a recent study has described patient-to-patient transmission and AR development in different *Achromobacter* species [334]. Therefore, these species should be included in preventive programs.

The genus *Agrobacterium* is a recognized group of soil and plant-pathogenic bacteria that has also been implicated in human opportunistic infections, particularly *Agrobacterium radiobacter* (also known as *Rhizobium radiobacter*). Infections caused by these bacteria include bacteraemia, peritonitis and urinary tract infections, and they have been frequently associated with the use of intravascular devices in immunocompromised patients [335–337]. Further, *A. radiobacter* has been recently described to cause ocular infections, and it was identified in polymicrobial keratitis cases [338]. Although there is not much information about the intrinsic AR of these bacteria, the presence of RND efflux pumps in the genus *Agrobacterium* [339,340] suggests that these bacteria may present low susceptibility to different drugs. Further, the finding of an *A. radiobacter* clinical isolates carrying different antibiotic-inactivating enzymes [341] indicate that this microorganism may possess a wide set of AR determinants.

*Aeromonas* are Gram-negative bacteria with an aquatic environmental primary habitat that have also been suggested to behave as opportunistic pathogens [342]. Although there are controversial data about the role of these bacteria in human pathogenesis [343–345], different studies have described a significant correlation between diseases and the production of different virulence factors, such as haemolysins and enterotoxins [346,347]. The principal sources of these infections are contaminated water and foods, mainly inadequately cooked seafood and oysters [348,349]. In particular, *Aeromonas intestinalis*, *Aeromonas enterica*, *Aeromonas crassostreae* and *Aeromonas aquatilis* have been recently identified as representative species of *Aeromonas* with pathogenicity for both humans and aquatic organisms [350,351]. *Aeromonas* spp. are difficult to treat due to their intrinsic resistance to β-lactams, which results from a high constitutive expression of the gene encoding the β-lactamase AmpC, the low permeability of their external membrane and the activity of several outer membrane proteins [351,352]. In addition, *Aeromonas* species can also acquire resistance to β-lactams, such as ampicillin, and drugs from other structural families, such as erythromycin, tetracycline or chloramphenicol, by the acquisition of ARGs [353,354]. Moreover, these bacteria may have importance in aquatic environments as reservoirs of ARGs [355,356]. In this regard, it is worth mentioning that the analysis of the bacterial lineages likely associated with the dissemination of ARGs in a wastewater treatment plant indicate that *Aeromonas* could be a hub for such dissemination [11].

*Erwinia* is a genus of Enterobacteriales ubiquitous in the environment, especially in aquatic ecosystems and soils [357]. It mainly comprises phytopathogenic species, such as *Erwinia amylovora*, the first pathogen shown to cause disease in plants (i.e., the fire blight); *Erwinia persinicus*, which infects a wide range of hosts (e.g., tomatoes, cucumbers and bean pods); or *Erwinia carotovora*, among others. Strikingly, these phytopathogens and other plant-associated non-pathogenic *Erwinia* species (i.e., *Erwinia billingiae* and *Erwinia tasmaniensis*) have been occasionally found infecting animals, including humans [358]. For instance, *E. carotovora* and *E. persinicus* have exhibited pathogenicity against invertebrate infection models [357,359], and the latter has also been isolated from a human urinary tract infection [360]; whereas, *E. billingiae* and other non-phytopathogenic *Erwinia* strains can cause cutaneous infections, septic arthritis, brain abscesses or bacteraemia in humans [361–364]. Since these examples are quite unusual, the AR determinants that

these species could harbour have not been sufficiently studied. However, there are reports about ARGs present in *E. amylovora*, like *strAB*, which codes for a phosphotransferase that confers resistance to streptomycin, and that has been likely acquired by non-pathogenic epiphytic bacteria also present in plant hosts [365]. In addition, some *E. amylovora* strains resistant to oxolinic acid, most likely mediated by chromosomal mutations, have been described [366]. Considering all this information, the potential of the *Erwinia* genus to become an opportunistic human pathogen, as other bacterial species described here, should be closely monitored.

Another Gram-negative genus within the *Enterobacteriaceae* family is *Pantoea*, which includes 20 species isolated from different aquatic and terrestrial environments. Although many *Pantoea* isolates are misidentified, they have been described in association with plants and animals; mainly insects, but also birds, fish, bears, ruminants and importantly, humans [367–369]. The ability of this bacterial group to compete and survive in different niches has made it attractive for biotechnological uses. Water and soil isolates have been used for industrial applications, as bioremediation, since they are able to degrade many products; or agricultural purposes because they compete with plant pathogens and induce plant defences [370,371]. Besides being a plant pathogen, *Pantoea* has been recently identified in nosocomial environments. Different *Pantoea* species have been isolated from both immunocompetent and immunocompromised patients from wounds, blood, skin, stool, cysts and abscesses, as well as from urethra, trachea and oropharyngeal swabs [368]. Opportunistic infections in humans caused by *Pantoea* include septicaemia, pneumonia, septic arthritis, wound infections and meningitis [372]. As it happens with other pathogens with an environmental origin, clinical and environmental isolates are phylogenetically indistinguishable. Even more, *Pantoea* species considered primarily plant pathogens can be isolated from humans [367,369]. The most prevalent species infecting humans are *Pantoea agglomerans* and *Pantoea septica* [369]. Other clinically-relevant species include *Pantoea dispersa*, causing bacteraemia and neonatal sepsis [373], *Pantoea brenneri* and *Pantoea conspicua*, isolated from human sputum and blood, respectively [374,375]. Besides, clinical reports demonstrated cases of pneumonia and death in children with comorbidities where the causative agent was identified as an MDR *P. agglomerans*, resistant to third-generation cephalosporins, carbapenems, aminoglycosides and ciprofloxacin [376]. However, *Pantoea*'s AR determinants are mostly unexplored. Recently, a study has found that a foodborne *P. agglomerans* isolate possesses RND, ABC and MFS antibiotic efflux pumps such as MdtABC, MsbA or EmrAB, and antibiotic target modifiers that provide resistance to antibiotics such as macrolides, fluoroquinolones, tetracyclines or aminoglycosides [372]. Accordingly, further studies are needed to validate the ARGs of this opportunistic pathogen.

Altogether, these data indicate that natural environments are an important primary source of opportunistic pathogens. Since humans, animals and natural environments are interconnected, One-Health approaches [8] are required to limit the spread and evolution of AR.

## 7. Ecological Role of Antibiotic Resistance Determinants Outside Clinical Settings

The environmental origin of different opportunistic bacteria indicates that the mechanisms of virulence and AR with a current role in human infection present a different and unique function in the natural environments where these bacteria emerge. Indeed, the fact that intrinsic ARGs may have other functional roles besides AR has been previously discussed [17,377–380]. While some of these functions deal with basic aspects of bacterial physiology, such as peptidoglycan recycling [381], some others are related to bacterial interactions with other elements of the biosphere and hence, have ecological value. This includes not only ARGs but also situations that trigger transient AR. For instance, it has been demonstrated that increased production of alginate, a key element for *P. aeruginosa* biofilm formation, protects this bacterial species against its protozoan predators in nature [382]. Concerning ARGs, bacterial MDR efflux pumps stand as relevant elements modulating bacterial interactions with the environment. These ARGs are ancient elements that extrude

not only antibiotics but also a wide range of non-antibiotic substrates. Further, the facts that efflux pumps are conserved within a species and between species [383,384], that their expression may be induced by host-produced compounds [246,385–392] (such as bile salts or fatty acids, plant-produced compounds or QS signals, from humans, plants and bacteria, respectively), and that these systems are able to extrude non-antibiotic substrates [393] (such as QS signals, bacterial metabolites, or plant-produced compounds [390,392,394–399]), indicates that they play important roles in the adaptation of bacterial physiology to changing environments. In this review, we discuss the role of efflux pumps outside clinical settings, focusing on bacterial interactions in the rhizosphere.

The rhizosphere is a natural ecosystem that comprises the plant roots and microbial community present in the surrounding soil. Within this ecosystem, soil bacteria and plants affect each other, leading to a feedback system that drives the ecology and evolution of both organisms [400]. Accordingly, the evolution of the microbial community is the result of either the trade-offs associated with overcoming the plants' defence or the specific benefits associated with the host plant colonization. In this sense, plants' roots, apart from providing mechanical support and allowing the absorption of water and nutrients by plants, exudate a wide array of natural products into the rhizosphere [401]. This extrusion modifies soil composition and provides both nutrients for bacterial growth and defensive secondary metabolites. Therefore, roots shape the composition and dynamics of microbial communities, as only bacteria capable of dealing with root exudates are present in the rhizosphere, but they also drive the evolution of plant pathogens [402]. The selection of more virulent mutants that can evade plant defences [403,404] and of mutants that present an improved capacity to metabolize plant-produced nutrients is the driving force of this evolution [405]. Even more, microorganisms present different mechanisms that allow them to deal with root exudates, such as the flavonoid-responsive family of RND efflux pumps. These mechanisms of resistance have been identified in different plant-associated bacteria such as *Agrobacterium tumefaciens* [340], *Pseudomonas syringae* [390,406], *E. amylovora* [392,407], *Bradyrhizobium japonicum* [408], *Xanthomonas axonopodis*, *Ralstonia solanacearum* [409], *S. maltophilia* [246] and *Sinorhizobium meliloti* [410].

As mentioned above, MexAB-OprM is an important MDR determinant of the human opportunistic pathogen *P. aeruginosa* [411,412], which contributes to its intrinsic resistance to several antibiotics (quinolones, macrolides, tetracycline, chloramphenicol and β-lactams) [413]. Besides, this pump is a relevant mechanism for acquiring AR in clinical settings [414] since *mexAB-oprM* overexpressing mutants are selected in the infected patients [415]. MexAB-OprM is also able to extrude monoterpenes and related alcohols present in the tea tree (*Melaleuca alternifolia*) [394], indicating a role in natural environments that was probably acquired before that of antibiotic resistance at clinical settings. In fact, plant flavonoids induce the expression of *mexAB-oprM* in *P. syringae*, the causal agent of bacterial speck in tomato plants, which allows colonization of these plants [390]. These compounds are inhibitors of motility and the type III secretion system in *P. syringae* via the GacS/GacA two-component system [416,417]. Therefore, one of the roles of MexAB-OprM in natural environments is extruding flavonoids to avoid the inhibition of virulence and hence, allowing the colonization of tomato plants. In fact, flavonoids also regulate the capacity of other plant-associated bacteria to colonize plants, such as *E. amylovora*, *A. tumefaciens, X. axonopodis* and *S. maltophilia*. These effectors are inducers and may also be substrates of efflux pumps [246,407,418,419]. These Red-Queen adaptive coevolution phenomena indicate that the original role of bacterial efflux pumps may be the extrusion of plant-derived anti-virulence compounds, among others. Therefore, the screening of natural or natural-like compounds that act as both inducers and substrates of efflux pumps of clinical relevance could serve to identify virulence inhibitors that could be potentially combined with antibiotics in new therapeutic strategies to control bacterial infections caused by environmental pathogens as *P. aeruginosa* [420].

Root exudates not only avoid colonization by pathogenic bacteria but also recruit nitrogen-fixing and growth-promoting bacteria [421]. Many plant species, mainly legumes,

present an intimate association with nitrogen-fixing bacteria and, again, the above-mentioned flavonoids are involved in establishing these associations [422]. This has been observed in *S. meliloti* [410,423] and *B. japonicum* [408], in which flavonoids are also inducers of efflux pumps. Additionally, roots also attract bacteria able to promote plant growth by the extrusion of carbohydrates, amino acids and benzoxazinoids [424,425]. Once again, efflux pumps may be mediating these associations.

Efflux pumps also play essential roles in bacteria-bacteria interactions within the host plant, where there is competition for space and nutrients. Cell-cell interactions are controlled by the QS system, which allows cooperation within a species to colonize a given environment and inter-species communication. In this regard, it is known that the AR determinants MexAB-OprM and MexCD-OprJ of *P. aeruginosa* [411,412,426] modulate QS-responses and host-pathogen interactions, either by the extrusion [427–430] or by the impaired production [431] of QS signals or their metabolic precursors. While bacteria from the rhizosphere produce QS signals to coordinate plant colonization [432], plants may secrete compounds similar to bacterial N-acyl-homoserine lactones (AHLs) through root exudation [433,434], something that is known as Quorum Quenching. For instance, the red seaweed *Delisea pulchra* produces halogenated furanones that interfere with the AHL regulatory system of several Gram-negative bacteria [435,436]. In addition, it is known that certain bacteria also possess the ability to quench QS by enzymatic degradation of AHL signals [437]. This is the case of a *Bacillus* acyl-homoserine lactonase enzyme able to hydrolyse the lactone bond of AHL compounds of the plant pathogen *E. carotovora* [438].

Finally, another relevant role of efflux pumps in bacteria-bacteria interactions within the plant host is the extrusion of antimicrobial compounds produced by other bacterial species. For example, *E. amylovora* and *P. agglomerans* (a biocontrol agent for fire blight) co-colonize rosaceous plants [439,440], but the last one impedes colonization of stigmas of apple and pear plants by *E. amylovora* by effectively inhibiting its growth [441]. However, this microorganism can reach high-density populations when the expression of *norM* is induced (at 18 °C [439]), indicating that this efflux pump extrudes antimicrobial compounds produced by *P. agglomerans* [396].

All in all, these data indicate that bacterial efflux pumps are much more than AR determinants. They are relevant elements for the physiology of microorganisms in natural ecosystems. In this sense, it is important to keep in mind that evolution is similar to a tinkerer [442], which produces new functions from old materials and not from scratch.

**Table 1.** Clinically relevant MDR efflux pumps in *P. aeruginosa*

| Efflux Pump | Main Regulators | Substrate Range | Resistance | References |
|---|---|---|---|---|
| MexAB-OprM | MexR, NalD, NalC | β-lactams (excepting imipenem), quinolones, macrolides, tetracyclines, chloramphenicol | IR *, AR **, TR *** | [384] |
| MexCD-OprJ | NfxB | Penicillin, cefepime, cefpirome, meropenem, quinolones, macrolides, tetracyclines, chloramphenicol | AR, TR | [413] |
| MexEF-OprN | MexT, MexS | Carbapenems, quinolones, chloramphenicol | AR, TR | [443] |
| MexXY-OprM | MexZ | Penicillin, cefepime, cefpirome, meropenem, quinolones, macrolides, tetracyclines, chloramphenicol, aminoglycosides | IR, AR, TR | [413] |

* Intrinsic (IR) ** Acquired (AR) and *** Transient (TR) antibiotic resistance.

**Table 2.** Main antibiotic resistance determinants encoded in *S. maltophilia* genome.

| Gene | Product | Drug Resistance | Type of Resistance | References |
|---|---|---|---|---|
| *L1* | Class B3 $Zn^{2+}$-dependent MBL | β-lactams (except monobactams) | IR *, TR *** | [208,211] |
| *L2* | Class A clavulanic acid-sensitive cephalosporinase | β-lactams | IR, TR | [209,211] |
| *aac(6′)-Iz* | N-Aminoglycoside acetyltransferase | Amikacin, tobramycin, sisomicin, netilmicin | IR | [214] |
| *aac(6′)-Iak* | N-Aminoglycoside acetyltransferase | Arbekacin, kanamycin, neomycin, sisomicin, tobramycin | IR | [213] |
| *aph(3′)-IIc* | Aminoglycoside phosphotransferase | Kanamycin, neomycin, paromycin, butirosin | IR | [215] |
| Sm*qnr* | Pentapeptide repeat protein | Quinolones | IR, AR ** | [218,219,240] |
| *smeYZ* | RND efflux pump | Aminoglycosides, tetracycline, leucomycin, SXT | IR, AR, TR | [247,444] |
| *smeDEF* | RND efflux pump | Fluoroquinolones, chloramphenicol tetracycline, tigecycline, macrolides, sulfamethoxazole, trimethoprim, SXT | IR, AR, TR | [207,225,229,445,446] |
| *smeGH* | RND efflux pump | β-lactams, fluoroquinolones, tetracycline, polymyxin B, ceftazidime | IR, AR | [221] |
| *smeIJK* | RND efflux pump | Aminoglycosides, tetracycline, ciprofloxacin, levofloxacin, leucomycin, minocycline | IR, AR | [222] |
| *smeOP* | RND efflux pump | Nalidixic acid, doxycycline, aminoglycosides, macrolides | IR | [223] |
| *smeVWX* | RND efflux pump | Quinolones, chloramphenicol, trimetho-prim/sulfamethoxazole | AR, TR | [225,226,247,248] |
| *smeABC* | RND efflux pump | Aminoglycosides, β-lactams and fluoroquinolones | AR | [238] |
| *smrA* | ABC efflux pump | Fluoroquinolones, tetracycline, doxorubicin | ND | [230] |
| *macABCsm* | ABC efflux pump | Aminoglycosides, macrolides, polymyxins | IR | [231] |
| *emrCABsm* | MFS efflux pump | Nalidixic acid, erythromycin, CCCP, tetrachlorosalicylanilide | IR | [232] |
| *fuaABC* | Fusaric acid tripartite efflux pump | Fusaric acid | TR | [249] |

* Intrinsic (IR) ** Acquired (AR) and *** Transient (TR) antibiotic resistance.

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# *Pseudomonas aeruginosa*: an antibiotic resilient pathogen with environmental origin

Pablo Laborda, Fernando Sanz-García, Sara Hernando-Amado and José Luis Martínez

*Pseudomonas aeruginosa*, a bacterium characterized for its low antibiotics' susceptibility, is one of the most relevant opportunistic pathogens, causing infections at hospitals and in cystic fibrosis patients. Besides its relevance for human health, *P. aeruginosa* colonizes environmental ecosystems; therefore the elements driving its infectivity and antibiotic resistance must be analyzed from a One-Health perspective. Although some epidemic clones have been described, there are not specific lineages linked to infections, suggesting that *P. aeruginosa* virulence and antibiotic resistance determinants evolved in nature to play functions other than infecting the human host and avoiding antimicrobial treatment. Herein, we review current information on the population structure of *P. aeruginosa* and on the functional role that its resistance and virulence determinants have in non-clinical ecosystems.

**Address**
Centro Nacional de Biotecnología, CSIC, 28049, Madrid, Spain

Corresponding author: Martínez, José Luis (jlmtnez@cnb.csic.es)

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## Introduction

Bacterial pathogens can be broadly classified into two categories, those capable of proficiently infecting immunocompetent humans and those, named opportunistic pathogens, which infect just patients with basal diseases, immunosuppressed or elder people. Commensal bacteria, regularly present in the human host, have been traditionally the main cause of opportunistic infections [1]. Nevertheless, the use of antibiotics, which inhibit the growth of commensals besides pathogens, has boosted the role in nosocomial infections of some bacteria with an environmental origin, and presenting low susceptibility to antibiotics [2], as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* or *Stenotrophomonas maltophilia*. All of them present wide genomes, which allow their adaptation to different habitats, from natural ecosystems to human hosts.

*P. aeruginosa*, the most prevalent opportunistic pathogen with environmental origin, is a major cause of nosocomial infections and produces chronic infections in cystic fibrosis and chronic obstructive pulmonary disease patients [3,4]. In addition, *P. aeruginosa* is a widely distributed environmental bacteria able to colonize a variety of habitats [5,6••], including human-contaminated ones, as sewage, oil spills, swimming pools, or biocide-polluted water [7•]. Although not deeply studied, its success in surviving in polluted ecosystems might be due to the same mechanisms and stress-response networks that contribute to the low susceptibility to antibiotics of this microorganism [8].

Given its ubiquitous distribution, its ability to infect/colonize a variety of hosts and its antibiotic resistance (AR) phenotype, *P. aeruginosa* infection should be considered as a prototype of One-Health problem [9••]. The selection of highly virulent clones or of variants presenting increased levels of resistance [10] may occur in any environment [11•], from which these variants could spread among any other habitat, including the human host.

## Population structure of *P. aeruginosa*: the role of epidemic clones in infection

The adaptation of a microorganism to different ecosystems can be the consequence of two situations. In the first one, the species can be in a process of radiative speciation [12], due to the acquisition or loss of genes and, eventually, the integration of these gained/lost genes within the bacterial physiology through mutation [13,14]. In this evolutionary process, adaptation to one habitat usually results in de-adaptation to another. As a consequence of those clone-specific genetic changes, the genomic structure of the species may consist of a large pangenome and a relatively small core genome. This usually ends up in the generation of novel species from the common ancestor. In the second situation, all members of ubiquitous species as *P. aeruginosa*, present large genomes containing multifunctional determinants that enable bacterial adaptation to different habitats and stress pressures. In this situation, evolution towards speciation is not an expected outcome [2].

*P. aeruginosa* presents a panmictic population structure where environmental and clinical strains are essentially indistinguishable [15]. This indicates that, although some clones are more proficient in causing infections and predominate in clinical settings, they are also present in natural environments. These epidemic lineages are not hence the result of a speciation process towards increasing virulence and a better capability for colonizing the habitat that the human host constitutes. This neither means that *P. aeruginosa* does not evolve during infection nor that different clones are fully equivalent in terms of their virulence. The analysis of *P. aeruginosa* isolates causing chronic infections in cystic fibrosis patients have shown common patterns of evolution [16]. However, these evolved lineages are less proficient for surviving outside the infected host, and disappear before fixation, providing an example of short sighted evolution [2]. Whole-genome phylogenetic analysis of geographically diverse environmental and clinical isolates of *P. aeruginosa* shows that most isolates can be clustered into two large clades, each one containing either *exoS* or *exoU*, encoding Type-III-Secreted toxins [17••]. To note that, while *exoS* belongs to *P. aeruginosa* core genome, *exoU* is present in a genomic island acquired through horizontal gene transfer [18]. Although each group was not found to have a higher prevalence in specific geographic areas or in specific environmental sources, a higher proportion of environmental isolates and most clinical isolates causing respiratory infections were in the *exoS* group, while those causing eye infections belonged to the *exoU* group. Further, a recent work has shown that, even within these broad groups, global physiological aspects as the activity of the Type III Secretion System (T3SS), the quorum sensing (QS) response or the capacity of acquiring foreign DNA, can vary depending on the clonal complex [19•]. The extent to which these group-discriminatory genes/physiological features are niche-adaptive remains to be uncovered.

Besides its capability for colonizing different habitats, the robustness of *P. aeruginosa* for dealing with different stressors can also be on the basis of its success as an opportunistic pathogen capable of spreading among a variety of ecosystems. *P. aeruginosa* is able to survive in water for several weeks in a dormant state, associated with a reduction in its metabolic activity, change in composition and permeability of bacterial membrane, condensation of DNA or a decrease of cell size [20], therefore being water a reservoir of this bacterium. Its persistence in dry surfaces, such as medical equipment, for months, even resisting disinfection procedures, has also been described [21]. Forming recalcitrant biofilms allows this bacterium to adhere to abiotic (as sinks or catheters) and biotic (as lung or plants' roots) surfaces and resist stresses, including the effect of antimicrobial compounds [22]. Moreover, the alternation of planktonic and biofilm lifestyle and the associated modulation of its behavior support *P. aeruginosa* endurance within fluctuating conditions [23].

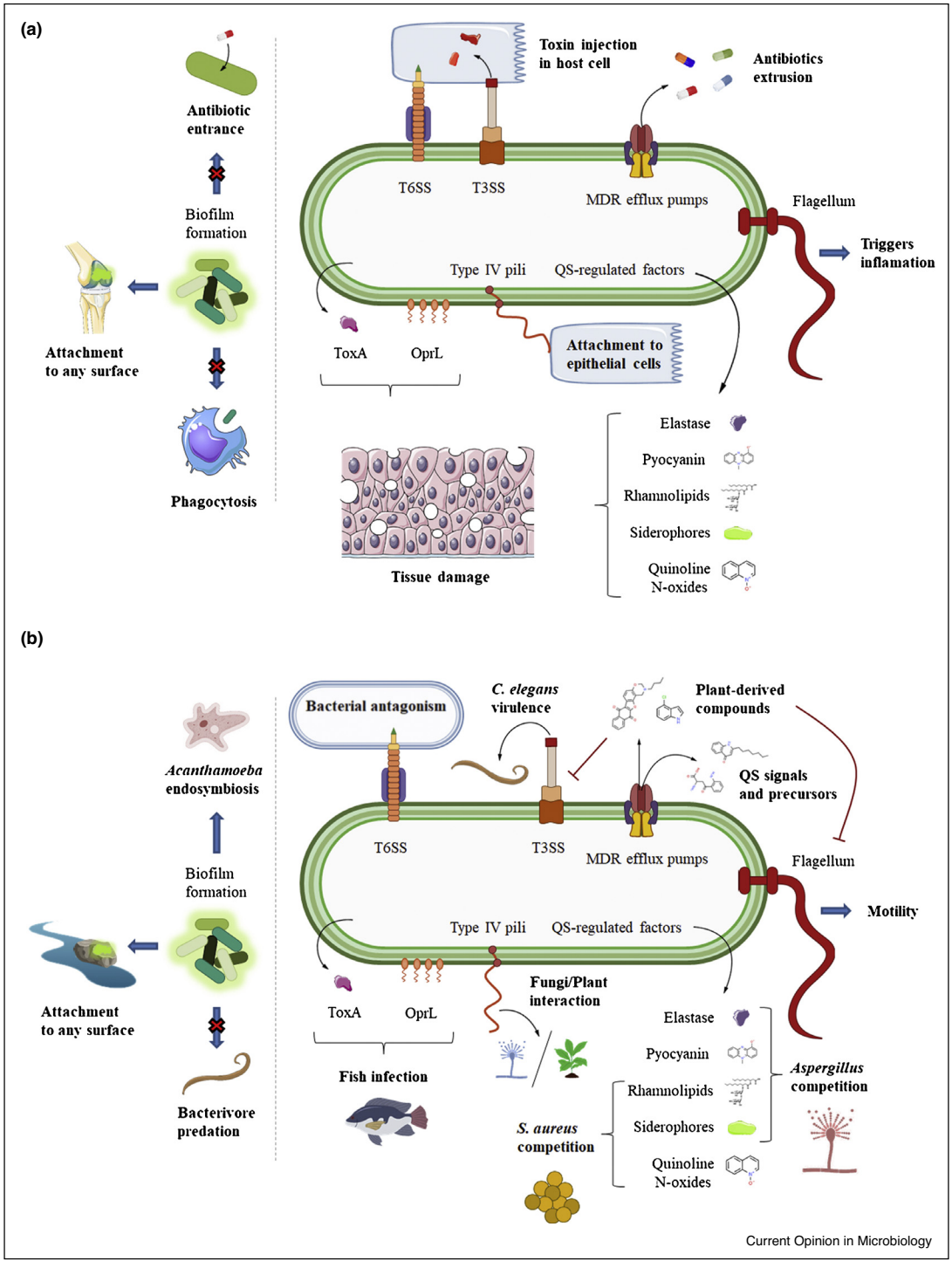
Despite the remarkable clonal diversity showed by clinical isolates, there are some epidemic clones frequently involved in outbreaks worldwide [24,25••]. Notably, lineages as clone C, which is very abundant in clinical settings, is also widely distributed in nature [7•]. Worryingly, the most currently widespread clones are usually associated with multidrug resistant infections and worse clinical outcomes [24]. Their AR phenotype, besides being of clinical relevance, may also be contributing to the prevalence of predominant clones, as C [7•] or ST235 [26], which usually show multidrug resistance (MDR). This phenotype may favor their spread in clinical settings, unless such resistance is associated with high fitness costs. Indeed, the analysis of ST235 isolates collected for 27 years suggests that the wide distribution of this clone, which emerged in 1984 with the introduction of anti-pseudomonal fluoroquinolones in clinical practice, may result from a low fitness cost of fluoroquinolone resistance mutations in the ST235 genetic background [25••].

In spite of the direct effect on the human host when infected with a *P. aeruginosa* epidemic clone, it has been recently proposed that a worse clinical outcome may also be a consequence of the alteration of the host microbiome, as a primary means of pathogenesis [27]. All these evidences support that the cause behind the prevalence of specific *P. aeruginosa* clones in clinics is multifactorial [28]. Further analysis is needed for understanding in depth the reasons behind the success of these epidemic clones.

## Virulence of *P. aeruginosa*, a Swiss knife to colonize a variety of ecosystems

Besides colonizing an ample set of habitats, *P. aeruginosa* is capable of infecting/colonizing several hosts, from unicellular eukaryotes to humans. While some of its virulence determinants are host specific, several others are not [29]. This feature suggests that the virulence of *P. aeruginosa* evolved to deal with inter-species interactions, including prey/predator relationships [30] in natural environments [31,32]. In agreement with this concept, most virulence determinants, which provide *P. aeruginosa* with the ability to infect different hosts, are encoded in its core genome. Despite these common characteristics, the virulence of individual strains may significantly differ [33]. This heterogeneity can be explained by differences in the accessory genome [34••]; particularly, in pathogenicity islands, as PAPI-1 and PAPI-2, present in the *exoU*-positive PA14 strain [35], and their encoded virulence factors – that is, type IVb pili or exotoxins –, that are present in some of the most virulent clones of *P. aeruginosa*. Aside from disparities in accessory genome, the reason behind the observed variations in virulence among different strains remains obscure in occasions,

**Figure 1**

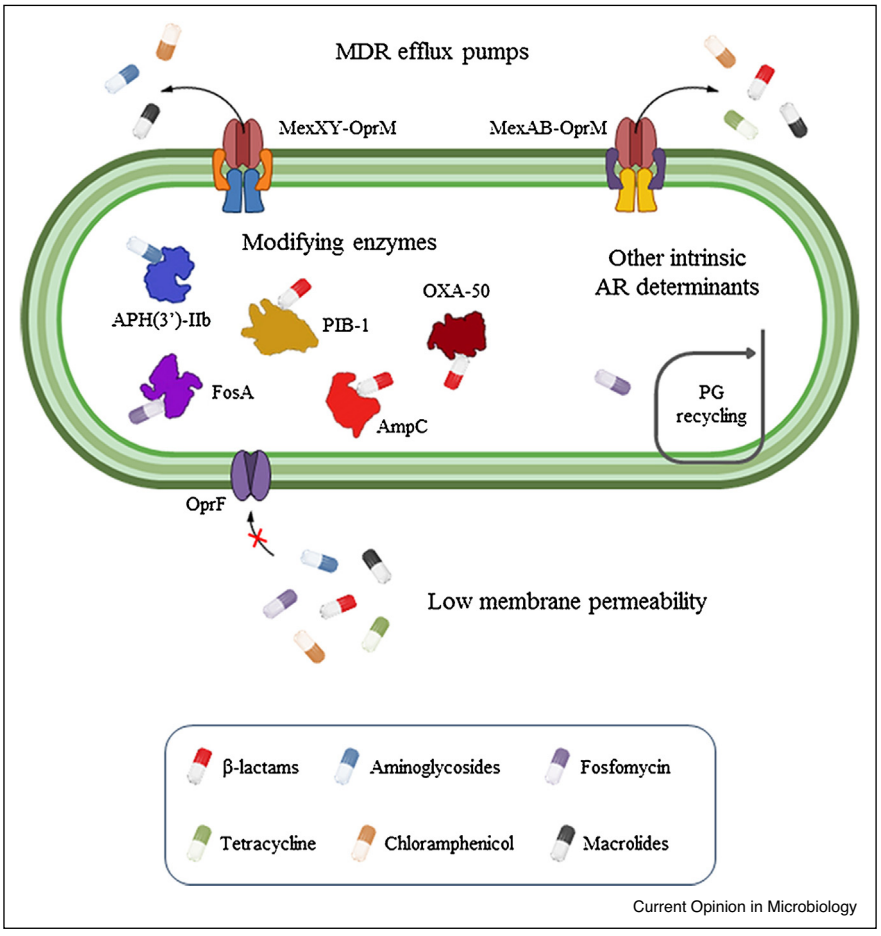



Functions of *P. aeruginosa* virulence determinants and MDR efflux pumps in natural, non-clinical ecosystems.
*P. aeruginosa* possesses a large set of virulence and antibiotic resistance determinants with important roles in infection. **(a)** Most of these determinants, not acquired by horizontal gene transfer, have evolved in natural ecosystems where they play roles different than infecting the human host or overcoming the action of antibiotics used in therapy. **(b)** Some of the virulence determinants, as the flagellum or the liposaccharide, are just structural elements that are recognized by the immune system and that can be involved in the colonization of nearby ecosystems, while other elements have eco-adaptive functions, as siderophores, which are needed when iron availability is low. Besides the response to abiotic stressors, *P. aeruginosa* antibiotic resistance and virulence determinants have important roles on cell-to-cell communication and on the interaction of *P. aeruginosa* with other organisms, from unicellular ones, to plants or worms. More details of these interactions are discussed in the text of the article.

though a correlation to core metabolism has been ascertained [36].

The assortment of *P. aeruginosa* virulence determinants is crucial for colonizing many ecosystems, beyond its role in infecting human hosts (Figure 1). For instance, OprL and ToxA facilitate fish infection by marine strains [37], and their QS regulation may divert from the one of human strains [38], which suggests this system to be adaptable to fluctuating conditions. Regarding other interactions, it has been described that the biofilm matrix and a specific *P. aeruginosa* exopolysaccharide impede *Caenorhabditis elegans* motility, preventing bacterivore predation [39]; while said biofilm is also important when *P. aeruginosa* thrives as an *Acanthamoeba*'s endosymbiont [40]. Moreover, some of these traits enable this opportunistic pathogen to outcompete other microorganisms. An example is the enhanced capacity of *P. aeruginosa wsp* mutants – which typically emerge when sharing niche with *Staphylococcus aureus* –, for killing this pathogen by the upregulation of virulence factors [41], several of them also displaying antifungal activity that may have ecological relevance, as in *Aspergillus–Pseudomonas* interplay during competition in airways [42]. Lastly, type VI secretion system (T6SS) is essential for bacterial antagonism [43], serving as a final example of the versatility of *P. aeruginosa* virulence for colonizing diverse habitats.

**Figure 2**




Schematic representation of the main intrinsic antibiotic resistance determinants in *Pseudomonas aeruginosa*.
Intrinsic antibiotic resilience of P. aeruginosa is determined by a low membrane permeability to the influx of antibiotics, caused by a closed conformation status of most OprF porin present in this bacterium [66]. An efficient extrusion of a huge number of different antibiotics by a set of efflux pumps, among which we may highlight MexAB-OprM and MexXY-OprM, also contributes to the low susceptibility to antibiotics of *P. aeruginosa* [67]. Another element playing an important role in intrinsic antibiotic resistance of this opportunistic pathogen is the presence of a diverse group of antibiotic modifying enzymes, such as β-lactamases as AmpC, PIB-1 or OXA-50, the aminoglycoside phosphotranferase APH(3′)-IIb and the fosfomycin modifying enzyme FosA [68–70]. Besides classical antibiotic resistance determinants, many other systems with a primary role in the bacterial physiology are also involved in intrinsic resistance, as the peptidoglycan recycling pathway whose inactivation renders fosfomycin hyper-susceptibility [71,72].

## The functional role of *P. aeruginosa* antibiotic resistance determinants in natural, non-clinical, ecosystems

The reduced susceptibility to several antibiotics of *P. aeruginosa* is mainly due to the low permeability of its envelopes and to the presence in its core genome of several genes encoding efficient AR determinants (Figure 2). Those genes are present in all (or most) *P. aeruginosa* strains. They constitute the intrinsic resistome, which is encoded in the bacterial core genome [44] and they have not been acquired as a consequence of the selective pressure exerted by antibiotics used in therapy, rather these intrinsic resistance genes had been selected in the natural environments that *P. aeruginosa* colonize. Among these resistance determinants, MDR efflux pumps stand as major elements contributing to intrinsic, acquired (when overexpressed as the consequence of mutations) and inducible AR in *P. aeruginosa*. These elements are highly conserved in any studied organism [45] and, besides contributing to AR, they are also involved in response to stress and in the maintenance of bacterial homeostasis [46]. Notably, the expression of genes encoding efflux pumps may be induced by host-produced compounds [47•] and non-antibiotic substrates, as QS signals, bacterial metabolites or plant-produced compounds, which are extruded by these ancient AR elements [48]. This feature supports that MDR efflux pumps have been selected in nature for functions beyond antibiotic detoxification. While abiotic stress response is one of such roles [8], the modulation of bacterial–host and bacteria–bacteria interactions in nature might also be among the natural functions of *P. aeruginosa* MDR efflux pumps. The *Pseudomonadaceae* group contains bacteria able to colonize plants and, for such colonization, an adaptive response to defensive compounds present in plant exudates is needed. One of the mechanisms for dealing with plant defense is the extrusion of those defensive compounds by efflux pumps from the flavonoid-responsive family, such as MexAB-OprM [49,50], which plays key roles in plants' colonization [50,51]. This efflux pump does not only contribute to intrinsic resistance to quinolones, macrolides, tetracycline, chloramphenicol and β-lactams in *P. aeruginosa* [52] but it also extrudes plant-derived compounds, such as indole-derivatives, flavonoids or coumarins [47•].

Besides their contribution to host-bacteria interactions, *P. aeruginosa* MDR efflux pumps are involved as well in QS intercellular communication system. Different works have shown the efflux pumps to modulate QS by disrupting the production of signaling molecules [53] or by extruding them [48]. Since the expression of several *P. aeruginosa* virulence determinants is QS-regulated, changes in the level of expression of MDR efflux pumps that can render AR may also alter *P. aeruginosa* virulence and metabolism [54,55], providing an evidence of the interplay among AR, virulence and general physiology of this bacterial pathogen. To note here that the evolution of these virulence and AR determinants, encoded in *P. aeruginosa* core genome, has occurred in natural, non-clinical ecosystems, likely long before the emergence of humankind as a species.

## Conclusions

*P. aeruginosa* is an environmental-borne opportunistic pathogen that harbours in its core genome a repertoire of AR and virulence determinants that have evolved in nature, where they play different roles in bacterial homeostasis, stress response, intercellular communication or bacteria–host interactions. Although there is no clear boundary between environmental and clinical isolates, there exist predominant MDR clones that cause infections worldwide, increasing the risks for humans of infections caused by them. Besides the recent acquisition of carbapenemases [56•], these clones tend to present a characteristic resistance mutational pattern. For instance, the ST175 MDR phenotype is mainly due to mutations in *oprD*, *mexZ*, *ampR*, *parC* and *gyrA* [57–59]. Novel strategies based on the use of anti-virulence compounds, either alone or in combination with antibiotics, have been proposed as alternative strategies to single antibiotic use, even in the case of MDR mutants [60,61].

Furthermore, evolution-based strategies, consisting in the identification of robust collateral sensitivity networks [62] or in knowing the original functions of MDR efflux pumps, have been proposed. An example of the latter is the finding of natural compounds that are both substrates and inducers of efflux pumps. These inducer compounds reduce *P. aeruginosa* virulence without increasing resistance, possibly due to competition between the virulence inhibitor and the antibiotic extruded by the same efflux pumps [47•].

While evolution-based approaches can feed the field of anti-*Pseudomonas* research, little is still known about the contribution of natural ecosystems in the evolution and spread of *P. aeruginosa* antibiotic resistant strains, despite the *bona fide* environmental niche of this microorganism. In this respect, subinhibitory concentrations of antibiotics, likely found in human-related habitats colonizable by *P. aeruginosa*, as wastewater treatment plants, can select MDR mutants presenting clinically relevant cross-resistance to antibiotics currently used in clinical practice [63•]. The identification of the minimal concentration of antibiotics – or of biocides – able to select antibiotic resistant mutants of *P. aeruginosa*, particularly in non-clinical ecosystems [64,65], and the determination of the concentration of antibiotics present in different natural habitats, is needed for quantitatively examining the risks of antibiotic pollution for the selection of *P. aeruginosa* MDR strains.

## Authors' contribution

All authors contributed to the design and writing of the article. Figures were drawn by PL. All authors read and approved the submitted version of the work.

## Conflict of interest statement

Nothing declared.

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## Declaration of Competing Interest

The authors report no declarations of interest.

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