



Epigenetics of Antimicrobial Resistance in Gram-Negative Bacteria

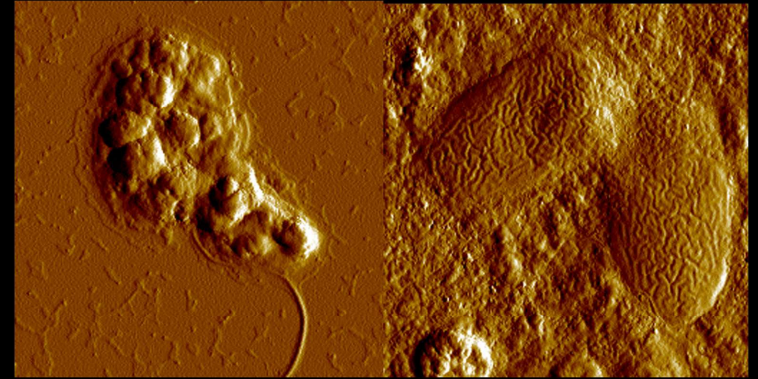
Ester Fusté i Domínguez



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EPIGENETICS OF ANTIMICROBIAL RESISTANCE IN GRAM-NEGATIVE BACTERIA

ESTER FUSTÉ DOMÍNGUEZ
L'HOSPITALET DE LLOBREGAT, 2012

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PhD THESIS ESTER FUSTÉ DOMÍNGUEZ



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DOCTORAL PROGRAM IN BIOMEDICINE

AUTHOR: ESTER FUSTÉ I DOMÍNGUEZ

DIRECTOR: MIQUEL VIÑAS CIORDIA

Barcelona, July 2012



**DEPARTMENT OF PATHOLOGY AND EXPERIMENTAL
THERAPEUTICS – IDIBELL**

Faculty of Medicine and School of Nursery

UNIVERSITY OF BARCELONA

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Miquel Viñas Ciordia, Catedràtic de Microbiologia del Departament de Patologia i Terapèutica Experimental de la Facultat de Medicina de la Universitat de Barcelona

FAIG CONSTAR,

Que la Tesi Doctoral presentada per Ester Fusté Domínguez i titulada “EPIGENETICS OF ANTIMICROBIAL RESISTANCE IN GRAM-NEGATIVE BACTERIA” ha estat desenvolupada per l'autora sota la meva supervisió.

Que el manuscrit compleix els requisits formals i conceptuals per a que pugui ser defensada davant del tribunal corresponent.

I per a que consti signa el present a Bellvitge, l'Hospitalet de Llobregat, el dia 24 de Juliol de 2012.

Signat Miquel Viñas Ciordia

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Doctoral Program in Biomedicine

Thesis submitted by Ester Fusté Domínguez for the degree of Doctor (Faculty of Medicine, University of Barcelona) including the mention of “European doctor” under the direction of Miquel Viñas Ciordia, Professor of Microbiology in the University of Barcelona.

Signed Ester Fusté Domínguez

L'Hospitalet de Llobregat, 24 July 2012

"L'essentiel est invisible pour les yeux."

"[...] dehors des grosses planètes comme la Terre, Jupiter, Mars, Vénus, aux quelles on a donné des noms, il y en a des centaines d'autres qui sont quelque-fois si petites qu'on a beaucoup de mal à les apercevoir au télescope. Quand un astronome découvre l'une d'elles, il lui donne pour nom un numéro.

Antoine de Saint-Exupéry
(*Le Petit Prince*)

A la meua família.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette superfamily
AFM	Atomic force microscopy
AMEs	Acquired aminoglycoside-modifying enzymes
APS	Ammonium persulfate
ATL	Tissue lysis buffer
BA	Boronic acid
BCA	Bicinchoninic agent
BHI	Brain heart infusion
°C	Celsius degrees
CAZ	Ceftazidime
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
CFU	Colony-forming units
CLA	Clavulanic acid
CLSI	Clinical Laboratory and Standards Institute
CT	Caprylate-thallos agar selective
CTX	Cefotaxime
Da	Daltons
DiphPc	Diphytanoylphosphatidylcholine
DMB_{SUP}	Supplemented minimal broth Davis
DMSO	Carbonyl cyanide m-chlorophenylhydrazone
DNA	Deoxyribonucleic acid
DR	Repeated sequences
EDTA	Ethylenedinitrilotetraacetic acid
EPI	Efflux pump inhibitor
EPO	Efflux pump overexpressed phenotype
ESBLs	Extended spectrum β -lactamases
EUCAST	European Committee on Antimicrobial Susceptibility Testing
g	Gravity
HTH	Helix-turn-helix-motif
IM	Inner membrane
K-W	Krustall Wallis test
LB	Luria broth

List of abbreviations

LPS	Lipopolysaccharide
M	Molar
MATE	Multidrug and toxic compound extrusion
MDR	Multidrug-resistant
MDRPA	Multidrug-resistant <i>Pseudomonas aeruginosa</i>
MFPs	Membrane fusion proteins
MFS	Major facilitator super family
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MIC	Minimal inhibitory concentration
min	Minutes
OD	Optical density
OD_{550/625}	Optical density at 550 nm or 625 nm
OM	Outer membrane
OMP s	Outer membrane proteins
O/N	Overnight
ORFs	Open reading frames
O/W	Over week-end
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulse-field gel electrophoresis
PS	Diphytanoylphosphatidylglycerol
QRDR	Quinolone-resistance- determining region
QS	Sufficient quantity
RNA	RiboNucleic acid
RND	Resistance-nodulation-division superfamily
rpm	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMR	Small multidrug resistance family

SSC	Saline-sodium citrate buffer
t	Time
TAE	Tris-acetate buffer
Taq	Thermo-resistant DNA polymerase
TBE	Tris-Borate-Edta buffer
TEMED	N,N,N',N'-Tetramethylethylenediamine
TSA	Tryptone soy agar
TSB	Tryptone soy broth
U	Units
V	Volts
v/v	Volume per volume
w/v	Weight per volume

LIST OF PUBLICATIONS

The five years period employed in the experimental work of this thesis has allowed the participation in the research which production can be seen in the following papers:

Fenosa A, Fusté E, Ruiz L, Veiga-Crespo P, Vinuesa T, Guallar V, Viñas M Role of TolC in *Klebsiella oxytoca* resistance to antibiotics. J Antimicrob Chemother 2009;63:668-674.

Veiga-Crespo P, Fusté E, Vinuesa T, Vinas M, Villa TG. The synergism between OM-proteins and antimicrobials. Antimicrob Agents Chemotherapy. 2011;55:2206-11.

Ruiz-Martinez L, Lopez-Jimenez L, Fusté E, Vinuesa T, Martinez JP, Vinas M. Class 1 integrons in environmental and clinical isolates of *Pseudomonas aeruginosa*. Int J Antimicrob Agents. 2011;38:398-402.

Ruiz-Martínez L, López-Jiménez L, d'Ostuni V, Fusté E, Vinuesa T, Viñas M. A mechanism of carbapenem resistance due to a new insertion element (ISPa133) in *Pseudomonas aeruginosa*. Int Microbiol. 2011;14:51-58.

Fusté E, Galisteo GJ, Jover L, Vinuesa T, Villa TG, Viñas M. Comparison of antibiotic susceptibility of old and current *Serratia*. Future Microbiol. 2012;7:781-6.

Fusté E, López-Jiménez L, Segura C, Vinuesa T, Villa TG, Gainza E and Viñas M. Carbapenem resistance in an endemic clone of *Pseudomonas aeruginosa*. (Submitted to Microbial drug resistance)

Viñas M, Fusté E, López L, Vinuesa T. Nanotechnology a tool for study and fight against biofilms. Invited Review to a Special Issue of The Open Microbiology. (manuscript in preparation)

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1. INTRODUCTION

1.1. EPIGENETICS

Epigenetics is an old word that was first used by Conrad Waddington in the early 40's. He pointed out that there is no simple relationship between a gene and its phenotypic effects. Epigenetics in Waddington's sense was not a synonym of developmental biology, but it could be considered a part of it. Waddington and others focused on understanding why many times genotype and phenotype variations are not associated, and phenotype differences do not necessary involve changes in genotype. However, the definition of epigenetics kept changing over time, and in 1982 a dictionary of biology defined it as "Pertaining to the interaction of genetic factors and the developmental processes through which the genotype is expressed in the phenotype." In 1983, Medawar described epigenetics with this sentence: "Genetic proposes: epigenetics disposes". An easily understandable definition of epigenetics could be something like: "epigenetics are changes in phenotype that do not involve changes in the underlying DNA". The more relevant change in the concept of epigenetics emerged in the decade of 1990, when genetic and non-genetic factors were considered to be essential for the control of gene expression that produces increasing phenotypic complexity during development. It was emphasized that other factors apart from classical DNA code were necessary and relevant in the transmission of hereditary information.

At the end of the 20th century, the word epigenetics became a synonym of epigenetic inheritance. Nevertheless, the main problem is that it is not easy to distinguish between genetic and epigenetic phenomena, although one can usually distinguish between DNA and non-DNA inheritance. In fact, there is no a general consensus about what the term epigenetics means, but an almost universal agreement. At present, genetics deals with the transmission and processing of information in DNA, whereas epigenetics deals with its interpretation and integration with information from other sources.

Jablonka & Lamb [1] wrote that examination of recent books and articles with epigenetics in their titles shows that the scope of the subject is far less narrow than some current definitions suggest.

Today's epigenetics importance is growing because private companies, as well as scientific community, are taking an active interest in what goes on beyond the DNA level and they are aware that epigenetics could introduce changes both in medicine and agriculture.

Epigenetics is also relevant in hereditary diseases, and it is known that in some cases the inherited disorder is caused by a mutation of the gene, but in others the defect may be epigenetic. As epigenetic effects can be transmitted to offspring it could be necessary to develop an epigenetic epidemiology. There are other aspects of epigenetics that may be relevant in preventing or curing diseases. As cells have sophisticated epigenetic mechanisms for avoiding or destroying genomic parasites, it could be possible to control and use these natural, epigenetic defence mechanisms to silence the foreign or endogenous genes associated with various diseases. Epigenetics also has implications for ecology, because organisms interact with each other and with their abiotic environment, and through these interactions they acquire epigenetic information, some of which may be inherited.

It is currently known that epigenetic inheritance is not limited to multicellular organisms; it is also found in unicellular organisms.

In 2007 epigenetics was defined as changes in the expression of genes that happen without a modification in DNA sequence. This can be understood as a bridge between genotype and phenotype [2, 3].

1.2. THE GENUS *Serratia*

1.2.1. HISTORICAL OVERVIEW

The story of this microorganism began in 1819 with the odd history of the bloody polenta. Bizio, a young pharmacist of the University of Padua named this bacterium. The name of the genus was chosen to honour to Serafino Serrati, a renowned Italian physicist who developed an early steamship engine. Noting that the pigment faded rapidly, he added the descriptor *marcescens*, derived from the Latin word that means “to decay.” However, Ferdinand Cohn, a distinguished microbiologist dated back his origin from the 4th century BC on the basis of Pitagoras credence showed in the satiric work *Vitarium auctio* written by Luciano de Samosata (170 AC). This bacterium has been related to an enormous quantity of miraculous due to the capacity of some *S. marcescens* strains to produce a red pigment called prodigiosin.

It is believed that the first reference of a *Serratia* contamination is the one explained by Quinto Curcio Rufo in his “Alejandro History”, where he explains that some soldiers saw blood drops in the bread during their conquest of Tiro. This fact was interpreted as a good prediction by a fortune-teller. Finally the conquest was successfully. This episode was two centuries later confirmed by Siculus. In the middle of the 19th century, Christian Ehrenberg described all the miraculous related with this bacterium, but he used the name of *Monas prodigiosa* instead of *S. marcescens*. The most known miraculous event occurred in Bolsena, which was decisive for the celebration of Corpus Christi. Raffaello Santi painted the fresco “The Mass of Bolsena”, from the Stanza d’Elidoro.



Fig. 1. “The Mass of Bolsena” from the Stanza d’Elidoro. Raffael.

In 1393, in the church of Wilsnack (Germany), the priest left three hosts for sick people in the altar during eight days. The previous night was raining, so next morning hosts were completely dry. However, environment was still wet and drops similar to blood appeared in the hosts scaring all the population. This anecdote point at a *S. marcescens* contamination, but in that time many pilgrims went to that church to be cured [4].

In the 20th century, *S. marcescens* was used as a marker due to his pigment and the belief that this bacterium was innocuous for human health. For instance, in one of the experiments, M.H. Gordon put around an empty room some Petri dishes with medium and after gargling with a *S. marcescens* culture, he recited Shakespeare texts to check the importance of the speaking in the transmission of diseases.

In 1920, Cumming contaminated with *Serratia* the throat, the lips and the gum of a group of soldiers to study the transmission of diseases. Later on, he recovered this bacterium from the hands and mouths of non-inoculated soldiers.

In 1950, the United States Navy conducted a secret experiment named "Operation Sea-Spray" in which some *S. marcescens* were released by bursting balloons of it over urban areas of the San Francisco Bay Area in California. Although the Navy claimed that bacteria were harmless, some patients at a local hospital developed very rare, serious urinary tract infections and one of these individuals died. Cases of pneumonia in San Francisco also increased after *S. marcescens* was released [5].

Since 1950, *S. marcescens* has steadily increased as a cause of human infection, with many strains resistant to multiple antibiotics [6].

The question is now: Which will be the following surprise that this prodigious creature will offer to us? [5]

1.2.2. CHARACTERISTICS OF THE GENUS

The genus *Serratia* belongs to the family *Enterobacteriaceae*, and it is comprised of a group of bacteria that are related both phenotypically and by DNA sequence [7].

Serratia are Gram-negative short rods that have a 0.5-0.8 μm in diameter and 0.9-2.0 μm in length. They are usually motile by peritrichous flagella and facultatively anaerobic having two types of metabolism: respiratory and fermentative. They grow well at 30 and 37 $^{\circ}\text{C}$. They catabolize D-Glucose and other carbohydrates with the production of acid and often gas. They are indole negative except *S. odorifera* strains, Simmons citrate test positive, Voges-Proskauer test usually positive except for *S. fonticola* and most of the strains produce DNase and hydrolyze gelatin and corn oil. Most of the species are also lysine decarboxylase positive, arginine dihydrolase negative and ornithine decarboxylase positive. They are not able to hydrolyze urea or produce H_2S and they don't usually use malonate. They have the capacity to reduce nitrates and ferment carbohydrates as maltose, D-mannose, salicin, sucrose and trehalose [8]. Figure 2 shows different images of *S. marcescens* from culture in Petri dish, scanning electron microscope, confocal microscope (dead-alive) and atomic force microscopy (AFM).

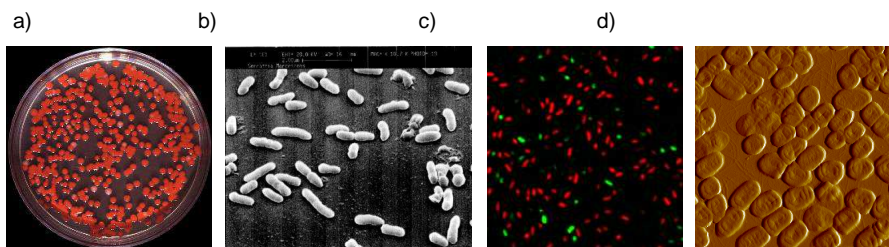


Fig. 2. a) *S. marcescens* pigmented colonies growth in TSA. b) *S. marcescens* obtained by Scanning Electron Microscope. c) *S. marcescens* from confocal microscope after staining with LIFE/DEAD stain and d) *S. marcescens* obtained by AFM.

Serratia species occupy different habitats and are mainly isolated from small mammals, water, plants and hospitalized patients.

Water is probably the principal habitat of *S. plymuthica*. However, many other species have been isolated from water. The 75% of 150 *Serratia* strains isolated from river water belonged to *S. marcescens* (F. Agbalika, F. Grimont, and P.D.A. Grimont, unpublished observations). In soil, *S. marcescens* might play a role in the biological cycle of metals by

mineralizing organic iron and dissolving gold and copper. Most of the strains isolated from soil and water are prodigiosin producers. This pigment is toxic to protozoa, so this may be an ecological advantage for them.

Plants are also a usual habitat for *Serratia* strains. *S. ficaria* is mainly found in figs, and *S. rubidaea* in coconuts. *Serratia* species were frequently found in plants (Grimont *et al.*, 1981). Vegetables, mushrooms, mosses and wet plants are the most important habitats where *Serratia* is found. It is sometimes found in grasses and less in trees and shrubs. The most common *Serratia* strains isolated from plants other than figs and coconuts are *S. liquefaciens* and *S. proteamaculans*. *S. marcescens* is rarely isolated from plants.

Insects are mainly associated with *S. marcescens*, *S. proteamaculans* and *S. liquefaciens*, which are considered potential insect pathogens. The rarity of *S. rubidaea* in insects might be explained by its inability to produce chitinase, a virulence factor for insect-associated *Serratia* species.

Serratia strains can be isolated from many vertebrates as cows, turtles, birds and wild rodents. Sometimes strains have been recovered from healthy vertebrates, but usually *Serratia* has been associated with chronic infections. For instance, red-pigmented strains are responsible for 0.2-1.5% of mastitis in cows. *Serratia* is not usually isolated from healthy people, whereas hospitalized patients are frequently colonized or infected. The opportunistic pathogen *S. marcescens* has been related to many infections. This fact will be widely described in subsequent sections [7].

1.2.3. TAXONOMY

The number of species that belong to the genus *Serratia* has increased from five species mentioned in the first edition of *The prokaryotes* to seven species described in *Bergey's Manual of Systematic Bacteriology* until ten species known at present. The *Serratia* species are: *S. marcescens*, *S. liquefaciens*, *S. proteamaculans*, *S. grimesii*, *S.*

plymuthica, *S. rubidaea*, *S. odorifera*, *S. ficaria*, *S. entomophila* and *S. fonticola*.

1.2.4. PATHOGENICITY FACTORS

S. marcescens is an opportunistic pathogen that causes infections in immunocompromised patients. The possible pathogenicity factors found in *Serratia* are described below:

Fimbriae: are proteinaceous appendages that are thinner and shorter than flagellum. Five types of fimbriae have been observed in *Serratiae*, and each strain can produce between one and three kinds of fimbrial hemagglutinin. The *type 1 fimbriae* produce mannose-sensitive hemagglutinin (MS-HA) and was found to be produced by all or almost all strains of *S. marcescens* whether environmental or clinical and also in some strains of other *Serratia* species. The production of MS-HA has been correlated with the ability of *S. marcescens* to attach to human bucal epithelial cells or to the human urinary bladder surface.

The *type 3 fimbriae* are associated with mannose-resistant hemagglutinin (MR/K-HA) and are produced by almost all *Serratia* strains. However, this type of fimbriae is more frequently produced by clinical strains than environmental strains. The *type FGH MR/PHA fimbriae* produced by strains of all species except *S. plymuthica* and *S. fonticola* are associated with mannose-resistant hemagglutinin and these fimbriae are immunologically related in the different species [9].

The *type F MR/P-HA fimbriae* produced by some *S. rubidaea* are associated with mannose-resistant hemagglutinin and are immunologically unrelated with other hemagglutinins.

The *type F MR/P-HA fimbriae* produced by some *S. fonticola* strains are also associated with mannose-resistant hemagglutinin.

Siderophores: Siderophores are produced by clinical and environmental *S. marcescens* strains. *Serratia* strains generally produce enterobactin but only rarely produce aerobactin.

O antigens: Although O-antigens cannot be considered truly pathogenicity factors, they are located in LPS surface, and it is known that O6 and O14 serotypes of *S. marcescens* are predominant in infections.

Extracellular enzymes: Enzymes that play a prominent role in the pathogenesis of experimental pneumonia and keratitis.

Plasmids: They probably play no role in the virulence in experimental models, but multiple drug resistance may affect the course and prognosis of infections. Non-pigmented strains of *S. marcescens* use to be more resistant because they often harbour resistance plasmids.

Relatively little is known about factors that contribute to *S. marcescens* pathogenesis within its host. However, some virulence factors of the human opportunistic *S. marcescens* have been recently identified by *in vivo* screening [7, 10].

1.3. THE GENUS *Pseudomonas*

Pseudomonas spp. are aerobic, non-spore-forming Gram-negative bacilli, which are usually motile by one or several polar flagella.

Pseudomonas is a genus including species that can use a wide range of organic and inorganic compounds and can live under diverse environmental conditions. For that reason, they are ubiquitous in soil and water, plants, animals and humans (some isolates may infect immunocompromised patients).

This genus is well known for its metabolic and genetic plasticity. They generally grow rapidly and are able to metabolize a huge number of substrates including toxic organic chemicals, such as aliphatic and aromatic hydrocarbons [11].

They possess a strictly respiratory metabolism with oxygen as the terminal electron acceptor; but they are capable grow anaerobically due to its ability to use nitrate, an alternative electron acceptor [12].

One important consequence of its metabolic diversity is that *Pseudomonas* spp., in particular *P. aeruginosa*, is common throughout the hospital setting and persist in many types of equipment, sinks, tubes, etc.

This characteristic certainly contributes to the opportunistic nature of opportunistic infections [13].

Pseudomonas species, particularly the human pathogen *P. aeruginosa*, not often colonize healthy humans. The throat, intact skin or stools of healthy individuals are densely colonized by normal flora, which do not include *Pseudomonas* species. Nevertheless, individuals that often received antibiotic therapies are at risk of gastrointestinal colonization with *P. aeruginosa* and consequently, due to this reservoir the patient can suffer septicemia caused by this microorganism. Patients undergoing anti-cancer chemotherapy or marrow ablation for bone marrow transplantation and patients with cystic fibrosis or receiving mechanical ventilation have greater risk to develop serious *Pseudomonas* infections [12].

1.3.1. TAXONOMY

Pseudomonas is a genus that was first proposed by Migula in 1894. It has been subjected to many taxonomic revisions and identification methodologies. In the early 1970s this genus was classified into five unrelated groups according to RNA-DNA hybridization studies: I, *Proteobacteria*; II, *Burkholderia* species; III, *Comamonas*, *Acidovorax*, and *Hydrogenophaga* genera; IV, *Brevundimonas* species; and V, *Stenotrophomonas* and *Xanthomonas* genera. Currently, there are about 160 species within *Pseudomonas* genus, of which few species have clinical significance.

1.3.2. PATHOGENESIS

P. aeruginosa is the most important causative agent of opportunistic infections among the genus *Pseudomonas*; this is the reason why we are basically going to focus on the pathogenesis of this particular microorganism [12,14]. It can cause both acute and chronic infections.

P. aeruginosa is now recognized as a common source of many community acquired and nosocomial infections. The main infections are the ones that involve cornea, skin, urinary tract and respiratory tract, although infections may occur in essentially all anatomical locations. Host

immunodeficiency, combined with a high incidence of antibiotic resistant strains, makes treatment of *P. aeruginosa* a serious medical challenge.

There are many factors involved in the pathogenesis of *P. aeruginosa*. Thus, the virulence of this opportunistic pathogen cannot be attributed to a single factor; it has to be considered multifactorial. Some of the factors relevant in *Pseudomonas* pathogenesis are adhesins, endotoxins, proteases, hemolysins and a type III secretion system.

The virulence determinants with known roles in pathogenesis are summarized below:

Adherence: The two important types of adhesins used by *P. aeruginosa* to colonize host tissues are fimbrial adhesins, such as type IV pili and type I fimbriae, and non-fimbrial adhesins, including lipopolysaccharide, flagella, outer membrane proteins and alginate.

Exotoxin A: It is the most potent exotoxin produced by almost all clinical *P. aeruginosa* isolates.

Proteases and phospholipases: *P. aeruginosa* produces several proteases including LasB elastase, LasA elastase, and alkaline protease. Las A, Las B and alkaline protease are associated with tissue damage in *P. aeruginosa* infections. Besides, it produces two homologous extracellular phospholipases, the hemolytic PlcH and the nonhemolytic PlcN, which has been demonstrated to be a virulence factor in a variety of *P. aeruginosa* infections.

Type III secretion: This system is a major determinant of virulence in *P. aeruginosa*. It is used to deliver four toxins that act in concert to inhibit phagocytosis and promote tissue destruction. It is expressed in response to a variety of environmental signals (low Ca^{2+} concentrations, serum and contact with eukaryotic cell surfaces).

Quorum sensing: *las* and *rhl* are the two quorum sensing systems used by *P. aeruginosa* for the coordination of gene expression with density of bacteria. Many of the genes regulated by quorum sensing are known virulence factors. However, as *P. aeruginosa* is considered an opportunistic pathogen, it is thought that quorum sensing plays an important function in the environment.

Biofilms: *P. aeruginosa* biofilms (surface-associated bacterial communities encased within a polymeric matrix) are mainly formed on the surfaces of medical devices, and can contribute to chronic infections. Moreover, inherent resistance of biofilms makes them difficult to treat and eliminate.

1.4. ANTIMICROBIAL AGENTS AND BACTERIAL RESISTANCE

1.4.1. β -LACTAMS

β -lactams are a group of antibiotics that contain a four-atom ring (β -lactam) that determine their most relevant characteristics i.e. mechanism of action (inhibition and synthesis of the wall), main mechanisms of resistance (β -lactamases) and low toxicity (strict inhibition of wall biosynthesis, since the structure do not exist in eukaryote cells). β -lactam antibiotics include penicillins, cephalosporins, carbapenems and monobactams.

Since 1920, when penicillin was discovered, β -lactams have been successfully used in the treatment of human infectious diseases. Due to their spectra of activity, effectiveness, low toxicity and wide therapeutic margin, they are the most used antimicrobial agents in the community, as well as in hospitals.

1.4.1.1. PENICILLINS

1.4.1.1.1. CLASSIFICATION AND STRUCTURE

Penicillins are natural or semisynthetic antibiotics containing the chemical nucleus 6-aminopenicillanic acid. The penicillins differ from one another in the substitution at position 6.

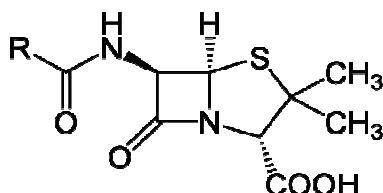


Fig. 3. Penicillin core structure.

1.4.1.1.2. TARGETS AND MECHANISM OF ACTION

Penicillins have the ability to inhibit a number of bacterial enzymes, specifically penicillin-binding proteins (PBPs) that are essential for peptidoglycan synthesis.

PBPs have enzymatic activity as carboxypeptidases and transpeptidases being directly responsible of the incorporation of new subunits into the growing peptidoglycan. Activity of penicillins is usually related to their ability to destroy bacterial wall by the triggering of membrane-associated autolytic enzymes. They can also inhibit endopeptidase and glycosidase enzymes, which are relevant in bacterial growth.

1.4.1.1.3. SPECTRUM OF ACTIVITY

Penicillins are active against most Gram-positive and many Gram-negative and on both aerobic and anaerobic organisms. Penicillin G is not active against enterococci, members of the family *Enterobacteriaceae*, *Pseudomonas* spp. or members of the *Bacteroides fragilis* group. Ampicillin and amoxicillin have similar spectra of activities than Penicillin G, and they are degraded by β -lactamase and are inactive against many *Enterobacteriaceae* and *P. aeruginosa*. Carboxypenicillins and ureidopenicillins are more stable against hydrolysis by the β -lactamases of *Enterobacteriaceae* and *P. aeruginosa*.

1.4.1.2. CEPHALOSPORINS

1.4.1.2.1. CLASSIFICATION AND STRUCTURE

Cephalosporins belong to β -lactam antibiotics and originally derived from *Acremonium*. They contain a 7-aminocephalosporanic acid nucleus, which consist of a β -lactam ring fused. Various substitutions at positions 3 and 7 alter their antibacterial activities and pharmacokinetic properties. The addition of a methoxy group at position 7 of the β -lactam ring results in a new group of compounds called cephamycins, which are highly resistant to a variety of β -lactamases.

Cephalosporins are classified in generations. The evolution of the generations is related with an increment of their spectrum of activity and intrinsic activity against Gram-negative organisms.

Figure 4 shows the chemical structure of cephalosporins.

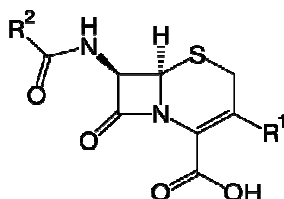


Fig.4. Cephalosporin core structure.

Classification of cephalosporins is shown in table 1.

Table 1. Cephalosporins classification

First generation (narrow-spectrum)	Second generation (expanded spectrum)	Third generation (broad-spectrum)	Fourth generation (extended spectrum)
Cefadroxil	Cefaclor	Cefdinir	Cefepime
Cefazolin	Cefamandole	Cefditoren	Cefpirome
Cephalexin	Cefonicid	Cefixime	
Cephaloridine	Ceforanide	Cefoperazone	
Cephalotin	Cefuroxime	Cefotaxime	
Cephapirin	Cefprozil	Cefpodoxime	
Cephradine	Loracarbe	Ceftazidime	
	Cefmetazole	Ceftibuten	
	Cefotetan	Ceftizoxime	
	Cefoxitin	Ceftriaxone	

1.4.1.2.2. TARGETS AND MECHANISM OF ACTION

Like penicillins, cephalosporins act by binding to penicillin-binding proteins (PBPs) of susceptible organisms, thereby interfering with synthesis of peptidoglycan of the bacterial wall. Cephalosporins also produce

bactericidal effects by triggering autolytic enzymes in the envelope of bacteria.

1.4.1.2.3. SPECTRUM OF ACTIVITY

First generation cephalosporins (narrow spectrum) have good activity against Gram-positive organisms and modest activity against Gram-negative organisms.

Second generation cephalosporins (expanded spectrum) are more active against Gram-negative bacteria than first generation cephalosporins because of their stability to β -lactamases, and less active against Gram-positive organisms. The activity also depends on the concrete antibiotic used.

Third generation cephalosporins (broad spectrum) are generally less active against Gram-positive cocci than the narrow spectrum agents, but they are more active against the *Enterobacteriaceae* and *P. aeruginosa*.

Fourth generation cephalosporins (extended spectrum) are active against stably derepressed class I β -lactamase mutants of *Enterobacteriaceae* and *P. aeruginosa*. Besides, these antibiotics penetrate well through the outer membrane of Gram-negative bacteria but they are inactive against enterococci or anaerobes.

1.4.1.3. MONOBACTAMS AND CARBAPENEMS

1.4.1.3.1. CLASSIFICATION AND STRUCTURE

Monobactams, such as aztreonam, are β -lactam compounds wherein the β -lactam ring is alone and not fused to another ring.

Carbapenems are β -lactams with a hydroxyethyl side chain in *trans* configuration at position 6 and lacking a sulphur or oxygen atom in the bicyclic nucleus (Figure 5). Doripenem, imipenem, meropenem and ertapenem are currently available for clinical use.

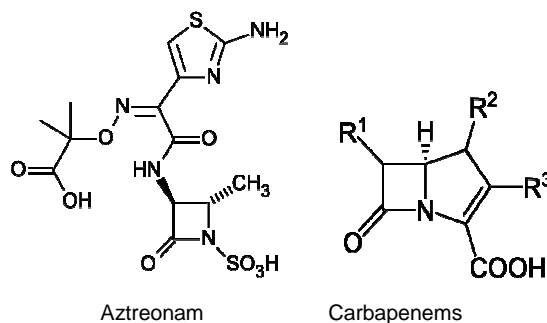


Fig. 5. Chemical structure of aztreonam and carbapenems.

1.4.1.3.2. TARGETS AND MECHANISM OF ACTION

Aztreonam binds to the PBP 3 of Gram-negative aerobes disrupting bacterial wall synthesis. It does not induce production of enzymes and it is not hydrolyzed by most common β -lactamases.

Carbapenems bind to PBP 1 and PBP 2 of Gram-negative and Gram-positive bacteria, elongating the cell and causing lysis.

1.4.1.3.3. SPECTRUM OF ACTIVITY

Aztreonam has significant activity against *Enterobacter* spp. and *S. marcescens*.

Carbapenems are active against aerobic Gram-positive species. More than 90% of *Enterobacteriaceae* are generally susceptible to carbapenems, including *S. marcescens*. Ertapenem is inactive against *Pseudomonas* and *Acinetobacter*. Carbapenems are the most potent β -lactams against anaerobes. At present, there are some *P. aeruginosa* strains resistant to imipenem.

1.4.1.3.4. RESISTANCE TO β -LACTAM ANTIBIOTICS

1. Penicillin-binding protein-mediated resistance

This kind of resistance takes several forms described below:

PBP overexpression: It is an uncommon mechanism of resistance where the high number of target molecules can result in resistance under the correct circumstances [12].

2. β -lactamase-mediated resistance

There are two β -lactamase classification systems currently used that are compared in table 2.

Table 2. β -lactamase classification

Bush-Jacoby-Medeiros system	Major subgroups	Ambler system	Main attributes
Group 1 cephalosporinases	-	C (cephalosporinases)	Usually chromosomal; resistance to all β -lactams except carbapenems; not inhibited by clavulanate
Group 2 penicillinases (clavulanic acid susceptible)	2a	A (serine β - lactamases)	Staphylococcal penicillinases
	2b	A	Broad-spectrum: TEM-1, TEM-2, SHV-1
	2be	A	Extended-spectrum: TEM and SHV variants predominantly
	2br	A	Inhibitor-resistant TEM
	2c	A	Carbapenem hydrolyzing
	2e	A	Cephalosporinases inhibited by clavulanate
	2f	A	Carbapenemases inhibited by clavulanate
Group 3 metallo- β -lactamase	3a	B (metalloenzymes)	Zinc-dependant carbapenemases
	3b	B	
	3c	B	
Group 4	-	Not classified	Miscellaneous enzymes, most not yet sequenced

β -lactamase mechanism: This mechanism has been studied in TEM-1 and SHV-2 Class A β -lactamase. Disruption of the amide bond is done in a two-step reaction where the negatively charged carboxylate group of the β -lactam antibiotic is attracted to the active site by the enzymes positively charged residues and then, β -lactam becomes acylated.

β -lactamases: These enzymes are produced in a constitutive or inducible manner. They can be encoded by chromosomal, plasmids, or transposon genes. Genes encoding β -lactamases of all known molecular classes (A through D) can be found as integrons-associated.

Integrans are genetic elements of variable lengths that contain a 5' conserved integrase gene (*int*), mobile antibiotic resistance genes (cassettes), and an integration site for the gene cassette, *attI* (att, attachment). Class 1, 2 and 3 integrans are common in Gram-negative bacteria. Integrans capture antibiotic resistance gene cassettes by using a site-specific recombination mechanism. They act both as natural cloning systems and as expression vectors [15]. The capture and spread of antibiotic resistance determinants by integrans underlie the rapid evolution of multiple-antibiotic resistance in Gram-negative clinical isolates [16]. Integrans carrying β -lactamases have been found in most Gram-negative bacteria.

β -lactamases classes:

Class A β -lactamases (Group 2b Penicillinases): The two commonly class A β -lactamases found in *Enterobacteriaceae* are TEM-1 and SHV-1, which are primarily penicillinases with diminished activity against cephalosporin substrates. They are the progenitors of the ESBLs and the inhibitor-resistant TEM β -lactamases now common in many hospitals [12].

Bush group 2be (ESBLs): Extended-spectrum cephalosporins (a group of potent β -lactams) are poor substrates for hydrolysis by group 2b enzymes. Mutations at critical amino acids resulted in an expansion of the spectra of these enzymes enabling them to hydrolyze extended-spectrum cephalosporins. The number of enteric Gram-negative rods possessing ESBLs has increased recently and this has obliged to modify the choice of antimicrobial therapies.

Non-TEM, non-SHV ESBLs: CTX-M β -lactamases commonly found in *Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Citrobacter freundii*, *Enterobacter* spp., and *S. marcescens* are the most important non-TEM, non-SHV β -lactamases. They hydrolyze cefotaxime and ceftriaxone better than they do ceftazidime. They are more readily inhibited by tazobactam than they are by clavulanic acid.

Inhibitor-resistant class A β -lactamases (Bush group 2br): This group is formed by amino acid mutated TEM and SHV conferring resistance to inhibition by β -lactamases inhibitors.

Class B β -lactamases (Bush group 3 enzymes): This class of β -lactamases is formed by metallo-enzymes that require zinc or another heavy metal for catalysis; their activities are inhibited by chelating agents such as EDTA. These β -lactamases confer resistance to a wide variety of β -lactam compounds, including carbapenems and cephamycins. They are resistant to inactivation by clavulanate, sulbactam and tazobactam. Some of them hydrolyse aztreonam. The majority of β -lactamases are chromosomally encoded, and their expression may be constitutive or inducible. VIM and IMP class B β -lactamases are active against most β -lactams and have been found in various Gram-negative clinical isolates. The majority of these enzymes are mobilized on integrons, transposons and mobile common regions.

Class C β -lactamases (Bush-Jacoby-Medeiros group 1): β -lactamases chromosomally encoded by almost all Gram-negative bacteria except *Salmonella* and *Klebsiella*. These enzymes are important in Gram-negative clinical isolates, including *S. marcescens*. Class C β -lactamases hydrolyze cephalosporins and penicillins. Most of them are resistant to inhibition by clavulanate, sulbactam and tazobactam. Some class C β -lactamases of Gram-negative bacteria are plasmid mediated.

Class D β -lactamases (oxacillin-hydrolyzing): OXA enzymes β -lactamases are included in this group. They confer resistance to a wide variety of penicillins and are weakly inhibited by clavulanic acid and totally inhibited by sodium chloride. The frequent location of class D genes on mobile genetic elements (plasmid or integrons) facilitates its spread. Several OXA β -lactamases are associated with an ESBL phenotype, and are important because some of them are capable to hydrolyze carbapenems. Although OXA carbapenases hydrolyze imipenem inefficiently, their presence in an organism with an active efflux pump or a porin mutation may confer clinically significant levels of resistance.

1.4.2. AMINOGLYCOSIDES

1.4.2.1. CLASSIFICATION AND STRUCTURE

Streptomycin was the first aminoglycoside introduced in 1944 for the treatment of serious Gram-negative infections. Structurally, each of these aminoglycosides contains two or more amino sugars linked by glycosidic bonds to an aminocyclitol ring nucleus.

There are two different groups of aminoglycosides. Streptomycin, neomycin, kanamycin, tobramycin and gentamicin belong to the group of natural aminoglycosides, and amikacin and netilmicin belong to the group of semisynthetic aminoglycosides (Figures 6 and 7).

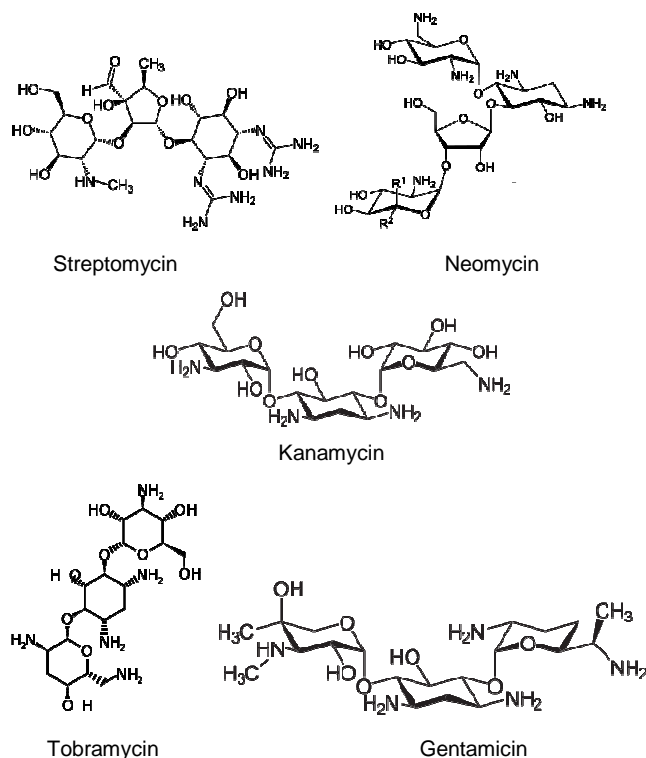


Fig. 6. Chemical structures of natural aminoglycosides.

Table 3. Differences in antimicrobial spectra among aminoglycosides.

Aminoglycoside	Resistant to :	Other particularities:
Kanamycin	<i>P. aeruginosa</i> Plasmid-mediated inactivating enzymes among other Gram-negative bacilli.	Used in mycobacterial infections in combination with other antimicrobial agents.
Streptomycin	Some <i>Enterobacteriaceae</i> .	Used as a single agent in <i>Francisella tularensis</i> and <i>Yersinia pestis</i> . Used in combination with tetracycline for brucellosis. Used in combination with penicillin or vancomycin for streptococci or enterococci infective endocarditis.
Gentamicin	Bacteria that produce modifying enzymes (except 6-acetyltransferase and 4'-adenyltransferase).	Active against <i>Mycobacterium tuberculosis</i> . Very active against <i>Serratia</i> spp.
Tobramycin	Bacteria that produce modifying enzymes (including 6-acetyltransferase and 4'-adenyltransferase). Variable resistance to 3-acetyltransferase producers.	Very active against <i>P. aeruginosa</i> .
Netilmicin	Many of the aminoglycoside-modifying enzymes. Most gentamicin-resistant <i>Serratia</i> , <i>Proteus</i> , <i>Providencia</i> and <i>Pseudomonas</i> isolates.	Active against most gentamicin and tobramycin-resistant <i>Enterobacteriaceae</i> . Less active against <i>P. aeruginosa</i> than gentamicin and tobramycin.
Amikacin	Some aerobic Gram-negative bacillus and <i>S. aureus</i> .	Active against most gentamicin and tobramycin-resistant <i>Enterobacteriaceae</i> . Used when tobramycin and gentamicin resistances are prevalent. Active against many <i>Mycobacterium</i> spp. and moderately active against <i>Haemophilus</i> spp. and <i>Neisseria</i> spp.

1.4.2.4. RESISTANCE TO AMINOGLYCOSIDES

There are three known mechanisms of resistance to aminoglycosides:

1. Reduction of intracellular aminoglycosides accumulation due to outer membrane permeability alteration, inner membrane transport decrease or active efflux increase.
2. Modification of the target site by mutation in the ribosomal proteins or 16S RNA.
3. Enzymatic modification of the drug.

All *S. marcescens* produce chromosomal AAC(6')-Ic enzyme that may slightly affect the activity of all aminoglycosides except streptomycin and gentamicin.

Gentamicin is the most active aminoglycoside against *Serratia*. However, other aminoglycosides can be used when isolates do not produce aminoglycoside-modifying enzymes.

Resistance to aminoglycosides in *P. aeruginosa* usually results from drug inactivation by plasmid or chromosome-encoded enzymes harbored by resistant strains.

Enzyme-independent resistance can be due to defects in uptake/accumulation of the drug [17].

1.4.3. QUINOLONES

1.4.3.1. CLASSIFICATION AND STRUCTURE

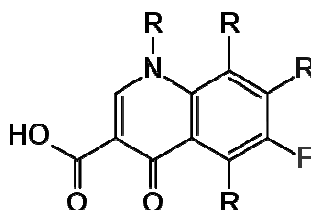
The era of quinolones began in the early 1960s when Leshner and associates discovered nalidixic acid during the synthesis of the antimalarial agent chloroquine [18,19].

Quinolones are a class of wide-spectrum agents which are usually classified by generations taking into account their spectrum of activity and clinical indications.

Table 4. Classification of quinolones by generations

First generation	Second generation	Third Generation	Four generation	Five generation
Nalidixic acid	Ofloxacin	Ciprofloxacin	Trovafloxacin	Garenofloxacin
Pipemidic acid	Enoxacin	Pefloxacin	Moxifloxacin	DX-619, WCK-771, WCK-1153, DW-286
Cinoxacin	Lomefloxacin	Levofloxacin	Gemifloxacin	
Flumequine		Sparfloxacin	Sitafloxacin	
		Grepafloxacin	Clinafloxacin	
		Gatifloxacin		

The chemical structure of fluoroquinolones (Fig. 8) is based on the 1,4-dihydro-4-oxo-pyridine molecule, which has a carboxylic acid substituent at position 3. This substituent with the carbonyl group at position 4 appears to be essential for the activity of these antimicrobial agents. First generation quinolones (except flumequine) lack the fluorine atom at position 6. The second-generation quinolones have a cyclic diamine at the position 7 and fluorine atom at position 6 in the quinolone nucleus, and the third generation differs from the previous generation in the substituents located at positions 1, 7 and 8 of the quinolone nucleus [17, 20].

**Fig. 8.** Basic structure of quinolones.

1.4.3.2. TARGETS AND MECHANISM OF ACTION

DNA gyrase and topoisomerase IV are the protein targets of quinolones. On the one hand, DNA gyrase is a tetrameric enzyme with two A subunits and two B subunits that catalyzes the negative supercoiling of DNA. The energy required for this process is obtained from the hydrolysis of ATP. DNA gyrase is known to play a significant role in both transcription and replication of DNA. On the other hand, topoisomerase IV is a tetrameric enzyme with two A subunits and two B subunits encoded by the *ParC* and *ParE* genes. It seems to be involved in decatenating daughter replicons following DNA replication.

At present, the mechanism of action of quinolones is not fully understood. There are some controversies, despite the fact that the mechanism of action has been carefully studied. It is accepted that quinolones have two binding domains, one interacting with DNA and the other with DNA gyrase. Complex formation of the DNA gyrase-quinolone-DNA has been associated to inhibition of DNA replication.

1.4.3.3. SPECTRUM OF ACTIVITY

First generation quinolones are substantially less potent *in vitro* and have narrower antibacterial spectra than newer generations, having enhanced activities against Gram-negative bacilli, except *P. aeruginosa*. They lack activity against Gram-positive cocci and anaerobes. These quinolones reach high concentrations in the urinary tract and subsequently they are widely used to treat urinary tract infections [17, 21].

Second generation quinolones exhibit striking potency against enteric Gram-negative bacilli, less activity against non-enteric Gram-negative bacilli, including *P. aeruginosa*, and generally marginal activity against staphylococci and anaerobes [18, 20]. These quinolones are mainly used to treat urinary tract infections, pyelonephritis, skin and soft tissue infections and sexually transmitted diseases [21].

The third generation quinolones have improved activity against Gram-positive cocci and anaerobic bacteria. They are mostly used for the treatment of chronic bronchitis, acute sinusitis and community pneumonia. The fourth generation quinolones have similar spectrum of activity than third generation quinolones. They exhibit activity against anaerobes. They are used for the treatment of nosocomial pneumonia, intraabdominal and gynecologic infections and the same infections to which the previous generations are used but urinary tract infections.

Finally, some of the fifth generation quinolones have antimicrobial activity against the same microorganisms than those of fourth generation, whereas others are specifically designed to fight against infections caused by multidrug-resistant bacteria [20].

1.4.3.4. RESISTANCE TO QUINOLONES

Both chromosomal and plasmid-mediated quinolone resistance have been described. Chromosomal mutations comprise mutations in the topoisomerase genes (*gyrA*, *gyrB*, *parC* and *ParE*) and mutations that produce a reduction in drug accumulation (decrease uptake or increase efflux).

There are many factors that can promote the emergence of resistance to quinolones, not only in the target pathogen, but also in the normal flora (gut, skin and throat). These factors can be categorized in three groups: dependent on the drug, dependent on the bacterium or dependent on the host and others. Table 5 shows the relevant factors to be considered in each group [17].

Table 5. Relevant factors favoring emergence of resistance to quinolones.

Factors dependent on the:		
Drug	Bacterium	Host and others
Concentration of the drug achieved in the infection site or epithelium.	Higher inoculum increase probability of spontaneous mutations.	Pharmacokinetics of the drug at the infection or colonization sites.
Time of exposure (concentration of the drug above the MIC).	Quorum sensing. (Control of virulence factors expression, the entry into stationary phase, the conjugal factor of transfer DNA, and spore formation and transformation competence).	Immune status.
Mutagenesis	Hypermutability.	Environment at the site infection such as pH, oxygen, nutrients, etc.
	Capability and capacity to form biofilm. (Bacterial density determines the gradients of nutrients and oxygen availability within the biofilm structure; bacteria located in deeper parts of biofilm have worst access to nutrients and oxygen; bacteria located in zones of the biofilm with high metabolic activity and oxygen concentration are better killed by antimicrobial agents).	
	Facility to acquire quinolone resistance. (Slow or no growth bacteria have increased resistance than exponential ones).	

Many authors have shown that in *S. marcescens*, like in other *Enterobacteriaceae* (but *E.coli*) a single substitution in the quinolone-resistance determining region (QRDR) is enough to produce high level resistance. However, higher levels of fluoroquinolone resistance can be achieved when there is cooperation between QRDR alterations and low permeability (or active extrusion) limiting the intracellular accumulation of the drug [22].

It was also demonstrated that when the Omp1 porin was absent in clinical isolates of *S. marcescens*, there was a prominent decrease in quinolone accumulation. Thus, porins play a key role in *S. marcescens* quinolone resistance [23].

Puig *et al.* indicated that susceptibility to antibiotics in *S. marcescens* was modified by analgesics, suggesting that when analgesics such as acetylsalicylate and antibiotics are administered together, their interaction can change the response of microorganisms to antibiotics [24]. Furthermore, it was shown that when concentration of salicylic acid augments, there is a diminishing in ciprofloxacin accumulation and a reduction in the porin content of the outer membrane. Resistance to ciprofloxacin and nalidixic acid was enhanced when bacteria grew in the presence of salicylic acid, supporting the idea that salicylate induces phenotypic resistance to quinolones in *S. marcescens* [25].

Subsequently, Begic and Worobec evidenced that high salicylate and sucrose concentrations, increased temperature and an acidic pH reduce the porins uptake and availability of norfloxacin (hydrophilic fluoroquinolone), leading to an increased resistance of *S. marcescens* to this antibiotic. Nevertheless, nalidixic acid (hydrophobic quinolone) susceptibility was not affected by the same conditions [26].

Mechanisms of quinolone resistance in non-fermentative Gram-negative bacteria are similar to the ones described for *Enterobacteriaceae*. The quinolone susceptible strains of *P. aeruginosa* (also *Acinetobacter baumannii* and *Stenotrophomonas maltophilia*) usually show higher baseline MICs for quinolones when compared with *Enterobacteriaceae*. In *P. aeruginosa*, the decreased permeability of the outer membrane together with several efflux pumps such as MexAB-OprM, MexCD OprJ, MexEF-OprN, MexXY-OprM and MexVW-OprM can affect fluoroquinolone susceptibility. The genome of this microorganism revealed the presence of more efflux pumps, although their potential role in antibacterial resistance has not been fully elucidated. Mutations in *gyrA* and *parC* genes can also contribute to increasing the level of resistance to quinolones. Fluoroquinolone minimal inhibitory concentration (MIC) can be modulated by overexpression of one or more efflux pumps in collaboration with mutations in the *gyrA* and *parC* genes [17].

1.4.4. POLYMYXINS

1.4.4.1. CLASSIFICATION AND STRUCTURE

Polymyxins are cationic peptides antimicrobials (Fig. 9) originally derived from *Bacillus polymyxa*. Only polymyxins B and E (colistin) are available for therapeutic use in humans.

Colistin is available since 1959 for the treatment of infections caused by Gram-negative bacteria. However, when early clinical reports suggested a high level of toxicity in the early 1970s, its use was reduced since less toxic aminoglycosides and other anti-pseudomonal agents were available. This is the reason for the reduced number of studies between 1970s until the mid-1990s [27].

There are two forms of colistin available: colistin, which is usually used as the sulphate salt, and colistimethate, which is used as sodium salt [28, 29].

Cationic peptides are amphipathic molecules that have both a hydrophobic face, comprising non-polar amino acid side-chains, and a hydrophilic face of polar and positively charged residues.

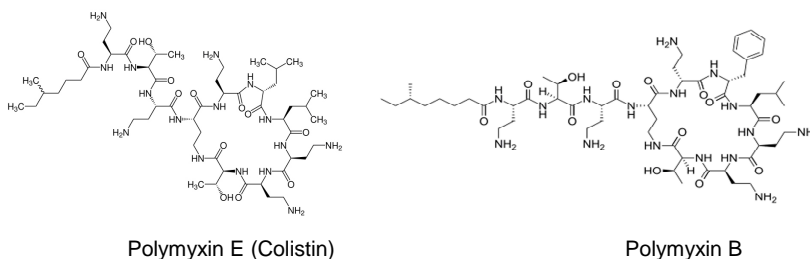


Fig. 9. Molecular structure of polymyxin E (colistin) and polymyxin B.

1.4.4.2. TARGETS AND MECHANISM OF ACTION

Polymyxins interact with LPS on the surfaces of Gram-negative bacteria, and are subsequently taken up via the self-promoted uptake pathway.

Firstly, these cationic peptides interact with divalent cation binding sites on LPS and competitively displace native divalent cations (Ca^{2+} or Mg^{2+}).

This displacement disrupts the normal barrier property of the outer membrane which permits the passage of molecules such as hydrophobic compounds, small proteins and/or antimicrobial agents. Moreover, it is promoted the uptake of the perturbing peptide itself (Fig. 10.a).

Bacteria are killed as a result of insertion of peptides into cytoplasmic membrane leading to channels formation and leakage of cytoplasmic molecules (Fig. 10.b). It is remarkable the fact that channels formation is favored by the large transmembrane potentials, high content of negatively charged lipids and lack of cationic lipids and cholesterol [30, 31].

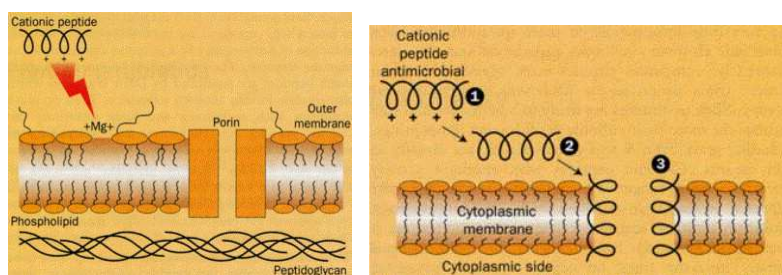


Fig. 10. a) Uptake of polymyxins across outer membrane of Gram-negative bacteria. b) Mechanism of bacterial killing by polymyxins [30]

1.4.4.3. SPECTRUM OF ACTIVITY

Polymyxins are only active against Gram-negative rods, particularly *Pseudomonas* spp.

Proteus, *Providencia*, *Serratia*, and *Neisseria* are generally resistant [12].

Colistin does not exhibit a wide antibacterial spectrum. It is basically active against common Gram-negative clinical isolates, including *Enterobacteriaceae* species and *Aeromonas*, but not *Vibrio* species, or some non-fermentative and fastidious Gram-negatives. Some bacteria are resistant to this antimicrobial, such as *Proteus* spp., *Providencia* spp., *Morganella morganii* and *S. marcescens*. However, the non-fermentative Gram-negative bacteria *P. aeruginosa* and *Acinetobacter* species are mainly susceptible, although some strains have recently developed resistance.

It is also noteworthy that many authors have shown that colistin sulphate has lower antimicrobial activity than sodium colistin methanesulphonate [28].

1.4.4.4. MECHANISM OF RESISTANCE

Although resistance is not as common as resistance to other families of antimicrobial agents, probably due to its low use over the past 50 years, some polymyxin resistant-bacteria have been identified. Modifications of LPS are the most common mechanisms of resistance to colistin, which is the initial site of action of this antimicrobial agent. Other mechanism of resistance to colistin have been described, for instance, tolerance to colistin in *P. aeruginosa* biofilms has been associated to metabolically active bacteria and controlled by *pmr* and *mexAB-oprM* [27].

A recent study showed that the opportunistic pathogen *P. aeruginosa* is able to become resistant in the presence of subinhibitory levels of polymyxins and certain antimicrobial peptides. It is believed that the PhoP-PhoQ and PmrA-PmrB two-component systems, which control this modification under low-Mg²⁺ conditions, are irrelevant in peptide mediated adaptive resistance. In 2010, a novel *P. aeruginosa* two-component regulator affecting polymyxin adaptive resistance, ParR-ParS (PA1799-PA1798) was identified and characterized by Fernández *et al.* [32]. They also demonstrated that this system is required for activation of the *arnBCADTEF* LPS modification operon in the presence of subinhibitory concentrations of colistin, leading to increased resistance to various polycationic antibiotics, including aminoglycosides.

1.5. EPIGENETIC INHERITANCE AND EVOLUTION OF ANTIBIOTIC RESISTANCE IN BACTERIA

In terms of epigenetic inheritance and evolution it is important to have in mind the concept of fitness or adaptedness of a given microorganism [33] and the concept of environment, which has been almost ignored for a long time in clinical microbiology.

Antibiotic resistance has to be understood as a multifactorial process where biochemical and genetic mechanisms as well as environmental processes involved in the selection and diffusion of genes of resistance and organisms have to be taken into account.

Baquero *et al.* considered that the term of antibiotic pressure should be understood within the larger concept of selective environment [34].

DNA mutations and plasmid uptake are not the only factors related to antibiotic resistance. It is necessary to bear in mind that evolution of antibiotic resistance by bacteria is, at least in part, the result of natural selection acting on variant phenotypes.

The phenotype is determined not only by the strict base sequence of DNA, but also by the variable expression patterns of the genes present.

In bacterial populations, phenotypic diversity is due to heritable epigenetic variations. That is, natural selection arises from differences in gene expression patterns.

A clear example of this selection is the exposure of an isogenic population to different antibiotics, where it can be observed how some individuals survive and form colonies while genetically identical neighbors die. In that case, it is necessary to look for other factors apart from stable DNA mutations to explain the high rate of survivors.

Several variables that have influence on the probability of appearance of individuals resistant to antimicrobial agents have been identified, including the number of target genes and the number of positions within those genes that can be mutated to confer resistance.

A few studies show that bacterial survival rates ramp higher by successive exposures to increasing concentrations of antibiotics, indicating the lack of stable mutations that confer resistance.

Only bacteria that have the capacity to epigenetically inherit the gene expression pattern of antibiotic resistance are able to divide and form colonies. In contrast, bacteria without this capacity would eventually die.

Unlike genetic mutations, epigenetic alterations are much more unstable. For that reason, epigenetic inheritance, such as maintenance of certain chromatin configuration and/or DNA methylation states, would give much higher reversion rates to antimicrobial sensitivity than mutations.

Stability of antibiotic resistance by bacteria depends on the antibiotic concentration and the time under selection. In other words, instability of antibiotic resistance state can disappear as the time under selection and antibiotics concentrations are increased.

Selective pressure and phenotypic diversity created by heritable epigenetic variation plays a key role in the evolution of antimicrobial resistance.

Adam *et al.* proposed that evolution of antibiotic resistance in microorganisms depend on two processes. The first is the variation in gene expression patterns, and the second is related to the epigenetic memory mechanisms that are able to maintain the gene expression state for numerous generations after a gene expression is established. Both DNA methylation patterns and DNA modifications inherited by bacteria are factors that can mediate transcriptional memory.

Natural selection is presumably provided by several mechanisms that are capable of performing heritable phenotypic variation. In the mentioned study it was also emphasized that epigenetic inheritance based evolution could be of medical relevance. It is though that when bacteria within a patient are exposed to a low dose of antibiotics, a subpopulation of bacteria survives. These survivors with low level resistance could then divide and form new bacteria for a sufficient period of time to accumulate more stable mutations in DNA resulting in an increase of the antibiotic resistance level [35].

1.6. ADAPTATIVE RESISTANCE

In contrast to intrinsic and acquired resistances, which are characterized by an irreversible phenotype and are independent of the presence of antibiotic or the environmental conditions, less is known about adaptative resistance. Fernández, *et al.* [36] defined this type of resistance as the induction of resistance to one or more antimicrobial agents in response to the presence of a specific signal. Mawer and Greenwood [37] showed that increase of resistance generally reverts upon removal of the triggered factor, although original level of resistance is not usually restored. This adaptation response is not only triggered by antibiotics, other signals such as pH, anaerobiosis or biofilm formation may play significant roles in determining antibiotic resistance and, they could be involved in a clinical failure of some antibiotic administration.

Although molecular mechanisms involved in adaptative resistance are not well understood, it is necessary a better comprehension about their role in the slow but constant increase in baseline MIC values to design more effective antibiotic strategies.

It is known that bacteria respond to changes in environment by modulating their gene expression and these changes can induce antibiotic resistance. Anaerobiosis is one of the important factors that trigger adaptative resistance. For instance, adaptation to anaerobic conditions allows *P. aeruginosa* to maintain a chronic infection and become more persistent and resistant to a broad range of antipseudomonal agents. Furthermore, some authors have demonstrated that aminoglycoside activity is reduced under anaerobic conditions. Thus, variation of oxygen concentration in the environment can have a great impact on adaptative responses.

Another important factor in microbial adaptative resistance is the cation concentration in the surrounding milieu. The presence of divalent cations, which have a role in stabilizing the outer membrane, can limit the self-promoted uptake of aminoglycosides, cationic peptides and polymyxins.

Other environmental factors that can modify antibiotic resistance in pathogenic bacteria are carbon source, pH and osmolarity.

Under certain environmental conditions, microorganisms exhibit coordinated multicellular behaviors that require cell-to-cell communication and are often controlled in part by quorum sensing signals. Microbes when forming biofilms give the best relevant example of social behavior. In this case, bacteria show phenotypic and transcriptional differences compared to their planktonic counterparts, and they mimic more the behavior of a multicellular organism [38]. One of the major complications associated with resistance and biofilms in the clinical environment is their high resistance to antibiotics and detergents, as well as to the immune system. Some studies seem to indicate that the conditions of biofilm production facilitate the acquisition of inheritable resistance traits. Many mechanisms are implicated in biofilms resistance such as restricted drug penetration, antimicrobial destroying enzymes limited or anaerobic growth at the base of biofilms, specific quorum sensing regulated resistance mechanisms for individual classes of antibiotics, the presence of persister bacteria and general stress responses.

In general, biofilms constitute a major threat in the clinical environment by acting as reservoirs of multidrug resistant pathogenic bacteria. There are evidences that the increase resistance of microorganisms in biofilms is due, at least in part to the characteristics and adaptations to the biofilm state. Swarming is also considered a social phenomenon that may contribute to failure of antimicrobial therapy due to adaptive resistance.

Brazas and Hancock [39] showed that bacterial gene expression can be regulated by the presence of certain concentrations of antimicrobial compounds. It is well known that antimicrobial susceptibility decreases after the first exposure to the antibiotic as a consequence of one or more adaptive changes.

Phenomenon of adaptive antimicrobial resistance affects almost all antibiotic families by different mechanisms. In the case of aminoglycosides, the mechanisms involved in this type of resistance are still not well understood. Recently, it has been demonstrated that genes

encoding the efflux pump MexXY are induced by aminoglycosides, but mutations in these genes do not lead to adaptative resistance.

Several studies have demonstrated that during the administration of fluoroquinolones in clinical use, its concentration often decrease causing the appearance of adaptative mutations. These subinhibitory concentrations reached during therapy are reflected in an increase of resistance and a possible therapeutic failure.

Bacteria have also developed mechanisms of adaptative resistance to β -lactams, mainly triggered by the presence of β -lactams themselves. For instance, in many Gram-negative bacteria, the chromosomally encoded β -lactamase is inducible by particular β -lactam antibiotics, and the expression of genes involved is lower in the absence of these antibiotics. When there is certain β -lactams in the environment, transcription of *ampC* and β -lactamase production is higher and there is an increase of drug degradation [40]. Moreover, β -lactam adaptative resistance is also affected by the exposure of a microorganism to subinhibitory concentrations of the drug, which allow the microorganism to adapt and become resistant against killing, by a follow-up exposure.

Bacteria can become more resistant due to environmental conditions and different physiological states (biofilms and swarming). It is necessary to pay attention to the small increases in resistance observed in clinic, because this probably indicates the emergence of adaptative resistance during the infection and possible troubles in the treatment.

1.7. BACTERIAL ENVELOPE

Bacterial envelope is a complex multilayered structure that serves to protect these organisms from their unpredictable and often hostile environment. There are principally two groups: Gram-negative envelope is formed by a thin peptidoglycan wall of bacteria, which itself is surrounded by an outer membrane (OM) containing lipopolysaccharide; Gram-positive envelope lacks an outer membrane and is surrounded by thick layers of peptidoglycan linked via teichoic acids. The characteristic shape of bacteria is due to the very large polymer called peptidoglycan. The OM is

essentially stapled to the underlying peptidoglycan by lipoproteins (Fig.11).

The periplasm is an aqueous compartment delimited by outer and inner membranes. Cellular compartmentalization allows Gram-negative bacteria to sequester potentially harmful degradative enzymes such as RNase or alkaline phosphatase.

The inner membrane (IM) in bacteria is a phospholipid bilayer with several proteins involved in energy production, lipid biosynthesis, protein secretion and transport of nutrients or waste products [41].

The outer membrane plays a key role in the physiology of Gram-negative bacteria in making them resistant to host defense factors. It also acts as an effective permeability barrier to many antibiotics. Although the presence of OM decelerates the diffusion of antibiotic, the small amount of antibiotic that penetrate can be inactivated by bacteria, and thus very high levels of resistance are easily established in Gram-negative bacteria [42, 43].

Due to the relevancy of this structure, it will be described in great detail in the following section.

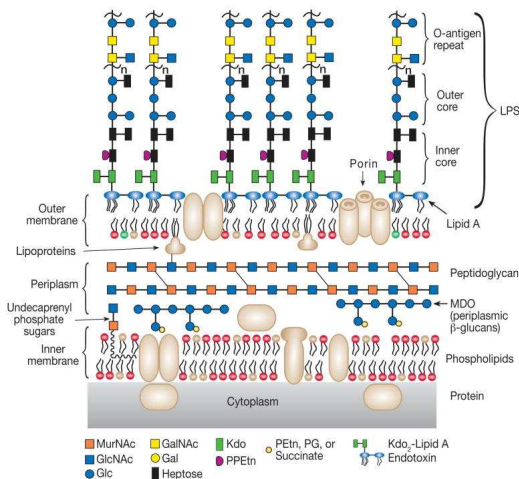


Fig. 11. Schematic representation of the envelopes of Gram-negative bacteria.

1.7.1. OUTER MEMBRANE OF GRAM-NEGATIVE BACTERIA

Gram-negative bacteria are surrounded by a thin peptidoglycan wall, which itself is surrounded by an outer membrane containing lipopolysaccharide. Biological membranes are constituted by lipid components and specific proteins that make each membrane unique. Bacteria survive thanks to the complex bacterial envelope that at the same time protects them and allows selective entry of nutrients and expulsion of waste products. Membranes do not simply serve to segregate different types of molecules, but also function as surfaces on which reactions can occur.

The OM is a distinguishing feature of Gram-negative bacteria, since Gram-positive bacteria lack it.

The OM contains phospholipids, but it is not a phospholipid bilayer.

The outer leaflet of the OM is composed of glycolipids, principally lipopolysaccharide (LPS) [44].

OM also contains proteins. There are basically two classes of OM proteins: lipoproteins and β -barrel proteins.

Almost all OM transmembrane proteins (OMPs) assume a β -barrel conformation. OMPs allow the passive diffusion of small molecules such as mono- and disaccharides and amino acids across the OM. LPS are molecules avidly bound especially if cations like Mg^{2+} are present to neutralize the negative charge of phosphate groups. They play a key role in the OM barrier function for hydrophobic molecules. This coupled with the fact that porins limit diffusion of hydrophilic molecules larger than 700 Daltons, making OM a very effective yet, selective permeability barrier.

1.7.2. PORINS

In 1976, Taji Nakae working in the laboratory of Hiroshi Nikaido discovered the outer membrane nonspecific channel proteins and proposed the word "porin" to describe them. There are two classes of proteins depending on their permeability properties for hydrophobic solutes: porins that have a certain solute specificity (not acting as specific

diffusion pores) and diffusion porins that form large-water filled channels through which solutes pass depending on their molecular mass or physico-chemical properties (acting as general diffusion pores) [45].

It has been described that expression profiles of porins are usually regulated by environmental conditions such as pH, temperature or nutrients [46].

Porin structures generally contain 14 (less common), 16 or 18 β -sheets depending on the protein family, and are typically closed by loops or cork-domain-like structures (α -helix). Most of the bacterial porins are oval-shaped and are laterally 3-3.5 nm in size and 5 nm in height for the monomer. The motif of structural architecture is the closure of the barrel by pairing of the first and the last β -strand in an anti-parallel way. The strands are connected by eight or nine long loops, facing the extracellular side, with seven or eight small turn-like structures in the periplasmic space. There are also aromatic girdles with tyrosine and phenylalanine residues in the outer and inner membrane boundaries, which could interact with lipid head groups or LPS molecules [47].

Structures of different porins of outer membrane are shown in figure 12.

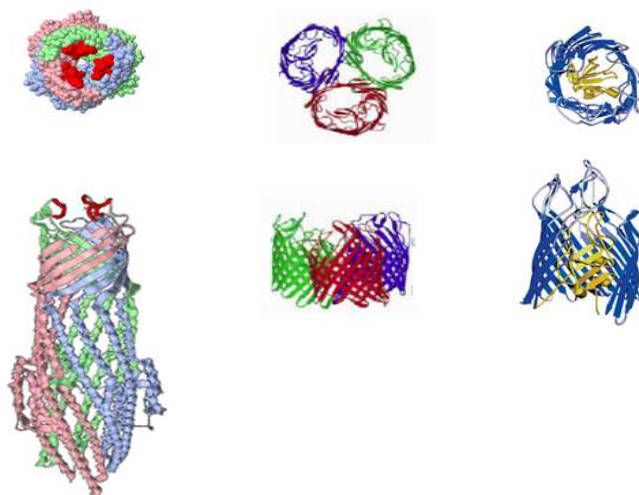


Fig.12. Structures of different porins of outer membrane. a) TolC protein of *K. oxytoca*. [88]
b) OmpA porin of *S. marcescens* [82] c) *FepA* porin of *E. coli* [48]

1.7.3. EFFLUX SYSTEMS

There are five families of bacterial efflux pumps which are ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family, and the resistance-nodulation-division (RND) superfamily, the most important efflux system in clinically-resistant Gram-negative bacteria. The latest, function as proton/drugs antiporters, and catalyze the active efflux of a wide variety of antibacterial substrates such as antibiotics and chemotherapeutic agents.

The main characteristic of RND transporters is that have large periplasmic domains and form tripartite complexes with the periplasmic adaptor proteins or membrane fusion proteins (AcrA and MexA) and OM channels (TolC and OprM).

Figure 13 shows the schematic drawing of tripartite RND multidrug efflux system of a Gram-negative bacterium.

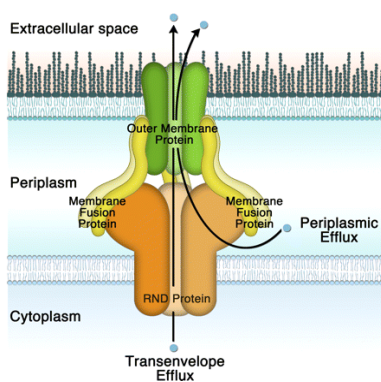


Fig. 13. Schematic drawing of tripartite RND multidrug efflux system of a the Gram-negative bacterium showing the OMP, the RND Protein and the MFP [49].

Membrane fusion proteins (MFPs) are essential domains in the assembly and function of the tripartite complex and may play an important role in the direct activation of the pump. They work as adaptor proteins in systems containing RND (AcrAB-TolC), MFS (EmrAB-TolC) and ABC (MacAB-TolC) pumps.

Outer membrane channel proteins are trimeric proteins represented by TolC and OprM. These proteins interact with other efflux components i.e. AcrA-TolC, AcrA-AcrB-TolC, AcrB-TolC, MexA-OprM and chimeric.

The importance of efflux pumps in Gram-negative bacteria is due to its relationship with antimicrobial resistance. Overexpression of efflux pumps and deficiencies of OMPs have a key role in bacterial drug-resistance and may be necessary for adaptative resistance to some antimicrobial agents such as aminoglycosides. Efflux pumps can also contribute to resistance in bacteria of animal and environmental origin, and efflux-mediated resistance has been usually found in animal pathogens and has been evident in environmental isolates.

Another role of efflux pumps has been described in biofilm resistance, where bacteria growing in biofilms seem to be more resistant or tolerant to antimicrobial agents than planktonic counterparts.

Drug efflux pumps can also be affected by different stimulus, including stress responses, colonization, virulence and quorum sensing, among others. Moreover, they can be phenotypically induced by small molecules, or substrates of efflux pumps and the expression of their genes can vary depending on the phases of growth [50].

1.7.4. LIPOPOLYSACCHARIDES (LPS)

The outer membrane of Gram-negative bacteria consists of an asymmetric double layer of polyanionic lipopolysaccharide molecules (outer leaflet) and glycerophospholipids (inner leaflet). Structurally, LPS is composed of three domains: lipid A, core oligosaccharide and the distal O antigen (Fig. 14).

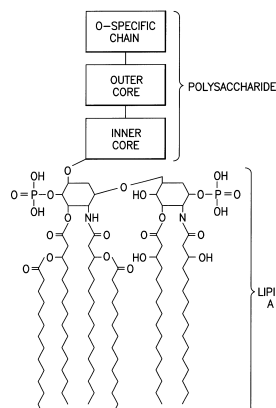


Fig. 14. General structure of Gram-negative LPS [51].

The outer core OS has a unique feature; it exists in two structurally distinct glycoforms, called “uncapped or smooth” and “capped or rough”.

The smooth glycoform is covalently attached to O-Ag, whereas the uncapped glycoform cannot be substituted with O-Ag.

To appreciate the complexity and heterogeneity of LPS, it is noteworthy that heterogeneity exists in the chain length of the O-Ag.

In general, core OS, which is the linker region between O-Ag and lipid A can be divided into two regions, namely the inner and the outer core.

LPS molecules are electrostatically linked by divalent cations (e.g., Mg^{2+} and Ca^{2+}). This structure can be weakened by removing divalent ions or replacing them with other cationic agents. This results in an increase of outer membrane permeability and sensitizes the bacteria to hydrophobic antibiotics, detergents, or dyes.

Beyond their structural role, LPSs mediate the interaction of bacteria with their environment and act as a protection against harmful molecules.

Moreover, LPSs acts as a major virulence factor. Some studies have pointed out that modification of lipid A of lipopolysaccharide (LPS) is a key component of adaptive antimicrobial resistance, such as peptide resistance [52, 53, 54].

1.8. INTEGRONS

Integrons were firstly described in the eighties as DNA elements that function as gene-capture and expression systems that incorporate open reading frames (ORFs) and convert them into functional genes [55, 56].

These genetic elements are able to acquire a variety of individual and unconnected genes via a site-specific recombination.

Integrons provide an integration site (*attI*) for the site specific integration of gene-cassettes, an integrase to mediate excision and orientation-specific integration of gene-cassettes, and a promoter *P_c* to ensure the expression of the operon [57, 58].

1.8.1. GENE CASSETTES: STRUCTURE AND FUNCTION

Gene cassettes are the smallest known form of mobilizable DNA. In contrast to other mobile DNA elements, gene cassettes are inserted at specific locations in the host genome defined by the presence of an integron [59, 60]. Although they may contain several genes, the main characteristic of these elements is that they all share a common structure.

A gene cassette is comprised of a single gene and a recombination site known as 59 base element (59-be or *attC* site), which is located downstream of the gene coding region. The 59-be sites vary in length and structure, but they are all bounded by a core site (GTTRRRY) at the recombinant crossover point and an inverse core site (RYYAAC) at the 3' end of the inserted gene (Fig. 15) [61, 62].

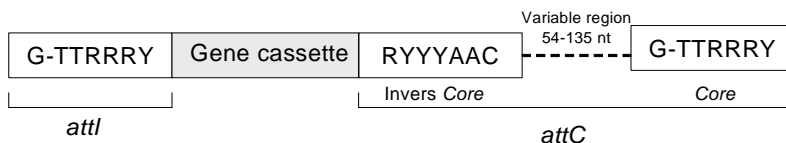


Fig. 15. Structure of a gene cassette. *attC* consist of *Invers Core* and *Core*, and are separated by a variable region. Extremes of the cassette are defined by two cores, *attI* (on the left) and *attC* (on the right). Recombination takes place between G of the first core and T of the second core. R= purine and Y= pyrimidine.

Cassettes vary considerably in total length from 262 to 1549 bp. This is largely because of differences in the size of the gene, as in most cassettes little non-coding sequence flanks the gene. Integrons of different isolates vary with regard to the type, number and order of genes cassettes they contain.

Site-specific recombination of a gene cassette is catalyzed by an integrase. In class 1 integrons the integrase gene *intI1* is located in the 5' conserved-segment (CS) of the integron, and it interacts with two recombination sites (be-59 of gene cassette and the *attI* site located in the 5'CS of the integron. Integrated cassettes are always in the same orientation of 5' end of the gene closest to the 5'CS of the integron. The insertion of additional cassettes may result in multi-cassette arrays containing two or more cassettes. Although gene cassettes are generally found inserted into an integron, they also transiently exist as free covalent circular molecules, very important in cassettes dissemination [63].

Class 1 integrons are the most common and well-characterized class of integrons, and are widely disseminated in animal and human clinical strains of the family *Enterobacteriaceae* [64]. Class 1 integrons are also common in clinical isolates of *P. aeruginosa*, but not in environmental strains [65].

Class 1 integrons contain the *attI1* recombination site, integrase gene and the promoter *Pc*. They are bounded by terminal inverted repeats, designated *IRi* and *IRt*. *IRi* is located at the 5' end of class 1 integrons and *IRt* is located at the 3' end [57].

Class 1 integrons contain a specific recombination site *attI1*, located near the *intI1* gene that is recognized by the integrase IntI1 and a promoter *Pc* that lies within the *intI1* gene that directs the transcription of inserted genes cassettes. Most class 1 integrons possess two CSs that flank either end of the gene cassette region. The 5'CS contains the *intI1* gene and a promoter *Pc* directed towards the integration site (Fig. 16).

The 3'CS usually contains the *qacEΔ1* and *sul1* genes and an ORF of unknown function (*orf5*).

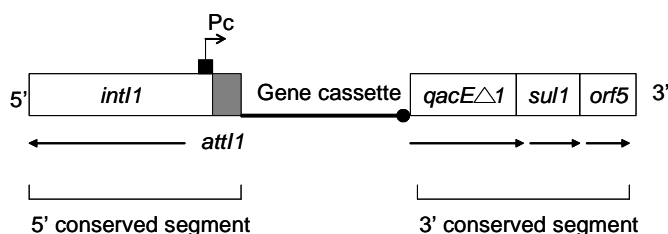


Fig 16. Basic structure of a class 1 integrons showing the 5' and 3' conserved segments. The *IntI1* gene (open box) and the *attI1* recombination site (grey box) are located in the 5'CS. The *qacEΔ1* gene encoding resistance to the quaternary ammonium compounds, the *sul1* gene encoding sulphonamide resistance and the ORF *orf5* are found in the 3'CS. The gene cassette is represented by a bold line and the associated 59-bp by a filled circle. The direction of gene transcription and the promoted region *Pc*, directed toward the integration site, are indicated.

1.8.2. ROLE OF INTEGRONS IN BACTERIAL DRUG RESISTANCE

Increasing attention has been given to integrons in the context of resistance to antimicrobials. In particular, much research effort has been devoted to integrons belonging to classes 1, 2 and 3, the classes most commonly associated with the spread of antibiotic resistance in pathogens. Surveys examining the prevalence of various genetic elements in multidrug-resistant strains invariably show a high correlation between the presence of a class 1 integron and particular antimicrobial resistance profiles. Integrons that contain gene cassettes provide a powerful vehicle for the rapid horizontal transfer of resistance across

bacterial populations and thus could contribute to the sudden increase in the prevalence of multidrug-resistant infections in a community. The distribution of identical genes in organisms isolated from people living in disparate geographic regions indicates that they were more likely infected by organisms already harboring such gene cassettes than originate in non-human reservoirs.

The variety of structures found amongst class 1 integrons after more than half a century of antibiotic usage bears testament to the genetic flexibility and adaptability of the bacterial genome under environmental stress, characteristics that make these microorganisms ultimate survivors [65].

Several studies in European hospitals have demonstrated that integron-positive isolates were statistically more likely to be resistant to aminoglycoside, quinolone and beta-lactam compounds, including third-generation cephalosporins and monobactams, than integron-negative isolates. Integron-positive isolates were also more likely to be multi-resistant than integron-negative isolates [66].

2. AIMS AND RESEARCH OBJECTIVES

Resistance to antimicrobials is a well-known phenomenon leading to difficulties in the treatment of infectious diseases. The genetic determinants of such resistance are in general well understood: plasmids, transposons, insertion sequences and integrons are the most frequently related genetic elements.

The word epigenetics refers to changes in the phenotype or in the gene expression caused by mechanisms other than underlying DNA sequence. In some cases these changes can remain for generations.

S. marcescens is an enterobacterium characterized by their ability to produce prodigiosin (a linear tripyrrolic pigment) which was, in former times extensively studied in our laboratory [67, 68, 69, 70]. In the last few years a renewed interest on prodigiosin due to its antitumor activities has emerged [71].

This bacterium can also produce other pigments derived from the catabolism of aromatic compounds [72, 73]. A prominent characteristic of *Serratia* is its natural (intrinsic) resistance to most antibiotics. At the same time *S. marcescens* is a relevant opportunistic pathogen which has been involved in several pathologies such as urinary tract infections prostheses infections, cellulitis, bacteremia and others. Virulence factors were investigated several years ago in part by our group [67, 74, 75, 76, 77].

On the other hand, *P. aeruginosa* is a Gram-negative bacterium considered one of the major nosocomial pathogens worldwide. It causes several infections such as wound and burn infections as well as respiratory tract infections mostly affecting cystic fibrosis patients. Our group has studied resistance in this bacterium since many years ago [14, 65].

Moreover, an increasing prevalence of infections caused by multidrug-resistant (MDR) isolates has been reported in many countries and is actually a cause of concern. Both, *P. aeruginosa* and *S. marcescens* are relevant nosocomial pathogens.

Some of the classic antimicrobials used to treat these pathogens are out-of-date and several of the new drugs available have already become targets for bacterial mechanisms of resistance.

The mechanisms leading to antimicrobial resistance have been investigated in our laboratory from many years ago [22, 65, 78, 79, 80, 81]. The role of the outer membrane as well as proteins involved have been also the focus of our research [82, 83, 84, 23, 85, 86, 87, 88, 89].

AIMS

Environmental conditions exert high pressure not only in the selection of genes encoding resistance to antibiotics or integron fixation in bacterial genomes or plasmids and other mobile elements transmission, but also in the expression of these potentialities that leads to resistance. Thus the role of epigenetics remains to be investigated. In addition it is well known that bacteria causing infections are naturally forming part of biofilms instead the planktonic way of life normally assumed to be in laboratory conditions.

The aim of this project was to explore unconventional mechanisms of antimicrobial resistance contributing to MDR phenotypes in both Gram-negative bacteria. The research itself has conditioned a series of new partial objectives which can be summarized as follows:

- Exploration of antimicrobial susceptibilities of three extensive groups of *S. marcescens* strains, a Gram-negative bacterium that belongs to *Enterobacteriaceae* family and which has a cosmopolitan distribution. The first group is formed by strains isolated and lyophilized in 1940/50 by Robert P. Williams's in Houston (*Robert P. Williams Serratia Collection*; UB); the second group is a collection of current *Serratia* isolated from environment, and finally, the third group is formed by clinical isolates of *Serratia* after 2000.
- Exploration of integron presence, and in the case, determination of their sequences and identification of genes encoding antimicrobial resistance. Also exploration of the eventual presence of ESBLs (Expanded Spectrum Beta-lactamases) in *S. marcescens*.

- Development of an experimental method to measure functionality of efflux pumps when bacteria grow in biofilm conditions and comparison with planktonic cultures.
- Evaluation of the actual role of efflux pumps in antimicrobial resistance of bacteria forming part of the biofilm to explore the importance of epigenetics on these proteins involved in antimicrobial resistance.
- Exploration of antimicrobial susceptibilities in multidrug-resistant *P. aeruginosa*, including colistin-resistant strains.
- Development of a bioassay to compare meropenem accumulation *P. aeruginosa* growth without antibiotic and *P. aeruginosa* treated with meropenem.
- Detection of eventual presence of integrons in multidrug-resistant *P. aeruginosa* and determination of sequences and identification of the genes encoding antimicrobial resistance.
- Study of the effects of drug-selective pressure in MDRPA and colistin-resistant *P. aeruginosa*.
- Evaluation of efflux pumps in MDRPA when synergic combinations of antimicrobial agents are used.
- Exploration of the contribution of outer membrane proteins in *P. aeruginosa* multidrug-resistance.
- Comparison of outer membrane proteins and lipopolysaccharide profiles of colistin-resistant and colistin-susceptible clinical isolates of *P. aeruginosa*.
- Investigation of the role of oprD porin in imipenem heteroresistance among clinical isolates of *P. aeruginosa*.

3. MATERIAL AND METHODS

3.1. BACTERIAL STRAINS

3.1.1. *S. marcescens*

A total of 79 strains of *Serratia* were examined. There were distributed into three different groups depending on their origin. The first group (G1) included 14 strains of *Serratia* isolated between 2007 and 2008 from environment, mainly from water samples, but also from vegetables and soil in the suburbs of Pallars Jussà, and the region surrounding Barcelona. The second group of *Serratia* (G2) consisted in 41 strains isolated and freeze-dried many years ago (1940/50) by Professor Robert P. Williams at Baylor College of Medicine (BCM), Houston Tx. These isolates were preserved and after Dr. Williams death donated by BCM to our laboratory. (*Robert P. Williams Serratia* Collection; BCM-UB); finally, the third group (G3) consisted in clinical strains isolated from patients at Laboratory Clinic L'Hospitalet (Barcelona).

Identification and preliminary antibiotic susceptibility testing was accomplished by using the Microscan system (Sacramento, CA, USA).

S. marcescens 2170 was used in experiments of efflux and biofilms [90]. Groups of *Serratia* strains used in this study are described in tables 6-8.

Table 6. List of environmental strains.

G1: Environmental <i>Serratia</i> strains (2007 and 2008). Own culture collection.	
Strain:	Source:
ES001	River water from Catalonia
ES002	Catalonia Irrigation channel
ES005	River water from Catalonia
ES007	Soil from Guatemala
ES008	River water from Catalonia
ES009	Soil from Guatemala
ES013	Pipe from Catalonia
ES014	Catalonia Irrigation channel
ES015	Pond from Catalonia
ES016	Stream from Catalonia
ES018	Spring from Catalonia
ES019	Fountain from Catalonia
ES020	Onion
ES021	Water from Metlini (Algeria)

Material and methods

Table 7. List of old strains.

G2: Robert P.Williams <i>Serratia</i> Collection; BCM-UB.	
Strain:	Source:
8PW	Unknown. University of Wisconsin
10PW	Unknown .University of Wisconsin
33PW	Unknown
34PW	Unknown
36aPW	Unknown. Texas (USA)
41PW	Soil from USA
42b PW	Environmental. Soil. 1956. Dept. Agriculture
44a PW	Environmental. Eggs. 1956. Dept. Agriculture
46b PW	Environmental. Eggs. 1956. Dept. Agriculture
49a PW	Environmental.Eggs. Dept. Agriculture
51b PW	Environmental.Eggs. Dept. Agriculture
54b PW	Environmental.Isolated from eggs. Dept. Agriculture
56b PW	Environmental. Fly. 1956
57a PW	Clinical. Throat. 1956
58a PW	Clinical. Sputum. 1958
59a PW	Clinical. Blood. 1958
60 PW	Clinical. Bronchial washing. 1956
62 PW	Clinical. Bronchial washing. 1956
63 PW	Clinical. Urine. 1956
65 PW	Clinical. Paracentesis fluid. 1956
66 PW	Clinical. Blood. 1956
73PW	Ulcer from a University Hospital
74aPW	Milk from Hygienic Inst. Kiln. 1927. Lister Institute, London
75PW	Hospital bread isolated in 1926 American Type Culture Collection, Chicago
76aPW	Human case of spinal meningitis. American Type Culture Collection.
77bPW	Water from a paper mill
80bPW	Cheese from Ithaca (USA)
82PW	Unknown. Received from American Type Culture Collection, Chicago in 1925
83PW	Unknown. Received from Oswaldo Cruz Institute (Brazil) in 1928
86PW	Unknown. Received from Kluver of the Technische Hoogeschool Laboratory Voor Microbiologii et Delft. Holland in 1929
87PW	Unknown. Received from Kluver of the Technische Hoogeschool Laboratory Voor Microbiologii et Delft. Holland in 1929
88PW	Unknown. Received from Kluver of the Technische Hoogeschool Laboratory Voor Microbiologii et Delft. Holland in 1929
90aPW	Unknown
119 PW	Environmental. Wine. 1956 from Dr. Breed's collection
121 PW	Environmental.Sugar cane. 1956
132 PW	Environmental. Tap water. 1956
137 PW	Environmental. Fruit fly. 1956
147 PW	Clinical. Hospital clothes. 1956
159a PW	Environmental. Sea water. 1956
230 PW	Clinical. Sputum. 1957
248aPW	Unknown

Table 8. List of current clinical strains.

G3: Clinical strains isolated after 2000. Laboratory Clinic L'Hospitalet (Barcelona).	
Strain:	Source:
1JO	Unknown
2JO	Unknown
3JO	Unknown
4JO	Exudate
5JO	Ulcer
6JO	Blood culture
7JO	Sputum
9JO	Sputum
10JO	Sputum
11JO	Exudate
12JO	Sputum
13JO	Exudate
14JO	Sputum
15JO	Ulcer
16JO	Ulcer
17JO	Ulcer
18JO	Urine
19JO	Urethral smear
20JO	Exudate
21JO	Exudate
22JO	Ulcer
23JO	Sputum
24JO	Blood culture
26JO	Ulcer

3.1.2. ISOLATION AND IDENTIFICATION OF ENVIRONMENTAL STRAINS

Environmental samples (mainly water and soil) were taken and stored at 4 °C until their processing in the laboratory. Samples were pre-enriched in nutrient broth with 4% NaCl, and incubated overnight at room temperature (about 20 °C) with shaking. Then, caprylate-thallos (CT) agar medium was used for selective isolation of *Serratia* species. This CT agar, derived from M70 minimal medium (Véron, 1975) [91]. A volume of 0.1 mL of the

pre-enrichment culture was streaked onto CT agar and then incubated at 30 °C for seven days.

Isolated colonies were streaked onto TSA to achieve the identification.

Identification of the isolates was done by using standard microbiological methods such as colonies shape and pigmentation, typical morphology of Gram-negative rod-shaped in Gram staining, negative reaction to oxidase, ability for gelatine liquation, production of DNase, citrate degradation (Simmons Citrate), inability to hydrolyze urea (Christensen's Urea Agar), inability to form indole from tryptophan and positivity to Voges-Proskauer test.

3.1.3. MULTIDRUG-RESISTANT *P. aeruginosa*

Four isolates of multidrug-resistant *P. aeruginosa* (MDRPA) from Hospital del Mar, Barcelona (Spain), were used. Identification and preliminary antibiotic susceptibility testing was accomplished by using the Microscan system (Sacramento, CA, USA). *P. aeruginosa* ATCC 27853 was used as a control strain.

These strains were used to explore the mechanisms of resistance leading to multidrug-resistance in *P. aeruginosa*.

Clinical isolates are described in the table below.

Table 9. Multidrug-resistant *P. aeruginosa* (MDRPA).

Strain:	Abbreviation:	Source:
2404459	459	Surgical wound
1449133	133	Sputum
2908162	162	Urine
1169527	527	Blood culture

Several colistin-resistant clinical isolates of *P. aeruginosa* from Hospital del Mar patients (Barcelona, Spain) were explored for mechanisms of resistance to colistin. As described previously, identification and preliminary antibiotic susceptibility testing was done by using the Microscan system.

Strains 5150328S (328S) and 5150328R (328R) were isolated from sputum of the same patient. Strain 5150328S was colistin-susceptible whereas 5150328R was colistin-resistant.

Table 10. Colistin resistant *P. aeruginosa*

Strain:	Abbreviation:	Source:
4811296	296	Urine
442362	362	Bronchial aspirate
1820017	017	Urine
2473493	493	Urine
1138264	264	Urine
2882025	025	Bronchial aspirate
5181401	401	Pharynx
5116732	732	Urine
4769780	780	Sputum
5150328R	328R	Sputum

Two more *P. aeruginosa* clinical isolates were explored. These strains were isolated from the same patient at the “Servei de Microbiologia” of the Hospital Universitari de Bellvitge (Barcelona, Spain). The strain PA110514 was isolated before the treatment with imipenem whereas strain PA116136 was isolated once the treatment was finished. Both isolates gave identical pulsed field gel electrophoresis (PFGE) profile and biochemical tests, suggesting their close relationship.

3.2. CULTURE MEDIA

3.2.1. TRYPTONE SOY AGAR (TSA)

Tryptone Soy Agar is a solid nutritive medium useful for cultivation of a wide variety of organisms.

Composition in g/L:	
Peptone from casein.....	15.0
Peptone from soymeal.....	5.0
Sodium chloride.....	5.0
Agar	15.0

40g/liter; adjust pH to 7.3 ± 0.2 at 25 °C. boiled and autoclaved.

3.2.2. TRYPTONE SOY BROTH (TSB)

Tryptic Soy Broth was also used in experiments in molecular genetics.

Composition in g/L:	
Peptone from casein.....	17.0
Peptone from soymeal	3.0
Sodium chloride.....	5.0
Dipotassium phosphate	2.5
Glucose.....	2.5

Suspended 30 g/liter (broth), autoclaved (15 min at 121 °C), adjusted pH to 7.3 ± 0.2 at 25°C.

3.2.3. MUELLER-HINTON BROTH (MHB)

Muller-Hinton Broth is a nutritive medium used for microdilution antibiotic susceptibility testing.

Composition in g/L:

Peptone from casein.....	17.5
Starch.....	1.5
Meat extract	4.0

21 g of powder to 1 L of distilled water sterilized by autoclaving at 121°C for 15 minutes.

For experiments with *P. aeruginosa*, the medium used was BBL™ Mueller-Hinton II broth cation adjusted (20-25 mg/L of calcium and 10-12.5 mg/L of magnesium).

3.2.4. MUELLER-HINTON AGAR (MHA)

Mueller-Hinton agar is a microbiological growth medium that is recommended for the disk diffusion method of antibiotic susceptibility testing. It is recommended by FDA, World Health Organization and NCCLS for testing most commonly encountered aerobic and facultative anaerobic bacteria in food and clinical material.

Composition in g/L:

Beef extract powder.....	2.0
Acid digest of casein	17.5
Starch.....	1.5
Agar	17.0

3.2.5. LURIA BROTH (LB)

Luria broth is standard and rich nutrient media used to grow bacteria.

Composition in g/L:

Pancreatic digest of casein.....	10.0
NaCl.....	5.0
Yeast extract.....	5.0
Glucose.....	1.0

3.2.6. SUPPLEMENTED MINIMAL BROTH DAVIS WITHOUT DEXTROSE (DMB_{SUP})

Minimal Broth Davis without dextrose and supplemented with 0.05% glucose and 0.05% casamino acids is the medium that was used in biofilm's experiments.

Composition in g/L:

Dipotassium Phosphate.....	7.0
Monopotassium Phosphate.....	2.0
Sodium Citrate.....	0.5
Magnesium Sulphate.....	0.1
Ammonium Sulphate.....	1.0
Glucose.....	0.5
Casamino Acids.....	0.5

3.2.7. PRE-ENRICHMENT MEDIUM FOR *Serratia* ISOLATION

This medium was used to pre-enrich environmental samples (mainly water) for the isolation of the *Serratia* strains.

Composition in g/L:

NaCl.....	4.0
Peptone from meat.....	5.0
Meat extract.....	3.0

3.2.8. CAPRYLATE-THALLOUS AGAR SELECTIVE FOR *Serratia* (CT)

This medium was used for the selective isolation of *Serratia* species. This CT agar, derived from M70 minimal medium (Véron, 1975) [91].

CT agar has the following composition and mode of preparation:

Two solutions designated A and B were prepared, autoclaved separately and mixed aseptically to yield a liter of the final medium.

Material and methods

Solution A contained:

Calcium Chloride Dihydrate	0.0147 g
Magnesium Sulphate Heptahydrate.....	0.123 g
Potassium Dihydrogen Phosphate.....	0.680 g
Dipotassium Phosphate Anhydrous.....	2.610 g
Trace element solution	10 mL
Caprylic (n-octanoic) acid	1.1 mL
Yeast extract 5% wt/vol solution	2 mL
Thalious sulphate	0.25 g
Distilled water up to	500 mL

The pH was adjusted to 7.2 with NaOH using a pH-meter and the solution was autoclaved at 110 °C for 20 min.

Solution B contained:

Sodium Chloride.....	7 g
Ammonium Sulphate	1 g
Agar	15 g

Trace element solution (Véron, 1975) contained the following components in g/L:

Phosphoric Acid	1.96
Ferrous Sulfate Heptahydrate.....	0.0556
Zinc Sulphate Heptahydrate	0.0287
Manganese Sulphate Tetrahydrate.....	0.0223
Copper Sulfate Pentahydrate.....	0.0025
Cobalt Nitrate Hexahydrate	0.003
Boric Acid	0.0062

This solution was stored unsterilized at 4° C.

The pH was adjusted to pH 7.2, and this agar base (solution B) was autoclaved at the same time and under the same conditions as solution A. After autoclaving, solution A and B were mixed aseptically and the resulting CT agar medium was poured into sterile plastic Petri dishes. The dishes were stored at 4 °C until used.

3.2.9. BRAIN HEART INFUSION BROTH WITH 10% GLYCEROL (BHI)

This medium was used for the growing of *Serratia* and *Pseudomonas* strains previous to the DNA extraction for the search of integrons. Glycerol was added afterwards.

Composition in g/L:	
Brain and heart infusion	17.5
Peptone	10.0
Glucose	2.0
Sodium Chloride	5.0
Disodium phosphate	2.5

3.2.10. CRYOPRESERVATION MEDIUM

This medium was used for the cryopreservation of bacterial strains at -80 °C. It was prepared by dissolving 3g of TSB in 80 mL of distilled water plus 20 mL of glycerol. The solution was autoclaved at 121 °C for 15 minutes.

3.3. SOLUTIONS AND OTHER COMPOUNDS USED

3.3.1. PHENOL SOLUTION

This solution was used for extraction of nucleic acids. Liquid phenol was equilibrated with an equal volume of buffer (usually Tris-HCl pH 8 at room temperature). Then, the mixture was stirred on a magnetic stirrer for 15 min and when the two phases were separated, the upper (aqueous) phase was aspirated. Afterwards, an equal volume of 0.1 M of Tris-HCl pH 8.0 was added to the phenol and the mixture was stirred as described previously. Extractions were repeated until the pH of the phenolic phase was >7.8.

Phenol solution was stored in a light-tight bottle for periods of up to 1 month [92].

3.3.2. CHLOROFORM-ISOAMYL ALCOHOL

This mixture was used in the extraction of chromosomal DNA. It consisted of 24:1 v/v that means the addition of 1 mL of isoamyl alcohol each 24 mL of chloroform.

3.3.3. SALINE-SODIUM CITRATE BUFFER 20X (SSC)

This solution was used in the extraction of chromosomal DNA, and contained:

Sodium Chloride	175.3 g
Sodium citrate.....	88.2 g
Distilled water.....	up to 1000 mL

All the ingredients were dissolved and pH was adjusted to 7.0 with a few drops of a 14N solution of hydrochloric acid. Then, solution was sterilized

by autoclave. Final concentrations were 3.0 M of sodium chloride and 0.3 M of sodium citrate.

3.3.4. PHOSPHATE-BUFFERED SALINE (PBS) PH 7.5

This buffer was used to maintain the correct osmolarity and keep the bacteria in an isotonic state.

The stock was prepared 10X concentrated, and the working solution was 1X.

Composition of the 10X concentrated solution in g/l was:

Sodium Chloride	80.0
Potassium Phosphate Monobasic	2.0
Di-Sodium Phosphate	11.44
Potassium Chloride	11.0
Distilled water.....	Up to a final volume of 1L

3.3.5. GLYCINE SOLUTION PH 3

This solution was used in fluorometry experiments to allow bacterial lysis.

The composition was:

Glycine.....	0.1 M
Hydrochloric acid	24 mM

It was prepared by mixing 50 mL of a 0.2 M glycine solution with 12 mL of a 0.2 M HCl solution and distilled water up to a final volume of 100 mL.

3.3.6. TRIS-ACETATE-EDTA 50X (TAE)

This solution was used in the preparation of agarose gels to determine the presence of integrons and as buffer in electrophoresis.

The stock was prepared 50X concentrated, and the working solution was 1X.

The composition was:

Tris.....	242.28 g
Acetic acid glacial	57.1 mL
0.5M EDTA (pH 8.0).....	100 mL
Distilled water up to.....	1 L

All the components were mixed until complete dissolution.

3.3.7. TRIS-BORATE-EDTA 50X (TBE)

This solution was used in the preparation of agarose gels to analyze amplicons obtained from the *oprD* gene region from several *P. aeruginosa* strains and as buffer in electrophoresis.

The stock was prepared 50X concentrated, and the working solution was 1X.

The composition was:

Tris.....	108.0 g
Boric acid.....	55 g
0.5M EDTA (pH 8.0).....	40 mL
Distilled water up to.....	1 L

All the components were mixed until complete dissolution.

3.3.8. TRIS-GLYCINE SDS GEL RUNNING BUFFER (5X) AND 30% ACRYLAMIDE STOCK SOLUTION

Tris-glycine SDS gel running buffer was used in SDS-PAGE analysis of LPS or OMPs.

Tris	15 g/L
Glycine.....	72 g/L
SDS	5 g/L

This buffer was equilibrated to a pH 8.3 and stored at 4 °C until its use. 30% acrilamide stock solution was used in SDS-PAGE gel electrophoresis.

Acrylamide.....	30 g
N,N'-methylbisacrylamide.....	0.8 g
Distilled H ₂ O.....	100 mL

This stock was stored at 4 °C in dark until its use.

3.3.9. SILVER STAINING SOLUTION

This solution was used in the silver staining protocol. It was prepared by mixing A and B solutions under constant shaking. Silver staining solution was discarded if argent precipitates were observed.

A SOLUTION:

AgNO ₃	0.5 g
Distilled H ₂ O.....	2.5 mL

B SOLUTION:

NaOH 1 N	1.4 mL
NH ₃ 30%	1 mL
Distilled H ₂ O.....	70 mL

3.3.10. SILVER STAIN DEVELOPER

The silver stain developer was used in the silver staining protocol.

Citric acid 10%	50 µL
Formaldehyde	50 µL
Distilled H ₂ O.....	up to 100 mL

3.3.11. STOPPING SOLUTION

This solution was used in the silver staining protocol.

Glacial acetic acid 10%	10 mL
Methylamine	3 drops
Distilled H ₂ O.....	up to 100 mL

3.3.12. PRESERVING SOLUTION

This solution was used in the silver staining protocol.

Ethanol	30% v/v
Glycerol	5% v/v
Distilled H ₂ O.....	65% v/v

3.3.13. PROTEINASE K

Proteinase K was used in bacterial DNA extraction.

The stock was prepared at 20 mg/mL:

Proteinase K	20 mg
Ultrapure H ₂ O	1 mL

All the compounds were added in the indicated order and shaken until complete dissolution. 50µL aliquots were stored at -20 °C.

3.3.14. RNase (10 mg/mL)

RNase was used in bacterial DNA extraction.

RNase.....	0.010 g
Tris-HCl.....	10 µL
5M Sodium Chloride.....	3 µL
Ultrapure H ₂ O	987 µL

All the ingredients were dissolved and sterilized at 100 °C for 15 min. The solution was cooled on ice and 20 µL aliquots were stored at -20 °C.

3.3.15. PIV SOLUTION

This solution was used in experiments of bacterial strain typing by PFGE.

Tris 1M pH 8	10 mL
NaCl 5M.....	200 mL
Distilled water	790 mL

Solution was sterilized by autoclave.

3.3.16. ST SOLUTION (LYSIS SOLUTION)

This solution was used in experiments of bacterial strain typing by PFGE.

Tris pH 8	0.6 mL
NaCl 5M.....	20 mL
EDTA 0.5 M pH 8.....	20 mL
10% Sodium deoxycholate.....	2 mL
10% Sarkosyl	5 mL
Distilled water	52.4 mL

Solution was sterilized by autoclave.

3.3.17. ES SOLUTION (FOR PROTEINASE K INCUBATION)

This solution was used in experiments of bacterial strain typing by PFGE.

EDTA 0.5 M pH 8.....	90 mL
10% Sarkosyl	10 mL

Solution was sterilized by autoclave.

3.3.18. TE SOLUTION

This solution was used in experiments of bacterial strain typing by PFGE.

Tris 1M pH 7.5	10 mL
EDTA 0.5 M pH 8	2 mL
Distilled water	988 mL

Solution was sterilized by autoclave.

3.3.19. RESTRICTION SOLUTION

This solution was used in experiments of bacterial strain typing by PFGE.

SpeI restriction enzyme.....	2.5 U
Bovin seric albumin (BSA).....	0.4 µL
Spe I buffer 1X	

3.3.20. 6X GEL-LOADING BUFFER

Gel-loading buffer was used to suspend samples for analysis of agarose gel analysis of DNA.

Bromophenol blue.....	0.25%
Xylene cyanol.....	0.25%
Ficoll or glycerol.....	30.0%
Distilled water	Up to 10 mL

3.3.21. MARKERS

Lambda DNA/HindIII is a marker suitable for sizing and quantifying linear double-stranded DNA molecules of between 125 bp and 23.1 kb. The marker is prepared by completely digesting lambda DNA (cI857, Sam7) with HindIII.

Gene Ruler 1 kb DNA Ladder is a marker suitable for sizing and quantifying linear double-stranded DNA molecules of between 250 bp and 10000 bp.

SDS-PAGE Molecular Weight Standard, Low Range is a marker suitable for sizing protein molecules of between 14400 and 97400 Da.

3.3.22. DYES

- Ethidium bromide: It was used to stain DNA in agarose gels, and prepared by adding 1 g of ethidium bromide in 100 mL of distilled water. It was kept at room temperature and dark conditions.
- Silver nitrate: It is an inorganic compound used for silver staining of proteins and LPS in SDS PAGE gels.
- LIVE / DEAD BacLight Bacterial Viability Kit for microscopy and quantification assays L7011: This kit that provides a two-colour fluorescence assay of bacterial viability was used to stain biofilms and analyzed them by Confocal Laser Scan Microscopy (CLSM). The working solution was prepared dissolving 1.5 μ L of SYTO 9 dye 3.34 mM solution in dimethyl sulfoxide (DMSO) plus 1.5 μ L of propidium iodide, 20 mM solution in DMSO in 997 mL of distilled water.

3.3.23. BACTERIAL EFFLUX PUMPS INHIBITORS

- Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP): It is an energy uncoupler that collapses the membrane energy and is able to collapse the energy required for drug transport [93,94]. It reduces the viability of the bacterium and cause death of bacteria via the dissipation of the proton-motive force of the membrane. CCCP is highly noxious and cytotoxic and is a substrate of bacterial efflux pumps [95]. It was used in the efflux experiments of *P. aeruginosa* or *S. marcescens* strains. The stock was prepared at a concentration of 10 mg/mL in methanol, and stored at -20 °C until its use.
- MC207,110 or phenylalanine arginyl β -naphthylamide (PA β N): It is a peptidomimetic that have the ability to inhibit the efflux of one or more antibiotics and restore antibiotic susceptibility of resistant bacteria. This molecule can be considered to exhibit a broad spectrum activity of efflux pump inhibition. However, not all antibiotic substrates for a given pump are potentiated by PA β N. Mechanism of action of this compound has indicated that PA β N is itself a substrate of efflux

- pumps. It is assumed that different antibiotics have dissimilar binding pockets within the transporter protein and that PA β N compete with antibiotics for binding in the substrate pocket to the potentiated antibiotic, but not to the binding site for the non-potentiated antibiotic (substrate-dependent inhibition) [96]. It was used in the efflux experiments of *P. aeruginosa*. Stock of PA β N was prepared at a concentration of 50 mg/mL in methanol and stored at -20 °C until its use.

3.3.24. OTHERS

- Ethylenedinitrilotetraacetic acid (EDTA): It is a chelator of divalent cations. It was used in experiments of colistin-resistant *P. aeruginosa*. Since lipopolysaccharides (LPS) molecules present in the outer membrane of Gram-negative bacteria are electrostatically linked by divalent cations such as Mg²⁺ and Ca²⁺, EDTA interferes with the Ca²⁺ and increases the permeability of the outer membrane, therefore sensitizing Gram-negative bacteria to certain antimicrobial agents such as colistin.
- LPS extraction kit: (Gentaur). It is a kit designed for the extraction of LPS from small volume of bacteria. It was used to analyze LPS of several colistin-resistant *P. aeruginosa* and control strains following manufacturer's instructions.
- Pierce[®] BCA Protein Assay Kit: (Thermo Scientific). It is a detergent-compatible formulation based on bicinchoninic agent (BCA) for the colorimetric detection and quantification of total protein. This method combines the reduction of Cu²⁺ to Cu¹⁺ by protein in an alkaline medium (biuret reaction) with the sensitive and colorimetric detection of the cuprous cation (Cu¹⁺) using a reagent that contains bicinchoninic agent. This kit was used to equal total protein of bacteria used in the bioassay for the measurement of meropenem accumulation in *P. aeruginosa*.

3.4. DETERMINATION OF BACTERIAL SUSCEPTIBILITIES TO ANTIMICROBIAL AGENTS

The three methods described in sections 3.4.2, 3.4.3 and 3.4.4 were used indistinctly for drug susceptibility testing.

3.4.1. PREPARATION OF ANTIMICROBIAL STOCKS

Antimicrobial agents used are shown in table 11.

Table 11. Stock solutions of antimicrobial agents.

Antibiotic	Concentration	Solvent [97]
β-lactam		
Penicillin	400 µg/mL	Sterile distilled water
Ampicillin	1 mg/mL	Phosphate buffer pH 8; 0.1 M
Amoxicillin	1000 µg/mL	Buffer phosphate pH 6; 0.1 M
Cefoxitin	100 mg/mL	Sterile distilled water
Ceftazidime	2000 µg/mL	Ca ₂ CO ₃ (10% of the ceftazidime used)
Cefotaxime	2000 µg/mL	Sterile distilled water
Ceftriaxone	100 mg/mL	Sterile distilled water
Aminoglycosides		
Kanamycin	100 mg/mL	Sterile distilled water
Gentamicin	100 mg/mL	Sterile distilled water
Streptomycin	100 mg/mL	Sterile distilled water
Tobramycin	10 mg/mL	Sterile distilled water
Rifampicin	6.25 mg/mL	Methanol
Tetracycline	500 µg/mL	Sterile distilled water
Carbapenems		
Meropenem	5,000 mg/L	Methanol, 0.1mmol/L NaOH
Imipenem	5,000 mg/L	Phosphate buffer pH 7.2; 0.1 M
Quinolones		
Ofloxacin	20 mg/mL	½ volume of water, then 0.1 mol/L NaOH
Ciprofloxacin	100 mg/mL	Sterile distilled water
Polymyxins		
Colistin	1000 mg/L	Cold Sterile distilled water

All the antibiotic stocks were stored at -20 °C until their use.

3.4.2. MINIMAL INHIBITORY CONCENTRATION (MIC) BY BROTH MICRODILUTION METHOD

Broth Microdilution test is a quantitative method to determine the minimal inhibitory concentration of each antibiotic. This procedure involves a *microtiter* tray containing standard twofold dilutions of antimicrobials agents to be inoculated with standardized microbial suspensions. Each *microtiter* tray can be prepared with several antimicrobial agents or with only a single agent.

The method used is the one described below [98]:

- Bacterial cultures were incubated at 30 °C (*Serratia*) or 37 °C (*Pseudomonas*) for approximately 18 hours.
- The cultures were then diluted 1:100 in Mueller-Hinton broth ($OD_{625nm}=0.08-0.1$)
- 5 μ L of the diluted culture (5×10^5 CFU/mL) were inoculated using a multi-channel pipette in each of the *microtiter* wells containing serial dilutions of the antimicrobial agent.
- *Microtiter* plates were incubated at 30 °C (*S. marcescens*) or 37 °C (*P. aeruginosa*) for 18 hours.
- MICs were determined as the lowest concentration of drug that inhibits more than 99% of the bacterial population.

Based on breakpoints defined by European Committee on Antimicrobial Susceptibility Testing (EUCAST) [99] or Clinical Laboratory and Standards Institute (CLSI) [98] bacterial strains were classified as sensible (S), intermediate (I) or resistant (R) to the tested antimicrobial agent.

3.4.3. DETERMINATION OF BACTERIAL SUSCEPTIBILITIES BY DISK DIFFUSION METHOD

Antimicrobial disk susceptibility test is a well standardized method for determining susceptibility of bacteria to antimicrobials [100].

Bacterial cultures were incubated at 30 °C (*Serratia*) or 37 °C (*Pseudomonas*) for approximately 18 hours.

- Inoculum was prepared by diluting the cultures 1:100 in Mueller-Hinton broth ($OD_{625nm}=0.08-0.1$, equivalent to $1-2 \times 10^8$ CFU/mL).
- Bacterial inoculum was applied to the surface of a large (150 mm diameter) Mueller-Hinton agar plate.
- Up to 12 commercially-prepared, fixed concentrations, paper antibiotic disks were placed on the inoculated agar surface.
- Plates were incubated for 16-24 h at 30 °C (*S. marcescens*) and 37 °C (*P. aeruginosa*) prior to determination of results.
- The zones of growth inhibition around each of the antibiotic disks were measured to the nearest millimeter. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium.
- The zone diameters of each drug were interpreted using the criteria published by the Clinical and Laboratory Standards Institute CLSI or EUCAST
- It is important to note that the results of the disk diffusion test are “qualitative,” in that a category of susceptibility (i.e., susceptible, intermediate, or resistant) is derived from the test rather than a MIC.

3.4.4. DETERMINATION OF BACTERIAL SUSCEPTIBILITIES BY DOUBLE DILUTION AGAR METHOD

The method of double dilution agar was used for drug susceptibility testing and is the one specified by CLSI [101]. It is described below:

- Bacterial cultures were incubated at 37 °C for approximately 18 hours.
- The cultures were then diluted 1:100 in Mueller-Hinton broth ($OD_{625nm}=0.08-0.1$).
- The Mueller-Hinton agar plates with appropriate antibiotic concentrations were inoculated using a Steers replicator, depositing 0.001 mL of a suspension of each adjusted culture (5×10^5 CFU/mL).
- Plates were incubated at 37 °C for 18-24 h.
- MICs were determined as the lowest concentration of drug that inhibits more than 99% of the bacterial population.

3.4.5. STATISTICAL ANALYSIS OF ANTIMICROBIAL SUSCEPTIBILITIES FOR THE THREE GROUPS OF *Serratia* STRAINS

Comparison of antimicrobial MICs between the three groups of *S. marcescens* strains were statistically analyzed using Kruskal-Wallis (K-W) test with Bonferroni correction for multiplicity, following the Holm sequential procedure.

Antimicrobials that showed significant differences in K-W test were analyzed by U Mann-Whitney test to check differences between couples of strain groups.

3.4.6. MICS OF COLISTIN WITH AND WITHOUT EDTA

MICs of colistin alone and in combination with EDTA were determined by twofold standard broth microdilution method (section 3.4.2). Colistin was tested at 11 concentrations (128-0.25 µg/mL), and EDTA at the concentration of 1 mM, based on previous studies [102]

3.5. ANALYSIS OF PRESENCE/ABSENCE OF INTEGRONS

The technique described by Lévesque *et al.* [58] was used to detect the presence or absence of class 1 integrons in *Serratia* or *Pseudomonas* strains.

The protocol followed is described below:

3.5.1. DNA EXTRACTION

- *Serratia* and *Pseudomonas* strains were grown O/N in 4 mL of brain heart infusion (BHI)-10% glycerol. *Serratia* was incubated at 30 °C and *Pseudomonas* at 37 °C.
- 200 µl of the culture was added to 800 µl of distilled water and boiled for 10 min.
- Bacterial suspension was then centrifuged at 12,000 x g for 2 min, and the supernatant was used for PCR.

3.5.2. POLYMERASE CHAIN REACTION (PCR)

PCR was carried out in 100 μ L volumes containing 30 μ L of template DNA and 70 μ L of mix.

Components for 100 μ L of mix:

Oligonucleotides	2.5 pmol
Taq DNA polymerase	1U
Deoxynucleoside triphosphate	200 μ M each
MgCl ₂	3 mM
Tris-HCl pH 8	10 mM
KCl Sulphate	50 mM
Gelatin	0.001%
Distilled water	QS

Primers:

Primers used were described by Lévesque *et al.* [58] and are designed from conserved segments in integrons allowing only the amplification of the integrated gene cassettes.

These are the sequences of the primers used:

Primer	Nucleotide Sequence (5' to 3')
5'CS	GGC ATC CAA GCA GCA AG
3'CS	AAG CAG ACT TGA CCT GA

Primers were purchased from Invitrogen (USA).

DNA amplification:

Previously to the amplification, DNA was pre-denaturalized using the hot start method:

- 12 min at 94 °C

To amplify the DNA in the thermal cycler, a three-step profile was used:

- 1 min of denaturation at 94 °C
- 1 min of annealing at 55 °C
- 5 min of extension at 72 °C

Total of cycles: 35. Five seconds were added to the extension time in each cycle.

All completed reactions were maintained at 4 °C.

3.5.3. VISUALIZATION OF PCR PRODUCTS

The PCR products were visualized in 0.7% (w/v) agarose gels in 1X TAE at 100 volts for 1.30 hours.

Composition of agarose gel 0.7% (w/v):

Agarose0.7 g
TAE..... 100 mL

3.5.4. GEL STAINING

Agarose gel staining is described in section 3.15.2.

3.6. DETECTION OF EXTENDED-SPECTRUM β -LACTAMASES (ESBL_S) BY BORONIC ACID (BA) DISK TEST AND THE CLSI CONFIRMATORY TEST

The method used for the detection of ESBLs in *S. marcescens* strains was the one described by Seok Hoon Jeong *et al.* [103]:

- Disks containing BA were prepared using commercially available antibiotic-containing disks (Oxoid Ltd., Basingstoke, Hampshire, England) as described by Coudron PE [104].
- 20 μ L of the phenylboronic acid (BA) solution (20g/L; Sigma-Aldrich Chemie GmbH, Germany) was dispensed onto each disk containing cefotaxime (CTX) (30 μ g) and ceftazidime (CAZ) (30 μ g) with and without clavulanic (CLA) (10 μ g).
- Disks were allowed to dry for 60 min and used immediately or stored in airtight vials with desiccant at 4 °C.
- Similar to the CLSI ESBL confirmatory test, a \geq 5mm increase in the zone diameter of CTX/CLA and/or CAZ/CLA disks tested in combination with BA (CTX/CLA/BA and/or CAZ/CLA/BA) versus CTX and/or CAZ disks containing BA (CTX/BA and/or CAZ/BA) was considered positive for ESBLs. CTX, CTX/CLA, CAZ and CAZ/CLA disks with BA were spaced over the Mueller–Hinton agar surface. Inoculated plates were incubated overnight at 30 °C in ambient air.

- The CLSI confirmatory test for ESBL production was also carried out on Mueller–Hinton agar using CTX and CAZ alone and with CLA.

3.7. BIOFILMS

3.7.1. FORMATION OF BIOFILMS

The strain used for the studies of biofilms was *S. marcescens* 2170.

Biofilms were obtained in the following conditions:

- Nutritional conditions: Davis minimal broth without dextrose, supplemented with 0.05% glucose and 0.05% of casamino acids.
- Material for biofilm formation: a methacrylate piece of 1 mm thickness and size of 1 x 1 cm².
- Procedure: submersion of methacrylate pieces in Petri dishes with 25 mL of DMB_{sup} and inoculation of the medium with 100 µL of an ON bacterial culture.
- Environmental conditions: incubation at 30 °C for 24 or 48 hours without shaking.

3.7.2. QUANTIFICATION OF BIOFILMS

The determination of the number of bacteria forming biofilms was a previous condition to measure both intake and efflux of antibiotics. Quantification of biofilms was done by following the method described below:

- Methacrylate pieces were withdrawn with sterile tweezers and cleaned three times by immersion in 1X PBS pH 7.4 for 10 seconds to eliminate planktonic bacteria.
- PBS was discarded in order to remove bacteria loosely attached to the methacrylate.
- Methacrylate pieces where the biofilm was formed were put into Eppendorf tubes and sonicated on ice (3x3 min) to aid dispersal of attached bacteria.

- To check the sonication efficiency, images of the methacrylate pieces were taken by AFM (Fig. 17).

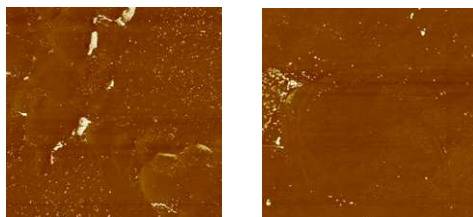


Fig. 17. Two AFM images of sonicated methacrylate pieces to check biofilm removal. Visualization in air by using the Atomic Force Microscope XE-70 (Park Systems) previously air dried at RT in a dust-free environment. Images were collected in Non-contact mode using pyramidal-shaped silicon cantilevers.

- To estimate the amount of bacteria in biofilms, optical densities at 550 nm were measured followed by serial dilution and TSA plating.
- After the counting of colonies, a relationship between absorbance (OD) and microbial concentration was established.

3.7.3. VISUALIZATION OF BIOFILMS

3.7.3.1. ATOMIC FORCE MICROSCOPY (AFM)

In order to check that bacteria were attached to the surface of the polymer, pieces of methacrylate were visualized in air by using the Atomic Force Microscope XE-70 (Park Systems). For visualization of biofilms under AFM, they were previously air dried at RT in a dust-free environment. All images were collected in Non-contact mode using pyramidal-shaped silicon cantilevers. The acquired data were converted into images and analysed by using XEP and XEI softwares (Park Systems).

Figure 18 shows AFM images of *Serratia* biofilms formed in methacrylate pieces and incubated at different times.

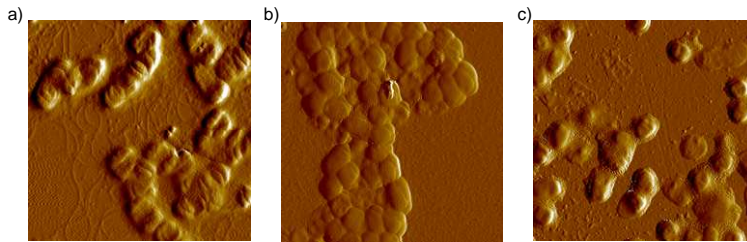


Fig. 18. AFM images of *Serratia* biofilms formed in methacrylate pieces and incubated at different times. Biofilms were visualized in air by using the Atomic Force Microscope XE-70 (Park Systems) previously air dried at RT in a dust-free environment. All images were collected in Non-contact mode using pyramidal-shaped silicon cantilevers. a) 24 h biofilm-formation. b) 48 h biofilm-formation. c) 72 h biofilm-formation

3.7.3.2. CONFOCAL MICROSCOPY

In order to check viability of bacteria in biofilms, they were prepared for confocal scanning laser microscopy (CLSM) by staining with the LIVE/DEAD Baclight viability probe, prepared according to manufacturer's specifications. Microscopic observation and image acquisition of biofilms was performed by using the Basic Scanning using AxioPlan 2 LSM 510 equipped with an argon ion laser at 483 nm of excitation and 543 nm band-pass filter for emission.

Figure 19. shows confocal microscopy images of *S. marcescens* biofilms.

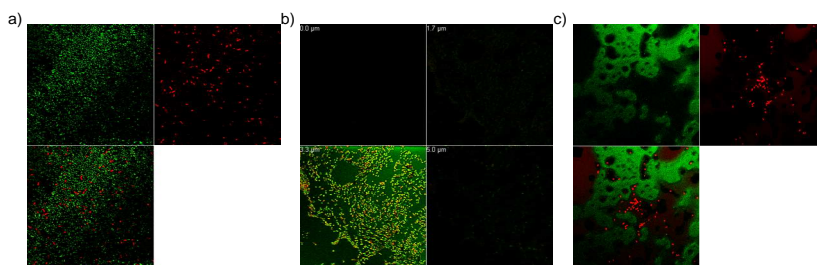


Fig. 19. Confocal images of *S. marcescens* biofilm at different times of incubation. Basic Scanning using AxioPlan 2 LSM 510 equipped with an argon ion laser at 483 nm of excitation and 543 nm band-pass filter for emission. X100 magnification. Stained 15 min in dark conditions and RT with 50 μ L LIVE/DEAD, and rinsed three times with PBSx1 pH 7.4. a) 24 h biofilm-formation. b) 48 h biofilm-formation. c) 96 h biofilm-formation.

3.8. EFFLUX EXPERIMENTS

3.8.1. GROWTH AND INHIBITION ASSAYS

3.8.1.1. BACTERIAL GROWTH CURVES

Bacterial growth curve was done for *P. aeruginosa* and *S. marcescens*.

The protocol is described below:

- Bacterial strains were inoculated in flasks containing 100 mL of TSB.
- The spectrophotometer was set to 550 nm and calibrated.
- The spectrophotometer was blanked inserting a test tube containing TSB into the sample holder.
- Incubation was at 37 °C for *P. aeruginosa* and 30 °C for *S. marcescens*. Cultures were shaken at 250 rpm until the OD was between 0.2-0.3.
- Every hour, a sample of 1 mL of each culture was removed and OD of the cultures were read and recorded.
- For direct growth measurements, serial dilutions and standard plate counts of each sample were done. A volume of 20 µL of each dilution (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} and 10^{-5}) were spread onto TSA and incubated at the appropriate temperature.
- The number of CFU/mL of the original culture for each of the time sets was determined by counting the plates containing between 30 and 300 CFU. Numbers were averaged and recorded only one for each time set.
- Counting of CFU growth in TSA allowed us to establish the relationship between OD and bacterial concentration.

3.8.1.2. INHIBITION ASSAYS WITH EFFLUX INHIBITORS

Growth inhibition assays for *S. marcescens* and *P. aeruginosa* were performed as described by Beyer *et al.* [105] with some modifications:

- Strains were inoculated at 1×10^7 to 2×10^7 CFU/mL (OD = 0.2 to 0.3) into flasks with 20 mL of TSB.

- Antibiotics were added at a concentration of one-fourth the determined MIC, either alone, with 10 µg/mL carbonyl cyanide 3-chlorophenylhydrazone (CCCP) or 40 µg/mL Phe-Arg-naphthylamide (PAβN). All strains were also grown with neither antibiotic nor CCCP/PAβN, with 10 µg/mL of CCCP alone or with 40 µg/mL of PAβN alone. Ciprofloxacin was the antibiotic tested for MDRPA strains, and colistin for colistin-resistant *P. aeruginosa* strains.
- Cultures were incubated at 37 °C (*P. aeruginosa*) or 30 °C (*S. marcescens*) and optical densities were determined at intervals of one hour at 550 nm (OD_{550 nm}).
- OD_{550 nm} was plotted on the Y axis and incubation times on the X axis.

3.8.1.3. QUINOLONE AND COLISTIN RESISTANCE

To determine the prevalence of efflux pump-mediated quinolone resistance in the MDRPA strains and efflux pump-mediated colistin resistance in colistin-resistant *P. aeruginosa*, MIC determinations were carried out in 96-well *microtiter* plates by twofold standard broth microdilution method in Mueller-Hinton broth either with or without a fixed concentration of the efflux pump inhibitor (EPI) Phe-Arg-naphthylamide (PAβN) using the method described by Kriengkauykiat *et al.* [106].

Ciprofloxacin concentration ranged between 128-0.25 µg/mL and colistin concentration ranged between 256-0.5 µg/mL. PAβN was used at a fixed concentration of 40 µg/mL based on previous studies [96].

Inoculums were adjusted to OD_{625 nm} = 0.08-0.1 equivalent to 1-2x10⁸ CFU/mL and diluted 1:10 (for inoculation volume of 5 µL) to get a final concentration of 5x10⁵ CFU/mL. *Microtiter* plates were incubated at 37 °C for periods of 18/24 hours.

3.8.1.4. MEXXY-OPRM EFFLUX PUMP

To determine the presence of a functioning MexXY-OprM efflux pump in the MDRPA strains, MIC of the aminoglycoside tobramycin was carried out in 96-well *microtiter* plates using a twofold standard broth microdilution

standard method in Mueller-Hinton broth in the absence and in the presence of 40 µg/mL of PAßN.

Tobramycin was tested at 11 concentrations (0.25-256 µg/mL).

3.8.1.5. ROLE OF EFFLUX PUMPS IN SYNERGIC COMBINATIONS OF ANTIMICROBIAL AGENTS

To determine the role of efflux pumps in the MDRPA strains when synergic combinations of antibiotics are used interaction between ceftazidime plus tobramycin and meropenem plus tobramycin alone and in combination with 40 µg/mL of the EPI PAßN were studied.

Meropenem and ceftazidime were tested at 11 concentrations (0.125 to 128 µg/mL) and these antibiotics were combined with different concentrations of tobramycin (0, 8, 16, 32, 64 and 128 µg/mL). *Microtiter* plates were incubated at 37 °C between 18 and 24 hours.

3.8.2. FLUOROMETRY EXPERIMENTS

3.8.2.1. CIPROFLOXACIN ACCUMULATION: SENSITIVITY OF THE METHOD

To explore if ciprofloxacin accumulation vary depending on the amount of bacteria, planktonic cultures of *S. marcescens* 2170 were used. The accumulation of quinolones was measured spectrofluorometrically by using the method described by Mortimer and Piddock [107] with some modifications [108].

The method used is described below:

- A volume of 100 mL of DMB_{SUP} was inoculated with 3 colonies of *S. marcescens* and incubated at 30 °C until OD₆₀₀ was 1.5.
- Bacteria were harvested by centrifugation at 8,000 rpm at room temperature for 15 min.
- The sediment was suspended in 100 mL 1X PBS pH 7.5 and centrifuged at 8,000 rpm at room temperature for 15 min.

- Sediment was suspended in 10 mL 1X PBS pH 7.5 and 2-fold serial dilutions were made. These dilutions were kept at 4 °C until his use.
- Number of bacteria (CFU) in each dilution was determined by serial dilutions from 1×10^{-1} to 1×10^{-6} , plating out 0.1 mL of the aliquots onto TSA.
- Bacterial dilutions were incubated at 30 °C for 10 min and 1 mL sample was removed (time 0).
- Ciprofloxacin was added to a final concentration of 10 µg/mL and 1 mL samples were removed at different time intervals. Incubation times with the antibiotic were 3 and 6 min.
- The samples were immediately diluted in 1 mL of ice-cold 1X PBS and centrifuged for 1 min at 4 °C and 11,000 rpm.
- Pellets were suspended in 1 mL of 0.1M glycine buffer at pH 3.0 and incubated at room temperature and dark conditions overnight to allow bacterial lysis.
- Thereafter, the suspensions were centrifuged at 20 °C for 15 min to remove bacterial debris.
- The fluorescence of supernatant was measured using the Fluoromax-4 spectrofluorometer at excitation and emission wavelengths of 279 and 447 nm, respectively.
- The concentration of ciprofloxacin in the supernatant was calculated by comparison with a standard curve for ciprofloxacin (0.02 to 10 µg/mL) in 0.1 M glycine hydrochloride (pH 3.0). Ciprofloxacin accumulation was expressed in ng of ciprofloxacin/mg bacteria.
- The experiment was repeated at least three times.

3.8.2.2. CIPROFLOXACIN ACCUMULATION/EFFLUX IN PLANKTONIC BACTERIA

The accumulation and efflux of ciprofloxacin was measured spectrofluorometrically by using the method described previously with the following modifications:

- A volume of 20 mL of DMB_{SUP} was inoculated with 3 colonies of *S. marcescens* and incubated at 30 °C ON.
- Then, the ON culture was diluted until the OD₅₅₀ was equaled to the OD₅₅₀ obtained for 24 h or 48 h biofilms formed in 1cm x 1cm methacrylate pieces.
- For both accumulation and efflux experiments, ciprofloxacin was added to a final concentration of 10 µg/mL and 1 mL samples were removed at different time intervals. Incubation times with the antibiotic or antibiotic plus CCCP were 0, 3 and 7.5 min.
- For efflux experiments, CCCP was added after 3 min of ciprofloxacin addition, at a final concentration of 10 µg/mL.
- The samples were immediately diluted in 1 mL of ice-cold 1X PBS and centrifuged for 1 min at 4 °C and 11,000 rpm.
- Pellets were suspended in 1 mL of 0.1M of glycine buffer at pH 3.0 and incubated at room temperature and dark conditions overnight to allow bacterial lysis.
- Thereafter, suspensions were centrifuged at 20 °C for 15 min to remove bacterial debris.
- The fluorescence of supernatant was measured using the Fluoromax-4 spectrofluorometer at excitation and emission wavelengths of 279 and 447 nm, respectively.
- The concentration of ciprofloxacin in the supernatant was calculated by comparison with a standard curve for ciprofloxacin in 0.1 M glycine hydrochloride (pH 3.0). Ciprofloxacin accumulation was expressed in ng of ciprofloxacin/mg bacteria.

- The experiment was performed at least three times to make sure of the reproducibility.

3.8.2.3. CIPROFLOXACIN ACCUMULATION/EFFLUX IN BIOFILMS

This experiment was designed to explore if active expulsion of antibiotics is or not dependent on the way that bacteria grow.

The method used is described below:

- 10 mL of TSB were inoculated with 3 colonies of the isolates and incubated overnight at 30 °C.
- 100 µL of the overnight cultures were used to inoculate Petri dishes containing 25 mL of DMB_{SUP} and sterile methacrylate pieces 1cm x 1cm x 0.1 cm in horizontal position. Cultures were incubated statically at 30 °C for 24 or 48h.
- Thereafter, methacrylate pieces where bacteria were attached forming the biofilm were gently removed by sterile tweezers and rinsed in a new Petri dish containing 25 mL of 1X PBS. This step was repeated three times.
- To determine the average of attached bacteria (CFU/cm²) in the methacrylate pieces at the beginning of experiment, 3 biofilms were sonicated for 3 min in tubes with 5 mL of 1X PBS. The OD₅₅₀ was measured and serial dilutions were made plating out 0.1 mL of the aliquots onto TSA.
- Washed biofilms were put in a new Petri dish containing 25 mL of sterile DMB_{sup} and 1 of them was removed (time 0).
- For both accumulation and efflux experiments, ciprofloxacin was added to a final concentration of 10 µg/mL and 1 mL samples were removed at different time intervals. Incubation times with the antibiotic or antibiotic plus CCCP were 0, 3 and 7.5 min.
- For efflux experiments CCCP was added after 3 min of ciprofloxacin addition, at a final concentration of 10 µg/mL.
- The samples were immediately put into tubes with 1 mL of ice-cold 1X PBS placed on ice.

- Tubes were gently inverted 3-4 times to wash biofilms, and 1X PBS was then removed.
- 1 mL of 0.1M of glycine buffer at pH 3.0 was added and incubated at room temperature and dark conditions overnight to allow bacterial lysis.
- Afterwards, tubes were sonicated for 3 min at room temperature and suspensions were replaced in 1 mL Eppendorf tubes and centrifuged at 20 °C for 15 min to remove bacterial debris.
- The fluorescence of supernatant was measured using the Fluoromax-4 spectrofluorometer at excitation and emission wavelengths of 279 and 447 nm, respectively.
- The concentration of ciprofloxacin in the supernatant was calculated by comparison with a standard curve for ciprofloxacin in 0.1 M glycine hydrochloride (pH 3.0). Ciprofloxacin accumulation was expressed in ng of ciprofloxacin/mg bacteria.

3.9. EFFECT OF DRUG-SELECTIVE PRESSURE IN ANTIMICROBIAL RESISTANCE

3.9.1. BIOASSAY FOR MEASUREMENT OF MEROPENEM ACCUMULATION IN MDRPA

- Long rod-shaped bacteria of *P. aeruginosa* formed in the presence of 4X meropenem MIC and short rods from cultures without antibiotic were washed with PBS pH 7.4 and resuspended in 30 mL of the same buffer.
- The total protein of both samples was equaled by BCA™ Protein Assay Kit and meropenem was added to a final concentration of 1 µg/mL while bacteria were centrifuged at 13,000 rpm for 1 min.
- The supernatant was used for the measurement of the meropenem unable to penetrate inside bacteria.
- Measures were done using a meropenem susceptible strain of *E. coli*. The standard curve ranged from 25 to 0 ng/mL of meropenem.

- After incubation of samples at 37 °C for approximately 2 hours, the optical density at 550 nm was measured. Optical densities of unknown samples were extrapolated to the standard curve to obtain the amount of meropenem inside and outside of long or short *P. aeruginosa*.

3.9.2. EFFECT OF DRUG-SELECTIVE PRESSURE IN COLISTIN RESISTANCE

To check if drug-selective pressure plays an important role in colistin resistance, several *P. aeruginosa* strains described in table 10 were chosen:

- *In vitro* experiments with strains 5181401, 4769780, 5150328R and 2473493 were done to explore the ability of bacteria to adapt to deficit of antibiotic and recover fitness on serial passage. Adaptation experiments were performed in 20 mL of MHB by subsequent serial passages in the absence of antibiotic for at least 20 times.
- The colistin-susceptible strain 5150328S and the colistin-resistant strains 5150328R and 2473493 were used to explore the ability of bacteria to adapt to higher concentrations of colistin. Adaptation experiments were performed in 20 mL of MHB and the concentration of colistin was doubled every passage. At the end of the experiment, colistin susceptibility of all the strains was indistinctly tested by using the method of broth microdilution method (section 3.4.2) or double dilution agar method (section 3.4.4).

3.9.3. EFFECT OF DRUG-SELECTIVE PRESSURE IN IMIPENEM RESISTANCE

To check if drug-selective pressure plays an important role in imipenem resistance, strain PA116136 was explored:

- Adaptation experiments were performed in 20 mL of MHB by subsequent serial passages in the absence of antibiotic.

- After 30 antibiotic-free passages antibiotic susceptibility was indistinctly tested by using the method of broth microdilution method (section 3.4.2) or double dilution agar method (section 3.4.4).

3.10. ANALYSIS OF OUTER MEMBRANE PROTEINS (OMPs)

3.10.1. OMPS ISOLATION

OMPs were obtained as described by Puig *et al.* [82]

- *P. aeruginosa* strains were grown in 10 mL of TSB at 37 °C O/W.
- The O/W cultures were added to 100 mL of TSB flasks and incubated at 37 °C with shaking (250 rpm) O/N.
- Cultures were centrifuged for 15 minutes at 7000 rpm and room temperature.
- Sediment was resuspended in 30 mL of ringer ¼ to wash bacterial cells.
- Cells were centrifuged at 7000 rpm for 15 min at room temperature.
- Pellet was resuspended in 30 mL HEPES 10 mM pH 7.4.
- Cells were disrupted at a pressure of 30 KPa (Constant cell disruption systems).
- Sedimentation of whole cells was done at 5,000 rpm for 15 min at 21 °C.
- Sedimentation of total membranes was done at 30,000 rpm for 1.30 h (ultracentrifuge) at 21 °C.
- Pellets were resuspended in 2 mL of SLS 1% in HEPES 10 mM pH 7.4
- Suspensions were incubated at RT for 20 min.
- Sedimentation of outer membranes was done at 30000 rpm for 60 min.
- Sediments were resuspended in 50 µL of sample buffer (4.0 mL of distilled water, 1.0 mL 0.5 M Tris-HCl pH 6.8, 0.8 mL of glycerol, 1.6 mL of 10% (w/v) SDS).
- Aliquots of 20 µL were kept at -20 °C.

- To remove lipids excess, 20 μL of each sample were resuspended in 200 μL of 10% SDS and centrifuged at 13000 rpm for 99 min.
- Pellets were resuspended in 50 μL of sample buffer with blue bromophenol and mercaptoethanol (4.0 mL of distilled water, 1.0 mL 0.5 M Tris-HCl pH 6.8, 0.8 mL of glycerol, 1.6 mL of 10% (w/v) SDS, 0.4 mL mercaptoethanol and bromophenol blue).
- For SDS-PAGE electrophoresis samples were boiled for 10 min and cooled 10 more min.

3.10.2. SDS-PAGE ANALYSIS OF OMPs

Sodium sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli's method [109] as modified by Ames *et al.* [110] The stacking and resolving gels were 4 and 12% acrylamide, respectively. Samples were loaded and run at RT in a Tris-glycine SDS gel running buffer (section 3.3.8). An Atto vertical electrophoresis cell was used. The gel was run at 70 V (10 mA) until stacking gel was finished and 110 V (15 mA) until the end.

RESOLVING GEL (12% acrylamide):

Distilled H ₂ O	1.67 mL
Tris-HCl 1.5 M, pH 8.8.....	1.25 mL
Acrylamide stock solution (section 3.3.8)	2 mL
SDS 10%.....	50 μL
Ammonium persulfate (APS) 10%	25 μL
TEMED	15 μL

STACKING GEL (4% acrylamide):

Distilled H ₂ O	3.05 mL
Tris-HCl 0.5 M, pH 6.8.....	1.25 mL
Acrylamide stock solution (section 3.3.8)	0.65 mL
SDS 10%.....	50 μL
Ammonium persulfate (APS) 10%	25 μL
TEMED	5 μL

Both solutions were degassed by vacuum for 15 minutes before APS and TEMED were added. Oxygen is a strong inhibitor of polymerization and it is better to remove it from the solutions.

3.10.3. OMPs STAINING PROTOCOL

- The gel was removed from the supports and stained with A solution (methanol, acetic acid and distilled water (5:1:5) + 1 g Coomassie blue) for 1 hour at 55 °C or O/N at RT.
- The gel was immersed in B solution (methanol, acetic acid and distilled water (5:1:5) until bands appeared.
- Gel was preserved in C solution (methanol, acetic acid and distilled water (1:1:18) until it was dried by vacuum using the Gel dryer model 543 (Bio-Rad).

3.10.4. OBSERVATION OF RESULTS

To compare OMPs profiles between strains, observation of results were done by visual analysis of the gels.

3.11. ANALYSIS OF LIPOPOLYSACCHARIDES (LPS)

LPS analysis was performed by using indistinctly the two methods for extraction described below:

3.11.1. LPS PREPARATION BY THE PROTEINASE K METHOD

The method of Hitchcock and Brown [111] was used to prepare LPS extracts with minor modifications: [112]

- All the isolates were grown overnight at 37 °C
- Bacteria were harvested by centrifugation at 13,000 x g for 2 min and resuspended in 50 µL of sample buffer which contained 66.6% H₂O, 16.6% Tris pH 6.8, 0.5 M, 13% glycerol and 6,6% 2-mercaptoethanol.
- After boiling for 10 min, 50 µL of proteinase K at 10 mg/mL were added.
- This was followed by incubation at 56 °C for a minimum of 3 h. This produced reasonably concentrations of LPS, except in a few cases in which the cell pellet was significantly small.
- Extracts were stored at -20 °C and thawed immediately before use.

3.11.2. MICROSCALE EXTRACTION OF LPS FROM BACTERIA

The LPS extraction kit (Gentaur, Belgium) was used to extract LPS from small volume of bacteria following the manufacturer's instructions.

- Bacterial cells were lysed by organic solution to disrupt and release in solution phospholipids and protein components of cell membrane.
- Purification of LPS among the released cell components was done with high salt concentration solution.
- Salts were briefly removed by a washing step for the obtaining of high quality LPS.

3.11.3. ANALYSIS OF LPS (SDS-PAGE)

SDS-PAGE was carried out according to Laemmli's method as modified by Ames *et al.* The stacking and resolving gels were 4 and 15% acrylamide, respectively. Samples (30 μ L) were loaded and run at RT in a Tris-glycine SDS gel running buffer (section 3.3.8). An Atto vertical electrophoresis cell was used. Constant current (10 mA) was applied for stacking gel and constant current (15 mA) for resolving gel.

RESOLVING GEL (15% acrylamide):

Distilled H ₂ O	2.4 mL
Tris-HCl 1.5 M, pH 8.8.....	2.4 mL
Acrylamide stock solution (section 3.3.8)	5.0 mL
SDS 10%.....	100 μ L
Ammonium persulfate (APS) 10%	50 μ L
TEMED	10 μ L

STACKING GEL (4% acrylamide):

Distilled H ₂ O	3.05 mL
Tris-HCl 0.5 M, pH 6.8.....	1.25 mL
Acrylamide stock solution (section 3.3.8)	0.65 mL
SDS 10%.....	50 μ L
Ammonium persulfate (APS) 10%	25 μ L
TEMED	5 μ L

Both solutions were degassed by vacuum for 15 minutes before APS and TEMED were added.

3.11.4. SILVER STAINING PROCEDURE

LPS were visualized by using previously described silver staining protocol [111] with minor modifications:

- After SDS-PAGE electrophoresis gel was removed from glass and placed for at least 2 hours (occasionally O/N) in a rinse solution of 5% v/v glacial acetic acid, 35% v/v absolute ethanol and 60% v/v distilled water shaking gently.
- Gel was treated with rinse solution plus sensitizer (0.7% of periodic acid) for 7 min.
- Sensitizing solution was replaced with distilled water and washed 3 times for 15 min with gently shaking.
- Water was replaced with silver staining solution (section 3.3.9). This solution was prepared immediately before its use, and gel was left for 10 min with shaking.
- Gel was washed with distilled water 3 times for 5 min.
- Silver stain developer solution (section 3.3.10) was added and left until sharp LPS bands appear.
- Development was stopped covering the gel with stopping solution for 10 min. (section 3.3.11)
- The silver stained gel was kept immersed into the preserving solution (section 3.3.12) until it was dried by vacuum using the Gel dryer model 543 (Bio-Rad).

3.11.5. OBSERVATION OF LPS PROFILES

To compare LPS profiles between strains, observation of results were done by visual analysis of the gels.

3.12. PULSED-FIELD GEL ELECTROPHORESIS

PFGE was performed by using the method described by Maslow *et al.* [113] and Schwartz *et al.* [114] with some modifications. Chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis were interpreted by criteria described elsewhere [115].

3.12.1. CHROMOSOMAL DNA EXTRACTION

- Microorganisms were grown in blood-agar at 37 °C for 18-24 hours. Colonies were resuspended in 200 µL of PIV and 5 µL were added to 1 mL of PIV solution to measure OD at 620 nm. Bacterial suspensions were adjusted in 1.5 mL Eppendorf tube with PIV solution by the following equation: PIV volume = (OD at 620 nm * 40 * 210) - 210.
- A volume of 100 µL of melted 1.0% Megabase agarose kept at 70 °C was added to new Eppendorf tube with 100 µL of the adjusted bacterial suspensions. Samples were mixed thoroughly by gently vortexing.
- 20 µL of the agarose-bacterial suspension mixture were dispensed on the top of a slide and covered by another slide.
- The agarose plugs were allowed to solidify at 4°C for 5 min.
- For the lysis of bacteria, the plugs were transferred to 10-mL tubes containing 1 mL of the cell lysis buffer (ST). Lysis was allowed to proceed for 5 hours at 37 °C.
- Lysis solution was discarded and samples were treated with proteinase K by adding 1 mL of ES solution prepared just before its use. Samples were then incubated at 50 °C in a water bath for approximately 18 hours (overnight).
- After lysis, the plugs were washed four times (30 min/wash) with TE, pH 8 with shaking. TE was discarded after each wash.

3.12.2. DNA DIGESTION

- Plugs were transferred to a tube containing 1X restriction buffer solution
- Then, the restriction enzyme mixture containing 2.5 U of Spel (Roche) was added to each tube.
- The plug slices were incubated at room temperature (23 to 25 °C) for 5 h.

3.12.3. ELECTROPHORESIS

- The electrophoresis conditions used during the developmental stage of this PFGE protocol consisted of a switch time of 0.25 s and a final switch time of 15 s (gradient of 6 V/cm and an included angle of 120).
- The gels were electrophoresed for 20 h at 14 °C (CHEF Mapper), in 0.5X TBE. After the electrophoresis run was completed, the gels were stained with 400 mL of ethidium bromide solution (50 µg/mL), and the band pattern was observed under UV illumination.

3.13. PLANAR BILAYER ASSAYS

The basic methods have been reported previously [116, 117, 175]. Membranes were made from 1% lipid (1% diphytanoylphosphatidylcholine (DiphPC) in n-decane or 0.2% diphytanoylphosphatidylglycerol (PS) plus 0.8% (DiphPC) in n-decane). Bilayers were painted across a 0.8 mm² hole in a Teflon divider separating two compartments containing 5 mL each of a bathing solution of 1 M KCl. Voltages were applied across this membrane through Ag/AgCl electrodes connected by a salt bridge, and the resultant current was boosted 10⁹-10¹⁰ fold by a current amplifier, and recorded on a Rikadenki strip chart recorder.

3.14. DNA EXTRACTION

Two methodologies were indistinctly used for the extraction of chromosomal DNA of *S. marcescens* or *P. aeruginosa*.

3.14.1. CHROMOSOMAL DNA EXTRACTION (LARGE SCALE)

The Marmur's method for bacterial DNA extraction was used for each strain as described below: [118]

- First, strain was inoculated in 5 mL of LB O/N at 30 °C without shaking.
- Then, 1 mL of the previous subculture was used to inoculate 50 mL of LB and incubated for 18 hours at 30 °C with shaking.
- Bacteria were pelleted by centrifugation at 10,000 rpm for 10 min at 4 °C and resuspended in 5 mL of 0.15 M NaCl and 0.01 M Tris pH 8. Mixture was kept on ice for 10 min.
- 1 mL of proteinase K (10 mg/mL) was added and sample was gently mixed. Lysis of bacteria was achieved by the addition of 263 µL of SDS 20% concentrated (1% final concentration) and incubated at 75-85 °C for 15 min shaking the mixture occasionally. Complete lysis was indicated by a rapid change in viscosity.
- Sample was cool at room temperature and 1.316 µL of sodium perchlorate 5M (final concentration 1 M) was added. Then the mixture was shaken and 3.29 mL of chloroform:isoamyl alcohol) 24:1 was added. After transferred it to glass-stoppered flasks, it was swirl to mix the two phases together, and shaken on a wrist-action shaker for 30 min at sufficient speed to get an emulsion.
- Emulsion was centrifuged at 10,600 rpm for 10 min in a refrigerated centrifuge at 4 °C.
- The upper aqueous layer was carefully pipetted from each tube, being careful not to collect any of the white precipitate (protein) at the interfaces between the two phases. The lysate extraction steps were repeated until the substantial protein layer at the surface had disappeared.
- The aqueous phase was placed in a beaker and slowly overlaid with cold 95% ethanol (an amount equal to about 2 volumes of the aqueous phase). The precipitated DNA was collected with glass stirring rod by gently stirring the two phases while spinning the rod.

The excess of ethanol was removed pressing the rods against the side of the beaker, and then the rod was placed in test-tube kept on ice.

- Spooled DNA was dissolved in 1,500 μL of 0.1X SSC
- 85 μL of 20X SSC was added to adjust the concentration to 1X.
- 17 μL of RNase mixture (5 mg/mL) was added and incubated at 37 $^{\circ}\text{C}$ for 30 min (final concentration 50 $\mu\text{g}/\text{mL}$)
- The mixture was transferred in a polypropylene flask, and 850 μL of phenol was added. Then, the mixture was shaken.
- 850 μL of chloroform:isoamyl alcohol was added and the mixture was shaken again. The emulsion was centrifuged at 5,000 rpm for 10 min (Sorvall RC 5C Plus Superspeed Centrifuge), and the aqueous phase was drawn off. The chloroform-isoamyl alcohol extractions were repeated until very little protein was observed between the two phases.
- In the aqueous phase (≈ 1.6 mL), 180 μL of 3 M sodium acetate (NaOAc 0,1125 vol.s) and 111 μL of isopropanol (0,625 vol.s) were added and the mixture was kept on the freezer for at least 2 hours.
- After that time, samples were centrifuged at 5,000 rpm for 10 min at 4 $^{\circ}\text{C}$ (Sorvall RC 5C Plus Superspeed Centrifuge)
- Supernatant was decanted and the soft pellet was washed with 3 mL of chilled ethanol. The sample was immediately centrifuged at 5,000 rpm for 10 min at 4 $^{\circ}\text{C}$ and supernatant decanted.
- Pellet was briefly dried by air drying or in a vacuum (5-10 min.).
- Pellet was resuspended by adding 2 mL of sterile distilled water.
- The DNA concentration was measured using the NanoDrop ND-1000 Spectrophotometer or a spectrophotometer ($A_{260/0,020} = [] \mu\text{g}/\text{mL}$)

3.14.2. RAPID CHROMOSOMAL DNA EXTRACTION

The commercial kit DNeasy® Blood & Tissue kit (Quiagen) was used for fast extraction of DNA.

Bacteria were grown in 10 mL of LB O/N at 30 °C (*S. marcescens*) or 37 °C (*P. aeruginosa*) without shaking, harvested by centrifugation for 10 min at 7,500 rpm. Pellet was suspended in 180 µL of tissue lysis buffer (ATL) included in the kit. Extraction was done following the manufacturer's instructions.

3.15. DNA METHODS

3.15.1. MEASUREMENTS OF DNA CONCENTRATION

Two methods were employed to assess both quantity and quality of DNA templates: the NanoDrop ND-1000 Spectrophotometer (improvement of the basic UV/Vis spectrophotometer) and agarose gel electrophoresis.

3.15.1.1. MEASUREMENTS OF PURITY OF DNA

NanoDrop ND-1000 spectrophotometer uses the surface properties of the sample to hold the sample in place during the measurement cycle, eliminating the need for cuvettes or capillaries. During each measurement cycle, the sample is assessed at two different path lengths (1.0 mm and 0.2 mm), resulting in a broad dynamic range (2ng/µL to 3700 ng/µL). The 0.2 mm path allows very concentrated absorbance measurements, so it is necessary to consume only 1 µL of the sample [119].

Measurements were made following this protocol:

- After nucleic acid extraction, the instrument was calibrated with 1 µL of ultrapure water.
- 1 µL of the sample was pipetted directly onto the lower optical (measurement) surface, and an upper optical fiber automatically engaged the sample, forming a liquid column of mechanically controlled path length (1 mm) that was held in place by the sample's surface tension. Light diffraction was reduced by direct contact of the sample with the optical surfaces. Measurement was then made and the NanoDrop software automatically determined the estimated DNA concentration in ng/µL.

- The sample was cleaned off with a lab wipe before loading the next sample.

3.15.1.2. AGAROSE GEL ELECTROPHORESIS

The method used is the one described by Sambrook *et al.* [92] with some modifications:

- The edges of a clean and dry plate were sealed with tape to make a mold. The mold was set on a horizontal section of the bench.
- The solution of agarose in TAE or TBE electrophoresis buffer was prepared by adding the amount of powdered agarose appropriate for the particular size fragments expected in DNA samples.
- The agarose solution was heated until the agarose was dissolved and cooled to 50 °C
- The desired comb was positioned above the plate to form the wells and warm agarose solution was poured into the mold.
- The gel was completely set at room temperature, and a small amount of electrophoresis buffer was poured on the top of the gel.
- Carefully the comb and the tape were removed and the gel was mounted in the electrophoresis tank.
- Enough electrophoresis buffer was added to cover the gel to a dept of approximately 1 mm.
- DNA samples were mixed with 0.20 volume of the desire 6X gel-loading buffer.
- Samples mixtures were slowly loaded in the slots of the submerged gel using a disposable micropipette. Size standards were loaded into slots on both the right and left sides of the gel.
- The lid of the gel tank was closed and the electrical leads attached. The constant applied voltage was between 50 and 100 V. The electrophoresis time varies with the size of DNA fragments and the applied voltage.

3.15.2. GEL STAINING

- The gel was stained in an aqueous ethidium bromide solution (0.5 µg/mL) for about 15-30 min in the dark, at room temperature.
- Then, the gel was rinsed in water plus MgCl₂ for 10-15 minutes.
- Finally, the gel was examined under UV light (302 nm) and photographed.

3.16. *oprD* IN CLINICAL ISOLATES OF *P. aeruginosa*

3.16.1. *oprD* AMPLIFICATION

To obtain genomic DNA of *P. aeruginosa* PA110514, AS1-4, AR1-4, PA116136 and the control strain ATCC 27853, the protocol used is described below:

- A single colony was used to inoculate 20 mL of LB and incubated ON at 37 °C and 250 rpm.
- Bacteria in 1 mL of the ON culture were harvested by centrifugation at 7,500 rpm at RT for 10 min.
- The pellet was suspended in 180 µL of ATL buffer of the DNeasy® Blood and Tissue kit (Quiagen, Germany) which was used to purify DNA following the manufacturer's instructions.
- *oprD* amplification was performed using a modified PCR assay previously described [120]. Reactions mixture of a volume of 50 µL contained 2 µL (1/10th volume) of genomic DNA, 0.5 µM of the OprDSEQF1 forward primer (5'-CTACGCAGATGCGACATGC-3') and 0.5 µM of the OpdRSEQR1 reverse primer (5'-CCTTTATAGGCGCGTTGCC-3') (Invitrogen, EE.UU), 1 U of Taq DNA polymerase (Fermentas, Lithuania) and 0.2 mM each deoxynucleoside triphosphate (Fermentas, Lithuania), 1X PCR buffer and 2 mM MgCl₂. Amplification was achieved in a Techne (United Kingdom) thermocycler model TC-312 as follows: 25 cycles, each consisting of 30 s at 95 °C, 30s. at 56 °C and 2 min at 72 °C. Final extension was performed at 72 °C for 10 min.

3.16.2. VISUALIZATION

PCR products were separated in agarose gels (1.5% w/v) in Tris-Borate buffer (TBE) at 150 volts for 1.5 h and visualized.

3.17. DNA SEQUENCING

3.17.1. DETERMINATION OF GENE SEQUENCES

DNA sequencing was performed by using an automated system, which use fluorescent-labeled dideoxynucleotides in dideoxynucleotide sequencing reactions. As the newly synthesized DNA strands are electrophoresed through a gel, they pass through a laser beam that excites the fluorescent label. The resulting emitted light is then detected by a photomultiplier and a computer collects and analyzes the data. The protocol is described below:

- PCR products were purified with a MinElute® PCR Purification Kit (Qiagen, Germany) prior to sequencing.
- An ABI PRISM BigDye® Terminator v.3.1 Cycle Sequencing Ready Reaction Kit and ABI PRISM® 3700 DNA Analyzer (Applied Biosystems, USA) were used for DNA sequencing. This PCR was performed for both primers (reverse and forward) in order to obtain a single strand in each tube.

3.17.1.1. PCR SEQUENCING

PCR was carried out in 20 μ L volumes containing 3 μ L of purified DNA and 17 μ L of mix.

Components for 20 μ L of mix:

Terminator reaction Mix.....	4 μ L
PCR product (10-30 ng/ μ l)	3 μ L
Primer diluted 1/10	1 μ L
Distilled water.....	12 μ L

3.17.1.2. DNA AMPLIFICATION

After 2 min of denaturalization at 96 °C, the following three-step profile was used:

- 0.5 min of denaturation at 96 °C
- 15 s of annealing at 50 °C
- 4 min of extension at 60 °C

Total of cycles: 25

All completed reactions were maintained at 4 °C.

3.17.1.3. PRECIPITATION OF DNA

When the amplification reaction finished, a rapid spin of the samples was performed, followed by precipitation of DNA:

- 63 µL of 95% ethanol were added to the sequencing reaction. The final volume was 100 µL.
- For DNA precipitation samples were mixed and left at RT for 15 min
- Samples were centrifuged at 14,000 rpm for 20 min
- The precipitate obtained in the previous step was washed two times with 70% ethanol and let it dry.
- Samples were stored at -20 °C until used.

Nucleotide sequencing was carried out at the Serveis Científico - Tècnics of the University of Barcelona on an ABI PRISM 3700 DNA analyser (PE Biosystems). Sequence chromatograms were analyzed with Chromas software (version 1.34).

3.17.21.4. COMPARISON AND ANALYSIS OF SEQUENCES

For comparison and identification of homologous sequences several bioinformatics programs were used.

The Basic Local Alignment Search Tool (BLAST) [121] and FASTA [122] were used for comparative analysis of nucleotide sequences against known sequences. ExPASy [123] (Expert Protein Analysis System), the

Material and methods

proteomics server of the Swiss Institute of Bioinformatics (SIB), was used to translate nucleotide sequences to protein sequences. ClustalW [124] was used for multiple sequence alignment of nucleotide sequences.

List of bioinformatics programs are in the table 12.

Table. 12. Bioinformatics programs used for the analysis and comparison of sequences.

Program:	Web page:
BLAST	National Center for Biotechnology Information, Bethesda, USA (http://www.ncbi.nlm.nih.gov)
EXPASy software	Swiss Institute of Bioinformatics, Geneva, Switzerland (http://www.expasy.org)
CLUSTALW v2	European Bioinformatics Institute, Hinxton, UK (www.ebi.ac.uk/Tools/msa/clustalw2/)

4. RESULTS AND DISCUSSION

4.1. COMPARISON OF ANTIBIOTIC SUSCEPTIBILITY OF OLD AND CURRENT *S. marcescens*

All the strains resulted to be sensitive to ceftazidime, cefotaxime, kanamycin, gentamicin, ofloxacin and ciprofloxacin and resistant to rifampicin, penicillin, ampicillin, amoxicillin and tetracycline. Among them, 95% were found to be sensitive to ceftriaxone, and 54% resistant to ceftiofloxacin. Old *Serratia* strains showed reduced susceptibility to streptomycin compared with their current relatives (Fig. 20).

It should be noted that cefotaxime and streptomycin were the only antimicrobials to show significant differences in the K-W test, so the U-Mann-Whitney test was applied to check differences between couples of strain groups. On the one hand, cefotaxime susceptibility had significant differences between G2 (old) and G3 (clinical). However, in both groups all *Serratia* strains were clinically sensitive. Furthermore, streptomycin showed significant differences between G1 (environmental) and G2 strains and those of G2 and G3 (Tables 13 and 14).

All strains studied were resistant to penicillin G, ampicillin, amoxicillin-clavulanic acid, cefazolin, cefamandole, polymixin B, rifampicin, glycopeptides, fusidic acid, lincosamides, streptogramins, colistin, daptomycin, linezolid and cefuroxime. Furthermore, they were susceptible to the third generation cephalosporins ceftazidime and cefotaxime. As the 5% resistance to ceftriaxone suggested the occurrence of Extended Spectrum β -lactamases (ESBL), we performed experiments to detect ESBL. None of the strains studied presented ESBL.

Several studies have shown that evolution of bacteria after the initial use (sometimes abuse) of antibiotics showed a constant tendency to increase MIC values. This was, at least in part, attributed to the emergence of new molecular mechanisms of resistance. Thus, it seemed to us pertinent to explore whether such mechanisms were already present in "old" strains. Most of the *Serratia* strains, especially those in G2, were streptomycin resistant, so we checked for the presence of integrons in the "old" strains isolated from 1940-1950. PCR amplification followed by gel

electrophoresis to visualize the amplification product showed that none of the strains examined presented class 1 integrons. However, when testing modern isolates (G1 and G3) they also completely lack class 1 integrons, this is consistent with the hypothesis that such mechanisms have arisen in response to the selective pressure of antibiotics. However, the lack of integrons in newer strains (G1 and G3) was relatively surprising since several reports have shown the occurrence of class 1 integrons in *Serratia* [125, 126]. It could be possible that such integrons are restricted to ESBL and CTX-M producing *Serratia* and they occur mostly in isolates from Asia. In Europe and US the occurrence of ESBL-producing *S. marcescens* carrying integrons is rare, although an outbreak of such a clone was described in an Italian Intensive Care unit with a mortality of 86 % [127]. Moreover, a singular clinical isolate SCH88050909 has been extensively studied [128]. This strain was isolated in Greece and presented three integrons. It was studied owing to its wide multiresistance, and has been used to explore several mechanisms of insertion and to demonstrate that the ant(299)-Ia *S.ma*.I2 gene cassette is a recombinationally active element [129].

The tendency to increase resistance has been demonstrated for *Streptococcus pneumoniae* and penicillin (from 0.04 µg/mL in 1940 to 0.12-1 µg/mL in 1980); *S. aureus* and vancomycin (from 0.25-0.5 µg/mL in 1986 to 32 µg/mL in 2002); *P. aeruginosa* and tobramycin (from < 2 µg/mL in 1980 to > 16 µg/mL in 2000) among others [36]. By contrast, in this study we found that resistance to antimicrobials was not so different in *Serratia* when old strains, isolated before the discovery of most of the antibiotics used nowadays, and current strains are compared. In other words, the proportion of strains exhibiting a given value of MIC is similar in groups G1, G2 and G3, although it is clear that MIC values are slightly higher in clinical isolates (Fig 20). In some cases, the resistance determinants becomes apparent much earlier than antibiotic discovery; for example, half of the strains had a MIC for cefoxitin of 16 µg/mL in both G1 (environmental) and G2 (old) groups despite this antibiotic was first used in 1977, almost 30 years after the isolation of the G2 microorganisms.

When other non-cell-wall active antimicrobials were considered, again our results indicate that overall susceptibility has not changed drastically over the last 50 years. This is true for all the antimicrobials tested except streptomycin. In this case old strains, isolated when the use of streptomycin was widespread, were much more resistant than the newer strains (Fig. 20). It also appears that the percentage of resistant *Serratia* strains has decreased since streptomycin was virtually withdrawn. Indeed, this has also occurred in other situations. For instance, in *P. aeruginosa* the resistance to imipenem (due to the loss of the only porin able to transport imipenem through the outer membrane) is reversible when selective pressure due to the presence of the antibiotic disappears. A case of an imipenem-resistant strain isolated from a patient in the course of a second episode after a recent treatment with imipenem gave us the possibility to explore and reproduce the phenomenon at laboratory scale. An insertion sequence ISPa133 was found. When the susceptible isolate (first episode) was cultured in the presence of imipenem, a resistant clone was detected. This clone had the ISPa133 inserted into the gene encoding OprD, but after repeated subcultures in absence of imipenem, the susceptible phenotype was restored [130]. These results indicate that, despite the multiple molecular mechanisms involving bacterial resistance, withdrawing the antibiotics tends to restore the original phenotypes, possibly since they are more effective in antibiotic-free conditions. However, when large families of antibiotics are considered, this advantage could be counteracted by cross-resistance. For instance, susceptibility to penicillin has not been recovered in most species, despite the fact that penicillin is no longer widely used. One reason could be that bacteria are selected by many modern cephalosporins that share the mechanisms of resistance with the old relatives. To a greater extent than most Gram-negative bacteria, *S. marcescens* exhibits characteristic patterns of antibiotic resistance within an individual hospital environment. Often, the resistance pattern of such “home strains” correlates with local antibiotic usage policy. This indicates that drug resistance plays a crucial role in

determining the biological success of this relatively noninvasive bacterium in hospital settings.

Metagenomics studies have demonstrated that antibiotic resistance genes are widely disseminated, which seems to be inconsistent with the idea that resistance to antibiotics is a new emergent phenomenon. On the contrary they suggest a long natural history of antimicrobial resistance. D'Costa *et al.* [131] analyzed DNA bacterial sequences recovered from Pleistocene and developed a study of the diversity of ancient antibiotic resistance sequences. Furthermore, they did a comparison of tridimensional structural models of ancient and contemporary enzymes responsible for vancomycin resistance.

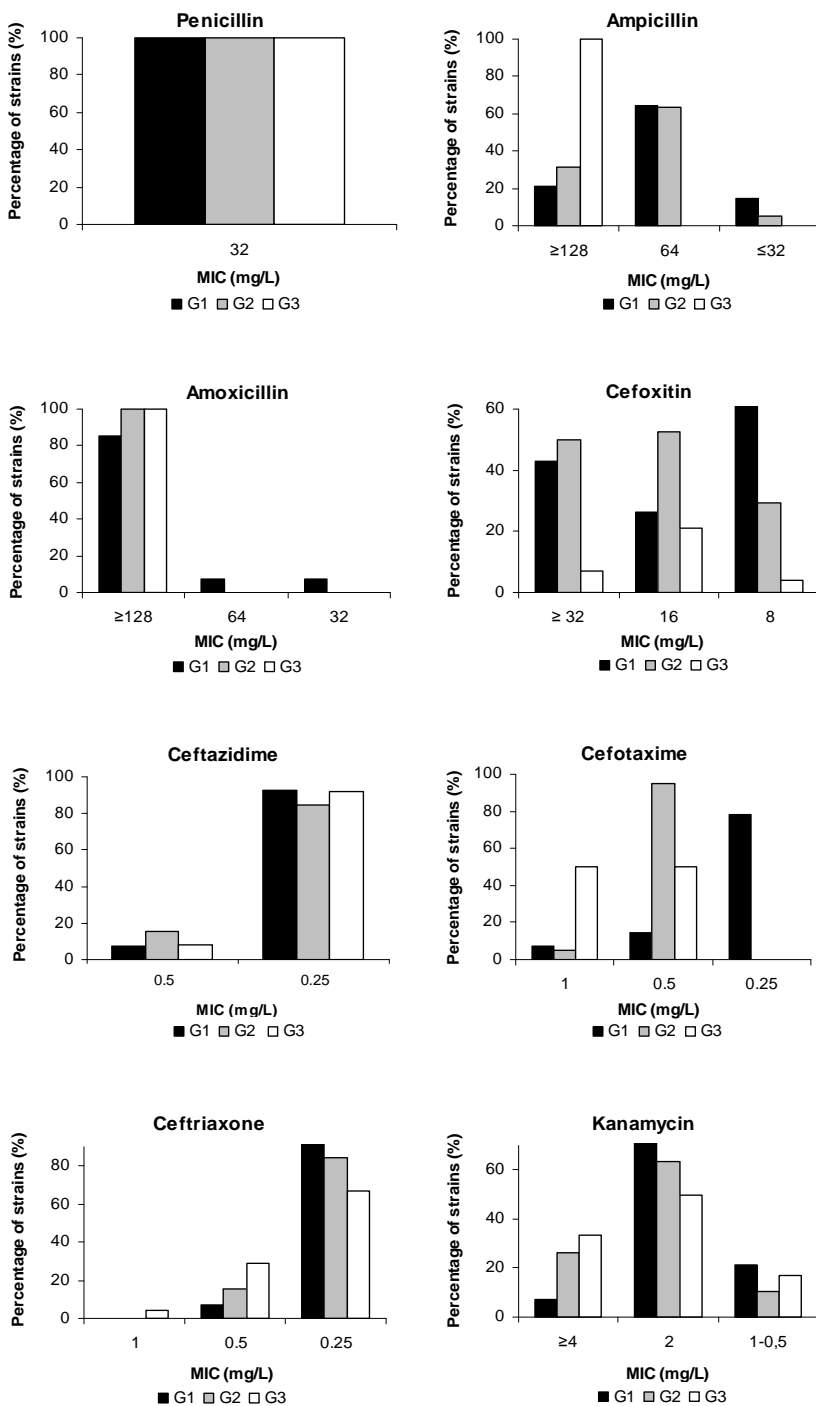
Genomics (the bacterial resistome) has allowed exploring the presence of genes encoding for antimicrobial resistance in the past. However, it is feasible that environmental conditions affecting antimicrobial susceptibility have not change significantly during the long lapse of time elapsed from Pleistocene to the first moiety of 20th Century. Therefore, studies based on phenotypical susceptibility, despite are not so frequent because the difficulty in having cultures, have a great interest, since as it has been pointed out, in many cases, the presence of the gene underlying a given function is necessary but not enough to ensure the expression of such function. In other words, when we have to study unculturable bacteria, metagenomics is undoubtedly the gold standard (and perhaps the unique method), but when bacteria can be cultured, interpretation of data can be made on the basis of both gene presence and phenotypic expression.

In fact isolates of G2 were obtained, cultured, identified and lyophilized at the beginning of antibiotic era before the discovery of most antimicrobials. The present work could be seen as a confirmation of the conclusion that resistance to antibiotics already existed in ancient times [132].

When results of MIC are evaluated in detail it becomes apparent that G3 isolates (modern and clinical) had slightly higher values of MIC i.e. 100% of isolates had a MIC of ampicillin higher than 128 mg/L, whereas in group G2 (ancient) 60 % had a MIC of 64 mg/L and only 35 % were higher than 128 and the additional 5% had a MIC of 32 mg/L. In summary, although

all isolates are clinically resistant to ampicillin, MIC values seemed to have a tendency to increase. A similar conclusion can be obtained when we evaluate data concerning other antimicrobials that do not act on the bacterial wall such as ciprofloxacin. In this case, all isolates were susceptible but among G1 MICs ranged from 0.002 to 0.04 mg/L, whereas G2 ranged from 0.002 to 0.08 and finally G3 had MICs higher than 0.004; the lower value in this group was 0.01 and maximal value reached 0.32 mg/L. This makes sense since clinical isolates include nosocomial populations submitted to antibiotic pressure.

The origin and spread of antimicrobial resistance is a main cause of concern and will be a research subject for many more years. It has been pointed out that control in antibiotic use has a great impact in delaying the emergence of resistance and even allows populations to evolve from resistance to susceptibility. This field of research has to be based on genomics since we lack original strains in many cases, but when available, old populations have to be phenotypically analyzed, as the role of heteroresistance, as well as adaptative responses of microorganisms (epigenetics) to antimicrobials, seem to play a key role in the process of resistance.



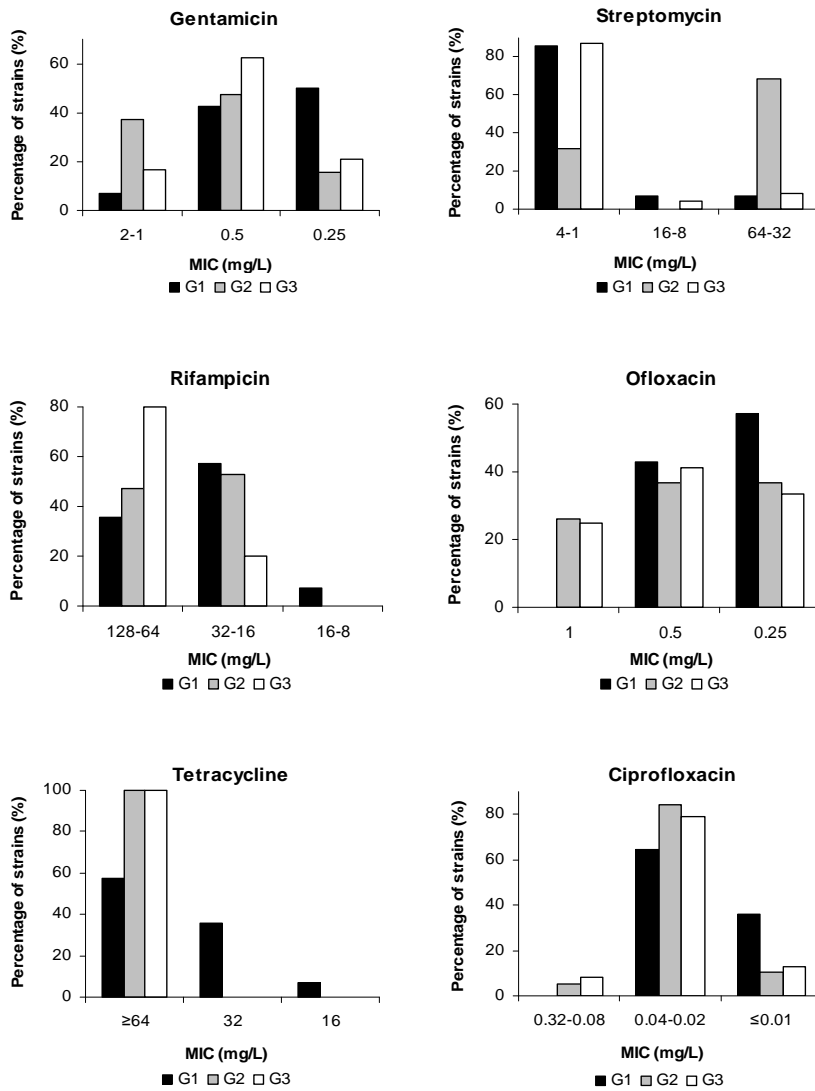


Fig. 20. Minimal inhibitory concentrations of tested antimicrobials showed as percentage of strains in the three groups of isolates studied.

Table 13. Kruskal-Wallis Tests

	chi-quadrat	p-value	tests	Sequential
Ceftriaxone	3.37016819	0.18542884	4	0.0125000
Cefotaxime	11.7698671	0.00278103	10	0.0050000
Cefoxitin	9.64345693	0.00805286	9	0.0055556
Ceftazidime	0.4677243	0.79147091	1	0.0500000
Ciprofloxacin	2.6782656	0.26207284	3	0.0166667
Gentamicin	6.1227156	0.04682407	7	0.0071429
Kanamycin	2.01085276	0.36588859	2	0.0250000
Ofloxacin	4.70536536	0.09511366	5	0.0100000
Rifampicin	5.0652131	0.07945166	6	0.0083333
Trimethoprim	9.37447766	0.00921209	8	0.0062500
Streptomycin	13.2119967	0.00135223	11	0.0045455

Table 14. U-Mann-Whitney Test

Cefotaxime	P.value
G1 vs G3	0.0974
G1 vs G2	0.1414
G2 vs G3	0.0008
Streptomycin	
G1 vs G3	0.7087
G1 vs G2	0.0049
G2 vs G3	0.0009

4.2. *Serratia* BIOFILM FORMATION AND FUNCTIONALITY OF EFFLUX PUMPS DEPENDING ON THE MODE OF BACTERIAL GROWTH

It is well known that sessile bacteria (those individuals forming part of biofilms) are phenotypically more resistant to antibiotics than planktonic bacteria [133, 134, 135, 136]. Despite in recent years the understanding of the molecular mechanisms involved in biofilm formation has greatly increased, there are still many details that remain unknown.

There is no a general agreement concerning a standard method adequate for the study of susceptibility in biofilms. In fact it is very difficult, if not impossible to compare results obtained from biofilm-growing bacteria. This is also true when bacteria are cultured and assayed under vastly different conditions [137]. Furthermore, we lack a widely accepted model for biofilm formation in the laboratory. Thus, we developed a laboratory procedure to obtain biofilms of *Serratia*.

Efflux pump activity can be measured by determining the accumulation rates of antimicrobials that can be extruded by the pump. Ciprofloxacin (and in general fluoroquinolones) are useful for this purpose since fluorescence allows to easily measure by spectrofluorimetry. Rates of ciprofloxacin accumulation in planktonic *Serratia* have been critically determined whereas almost nothing is known concerning accumulation of ciprofloxacin by bacteria growing in biofilm.

This part of our work was devoted to search an experimental procedure to check and evaluate efflux active mechanisms involved in fluoroquinolone resistance when bacteria live as biofilm. This can contribute to the understanding of the degree of involvement of efflux systems in antimicrobial resistance, as well as to compare data from planktonic and biofilm-forming bacteria.

4.2.1. BIOFILMS OF *S. marcescens*

4.2.1.1. SESSILE BACTERIA

S. marcescens biofilms formed on methacrylate pieces by using the method herein described (3.7) contained approximately 1×10^7 bacteria/cm² of methacrylate after 24 h incubation. For 48 h biofilms the average amount was 2×10^7 bacteria/cm² of methacrylate.

4.2.1.2. SENSITIVITY OF THE METHOD TO MEASURE CIPROFLOXACIN ACCUMULATION

To explore antibiotic uptake by bacterial biofilms, a preliminary study was done to check the sensitivity of the method used to measure ciprofloxacin accumulation in planktonic cultures of *S. marcescens*. The method analyzed is the one described in section 3.8.2.1.

The objective was to determine if ciprofloxacin accumulation changes depending on the density of microorganisms. Ciprofloxacin accumulation was determined at increasing bacterial densities (*S. marcescens* 2170) by sampling at 0, 3 and 6 min and subsequently by spectrofluorometry measurements.

Figures 21.a and 21.b show the rates of accumulation of ciprofloxacin by different densities of wild-type *S. marcescens* 2170.

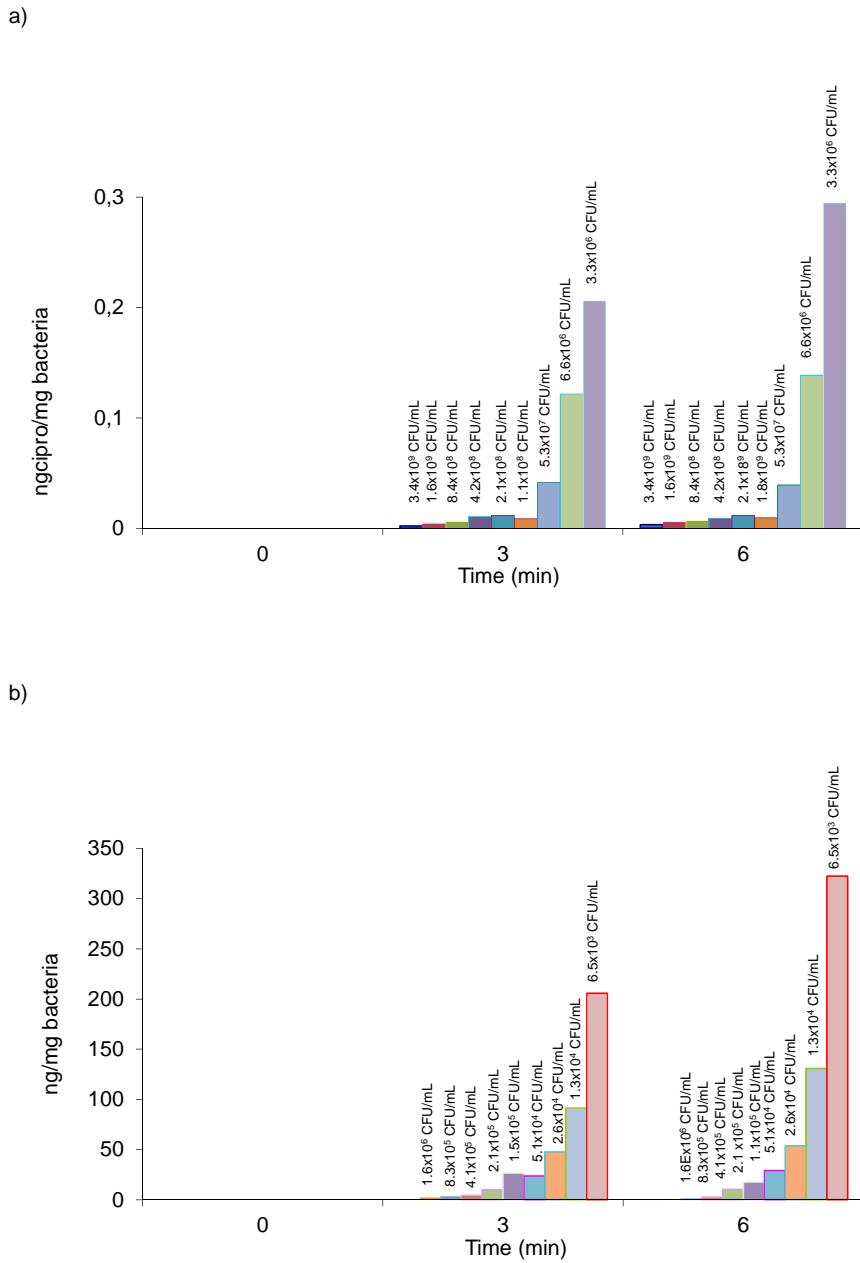


Fig. 21. Fluoroquinolone accumulation by *S. marcescens* 2170 (wild type). a) From 3.4x10⁸ to 3.3x10⁶ CFU/mL; b) From 1.6x10⁵ to 6.5x10³ CFU/mL

Fluorescence values of the supernatants collected from different concentrations of bacteria revealed that accumulation of ciprofloxacin (ng ciprofloxacin/mg bacteria) depends on the density of microorganisms in the assay. Although it is well known that planktonic *Serratia* accumulates ciprofloxacin proportionally to time [22, 79] the total amount of accumulated ciprofloxacin decreases as the bacterial number increases. In other words, accumulation of antibiotic in diluted bacterial cultures is higher than in more dense cultures. Then it becomes clear that in order to compare antibiotic uptake by planktonic and sessile bacteria, the achievement of isonomic populations should be established first. This is consistent with the idea that small populations will have higher availability of the drug at identical concentrations.

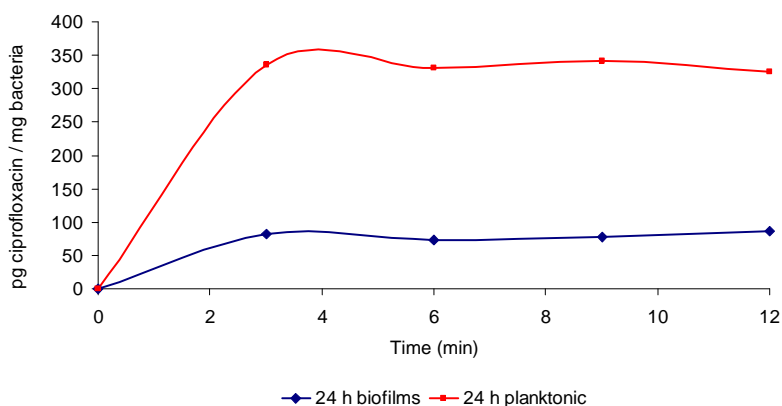
4.2.1.3. MEASUREMENT OF CIPROFLOXACIN ACCUMULATION IN PLANKTONIC AND SESSILE *S. marcescens*

Standard bacterial suspensions from both planktonic and sessile forms were prepared as indicated in 3.7.

Ciprofloxacin accumulation by planktonic and sessile *S.marcescens* 2170 is shown in figures 22.a and 22.b.

Both demonstrate that ciprofloxacin accumulation is higher in planktonic than in sessile forms and that it takes place during the first three minutes.

a)



b)

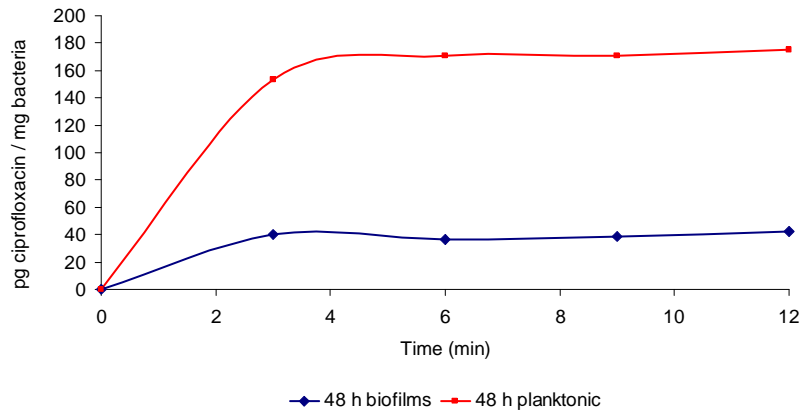


Fig 22. Fluorometric assay for ciprofloxacin accumulation in *S. marcescens* 2170. a) Ciprofloxacin accumulation in 24 h biofilms and planktonic bacteria. b) Ciprofloxacin accumulation in 48 h biofilms and planktonic bacteria.

Piddock *et al.* [164] suggested that accumulation of most quinolones in bacteria proceed by simple diffusion, thus the apparent saturation of ciprofloxacin after 3 minutes could be attributed to a fast diffusion of this antibiotic through *Serratia* envelopes.

It has also been described that ciprofloxacin easily penetrates into bacterial biofilms [138], furthermore the efficiency of antimicrobials can be modified since a certain rate of deactivation of biological activity in the biofilm could take place. Data reported here suggest that the rate of ciprofloxacin deactivation could be higher in biofilms than in planktonic bacteria, leading to a smaller accumulation of the antibiotic in bacterial biofilms. This also reinforces the idea that antibiotic uptake could be under a certain degree of control by some epigenetic mechanism.

4.2.1.4. MEASUREMENT OF CIPROFLOXACIN EFFLUX IN PLANKTONIC AND BIOFILM *S. marcescens* 2170

Preliminary experiments performed to measure ciprofloxacin accumulation in both planktonic and sessile bacteria demonstrated the different capability to accumulate this antimicrobial (Fig. 22). Subsequently, in order to quantify efflux activity, a set of experiments was performed using the efflux pump inhibitor CCCP.

Since ciprofloxacin accumulation was different in biofilms and planktonic bacteria, the evaluation of ciprofloxacin efflux in the presence and absence of an EPI was performed. It has been demonstrated that de-energized bacteria (by CCCP) have the efflux mechanisms partially inactivated, thus, accumulation of antimicrobials increases [139, 79]. However the final outcome depends not only on the efflux and entry but also on the effective inhibition of the target enzymes [56, 140].

CCCP was able to increase ciprofloxacin accumulation in planktonic cultures to a much higher extend than in bacterial biofilms (Fig. 23). In fact, it seems that effect of CCCP is negligible in biofilms.

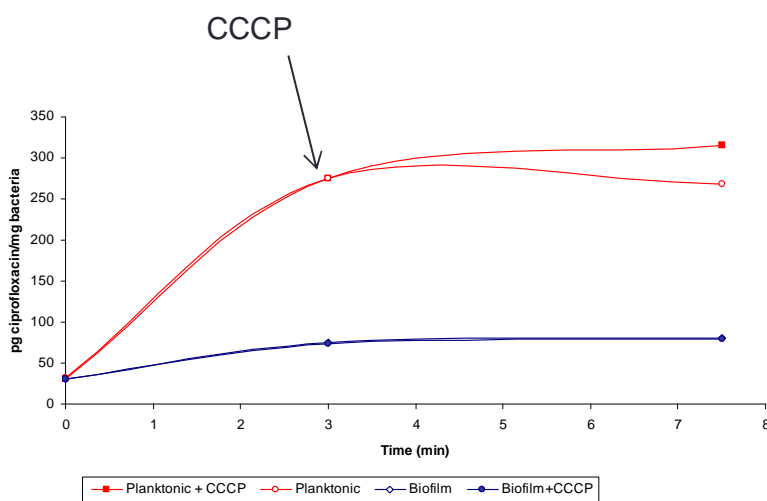


Fig 23. Fluorometric assay for ciprofloxacin efflux in biofilms and planktonic cultures of *S. marcescens* 2170 with and without the efflux pump inhibitor CCCP.

This showed that differences in susceptibility of planktonic and sessile bacteria, could be due, at least in part to differences in efflux pumps expression i.e. they play a crucial role in planktonic but they are irrelevant in sessile forms. On the contrary, Kvist *et al.* [141] proposed that efflux pumps are highly active in biofilms. In fact up to twenty efflux and transport genes were upregulated during biofilm growth. These authors also observed significant reductions in biofilm formation when they added EPIs. Several different EPIs had this capacity. However, they did not measure the antimicrobial efflux. Thus, one can assume that efflux pumps role in increasing antimicrobial resistance in liquid is directly due to the extrusion activity. On the contrary the role of efflux pumps on resistance of sessile bacteria could be due to the key role that extrusion machineries play in the building of biofilm itself, and individual resistance to antimicrobials is due in biofilm to the particularities of bacterial life in biofilms more than a truly extrusion activity.

The knowledge on the exact role of efflux pumps on biofilm growth is still limited and many studies of Gram-negative bacteria efflux mechanisms have produced discordant results. For instance, some studies have related efflux pumps with antibiotic resistance in biofilms and specific mechanisms of resistance [142,143], whereas others have found that pumps do not play a significant role [144]. Lay *et al.* [145] have explored the eventual association between swarming and widespread antimicrobial resistant phenotype by using several Gram-negative bacteria including *S. marcescens*. They found that in general swarm bacteria are more resistant to antimicrobials than their planktonic counterparts, but RND-type efflux pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM) did not appear to be relevant for the resistance of swarm cells. Consequently, they suggested that antimicrobial resistance is a general feature of bacterial multicellularity and proposed swarming motility (a form of social behavior like biofilm) as a useful model to investigate biofilm antibiotic resistance.

Moreover, it has been pointed out that the expression of bacterial efflux pumps is probably controlled by multiple factors such as growth rate,

accumulation of metabolites, deficient nutrition and altered permeability (huge amount of protective extracellular polysaccharides among others). Accurate measurements of efflux by bacteria living in biofilms remains unavailable, thus, the need of improvements of the methods should be envisaged.

4.3. CARBAPENEM RESISTANCE IN CLINICAL ISOLATES OF *P. aeruginosa*

4.3.1. DRUG SUSCEPTIBILITY

Values of MIC for ATCC 27853 and MDRPA strains of several antimicrobial agents are shown in Table 15. Clinical breakpoints are listed below the table in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations.

Table 15. MICs (mg/L) of ATCC 27853 and MDRPA determined by microdilution method.

Drug	Family	Strains				
		459	133	162	527	ATCC ₂₇₈₅₃
MIC (mg/L)						
Meropenem	Carbapenem	8	8	8	8	1
Imipenem	Carbapenem	8	8	8	8	2
Ciprofloxacin	Fluoroquinolone	64	64	64	64	0.5
Tobramycin	Aminoglycoside	32	64	64	64	0.5
Ceftazidime	Cephalosporin	128	64	64	64	4

Clinical breakpoints, according to European Committee on Antimicrobial Susceptibility Testing (EUCAST): Meropenem, susceptible ≤ 2 mg/L, resistant > 8 mg/L; Imipenem, susceptible ≤ 4 mg/L, resistant > 8 mg/L; Ciprofloxacin, susceptible ≤ 0.5 mg/L, resistant > 1 mg/L; Tobramycin, susceptible ≤ 4 mg/L, resistant > 4 mg/L; Ceftazidime, susceptible ≤ 8 mg/L, resistant > 8 mg/L.

4.3.2. PFGE ANALYSIS

PFGE analysis showed that there was a remarkable homogeneity among the isolates and had very closely related Spel patterns (Fig. 24).

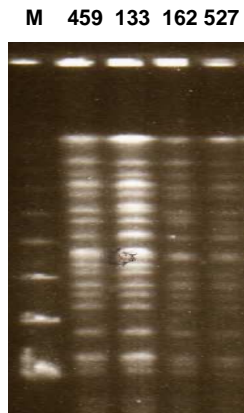


Fig 24. Pulse Field Gel Electrophoresis of MDRPA

4.3.3. OUTER MEMBRANE PROTEINS (OMPs)

OMPs were visualized in all the strains. No noticeable differences in OMP electrophoretic profiles were found between the MDRPA strains and ATCC 27853. However, MDRPA strains showed a reduction of OprD porin (46 kDa) expression when compared with that of ATCC 27853 (Fig. 25).

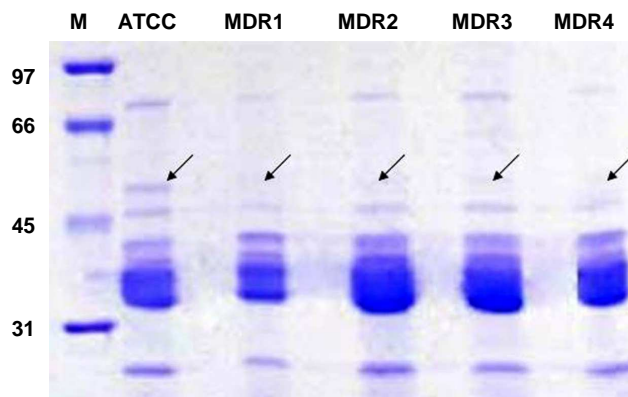


Fig. 25. SDS-PAGE of OMPs of *P. aeruginosa*. M: standard molecular mass OprD is indicated by arrows.

4.3.4. MEROPENEM ACCUMULATION

In the presence of high-concentration (4xMIC) of meropenem, *P. aeruginosa* was able to survive forming long rods; when meropenem pressure was suppressed, long-rods recovered their original shape (Fig. 26).

Accumulation of meropenem was 15% higher in regular morphological rods than in long ones.

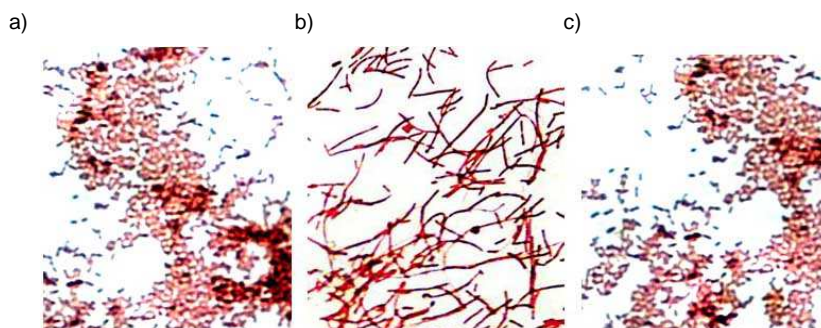


Fig. 26. Effects of high-concentration (4xMIC) meropenem depending on *P. aeruginosa* shape. a) *P. aeruginosa* rods grown without antibiotic. b) *P. aeruginosa* rods grown with 4xMIC of meropenem. c) *P. aeruginosa* rods after removing antibiotic.

4.3.5. CLASS 1 INTEGRONS

PCR amplification from MDRPA yielded amplicons of 750 bp (Fig. 27). Class 1 integrons detected in all of the MDRPA strains tested were sequenced and resulted to be identical. The cassette contained the *aadB* (also called *ant(2'')-Ia*) aminoglycoside resistance gene that confers gentamicin, kanamycin and tobramycin resistance (Fig. 28).

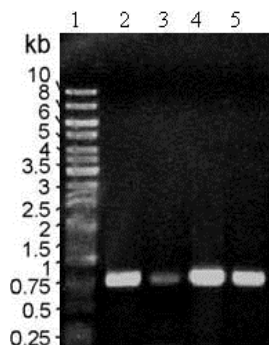


Fig. 27. PCR amplification, using the 5'-CS and 3'-CS primers, of variable regions of integrons from MDRPA isolates. The PCR products were separated by electrophoresis in 0.7% agarose. Lane 1, 1-kb DNA ladder; lane 2, *P. aeruginosa* 459; lane 3, *P. aeruginosa* 162; lane 4, *P. aeruginosa* 527 and lane 5, *P. aeruginosa* 133.

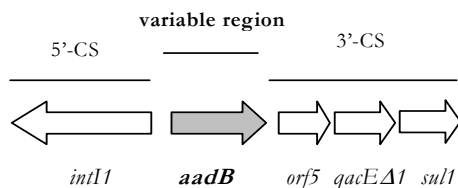


Fig. 28. Schematic representation of class 1 integron with an *aadB* gene cassette; arrows show direction of transcription.

4.3.6. EFFLUX PUMP-MEDIATED FLUOROQUINOLONE RESISTANCE

All the strains had a MIC of ciprofloxacin of 64 mg/L and subsequently should be considered clinically resistant. Those strains which MIC of ciprofloxacin decreases at least two fold after addition of PAβN are known as efflux pump overexpressed phenotype (EPO). We have tested such reduction in the strains studied. Results are indicated in figure 29.

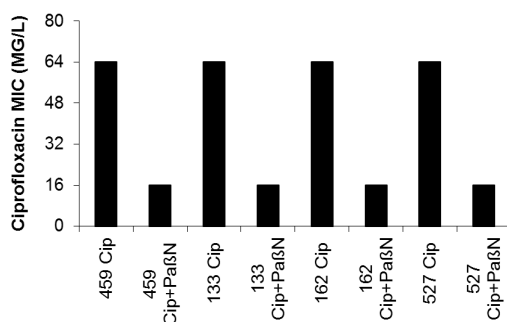


Fig. 29. MIC reduction by the efflux pump inhibitor PAβN; all the strains were tested over the same range of ciprofloxacin concentrations (0.125 to 128 µg/mL) with and without PAβN, and showed a 4-fold decrease in MIC with the EPI.

4.3.7. DETECTION OF A FUNCTIONING MEXXY-OPRM EFFLUX PUMP

All MDRPA strains, exhibiting high tobramycin MIC values, showed outstanding antagonism with PAβN (at 40 µg/mL). MIC values increased at least 4-fold as can be seen in Fig. 30.

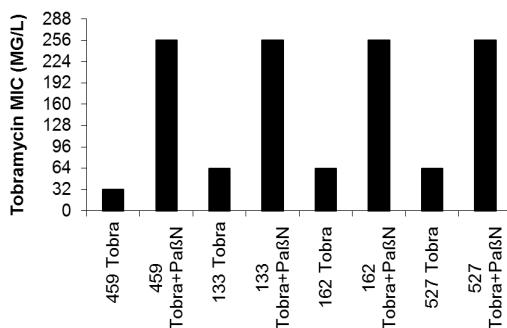


Fig. 30. Effect of the efflux pump inhibitor PAβN on tobramycin susceptibility; all the strains were tested over the same range of tobramycin concentrations (0.25 to 256 µg/mL), and showed antagonism with the PAβN.

4.3.8. ANTIBIOTICS COMBINATIONS AND EFFLUX

Many reports have suggested the convenience of the rescue of “old” antibiotics to build synergistic combinations of different antimicrobials that can be useful in the treatment of infections due to multidrug-resistant

microorganisms. Thus, we performed experiments to explore the convenience of such approach. First, a measure of the MICs reduction in the presence of PA β N was done. Slight reductions of MIC values of ceftazidime and meropenem were observed in the presence of PA β N without tobramycin (Fig. 31). Furthermore combinations of pairs of antibiotics were tested.

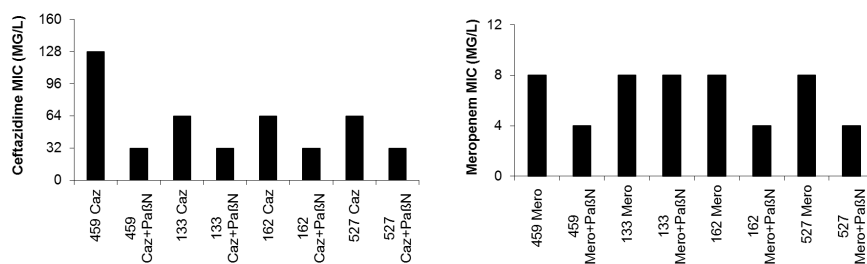


Fig. 31. Effect of the efflux pump inhibitor PA β N on ceftazidime and meropenem susceptibility; all the strains were tested over the same range of ceftazidime and meropenem concentrations (128-0.125 μ g/mL) with and without PA β N, and showed a trivial decrease in MIC with the EPI.

As we pointed out before tobramycin antagonizes PA β N irrespective of its concentration. Combinations of tobramycin with either ceftazidime or meropenem resulted to be synergistic. On the other hand when PA β N was added (40 μ g/mL) significant increased MIC values were determined, demonstrating again the antagonism between PA β N and tobramycin (Table 16).

Results and discussion

Table 16. MICs of combinations of antibiotics with and without PAβN.

Fixed concentration of tobramycin (µg/ml)	PAβN (µg/ml)	Meropenem MIC (µg/ml) Concentrations tested: 256-0.125 (µg/ml)				Ceftazidime MIC (µg/ml) Concentrations tested: 256-0.125 (µg/ml)			
		Isolates				Isolates			
		459	133	162	527	459	133	162	527
0	0	8	8	8	8	128	64	64	64
0	40	4	8	4	4	32	32	32	32
8	0	0.5	1	1	1	4	2	4	4
8	40	8	8	8	16	32	32	64	32
16	0	0.125	0.5	0.25	0.5	0.125	0.5	1	1
16	40	8	8	8	8	32	32	32	32
32	0	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
32	40	8	8	8	8	8	32	32	32
64	0	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
64	40	8	8	8	16	16	32	32	16
128	0	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
128	40	8	4	8	8	16	32	32	16

The high incidence of *P. aeruginosa* resistant to multiple drugs, the difficulties in treating infections due to such bacteria and the extensive use of antibiotics, leading to spread of resistance genes make this species in the center of objectives to control the spread, avoid infections, enlarge treatment strategies, and reduce mortality [146]. Table 15 shows that the four *P. aeruginosa* isolates presented high resistance to all the antimicrobial drugs tested and were therefore considered as MDRPA, i.e., resistant to at least three different classes of antimicrobial agents, mainly aminoglycosides, carbapenems, antipseudomonal penicillins, quinolones, and cephalosporins [147].

Carbapenems, such as meropenem and imipenem, are potent antimicrobial agents used to treat infections due to MDRPA isolates. These antibiotics bind to critical penicillin-binding proteins, disrupting the growth and structural integrity of walls of bacteria. They provide better anaerobic and Gram-negative coverage than other beta-lactams and their stability against extended-spectrum beta-lactamases (ESBLs) makes them an effective treatment option. Both antibiotics have broad-spectrum activity, including activity against non-fermenting and Gram-negative bacteria.

However, among non-fermenting Gram-negative bacteria, resistance to imipenem and meropenem is increasing [148]. Several mechanisms conferring *P. aeruginosa* resistance to carbapenems have been reported, such as diminished permeability, overexpression of the intrinsic efflux systems and production of carbapenem hydrolyzing β -lactamases [149].

Additionally, the observation that *P. aeruginosa* survives in the presence of high concentrations of meropenem (4xMIC) by forming long rod-shaped bacteria and its capability to return to the original shape when the antimicrobial was removed suggested that accumulation of meropenem probably differs depending on bacterial shape. Experiments to measure meropenem accumulation revealed that only a 15 % difference could be demonstrated. Meropenem has a great affinity for the PBP3. This protein is involved in the final steps of the wall building during bacterial division. When PBP3 is blocked the separation of daughter bacteria does not take place as it has been shown in other works. This leads to the formation of long filaments having a non-functional PBP3 but able to survive for a relatively long time periods, and also capable to restore original morphotypes.

The reduced OprD expression in MDRPA compared with the wild-type strain (ATCC 27853) could be related with the decreased susceptibility to both imipenem and meropenem. However, it is difficult to measure the quantitative role of the OM and their porins when an antibiotic is applied *in vivo*, since both the bacterial physiological states and the OM protein expression strongly depend on the environmental conditions [89]. Rodríguez-Martínez *et al.* [150], reported that *OprD* gene downregulation and OprD protein inactivation contributed to resistance to imipenem and reduced susceptibility to meropenem. Whereas OprD inactivation alone is the source of resistance to imipenem, the mechanisms leading to meropenem resistance seem to be more complex and are very likely multifactorial, involving overproduction of AmpC or overexpression of the efflux pumps MexAB-OprM, MexXY-OprM, and MexCD-OprM. The efflux systems MexAB-OprM and MexXY-OprM directly affect meropenem activity in *P. aeruginosa* [151]. Ikonomidis *et al.* [152] found that

heterogeneous populations had upregulation of efflux with increased levels of transcription of *mexB* and *mexY* genes and low intensity of oprD protein band (46 kDa) compared to native populations [152].

Strains 459, 133, 162 and 527 also produced OXA-1 and OXA-2 enzymes, which contribute to carbapenem resistance. (Segura *et al.*, unpublished data).

Our results from the study of efflux pumps using PA β N suggested that all the MDRPA strains belong to a phenotype that overexpresses MexAB-OprM and contain a functioning MexXY-OprM efflux pump. Moreover, overexpression of MexXY-OprM has been related with carbapenem resistance. Mao *et al.* [163] demonstrated that PA β N antagonizes the activity of aminoglycosides only in strains that contained a functioning MexXY-OprM, and the degree of antagonism was higher in strains with a high level of MexXY-OprM expression. Structural changes in target enzymes and active efflux are the major mechanisms that lead to fluoroquinolone resistance in *P. aeruginosa*.

Two mutations were also found in the strains studied, one in *parC* and one in *gyrA* (Segura *et al.*, unpublished data). These results correlate with quinolone resistance in *Enterobacteriaceae* being generally caused by a cooperative effect between mutations in the genes *gyrA* and *parC* and efflux pumps, mostly *acrAB* [152, 88, 153, 154].

The basis of multiple resistance of *P. aeruginosa* is complicated because several mechanisms are involved on it. Class 1 integrons have been strongly associated with the presence of MDRPA. Nemeč *et al.* found that among 108 clinical isolates of *P. aeruginosa*, the ones that were resistant to less than three agents were *intI1*-negative, whereas almost all those resistant to more than two agents were *intI1*-positive [155].

All of the MDRPA strains examined, which belong to serotype O4, had a class 1 integron with the gene cassette array *aadB*. The *aadB* product is aminoglycoside (2'') adenylyltransferase (ANT(2'')-Ia), associated with resistance to kanamycin, gentamicin, and tobramycin [156].

This type of integron was previously found in our laboratory in some clinical *P. aeruginosa* strains from Bellvitge Hospital [65], however, this

kind of integrons has not only been found in Spain. In a recent study from Iran, 78% of 41 MDRPA isolates from five hospitals had an integron containing the *aadB* gene and amplifications of internal variable regions (IVRs) of class 1 integrons confirmed a high prevalence of class 1 integrons with limited diversity of gene cassette arrays including *aadB*, *aadA6-orfD*, and *blaOXA10-aacA4* [157].

Syrmis *et al.* observed a high prevalence of the class 1 integron-associated *aadB* gene cassette in *P. aeruginosa* isolates from an Australian cystic fibrosis patient population. However, some of the strains that possessed the *aadB* gene were tobramycin sensitive. Thus, position of the *aadB* gene alone does not confer tobramycin resistance [158]. In Brazil, 106 *P. aeruginosa* clinical isolates were analyzed and none of the class 1 integrons detected had the *aadB* gene cassette [159].

Other studies showed that some class 1 integrons with more than one gene of resistance also contained the *aadB* gene. For instance, many MDRPA isolates (serotype O4) from various hospitals in the Czech Republic and Hungary shared an integron variable region with the gene-cassette array of *aadB-aadA13*, which may indicate recent international dissemination. In China and Iran, several *P. aeruginosa* clinical isolates had the *aadB* gene cassette array [155, 157, 160].

Although integron associated gene cassettes harbored by *P. aeruginosa* can vary between different geographic locations [158], it is important to take clonal dissemination of the MDRPA strains into account and establish effective measures to prevent their transmission. In addition to integrons, multiresistant bacteria serve as hosts for other genetic elements such as transposons and plasmids. These elements are involved in the development of antibiotic resistance phenotypes and consequently bacteria that contain these elements could be extremely effective vehicles for the dissemination of these resistance elements [146].

4.4. CCCP TOXICITY FOR *P. aeruginosa*

Antimicrobial resistance of *P. aeruginosa* has been shown to be due to several mechanisms and particularly to a series of active efflux pumps such as MexAB-OprM, MexXY-OprM, MexCD-OprJ. When analyzing the actual role of efflux mechanisms in determining bacterial susceptibility it is crucial not only to measure the levels of expression of genes involved, but also to determine the efflux activity by comparing antimicrobial accumulation on bacteria both in the presence and absence of efflux pump inhibitors (EPIs). Among EPIs, the most currently used is carbonyl cyanide m-chlorophenylhydrazone (CCCP). CCCP is a proton ionophore acting as an inhibitor of proton motive force. CCCP is currently used to measure active efflux in Gram-negative bacteria including *P. aeruginosa* [161, 162].

In the course of our work we tried to measure the active efflux in different environmental conditions by using the method of Beyer *et al.* with some modifications (section 3.8) [105].

In experiments with CCCP (10 mg/L concentration), the results apparently showed a predominant role of efflux pumps. However, when comparing with controls in the absence of antibiotics, lethality was indistinguishable, demonstrating that CCCP is highly toxic for *P. aeruginosa* (Fig. 32).

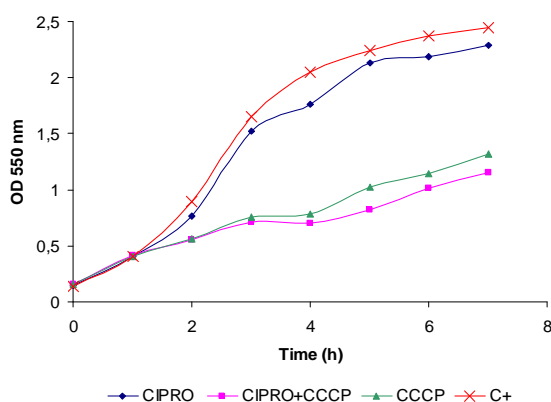


Fig. 32. Growth inhibition assay of a MDR *P. aeruginosa* using CCCP as efflux pump inhibitor. C+ is the control of *P. aeruginosa* without antibiotic nor CCCP.

When inhibition experiments were repeated using the efflux pump inhibitor PA β N (40 mg/L concentration), that competes with antibiotics for binding to the same target or generates steric hindrances that impairs antibiotic binding the results showed active efflux and it was easy to demonstrate extrusion for the different antibiotics assayed but for tobramycin which antagonizes with PA β N [163] (Fig. 33).

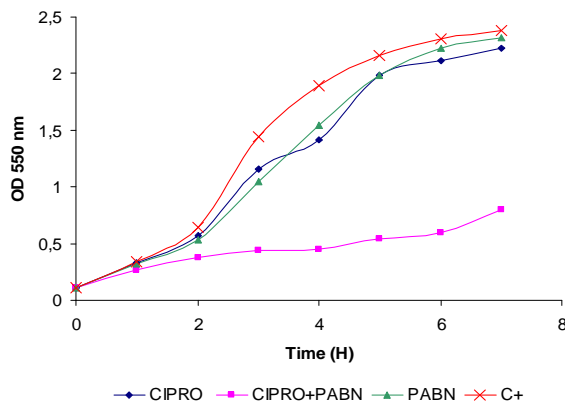


Fig. 33. Growth inhibition assay of a MDR *P. aeruginosa* using PA β N as efflux pump inhibitor.

We were able to demonstrate that CCCP is toxic for *P. aeruginosa* since it inhibits their growth. Therefore its use should be avoided in experiments done with *P. aeruginosa*. Furthermore, although we did not perform experiments it is possible that it should be avoided when studying other respiratory bacteria. It is feasible that the effect of inhibition of proton motive force in a strictly respiratory organism produces such a metabolic alteration that no data obtained in these conditions can be considered as reliable enough. Piddock *et al.* already pointed that accumulation of most quinolones in *E. coli* and *S. aureus* proceeds by simple diffusion, but that *P. aeruginosa* behaves differently. They observed that using CCCP, apparent saturation of quinolone accumulation by *P. aeruginosa* was faster than in other species such as *E. coli* and *S. aureus*. They suggested this could be due to more than one factor such as experimental artefacts because of long periods of time exposure, lysis of

P. aeruginosa due to the chelation of divalent cations in the presence of high quinolone concentrations or transport of quinolones into *P. aeruginosa* mediated by a cytoplasmic membrane carrier protein. Less likely, the apparent saturation of quinolone accumulation could be attributed to structural differences of bacteria [164].

We discourage the use of CCCP and we believe that special caution should be exercised when analyzing reports in which results were obtained from experiments using CCCP with aerobic microorganisms [165, 166, 167]. Furthermore the eventual role of active respiration in the extrusion measurements in facultative microbes should be also revised.

4.5. RESISTANCE TO COLISTIN IN *P. aeruginosa*

4.5.1. DRUG SUSCEPTIBILITY TESTING

Preliminary antibiotic susceptibility testing of *P. aeruginosa* clinical isolates was done initially by using the MicroScan system. Table 17. shows the values, and Table 18 shows their antibiotic susceptibility patterns.

Table 17. MICs obtained by using the MicroScan System for *P. aeruginosa* clinical isolates.

Drug	Isolates					
	296	362	017	493	264	025
MICs (mg/L)						
Amikacin	32	>32	32	>32	≤16	32
Aztreonam	8	8	8	8	8	8
Ceftazidime	16	8	16	16	8	8
Ciprofloxacin	>2	>2	>2	>2	>2	>2
Cefepime	16	16	16	16	16	>16
Gentamicin	>8	>8	>8	>8	>8	>8
Imipenem	>8	>8	>8	>8	8	>8
Pip-Taz	32	16	32	>64	≤16	32
Tobramycin	>8	>8	>8	>8	>8	>8
Levofloxacin	-	>4	>4	>4	-	-
Meropenem	-	>8	8	16	-	8
Colistin	4	>4	4	>4	8	>4

Pip-Taz= Piperacillin/Tazobactam; - No results from MicroScan system

Table 18. Antibiotic susceptibility patterns of *P. aeruginosa* clinical isolates to the antimicrobial agents tested by MicroScan System. S= clinically susceptible; R= clinically resistant; I= clinically intermediate;

Antibiotic	EUCAST/CLSI	Isolates					
		296	362	017	493	264	025
AMK	EU	R	R	R	R	S	R
	CL	I	R	I	R	S	I
ATM	EU	I	I	I	I	I	I
	CL	S	S	S	S	S	S
CAZ	EU	R	S	R	R	S	S
	CL	I	S	I	I	S	S
CIP	EU	R	R	R	R	R	R
	CL	R	R	R	R	R	R
CPM	EU	R	R	R	R	R	R
	CL	I	I	I	I	I	I
GENTA	EU	R	R	R	R	R	R
	CL	R	R	R	R	R	R
IMI	EU	R	R	R	R	R	R
	CL	R	R	R	R	R	R
PIP-TAZ	EU	R	S	R	R	S	R
	CL	-	-	-	-	-	-
TOBRA	EU	R	R	R	R	R	R
	CL	R	R	R	R	R	R
LEVO	EU	-	R	R	R	-	-
	CL	-	R	R	R	-	-
MERO	EU	-	R	I	R	-	I
	CL	-	R	I	R	-	I
COLIS	EU	S	R	S	R	R	R
	CL	I	R	I	R	R	R

EU= Clinical breakpoints by EUCAST; CL= Clinical breakpoints by CLSI

AMK= amikacin; ATM= aztreonam; CAZ= ceftazidime; CIP= ciprofloxacin; CPM= cefepime; GENTA= Gentamicin; IMI= imipenem; PIZ-TAZ= Piperacillin/Tazobactam; TOBRA= tobramycin; LEVO= levofloxacin; MERO= meropenem; COLIS = colistin

Then, these strains and four more clinical isolates of *P. aeruginosa* isolated in the *Laboratori de referència de Catalunya* as colistin-resistant isolates plus a colistin-susceptible strain, were used to explore the

resistance to colistin. This was done by adding EDTA and subsequent MIC determination.

MICs of colistin, EDTA and colistin plus 1 mM of EDTA are shown in table 19.

Table 19. Minimum inhibitory concentration of EDTA, colistin and colistin plus 1 mM of EDTA

Strain:	MICs of EDTA (mM)	MICs of Colistin ($\mu\text{g/mL}$)	MICs of Colistin ($\mu\text{g/mL}$) plus 1 mM EDTA
296	20	8	<0.0625
362	20	8	<0.0625
017	20	8	<0.0625
493	20	32	<0.0625
264	20	8	<0.0625
025	20	8	<0.0625
401	20	8	<0.0625
732	20	8	<0.0625
780	20	8	<0.0625
328S	20	0.5	<0.0625
328R	20	16	<0.0625

These results clearly show that addition of EDTA enhances the susceptibility of *P. aeruginosa* to colistin, whereas MIC values decreased more than 100-fold in the presence of 1 mM of EDTA.

Both EDTA and colistin are considered OM permeabilizers [54]. Mechanisms of action of polymyxins and EDTA are quite different: whereas EDTA acts through cation chelation (mostly divalent cations), polymyxins in general produce disruption of outer membrane (blebs) although they can be taken up by some negatively-charged site in the external layers [168]. Stability of lipid bilayers is based on the role of divalent cations (Mg^{2+} and Ca^{2+}). Polymyxins, and particularly colistin, compete with divalent cations, thus, phospholipids tend to bound to polycation instead its normal divalent cation.

The enhancement of colistin action by EDTA could be explained on the basis that EDTA chelates divalent cation Ca^{2+} and Mg^{2+} and induces drastic disorganization of the OM structure. Thus colistin can act in the absence of their normal competitors. The final outcome resulted in a

complete disorganization of the membranes and obviously it facilitates that colistin can reach the cytoplasmic membrane much easier [169].

This is consistent with the idea that LPS composition, length and electric charges can be significant in the appearance of colistin-resistant variants as well as resistant derivatives to other polycationic compounds. Moreover, the eventual role of total amount of LPS on polymyxin susceptibility should be carefully analyzed.

4.5.2. PFGE

Strains 328S and 328R were isolated from the sputum of the same patient being one of them susceptible and the other resistant. Initially we speculated that such phenomena could be also due to some kind of epigenetic mechanism, thus, in order to explore eventual genetic relationship between strains PFGE analysis was performed. Results showed a significant degree of genetic diversity between both strains (Fig. 34). This indicates that at least in this case resistance did not seem to be caused by an epigenetic mechanism, unless the transient effect of the polycation modifies drastically the expression of a big number of genes. Further experimental work to perform transcriptomics should be done before one can obtain some conclusions.

M 328S 328R

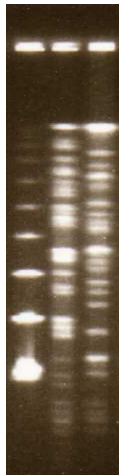


Fig 34. PFGE profiles of *SpeI*-digested chromosomal DNA of MDRPA

4.5.3. ROLE OF SELECTIVE PRESSURE ON COLISTIN RESISTANCE EMERGENCE

Four clinical isolates of MDRPA (all resistant to colistin) were used to test whether it was possible to reverse the resistance when the drug-selective pressure is removed. Susceptibilities to various antibiotics were tested by broth microdilution method at the beginning of the experiment and after several serial passages in antibiotic-free media.

Moreover, strains 328S, 328R and 493 were used to explore the ability of bacteria to become adapted to higher concentrations of colistin. To do this, bacteria were cultivated under conditions in which antibiotic concentration was gradually increased. Results are shown in tables 20 and 21.

Table 20. Evolution of MIC values of *P. aeruginosa* clinical isolates in antibiotic-free conditions.

Isolates									
		401	780		328R		493		ATCC 27853
MICs by broth microdilution method (mg/L)									
Drug	B	A	B	A	B	A	B	A	QC
Meropenem	8	8	1	1	8	8	16	16	1
Ciprofloxacin	128	64	32	16	64	64	32	32	0.12
Tobramycin	128	64	64	32	128	128	128	128	0.5
Gentamicin	>256	>256	>256	>256	>256	>256	>256	>256	0.5
Ceftazidime	32	32	8	8	16	16	8	8	1
Colistin	4	1	4	2	16	2	32	8	1

B = MICs at the beginning A = MICs after several passages without antibiotic

These results suggest that adaptative resistance evolve much faster for colistin than for the rest of the antimicrobial agents tested. After subsequent drug-free passages, there was a four-fold/ eight-fold reduction in colistin MIC, while only a slight reduction was seen in ciprofloxacin MIC, and no reduction in meropenem, tobramycin, gentamicin and ceftazidime MICs.

Table 21. Evolution of colistin-MIC values of *P. aeruginosa* clinical isolates in both antibiotic-free conditions and increasing concentrations of colistin.

Isolates	t=0	MICs (mg/L)	
		20 passages antibiotic-free	20 passages increasing colistin concentration
328S	0.5	-	1000
328R	16	2	2000
493	32	8	2000

Effect of colistin on *P. aeruginosa* was analyzed by comparing morphological and topographical changes. Colistin-susceptible (328S) and colistin-resistant (328R) strains were examined by atomic force microscopy (AFM).

Imaging of both colistin-susceptible and colistin-resistant *P. aeruginosa* after incubation for 36 hours in MHA and MHA supplemented with different concentrations of colistin (0, 4 or 500 µg/mL) were obtained. Visualization showed an increase in outer membrane damage as the concentration of this antimicrobial agent was higher (Fig. 35).

Furthermore, substantial topographical changes such as marked bulges and surface deformations were observed in bacteria upon treatment with 500 µg/mL of colistin (Fig. 35). These alterations were consistent with a remarkable increase in surface roughness of bacteria growth with 500 µg/mL of colistin (24.93 ± 2.1 for strain 328S and 28.58 ± 2.3 for strain 328R) compared with untreated bacteria or bacteria growth with low concentrations of colistin (9.22 ± 3.9 for strain 328S growth without colistin; 11.21 ± 1.8 for strain 328S growth with 4 µg/mL of colistin and 16.9 ± 2.3 for strain 328R growth with 4 µg/mL of colistin).

Mortensen *et al.* [170] evaluated the changes in *P. aeruginosa* morphology and nanomechanical properties due to exposure to colistin in a liquid environment, and found that treatment with this cationic peptide caused an increase in the rigidity of the bacterial cell wall and their surface changed morphologically from smooth to wrinkled. These morphological changes were suggested to be due to the loss of surface

proteins or LPS since lipopolysaccharide is necessary for the initial interaction needed for colistin uptake.

Soon *et al.* [171, 172] observed that surface of colistin-resistant *A. baumannii* strains exposed to high concentration of colistin (32 µg/mL) were smoother than colistin-susceptible ones when exposed to the same concentration of colistin. In our case, surface roughness of both 328R and 328S *P. aeruginosa* able to growth at 500 µg/mL of colistin do not exhibit remarkable differences.

These authors already pointed out that surface of colistin-resistant *A. baumannii* when treated with colistin exhibit similar alterations than the one of susceptible strains. In other words, the ability of colistin to interact with the OM seems to be independent from the final output of antimicrobial/bacteria interaction. This is in agreement with the results observed in intrinsically resistant species. For instance, *S. marcescens*, which is naturally resistant to polymyxins can modify some behaviors in the presence of colistin and related antimicrobials. Viñas *et al.* [173], demonstrated that addition of polymyxin enhances the synthesis of prodigiosin in the presence of L-proline in non proliferating cultures. This strongly suggests that injuries in outer membrane are needed for antimicrobial effect of polymyxins but are not enough to limit the viability of treated bacteria. In summary, although it seems that interaction between colistin and LPS is the first step of colistin antimicrobial action, it becomes apparent that bactericidal effect is due to the injuries induced in cytoplasmic membrane. Thus, LPS role in resistance is probably a consequence of its capacity to prevent the entry of colistin and the subsequent interaction between the antimicrobial and the membrane.

Our AFM results obtained for *P. aeruginosa* confirmed this idea, since AFM images of colistin-susceptible and -resistant strains revealed similar surface disruptive effects.

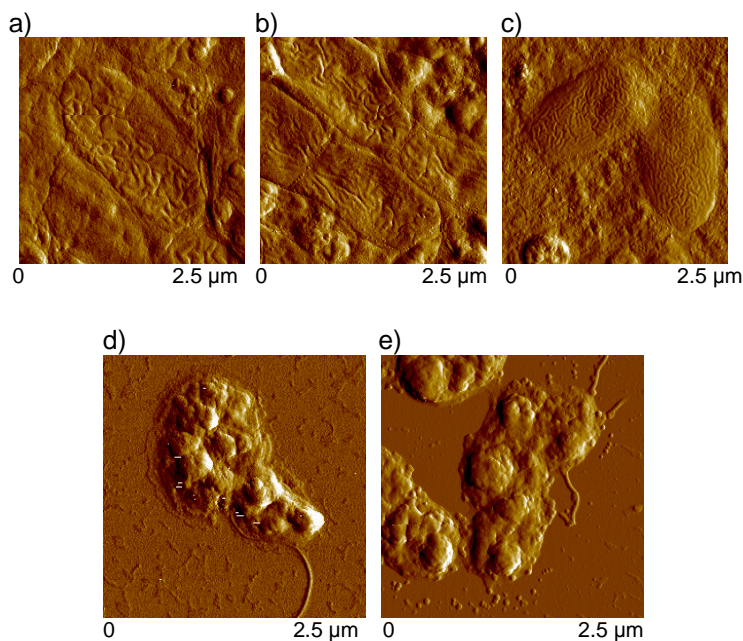


Fig. 35. AFM amplitude images of colistin-susceptible, colistin-resistant and ATCC 27853 *P. aeruginosa*. Bacteria were visualized in air by using the Atomic Force Microscope XE-70 (Park Systems) previously air dried at RT in a dust-free environment and images were collected in Non-contact mode using pyramidal-shaped silicon cantilevers. a) *P. aeruginosa* 328S growth without colistin; b) *P. aeruginosa* 328S growth with 4 mg/L of colistin; c) *P. aeruginosa* 328R, growth with 4 mg/L of colistin; d) *P. aeruginosa* 328S, growth with 500 mg/L of colistin and e) *P. aeruginosa* 328R, growth with 500 mg/L of colistin.

Due to these morphological consequences of colistin action on the surface properties of treated bacteria, it seemed of interest to explore the composition of LPS in order to compare LPS molecules from resistant and susceptible isolates and or derivatives. Figure 36 shows that LPS profiles of “original” clinical isolates, isolates after several passages without antibiotic and isolates that survived a concentration of colistin of 500 µg/mL were considerably different from that of ATCC 27853. O-side chain length from the control strain (ATCC 27853) resulted to be shorter than those from clinical isolates. On the contrary LPS profiles of both 328S at the beginning grown without colistin, after several serial passages by increasing colistin concentrations up to 500 µg/mL, grown with 4mg/mL of

colistin, and after twenty passages without colistin did not show noticeable differences with its correspondent resistant derivative (328R) after several serial passages increasing colistin concentration until 500 µg/mL, at the beginning growth with 4mg/mL of colistin, after twenty passages without colistin; and after several serial passages increasing colistin concentration until 500 µg/mL. Apparent differences are due to the differences in LPS concentration. Wells in the electrophoresis gels were filled with equivalent samples as determined by protein concentration (Bradford), thus, it seems that in the presence of colistin the amount of LPS is reduced and that at high concentrations (500 µg/mL) this reduction is drastic. This would be in agreement with conditions similar to the ones already described as heteroresistance. If one assume that both bacterial models (one with a regular LPS and another with a scarced LPS) already exists in the primary isolate, then the mechanism should be interpreted as a strict phenomenon of heteroresistance. In conditions in which scarced LPS are better adapted the rest is rapidly eliminated and replaced by resistant biotype. Whereas in non-selective conditions both models will survive. This is also in agreement with the fact that 328R and 328S were both isolated simultaneously from the same patient. We thus, hypothesize a heteroresistance phenomenon to explain colistin-resistance in *Pseudomonas aeruginosa*. A few months ago Bergen *et al.* [174] also pointed out a similar conclusion based on the study of synergism between imipenem and colistin.

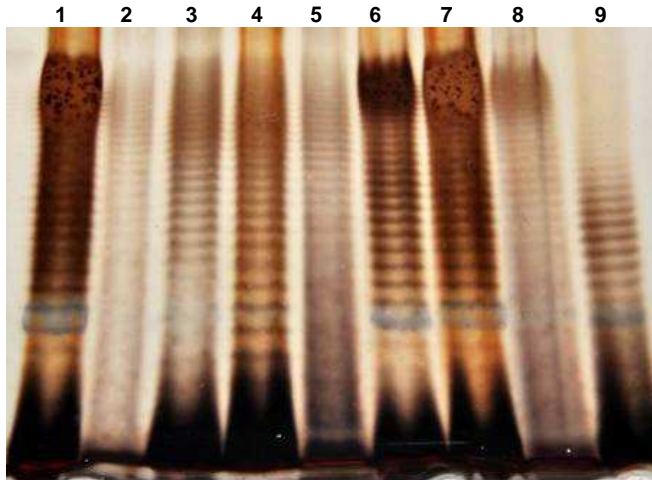


Fig. 36. SDS-PAGE analysis of *P. aeruginosa* LPS. 1) 328S at the beginning growth without colistin; 2) 328S after several serial passages increasing colistin concentration until 500 $\mu\text{g}/\text{mL}$; 3) 328S at the beginning growth with 4 mg/mL of colistin; 4) 328S after twenty passages without colistin; 5) 328R after several serial passages increasing colistin concentration until 500 $\mu\text{g}/\text{mL}$; 6) 493 at the beginning growth with 4 mg/mL of colistin; 7) 493 after twenty passages without colistin; 8) 493 after several serial passages increasing colistin concentration until 500 $\mu\text{g}/\text{mL}$; 9) ATCC 27853

In order to see eventual differences between both strains in protein-profiles, we examined OMPs by PAGE. No noticeable differences in OMP electrophoretic profiles were observed. Thus it seems unlikely that OMPs could play any significant role in colistin resistance.

4.5.4. CONTRIBUTION OF EFFLUX PUMPS IN COLISTIN RESISTANCE

For the analysis of the contribution of efflux pumps in colistin resistance, the two colistin-resistant isolates 328R and 493 were used.

In the course of the study we tried to measure the active efflux by using the method of Beyer *et al.* with some modifications [105]. Inhibition experiments were performed with colistin (1/4 MIC) and colistin (1/4 MIC) plus PA β N (40 $\mu\text{g}/\text{mL}$). The efflux pump inhibitor CCCP was not used, as we previously demonstrated its toxicity for *P. aeruginosa*.

The results showed (Fig. 37) that colistin efflux does not exist, and therefore, it seems reasonable to suggest that efflux pumps do not play any role in resistance to colistin.

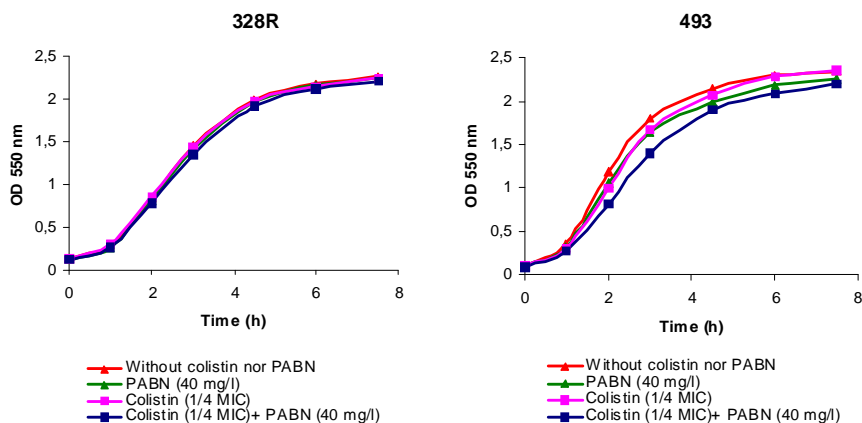


Fig. 37. Growth inhibition curves of colistin-resistant *P. aeruginosa* at a concentration of $\frac{1}{4}$ MIC of colistin in the presence and absence of PABN. a) *P. aeruginosa* 328R b) *P. aeruginosa* 493

4.5.5. MODEL MEMBRANE STUDIES

A possible approach to enlarge knowledge of the intimate mechanism of colistin action is to explore membrane damaging by electrophysiology. During the period of this research we had the possibility to initiate the exploration of colistin action on the artificial membranes by using the single channel conductance measurements technique. Membranes were constructed as described previously, using different proportions of the zwitterionic lipid DiphPC and the anionic lipid PS, both in n-decane. In Fig. 38, some illustrative recordings of the channel-forming behavior of colistin in 1% DiphPC/n-decane membranes are presented.

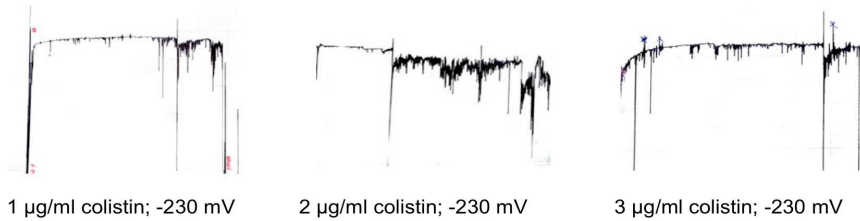


Fig. 38. Chart recorder tracings of conductance events that occurred upon the addition of different concentrations of colistin to the solution (1 M KCl) bathing a planar lipid bilayer. The indicated voltages were applied. 1% DiphPC/n-decane membranes. Approximately 3 min of chart recordings is shown.

Fig. 39. shows chart recording tracings of conductance events after addition of different concentrations of colistin in 0.8% DiphPC plus 0.2% PS/n-decane membranes.

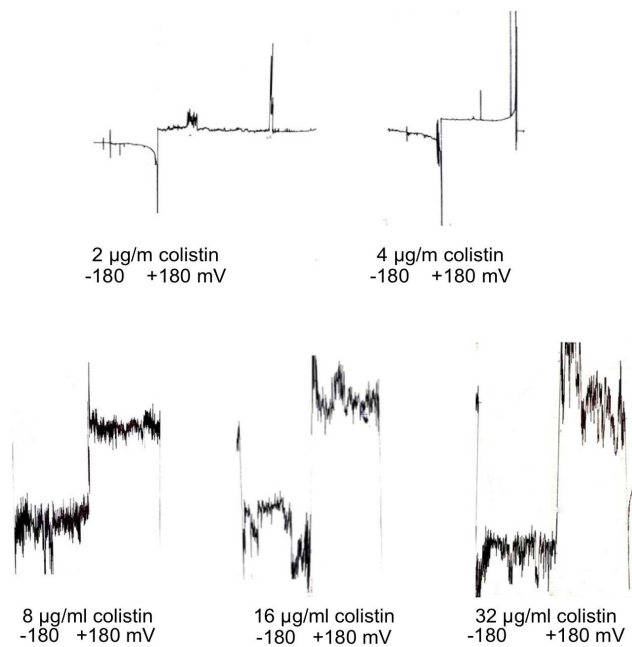


Fig. 39. Chart recorder tracings of conductance events that occurred upon the addition of different concentrations of colistin to the solution (1M KCl) bathing a planar lipid bilayer (0.8% DiphPC in n-decane). The indicated voltages were applied. Approximately 3 min of chart recordings is shown. Approximately 3 min of chart recordings is shown.

Similar to other studies with antimicrobial peptides [175], we observed that these peptides required large concentrations (approximately 1 $\mu\text{g}/\text{mL}$ or more) to permit the observation of reasonable numbers of channels. All the studies in the present work were performed using concentrations of colistin between 1 and 32 $\mu\text{g}/\text{mL}$. Certain generalizations could be made regarding the observed activities.

First, it is required a high voltage across the membrane. In general, voltages above ± 180 mV initiated conductance events. When the applied voltage was subsequently decreased to ± 130 mV, conductance events were still observed but often at a lower frequency.

Second, contrary to other studies [175], conductance events in DiphPC planar bilayer membranes were initiated by both positive and negative applied voltages (e.g., ± 180 mV). Other studies has obtained similar results for cationic antimicrobial peptides such as tritrypticin peptide [176], exhibiting ionic channel forming activity in both negative and positive voltages.

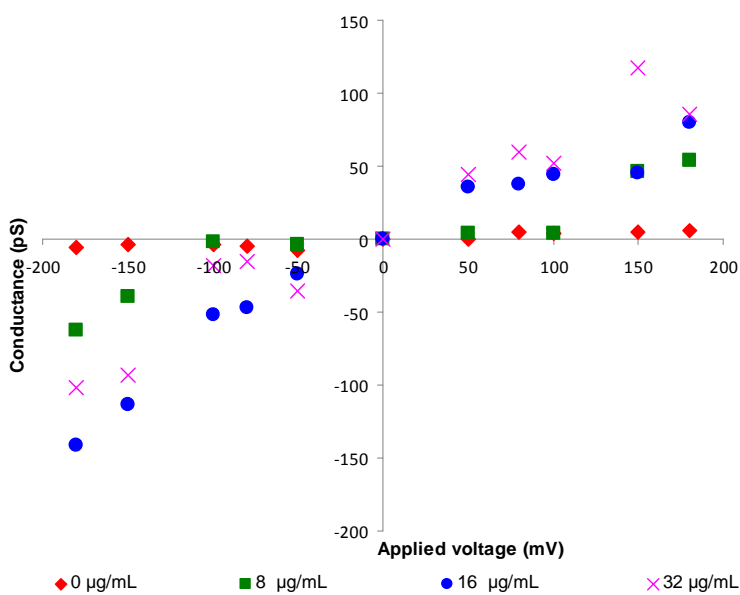
Third, for the same concentrations of colistin, conductance events in DiphPC/n-decane planar lipid bilayers were observed less frequently and were less relevant than in planar lipid bilayers containing negatively charged lipid PS. This result seems reasonable given the positive charge on the peptide.

Fourth, most of the events observed involved very rapid conductance alterations which were widely variable in magnitude (Figures 38 and 39). There were some situations where substantial increases in transmembrane conductance were observed for a short period of time, followed by a fast return to the conductance base line (Fig. 39).

Fifth, some of the experiments performed clearly showed that channel-like conductance events increase with higher concentrations of colistin (Fig. 40.a). However, the repeatability of these experiments was low, obtaining some contradictory results (Fig. 40.b). This would indicate the need to refine the method to obtain reliable data.

40.a)

Voltage (mv)	Concentration ($\mu\text{g/mL}$)			
	0	8	16	32
0	0	0	0	0
50	0	4	36	44
-50	-8	-4	-24	-36
80	5		38	60
-80	-5		-48	-15
100	4	4	44	52
-100	-4	-2	-52	-18
150	5	46	45	117
-150	-4	-40	-114	-94
180	5	54	80	85
-180	-5	-63	-141	-102



40.b)

Voltage (mv)	Concentration ($\mu\text{g/mL}$)			
	0	8	16	32
	Conductance (pS)			
0	0	0	0	0
50	8	4	12	-4
-50	0	-4	-8	-8
80	5	2	8	5
-80	-5	-2	-10	5
100	4	2	2	4
-100	-2	-6	-10	-6
150	4	58	17	4
-150	-4	-4	-22	-4
180	4	26	5	4
-180	-4	-28	-11	-6

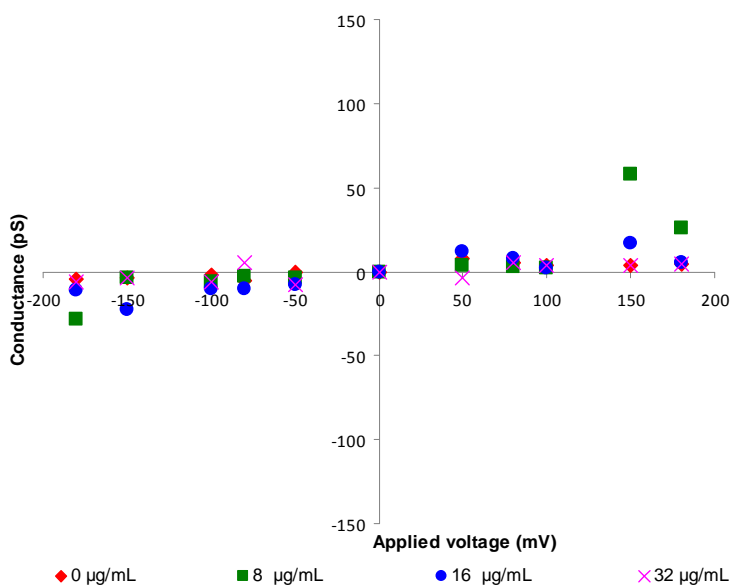


Figure 40.a and 40.b. Relationship between conductance and voltage of two independent experiments with 0.8% DiphPC plus 0.2% PS/n-decane and increasing concentrations of colistin.

It was also observed that conductance events begin at lower voltages when the concentration of colistin is higher. Therefore, in membranes constructed from 0.8% DiphPC plus 0.2% PS/n-decane and a concentration of colistin of 8 $\mu\text{g/mL}$, no remarkable conductance events were observed until voltages equal or superior to +150 mV, meanwhile for colistin concentrations of 32 $\mu\text{g/mL}$ increments of conductance started at +50 mV (Fig. 40).

Polymyxins are old antimicrobials whose use was reintroduced a few years ago to treat severe infections caused by *P. aeruginosa*. Then, it seemed a promising group of molecules; however, adaptative resistance was promptly described.

Many studies have been recently published with the aim to elucidate the main factors involved in this type of resistance. Modifications in the lipid A region of the LPS molecule as well as the recently identified two-component regulator ParR-ParS [32], mutations in *phoQ* and PmrB [177, 178] have been suggested to play a decisive role in the acquisition of colistin-resistance. Moreover, ParR-S seems to play a significant role in determining resistance to at least four classes of antimicrobials (polymyxins, aminoglycosides, β -lactams and fluoroquinolones) and its role seems to be played through the modification of three mechanisms of resistance (LPS modification, increase in drug efflux and reduction in porin pathway) and also by modifying the regulation of MexXY/OprM [179]. According to our results, LPS is modified, but not efflux machinery function. If there is some alteration in the protein pathway we were unable to visualize in electrophoresis analysis of porins.

Our work suggests that the use of colistin for multidrug-resistant *P. aeruginosa* may contribute to the emergence of a colistin-heteroresistant phenotype. The presence of subpopulations in primary isolates with less amount of LPS and consequently less sites for polymyxin binding might proliferate preferentially during the colistin treatment and lead to the treatment failure or recurrence infection. Thus, treatments with colistin concentrations below the MIC of the microorganisms might increase the emergence of resistant bacteria during exposure to the drug.

The optimization of colistin administration regimens with appropriate higher dosages to suppress amplification of the colistin-resistant subpopulations or the use of synergic combinations of antimicrobial agents might significantly reduce the treatment failure.

Conductance measurements in planar lipid membranes can be considered a useful tool for the study of the channel forming activity by both antimicrobial peptides and bacterial porins. One of the main advantages of these assays is its specificity and sensitivity, although in the case of antimicrobial peptides, the required concentration to observe a reasonable number of conductance events is larger (for example $\geq 8 \mu\text{g/mL}$ for colistin; $\geq 1 \mu\text{g/mL}$ for Gramidicin [175]) than the concentration required for the analysis of porins (for example 5 ng/mL for *K. oxytoca* [180]; 50 ng/mL for the 28 kDa cell wall channel protein of *Streptomyces griseus* [181]).

In the present study, the addition of colistin in 0.8% DiphPC (zwitterionic lipid) plus 0.2% PS (negatively charged lipid)/n-decane planar lipid bilayers induced irregular fluctuations of current.

In general, increments in conductance for colistin and other antimicrobial peptides are irregular, unstable and present fast openings and closings of pores. This is in contrast to the conductance events observed for the majority of channel-forming proteins, which tend to be regular, stable and in the form of step (stepwise manner).

Cationic peptides are generally able to interact electrostatically with the negatively charged headgroups of bacterial phospholipids and then insert into the cytoplasmic membrane, forming conductance events which are proposed to lead to the leakage of cell contents and cell death [175].

In accordance with the general model proposed by Matsuzaki *et al.* [182], antimicrobial peptides bind to the outer leaflet of model membranes and flip inward, carrying lipids with them and creating brief disruptions in permeability.

Besides, Wu *et al.* [175] suggested that these compounds contain irregular aggregates of peptide molecules within the membrane that will form in a concentration- and voltage-dependent manner creating informal

aqueous channels. These informal aqueous channels probably allow the passage of at least ions and possibly larger molecules. As long as these supramolecular complexes are of variable size and stability, this would explain the observed variations in both the magnitude and duration of conductance events observed in our and other planar bilayer studies.

4.6. IMIPENEM RESISTANCE IN CLINICAL ISOLATES OF *P. aeruginosa*

4.6.1. MINIMUM INHIBITORY CONCENTRATIONS

Antimicrobial susceptibilities were determined for strains PA110514 and PA116136. Besides, to study the stability of the resistant phenotype in the absence of antimicrobial selective pressure, a single colony of the imipenem-resistant *P. aeruginosa*, PA116136, was serially transferred 30 times on antibiotic-free medium (TSB) incubated overnight at 37 °C.

Susceptibilities to imipenem and colistin were tested by both methods of disk diffusion test and broth microdilution test, and susceptibilities for the rest of antimicrobial agents were tested by the method of broth microdilution test. Results are in tables 22 and 23.

Table 22. Susceptibilities to antimicrobial agents tested by broth microdilution test

Antibiotics	PA110514		PA116136			
	Before imipenem treatment		After imipenem treatment and before passages without antibiotic		After 30 passages without antibiotic	
	MIC (µg/mL)	Suscept.	MIC (µg/mL)	Suscept.	MIC (µg/mL)	Suscept.
Tobramycin	0.5	S	0.5	S	0.5	S
Gentamicin	0.5	S	1	S	1	S
Meropenem	4	S	4	S	4	S
Ciprofloxacin	0.12	S	0.12	S	0.12	S
Ceftazidime	1	S	1	S	1	S
Imipenem	4	S	16	R	16	R
Colistin	0.5	S	1	S	1	S

S= Susceptible, I= Intermediate and R= Resistant by CLSI M100-S20

Results and discussion

Table 23. Susceptibilities to antimicrobial agents tested by disk diffusion test

Antibiotics	PA110514		PA116136			
	Before imipenem treatment		After imipenem treatment and before passages without antibiotic		After 30 cultures without antibiotic	
	Ø (mm)	Susceptibility	Ø (mm)	Susceptibility	Ø (mm)	Susceptibility
Imipenem 10 µg	29*	S*	13	R	16	R
Colistin 10 µg	20	S	20	S	16	S

S= Susceptible, I= Intermediate and R= Resistant by CLSI M100-S20. (*) Some “satellite” colonies were observed at higher concentration of colistin.

When susceptibility to imipenem 10 µg was tested for PA110514, it was observed that few bacteria were able to resist higher concentrations of imipenem. These observations indicated the possible heterogeneity of the strain.

Some of the satellite colonies within imipenem inhibition zones (red arrow in Fig. 41) were isolated and named as AR1, AR2, AR3 and AR4, and some of the bacteria grown at lower concentration of imipenem were also isolated and named as AS1, AS2, AS3 and AS4 to explore the evolutionary mechanism by which the clinical isolate PA110514 yields imipenem-resistant derivatives such as PA116136, AR1, AR2, AR3 and AR4, or imipenem-susceptible derivatives such as AS1, AS2, AS3 and AS4.

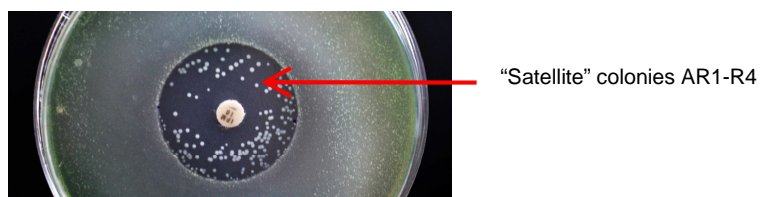


Fig. 41. Susceptibility to imipenem 10 µg of PA110514

Using the method of disk diffusion test AS1, AS2, AS3 and AS4 had the same antibiogram than strain PA110514, and using the method of broth

microdilution test, all of them had a MIC for imipenem of 4 µg/mL, again the same result than the one obtained for the original strain PA110514. However, using the method of disk diffusion test, AR1, AR2, AR3 and AR4 had the same antibiogram than strain PA116136, which was isolated once the patient finished the treatment with imipenem (Fig. 42). Using broth microdilution test, all these isolates had a MIC for imipenem of 16 µg/mL, again the same result than the one obtained for the original strain PA116136.

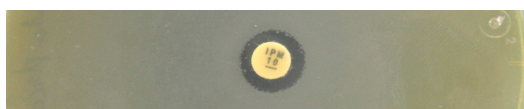


Fig. 42. Disk diffusion test to imipenem 10 µg in strain PA116136. Results are the same for isolates AR1, AR2, AR3 and AR4.

4.6.2. OUTER MEMBRANE PROTEIN PROFILES

Electrophoretic comparisons of OMPs demonstrated that the susceptible strain PA110514 and their susceptible derivatives (AS1-AS4) showed a band corresponding to the porin OprD in the outer membrane that it was not visible in the resistant strain PA116136 and their derivatives (Fig. 43).

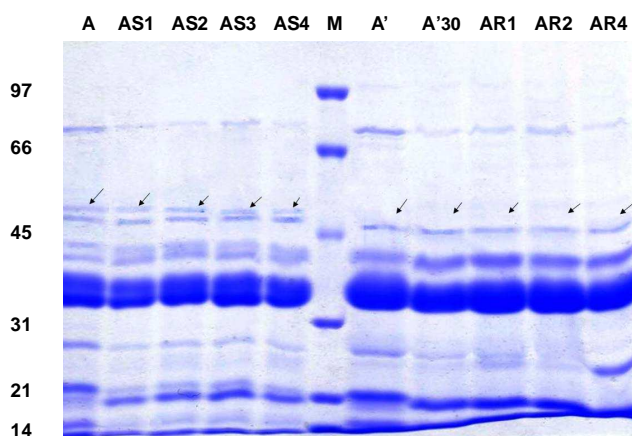


Fig. 43. SDS-PAGE analysis of Outer Membrane Proteins (OMPs) of *P. aeruginosa*. M: Molecular mass standards in kDa. Position of OprD is indicated by arrows on the left. A: PA110514. A': PA116136. A'₃₀: PA116136 after 30 cultures without antibiotic.

4.6.3. *oprD* AMPLIFICATION

To investigate the mechanism responsible for the loss of OprD expression, *oprD* genes of PA110514, PA116136 and their derivatives were amplified, sequenced and their sequences compared.

Size of amplicons of *oprD* gene region from PA110514, AR1, AR2, AR3, AR4, AS1, AS2, AS3 and AS4 and PA116136 were larger than that of strain ATCC 27853 (Fig. 44). The predicted length of *oprD* is 1586 bp, while the amplicons from PA110514, AS1, AS2, AS3 and AS4 were 3000 bp and from PA116136, AR1, AR2, AR3 and AR4 were 2000 bp.

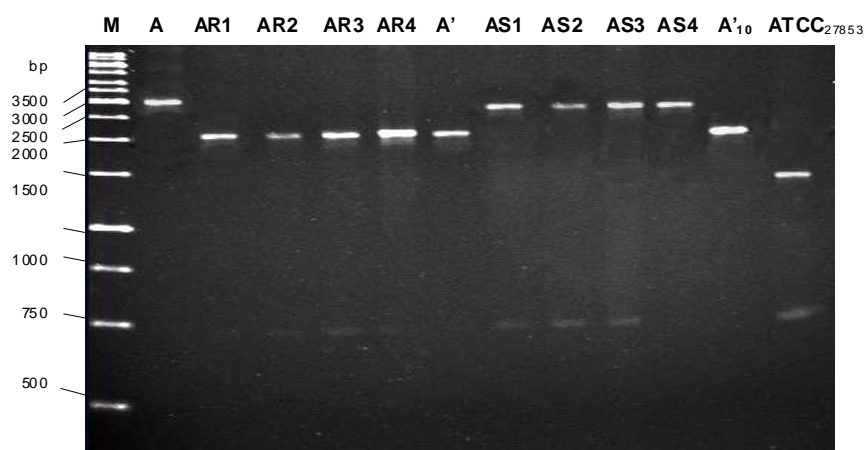


Fig. 44. PCR amplification products obtained with primers OprDSEQF1 and OprDSEQR1. Fragments were separated by electrophoresis through a 1.5% agarose gel. M: *GeneRuler*TM 1 kb DNA Ladder (Fermentas, Lithuania). A: PA110514. A': PA116136. A'₃₀: PA116136 after 30 cultures without antibiotic. ATCC₂₇₈₅₃= strain ATCC 27853, used as control.

Prior work in our laboratory [130] revealed that amplicons of PA110514 and PA116136 had a long insert, 1300 bp approximately. The same insert was later found in amplicons of A'₃₀, AS1, AS2, AS3, AS4, PA116136, AR1, AR2, AR3 and AR4. In all amplicons, insert had 59.6% G+C content. This insert was designated as ISPa133 and showed high homologies with the mobile elements included into the IS3 family of insertion sequences.

The similarities were not only in terms of the length of the ISPa133, that for members of the IS3 family is typically between 1200 and 1550 bp, but also due to the presence of two characteristic open reading frames (ORF), *orfA* and *orfB*, which did not overlap. The insertion element was flanked by 25-bp terminal inverted repeats (IRs), with TG at the 5'end and CA at the 3'end, as in other families of the IS3 family. However, short directly repeated sequences (DR) of the target DNA flanking the IS, were not detected on ISPa133 insertion.

The first ORF (*orfA*) encoded a transposase. In the search for conserved domains [183], we determined that the protein includes a helix-turn-helix motif (HTH), a family of DNA-binding domains unique in bacteria, and that it showed high identity to the transposase 8 family, some of which are members of the IS3 family. The second ORF (*orfB*) encoded the central catalytic domain of an integrase (Fig. 45).

```

0001 ttgaactaggtctaattggagcggacaccccaggtgaagcctcactgaggcattggag
0061 gtgttcatgaccaagaagacgcgacgtcggctactccgaccaattcaaggctgaggcggtc
      M T K K T R R R Y S D Q F K A E A V
0121 aacatggttcgaggagagggttatccatctccgaggctgccaggcggctggatcgat
      N M V R G E G Y A I S E A A R R L D I D
0181 cgcagcctgctagatcgctggtgcgccagcaacgtgaccgggaggatggtgtgactgac
      R S L L D R W C R Q Q R D R E D G V T D
0241 gaccaggcagatgagcgggatccgagatcaagaaacttcgcaagaagttcgtaaagtg
      D Q A D E R D A E I K K L R E E V R K L
0301 cggattgaaaaggaggttttaaaaaggccacggcctctctcgcgagagagtgagctga
      R I E K E V L K K A T A F F A R E S S *
0361 gataccagttcatcgagtcgggagaaggcctacttcccagtgggcgtgctgtgccgtgtca
0421 tggacgtcagccgagtgcttctatgctggtcagcgtgaaccgatgagcagcgc
      M D V S R S G F Y A W R Q R E P D E Q R
0481 agtgcctgcaccgggagggtcaaggacatccacgatcagaaacgtgtagctatgggagcc
      Q C L H R E V K D I H D Q K R G S Y G S
0541 ggcgaaatggccaaggaactccgtcgcagaggtcatgcggtcgtctatcaggcgcgta
      R R M A K E L R R R G H A V G R Y Q A R
0601 gcctgatgcaggaagcgggtgctgcctgccgacagcgtcggcctaccggcatacagcgg
      S L M Q E A G V A C R Q R R R Y R H T T
0661 acagcgaccacgggctgccagtggtcccaacctgctcaagcggcagttcacggctcctg
      D S D H G L P V A P N L L K R Q F T V P
0721 agcccaatcagcgtgggtggcggacatcactgccatttggaccttgaaggctgggtg
      E P N Q D E R V A D I T A I W T L E G W L
0781 tacctggcggcctgctggaacctctacgacccgacagggtcatcggctgggacctggccgac
      Y L A A V L D L Y D R Q V I G W A M A D
0841 catatgaagcgtcactcaccctggacgacctggagatggcaattggccgacggcct
      H M K T S L T L D A L E M A I G R R R P
0901 ccccgggagtgctccaccactcgggtcggcggcagcccgtacgcctctcgtgcgtatcgc
      P R G V L H H S D R G S Q Y A S R A Y R
0961 gaacgactggcaagcacgggttccaggcctcctatgagccgcaaggggaaactgttgggac
      E R L A K H G F Q A S M S R K G N C W D
1021 aatgcggtcatggagccttctcggctctgaagagcgaatggctggagggacagcgg
      N A V M E R F F G S L K S E W L E G Q R
1081 tattggaccgcccagtcagcggcggcagcgtcgtgacctatcagatggagatcaac
      Y W T R Q S A R R D V V T Y I E M E Y N

```

Transposase
orfA

Integrase-like
orfB

```

1111 agctgccggctccactcgacccttggtaccacacgccgagggagatcgagaaaatcgcg |
      S C R L H S T L G Y H T P R E I E K I A
1201 gaagcagcttaataaaaagtgtccgctacgacttgaccagaacatccctgtacgggtgctga | OprD
      E A A *
1261 actcgaagacatctatcgtcag

```

Fig. 45. Nucleotide sequence of the IS3-like element, ISPa133, present in PA110514, PA116136, A'₃₀, AR1, AR2, AR3 and AR4, AS1, AS2, AS3 and AS4. The underlined sequences represent the 25-bp inverted repeats (IRs). Start codons of both orfs encoded by the mobile element are in red; the amino acid sequence of the two open reading frames are shown below the sequence. Sequence of OprD is indicated in green.

Integrase mediates the integration of a DNA copy of the viral genome into the host chromosome. The enzyme is composed of three domains. The amino-terminal zinc-binding domain (pfam02022) is the central catalytic domain. The carboxyl terminal domain is a non-specific DNA-binding domain (pfarn00552). The catalytic domain acts as an endonuclease when two nucleotides are removed from the 3' ends of the blunt-ended viral DNA resulting from reverse transcription. This domain also catalyzes the DNA-strand-transfer reaction of the 3' ends of the viral DNA to the 5' ends of the integration site.

The large insert located in PA116136, A'₃₀, AR1, AR2, AR3, AR4, AS1, AS2, AS3 and AS4 was 99% identical to that found in PA110514. However, the location of IS element into oprD was different. As we previously described, in PA110514, ISPa133 was located 56 nucleotides upstream of the translational start codon, the same position where this insert was later found in AS1, AS2, AS3 and AS4.

However, in PA116136 and A'₃₀, the insert was located further down, immediately before the nucleotide 697, replacing them and therefore removing the first 232 amino acids of the porin OprD. In AR1, AR2, AR3 and AR4 the insert was located some nucleotides before, removing the first 222 amino acids of the porin OprD.

Schematic representation of the location of IS elements with respect to the *oprD* gene of *P. aeruginosa* PAO1 is shown in figure 46, and alignments of the amino acid sequences of OprD protein are shown in figure 47.

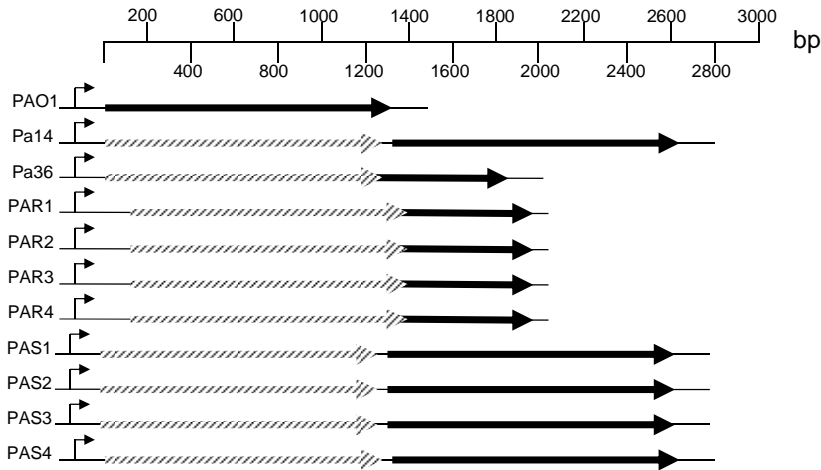


Fig. 46. Schematic diagram of the location of IS elements with respect to the *oprD* gene of *P. aeruginosa* PAO1. The solid arrow represents the *oprD* structural gene while the striped arrow represents the ISPa133.

		L1	
PaO1	MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNRYFNDRGKSGSG	60	
PA110514	MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNRYFNDRGKSGSG	60	
PA116136	-----	60	
A ₃₀	-----	60	
AS1, 2, 3, 4	MKVMKWSAIALAVSAGSTQFAVADAFVSDQAEAKGFIEDSSLDLLLRNRYFNDRGKSGSG	60	
AR1, 2, 3, 4	-----	60	
		L2	
PaO1	DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKTGTGNLPVMNDGKPRDDY	120	
PA110514	DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKSGTGNLPVMNDGTPRDDY	120	
PA116136	-----	120	
A ₃₀	-----	120	
AS1, 2, 3, 4	DRVDWTQGFLTTYESGFTQGTVGFGVDAFGYLGLKLDGTSDKSGTGNLPVMNDGTPRDDY	120	
AR1, 2, 3, 4	-----	120	
		L3	
PaO1	SRAGGAVKVRISKTKLKWGEMQPTAPVFAAGGSRLFPQTATGFQLOSSEFEGLDLEAGHF	180	
PA110514	SRAGGAVKVRISKTKLKWGEMQPTAPVFAAGGSRLFPQTATGFQLOSSELEGLDLEAGHF	180	
PA116136	-----	180	
A ₃₀	-----	180	
AS1, 2, 3, 4	SRAGGAVKVRISKTKLKWGEMQPTAPVFAAGGSRLFPQTATGFQLOSSELEGLDLEAGHF	180	
AR1, 2, 3, 4	-----	180	
		L4	
PaO1	TEGKEPTTVKSRGELYATYAGETAKSADFIGGRYAITDNL SASLYGAELEDIYRQYYLNS	240	
PA110514	TEGKQGTTTKSRGELYATYAGETAKSADFIGGRYAITDNL SASLYGAELEDIYRQYYLNS	240	
PA116136	-----YRQYYLNS	240	
A ₃₀	-----YRQYYLNS	240	
AS1, 2, 3, 4	TEGKQGTTTKSRGELYATYAGETAKSADFIGGRYAITDNL SASLYGAELEDIYRQYYLNS	240	
AR1, 2, 3, 4	-----SLYGAELEDIYRQYYLNS	240	

Results and discussion

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                                L5
Pa01  NYTIPLASDQSLGFDFNIYRTNDEGKAKAGDISN  TTWSLAAAYTLDAHTFTLAYQKVHGD  300

                                L6
Pa01  QPFDYIGFGRNGSGAGGDSIFLANSVQYSDFNGPGEKSWQARYDLNLASYGVPGLTFMVR  360

                                L7
Pa01  YINGKDIDGTKMSDNNVGYKNGYGEDGKHHETNLEAKYVVQSGPAKDLSFRIRQAWHRA  420

                                L8
Pa01  NADQEGEQNEFRLIVDYPLSIL

```

Fig. 47. Alignment of the amino acid sequence of OprD protein of PA110514, PA116136, A'₃₀, PA110514R1 (AR1), PA110514R2 (AR2), PA110514R3 (AR3) and PA110514R4 (AR4), PA110514 (AS1), PA110514S2 (AS2), PA110514S3 (AS3) and PA110514S4 (AS4). In bold and underlined is indicated loops (L) position. Hyphens indicate absence of amino acids.

As can be seen in the previous figure, insert ISPa133 in PA110514, AS1, AS2, AS3 and AS4 is located before *oprD* gene, thus OprD is not truncated and consequently, imipenem can enter inside bacteria via this porin. Nevertheless, OprD protein of PA116136 and A'₃₀ begin in the last amino acid of the loop 4 and OprD protein of AR1, AR2, AR3 and AR4 begins in the third amino acid of the same loop, therefore, this protein is not able to arrive to the membrane and imipenem is not able to enter inside bacteria. Carbapenems are a class of β -lactam antibiotics with good antimicrobial activity against *P. aeruginosa*; they are often used as a last resource to treat infections due to multidrug-resistant strains [184]. However, the emergence and spread of acquired carbapenem resistance have challenged therapeutic and control efforts [185], thus, a better understanding of the molecular mechanisms underlying resistance is needed. Moreover, it seems likely that several mechanisms are involved in carbapenem resistance [186]. The two clinical isolates of this study, PA110514 and PA116136 and their susceptible and resistant derivatives, were identical in their PFGE profiles and in biochemical tests, suggesting their close relationship. The unique noticeable difference between the isolates was in their susceptibility to imipenem. We hypothesized that this difference involves OMPs. This is reinforced by the results of SDS-PAGE, which revealed the loss of OprD in PA116136 and derivatives. To

investigate the mechanism responsible for the loss of the porin, the *oprD* gene of each strain was amplified and sequenced. Examination of these sequences revealed the presence of a new insertion sequence, ISPa133, located 56 nucleotides upstream of the *oprD* start codon in PA110514, AS1, AS2, AS3 and AS4. However, since this isolate is imipenem-susceptible, the presence of ISPa133 has no obvious effect on the upstream regulatory region of the gene, as *oprD* expression was normal (Fig. 37) as was the expression of the porin OprD. By contrast, in PA116136 and A'₃₀ the insertion element (99% identity) is located immediately before nucleotide position 696, which causes the removal of the first 232 amino acids of OprD and in AR1, AR2, AR3 and AR4 the insert was located some nucleotides before, removing the first 222 amino acids of the porin OprD.

Subsequently, the protein was not expressed and was not detectable in the gels (Fig. 36). Since loss of the protein prevents the entrance of imipenem, strain PA116136, A'₃₀, AR1, AR2, AR3 and AR4 are fully resistant to the antibiotic.

To date, the presence of ISs in OprD has been reported only once, although these elements have been detected in other genes of *P. aeruginosa* [187, 188, 65]. In those cases, the ISs were described in resistant isolates and thus assumed to be the cause of resistance, either via gene activation or by inducing the high level expression of a potential resistance gene.

Based on the findings of this study, we propose that the identified insertion element ISPa133 acts as a switch, depending on the degree of selective pressure exerted by imipenem. In the absence of selective pressure, as was the case during isolation of strain PA110514 from an infected, untreated patient, OprD is found on the OM and the position of ISPa133 in the genome has no effect on *oprD* expression; however, selective pressure exerted by antibiotic therapy results in the selection of strains in which ISPa133 moves within the *oprD* gene, such that, in the case of strain PA116136, the first 232 amino acids are removed, thus preventing expression of the gene. A high rate of ISPa133 jumping would

therefore provide a major selective advantage, one that allows the bacterium to survive in the presence of carbapenems.

It has been proposed that ISs without DRs may simply result from the homologous inter- or intra-molecular recombination between two IS elements, each with a different DR sequence, or from the formation of adjacent deletions arising from duplicative intramolecular transposition [189]. For ISPa133, this could explain the lack of DRs as well as the absence of a crossover region between *orfA* and *orfB*. As shown in other species, experiments mimicking natural conditions can provide insight into population phenomena [190]. This was the aim of experiments in which strain PA110514 was submitted to selective pressure by imipenem in culture flasks in order to obtain spontaneous resistant mutants in vitro with properties similar or identical to those of the strains isolated from a patient hospitalized with a *P. aeruginosa* infection. Indeed, mutants with levels of resistance similar to those of strain PA116136 were easily recovered. Moreover, when the experiments were prolonged, the MICs of the isolates were even higher (16 µg/mL) [130]. According to the above-proposed mechanism of action, removal of the selective pressure exerted by the antibiotic should restore bacterial susceptibility to carbapenems through the expression of the full-length OprD. However, after 30 passages without antibiotic, susceptibility to imipenem was not restored, and electrophoretic OMPs did not show the band corresponding to the porin OprD in the outer membrane. These results point out that recovery of susceptibility to imipenem is slower than acquisition of resistance, since the selective advantage conferred by imipenem resistance in the presence of the antimicrobial is strong whereas OprD expression is likely evolutionarily advantageous only under certain and unknown environmental conditions.

5. CONCLUSIONS

5. CONCLUSIONS

1. The resistome of *Serratia marcescens* did not change significantly during the antibiotic era.
2. Antibiotic withdrawing tends to restore original susceptible phenotypes, irrespective to the molecular mechanism involved in resistance.
3. None of *Serratia* strains studied presented integrons, any extended spectrum β -lactamases.
4. Phenotypical determination of susceptibilities of old strains inactive during the last 60 years have confirmed results obtained by metagenomics i.e. the genes of resistance already existed before antibiotics discovery and use.
5. Multiresistant *Pseudomonas aeruginosa* harbored class 1 integrons containing a cassette encoding aminoglycoside adenyltransferase (*aadB*).
6. Multiresistant *Pseudomonas aeruginosa* overexpressed MexAB-OprM and MexXY efflux machinery.
7. CCCP use should be avoided in experiments performed with *P. aeruginosa* and probably in other aerobic bacteria.
8. Meropenem induces the formation of aberrant long rods which can survive, accumulate less antibiotic than normal bacteria, and can revert to normal forma when antibiotic pressure disappears.
9. Colistin, the last therapeutic option to fight against *Pseudomonas* infections in cystic fibrosis patients, is normally active although cases of resistance have arisen recently.
10. Resistance to colistin seems to be mediated by lipopolysaccharide singular properties.
11. Colistin induces injuries in lipid bilayers, which can be studied by means of planar black lipid bilayer techniques. Preliminary results showed the ability of colistin to induce transient channels in the bilayers, with some dependence to voltage.

Conclusions

12. Recovery of susceptibility to imipenem is slower than acquisition of resistance, since the selective advantage conferred by imipenem resistance in the presence of the antimicrobial is strong whereas OprD expression is likely evolutionarily advantageous only under certain and unknown environmental conditions.

**“Now this is not the end.
It is not even the beginning of the end.
But it is, perhaps, the end of the beginning.”**

Winston Churchill. London, 10 November 1942.

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6. PUBLICATIONS

Role of TolC in *Klebsiella oxytoca* resistance to antibiotics

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Objectives: The Gram-negative human pathogen *Klebsiella oxytoca* is often resistant to several antibiotics such as fluoroquinolones, erythromycin, tetracycline, chloramphenicol and others. The aim of this study was to look at the mechanisms leading to this resistance and particularly the role of TolC and efflux mechanisms in determining resistance.

Methods: Ciprofloxacin accumulation was measured spectrofluorometrically. Growth inhibition assays were performed in the presence or absence of carbonyl cyanide *m*-chlorophenylhydrazone (10 mg/L, final concentration). The genome of *K. oxytoca* was analysed for the existence of loci encoding *tolC* by PCR using primers for the *Enterobacter aerogenes tolC* gene and subsequently sequenced. A plasmid named pUC18TolC was constructed and inserted into *Escherichia coli* C600*tolC*,Tn5, and the function of TolC was analysed. The structure modelling was performed using the Modeller program.

Results: The existence of the AcrAB efflux mechanism was demonstrated in the species, and a TolC-like protein, a channel-forming protein at the external membrane that allows the extrusion of antibiotics by the AcrAB efflux pump, was cloned, sequenced and a model proposed.

Conclusions: *K. oxytoca* express a functional TolC that lacks a fragment of six amino acids characteristic of the external loops of TolC in *E. coli*. This makes this species resistant to a few colicins.

Keywords: antimicrobial agents, *Klebsiella* spp., antibiotic accumulation, efflux, Enterobacteriaceae, modelling

Introduction

Klebsiella is an opportunistic pathogen leading to severe diseases, and it can also contaminate food, enteral feedings and infusion fluids, leading to common-source outbreaks.¹ *Klebsiella oxytoca* carries a constitutive β -lactamase that confers low-level resistance to penicillins but no significant resistance to other β -lactams.² Yigit *et al.*³ provided evidence that a *K. oxytoca* strain carried a gene encoding a carbapenemase on a mobile element. *Klebsiella* synthesizes two major porins, OmpK35 and OmpK36 (homologues of OmpF and OmpC), and a quiescent porin, OmpK37.⁴ Efflux causes decreases in the intracellular concentrations of drugs that can eventually lead to subtoxic levels, thus inducing phenotypic resistance.⁵ In Enterobacteriaceae, the major multidrug transporter is AcrB, which belongs to the

resistance-nodulation-cell division (RND) superfamily.⁶ The RND efflux pumps usually need two other proteins to be functional, i.e. to facilitate the efflux of noxious compounds directly to the external medium.⁷ In *Escherichia coli*, this efflux machinery is formed by an inner membrane transporter, AcrB, a periplasmic membrane fusion protein, AcrA, and an outer membrane channel protein, TolC.^{8,9} TolC plays a key role in the import of antibacterial proteins such as colicin E1 and participates in other secretion mechanisms, such as secretion of α -haemolysin, and in the regulation of porin levels.^{10–12} TolC, but not AcrB, mediates the uptake of multidrug-resistant *Salmonella enterica* serovar Typhimurium into target intestinal cells; it is also known for its role in bile salt resistance, reinforcing the interest in targeting research towards TolC for fighting multidrug-resistant Gram-negative bacteria.¹³

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ToIC of *K. oxytoca*

Multiresistant strains of *Klebsiella* have emerged since 1980, particularly strains producing extended-spectrum β -lactamases.^{14,15} Alternative antibiotic compounds (such as quinolones) have therefore become necessary. Resistance to quinolones (by alteration in the GyrA subunit of DNA gyrase and/or in the ParC subunit of DNA topoisomerase IV) in the hospital setting has been reported and is a matter of concern.¹⁶ An additional mechanism involving active efflux has been found in *E. coli* strains with high levels of resistance and is associated with both multiple mutations in the topoisomerase genes and overexpression of the AcrAB multidrug efflux system.¹⁷ Disruption of genes encoding multidrug efflux pumps resulted in a remarkable decrease in resistance to multiple antimicrobial agents.¹⁸ Two multidrug efflux pumps have been reported in *K. pneumoniae*, AcrAB and QacE; recently, a new efflux pump (KmrA) has been identified.¹⁹ In this work, we explore some of the mechanisms leading to resistance in *K. oxytoca* as well as characterize the outer membrane protein ToIC as a member of the efflux pumps.

Materials and methods

Bacterial strains and plasmids, media and growth conditions

K. oxytoca HUB 179213 was isolated from the urine of a 75-year-old man affected by a urinary tract infection at the Hospital Universitari de Bellvitge (University of Barcelona). Identification as well as preliminary antibiotic susceptibility testing were accomplished using the Microscan system (Sacramento, CA, USA). Strain C600*tolC*:Tn5, having a kanamycin cassette inserted into its *tolC* gene, derived from *E. coli* C600, was used in cloning experiments (a gift from Elisabeth Pradel). Plasmid pUC18ToIC was obtained from pUC18 and carries the *tolC* gene of *K. oxytoca*.

For experiments reported here, bacteria were cultured in Luria–Bertani (LB) broth and agar, Trypticase soy broth (TSB) or Trypticase soy agar (TSA) depending on the experiment. Bacteriological media were purchased in dehydrated form from Scharlau (Barcelona, Spain).

Drug susceptibility testing

The MICs of antimicrobial agents were determined by microdilution in Mueller–Hinton broth, according to the guidelines of the CLSI (formerly NCCLS).

The following antimicrobials were tested: erythromycin, tetracycline, chloramphenicol, nalidixic acid, ciprofloxacin, norfloxacin, novobiocin, ofloxacin, rifampicin, enrofloxacin, gentamicin, imipenem, meropenem, ceftriaxone, cefotaxime, acriflavin and Crystal Violet. MICs were also determined in the presence of a fixed concentration of the efflux pump inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). All antibiotics were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Accumulation of ciprofloxacin

Ciprofloxacin accumulation was measured spectrofluorometrically using the method described by Mortimer and Piddock²⁰ with some modifications.²¹ Isolates were incubated at 37°C in TSB until A_{600} reached values from 0.5 to 0.7. Bacteria were harvested by centrifugation (9000 g) at room temperature, washed and concentrated 10-fold in PBS, pH 7.5. Bacteria were incubated for 10 min at 37°C and ciprofloxacin added to a final concentration of 10 mg/L. After the addition of ciprofloxacin, 1 mL samples were removed at

different time intervals. Three minutes after the addition of ciprofloxacin, the efflux pump inhibitor CCCP (final concentration 100 mM) was added when necessary. The samples were immediately diluted in 1 mL of ice-cold PBS and centrifuged for 1 min at 11000 rpm in a microcentrifuge. Pellets were resuspended in 1 mL of 0.1 M glycine buffer at pH 3.0 and finally incubated at room temperature overnight to allow bacterial lysis. Thereafter, the suspensions were centrifuged at 20°C for 15 min to remove bacterial debris. The fluorescence of the supernatant was measured using an SLM Aminco 8100 spectrofluorometer (SLM-Aminco, Urbana, IL, USA).

The growth inhibition assays were performed as described by Beyer *et al.*²² with some modifications. *K. oxytoca* strains were inoculated at 1×10^6 – 2×10^6 cfu/mL into tubes containing TSB with antibiotics at concentrations one-fourth the previously determined MIC, either in the presence or absence of CCCP (10 mg/L final concentration). Bacteria were incubated at 37°C, and optical density values at a wavelength of 550 nm were registered over ~8 h. The OD₅₅₀ values were measured after 7 h, and results plotted.

PCR amplification

Genomic DNA of *K. oxytoca* was analysed for the existence of loci encoding *tolC*. Specific primers for the *Enterobacter aerogenes tolC* gene were designed and used in an attempt to amplify *K. oxytoca tolC*. PCRs were carried out in 50 μ L volumes containing 10 μ L of 10 \times PCR buffer (100 mM Tris–HCl pH 9.0, 500 mM KCl, 1.5 mM MgCl₂ and 0.2 mM of each dNTP), 25 pmol of each of the *tolC* forward primer (5'-AGCACATCTA GATCACGCACC-3') and the *tolC* reverse primer (5'-TCGG CGTTCTGATCTAGACAA-3'), 1 U of *Taq* DNA polymerase (Fermentas Vilnius, Lithuania), 1 μ L of *K. oxytoca* DNA and 24 μ L of sterile distilled water. Thermal cycling reaction parameters included an initial denaturation at 94°C for 5 min, followed by 25 cycles of 94°C for 1 min, 51°C for 1 min and 4 min of extension at 72°C, and a final extension at 72°C for 10 min. All completed reactions were maintained at 4°C. The PCR products were visualized using 0.7% agarose gel electrophoresis (85 V for 2 h); gels were stained with ethidium bromide.

DNA sequence analysis

Any amplified PCR products of interest were purified with the MiniElute PCR Purification Kit (Qiagen, West Sussex, UK) according to the guidelines of the manufacturer. Each gel-purified product was sequenced using BigDye Terminator (version 3.1; Applied Biosystems, Foster City, CA, USA). Sequences were initially compared with the current GenBank sequence databases using the BLAST suite of programs.²³

Cloning and plasmid construction

Restriction digestion, ligation and *E. coli* transformation were performed as described by Sambrook *et al.*²⁴ The full-length *tolC* gene of *K. oxytoca* was digested with the *Xba*I restriction enzyme, followed by insertion into pUC18 at the *Xba*I site. The ensuing plasmid, named pUC18ToIC, was inserted into *E. coli* C600*tolC*:Tn5 resulting in the strain named C600pUC18ToIC. For transformation, a single colony of C600*tolC*:Tn5 was inoculated in 2 mL of LB broth and grown while being shaken overnight at 37°C. The culture was diluted 1/50 in 50 mL of LB broth and grown while being shaken at 37°C for 6 h. The culture was then centrifuged and washed four times with 20 mL of 10% ice-cold glycerol. The cell

pellet was suspended in 9 mL of 10% glycerol and maintained on ice. A volume of 70 μ L of the competent bacterial suspension was mixed with 5 μ L of plasmid DNA (from 0.1 to 1 μ g of DNA) in water, chilled on ice for 5 min and pulsed using a Cell-Porator (Pharmacia LKB-ECPS 3000/150) at 150 μ F and 1400 V. Cells were transferred immediately to 1 mL of LB broth and grown while being shaken at 37°C for 1 h. The culture was then centrifuged, and the pellet was suspended in 0.1 mL of LB broth. Aliquots of suspended bacteria were spread on LB agar plates containing appropriate antibiotics.

Structure modelling

The structure modelling was performed using Modeller, a program to compare modelling of three-dimensional (3D) protein structures.²⁵ The user provides an alignment of the sequence to be modelled with known related structures, while Modeller automatically calculates a model containing all non-hydrogen atoms. Modeller implements comparative protein structure modelling by the satisfaction of spatial restraints^{26,27} and performs many additional tasks, including *de novo* modelling of loops in protein structures, optimization of various models of protein structure with respect to a flexibly defined objective function, multiple alignment of protein sequences and/or structures, clustering, searching of sequence databases, comparison of protein structures, etc. Once the target template was constructed, Modeller calculated a 3D model of the target. A script was used to generate the most similar model of TolC based on the template structure, in this case TolC of *E. coli*. The model was viewed by Pymol, a program that reads the PDB format.²⁸ Then a loop refinement was done using ModLoop, a web server for automated modelling of loops in protein structures. This server relies on the loop modelling routine in Modeller that predicts the loop conformations by the satisfaction of spatial restraints, without relying on a database of known protein structures.²⁹

Colicin susceptibility

Colicin was prepared from colicinogenic bacteria grown in TSB at 37°C with aeration to early log phase, induced with 0.4 μ g of mitomycin per mL and incubated for 3 h at 37°C with aeration. The cells were collected by centrifugation at 10000 g, and the supernatant, containing colicin E1, E2 and E3, was precipitated with ammonium sulphate. The susceptibility to the three colicins was determined by spotting 2-fold serial dilutions of the colicin stock solution on bacterial lawns. Killing zones were recorded after 6–8 h of incubation at 37°C.

TolC purification

TolC protein was expressed and purified as follows. Cultures (1 L) of an *E. coli* strain harbouring a plasmid containing the *tolC* gene from *K. oxytoca* were prepared, and cells were harvested by centrifugation and broken using a French Press. The membranes were collected (50000 g for 1 h) and washed three times in 20 mM HCl–Tris pH 7.4/20 mM MgCl₂/0.5% Triton X-100 (Merck). TolC was solubilized in 20 mM HCl–Tris pH 7.4/20 mM MgCl₂/5% Triton X-100/10% glycerol. After resuspension, insoluble material was removed by centrifugation at 50000 g for 20 min. The supernatant was placed on a Q Sepharose anion exchange column (5 mL HiTrap Q Pharmacia) and eluted with a NaCl gradient in 20 mM HCl–Tris pH 7.4/0.5% Triton X-100. TolC was then pure.

Results

Antibiotic susceptibility testing

The results of the antibiotic susceptibility tests (MICs) are reported in Table 1. MIC values obtained in the presence of the efflux pump inhibitor CCCP were lower than the MIC values obtained without CCCP.

Accumulation of ciprofloxacin

As *K. oxytoca* 179213 showed high quinolone resistance (Table 1), the intracellular accumulation of ciprofloxacin was measured in order to identify the existence of an active efflux pump. This was done in the presence and absence of the membrane energy uncoupler CCCP. Since quinolone efflux is energy-dependent, incubation with CCCP indicated the role of an active efflux pump in the intracellular concentration of ciprofloxacin. Figure 1 illustrates

Table 1. MICs (mg/L) of different antimicrobial agents for *K. oxytoca* HUB 179213

Antimicrobial agent	HUB 179213	HUB 179213+CCCP
Erythromycin	140	18.75
Tetracycline	250	53.15
Chloramphenicol	64.3	3.5
Nalidixic acid	27.3	1.95
Ciprofloxacin	12.5	0.23
Norfloxacin	30	1.17
Novobiocin	62.5	2.5
Ofloxacin	65	17.5
Rifampicin	165	23.5
Enrofloxacin	50	4.5
Gentamicin	31	2.25
Imipenem	0.45	0.45
Meropenem	0.97	0.97
Ceftriaxone	22.5	22.5
Cefotaxime	7.81	7.81
Acriflavin	62.5	0.25
Crystal Violet	31.25	0.55

CCCP, carbonyl cyanide *m*-chlorophenylhydrazine.

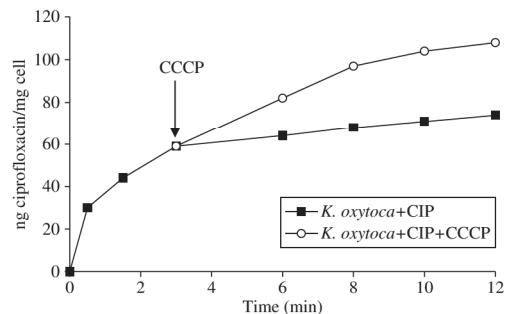


Figure 1. Ciprofloxacin (CIP) accumulation by *K. oxytoca* HUB 179213 in the presence or absence of CCCP.

TolC of *K. oxytoca*

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K.o ESRNLSLLQARLSQDLAREQIRQAQDGHLPPTLNLNASSGVSNRRYSGSKSIS-----QD..... 294
E.a ENRNLSLLQARLNQDLAREQIRQAQDGHLPPTLDLNASTGVSNRRYSGSKNIS-----QD..... 294
S.e ENRNLSLLQARLSQDLAREQIRQAQDGHLPPTLNLTATSTGISDTSYSGSKTNSAQY---DD..... 297
E.c EKRNLSLLQARLSQDLAREQIRQAQDGHLPPTLDLTASSGISDTSYSGSKTRGAAGTQYDD..... 300

K.o .....ELNIKSALGTLNEQDLVALNNTLGKSIPTSPDSVAPENPQQDASADGYSNTAAA---KPA 471
E.a .....ELNIKSALGTLNEQDLIALNNTLGKAIPTSPDSVAPENPQQDAAADGYANTASA---QPA 471
S.e .....QLNIKYALGTLNEQDLLALNNTLGKPIPTSPESVAPETPEQDAAADGYNHSAAPAVQPT 480
E.c .....QLNIKSALGTLNEQDLLALNNALSKPVSTNPENAVPQTPEQNAIADGYAPDSPAPVVQQT 480

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Figure 2. Alignment of the sequence of the protein TolC of *K. oxytoca* HUB 179213 (K.o), *E. aerogenes* (E.a) (GenBank accession number AJ306390), *S. enterica* (S.e) (GenBank accession number AL627278) and *E. coli* (E.c) (GenBank accession number X54049). Major differences are underlined.

the results obtained. A low level of intracellular accumulation of ciprofloxacin was found in the absence of CCCP. In contrast, a noticeable increase in intracellular drug concentration was observed in the presence of the inhibitor. The addition of CCCP enhanced ciprofloxacin's effect on bacterial growth; at concentrations of 0.10 mg/L, growth was almost completely inhibited in the presence of CCCP, whereas growth was unaltered by the fluoroquinolone alone (data not shown). Thus it can be assumed that the activity of the efflux mechanism was significantly inhibited by the addition of the energy uncoupler CCCP.

K. oxytoca tolC cloning and sequencing

Using primers generated from the *tolC* sequence of *E. aerogenes*, PCR amplification yielded a 2.1 kb fragment carrying the complete *tolC* gene of *K. oxytoca*, which was cloned and sequenced (GenBank DQ838802). *E. coli* C600*tolC*,Tn5 expressing *tolC* was used to determine the sequence. The deduced amino acid sequence of the *tolC* gene of *K. oxytoca* HUB 179213 was compared with the sequences of the TolC protein of *E. aerogenes*, *S. enterica* and *E. coli* (Figure 2). Nucleotide sequence analysis of the 2127 base PCR fragment indicates that the *tolC* gene from *K. oxytoca* encodes an open reading frame of 1457 nt. A putative promoter sequence with -10 (TACCAAT), Pribnow box and -35 (TTGACAT) regions lies 50 nt upstream of the start codon (ATG). The presumed initiator methionine is preceded by a canonical ribosome-binding site (GGAGGA). The deduced amino acid sequence encoded by the 2127 nt sequence of the *tolC* gene of *K. oxytoca* HUB 179213 showed a high degree of identity with several TolC proteins: 92% identity with the TolC protein of *E. aerogenes*, 81% identity with the TolC protein of *S. enterica* and 79% identity with the TolC protein of *E. coli* (Figure 2). The most apparent difference was localized between amino acids 286 and 293, where *K. oxytoca* HUB 179213 showed a six amino acid deletion; in addition, there was a three amino acid deletion between amino acids 465 and 469.

K. oxytoca tolC can functionally complement an *E. coli* tolC mutant

TolC mutants of *E. coli* are hypersensitive to detergents such as SDS and a variety of hydrophobic agents such as novobiocin.³⁰ The *tolC* gene of *K. oxytoca* inserted into plasmid pUC18 (pUC18TolC) was transformed into an *E. coli* *tolC*-deleted strain

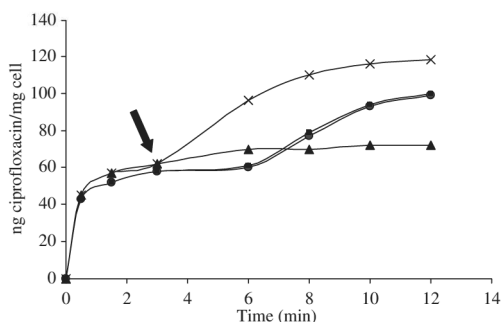


Figure 3. Ciprofloxacin accumulation by *E. coli* C600*tolC*,Tn5 in the presence (filled squares) or absence (filled circles) of CCCP and *E. coli* C600pUC18TolC in the presence (crosses) or absence (filled triangles) of CCCP (arrow indicates CCCP addition).

(C600*tolC*,Tn5) and transformants were selected on LB agar plates containing SDS and appropriate antibiotics. We examined the resistance to ciprofloxacin due to the efflux pump of this strain, named C600pUC18TolC, by measuring intracellular accumulation of ciprofloxacin in the presence of the membrane energy uncoupler CCCP. The results for strains *E. coli* C600*tolC*,Tn5 and C600pUC18TolC are shown in Figure 3. A low level of intracellular accumulation of ciprofloxacin was found in the presence and absence of CCCP for C600*tolC*,Tn5. However, an increase in intracellular ciprofloxacin concentration by strain C600pUC18TolC was obtained in the presence of the inhibitor. These results pointed to the actual role of the TolC protein in the efflux of antibiotic out of the cell, and, moreover, that *K. oxytoca* *tolC* was able to complement the *E. coli* *tolC* mutant to drug resistance.

Structural model of TolC

The closest homology (87%) was seen with Iek9 (TolC of *E. coli*). The TolC amino acid sequence was threaded to the Iek9 crystal structure based on the alignment. The first 24 residues and the last 39 residues of TolC were excluded since there are no corresponding residues in the Iek9 structure. The alignment already shows that the parts of the sequence that form β -strands in Iek9 from *E. coli* are highly conserved. The highest variability was found in regions forming the extracellular loops (Figure 4).

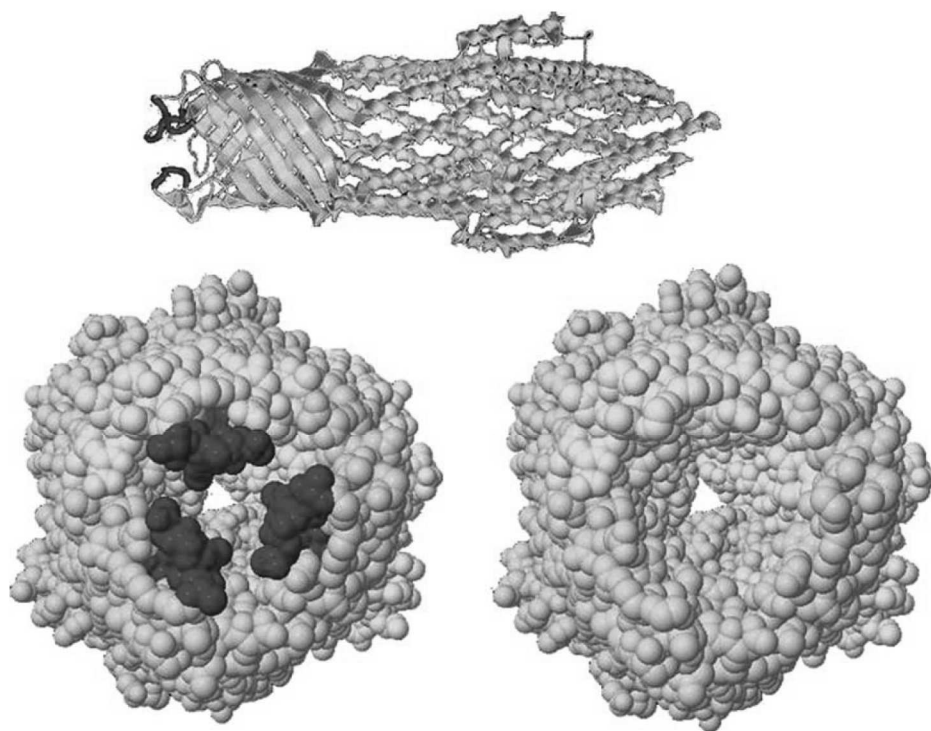


Figure 4. Model of the trimeric TolC of *K. oxytoca*: the whole molecule (top) and a comparison between the mouths of the channel of *E. coli* TolC (left) and *K. oxytoca* TolC (right). The main difference (if not the only) is due to the lack of external loops (red) surrounding the mouth of the channel. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

It has been shown that these regions are responsible for colicin attachment in *E. coli* (namely ColE1). C-600 was susceptible to colicin E1, whereas the clones *E. coli* C600*tolC*, Tn5 and C600pUC18*TolC* as well as *K. oxytoca* HUB 179213 were resistant to the three colicins tested (ColE1, ColE2 and ColE3).

Discussion

Species of the genus *Klebsiella* are naturally resistant to many β -lactams due to their ability to produce β -lactamases (mostly chromosomal SHV); ~40% of strains harbour a plasmid encoding SHV-5.⁵ The data in Table 1 show that *K. oxytoca* HUB 179213 is resistant to several commonly used antibiotics and exhibits high resistance to quinolones, reaching MICs >4 mg/L (according to the recommendations of the CLSI, formerly the NCCLS)³¹. The actual role of efflux in determining resistance in *K. oxytoca* is unknown. When determining MICs of a wide series of antimicrobial agents, MIC values became strongly affected by the efflux pump inhibitor CCCP, except in the case of imipenem, meropenem, ceftriaxone and cefotaxime (Table 1). Strain HUB 179213 exhibited high-level resistance to fluoroquinolones. It has been shown that quinolone resistance in Enterobacteriaceae is generally caused by a cooperative effect between mutations in the genes *gyrA* and *parC* and efflux pumps, mostly *acrAB*. The measurements of the accumulation of

quinolones by this bacterium either with or without the energy uncoupler CCCP suggested that an active efflux mechanism significantly contributes to fluoroquinolone resistance in the isolate *K. oxytoca* HUB 179213. In addition, sequences of *gyrA* and *parC* genes cannot explain the resistance of the strain to quinolones. The inhibition assays demonstrated that the presence of the inhibitor CCCP at sublethal concentrations of ciprofloxacin resulted in complete inhibition of growth. A similar experiment using the strain *K. pneumoniae* CECT 434, a fully susceptible strain, was unable to detect differences between curves obtained with or without CCCP. The presence of *acrAB* was tested by PCR amplification by using primers constructed on the basis of *acrA* and *acrB* sequences of *E. coli*. Two amplicons of 1.2 kb (*acrA*) and 3.15 kb (*acrB*) were obtained, showing that the resistant strains harbour genes of the efflux machinery.

The outer membrane protein involved in the function of AcrAB has been demonstrated to be the TolC protein in several Enterobacteriaceae. However, up to now, almost nothing has been published concerning the actual role of TolC or the TolC-like protein in the genus *Klebsiella*. An amplicon of 2.2 kb was obtained by PCR (almost identical in size with that obtained in *E. aerogenes*) (data not shown). A recombinant plasmid containing *tolC* was able to complement a *tolC* mutant of *Enterobacter cloacae*. The sequence encoded by the *tolC* from *K. oxytoca* was obtained and compared with the sequence encoded by *tolC* of other enterobacteria such as *E. aerogenes*, *E. coli* and *S. enterica*

TolC of *K. oxytoca*

by using BLAST (Figure 2). The sequence was submitted to GenBank (accession number DQ838802). The most significant differences can be found between amino acids 280 and 290, in which *K. oxytoca* lacks six amino acids forming part of the loop exposed to the external side of the outer membrane. It was supposed that this region would play some role in the attachment of colicins and bacteriophages. Results of colicin susceptibility revealed that *K. oxytoca* is resistant to ColE1, which is known to use an external part of TolC as a receptor.

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This paper is dedicated to the recently retired microbiologists Richard and Sheila Hull. We thank Elisabeth Pradel (Enveloppe Bactérienne, Perméabilité et Antibiotiques, Université de la Méditerranée, France) for TolC⁻ *Enterobacter* strains and Richard Hull (Baylor College of Medicine, Houston, TX, USA) for kindly supplying colicinogenic strains of *E. coli*.

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Transparency declarations

None to declare.

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Synergism between Outer Membrane Proteins and Antimicrobials[∇]

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Antibiotic-resistant bacteria are becoming one of the most important problems in health care because of the number of resistant strains and the paucity of new effective antimicrobials. Since antibiotic-resistant bacteria will continue to increase, it is necessary to look for new alternative strategies to fight against them. It is generally accepted that Gram-negative bacteria are intrinsically less susceptible than Gram-positive bacteria to antimicrobials. The main reason is that Gram-negative bacteria are surrounded by a permeability barrier known as the outer membrane (OM). Hydrophilic solutes most often cross the OM through water-filled channels formed by a particular family of proteins known as porins. This work explores the possibility of using exogenous porins to lower the required amounts of antibiotics (ampicillin, ciprofloxacin, cefotaxime, clindamycin, erythromycin, and tetracycline). Porins had a bactericidal effect on *Escherichia coli* cultures, mainly in the logarithmic phase of growth, when combined with low antibiotic concentrations. The use of different antibiotic-porin mixtures showed a bactericidal effect greater than those of antibiotics and porins when used separately. It was possible to observe different behaviors according to the antibiotic type used.

It is generally accepted that Gram-negative bacteria are intrinsically less susceptible to antimicrobials than Gram-positive bacteria. The main reason is that Gram-negative bacteria are surrounded by a permeability barrier known as the outer membrane (OM), and the biological significance of such a structure is quite high. The analysis of prokaryote phylogeny using signature sequences in proteins revealed that a major phylogenetic division possibly exists between organisms with double-membrane envelopes (so-called diderms) and those with only a single cytoplasmic membrane (monoderms) (3). According to Nikaido (11), it is likely that the most important function of the OM in Gram-negative bacteria is to serve as a selective (protective in many environments) permeation barrier.

Hydrophilic solutes most often cross the OM through water-filled channels formed by a particular family of proteins named porins. For example, in *Escherichia coli*, all β -lactams utilize predominantly the porin pathway. It is generally assumed that most porins are nonspecific; thus, it should be expected that many small, hydrophilic molecules would also utilize this pathway. However, porin channels may be very narrow, and consequently they can act as an effective barrier to the penetration of large or hydrophobic compounds. The OM contributes significantly to generate a variety of intrinsic resistance to antimicrobials, even in wild-type bacteria. Furthermore, the level of resistance can be increased by genetic or physiological alterations that lower the permeability of this membrane.

The importance of the role of the OM in determining low susceptibilities is enhanced by the fact that molecular mechanisms leading to antimicrobial resistance are strongly synergis-

tic. Decreases in OM permeability do not necessarily result in large increases in MIC values if the bacterium produces an enzyme able to inactivate the antibiotic, and changes in permeability strongly modify the internal concentration of the antimicrobial. This in turn would increase the efficacy of the enzyme.

In some cases the restrictions posed by the OM to antibiotic uptake are enough to determine a true resistance. A good example is *Pseudomonas aeruginosa*, a Gram-negative bacterium whose OM exhibits such a low permeability that the species is intrinsically resistant to many antimicrobials. The antibiotic of choice to treat *P. aeruginosa* infections is therefore imipenem, because it crosses the *P. aeruginosa* OM, primarily via the OprD-specific channel. Mutants with decreased expression of this protein become predominant during imipenem therapy, since OprD is not important for the uptake of most nutrients. These resistant variants could be regarded as a simple result of loss of OM permeability.

It is difficult to measure the quantitative role of the OM when an antibiotic is applied *in vivo*, since both the bacterial physiological state and the OM protein expression strongly depend on the environmental conditions.

TolC is an OM channel protein (14, 15). TolC plays a key role in the movement of antibacterial proteins, secretion mechanisms, and regulation of porin levels, and it is involved in multidrug resistance in Gram-negative bacteria such as *Klebsiella oxytoca* (2, 6, 7, 12, 17).

In this study we explored *in vitro* the eventual usefulness of porins as adjuvants to improve the access of antimicrobials to their targets for the treatment of diseases caused by resistant bacteria.

MATERIALS AND METHODS

Bacterial strains and media. *K. oxytoca* HUB 179213 (2) was used as the source of porins. *E. coli* TOP10 (Invitrogen) and *E. coli* TOP10 transformed with the pUC19 vector (Invitrogen) were used in experiments to measure suscepti-

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to mixtures of antibiotics and porins prepared under different conditions. *E. coli* C600+ and *E. coli* C600 *tolC* Tn5 (*E. coli* C600-) expressing *tolC* of *K. oxytoca* (2) were used in a series of experiments to explore the eventual effect of the presence of additional pore-forming proteins in the OM.

Crude porin preparation. Whole bacterial proteins and OM proteins (OMPs) were obtained as described elsewhere (13). To visualize proteins, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a modification of the Laemmli method (8). Gels were stained with 0.25% Coomassie brilliant blue, destained, and dried using the Bio-Rad Miniprotean II and Bio-Rad 543 systems (Bio-Rad Laboratories S.A., Madrid, Spain) for electrophoresis and gel drying, respectively.

TolC purification. TolC protein was expressed and purified as follows (2). Cells from 1-liter cultures of *E. coli* C600 *tolC* Tn5 were harvested by centrifugation and broken using a French press. The membranes were collected by centrifugation (50,000 × g, 1 h) and washed three times in 20 mM Tris-HCl (pH 7.4), 20 mM MgCl₂, and 0.5% Triton X-100 (Merck). TolC was solubilized in 20 mM Tris-HCl (pH 7.4), 20 mM MgCl₂, 0.5% Triton X-100, and 10% glycerol. After resuspension, insoluble material was removed by centrifugation (50,000 × g, 20 min) and the supernatant was placed on a 5-ml Q Sepharose anion-exchange column (Hitrap Q; Pharmacia) and eluted with a NaCl gradient in 20 mM Tris-HCl (pH 7.4) and 0.5% Triton X-100. TolC was then considered to be pure, as could be determined by SDS-PAGE.

Optimization of porin-antibiotic mixture conditions. *E. coli* TOP10 and *E. coli* TOP10/pUC19 were used in experiments to determine their susceptibility to porin-antimicrobial mixtures prepared under diverse conditions (time and temperature of incubation) in order to establish their optimal effectiveness. Mixtures (1 μg/ml of porin preparation and 500 μg/ml of ampicillin) were incubated for 0, 15, 30, 60, or 120 min at 4, 25, and 30°C. After incubation, the mix was added to *E. coli* TOP10 and TOP10/pUC19 cultures. The effect on the bacteria in either the logarithmic or stationary phase of growth was studied. The cultures were maintained for 24 h at 37°C in a rotary shaker at 180 rpm, and then the optical density at 600 nm (OD₆₀₀) was determined. Experiments were carried out three independent times, and all measurements were carried out in triplicate. Negative-control experiments were performed simultaneously using bacteria with porin alone, bacteria with antibiotics alone, and bacterial cells without treatment.

After determination of the MICs of the antibiotics, the antibiotic concentrations in porin mixes for final experiments were chosen so as to be lower than the MICs. The final concentrations were as follows: tetracycline, 10 μg/ml; cefotaxime, 0.5 μg/ml; ampicillin, 75 μg/ml; ciprofloxacin, 5 μg/ml; erythromycin, 30 μg/ml; and clindamycin, 1 μg/ml. The porin concentration was 1 μg/ml in all cases. The incubation conditions for porin-antibiotic mixtures were 15 min and 60 min at 30°C. The porin-antibiotic mixtures plus cells were maintained for 24 h at 37°C in a rotary shaker at 180 rpm, and then the OD₆₀₀ was determined. Experiments were carried out three independent times, and all measurements were carried out in triplicate together with those for the negative controls as described above.

Incorporation of exogenous porins. Incorporation of exogenous labeled porins into the OM of *E. coli* was studied using porins previously labeled with fluorescein isothiocyanate (FITC) as described elsewhere, with few modifications (1). Labeled porins were finally dialyzed against 10 mM Tris-HCl buffer (pH 8.5) and for 24 h against 10 mM Tris-HCl (pH 8), 2 mM EDTA, and 2% Genapol to eliminate the free FITC using a Slide-A-Lyzer dialysis cassette (molecular mass cutoff, 3,500 Da) (Thermo Scientific).

Starter cultures of *E. coli* C600+ were used to inoculate fresh LB medium (1:100, vol/vol) containing 1 μg/ml of labeled porins (PF). The cultures were maintained for 24 h at 37°C. Samples were withdrawn at 0, 5, 10, 20, 30, 40, and 50 min and at 1, 3, 5, 7, and 24 h. At each time, samples were centrifuged and the pellets resuspended and maintained in fresh porin-free LB medium for an additional 24-h period at 37°C. After the cultures were cooled, samples were taken during the following hour at 0, 5, 10, 20, 30, 40, and 50 min and then at 25, 27, 29, 31, and 48 h. Control experiments were done with *E. coli* C600+ (CV), *E. coli* plus free FITC (CVF), *E. coli* plus 1 μg/ml of labeled porins and 0.02% sodium azide (PFA), dead *E. coli* (CM), dead *E. coli* with free FITC (CMF), and dead *E. coli* with 1 μg/ml of labeled porins (CMPF). Dead cells were obtained after thermal shock at 60°C for 15 min.

Fluorescence was measured as follows. Samples were centrifuged at 6,000 rpm for 5 min. The pellets were washed twice in 500 μl of phosphate-buffered saline (PBS) (pH 7.4) (Sigma-Aldrich) and suspended in 400 μl of 50 mM Tris-HCl (pH 8.5). Volumes of 350 μl were dispensed in 96-well plates (Nunc). The fluorescence was measured in an LS50B luminescence spectrometer (Perkin-Elmer) with a numeric aperture of 2.5, an excitation wavelength of 490 nm, and an emission wavelength of 520 nm.

All experiments were carried out three independent times, and all samples

TABLE 1. Antibiotics and concentrations tested

Antibiotic	Concn range assayed (μg · ml ⁻¹)	MIC (μg · ml ⁻¹) for <i>E. coli</i> strain:		Concn used with porins (μg · ml ⁻¹)
		C600+	C600-	
Tetracycline	0-900	700	800	10
Cefotaxime	0-10	10	10	0.5
Ampicillin	0-500	>500	>500	75
Ciprofloxacin	0-30	25	30	5
Erythromycin	0-300	200	250	30
Clindamycin	0-40	>40	>40	1

were measured in triplicate. Microscopy was performed using a Jenamed (Carl Zeiss) fluorescence microscope.

Effects of TolC porin on eukaryotic cells. Different TolC concentrations (0.25, 0.5, 0.75, 1, and 1.25 μg/ml) were added to monolayers of Vero cells maintained in Dulbecco's modified Eagle medium (DMEM) at 80% confluence. Cultures supplemented with equivalent volumes of porin buffer (10 mM Tris-HCl [pH 8], 2 mM EDTA, and 2% Genapol) were used as controls. Cells were maintained for 7 days in a CO₂ incubator. Cell cultures were monitored daily, and cell viability was determined by trypan blue staining.

RESULTS

Determination of the MIC. Analysis of the MICs of the different antibiotics used showed differences between *E. coli* C600+ and *E. coli* C600- (Table 1). *E. coli* C600+ was generally less susceptible than *E. coli* C600-, except to cefotaxime. MIC values were used to select the lowest concentrations of antibiotics in order to perform later experiments. The lowest selected concentrations for further experiments were unable by themselves to inhibit the growth of *E. coli* strains.

Determination of incubation conditions for porins and antibiotics. A series of experiments was carried out to determine the optimum temperature as well as the time of coinoculation of porins and antibiotics. The influence of the bacterial growth phase was also analyzed. These experiments were carried out with *E. coli* TOP10, *E. coli* TOP10/pUC19, and ampicillin.

It was observed that, as expected, cells carrying the plasmid exhibited greater resistance to ampicillin than the TOP10 cells. The mix of porins and antibiotics showed a greater effect on the cells than that observed when the antibiotic or the porins were tested independently. The temperature and the time of incubation had a direct effect on the efficiency of the treatment. Optimal effects were attained with a period of incubation of porins and antimicrobials of between 15 and 60 min at 30°C prior to addition to the *E. coli* cultures. The relationship between the efficiency of the treatment and the growth phase was clear. When the mixtures were added to fresh cultures, an inhibition of the growth was observed; in contrast, a slight effect was observed on bacteria at the stationary phase of growth (results not shown). Based on these experimental results, we performed further porin-antibiotic experiments at 30°C and with coinoculation periods of 15 min and 1 h.

Effects of the mixtures of porins and antibiotics. Strains C600 and C600+ exhibited similar susceptibilities to antimicrobials. Addition of crude porin preparations to exponentially growing cultures resulted in an inhibition of growth and death, whereas addition to cultures in the stationary phase did not have an effect (Fig. 1). Bacteria were able to grow in the presence of antimicrobial concentrations below the MIC val-

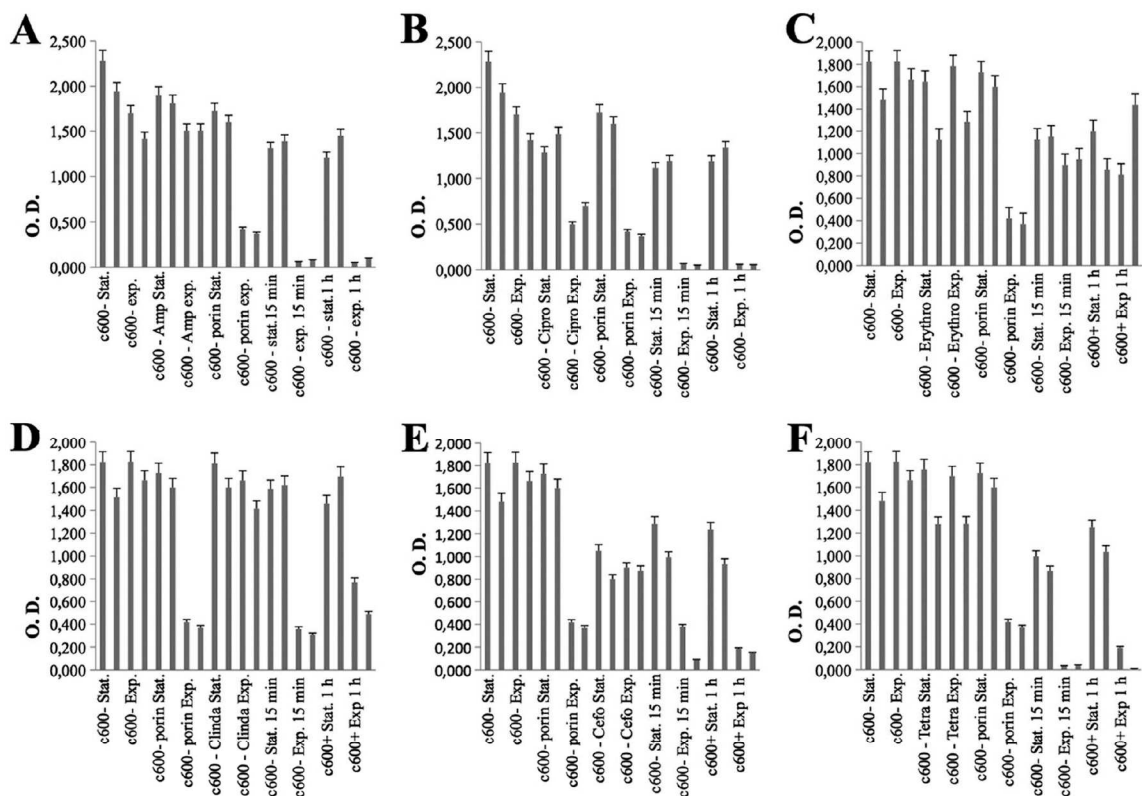


FIG. 1. Effects of porins, antibiotics and mixtures of porins and antibiotics on *E. coli* C600+ and C600-. (A) Ampicillin. (B) Ciprofloxacin. (C) Erythromycin. (D) Clindamycin. (E) Cefotaxime. (F) Tetracycline. All incubations were at 30°C. Stat., cells in stationary phase of growth; Exp., cells in exponential phase of growth; porin: crude porins; Amp, ampicillin; Cipro, ciprofloxacin; Erythro, erythromycin; Clinda, clindamycin; Cefo, cefotaxime; Tetra, tetracycline.

ues. However, the rates of growth were significantly lower than those in control cultures. A very significant reduction in growth rates occurred when porins and antibiotics were added together in almost all of the tested cases (Fig. 1). The response when antibiotics and porins were added simultaneously was different for each antibiotic.

(i) **Ampicillin.** The effect of ampicillin and porins on bacterial cultures is shown in Fig. 1A. The cultures inoculated with exponentially growing bacteria exhibited much greater susceptibility to ampicillin in the presence of porins than those inoculated with stationary-phase bacteria. In the exponential phase of growth, the inhibitory effect was almost complete. This effect was not observed when ampicillin was added alone.

(ii) **Ciprofloxacin.** Cultures inoculated with exponentially growing bacteria were much more susceptible to ciprofloxacin than those inoculated with stationary-phase bacteria (Fig. 1B). The effect of porin addition drastically enhanced the antimicrobial action of this antibiotic in cultures with exponential-phase bacteria, whereas the effect was much less apparent, but still detectable, in those inoculated with stationary-phase bacteria.

(iii) **Erythromycin.** No clear effects were observed when the porins were incubated with erythromycin (Fig. 1C). At 15 min of incubation, the inhibitory effect was greater in exponential-phase than in stationary-phase cultures. However, the latter values were higher than those for the controls. In the case of 60-min incubations, the behavior of the samples was too variable for conclusions to be drawn.

(iv) **Clindamycin.** When porins and clindamycin were used together, no effects of the mixture on the ability of cultures to grow were observed. The OD values were almost identical to those observed with crude porins in both stationary- and exponential-phase cultures (Fig. 1D).

(v) **Cefotaxime.** The observed effects with cefotaxime were similar to those obtained for other antibiotics such as ampicillin or ciprofloxacin but with a lower magnitude. C600- showed a greater sensitivity to cefotaxime plus porins than C600+ in both stationary- and exponential-phase cultures (Fig. 1E).

(vi) **Tetracycline.** The effect of tetracycline on an actively growing culture was evident when the mixtures were incubated for 15 or 60 min. The effect on stationary-phase culture was also evident (Fig. 1F).

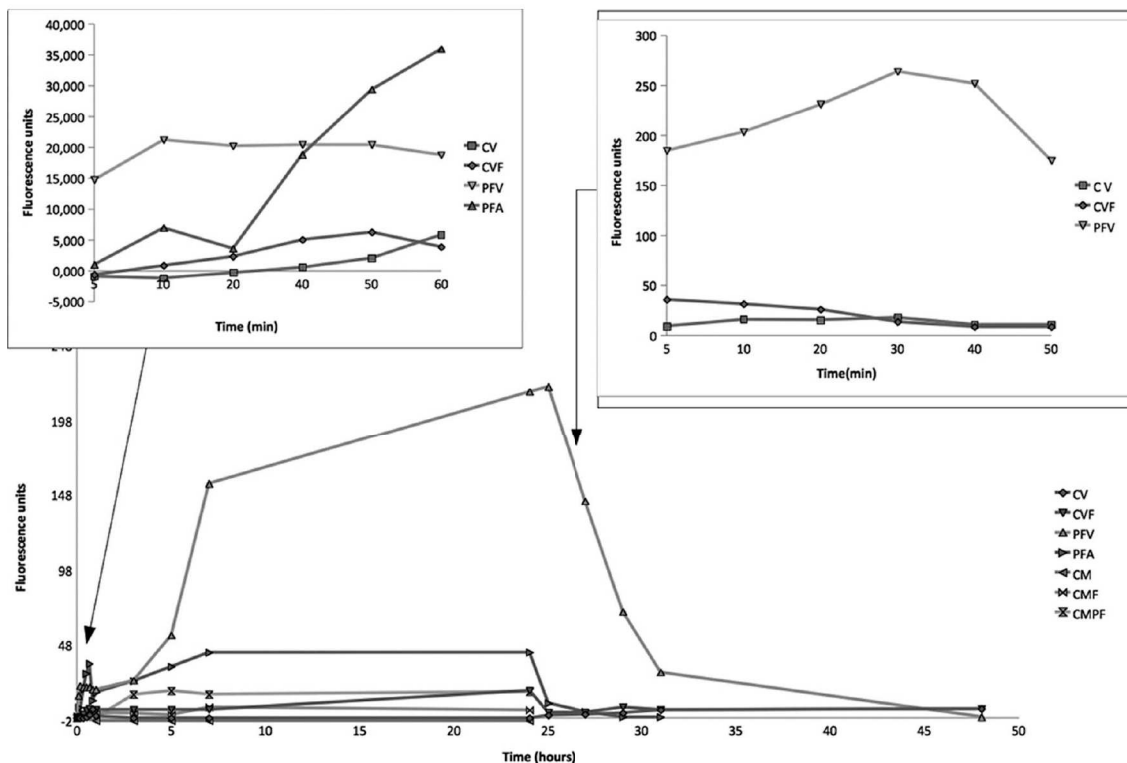


FIG. 2. Incorporation of exogenous FITC-labeled porins into the cell wall of *E. coli* during 48 h. The variation of the registered fluorescence during the first hour of the culture is shown in the left upper box. The discontinuous line marks the refreshment of the culture medium and the elimination of the FITC-porins from it. The variation in fluorescence in the first hour after that is summarized in the right upper box. CV, live cells; CVF, live cells in the presence of free FITC; PFV, FITC-porins plus live cells; PFA, FITC-porins plus live cells plus sodium azide; CM, dead cells; CMF, dead cells in the presence of free FITC; CMPF, FITC-porins plus dead cells.

Incorporation of exogenous porins into the cell wall of *E. coli*. The labeling of porins with FITC made it possible to observe that only viable cells were able to incorporate the porins, presumably into the outer membrane of the cell wall, since only in these cases there was an increase in the measurement of fluorescence that paralleled the cellular growth. When the labeled porins were added to dead cells or under inhibitory growth conditions (0.0020% sodium azide present in the medium), no increase in fluorescence was recorded (Fig. 2). The same pattern was observed during the first hour of the culture. When the fluorescence was measured in the controls with free FITC, no signals were registered.

When cells were examined by fluorescence microscopy, it was observed that the field of cells was coincident with the field of fluorescence spots and the number of spots increased with the age of the bacterial culture, whereas no spots were detected in negative controls (Fig. 3).

In order to check the effect of active growth on the exogenous incorporation of labeled porins, a complementary experiment was carried out. When the labeled porins were removed from the culture after 24 h and fresh medium was added to the cells, the level of fluorescence decreased inversely with the cell number (Fig. 2).

Effects of TolC porin on eukaryotic cells. The concentrations tested did not affect the viability of the Vero cells, as no significant differences were observed between the proportions of cells alive or dead in control and in porin-treated cultures. Under the three conditions tested, the increase in cell death was related to the duration of the cultures and the overgrowth of the Vero cells.

DISCUSSION

Antibiotic resistance is becoming more frequent every day, and thus the number of resistant pathogens increases. On the



FIG. 3. Fluorescence microscopy images of *E. coli* C600+ that had incorporated labeled porins into the OM of the cell wall (magnification, $\times 60$). The visible light showed that the field of cells was coincident with the field of fluorescence spots.

other hand, the number of new approved active compounds able to be used as antibiotics is smaller every year (16). During the last decade, the U.S. Food and Drug Administration (FDA) has approved only four new antibiotics. The resistance phenomenon has an enormous cost for health care systems around the world, which is estimated to be 4,000 million dollars per year (5). The use of high antibiotic concentrations is not a desirable alternative due to the side effects. Knowledge of the mechanisms of antibiotic uptake by bacterial cells may help in the development of more effective strategies in the fight against resistant bacteria.

The relationship between TolC from *K. oxytoca* and antibiotics has been previously described (2). Results from the experiments carried out during the current work showed that the close relationship between TolC, antibiotics, porins can be employed both to rescue old-fashioned antibiotics for clinical use and to allow the use of lower effective doses.

The TolC protein from *K. oxytoca* showed a lytic effect on *E. coli* cells when it was exogenously added, especially with young cultures in the exponential growth phase. A smaller effect was observed with old cultures. This fact could suggest that porins become inserted into the OM much more effectively in young bacteria than in old ones. When the cells are growing actively and quickly, the cell wall has a weaker architecture than in stationary-phase cells. Perhaps this could explain the different behavior of the cultures.

The influence of the growth status of the cells on the incorporation of the exogenous porins is reinforced by results from the fluorescence experiments. It was possible to detect the incorporation and the increase of the FITC signal only in actively growing cultures. Neither dead cells nor azide-inhibited cells could incorporate labeled porins into the OM. Also, it was suggested that the incorporation was specific and that the differences observed were not due to free FITC linked to cell surface molecules.

The linkage between the porins and antibiotics was determined by the incubation conditions, mainly the temperature, but more experiments must be carried out in order to determine the exact nature of the linkage between the two molecules.

There have been numerous studies on efflux pump systems involved in antibiotic resistance. In *Enterobacteriaceae*, the major multidrug transporter is AcrB, which belongs to the resistance-nodulation-cell division (RND) superfamily (10). TolC is the OM protein involved in the AcrAB efflux system together with AcrB and AcrA, a periplasmic membrane fusion protein (14). The relationship between TolC from *K. oxytoca* and antibiotic transporters seems to be clear (2). Different efflux pumps have been described for *Klebsiella*, with AcrAB being the most important one (4, 9), mainly in the efflux of quinolones. It has been shown that quinolone resistance in *Enterobacteriaceae* is generally caused by a cooperative effect between mutations in the genes *gvrA* and *parC* and efflux pumps, mostly AcrAB. This system is also present in *K. oxytoca* (2). The observed differences between the types of antibiotics must be explained according to differences in systems for transport across the *E. coli* cell wall. The greatest antibacterial action was attained by using mixtures of porins and ampicillin (a β -lactam), ciprofloxacin (a fluoroquinolone), or tetracycline (a polyketide), whereas the mixtures of clindamycin (a lincos-

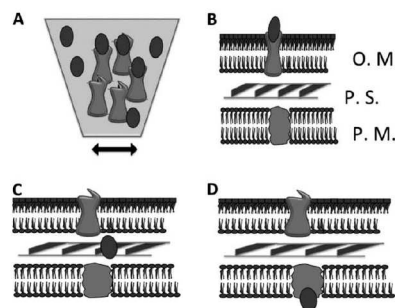


FIG. 4. Putative pathway of the mixture of exogenous porins and antibiotics across the cell walls. (A) Incubation of porins and antibiotics with shaking at 37°C. The linkage between antibiotic and porin is established in this step. (B) Insertion in the OM. P. S., periplasmic space; P.M., periplasmic membrane. (C) Release of antibiotics into the periplasmic space. (D) Uptake of antibiotics into the cytoplasm using the natural mechanisms of the cell.

amide) and erythromycin (a macrolide) with porins were not effective. The results obtained are consistent with previous reports on the implication of TolC in the transport of β -lactams and quinolones, as well as with the low ability of β -lactams and quinolones to act on bacteria in the stationary phase of growth.

TolC did not show any lethal effects on eukaryotic cells, at least at the concentrations tested. The lack of effect on mammalian cells could reinforce the potential application of porins as therapeutic adjuvants.

According to the results from the experiments presented here, the following hypothesis is proposed: (i) during incubation, the porin and the antibiotics establish an intermolecular linkage; (ii) the antibiotic-loaded porins incorporate into the bacterial OM and release their cargo into the periplasmic space; (iii) the antibiotics are taken up via classical mechanisms for *E. coli*; and (iv) the antibiotics exert their biochemical antibacterial action (Fig. 4).

The use of porins as adjuvants in antibiotic therapy may have a role in the fight against bacterial pathogens, particularly as far as lowering the effective dose and allowing the reintroduction in the clinic of older active drugs such as penicillins. This in turn could reduce or avoid undesirable side effects. The use of exogenous porins could minimize the impact on the natural microbiota of the treated organism, since bacteria causing acute infectious processes grow exponentially whereas natural populations forming part of the normal microbiota remain in states evoking a stationary phase.

Although it is obvious that additional experiments need to be carried out in order to clarify the exact mechanisms of porin incorporation into the cell wall and the relationships between antibiotics and porins, the present paper contributes important initial groundwork in this area.

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Class 1 integrons in environmental and clinical isolates of *Pseudomonas aeruginosa*[☆]

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ABSTRACT

The aims of this study were to ascertain the presence and spread of class 1 integrons amongst environmental and clinical isolates of *Pseudomonas aeruginosa* and to characterise their variable regions. A total of 76 isolates (56 clinical and 20 environmental) were studied. The presence of plasmids was explored, and polymerase chain reaction (PCR) was used for integron detection. All amplicons were sequenced. PCR detected class 1 integrons in 26 of the 56 clinical isolates; environmental isolates were integron-free. No plasmids were found, thus all the integrons found are possibly on the chromosome. Most isolates presented one amplicon, except PA110514 and PA116136, which showed two PCR products each. Variable regions revealed that 18 strains carried only one gene involved in aminoglycoside resistance, whereas in 3 strains gene cassettes were not found. The most prevalent cassettes amongst isolates were those encoding aminoglycoside adenylyltransferase B (*aadB*). Several of the strains had acquired the same or a highly similar cassette array as those detected in geographically distant *P. aeruginosa*. This finding suggests that contact with bacterial reservoirs contributes to the evolution of this pathogen towards multiresistance. Empty structures found may represent a reservoir increasing the capacity to adapt to the environment. However, these integrons are not retained when the selective pressure disappears. It is hypothesised that integrons containing gene cassettes are crucial vehicles for the rapid horizontal transfer of resistance. If this is so, reduced use of antibiotics may lead to a significant decrease in the carriage of integrons amongst *P. aeruginosa* strains.

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1. Introduction

The opportunistic pathogen *Pseudomonas aeruginosa* is noted not only for its metabolic versatility and its exceptional capacity to adapt to and colonise a wide variety of ecological environments (water, soil, rhizosphere, animals), but also for its intrinsic resistance to a broad range of antimicrobial agents. Infections by *P. aeruginosa* are often difficult to treat because of its virulence and

the relatively limited availability of effective antimicrobial agents. Nosocomial infections caused by *P. aeruginosa* frequently occur in ventilated and immunocompromised patients in Intensive Care Units [1,2]. Opportunistic infections caused by this pathogen can result from immunosuppression, which enhances the virulence of strains with low pathogenicity. Several studies have addressed the structure of *P. aeruginosa* populations [3–5].

Multiresistance of *P. aeruginosa* has been extensively described and involves components such as outer membrane proteins (OMPs), β -lactamases and other antibiotic-modifying enzymes, and efflux pumps. In the course of a population study of *P. aeruginosa* isolated in the hospital setting, we explored the basis of the resistance shown by this pathogen.

Increasing attention has been given to integrons in the context of resistance to antimicrobials. In particular, much research effort has been devoted to integrons belonging to classes 1, 2 and 3, the classes most commonly associated with the spread of antibiotic resistance in pathogens. Whilst integrons generally pose a problem for the management of spread of resistance, they are especially common in pathogenic bacteria [6]. Surveys examining the prevalence of various genetic elements in multidrug-resistant strains invariably

[☆] Nucleotide sequences accession nos.: The nucleotide sequences reported in this paper have been submitted to GenBank databases. The accession nos. for all the sequences are: In141, HM367607; In361, HM367608; InB, HM367609; In17, HM367610; In18, HM367611; In19, HM367612; In113, HM367613; In119, HM367614; In122, HM367615; In128, HM367616; In130, HM367617; In133, HM367618; In134, HM367619; In135, HM367620; In169, HM367621; In176, HM367622; In177, HM367623; In187, HM367624; In193, HM367625; InP18, HM367626; In199, HM367627; InP1, HM367628; and InP4, HQ157204.

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show a high correlation between the presence of a class 1 integron and particular antimicrobial resistance profiles. Most notable amongst these is resistance to ampicillin and streptomycin, and particularly to trimethoprim and sulfamethoxazole (SMX) [6,7]. In the case of class 1 integrons, SMX resistance is normally derived from *sul1*, which is located in a region downstream of the integron called the 3'-conserved segment (3'-CS), a region present in most class 1 integrons isolated from clinical environments [8,9]. Consequently, SMX resistance is a common feature of strains that carry class 1 integrons. The class 1 integrons that carry drug resistance genes in clinical isolates have a relatively conserved structure, which commonly comprises two conserved DNA sequences (5'-CS and 3'-CS) separating a variable region where mobile gene cassettes are located [10]. This arrangement has been used as a polymerase chain reaction (PCR) tool for the simple recovery of cassette arrays irrespective of knowledge of the many and varied cassettes that may be present [11,12]. In addition to recovering and analysing cassettes from specific isolates, PCR has also been used as a tool in broader epidemiological studies [13]. However, this method has some limitations. First, some class 1 integrons from clinical isolates do not have part or all of the 3'-CS, or alternatively they may carry a very large array of cassettes, thereby preventing detectable amplification. Either outcome can generate a false-negative result. In addition, PCR array length analysis alone underestimates cassette diversity since some array combinations may be similar or even identical in length [14]. Also, given that lateral gene transfer is mediated by a number of types of elements, the combinatorial exchange and spread of integron cassette arrays can occur in a variety of ways, including homologous recombination, transposition and even non-integron-mediated site-specific recombination. Thus, research into integron cassette arrays in the absence of context may not give a truly accurate picture of the processes that influence the spread of resistance genes in pathogenic bacteria [9].

Here we detected and characterised the integrons carried by a group of *P. aeruginosa* isolates of clinical and environmental origin [5].

2. Materials and methods

2.1. Bacterial strains

A total of 56 clinical and 20 environmental *P. aeruginosa* isolates were studied. Clinical strains were isolated by the Servei de Microbiologia of the Hospital Universitari de Bellvitge (Barcelona, Spain). Environmental isolates were obtained from samples of water and fomites in the hospital setting, as described elsewhere [5]. All bacteriological media were obtained from Scharlab, S.L. (Barcelona, Spain).

2.2. Minimum inhibitory concentration determination

Antimicrobial susceptibility testing was performed using the microdilution method in Mueller–Hinton broth following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [15,16]. The following antimicrobials were tested: piperacillin; piperacillin/tazobactam; ticarcillin; aztreonam; gentamicin; tobramycin; amikacin; trimethoprim/sulfamethoxazole; ciprofloxacin; ofloxacin; ceftazidime; cefepime; meropenem; imipenem; and ampicillin. Antibiotics were purchased from Sigma Chemical Co. (St Louis, MO), except for cefepime, which was from Bristol-Myers Squibb (Rueil-Malmaison, France). For some strains, the disk diffusion method with Mueller–Hinton agar was also performed. Disks were purchased from Oxoid Ltd. (Basingstoke, UK). Strains were classified as susceptible or resistant following the CLSI guidelines [15].

2.3. Plasmid isolation

Two strategies were used to obtain plasmids from the strains. In the first, conventional plasmids were detected by the alkaline lysis method described by Feliciello and Chinali [17]. Briefly, cells were lysed with sodium hydroxide/sodium dodecyl sulphate (NaOH/SDS), followed by precipitation of the cell lysate with 2 M potassium acetate/1 M acetic acid, and precipitation of the resulting supernatant with isopropanol. Finally, the precipitate was treated with RNase and was once again precipitated with isopropanol. The second method, for detecting megaplasmids, consisted of pulsed-field gel electrophoresis (PFGE) of genomic DNA using the method described by Barton et al. [18]. Briefly, bacteria were embedded in agarose gel and were lysed using a rapid protocol. After this, plugs were incubated with S1 nuclease (Fermentas, Vilnius, Lithuania) and were subjected to PFGE in agarose gels in a CHEF-DR III unit (Bio-Rad, Hercules, CA) for 14 h at 14 °C and 6 V/cm with pulse times of 45 s, and for 6 h at 14 °C and 6 V/cm with pulse times of 25 s. Electropherograms were visualised by ethidium bromide staining as described elsewhere [5].

2.4. Detection and analysis of integrons

DNA extraction for integron detection was performed by a modification of the method described by Lévesque et al. [11]. Briefly, strains were grown overnight in 10 mL of brain–heart infusion (Scharlab, S.L.) with 10% glycerol in the presence of a selective antibiotic at 37 °C. After diluting the culture 1:5 with distilled water, the bacterial suspension was boiled for 10 min and was then centrifuged at 12 000 × g for 2 min. The supernatant was used for PCR analysis. PCR was carried out in a 100 µL volume containing 250 µM dNTP (Fermentas), 2.5 pmol of each primer (5'CS, 5'-GGCATCCAAGCAGCAAG-3'; and 3'CS, 5'-AAGCAGACTTGACCTGA-3') (Invitrogen, Camarillo, CA), 1× PCR buffer, 3 mM MgCl₂, 1 U *Taq* DNA polymerase (Fermentas) and 30 µL of freshly prepared bacterial suspension. Amplification was achieved as follows: initial denaturation at 94 °C for 12 min, followed by a three-step profile of 94 °C for 1 min, 55 °C for 1 min and 5 min of extension at 72 °C for a total of 35 cycles; 5 s were added to the extension time at each cycle. A final extension at 72 °C was carried out for 5 min and following this step all completed reactions were maintained at 4 °C. All reactions were performed in a GeneAmp® PCR System 2700 thermal cycler (Applied Biosystems, Foster City, CA). PCR products were electrophoresed at 100 V for 1.30 h on a 0.7% (w/v) Tris–borate–EDTA buffer (TBE) agarose gel and viewed by ethidium bromide staining as described elsewhere.

2.5. DNA sequencing and analysis of sequence data

Sequence data for the amplicons were obtained with primers 5'CS and 3'CS. For isolates PA110514, PA116136, P18 and P4, it was necessary to design additional internal primers in order to amplify variable regions of integrons [for cassettes InA1/InP18, primers 5'-A18L (GTGCAGAGAATGATCAGC), 5'-A18L2 (CCTCCACATCGTG-GAA) and 5'-A18LR (TCTGTGGCGATGCACCA); and for cassette InP4, primer 5'-A4L (CTCCATAAGGCATTGAGCA)]. PCR products were purified with a MinElute® PCR Purification Kit (QIAGEN, Crawley, UK) prior to sequencing. An ABI PRISM BigDye® Terminator v.3.1 Cycle Sequencing Ready Reaction Kit and ABI PRISM® 3700 DNA Analyzer were used (Applied Biosystems). Database searches were conducted using the basic local alignment search tool (BLAST) [19].

3. Results and discussion

The genetic relationship between some of the isolates studied was analysed previously both by PFGE and polyacrylamide gel elec-

Table 1
Susceptibility of 76 *Pseudomonas aeruginosa* isolates to the antimicrobial agents tested.

Susceptibility	% of isolates									
	TZP	CAZ	FEP	ATM	IPM	MEM	GEN	TIC	AMP	CIP
Susceptible	80.3	51.3	56.6	60.5	40.8	65.8	48.7	57.9	82.9	53.9
Intermediate	11.8	26.3	31.6	7.9	5.3	5.3	11.8	2.6	7.9	1.0
Resistant	7.9	22.4	11.8	31.6	53.9	28.9	39.5	39.5	9.2	45.1

TZP, piperacillin/tazobactam; CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; IPM, imipenem; MEM, meropenem; GEN, gentamicin; TIC, ticarcillin; AMP, ampicillin; CIP, ciprofloxacin.

trophoresis (PAGE) of OMPs showing a first cluster including four strains (three of clinical and one of environmental origin), a second cluster that also included clinical and environmental isolates, as did a third cluster.

An additional cluster with a high degree of diversity was formed only by environmental isolates, although distance suggested a poor relationship amongst them. Thus, measurement of genetic diversity by PAGE of OMPs disagrees with results from PFGE. Assuming that the outer membrane constituted a permeability barrier with a key role in antibiotic resistance, only some OMP combinations were expected to survive in hospitalised patients. Therefore, in this case selective pressure acted in the opposite manner, leading to lower diversity amongst clinical isolates, although genetic diversity as measured by PFGE was higher in this group [5].

Susceptibility to antimicrobials as percentage resistant, intermediate and susceptible strains is shown in Table 1. When the presence of plasmids was explored either by alkaline lysis or S1-PFGE, all strains gave negative results. Thus, although we cannot confirm that these isolates are plasmid-free, it is feasible that the presence of plasmids is rare, which suggests that all the integrons found are on the chromosome.

The search for class 1 integrons by PCR using primers 3'CS and 5'CS, which led to the amplification of only the variable region (gene cassettes), resulted in the detection of class 1 integrons in 26 of the 56 clinical isolates.

Environmental strains were free of resistance and were completely integron-free. This observation may be a consequence of the lower capacity of these strains to acquire integrons or may simply imply that they do not interact with integron-bearing organisms. The prevalence of class 1 integrons amongst isolates decreases significantly as their origin lies progressively further from human influence. We thus conclude that antibiotics exert the main pressure that selects for and maintains the integrons.

Amongst integrons, class 1 integrons have received the greatest attention. They have been proposed to be largely involved in the dissemination of antibiotic or disinfectant resistance amongst clinical isolates; in fact, ca. 75% of clinical isolates carry at least one integron of this class [8,9].

The largest amplicon (3000 bp) was found in strain P4, whereas in P1 and P18 amplicons of ca. 2500 bp were detected, whilst isolates cc, cd and 28 gave amplicons of ca. 1000 bp. Isolates 7, 19, 22, 30, 33, 69, 76, 77, 87, 93, 99, cm, cn, co and B' had integrons giving amplicons of ca. 750 bp. Isolates cb, 17 and 26 showed the smallest amplicons (ca. 200 bp). Finally, PA110514 and PA116136 (an imipenem-resistant derivative of PA110514) showed two PCR products in each strain, thereby suggesting the presence of two integrons in each; the amplicon lengths corresponding to the sizes of the variable regions were 2500 bp and 400 bp. Gene cassettes were not detected in all the integrons (Table 2).

Direct sequencing of the variable region revealed that 18 strains carried only one resistance gene involved in aminoglycoside resistance. The most prevalent cassettes amongst isolates were those encoding aminoglycoside adenyltransferase (*aad*).

The cassette most commonly found in integrons was *aadB* (strains 7, 19, 22, 30, 33, 69, 76, 77, 87, 93, 99, cm, cn,

co and B'). This cassette encodes for the aminoglycoside-2'-*O*-adenyltransferase that confers resistance against kanamycin, gentamicin and tobramycin. *aadA7* was present in three strains (cc, cd and 28) and encodes for aminoglycoside adenyltransferase conferring resistance to streptomycin and spectinomycin. Sequencing revealed a high prevalence of genes conferring resistance to streptomycin and spectinomycin in the integrons. The former is used in clinics (e.g. to treat tuberculosis) and the second has been widely used as a growth promoter in food-producing animals. These uses thus result in an increase in selective pressure [20,21].

Sequencing of amplicons from strains P1, P4, PA110514, PA116136 and P18 revealed that the variable region contained a cassette array with various open reading frames (ORFs). The cassette array of P1 had three ORFs, all involved in aminoglycoside resistance; *aadB* encoding aminoglycoside-2'-*O*-adenyltransferase that confers resistance to kanamycin, tobramycin and gentamicin; and *aadA11* encoding aminoglycoside-3'-adenyltransferase that confers resistance to streptomycin and spectinomycin. The third, named *orfE*, encodes an aminoglycoside-2'-adenyltransferase that belongs to the conserved bacterial family pfam10706 and superfamily cl11303. The proteins included in these two families are involved in resistance to kanamycin, gentamicin and tobramycin and remove the synergism between aminoglycosides and cell wall-active agents. This cassette array also occurs in the integron In2345 (AY758206) of *P. aeruginosa* strain PA2345, isolated in the University Hospital of Besançon (France) [22], and both integrons share 100% identity for *aadB* and *orfE* and 98% for *aadA11*. Furthermore, the high similarity between sequences from bacterial isolates from different geographic regions suggests frequent global and cross-species spread of this cassette complex.

Strain P18 contained a cassette array with three ORFs, *orfL*, *tetR* and *orfJ*, in that order, and its variable region was identical to that of the largest amplicon present in strains PA110514 and PA116136. *orfL* encodes a phage integrase-like protein with an identity of 79% with the protein already described in plasmid pKLC102 [23]. This *orf* is overlapped with *tetR*, which belongs to the TetR family of transcriptional regulators involved in the transcriptional control of multidrug efflux pumps, pathways for the biosynthesis of antibiotics, response to osmotic stress and toxic chemicals, control of catabolic pathways, differentiation processes and pathogenicity [24]. Cassettes are preferentially integrated adjacent to the *attI1* site, but recombination events do not always occur as predicted (for example because of unusual recombination between an *attI* site and an *attC* site), thus creating a potential fusion of two adjacent gene cassettes [25]. *orfJ* encodes a hypothetical protein with conserved domains belonging to the family pfam06977 and to the superfamily cl06158. These two families represent a conserved region ca. 100 residues long within a number of hypothetical bacterial proteins that may be regulated by SdiA, a member of the LuxR family of transcriptional regulators. *orfJ* is inserted in an inverted position with regard to the promoters, thus there may be no transcription of the putative protein.

The smallest amplicon present in strains PA110514 and PA116136 was an integron that lacked integrated gene cassettes

Table 2

Characteristics of integrons and their gene cassettes identified in the integron-carrying clinical isolates.

Isolate	Amplicon length (bp)	Integron	Cassette(s)	Protein encoded
Cc	1000	InI8	<i>aadA7</i>	Aminoglycoside adenylyltransferase
Cd	1000	InI9	<i>aadA7</i>	Aminoglycoside adenylyltransferase
28	1000	InI28	<i>aadA7</i>	Aminoglycoside adenylyltransferase
7	740	InI7	<i>aadB</i>	Aminoglycoside-2'-O-adenylyltransferase
Cm	740	InI13	<i>aadB</i>	Aminoglycoside-2'-O-adenylyltransferase
Cn	740	InI34	<i>aadB</i>	Aminoglycoside-2'-O-adenylyltransferase
Co	740	InI35	<i>aadB</i>	Aminoglycoside-2'-O-adenylyltransferase
19	740	InI19	<i>aadB</i>	Aminoglycoside-2'-O-adenylyltransferase
22	740	InI22	<i>aadB</i>	Aminoglycoside-2'-O-adenylyltransferase
30	740	InI30	<i>aadB</i>	Aminoglycoside-2'-O-adenylyltransferase
B'	740	InB	<i>aadB</i>	Aminoglycoside-2'-O-adenylyltransferase
33	750	InI33	<i>aadB</i>	Aminoglycoside-2'-O-adenylyltransferase
69	750	InI69	<i>aadB</i>	Aminoglycoside-2'-O-adenylyltransferase
76	750	InI76	<i>aadB</i>	Aminoglycoside-2'-O-adenylyltransferase
77	750	InI77	<i>aadB</i>	Aminoglycoside-2'-O-adenylyltransferase
87	750	InI87	<i>aadB</i>	Aminoglycoside-2'-O-adenylyltransferase
93	750	InI93	<i>aadB</i>	Aminoglycoside-2'-O-adenylyltransferase
99	750	InI99	<i>aadB</i>	Aminoglycoside-2'-O-adenylyltransferase
cb	200	InI2	No gene	–
17	200	InI17	No gene	–
26	200	InI26	No gene	–
P1	2500	InP1	<i>aadB</i> <i>orfE</i> <i>aadA11</i>	Aminoglycoside-2'-O-adenylyltransferase Aminoglycoside-2''-adenylyltransferase Aminoglycoside-3'-adenylyltransferase
P18	2500	InP18	<i>orfL</i> <i>tetR</i> <i>tetR</i>	Putative phage integrase TetR TetR
P4	3000	InP4	<i>orfJ</i> <i>aac(6')-II</i> <i>bla_{P1b}</i> <i>aadA2</i>	Putative protein, function unknown Aminoglycoside 6'-N-acetyltransferase β-Lactamase Aminoglycoside-3''-O-adenylyltransferase
PA110514	2500	InI41	<i>orfL</i> <i>tetR</i> <i>orfJ</i>	Putative phage Integrase TetR Putative protein, function unknown
PA116136	400	InI42	No gene	–
	2500	InI361	<i>orfL</i> <i>tetR</i> <i>orfJ</i>	Putative phage integrase TetR Putative protein, function unknown
	400	InI362	No gene	–

but contained 5'-CS and 3'-CS, like the one in strains cb, 17 and 26. These empty structures may represent a reservoir that could confer bacteria the capacity to adapt rapidly to the environment by means of the acquisition of antibiotic resistance genes, amongst others, thus allowing selective advantage. Alternatively, the lack of integrated genes cassettes may be a consequence of the excision of previously integrated cassettes from the integron when antibiotic selective pressure is diluted in the environment, thus integrons could be in contact with, and even be acquired by, any strain, but not retained when selective pressure disappears. Recently it has been demonstrated that the expression of gene cassettes is regulated by an SOS response control [26] and antibiotic resistance genes can be silenced at no biological cost until they are required. However, these 'empty' structures appear to be indicators of the absence of sustained antimicrobial pressure.

The integron carried by P4 (Fig. 1), designated InP4 from its gene content, included *aacA4*, also called *aac(6')-II* (aminoglycoside 6'-N-acetyltransferase), downstream of the *utl1* recombination site,

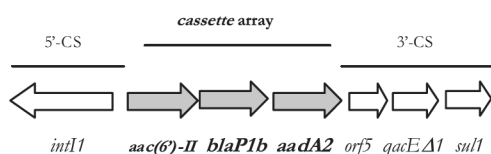


Fig. 1. Schematic representation of the cassette array contained in the variable region of the integron found in *Pseudomonas aeruginosa* P4. Inserted gene cassettes are indicated by grey arrows. The arrows show the direction of transcription of genes.

directly followed by the β-lactamase gene *bla_{P1b}* coding for PSE-1/CARB-2, and the adenylyltransferase gene *aadA2*. The AAC(6')-II family of enzymes provide resistance to tobramycin, netilmicin, kanamycin and gentamicin. AAC(6')-II is not only the most common AAC(6') but also the most common AAC in *P. aeruginosa* and is thus a significant determinant of gentamicin and tobramycin resistance in this organism. *bla_{P1b}* coding for PSE-1/CARB-2 carbapenemase confers resistance to the β-lactams piperacillin, carbenicillin and ticarcillin. The last gene, *aadA2*, encodes for aminoglycoside-3''-O-adenylyltransferase that confers resistance to streptomycin and spectinomycin. It has been argued that the common use of β-lactams and aminoglycosides for the clinical treatment of humans has contributed to the simultaneous presence of gene cassettes encoding β-lactamases and aminoglycoside-modifying enzymes in the same integron. Furthermore, the genes of this cassette, and also the complete cassette array, show high sequence identity (99%) with strains from distant countries such as China (*P. aeruginosa* strain PA466, GenBank accession no. FJ817423.1), Portugal (*P. aeruginosa* In99 and In100; GenBank accession nos. DQ219465.1 and AY560837.1 [27]) and Italy [28], amongst others.

The observation that geographically diverse *P. aeruginosa* strains have acquired the same or a highly similar cassette array suggests that contact with yet unidentified bacterial reservoirs contributes to the evolution of this pathogen towards multiresistance in Europe. This contribution is possibly by means of horizontal transfer of the complete integron structure. Antibiotics are used on a large scale in other ecological niches, for instance in animals for food production. In this context, these antibiotics may also contribute as sources or reservoirs of integrons carrying resistance genes [29].

Moreover, several studies have examined normal commensal microbiota from apparently healthy people, and high resistance rates to several antimicrobial agents have been observed. Thus, these bacteria could act as a reservoir for drug resistance genes recruited by pathogens under antibiotic pressure [30].

Integrations that contain gene cassettes provide a powerful vehicle for the rapid horizontal transfer of resistance across bacterial populations and thus could contribute to the sudden increase in the prevalence of multidrug-resistant infections in a community. The distribution of identical genes in organisms isolated from people living in disparate geographic regions indicates that they were more likely infected by organisms already harbouring such gene cassettes that originate in non-human reservoirs. That is, there appears to be a global 'epidemic' of mobile drug resistance genes, possibly spread by globalisation of trade.

The variety of structures found amongst class 1 integrons after more than half a century of antibiotic usage bears testament to the genetic flexibility and adaptability of the bacterial genome under environmental stress, characteristics that make these microorganisms ultimate survivors.

In conclusion, we propose that class 1 integrons are transient elements that foster antibiotic resistance in clinical environments, but not in the absence of antibiotic selective pressure. If this were the case, a decrease in antibiotic usage may lead to a significant reduction in the carriage of integrons amongst *P. aeruginosa* strains.

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A mechanism of carbapenem resistance due to a new insertion element (ISPa133) in *Pseudomonas aeruginosa*

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Summary. This study explored the evolutionary mechanism by which the clinical isolate PA110514 yields the imipenem-resistant derivative PA116136. Both isolates were examined by PFGE and SDS-PAGE, which led to the identification of a new insertion sequence, ISPa133. This element was shown to have distinct chromosomal locations in each of the original isolates that appeared to explain the differences in imipenem susceptibility. In strain PA110514, ISPa133 is located 56 nucleotides upstream of the translational start codon, which has no effect on expression of the porin OprD. However, in strain PA116136 ISPa133 is located in front of nucleotide 696 and, by interrupting the coding region, causes a loss of OprD expression, thus conferring imipenem resistance. In vitro experiments mimicking the natural conditions of selective pressure yielded imipenem-resistant strains in which ISPa133 similarly interrupted *oprD*. A mechanism is proposed whereby ISPa133 acts as a mobile switch, with its position in *oprD* depending on the degree of selective pressure exerted by imipenem. [Int Microbiol 2011; 14(1):51-58]

Keywords: *Pseudomonas aeruginosa* · protein OprD · carbapenems · imipenem · insertion elements · antimicrobial resistance

Introduction

Infections by *Pseudomonas aeruginosa* are a serious clinical problem, particularly in immune compromised hosts in hospital settings [9,29,33]. Moreover, the treatment of these infections is often difficult because of the limited number of effective antimicrobial agents, due to the intrinsic resistance of *P. aeruginosa* strains and their different modes of growth [10]. These properties reflect the synergy between the bac-

terium's low outer-membrane permeability [2,6,38], its chromosomally encoded AmpC β -lactamase [14], and its broadly specific drug efflux pump [11,17,22,23]. Furthermore, *P. aeruginosa* readily acquires resistance to most antimicrobials through mutations in its chromosomal genes and through extrachromosomal elements carrying resistance determinants [14,25]. Although there are several antimicrobials (carbapenems, cefepime, ceftazidime, tobramycin and amikacin) that continue to be effective against *P. aeruginosa*, in the last few years the bacterium's increasing resistance to many others has been reported [4,13,18,28,30].

Carbapenems are a class of β -lactam antibiotics with good antimicrobial activity against *P. aeruginosa* but the emergence and spread of acquired carbapenem resistance in this species have challenged the success of therapeutic and control efforts. Since carbapenems, especially imipenem, are

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widely used in the clinical setting [26], investigation of the molecular mechanisms leading to resistance is crucial. Imipenem resistance can involve low permeability, the activity of an inducible β -lactamase [15], and multidrug efflux systems, but the most widely accepted mechanism involves the loss of the porin OprD from the outer membrane [37], which can occur at the transcriptional or translational level or through the emergence of mutations in the *oprD* gene [20]. For example, in one report, the down-regulation of *oprD* transcription in clinical isolates of *P. aeruginosa* was shown to have occurred by a mechanism involving inactivation of the gene due to the presence of insertion sequence elements (IS) [36]. Additional mechanisms of carbapenem resistance include repressed OprD expression, as is the case in *nfxC*-type mutants, which simultaneously overexpress the MexEF-oprN efflux pump.

The present study resulted from the isolation of two strains of *P. aeruginosa* from the same patient in the course of a chronic respiratory infection. The first strain was obtained soon after the patient was admitted to the University Hospital of Bellvitge (L'Hospitalet, Barcelona, Spain). The infection was successfully treated with imipenem. The second strain was obtained 6 days later, from the patient's return. Pulsed-field gel electrophoresis (PFGE) profiles of the two isolates were identical, as were the results of biochemical tests aimed at their identification. However, determination of antibiotics susceptibility demonstrated that the isolates were identical for all antibiotics tested except imipenem: the first isolate was imipenem susceptible whereas the second was imipenem resistant. Thus, aim of this study was to search for underlying changes in the isolates' DNA that could account for the difference in imipenem susceptibility, starting from the hypothesis that resistance was mediated by a loss of OprD expression on the outer membrane.

Materials and methods

Bacterial strains. The two studied clinical isolates of *P. aeruginosa* were isolated from a patient at the Servei de Microbiologia of the Hospital Universitari de Bellvitge. PA110514 was isolated before imipenem treatment was started, and strain PA116136 after the patient had received the full course of the antibiotic. *P. aeruginosa* PA9 was used as the positive control for amplification of the *oprD* gene. Strains PA132325 and PA138635, susceptible and resistant to imipenem, respectively, were used as controls in preparing extracts of outer membrane proteins (OMPs). *P. aeruginosa* strains PAFL2, PAFL4, PAFL8, PAFL12, and PAFL16 are the resistant mutants obtained in this study. All of the bacterial strains were cultured in trypticase soy broth (TSB) or on trypticase soy agar (TSA). Bacteriological media were purchased from Scharlab (Barcelona, Spain).

Pulsed-field gel electrophoresis. To compare the different strains, PFGE was carried out as follows: DNA was extracted and purified as described elsewhere [28,31]. *SpeI*, a low-frequency restriction enzyme, was used according to the manufacturer's specifications (New England Biolabs, Beverly, MA, USA). The *SpeI* DNA restriction fragments were separated in a CHEF-DR III unit (Bio Rad, Hercules, CA, USA) for 20 h at 14°C and 6 Volts/cm, with pulse times ranging from 0.5 to 25 s. Strain relatedness was assigned in accordance with published criteria [32]. *Pseudomonas aeruginosa* PAO1 was used as the control in sequence analysis (accession number Z14065.1)

Minimum inhibitory concentration (MIC) determinations.

Antimicrobial susceptibility was tested using the microdilution method, with Mueller-Hinton broth (Scharlab, Barcelona, Spain), according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). The following antimicrobials were tested: piperacillin, piperacillin/tazobactam, ticarcillin, aztreonam, gentamicin, tobramycin, amikacin, trimethoprim/sulfamethoxazol, ciprofloxacin, ofloxacin, ceftazidime, cefepime, imipenem, and meropenem. The antibiotics were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Outer membrane preparations and SDS-PAGE.

Whole bacterial proteins and OMPs were obtained as described elsewhere [24,27]. SDS-PAGE was performed, using a modification of the method of Laemmli, in a Bio-Rad apparatus (Mini-Protein II). Gels were stained with 0.25% Coomassie brilliant blue, destained, and then dried on a gel dryer (Biorad 543) [8,12].

Gene *oprD* amplification.

To obtain *P. aeruginosa* genomic DNA, 5 ml of Luria broth inoculated with a single colony was incubated overnight at 37°C. Bacteria were harvested by centrifugation and suspended in 180 μ l of ATL buffer from the DNeasy Tissue kit (Qiagen, Germany), which was used for DNA purification following the manufacturer's instructions. A modified version of the PCR assay previously described [36] was used to amplify *oprD*. Reaction mixtures had a final volume of 50 μ l and contained 2 μ l (1/10th volume) of genomic DNA, 0.5 μ M of the OprDSEQF1 forward primer (5'-CTACGCAGATGCGACATGC-3'), 0.5 μ M of the OprDSEQR1 reverse primer (5'-CCTTTATAGCGCGTTGCC-3') (Invitrogen, USA), 1 U of Taq DNA polymerase (Fermentas, Vilnius, Lithuania), 0.2 mM of each deoxynucleoside triphosphate (Fermentas, Lithuania), 1 \times PCR buffer, and 2 mM MgCl₂. Amplification was achieved in a Techne (Staffordshire, UK) thermocycler model TC-312 during 25 cycles, each consisting of 30 s at 95°C, 30 s at 56°C, and 2 min at 72°C. Final extension was performed at 72°C for 10 min. PCR products were separated by agarose-gel (1.5% w/v) electrophoresis in Tris-borate buffer (TBE) at 9 Volts/cm for 1.5 h and visualized using ethidium bromide.

DNA sequencing and analysis of sequence data.

Sequence data for *oprD* were obtained by using the primers OprDSEQF1 and OprDSEQR1. PCR products were purified with the MinElute PCR purification kit (Qiagen, Germany) prior to sequencing. The ABI PRISM BigDye Terminator (version 3.1) Cycle Sequencing Ready Reaction kit and ABI PRISM(R) 3700 DNA analyzer were used (Applied Biosystems, USA). Database searches were conducted using the basic local alignment search tool (BLAST) [1] and multiple alignments carried out with CLUSTAL W [34].

In vitro generation of imipenem-resistant mutants of *Pseudomonas aeruginosa*.

Approximately 10⁵ *P. aeruginosa* PA110514 cells were used to inoculate 20 ml of MHB medium with imipenem concentrations ranging from 0.5 to 16 μ g/ml. After 24 h of incubation at 37°C, resistant derivatives (MICs = 6–16 μ g/ml) were selected by spreading

Table 1. Susceptibilities to antimicrobial agents tested against *Pseudomonas aeruginosa* PA110514 and PA116136

Antibiotics	Strain PA110514		Strain PA116136	
	MIC*	Susceptibility	MIC	Susceptibility
piperacillin	<16	S	<16	S
piperacillin/tazobactam	<16	S	<16	S
ticarcillin	<16	S	<16	S
aztreonam	2	S	2	S
gentamicin	<4	S	<4	S
tobramycin	<4	S	<4	S
amikacin	<8	S	<8	S
trimethoprim/sulfamethoxazol	<2/38	S	>2/38	R
ciprofloxacin	<0.12	S	<0.12	S
ofloxacin	<0.5	S	<0.5	S
ceftazidime	<1	S	<1	S
cefepime	<1	S	<1	S
imipenem	<1	S	>8	R
meropenem	<4	S	<4	S

*MICs are expressed in µg/ml.

0.01 ml of the overnight culture onto MHA plates (Scharlab, Barcelona, Spain) containing the appropriate concentration of imipenem. Repeated exposure to the antibiotic was continued with the most resistant derivatives until an increase in the minimum inhibitory concentration (MIC) of imipenem to 16 µg/ml was achieved.

Nucleotide sequences accession numbers. The nucleotide sequences reported herein have been submitted to GenBank databases. The accession numbers for the ISPa133 found in strains PA110514 and PA116136 are FJ387165.2 and FJ387166.2, respectively.

Results

Minimum inhibitory concentration determinations. Antimicrobial susceptibilities were determined in MIC tests, with the results shown in Table 1. *P. aeruginosa* strain PA110514 was found to be susceptible to all tested antibiotics while *P. aeruginosa* strain PA116136 was resistant to trimethoprim/sulfamethoxazole and imipenem.

Outer-membrane protein profile. Electrophoretic comparisons of OMPs from both strains demonstrated, as expected, that the susceptible strain, *P. aeruginosa* PA110514, but not the resistant strain, PA116136, showed a visible band corresponding to the porin OprD in the outer membrane (Fig. 1). To investigate the mechanism responsible for the loss of OprD expression, the *oprD* genes of both

strains were amplified and sequenced and their sequences compared.

Gene *oprD* amplification. The amplicons obtained from the *oprD* gene region of strains PA110514 and PA116136 were larger than that of strain PA9 used as positive control (Fig. 2). The predicted length of *oprD* is 1586 bp, while the amplicons from PA110514 and PA116136 were 3000 and 2000 bp, respectively. Sequence analysis of both amplicons revealed the presence of a long insert, approximately 1300 bp, with a 59.6% G + C content. The insert, designated ISPa133, showed high homologies with the mobile elements of the IS3 family of insertion sequences. The similarities were not only in terms of the length of the ISPa133, which for members of the IS3 family is typically between 1200 and 1550 bp, but due to the presence of two characteristic open reading frames (ORF), *orfA* and *orfB*, which, unusually, did not overlap (Fig. 3). The insertion element was flanked by 25-bp terminal inverted repeats (IRs), with TG at the 5' end and CA at the 3' end, as in other members of the IS3 family. Another general feature of IS elements is that, on insertion, most generate short directly repeated sequences (DR) of the target DNA flanking the IS, but these were not detected in ISPa133.

The first ORF (*orfA*) encodes a transposase (Fig. 3). In the search for conserved domains [16], we determined that

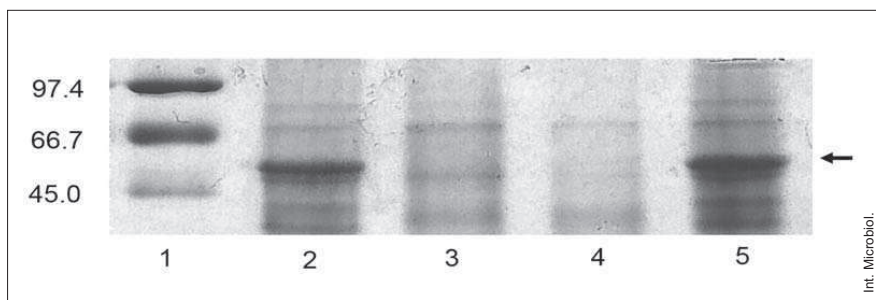


Fig. 1. Outer membrane protein profiles. Black arrows indicate the position of OprD. Lane 1: SDS-PAGE standards, (Low Range, Bio-RAD, USA); lane 2: strain PA110514; lane 3: strain PA116136; lane 4: strain PA138635 (imipenem-resistant control); lane 5: strain PA132325 (imipenem-susceptible control).

the protein includes a helix-turn-helix motif (HTH), a family of DNA-binding domains unique in bacteria, and that it showed high identity to the transposase 8 family, some of which are members of the IS3 family. The second ORF, *orfB*, encodes the central catalytic domain of an integrase from the *rve* super-family [http://pfam.sanger.ac.uk/family PF00665]. Integrase mediates the integration of a DNA copy of the viral genome into the host chromosome. The enzyme is composed of three domains. The amino-terminal zinc-binding domain (pfam02022) is the central catalytic domain. The carboxyl terminal domain is a non-specific DNA binding domain (pfam00552). The catalytic domain acts as an endonuclease when two nucleotides are removed from the 3' ends of the blunt-ended viral DNA resulting from reverse transcription. This domain also catalyzes the DNA-strand-transfer reaction

of the 3' ends of the viral DNA to the 5' ends of the integration site.

While the large insert located in PA116136 was 99% identical to that found in PA110514, its location in *oprD* was different. In PA110514, ISPa133 was located 56 nucleotides upstream of the translational start codon, but in PA116136 it occurred immediately before nucleotide position 697, effectively replacing this nucleotide and causing the removal of the first 232 amino acids of the porin OprD (Fig. 4).

In vitro generation of imipenem-resistant mutants of *Pseudomonas aeruginosa*. Repeated exposure of *P. aeruginosa* PA110514 to imipenem allowed the selection of several defective OprD mutants. When *oprD* was amplified using DNA from cultures resistant to imipen-

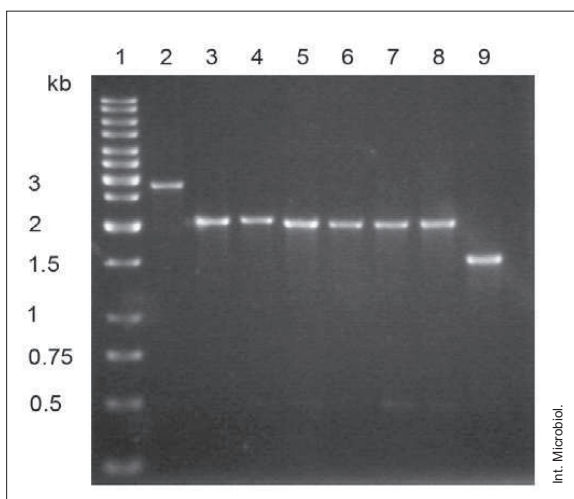


Fig. 2. PCR amplification products obtained with primers OprDSEQF1 and OprDSEQR1. Fragments were separated by electrophoresis through a 1.5% agarose gel. Lane 1: GeneRuler 1 kb DNA Ladder (Fermentas, Vilnius, Lithuania). Lane 2: strain PA110514. Lane 3: strain PA116136. Lane 4: strain PAFL2. Lane 5: strain PAFL4. Lane 6: strain PAFL8. Lane 7: strain PAFL12. Lane 8: strain PAFL16. Lane 9: strain PA9, used as positive control.

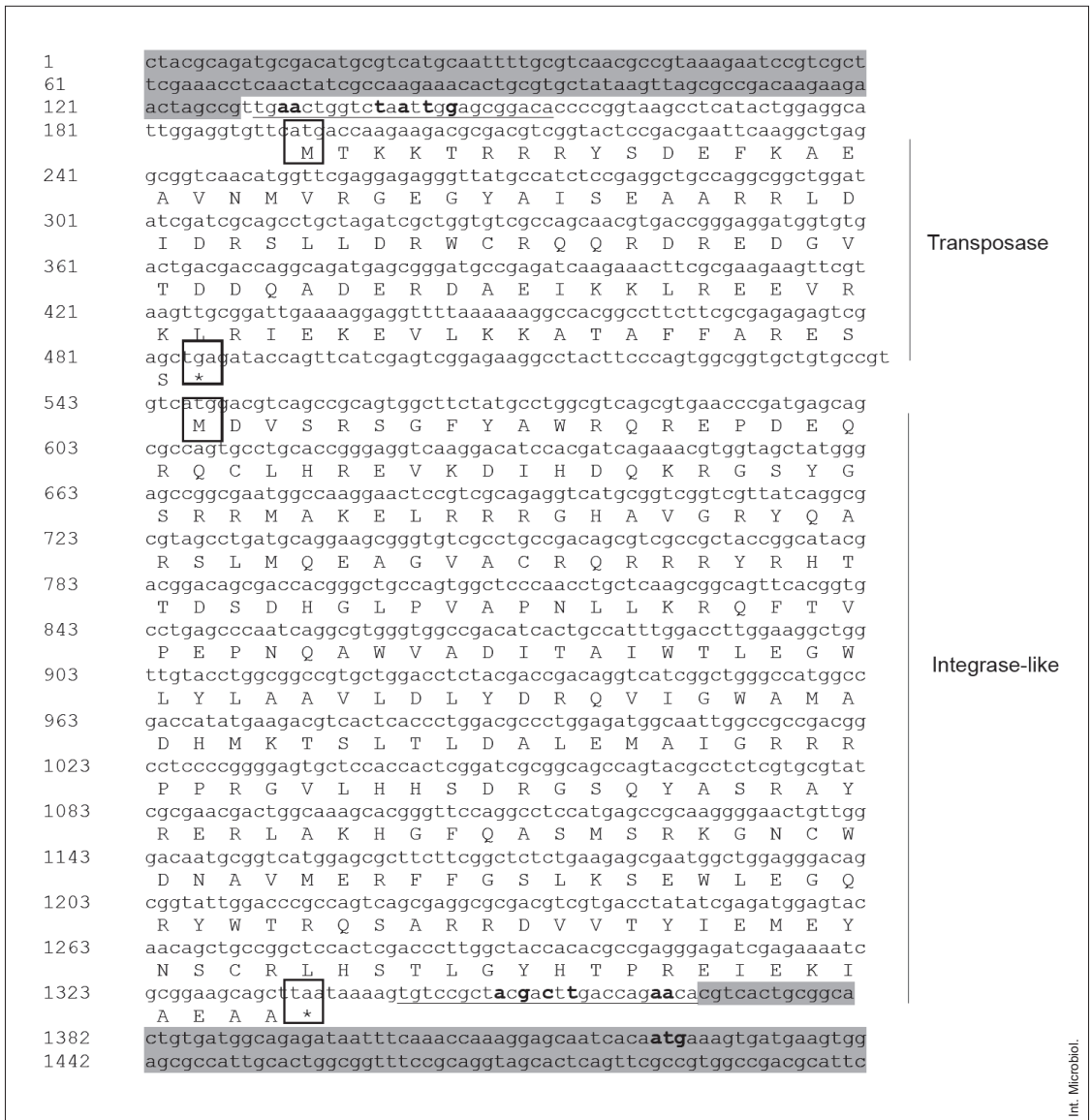


Fig. 3. Nucleotide sequence of de novel IS3-like element, ISPa/33, present in strain PA110514 and its proposed derivative PA116136. The underlined sequences represent the 25-bp inverted repeats (IRs). Bold letters within the IRs indicate mismatches between left and right IRs. Translational start and stop codons of both *orfS* encoded by the mobile element are boxed; the amino acid sequence of the two open reading frames are shown below the sequence. Shaded regions represent the sequence of the *oprD* gene flanking the IS element.

em concentrations 2, 4, 8, 12, and 16 times higher than the MIC value for PA110514, an amplicon of 2000 bp was obtained, i.e., identical in size to the amplicon of the resistant clinical isolate PA116136. Sequencing of the amplicon

revealed the presence of ISPa/33. The position of the insertion element in the mutants was not the same as in PA110514 and PA116136. In the mutant strain PAFL2, ISPa/33 was located immediately before nucleotide position 667, replac-

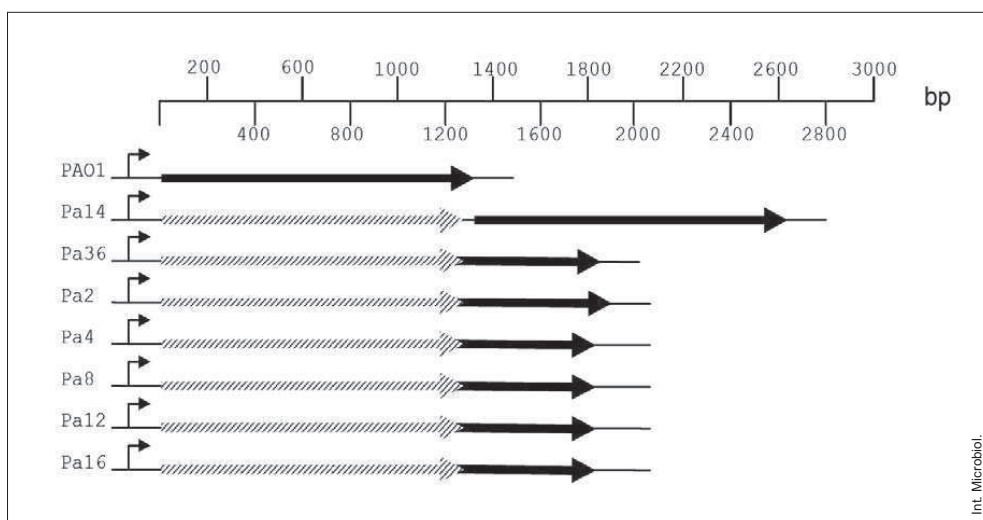


Fig. 4. Schematic diagram of the locations of the ISPa133 element with respect to the *oprD* gene. The solid arrow represents the *oprD* structural gene while the striped arrow represents ISPa133.

ing the first 222 amino acids of the porin, but in PAFL4 and in the rest of resistant strains, it was located just before nucleotide 703, causing the removal of the first 234 amino acids of OprD (Fig. 4).

Discussion

Carbapenems are a class of β -lactam antibiotics with good antimicrobial activity against *P. aeruginosa*; as such, they are often used as a last resort in infections due to multidrug-resistant strains of the bacterium [21]. However, the emergence and spread of acquired carbapenem resistance have challenged therapeutic and control efforts [5], necessitating a better understanding of the molecular mechanisms underlying resistance. Moreover, it seems likely that several mechanisms are involved in carbapenem resistance [7].

The two clinical isolates of this study, PA110514 and PA116136, were identical in their PFGE profiles and in biochemical tests, suggesting their close relationship. The unique noticeable difference between the isolates was in their susceptibility to imipenem. Our hypothesis, that this difference involved the OMPs, was supported by the results of SDS-PAGE, which revealed the loss of OprD in PA116136 (Fig. 1) and suggested that this strain was a PA110514 derivative. To investigate the mechanism responsible for the loss

of the porin, the *oprD* gene of each strain was amplified and sequenced. Examination of these sequences revealed the presence of a new insertion sequence, ISPa133, located 56 nucleotides upstream of the *oprD* start codon in PA110514. However, since this isolate is imipenem-susceptible, the presence of ISPa133 has no obvious effect on the upstream regulatory region of the gene, as *oprD* expression was normal (Fig. 1) as was the expression of the porin OprD. By contrast, in PA116136, the insertion element (99% identity) is located immediately before nucleotide position 696, which causes the removal of the first 232 amino acids of OprD. Consequently, the protein is not expressed and was not detectable in the gels (Fig. 1). Since loss of the protein prevents the entrance of imipenem, strain PA116136 is resistant to the antibiotic.

To date, the presence of ISs in OprD has been reported only once, although these elements have been detected in other genes of *P. aeruginosa* [3,4,28]. In those cases, the ISs were described in resistant isolates and thus assumed to be the cause of resistance, either via gene activation or by inducing the high level expression of a potential resistance gene. Based on the findings in the two clinical isolates analyzed in this study, we propose that the newly identified insertion element ISPa133 acts as a switch, depending on the degree of selective pressure exerted by imipenem. In the absence of selective pressure, as was the case during isolation of strain PA110514 from an infected, untreated patient, OprD is found

on the OM and the position of ISPa133 in the genome has no effect on *oprD* expression; however, selective pressure exerted by antibiotic therapy results in the selection of strains in which ISPa133 moves within the *oprD* gene, such that, in the case of strain PA116136, the first 232 amino acids are removed, thus preventing expression of the gene. A high rate of ISPa133 jumping would therefore provide a major selective advantage, one that allows the bacterium to survive in the presence of carbapenems.

It has been proposed that ISs without DRs may simply result from the homologous inter- or intra-molecular recombination between two IS elements, each with a different DR sequence, or from the formation of adjacent deletions arising from duplicative intramolecular transposition [35]. For ISPa133, this could explain the lack of DRs as well as the absence of a crossover region between *orfA* and *orfB*.

As shown in other species, experiments mimicking natural conditions can provide insight into population phenomena [19]. This was the aim of our experiments in which strain PA110514 was submitted to selective pressure by imipenem in culture flasks in order to obtain spontaneous resistant mutants in vitro with properties similar or identical to those of the strains isolated from a patient hospitalized with a *P. aeruginosa* infection. Indeed, mutants with levels of resistance similar to those of strain PA116136 were easily recovered. Moreover, when the experiments were prolonged, the MICs of the isolates were even higher (16 µg/ml).

Note that, according to the above-proposed mechanism of action, removal of the selective pressure exerted by the antibiotic should restore bacterial susceptibility to carbapenems through the expression of the full-length OprD; whether this is the case remains to be determined. However, this change would not be expected to occur as quickly as the acquisition of resistance, since the selective advantage conferred by imipenem resistance in the presence of the antimicrobial is severe whereas OprD expression is likely to be evolutionarily advantageous only under certain environmental conditions.

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Comparison of antibiotic susceptibility of old and current *Serratia*

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Aims: We explored changes in antibiotic susceptibility of *Serratia marcescens* in the last 50 years by comparing isolates collected between 1945 and 1950, and current isolates. **Materials & methods:** Isolates were divided into three groups: environmental, clinical and 'old'. Susceptibility was determined by microdilution. Class 1 integrons were determined by PCR. Statistical analysis was conducted using the Kruskal–Wallis (K–W) tests with Bonferroni correction for multiplicity. Antimicrobials showing differences in the K–W test were analyzed by Mann–Whitney U test. Differences were considered significant when $p < 0.05$. **Results:** All isolates were sensitive to ceftazidime, cefotaxime, kanamycin, gentamicin, ofloxacin and ciprofloxacin, and resistant to rifampicin, penicillin, ampicillin, amoxicillin, tetracycline, amoxicillin–clavulanic acid, cefazolin, cefamandole, polymyxin B/colistin, fusidic acid, lincosamides, streptogramins, daptomycin, linezolid and cefuroxime. Old isolates exhibited reduced susceptibility to streptomycin. Cefotaxime and streptomycin showed significant differences in the K–W test. None of the strains studied presented ESBL. Resistance to antimicrobials was not drastically different in *Serratia* when old and current strains were compared. **Conclusion:** Despite the multiple molecular mechanisms involved in bacterial resistance, withdrawing the antibiotics tends to restore the original phenotypes. Results from this report essentially confirm the conclusion obtained through metagenomic analysis that resistance to antibiotics already existed in ancient times.

Resistance to antimicrobials is an important public health problem in developed countries. *Serratia* are a genus of Gram-negative bacilli (family: Enterobacteriaceae), which occupy various habitats, mainly water, plants, soil, small mammals and hospitalized patients. The species *Serratia marcescens* causes both opportunistic and nosocomial infections, and it has been demonstrated to be involved in respiratory and urinary tract infections, septicemia, meningitis, and ocular and wound infections, among others [1–4]. It shows intrinsic resistance to many antimicrobial agents and expresses a wide variety of virulence factors [5,6]. It is generally accepted that restraint in antibiotic usage is essential to prevent the development of resistance to new antibiotics. This acceptance is based on the assumption that massive prescription, as well as extensive unregulated usage, leads to the development and spread of antibiotic resistance, which has had enormous clinical consequences. It is unusual to have access to a large collection of bacterial strains

that were isolated before this selective pressure of antimicrobials occurred. In the 1980s, one of the authors (MV) collaborated with Robert P Williams at Baylor College of Medicine (TX, USA). Dr Williams had a collection of strains that were lyophilized between 1940 and 1950 in the USA, which we were able to study. A total of 79 *Serratia* strains were examined.

Materials & methods

The strains were divided into three groups, depending on their origin. The first group (G1) included 14 strains of *Serratia* isolated between 2007 and 2008 from the environment, mainly from water, but also from vegetables and soils in Pallars Jussà (Spain), and the region surrounding Barcelona. The second group of *Serratia* (G2) included a total of 41 strains from the Robert P Williams *Serratia* collection. Finally, the third group (G3) was formed from clinical isolates of *Serratia* collected in Laboratory Clinic L' Hospitalet (Spain) in the

Keywords

- antimicrobial resistance
- bacterial evolution ■ MIC
- *Serratia*

last few years. For the experiments reported here, bacteria were cultured at 30°C in Luria–Bertani broth and agar, Trypticase™ soy broth (Beckton Diagnostic, NJ, USA) or Trypticase soy agar (Beckton Diagnostic), brain–heart infusion with 10% glycerol infusion (Difco, UK) and Mueller–Hinton (MH) broth and agar, depending on the experiment. Media were purchased in dehydrated form from Scharlau (Spain). Environmental samples were first pre-enriched in nutrient broth with 4% NaCl, and then incubated overnight at room temperature with shaking. Caprylate-thallos (CT) agar medium was then used for the selective isolation of *Serratia* species. CT agar was derived from M70 minimal medium, as proposed by Starr *et al.* [7]. A volume of 0.1 ml of the pre-enrichment culture was streaked onto CT agar and then incubated at 30°C for 7 days. Isolated colonies grown were streaked onto Trypticase soy agar to achieve the identification of *serratiae* from other Enterobacteriaceae. Identification was achieved with the following tests: oxidase test, DNase test, pigmentation, form and size of colonies, Gram staining, Simmons citrate, Indol, Voges Proskauer, gelatinase and urease. Clinical strains were isolated from patients affected by different infections at Laboratori Clinic L'Hospitalet (Spain). Identification and preliminary antibiotic susceptibility testing was accomplished by using the MicroScan system (Siemens Healthcare Diagnostics, CA, USA).

Drug susceptibility testing

Drug susceptibility testing was performed by determination of the MIC of antimicrobial agents using microdilution in MH broth, according to the guidelines of the Clinical Laboratory Standards Institute. Penicillin, ampicillin, amoxicillin, rifampicin, cefoxitin, ceftazidime (CAZ), cefotaxime (CTX), ceftriaxone, ofloxacin, ciprofloxacin, kanamycin, gentamicin, streptomycin, amoxicillin plus clavulanate and tetracycline (Sigma Chemical Co., MO, USA) were tested. To perform detection of class 1 integrons, bacteria were grown in 4 ml of brain–heart infusion with 10% glycerol at 30°C overnight. Subsequently, 200 µl of the culture was added to 800 µl of distilled water and boiled for 10 min. Bacterial suspension was then centrifuged at 12,000 *g* for 2 min, and the supernatant was used for PCR. The PCRs were carried out in 100-µl volumes containing 30 µl of template DNA and 70 µl of mix (2.5 pmol oligonucleotides, 1 U Taq DNA polymerase, 200 µM of each deoxynucleoside triphosphate,

3 mM MgCl₂, 10 mM Tris-HCl pH 8.5 mM KCl, 0.001% gelatin and distilled water). Primers used were described by Lévesque *et al.* [8]. All PCR products used were purchased from Fermentas (Lithuania) except the primers, which were purchased from Invitrogen (CA, USA). To amplify DNA in the thermal cycler, we used a three-step profile: 1 min of denaturation at 94°C; 1 min of annealing at 55°C; and 5 min of extension at 72°C for a total of 35 cycles [8,9]. All completed reactions were maintained at 4°C. The PCR products were visualized using 0.7% agarose gel electrophoresis (85 V for 2 h); gels were stained with ethidium bromide. As positive control, two strains of *Pseudomonas aeruginosa* with a 1 kb integron were used.

The method used for the detection of ESBL was described by Jeong *et al.* [10]. Briefly, disks containing phenylboronic acid (BA) made by using commercially available antibiotic-containing disks (Oxoid Ltd., UK) were prepared as described by Coudron [11]; a volume of 20 µl of the BA solution (20 g/l; Sigma-Aldrich Chemie GmbH, Germany) was dispensed onto each disk containing CTX (30 µg) and CAZ (30 µg) with and without clavulanate (10 µg). Disks were dried for 60 min and immediately used or stored at 4°C in airtight vials containing a desiccant. Similarly to the CLSI ESBL confirmatory test, any ≥5 mm increase in the zone diameter of CTX/CLA and/or CAZ/CLA disks tested in combination with BA (CTX/CLA/BA and/or CAZ/CLA/BA) versus CTX and/or CAZ disks containing BA (CTX/BA and/or CAZ/BA) was considered positive for ESBLs. CTX, CTX/CLA, CAZ and CAZ/CLA disks with BA were spaced over the MH agar surface. Inoculated plates were incubated overnight at 30°C in air. The CLSI confirmatory test for ESBL production was also carried out on MH agar using CTX and CAZ alone and with CLA [10].

Statistical analysis

Comparisons of MICs were statistically analyzed using the Kruskal–Wallis (K–W) tests with Bonferroni correction for multiplicity, following the Holm sequential procedure. Antimicrobials that showed significant differences in the K–W test were analyzed by Mann–Whitney U test to check differences between couples of strain groups. Differences were considered significant when $p < 0.05$.

Results & discussion

All the strains were sensitive to CAZ, CTX, kanamycin, gentamicin, ofloxacin and ciprofloxacin,

and resistant to rifampicin, penicillin, ampicillin, amoxicillin and tetracycline. Among them, 95% were found to be sensitive to ceftriaxone, and 54% were resistant to ceftiofloxacin. Old *Serratia* strains showed reduced susceptibility to streptomycin compared with their current relatives. It should be noted that CTX and streptomycin were the only antimicrobials to show significant differences in the K-W test, so the U-Mann-Whitney test was applied to check differences between couples of strain groups.

On the one hand, CTX showed significant differences between G2 and G3; however, in both groups all *Serratia* strains were clinically sensitive. Furthermore, streptomycin showed significant differences between strains of G1 and G2 and those of G3 and G2.

All strains studied were resistant to penicillin G, ampicillin, amoxicillin-clavulanic acid, cefazolin, cefamandole, polymyxin B, colistin, rifampicin, glycopeptides, fusidic acid, lincosamides, streptogramins, daptomycin, linezolid and ceftiofloxacin. Furthermore, they were susceptible to the third-generation cephalosporins CAZ and CTX. The 5% resistance to ceftriaxone suggested the occurrence of ESBL and therefore we performed experiments to detect ESBL studied. None of the strains studied presented ESBL.

Several studies have demonstrated that evolution of bacteria after the initial use (sometimes abuse) of antibiotics shows a constant tendency to increase MIC values [12]. This was, at least in part, attributed to the emergence of new molecular mechanisms of resistance. Thus, it seemed to us pertinent to explore whether such mechanisms were already present in 'old' strains. Most of the *Serratia* strains, especially those in G2, were streptomycin resistant, so we checked for the presence of integrons in the 'old' strains isolated from 1940–1950. PCR amplification followed by gel electrophoresis to visualize the amplification product showed that none of the strains examined presented class 1 integrons. However, when testing modern isolates (G1 and G3), we found that they also completely lack class 1 integrons, which is consistent with the hypothesis that such mechanisms have arisen in response to the selective pressure of antibiotics. However, the lack of integrons in newer strains (G1 and G3) was relatively surprising, since several reports have shown the occurrence of class 1 integrons in *Serratia* [13,14]. It could be possible that such integrons are restricted to ESBL and CTX-M producing *Serratia* and they mostly occur in isolates from Asia. In Europe

and the USA the occurrence of ESBL-producing *S. marcescens* carrying integrons is rare, although an outbreak of such a clone was described in an Italian intensive care unit with a mortality of 86% [15]. Moreover, a singular clinical isolate SCH88050909 has been extensively studied [16]. This strain was isolated in Greece and presented three integrons. It was studied owing to its wide multiresistance, and has been used to explore several mechanisms of insertion and to demonstrate that the *ant(299)-Ia* S.ma.12 gene cassette is a recombinationally active element [17].

The tendency to increase resistance has been demonstrated for *Streptococcus pneumoniae* and penicillin (from 0.04 µg/ml in 1940 to 0.12–1 µg/ml in 1980); *Staphylococcus aureus* and vancomycin (from 0.25–0.5 µg/ml in 1986 to 32 µg/ml in 2002) and *P. aeruginosa* and tobramycin (from <2 µg/ml in 1980 to >16 µg/ml in 2000), among others [18]. By contrast, in this study we found that resistance to antimicrobials was not so different in *Serratia* when old strains, isolated before the discovery of most of the antibiotics used today, and current strains are compared. In other words, the proportion of strains exhibiting a given value of MIC is similar in groups G1, G2 and G3, although it is clear that MIC values are slightly higher in clinical isolates (FIGURE 1). In some cases, the existence of resistance determinants becomes apparent much earlier than antibiotic discovery; for example, half of the strains had a MIC for ceftiofloxacin of 16 µg/ml in both G1 (environmental) and G2 (old) groups, despite the fact that this antibiotic was first used in 1977, almost 30 years after the isolation of the G2 microorganisms. When other non-cell-wall-active antimicrobials were considered, again our results indicated that overall susceptibility has not changed drastically over the last 50 years. This is true for all the antimicrobials tested except streptomycin. In this case, old strains, isolated when the use of streptomycin was widespread, were much more resistant than the newer strains (FIGURE 2). It also appears that the percentage of resistant *Serratia* strains has decreased since streptomycin was virtually withdrawn. Indeed, this has also occurred in other situations. For instance, in *P. aeruginosa*, the resistance to imipenem (due to the loss of the only porin able to transport imipenem through the outer membrane) is reversible when selective pressure due to the presence of the antibiotic disappears. A case of an imipenem-resistant strain isolated from a patient in the course of a second episode after a recent treatment with imipenem provided the possibility to explore and reproduce

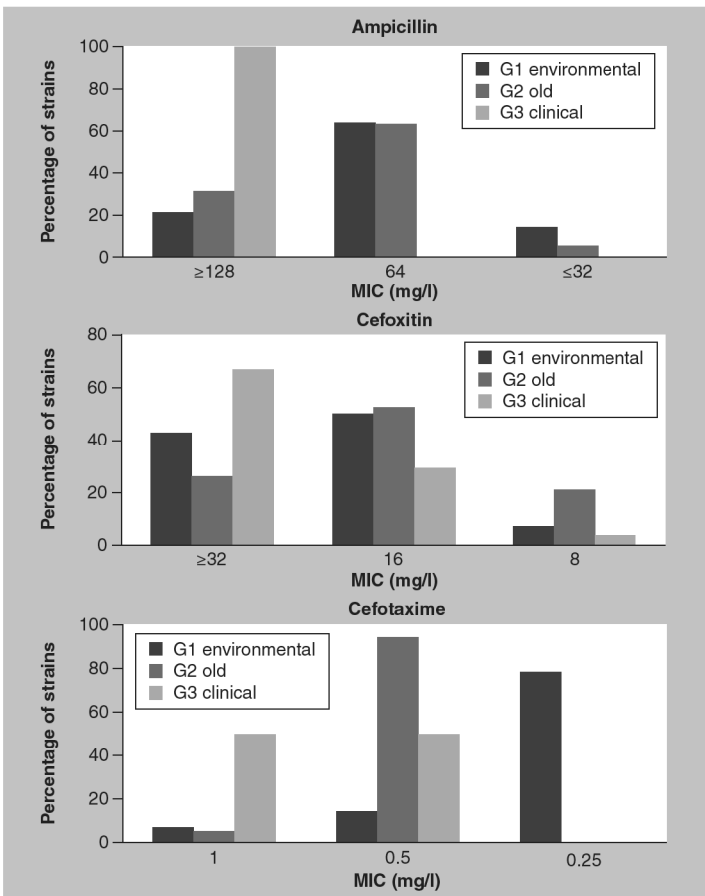


Figure 1. Comparison of MIC values for ampicillin, cefoxitin and cefotaxime values in the three groups. Highly similar profiles were seen in all other antibiotics tested apart from penicillin, to which all isolates were fully resistant.

the phenomenon at laboratory scale, (an insertion sequence *ISPa133* was found). When the susceptible isolate (first episode) was cultured in

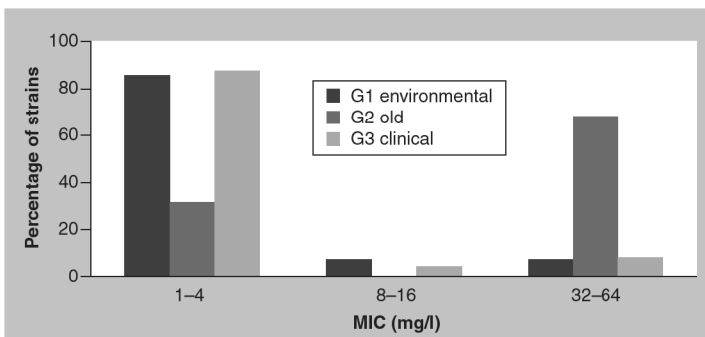


Figure 2. MICs of streptomycin shown as percentage of strains in three groups of isolates studied.

the presence of imipenem, a resistant clone was detected. This clone had the *ISPa133* inserted into the gene encoding *OprD*, but after repeated subcultures in absence of imipenem, the susceptible phenotype was restored [19]. These results indicate that, despite the multiple molecular mechanisms involved in bacterial resistance, withdrawing the antibiotics tends to restore the original phenotypes, possibly since they are more effective in antibiotic-free conditions. However, when large families of antibiotics are considered, this advantage could be counteracted by cross-resistance. For instance, susceptibility to penicillin has not been recovered in most species, despite the fact that penicillin is no longer widely used. One reason could be that bacteria are selected by many modern cephalosporins that share the mechanisms of resistance with the old relatives. To a greater extent than most Gram-negative bacteria, *S. marcescens* exhibits characteristic patterns of antibiotic resistance within an individual hospital environment. Often, the resistance pattern of such 'home strains' correlates with local antibiotic usage policy. This indicates that drug resistance plays a crucial role in determining the biological success of this relatively noninvasive bacterium in hospital settings.

Metagenomics studies have demonstrated that antibiotic resistance genes are widely disseminated, which seems to be inconsistent with the idea that resistance to antibiotics is a new emergent phenomenon. On the contrary, they suggest a long natural history of antimicrobial resistance. D'Costa *et al.* analyzed DNA bacterial sequences recovered from the Pleistocene period and developed a study of the diversity of ancient antibiotic resistance sequences [20]. Furthermore, they conducted a comparison of tridimensional structural models of ancient and contemporary enzymes responsible for vancomycin resistance.

Genomics (the bacterial resistome) has allowed exploration for the presence of genes encoding antimicrobial resistance in the past. However, it is feasible that environmental conditions affecting antimicrobial susceptibility have not changed significantly during the long period of time elapsed from the Pleistocene to the first part of the 20th century. Therefore, studies based on phenotypical susceptibility, despite not being so frequent because of the difficulty in obtaining cultures, are of great interest since, as it has been pointed out, in many cases the presence of the gene underlying a given function is necessary but not sufficient to ensure the expression of such a function. In other words, when we have to study

unculturable bacteria, metagenomics is undoubtedly the gold standard (and perhaps the unique method), but when bacteria can be cultured, interpretation of data can be made on the basis of both gene presence and phenotypic expression.

In fact, isolates of G2 were obtained, cultured, identified and lyophilized at the beginning of antibiotic era before the discovery of most antimicrobials. The present work could be seen as a confirmation of the conclusion that resistance to antibiotics already existed in ancient times [21].

When results of MIC are evaluated in detail it becomes apparent that G3 isolates (modern and clinical) had slightly higher values of MIC; for example, 100% of isolates had a MIC of ampicillin higher than 128 mg/l, whereas in group G2 (old) 60% had a MIC of 64 mg/l and only 35% were higher than 128, and the additional 5% had a MIC of 32 mg/l. In summary, although all isolates are clinically resistant to ampicillin, MIC values seemed to have a tendency to increase. A similar conclusion can be obtained when we evaluate data concerning other antimicrobials that do not act on the bacterial wall such as ciprofloxacin. In this case, all isolates were susceptible, but among G1 MICs ranged from 0.002 to 0.04 mg/l, whereas G2 ranged from 0.002 to 0.08 and finally all G3 isolates had MICs higher than 0.004; the lower value in this group was 0.01 and the maximum value reached was 0.32 mg/l. This makes sense, since clinical isolates include nosocomial populations exposed to antibiotic pressure.

Future perspective

The origin and spread of antimicrobial resistance is a main cause of concern and will be a research subject for many more years. It has been pointed

out that control in antibiotic use has a great impact in delaying the emergence of resistance and even allows populations to evolve from resistance to susceptibility. This field of research has to be based on genomics since we lack original strains in many cases, but when available, old populations have to be phenotypically analyzed, as the role of heteroresistance, as well as adaptive responses of microorganisms (epigenetics) to antimicrobials, seem to play a key role in the process of resistance.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- Antimicrobial resistance of current strains is not markedly different from that of strains isolated in the preantibiotic era.
- A certain increase of MICs has taken place.
- Results based on culture and conventional microbiology confirmed the conclusions obtained from metagenomics.
- Interpretation of data on the basis of both gene presence and phenotype expression improves the power of analysis.

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Carbapenem resistance in an endemic clone of *Pseudomonas aeruginosa*

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The prevalence of Gram-negative multidrug-resistant *Pseudomonas aeruginosa* (MDRPA) has increased in recent years. Clonal dissemination of this antibiotic-resistant pathogen is a major concern worldwide. The aim of this study was to explore carbapenem mechanisms leading to this resistance in an endemic clone. Outer membrane proteins were resolved in SDS-PAGE electrophoresis after outer membranes isolation. A bioassay method was used to measure meropenem accumulation depending on bacterial shape (bacilli or filaments). PCR was used for class 1 integron detection. Efflux pumps were studied for several antimicrobial agents and synergic combinations in the presence or absence of Phe-Arg- β -naphthylamide (PABN) at a final concentration of 40 $\mu\text{g/mL}$. MDRPA showed a reduction of OprD porin in OMP electrophoretic profiles. Accumulation of meropenem was 15% higher in bacilli than in filaments. All the strains had a class 1 integron with a cassette encoding aminoglycoside adenylyltransferase B (*aadB*). Overexpression of the efflux pump MexAB-OprM and a functional MexXY-OprM were detected in all the strains explored.

Key words: Multidrug-resistant *Pseudomonas aeruginosa* (MDRPA), carbapenem resistance, OprD, integrons, morphological changes and efflux pumps.

Introduction

Pseudomonas aeruginosa is a major nosocomial pathogen worldwide. It causes several infections, such as wound and burn infections as well as respiratory tract infections that mostly affect cystic fibrosis patients. Moreover, increasing prevalence of infections caused by multidrug-resistant isolates has been reported in many countries and is now a cause of concern [1, 2]. Some of the classic antimicrobial drugs used to treat these pathogens are out of date and bacterial mechanisms of resistance have already affected the efficacy of several of the newly-available drugs [3]. High-level antibiotic resistance in *P. aeruginosa* involves several mechanisms including overexpression of active efflux systems, the production of modifying enzymes, a decrease in outer membrane permeability and structural alterations of topoisomerases II and IV which are involved in quinolone resistance [4].

One of the main mechanisms of carbapenem resistance in *P. aeruginosa* is the reduction of outer membrane permeability through alterations in or decreased production of outer membrane protein OprD. This outer membrane porin allows entry of carbapenems, especially imipenem, into the cell [5].

While imipenem does not have any influence on the bacterial shape of *P. aeruginosa*, meropenem, at high concentrations it induces the formation of bacterial filaments. Previous in vitro studies have shown that morphological changes induced by meropenem in *P. aeruginosa* depend on the concentration and the exposure period [6, 7]. Furthermore, Horii *et al.* [8] demonstrated that meropenem induces the release of greater amounts of endotoxin than other carbapenems. This phenomenon is correlated with bacterial shape just before cell lysis. We measure the accumulation of meropenem in cells depending on the cell shape.

In *P. aeruginosa*, resistance to carbapenems, other β -lactam antibiotics and aminoglycosides, has been strongly related to enzyme production. Production of the following enzymes is particularly noteworthy: β -lactamase such as AmpC β -lactamase, class A carbenicillin hydrolyzing

β -lactamases (PSE-1 (CARB-2); PSE-4 (CARB-1); CARB 3 and CARB-4)), class A ESBLs (TEM, SHV, PER, VEB, GES/IBC and BEL types), class D β -lactamases (oxacillinases), carbapenemases or MBLs (IMP, VIM, SPM and GIM types) and aminoglycoside-modifying enzymes or AMEs (APHs, AADs or ANTs and AACs). Some of the antibiotic resistant genes found in multidrug-resistant *P. aeruginosa* (MDRPA) had been reported to occur as a cassette carried by class 1 integron [9]. The current study explored the presence of these genetic elements that contribute actively to the dissemination of resistance determinants to aminoglycosides and β -lactams among gram-negative species, and notably among *P. aeruginosa* multi-resistant isolates.

Other mechanisms are needed in addition to outer membrane barrier low permeability, hydrolysis by β -lactamase and genetic elements to explain the intrinsic and acquired resistance in *P. aeruginosa*. Multidrug efflux makes a major contribution to the resistance of gram-negative bacteria. Several studies with clinical isolates including epidemic clones support the role of the drug efflux pumps in multidrug-resistant (MDR) strains [10, 11]. Moreover, elevation of their expression levels can frequently produce high levels of antibiotic resistance [12].

The ten *P. aeruginosa* pumps (excluding the metal cation transporters) that belong to the resistance–nodulation–division (RND) family are: MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY, MexJK, MexGHI-OpmD, MexVW, MexPQ-OpmE, MexMN, and TriABC [10, 11]. Nowadays, a new molecular route for fighting against the increasing problem of antibiotic efflux is via efflux pump inhibitors (EPIs) to block efflux and restore drug susceptibility to resistant clinical strains. The recently discovered inhibitor PA β N, which is highly effective against *P. aeruginosa* efflux mechanisms, has an interesting efflux-inhibitory capacity associated with a reversal of fluoroquinolone resistance and has potent broad-spectrum activity [13-15]. Some of the mechanisms leading to resistance in four multidrug-resistant *P. aeruginosa* belonging to an endemic clone at the Hospital del Mar (Barcelona) were studied.

Materials and methods

Bacterial strains, media and antibiotics

Four MDRPA isolates belonging to an endemic clone selected between 2005 and 2008 in Hospital del Mar (Barcelona, Spain) were considered. These clinical strains were isolated from a blood culture, urine, a surgical wound and sputum. *P. aeruginosa* ATCC 27853 was also used as a test strain.

Ceftazidime, ciprofloxacin and the EPI, Phe-Arg-naphthylamide (MC-207,110, also termed PAβN) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Meropenem was from Astra Zeneca UK Ltd. (Macclesfield, UK), imipenem was from Merck (Rahway, NJ, USA) and tobramycin was obtained from Fagron Iberica, S.A.U (Barcelona, Spain). All the bacteriological media were obtained from Scharlab (Barcelona, Spain).

Drug susceptibility testing

Susceptibility to antimicrobial agents was determined using the broth microdilution method in Mueller-Hinton broth as recommended by the CLSI and expressed as MIC (minimal inhibitory concentration) [16]. The breakpoints were those recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [17].

Outer membrane (OM) isolation

Outer membranes were isolated from 100 mL of stationary-phase cultures. The cells were harvested, and the cell pellet resuspended in a 30 mL of Ringer 1/4. The cells were centrifuged at 6,000 x g at room temperature for 15 min and the pellet was resuspended in 30 mL HEPES 10 mM pH 7.4. Bacterial cells were disrupted at a pressure of 30 kPa (constant cell disruption systems) followed by a sedimentation of whole cells at 3,000 x g for 15 min at 21°C. Sedimentation of whole membranes was performed at 60,000 x g for 90 min at 21°C.

The pellets were resuspended in 2 mL of SLS 1% in HEPES 10 mM pH 7.4, and the suspensions were incubated at room temperature for 20 min. Sedimentation of the outer membranes was performed at 60,000 x g for 60 min and the pellets were resuspended in sample buffer.

Proteins were resolved by SDS-PAGE electrophoresis following a modification of the Laemmli method. Outer membrane proteins (OMPs) were compared with a size standard marker purchased from Bio-Rad (Hercules, CA, USA).

Bioassay for the measurement of meropenem accumulation

Long rods of *Pseudomonas aeruginosa* formed in the presence of four times the MIC and short rods from cultures without an antibiotic were washed with PBS pH 7.4 and resuspended in 30 mL of the same buffer. The total protein in both samples was measured using a Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, USA).

Meropenem was added while the cells were centrifuged at 20,000 x g for 1 min. The supernatant was used for the measurement of the meropenem that did not penetrate the cells.

Measurements were taken using a meropenem susceptible strain of *Escherichia coli*. The standard curve ranged from 25 to 0 ng/mL of meropenem. After incubation of the samples at 37°C for approximately 2 hours, the optical density at 550 nm was measured.

Detection of class 1 integrons and PCR amplification

Bacteria were grown in 4 mL of brain heart infusion (BHI; Difco, Detroit, MI, USA)-10% glycerol at 30 °C overnight. Then, 200 µl was added to 800 µl of distilled water and boiled for 10 min. The bacterial suspension was then centrifuged at 12,000 x g for 2 min, and the supernatant was used for PCR.

PCR was performed in 100-µl volumes containing 30 µl of template DNA and 70 µl of the mix (2.5 pmol oligonucleotides, 1 U Taq DNA polymerase, 200 µM deoxynucleoside triphosphate, 3 mM MgCl₂, 10 mM Tris-HCl pH 8, 50 mM KCl, 0.001% gelatin and distilled water). The

primers used were those reported by Lévesque *et al.* [18]. All the PCR products used were purchased from Fermentas (Vilnius, Lithuania) except the primers, which were purchased from Invitrogen (Camarillo, CA, USA). To amplify the DNA in the thermal cycler, we used a three-step profile: 1 min of denaturation at 94°C, 1 min of annealing at 55°C, and 5 min of extension at 72°C for a total of 35 cycles. All completed reactions were maintained at 4°C. The PCR products were visualized using 0.7% agarose gel electrophoresis (85 V for 2 h). The gels were stained with ethidium bromide.

DNA sequencing and submission

MinElute PCR Purification Kit (Qiagen, Crawley, UK) was used to purify the PCR products obtained for integron variable regions (IVRs). The purified amplicons were sequenced using the *ABI PRISM BigDye® Terminator version 3.1 Cycle Sequencing Ready Reaction Kit* and *ABI PRISM(R) 3700 DNA Analyzer* (Applied Biosystems, Foster City, CA, USA).

The sequences were compared using online blast software (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Study of efflux pump-mediated fluoroquinolone resistance

To establish the prevalence of efflux pump-mediated fluoroquinolone resistance in the MDRPA strains, MICs were determined in 96-well microtiter plates using a twofold standard broth microdilution method [19] in Mueller-Hinton broth either with or without a fixed concentration of EPI Phe-Arg-naphthylamide (PAβN) as described by Kriengkauykiat *et al.* [15].

Ciprofloxacin was tested at 11 concentrations (128-0.25 µg/ml) and PAβN at 40 µg/ml, based on previous studies [14]. A confirmation test was also performed during our research.

Detection of a functioning MexXY-OprM efflux pump

To detect the presence of a functioning MexXY-OprM efflux pump in the MDRPA strains, the MIC of aminoglycoside tobramycin was determined in 96-well microtiter plates using a twofold standard broth microdilution method in Mueller-Hinton broth in the absence and in the presence of 40 µg/mL of PAβN [20].

Effect of the efflux pump inhibitor PAβN on ceftazidime and meropenem MICs alone and in combination with tobramycin

A checkerboard titration assay was performed to assess the interaction between ceftazidime and meropenem plus tobramycin (concentrations ranging from 0 to 128 µg/ml), alone and in combination with 40 µg/mL of PAβN.

Results

Drug susceptibility testing

MIC data for the ATCC 27853 and MDRPA strains for several antimicrobial agents are shown in Table 1. Clinical breakpoints are listed below the table in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations [17].

Analysis of outer membrane proteins (OMPs)

OMPs were analyzed in all the strains. No great differences in OMP electrophoretic profiles were observed between the MDRPA strains and ATCC 27853. MDRPA showed a reduction of OprD porin (46 kDa) in comparison to ATCC 27853 (Fig. 1). This protein is a specific porin which facilitates the uptake of basic amino acids and imipenem [21].

Bioassay for measuring meropenem accumulation

In the presence of high-concentration (4xMIC) meropenem, *P. aeruginosa* was able to survive in long, undivided rods; when the meropenem antibiotic pressure was eliminated, the rods returned to their original shape. (Fig. 2)

Accumulation of meropenem was 15% superior in short rods than in long ones. Therefore, accumulation of meropenem revealed marked differences between regular bacilli and filaments.

Detection of class 1 Integrons

PCR amplification from all MDRPA endemic clones yielded a product of 750 bp (Fig. 3). The class 1 integrons mapped from all the MDRPA strains were identical and carried only one resistance gene involved in aminoglycoside resistance (Fig. 4).

The cassette contained the *aadB* (also called *ant(2'')-Ia*) aminoglycoside resistance gene that confers gentamicin, kanamycin and tobramycin resistance.

Efflux pump-mediated fluoroquinolone resistance

All the strains had a ciprofloxacin MIC of 64 mg/L and were interpreted as resistant according to EUCAST [17]. The efflux pump overexpressed phenotype (EPO) was defined with at least a twofold decrease in ciprofloxacin MIC when tested in the presence of the EPI PA β N [22] and was observed in all the MDRPA strains. Table 2 shows the decrease in ciprofloxacin MIC in the presence of 40 mg/L the EPI PA β N.

Detection of a functioning MexXY-OprM efflux pump

All the MDR strains, which had a high tobramycin MIC, showed outstanding antagonism in the presence of 40 μ g/mL of the EPI, PA β N. There was a minimum 8-fold increase in tobramycin MIC when combined with this EPI (Table 2).

Effect of PA β N on ceftazidime and meropenem MIC alone and in combination with tobramycin

A trivial reduction in ceftazidime and meropenem MICs was observed in the presence of PA β N. Regardless of the β -lactam concentration, the combination of tobramycin and ceftazidime or meropenem presented similar antagonism in the presence of PA β N (Table 2).

Discussion

P. aeruginosa is considered one of the more problematic drug-resistant pathogens, and many strains have developed resistance to multiple drugs, probably due to the large amount of antibiotics used to treat humans, farm animals and in aquiculture [23, 24]. It is extremely important to control the spread of multiresistant bacteria to avoid infections, increase the treatments that are available for patients and reduce patient mortality [25]. Table 1 shows that the four *P. aeruginosa* strains used presented high resistance to all the antimicrobial drugs tested in the present study and were therefore considered as MDRPA, i.e., resistant to at least three different classes of antimicrobial agents,

mainly aminoglycosides, carbapenems, antipseudomonal penicillins, quinolones, and cephalosporins [26].

Carbapenems, such as meropenem and imipenem, are potent antimicrobial agents of infections due to MDRPA isolates. These antibiotics bind to critical penicillin-binding proteins, disrupting the growth and structural integrity of bacterial cell walls. They provide better anaerobic and Gram-negative coverage than other beta-lactams and their stability against extended-spectrum beta-lactamases (ESBLs) makes them an effective treatment option. Both antibiotics have broad-spectrum activity, including activity against non-fermenting and Gram-negative bacteria.

However, among non-fermenting Gram-negative bacteria, resistance to imipenem and meropenem is increasing [27]. Several mechanisms that confer *P. aeruginosa* resistance to carbapenems have been reported, such as diminished permeability, overexpression of the intrinsic efflux systems and production of carbapenem hydrolyzing β -lactamases [28].

Additionally, the observation that *P. aeruginosa* survives in the presence of high concentrations of meropenem (4xMIC) by forming long undivided rods and its subsequent return to the original shape when the pressure was eliminated suggests that accumulation of meropenem probably differs depending on bacterial shape.

Our study revealed marked differences between regular bacilli and filaments.

These morphological changes could contribute to explaining the lack of success of antibiotic treatments, since long rods can survive treatment and become an origin of recidivisms when “normal” morphology is restored.

The reduction of OprD porin seen in all of the endemic clones compared to the wild-type strain (ATCC 27853) could be related with the decreased susceptibility to both imipenem and meropenem [4]. However, it is difficult to measure the quantitative role of the OM and their porins when an antibiotic is applied *in vivo*, since both the bacterial physiological states and the OM protein expression strongly depend on the environmental

conditions [29]. Rodríguez-Martínez *et al.* [30], reported that *OprD* gene downregulation and OprD protein inactivation contributed to resistance to imipenem and reduced susceptibility to meropenem. Whereas OprD inactivation alone is the source of intermediate susceptibility or resistance to imipenem, the mechanisms leading to meropenem resistance seem to be more complex and are very likely multifactorial, involving overproduction of AmpC or overexpression of the efflux pumps MexAB-OprM, MexXY-OprM, and MexCD-OprM. The efflux systems MexAB-OprM and MexXY-OprM directly affect meropenem activity in *P. aeruginosa* [31]. Ikonomidis *et al.* [32] found that heterogeneous populations had upregulation of efflux with increased levels of transcription of *mexB* and *mexY* genes and low intensity of oprD protein band (46 kDa) compared to native populations [4]. The aforementioned strains also produced OXA-1 and OXA-2 enzymes, which contribute to carbapenem resistance. (Segura *et al.*, unpublished data).

Our results from the study of efflux pumps using the EPI, PA β N suggested that all the MDRPA strains belong to a phenotype that overexpresses MexAB-OprM and contain a functioning MexXY-OprM efflux pump. However, structural changes in target enzymes and active efflux are the major mechanisms that lead to fluoroquinolone resistance in *P. aeruginosa*. Moreover, overexpression of MexXY-OprM has been related with carbapenem resistance. Mao *et al.* [20] demonstrated that PA β N antagonizes the activity of aminoglycosides only in strains that contained a functioning MexXY-OprM efflux pump, and the degree of antagonism was higher in strains with an elevated level of MexXY-OprM expression.

Furthermore, two mutations were found in the strains studied, one in *parC* and one in *gyrA* (Segura *et al.*, unpublished data). These results correlate with quinolone resistance in Enterobacteriaceae being generally caused by a cooperative effect between mutations in the genes *gyrA* and *parC* and efflux pumps, mostly *acrAB* [4, 33, 34, 35].

The multiple resistance mechanism of *P. aeruginosa* is extremely complicated, and many mechanisms are involved on it, including

integrations and gene cassette-mediated resistance [36]. Class 1 integrations have been strongly associated with the presence of MDRPA, as indicated by the fact that all the isolates that are not susceptible to less than three agents were *intl1*-negative, whereas almost all those non-susceptible to more than two agents were *intl1*-positive [37].

All of the MDRPA strains examined, which belong to serotype O4, had a class 1 integration with the gene cassette array *aadB*. The *aadB* product is aminoglycoside (2") adenylyltransferase (ANT(2")-Ia), associated with resistance to kanamycin, gentamicin, and tobramycin [38].

This type of integration was previously found in our laboratory in some clinical *P. aeruginosa* strains from Bellvitge Hospital, in Barcelona [39], however, this kind of integration has not only been found in Spain. In a recent study from Iran, 78% of a number of 41 MDRPA isolates from five hospitals had an integration containing the *aadB* gene and amplifications of internal variable regions (IVRs) of class 1 integrations confirmed a high prevalence of class 1 integrations with limited diversity of gene cassette arrays including *aadB*, *aadA6-orfD*, and *blaOXA10-aacA4* [40].

Syrmis *et al.* observed a high prevalence of the class 1 integration-associated *aadB* gene cassette in *P. aeruginosa* isolates from an Australian cystic fibrosis patient population. However, some of the strains that possessed the *aadB* gene were tobramycin sensitive. Thus, position of the *aadB* gene alone does not confer tobramycin resistance [41]. In Brazil, 106 *P. aeruginosa* clinical isolates were analyzed and none of the class 1 integrations detected had the *aadB* gene cassette [42].

Other studies showed that some class 1 integrations with more than one gene of resistance also contained the *aadB* gene. For instance, many MDRPA isolates (serotype O4) from various hospitals in the Czech Republic and Hungary shared an integration variable region with the gene-cassette array of *aadB-aadA13*, which may indicate recent international dissemination. In China and Iran, several *P. aeruginosa* clinical isolates had the *aadB* gene cassette array [37, 40, 43].

Although integration associated gene cassettes harbored by *P. aeruginosa* can vary between different geographic locations [41], it is important to

take clonal dissemination of the MDRPA strains into account and establish effective measures to prevent their transmission. In addition to integrons, multiresistant bacteria serve as hosts for other genetic elements such as transposons and plasmids. These elements are involved in the development of antibiotic resistance phenotypes and consequently bacteria that contain these elements could be extremely effective vehicles for the dissemination of these resistance elements [25].

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Nucleotide sequences accession numbers

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Transparency declarations

None to declare.

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Table 1. MICs (mg/L) of ATCC 27853 and MDRPA strains determined by a microdilution method.

Drug	Family	Strains					ATCC 27853
		459	133	162	527		
		MIC (mg/L)					
Meropenem	Carbapenem	8	8	8	8	8	1
Imipenem	Carbapenem	8	8	8	8	8	2
Ciprofloxacin	Fluoroquinolone	64	64	64	64	64	0.5
Tobramycin	Aminoglycoside	32	64	64	64	64	0.5
Ceftazidime	Cephalosporin	128	64	64	64	64	4

Clinical breakpoints, according to European Committee on Antimicrobial Susceptibility Testing (EUCAST): Meropenem, susceptible ≤ 2 mg/L, resistant > 8 mg/L; Imipenem, susceptible ≤ 4 mg/L, resistant > 8 mg/L; Ciprofloxacin, susceptible ≤ 0.5 mg/L, resistant > 1 mg/L; Tobramycin, susceptible ≤ 4 mg/L, resistant > 4 mg/L; Ceftazidime, susceptible ≤ 8 mg/L, resistant > 8 mg/L.

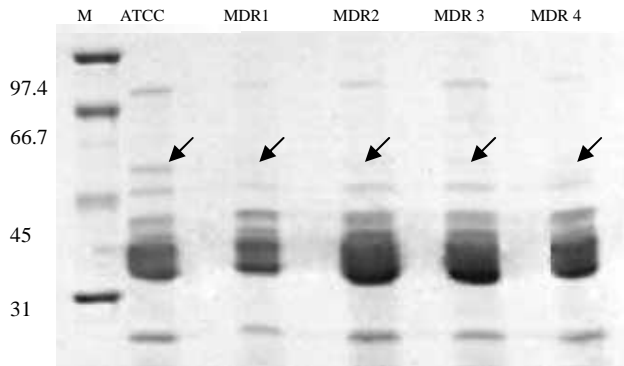


Figure 1. SDS-PAGE analysis of Outer Membrane Proteins (OMPs) of *P. aeruginosa*. Positions of molecular mass standards in kDa are shown on the left. Position of OprD is indicated by arrows on the left.

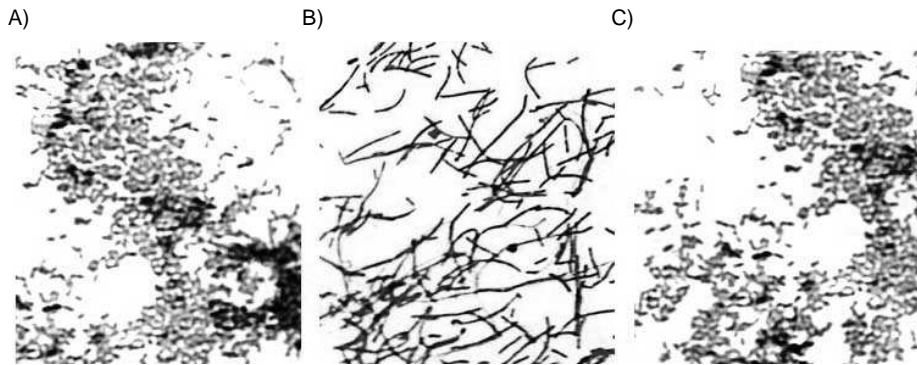


Figure 2. Effects of high-concentration (4xMIC) meropenem on *P. aeruginosa* cell shape. (A) *P. aeruginosa* rods grown without antibiotic. (B) *P. aeruginosa* rods grown with 4xMIC of meropenem. (C) *P. aeruginosa* rods after elimination of antibiotic pressure.

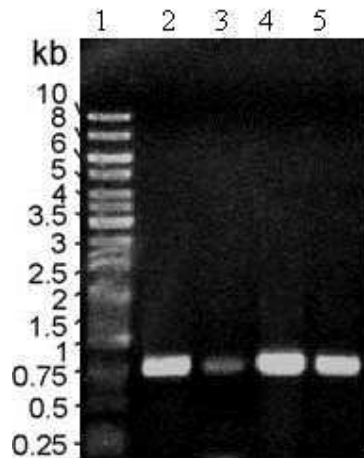


Figure 3. PCR amplification, using the 5'-CS and 3'-CS primers, of variable regions of integrons from MDRPA isolates. The PCR products were separated by electrophoresis in 0.7% agarose. Lane 1, 1-kb DNA ladder; lane 2, *P. aeruginosa* 459; lane 3, *P. aeruginosa* 162; lane 4, *P. aeruginosa* 527 and lane 5, *P. aeruginosa* 133.

Drug	Family	Strains				
		2404459	1449133	2908162	1169527	ATCC 27853
		MICs (mg/L)				
Meropenem	Carbapenem	8	8	8	8	1
Imipenem	Carbapenem	8	8	8	8	2
Ciprofloxacin	Fluoroquinolone	64	64	64	64	0.5
Tobramycin	Aminoglycoside	32	64	64	64	0.5
Ceftazidime	Cephalosporin	128	64	64	64	4

Clinical breakpoints according to European Committee on Antimicrobial Susceptibilities Testing (EUCAST): Meropenem, susceptible ≤ 2 mg/L, resistant >8 mg/L; Imipenem, susceptible ≤ 4 mg/L, resistant >8 mg/L; Ciprofloxacin, susceptible ≤ 0.5 mg/L, resistant > 1 mg/L; Tobramycin, susceptible ≤ 4 mg/L, resistant > 4 mg/L; Ceftazidime, susceptible ≤ 8 mg/L, resistant > 8 mg/L;

Table 2. MICs of synergic combinations of antibiotic with and without PA β N

Antibiotics	PA110514		PA116136			
	Before imipenem treatment		After imipenem treatment and before passages without antibiotic		After 30 passages without antibiotic	
	MIC (μ g/mL)	Suscept.	MIC (μ g/mL)	Suscept.	MIC (μ g/mL)	Suscept.
Tobramycin	0.5	S	0.5	S	0.5	S
Gentamicin	0.5	S	1	S	1	S
Meropenem	4	S	4	S	4	S
Ciprofloxacin	0.12	S	0.12	S	0.12	S
Ceftazidime	1	S	1	S	1	S
Imipenem	4	S	16	R	16	R
Colistin	0.5	S	1	S	1	S

In vitro activity of ceftazidime and meropenem in combination with tobramycin or ciprofloxacin in a clone of multiresistant *Pseudomonas aeruginosa*

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Abstract. Therapeutic options to fight against infections caused by multiresistant (MR) *Pseudomonas aeruginosa* strains are restricted to a few antimicrobials such as colistimethate and amikacin. The purpose of this study was to compare in vitro synergy testing by epsilometric (E-test) and the checkerboard (CB) methods with time-kill analysis in MR *P. aeruginosa* clinical isolates. Four isolates belonging to a MR endemic clone were selected. Their resistance mechanisms were studied. Susceptibility to ceftazidime (CAZ) and meropenem (MEM) in combination with tobramycin (TOB) and ciprofloxacin (CIP) were tested. Synergy was consistently detected in CAZ plus TOB as well as in MEM plus TOB combinations. The E-test method was comparable to CB method. Synergy and bactericidal activity were observed at 1/4 or 1/8 MIC TOB concentration combined with 1 MIC of CAZ or 1 MIC of MEM by time kill curves, with slight differences in the two isolates tested. These findings indicate the possibility of designing therapies based on combinations of a β -lactam and an aminoglycoside as a therapeutic option in infections caused by MR *P. aeruginosa*.

Keywords *Pseudomonas aeruginosa*, synergy, time-kill, FICI, SPBI

1. Introduction

Pseudomonas aeruginosa is naturally resistant to a wide variety of antimicrobials and can easily become resistant to many more; this constitutes a serious and growing therapeutic challenge, particularly in hospital setting. In addition, the natural capacity of *P. aeruginosa* to survive in adverse conditions and their minimal nutritional requirements, as well as their cosmopolitan distribution, enables this bacterium to be a silent nosocomial inhabitant.

A large number of publications have pointed out the increasing resistance rates, with special concern on the increase of isolation of multiresistant and panresistant strains [1, 2, 3].

As a result, endemic situations with variable incidences have emerged in many hospitals [4, 5, 6]. The lack of pipeline antipseudomonal agents available get the situation worse at short-term.

This has enhanced the interest in exploring the use of associations of several antibiotics and also in the rescue of old antimicrobials whose use had ceased several decades ago because of its potential toxicity; some of them have been used and assayed in several antibacterial combinations to achieve synergy [7].

The aim of this study was to assess synergistic effect of several antibiotic combinations as well as to explore bactericidal effect on *P. aeruginosa* isolates belonging to this endemic clone, thus, we explored the combinations of ceftazidime and meropenem with tobramycin and ciprofloxacin in four representative multiresistant *P. aeruginosa* isolates belonging to a nosocomial endemic clone of the Hospital del Mar, in Barcelona. Preliminary experiments to explore the mechanisms of resistance in these isolates were also

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2. Materials and methods

Bacterial strains: Four representative MR *P. aeruginosa* isolates belonging to an endemic clone from the Hospital del Mar at Barcelona (Spain) were selected.

Susceptibility tests: Susceptibility tests were done by diffusion or microdilution using the gram-negative breakpoint panel 38 for non-fermenter gram-negative bacilli of MicroScan® Walkaway system (Siemens Diagnostic Inc., CA) [Clinical Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: Twenty-first Informational Supplement M100-S21. CLSI, Wayne, PA, USA, 2011. Clinical Laboratory Standards Institute. Performance Standards for Antimicrobial Disk Susceptibility Tests -Tenth Edition; Approved Standard M02-A10. CLSI, Wayne, PA, USA, 2009.].

Clonal identification: Serotyping and pulse-field gel electrophoresis (PFGE) were done. DNA for PFGE was digested with 40 U of *xbal*.

Characterization of the mechanisms of resistance involved: Expanded spectrum β -lactamases (ESBLs), AmpC β -lactamases (AmpCs), oxacillinases (OXA-1t, OXA-2t, OXA-10t, OXA-20t, OXA-23t, OXA-24t, OXA-46, OXA-48, OXA-51t and OXA-58t), carbapenemases (GES, IMI, KPC, NMC, SME, GIM, IMP, SPM, VIM), aminoglycosides (ANT-2"IIa, ACC-3'-IIa, ACC-6'-Ia, ACC-6'-Ib, ACC-6'-Ic, ANT-4"-IIa, AAC-3'-Ia, AAC-3'-Ib, AAC-2'-Ia), and quinolones (*parC*, *gyrA*) were determined by PCR and sequencing (Macrogen Inc, Seoul, Korea) using the primers described in table 1 or in Aragon *et al.* [8]

Outer membrane proteins (OMPs). OMP extracts obtained and electrophoresed as previously described [9]

Mechanisms of efflux. The growth inhibition assays were performed as described [10] with some modifications. *P. aeruginosa* isolates were inoculated at $1 - 2 \times 10^6$ cfu/ml into tubes containing TSB with antibiotics at concentrations one-fourth the previously determined MIC, either in the presence or absence of PABN (10 mg/L final concentration). Bacteria were incubated at 37°C, and optical density values at a wavelength of 550 nm were registered over 8 hours. The OD₅₅₀ values were measured after 7 h.

Combination of Antibiotics: Antibiotic powders were provided by their manufacturers (Ceftazidima Combino Pharm®, Spain; Meropenem AstraZeneca, Spain; Tobramycin sulfate Fagron® Ciprofloxacin Combino Pharm, Spain).

Synergy tests: Synergy testing was performed using the checkerboard [11] and the epsilometric (E-test) methods [12]. Determinations by E-test were performed by duplicate, since the variation of concentration range on E-test strips, a MIC-to-MIC placement of the strips was easier to perform and seemed to give

a more accurate diffusion of the two drugs [12]. E-test strips were placed on the bacterial lawn sequentially, the first E-test strip (strip A) was incubated for 1 h at room temperature, then removed, and the second E-test strip (strip B) was added immediately over the imprint of the strip A. Plates were incubated for 18 h at 37°C. Respective MIC strips/scales were used to read MICs by placing them in each gradient's position. The summation operator Fractional Inhibitory Concentration Index (FICI) was calculated for each set of MICs, and the mean FICI was used to compare with the checkerboard test. High-off scale MICs (>256 mg/L) were converted to the next two-fold dilution (512 mg/L). The following formulas were used to calculate the FICI: (i) FIC of drug A = MIC of drug A in combination/MIC of drug A alone; (ii) FIC of drug B = MIC of drug B in combination/MIC of drug B alone; (iii) FICI = FIC of drug A + FIC of drug B. Synergy was defined by a FICI ≤ 0.5 . Antagonism was defined by a FICI ≥ 4 . Values of FICI between 0.5 and 1 were termed additive and those from 1 to 4 indifferent [13].

The Susceptible Breakpoint Index (SBPI) [14] was also calculated. SBPI = (susceptible breakpoint A/MIC of A in combination) + (susceptible breakpoint B/MIC of B in combination). A SBPI of 2 indicates that the MICs of antimicrobials A and B in combination are either equivalent to their respective breakpoints or that the combination MIC of one of the antimicrobials is lower than its susceptible breakpoint.

Time-kill analysis: Time-kill assays were performed by the broth macrodilution technique [11] only for those antibiotic combinations showing synergy by both checkerboard and E-test. Each organism was tested against each antimicrobial agent, alone and in combination. The combinations tested against each organism were the β -lactam (CAZ or MEM) with TOB (i.e., CAZ plus TOB, MEM plus TOB). The concentrations of each antimicrobial agent tested alone or in combination were 1, 1/2, 1/4 and 1/8 of MIC values. Volumes of 10 mL tubes inoculated at 6×10^5 cfu/ml were incubated at 37°C aliquots of 0.1 ml were withdrawn from each tube at 0, 6, and 24 h, and 10-fold dilutions were prepared and inoculated onto blood agar plates. Plates were incubated for 24 h/48 h at 37°C and colony counted, lower limit of detection was 40 cfu/ml.

Synergy was defined as decreases $\geq 10^2$ cfu/ml (≥ 2 -log 10) at 6 or 24 h in the combination compared with that of the most active single agent. Indifference as a ≤ 10 -fold change in colony count at 6 or 24 h in the combination compared with that of the most active single agent and Antagonism as a 100-fold increase in colony count at 6 or 24 h in the combination compared with that of the most active drug alone. Bactericidal activity was defined as a $\geq 3 \log_{10}$ cfu/ml decrease in the starting inoculum. *P. aeruginosa* ATCC 27853

was used as quality control strain in all susceptibility tests by microdilution technique, in all checkerboards, time-kill tests, and in every lot of E-tests strips. ATCC 27853 is a susceptible strain (CAZ 1 µg/ml; MEM 0.25 µg/ml; TOB 0.5 µg/ml and CIP 0.25 µg/ml)

3. Results

MicroScan® susceptibility results expressed in MIC values (mg/L) were: Aztreonam 16; Ceftazidime 16 and >16; Cefepime 16; Piperacilline + tazobactam 32 and 64; Imipenem 8 and >8; CIP >2; Gentamicin >8; TOB >8; Amikacine 8 and 16; Colistine ≤2.

The four selected representative multiresistant *P. aeruginosa* gave identical PFGE pattern and all belonged to the O:4 serotype. In all of them the same resistance mechanisms were detected: bla-OXA-1-type, bla-OXA-2-type, ant(2'')-Ia and ant(4'')-IIb. Moreover two mutations on parC (Leu87Trp and Leu168Gln) one in gyrA (Asn87Asp) as well as an important reduction of OmpD porin expression were detected.

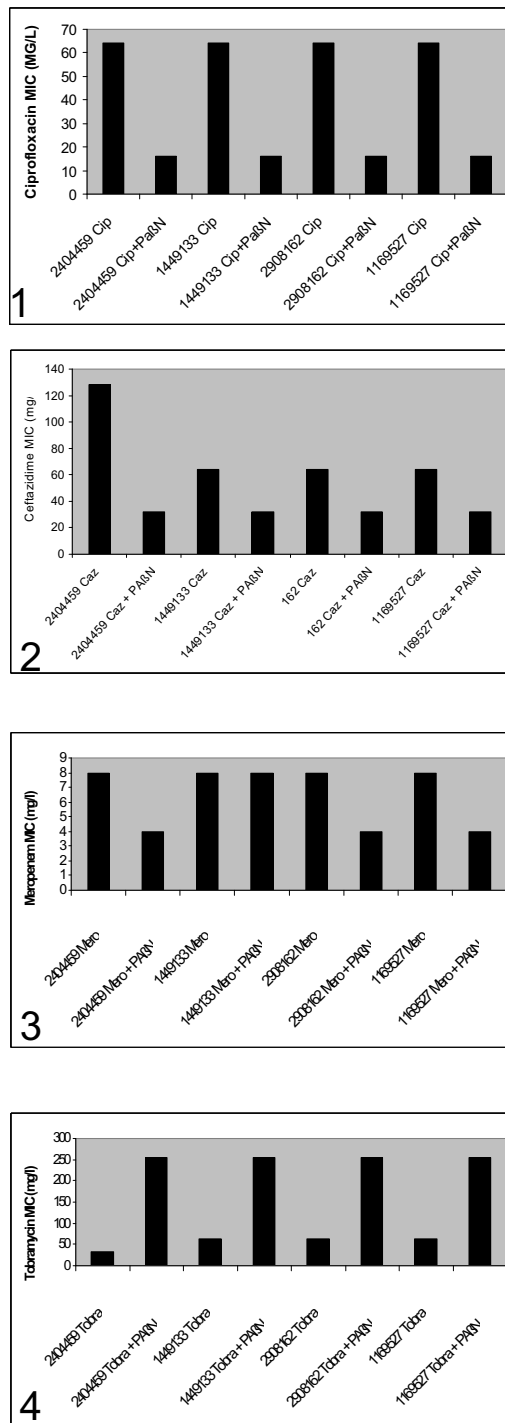
Fig 1 shows the effect of inhibition of efflux by the efflux pump inhibitor (EPI) PABN on (1) CIP susceptibility showing 4-fold decrease in MIC with the EPI; (2) CAZ susceptibility to which 2 and 4-fold decrease in MIC with the EPI were measured; (3) MEM susceptibility demonstrating 0 and 2-fold decrease in MIC with the EPI and finally (4) TOB in this case the antagonism between TOB and the EPI does not allow to measure the effect.

By both checkerboard and E-test, combinations of CAZ and MEM with TOB resulted to be synergistic against the isolates (Table 2). The combinations with CIP resulted to be additive or indifferent by the two methods, in the two isolates tested (Table 2). SBPI values were in concordance with FICI interpretation except in MEM + TOB combination.

Time-kill experiments done showed in two of the isolates synergy in the TOB-β-lactam combinations. CAZ+TOB as well MEM+TOB combinations showed synergy and bactericidal activity at different concentrations (Table 3).

Fig 1. Effect of inhibition of efflux by the efflux pump inhibitor (EPI) PABN on (1)ciprofloxacin susceptibility; all the strains were tested over the same range of ciprofloxacin concentrations (0.125 to 128 µg/ml), and showed 4-fold decrease in MIC with the EPI; (2) ceftazidime the strains were tested over the same range of ceftazidime concentrations (0.125 to 128 µg/ml), and showed 2 and 4-fold decrease in MIC with the EPI; (3) meropenem susceptibility, strains were tested over the same range of meropenem concentrations (0.125 to 128 µg/ml), and showed 0 and 2-fold decrease in MIC with the EPI and (4) tobramycin susceptibility. All the strains were tested over the same range of tobramycin concentrations (0.125 to 128 µg/ml), in this case the

antagonism between tobramycin and the EPI does not allow to measure the effect



Enzyme	Primer name	Primer sequence	GenBank number
OXA-1t	OXA1/4	CAGCAGCCAGTGCATC	J02967
	OXB1/4	TCCTGTAAGTGGCGACAC	
OXA-2t	OXA-2 up	GCCAAAGGCACGATGTTGT	X07260
	OXA-2 dn	ATAGAGCGAAGGATTGCCCG	
OXA-10t	OXA-10 up	GAGTTCTCTGCCGAAGCCG	J03427
	OXA-10 dn	GCCACCAATGATGCCCTCAC	
OXA-20t	OXA-20 up	CAGCTGTTGACTTGTCTCTC	AF024602
	OXA-20 dn	CGGATTGAAGAATAGCACGGC	
OXA-23t	OXA-23 up	CTTGCTATGTGGTGTCTTCT	AJ132105
	OXA-23 dn	CATTACGTATAGATCCGGGC	
OXA-24t	OXA-24 up	CTCTCAGTGCATGTTCATCT	AJ239129
	OXA-24 dn	CGAATAGAACCAGACATTCC	
OXA-46	OXA-46 up	ATGGCAATCCGATTCTCAC	AJ969237
	OXA-46 dn	TTAGTTGGGTGGCAATGCGT	
OXA-48	OXA-48 up	CGTTATGCGGTATTAGCCCTTAT	AY236073
	OXA-48 dn	TTTTTCCTGTTGAGCACTCTTT	
OXA-51t	OXA-51 up	ATGAACATTAACCACTCT	AJ309734
	OXA-51 dn	TTAAGGGAGAACGCTACA	
OXA-58t	OXA-58 up	CTTGTGCTGAGCATAGTATGAG	EU642594
	OXA-58 dn	ACCAATACGTTGCAATTAC	
GES	GES-1-up	ATGGCCTTCAATCACGCAC	GU831563
	GES-1-dn	CTATTTGTCCGTGCTCAGG	
IMI	IMI-up	GTCACCTAATGTAAAACC	U50278
	IMI-dn	TTAAGTTATCAATTGCG	
KPC	KPC-up	TGCTACTGTATCCCGCTC	AF297554
	KPC-dn	TTACTGCCCTGTGACGCC	
NMC	NMCA-up	GTCACCTAATGTAAAAGCA	Z21956
	NMCA-dn	GGTTATCAATTGCAATTC	
SME	SME-up	CAATTGCCTGAATTGCAAT	AY584237
	SME-dn	CGGCTTCATTTTGTGTTA	
GIM	GIM-up	ACTTGTAGCGTTGCCAGC	AJ620678
	GIM-dn	AATCAGCCGACGCTTCAG	
IMP	IMP up	GAAGGCGTTTATGTCATAC	DQ842025
	IMP dn	GTAAGTTCAAGAGTGTGTC	
SPM	SPM-1 A	CTGCTTGGATTCATGGGCGC	DQ145284
	SPM-1 B	CCTTTTCCGGACCTTGATC	
VIM	VIMB	ATGGTGTGTTGTCGATATC	DQ489717
	VIMF	TGGCCATTCAGCCAGATC	
ANT-2 ^{la}	ANT-2 ^{la} FW	ACGCCGTGGTTCGATGTTGATGT	X04555
	ANT-2 ^{la} R	CTTTTCCGCCCGAGTGAGGTG	
ACC-3 ^{la}	AAC-3 ^{la} FW	GGCAATAACG-	X13543

		GAGGCGCTTCAAAA	
	AAC-3 ^{la} R	TTCCAGGCATCCGCATCTCATAAG	
ACC-6 ^{la}	ACC-6 ^{la} FW	ATGAATTATCAAATTTGTG	M18967
	ACC-6 ^{la} R	TTACTCTTTGATTAAGT	
ACC-6 ^{lb}	ACC-6 ^{lb} FW	CAAAGTTAGGCATCACA	M21682
	ACC-6 ^{lb} R	ACCTGTACAGGATGGAC	
ACC-6 ^{lc}	ACC-6 ^{lc} FW	CTACGATTACGTCAACGGTGTG	M94066
	ACC-6 ^{lc} R	TTGTTCCGCCACTCTCTGACC	
ANT-4 ^{la}	ANT-4 ^{la} FW	CCGGGGCGAGGGCAGTGC	M98270
	ANT-4 ^{la} R	TACGTGGGGGGATT- GATGGGAACC	
AAC-3 ^{la}	AAC-3 ^{la} FW	GCAGTCCGCCTAAAACAAA	X15852
	AAC-3 ^{la} R	CACCTTCTCCCGATGCCCAACTT	
AAC-3 ^{lb}	AAC-3 ^{lb} FW	GCAGTCCGCCTAAAACAAA	L06157
	AAC-3 ^{lb} R	GGATCGTCAACCGTAGTCTGC	
AAC-2 ^{la}	AAC-2 ^{la} FW	AGAAGCGCTTACGATTATTA	L06156
	AAC-2 ^{la} R	GACTCCGCCTTCTTCTCAA	
parC	Ps parC up	CTATCTGAACATTCATGTACGT	AE004091
	Ps parC dn	ACGCGACTTCCCGAGGTG	
gyrA	Ps gyrA up	ATCGTCGGCGCGCCCTGCCG	AE004091
	Ps gyrA dn	GGGGTTGTCCATCAGCGCCA	

Table 1. Primers used to detect different genes involved in antimicrobial resistance.

4. Discussion

In our hospital a prolonged endemism of MR *P. aeruginosa* has been observed. Colistine resistant or intermediate isolates were encountered, albeit in low number; the treatment of infections caused by such isolates is difficult and nowadays restricted to colistine and amikacine.

On the other hand we tried to validate synergy testing by E-test as a rapid and easier tool to demonstrate synergy compared with the CB. Studied isolates showed resistance against all available antipseudomonal antibiotics except amikacin and colistine. Antibiotics for synergy testing were selected after reviewing the literature on the potentially active and less toxic antibiotic combinations for MR *P. aeruginosa*.

The resistance pattern and the lack of carbapenemases and extended spectrum betalactamases suggested that resistance was mainly due to a derepression of AmpC enhanced by OXA type betalactamases, up-regulation of efflux system showed by the pump inhibitor effect of PABN, reduction of OprD expression and alteration of topoisomerases and two aminoglycoside-modifying enzymes encoding resistance to kanamycin, gentamycin and tobramycin.

Synergy, was observed in combinations of CAZ and MEM with TOB both by E-test and checkerboard test. In the present study, as stated by other authors [12], checkerboard and E-test methods yielded equivalent results, making the E-test, by its simplicity, a suitable method in clinical laboratories for synergy studies. The SBPI values were in concordance with FIC index in all combinations but for MEM+TOB. In some combinations SPBI values were higher when CLSI susceptibility breakpoints instead EUCAST [http://www.eucast.org/clinical_breakpoints] were used. The SBPI was more discriminatory than FIC because it uses the susceptible breakpoint MICs and likely has more clinical relevance [14].

Time-kill results showed synergy in most of the combinations. Bactericidal activity was also observed in a lesser extent. Synergy and bactericidal activities observed with TOB concentrations lower than MIC values when combined with CAZ or MEM at 1 MIC. The bactericidal activity at 6 hours but not at 24 hours observed in most of meropenem combinations could be the consequence of meropenem degradation and the low TOB concentration that can't prevent regrowth.

In *P. aeruginosa*, synergistic activities between β -lactam and aminoglycosides were described previously [15, 16], but variable synergy rates were de-

scribed depending on the β -lactam antibiotic and on the characteristics of the *P. aeruginosa* strains included (susceptible, resistant or multiresistant strains). The mechanism of synergy between β -lactam and aminoglycosides is believed to be due to the increase of aminoglycoside penetration due to the activity of the β -lactam and, in turn, to the increase of entry of the β -lactam due to the cationic displacement caused by aminoglycosides [17]. The mechanisms of resistance have a critical role in the interaction of the different antibiotics and the absence of synergy observed in the ciprofloxacin combinations, unlike other studies, may be due to the presence of a resistance mechanism in our isolates [18]. It should be noticed that in our experiments synergy with antibiotics whose resistance mechanisms are chromosomally encoded resulted to be less evident than for those antibiotics whose resistance mechanisms are related to acquisition of genetic material.

Meropenem is commonly prescribed in nosocomial infections when the presence of *P. aeruginosa* is suspected. A peak plasma concentration of 53-62 mg/L were yielded after a dose of 1g meropenem in ICU patients [19], which is higher than all MIC values observed in the present study.

Isolate number	CAZ+TOB		MEM+TOB				CAZ+CIP		MEM+CIP			
	E-test		Check. test		E-test		Check. test		E-test		Check. test	
	^a FICI	^b SBPI	FICI	FICI	SBPI	FICI	FICI	SBPI	FICI	FICI	SBPI	FICI
1449133	0.36	2.12	0.34	0.35	1.33/0.83	0.31	1-1.5	0.69/0.67	1	>1	≤0.19/≤0.17	1
2404459	0.25	2.12	0.25	0.41	1.12/0.62	0.5	ND	ND	ND	ND	ND	ND
1169527	0.31	1.83	0.37	0.25	1.66/0.99	0.37	2	0.26/0.25	1	1-1.5	0.19/0.17	1
2908162	0.29	2.33	0.37	0.5	1.12/0.62	0.37	ND	ND	ND	ND	ND	ND

Table 2. In vitro interaction between ceftazidime and meropenem with tobramycin and ciprofloxacin, expressed by means of FICI (checkerboard and E-test methods) and SBPI. FICI: Synergy ≤ 0.5 ; Additive $>0.5 - 1$; Indifferent: $>1 - <4$; Antagonism ≥ 4 ; b: SBPI: Susceptible Breakpoint Index: SBPI CLSI/ SBPI EUCAST if different values were present; ND: Not done

Meropenem shows a time-dependent killing above MIC activity and its administration in continuous infusion could improve clinical results. Both strategies have shown concentrations above MIC in clinical studies [20]. Only maximum serum concen-

trations of ceftazidime administered in intermittent intravenous administration in human experiences [21] were in excess of the all MIC values of the studied isolates.

The pharmacodynamic profile of the aminoglyco-

Antibiotic combinations	Isolate number			
	1449133		1169527	
1 TOB + 1 CAZ	S	B	S	B
1/2 TOB + 1 CAZ	S	B	S	B
1/4 TOB + 1 CAZ	S	B	S	B
1/4 TOB + 1/2 CAZ	S	NB	S	NB
1/8 TOB + 1 CAZ	S	B	S	NB
1 TOB + 1 MEM	S	B	S	B
1/2 TOB + 1 MEM	S	NB	S	B
1/4 TOB + 1 MEM	NS ^a	NB	S	NB ^b
1/8 TOB + 1 MEM	NS	NB	S	NB ^b

Table 3. Time-kill results at 24 h. Antibiotic combinations expressed as MIC's fractions Synergy: S; Bactericidal activity: B; No synergy: NS; No Bactericidal: NB. Microdilution MIC values (mg/L): 1449133 CAZ 64, MEM 8, TOB 128; 1169527: CAZ 16, MEM 8, TOB 64; Synergy: ≥ 2 -log₁₀ decrease in colony count in the combination compared with that of most active single agent; Bactericidal: ≥ 3 log₁₀ cfu/ml decrease in the starting inoculum. a: Synergy at 6 h; b: Bactericidal at 6 h

sides has been characterized both *in vitro* and *in vivo*. Since these antibiotics eliminate bacteria more rapidly when their concentrations are above the

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MIC of the bacteria, their killing activity is referred to as concentration or dose-dependent bactericidal activity [22]. Concentrations of 8 to 10 times MIC value have been proposed to achieve the optimal bactericidal activity of aminoglycosides [23]. Once-daily dose of nearly 7mg/Kg tobramycin has been associated with peak concentrations of 30 mg/L in adult and pediatric patients with cystic fibrosis [24, 25]. This concentration matches with 1/2, 1/4 and 1/8 MIC values of tobramycin included in our *in vitro* study. Synergy and bactericidal activity against 1449133 *P. aeruginosa* isolate was mainly observed with these MIC tobramycin values combined with 1 MIC ceftazidime at 6h and 24h assay. However, tobramycin and meropenem combinations showed better results at 6 h, possibly, as stated above, as a consequence of meropenem degradation.

Recently, a mathematical simulation in combination therapy based on quantitative methods in a neutropenic murine pneumonia model have shown high consistence with the predictions of this *in vitro* model [26].

Other *in vitro* studies as antibiotic degradation, more antibiotic combinations and also more isolates with different resistance mechanisms are in progress before initiating the studies in animal models in order to find a combination active on *P. aeruginosa* MR.

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