Universidade do Minho
Escola de Engenharia

Idalina Goreti Maia Machado

Proteomic and phenotypic view of *Pseudomonas aeruginosa* resistance due to antimicrobial stress

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Proteomic and phenotypic view of *Pseudomonas aeruginosa* resistance due to antimicrobial stress

Doctoral Dissertation for PhD degree in
Chemical and Biological Engineering

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“Para ser grande, sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.
Assim como em cada lago a lua toda
Brilha, porque alta vive.”

Ricardo Reis

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if there was only one place, it would be by your side.
coz just with you on my side, I can make it right
would you share all your dreams with me for the rest of our lives?

Thank you to all of you.

SCOPE AND OUTLINE OF THE THESIS

The present thesis reports the works performed at The Biofilm Group Lab at IBB - Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, Universidade do Minho, Braga, Portugal, and at Thierry Jouenne BRICs Group, Laboratory "Polymères, Biopolymères, Surfaces", UMR 6270 CNRS, at the University of Rouen, France.

This thesis is organized into six chapters. The first chapter briefly reviews relevant aspects of *Pseudomonas aeruginosa* virulence factors and biofilm formation and summarizes the state-of-the-art knowledge on antimicrobial resistance in planktonic and biofilm cells. In Chapter 2, the microorganisms, materials and methods used in the work presented herein are summarized. Chapter 3 reports some studies with *P. aeruginosa* clinical isolates, regarding their motility, biofilm-formation ability and associated biofilm antimicrobial tolerance (Chapter 3.1), as well as on the biofilm recovery after selective pressure of antibiotics (Chapter 3.2). The fourth chapter focuses on the phenotypic characterization of the adaptive resistance induced in bacteria in the planktonic (Chapter 4.1) and biofilm state (Chapter 4.2). Chapter 5 resumes all the proteomic studies performed throughout this work, which comprises the outer membrane proteins characterization of planktonic (Chapter 5.1) and biofilm-entrapped *P. aeruginosa* (Chapter 5.2) after adaptation to antimicrobials. Finally Chapter 6 provides general conclusions of the work presented in this thesis and proposes future research lines.

ABSTRACT

Several studies have recognised that bacteria present higher resistance to antimicrobials when switching from planktonic to biofilm mode of life. However, the phenotypic and proteomic changes associated with the reduced susceptibility of biofilms to antimicrobials are still poorly described.

The goal of this work was to extend the understanding about the biofilm-augmented tolerance of *Pseudomonas aeruginosa*, an opportunistic pathogen that exhibits intrinsic resistance to several antimicrobial agents, and shows great aptitude to acquire adaptive resistance to environmental pressures. The experimental analysis was two-fold: a phenotypical examination comprising the initial adhesion ability, biofilm formation and susceptibility to antimicrobial agents, and a proteomic characterisation of the outer membrane (OM) proteins in planktonic and biofilm modes of growth. In order to best reproduce real-world situations and explore cell adaptive resistance, the study covered several *P. aeruginosa* strains, namely: a reference strain (ATCC 10145), an endoscope surface isolate, four clinical isolates inherently exposed to real pressures, and a laboratory-adapted strain obtained *in vitro* by sequential exposure to increasing concentrations of benzalkonium chloride (BC). As biofilms can comprise more than one species, and the interactions between microorganisms can enhance biofilm survival and resistance, *Escherichia coli* was used to form mixed biofilms. The phenotypical examination accounted for susceptibility to the BC and antibiotics Ciprofloxacin (CIP) and Gentamicin (GM) whilst the proteome of the OM of BC-adapted *P. aeruginosa* was characterised in planktonic and biofilm modes of growth.

Motility has been pinpointed as an important factor for *P. aeruginosa* adhesion and biofilm development, this trait being also associated with increased microorganism virulence. Each *P. aeruginosa* clinical isolate revealed specific ability to develop biofilm and to move along a surface, as well as specific biofilm response to the presence of BC and CIP. However, it was not possible to clearly establish direct relationships between higher motility and biofilm formation ability and increased resistance. The variability of behaviour observed between the clinical isolates has called attention to the need to develop and adopt standardized operational procedures to evaluate biofilm formation capacity, as well as its sensitivity to in-use antimicrobials.

Bacteria aptitude to become dormant when exposed to antimicrobial agents was also characterized as a possible mechanism of virulence. Biofilms formed by the endoscope-isolated *P. aeruginosa* were exposed to intermittent cycles of high-doses of CIP and GM to assess the so-called post-antibiotic effect. At the end of each stress cycle, the remaining cells were able to regrowth and reconstitute the biofilm, achieving values of the same magnitude of those obtained when biofilms were developed in the absence of antibiotics. So, it is conceivable to think that biofilm-growing *P. aeruginosa* strains possess high capacity to survive to external antibiotic pressure. Indeed, it is believed that such pressure acts as a selector of persister cells that are able to recolonize the surface contributing to increased biofilm recalcitrance.

The effect of antimicrobials on *P. aeruginosa* early-adhesion was detailed by studying the impact of BC residue deposition on the adhesion surfaces, as well as on the bacteria surface properties. Results showed that the presence of BC alters the thermodynamic properties of bacteria and adhesion surfaces, favouring adhesion and promoting biofilm formation. So, to prevent bacterial outbreaks and disinfection practice failures, special care should be taken with the design of effective sanitation programs avoiding antimicrobial residues accumulation.

The existence within a biofilm of multiple bacterial species can assist interspecies relationships, which might stimulate biofilm development and increase stability, and thus augment resistance. Studies with *P. aeruginosa* and *E. coli* showed that the continuous exposure of biofilms to sub-lethal concentrations of BC induced an adaptive response of both bacteria, stimulating biofilm formation and the overproduction of a denser and cohesive matrix. Data emphasised that the use of inappropriate doses of antimicrobials can, instead of kill sessile microorganisms and remove them from the surfaces, lead to the development of biofilms encompassing more virulent bacteria.

Resistance mechanisms are often related with changes in membrane permeability and differential expression of efflux-pumps and porins. The proteomic analysis of the outer membrane (membranome) of BC-adapted *P. aeruginosa* revealed differences in the expression of a small number of proteins after planktonic adaptive selection. Results suggest that planktonic bacterial adaptation does not mobilize transport systems at membranome level. Biofilm proteomic studies revealed that adaptation to BC and CIP promotes the down-regulation of several proteins, suggesting a shared resistance response of *P. aeruginosa* to the action of both antimicrobials. The differentially expressed proteins are related to: pilus formation (type IV pilus biogenesis), which affects motility and adhesion; membrane vesicle production (putative tail sheath protein and bacteriophage protein), which is known to contain active toxins and other virulence factors; and unknown function (major capsid protein), encoded by genes belonging to the genomic island PAGI-6, a prophage that has undergone multiple recombination and deletion events resulting in altered virulence. The different expression of these proteins may be on the basis of the emergence of small colony variants or sticky strains in biofilms, the increased *P. aeruginosa* virulence emphasised by higher adhesion, hydrophobicity, swimming and twitching motilities and also biofilm ability to survive under chemical stress conditions.

This work contributed significantly to the phenotypic and proteomic understanding of *P. aeruginosa* biofilm tolerance. It was concluded that resistance is strain-dependent and in close association with the surrounding environmental stresses. Moreover, the exposure to environmental stressors dictated *P. aeruginosa* recalcitrance and enhancement of bacteria virulence factors. This knowledge will undoubtedly assist the development of new and more effective strategies to control biofilm formation and treatment regimens to eradicate biofilm-growing bacteria.

RESUMO

Nos últimos anos, vários estudos têm demonstrado que as bactérias quando crescidas na forma de biofilme apresentam uma resistência antimicrobiana superior à observada em crescimento planctónico. Contudo, as alterações fenotípicas e proteómicas associadas à diminuição de susceptibilidade aos produtos antimicrobianos das bactérias quando constituídas em biofilme ainda não estão completamente caracterizadas e descritas.

Neste cenário, este trabalho teve como objectivo contribuir para o conhecimento acerca do aumento da tolerância aos produtos antimicrobianos oferecida pelos biofilmes formados por *Pseudomonas aeruginosa*, uma bactéria patogénica que apresenta frequentemente elevada resistência intrínseca a agentes antimicrobianos e grande aptidão para adquirir resistência adaptativa em resposta a pressões ambientais externas.

De modo a compreender o comportamento de bactérias em biofilmes, quando expostos a pressões ambientais, e obter informação acerca dos mecanismos que podem estar na base da tolerância aumentada dos biofilmes, procedeu-se à caracterização fenotípica e proteómica de biofilmes de *P. aeruginosa* expostos a diferentes produtos antimicrobianos. Com o intuito de melhor reproduzir situações reais e explorar a resistência adaptativa exibida por bactérias, este trabalho incluiu várias estirpes de *P. aeruginosa*: uma de referência (ATCC 10145), uma isolada de um endoscópio, quatro isolados clínicos inerentemente expostos a pressões reais, e uma estirpe adaptada em laboratório por exposição sequencial a concentrações crescentes de cloreto de benzalcónio (BC). Uma vez que, naturalmente, os biofilmes podem ser compostos por mais do que uma espécie de microrganismo e que a interação entre eles pode favorecer a capacidade de sobrevivência e a tolerância a produtos antimicrobianos, uma estirpe de *Escherichia coli* foi também estudada de modo a desenvolver biofilmes mistos.

A caracterização fenotípica envolveu, a determinação da susceptibilidade a BC e aos antibióticos, ciprofloxacina (CIP) e gentamicina (GM), enquanto que a análise proteómica compreendeu a caracterização das proteínas da membrana externa de bactérias de *P. aeruginosa* adaptadas a CB em crescimento planctónico e em biofilme.

Um factor importante associado ao aumento da virulência de *P. aeruginosa*, com grande influência na adesão e formação de biofilme é a capacidade de locomoção ou motilidade. Cada isolado clínico de *P. aeruginosa* estudado revelou especificidade na sua capacidade de desenvolver biofilmes e de se mover à superfície, bem como na resposta dos biofilmes à presença de BC e CIP. Contudo, não foi possível estabelecer uma relação evidente direta entre a maior capacidade de locomoção, formação de biofilme, e aumento de resistência. A variabilidade de comportamentos observada alertou para a necessidade de se desenvolver e adotar procedimentos de operação padronizados para avaliar a capacidade de formação de biofilme, bem como a sua sensibilidade a produtos antimicrobianos de uso comum.

Um outro factor de virulência bastante importante é a capacidade de as bactérias se tornarem dormentes quando expostas a produtos antimicrobianos. De modo a estudar esta característica, biofilmes desenvolvidos pela estirpe isolada do endoscópio foram expostos a ciclos intermitentes de elevadas doses de CIP e GM, determinando-se assim o efeito pós-antibiótico. No final de cada ciclo, as bactérias que sobreviveram foram capazes de recrescer e reconstituir o biofilme, alcançando valores da mesma grandeza que os biofilmes

desenvolvidos na ausência de antibióticos. Os resultados obtidos permitem especular que bactérias de *P. aeruginosa* quando crescidas em biofilme apresentam capacidade superior de sobreviver a pressões externas exercidas por antibióticos. Este factor de stress atuou assim como seletor de bactérias persistentes capazes de recolonizar superfícies o que poderá contribuir para o aumento da recalcitrância de biofilmes.

De modo a determinar qual a ação de produtos antimicrobianos na adesão inicial de *P. aeruginosa* e se a presença destes poderá contribuir para o aumento da resistência, foi estudado o impacto da deposição de resíduos de BC nas propriedades das superfícies das bactérias e das superfícies de adesão. Os resultados obtidos mostraram que a presença de BC causa modificações nas propriedades termodinâmicas de ambas as superfícies. Estas alterações favoreceram a adesão, promovendo a formação de biofilme, especialmente da estirpe adaptada a BC. Assim, de modo a prevenir surtos bacterianos e más práticas de desinfecção, devem ser tomadas precauções especiais na definição e implementação de procedimentos de limpeza eficientes, evitando a deposição de resíduos de produtos antimicrobianos.

A presença de múltiplas espécies de microrganismos na população que constitui o biofilme pode favorecer a relação entre as espécies, que poderá promover o desenvolvimento do biofilmes e aumentar a sua estabilidade, desenvolvendo assim a sua resistência. Os estudos efectuados com biofilmes mistos de *P. aeruginosa* e *E. coli* mostraram que a exposição contínua a concentrações sub letais de BC induziu uma resposta adaptativa dos biofilmes. Esta adaptação provocou um aumento da capacidade de formação de biofilme, e a elevada produção e coesão da matriz polimérica. Os dados obtidos enfatizaram que o uso de doses inapropriadas de produtos antimicrobianos poderá, em vez de inativar os microrganismos e eliminá-los das superfícies, promover o desenvolvimento de biofilmes constituídos por bactérias mais virulentas.

Os mecanismos de resistência estão frequentemente associados a alterações da permeabilidade da membrana e expressão diferencial de proteínas de bombas de efluxo e porinas. Assim, de modo a compreender se a estirpe adaptada a BC apresenta alterações a nível proteico, vários estudos proteómicos foram efectuados. A análise proteómica das proteínas da membrana (membranoma) de bactérias de *P. aeruginosa* adaptadas a BC em crescimento planctónico revelou diferenças na expressão de um reduzido número de proteínas após seleção por adaptação. Os resultados obtidos sugerem que a adaptação bacteriana a BC em estado planctónico não parece mobilizar sistemas de transporte ao nível da membrana externa. Os perfis proteicos das bactérias de *P. aeruginosa* adaptadas em biofilme mostraram que, a adaptação a BC e a CIP causou a subexpressão de algumas proteínas. A expressão comum poderá indicar uma possível resposta semelhante da bactéria quando exposta a ambos os produtos. As proteínas diferencialmente expressas estão relacionadas com: formação de pili ("type IV pilus biogenesis") afecta a motilidade e adesão, ii) produção de vesículas da membrana ("putative tail sheath protein" e "bacteriophage protein"), codificadas por genes que fazem parte de uma ilha genómica (PAGI-6), um profago que sofreu eventos de recombinação e deleção, causando alterações de virulência. A diferente expressão de proteínas de tal importância, poderá estar na base da emergência de variantes de colónias pequenas ou mucoides em biofilmes, aumentando assim a expressão de factores de virulência de *P. aeruginosa* tais como adesão, hidrofobicidade, capacidade de locomoção e a capacidade de sobrevivência de biofilmes em condições de stress químico.

Os dados obtidos ao longo deste trabalho contribuíram para a compreensão fenotípica e proteómica da tolerância de *P. aeruginosa* quando constituída em biofilme. A resistência está dependente da estirpe e em

estreita relação com o ambiente externo e a exposição a estes factores externos de stress poderá interferir com a recalcitrância da bactéria *P. aeruginosa* e aumento dos seus factores de virulência.

A compreensão de tais alterações permitirá o desenvolvimento de novas e mais eficientes técnicas de controlo da formação de biofilme e dos procedimentos para erradicar bactérias associadas em biofilme.

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ABBREVIATION AND ACRONYMS

Abbreviation/ Acronym: term

θ_W : Water contact angle
θ_F : Formamide contact angle
θ_B : α-bromonaphthalene contact angle
ΔG_{iwi}^{TOT} **:** Free energy of interaction with water (w)
ΔG_{bsb}^{TOT} **:** Free energy of adhesion between bacteria (b) and surface (s)
γ^{AB} **:** Lifshitz-van der Waals component of the surface free energy;
γ^{+} **:** electron acceptor parameter
γ^{-} **:** electron donor parameter
µg: microgram
µL: microliter
µm: micrometer

2-D: Second dimensional gel
2DE: Two-dimensional gel electrophoresis
A_{490}: Absorbance at 490 nm
A_{570}: Absorbance at 570 nm
A_{640}: Absorbance at 640 nm
ASB 14: Amidosulfobetaine-14
ATCC: American Type Culture Collection
BC: Benzalkonium chloride
BCA: Bicinchoninic acid
cfu: Colony forming units
CHAPS: 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHX: chlorhexidine diacetate
CIP: Ciprofloxacin
CV: Crystal violet
DLVO: Theory of Derjaguin, Landau, Verwey and Overbeek
DNA: Deoxyribonucleic acid
E. coli: *Escherichia coli*
eDNA: extracellular DNA
EDTA: Ethylene diamine tetracetic acid
e.g.: (*exempli gratia*) for example
EPS: Extracellular polymeric substances
et al.: (*et alli*) and others
Fig.: Figure
GM: Gentamicin
h: hour
i.e.: (*id est*) That is
IEF: isoelectric focusing
IPG: Immobilized pH gradient
j_0: initial deposition rate

Kg: kilogram
l: liter
LB: Luria Bertani Broth Miller
log_{10}: logarithm with base 10
LPS: Lipopolysaccharides
M: molar
MBC:
mg: milligram
MH: Mueller-Hinton Broth
MIC:
min: minute
mL: milliliter
mM: milimolar
MS/MS: mass spectrometry/mass spectrometry
MV: membrane vesicles
n_{30}: Number of adhering microorganisms after 30 minutes
n_{60}: number of adhering microorganisms after PBS passage
NCCLS: National committee for clinical laboratory standards
OD: Optical density
OM: Outer membrane
OMP: Outer membrane proteins
p: probability
PAE: Post-antibiotic effect
P. aeruginosa : *Pseudomonas aeruginosa*
PBS: Phosphate buffered saline
PEL: glucose-rich polymer
pH: potential hydrogen
p*I*: Isoelectric point
PMS: Phenazine methosulphate
***post hoc*:** after this
PPFC: Parallel plate flow chamber
PS: Polystyrene
PSL: mannose-rich polymer
QAC: Quaternary ammonium compounds
rpm: revolutions per minute
s: Second
SD: Standard deviation
SEM: Scanning electron microscopy
TSA: Tryptic soy agar
TSB: Tryptic soy broth
UP: ultrapure
vs: (versus) against
XTT: 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt

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Scientific Output

Papers in peer reviewed journals

Machado I., Lopes S. P., Sousa A. M., Pereira M. O.. Adaptive response of single and binary *Pseudomonas aeruginosa* and *Escherichia coli* biofilms to benzalkonium chloride. Journal Basic Microbiology. DOI 10.1002/jobm.201100137.

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Abstracts in conferences

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Machado I., Lopes H., Lopes S. P., Alves D., Pereira M. O.. Biological cost of antibiotic pressure in *Pseudomonas aeruginosa* biofilm phenotype. 20th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Vienna, Austria, April 2010.

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Machado I., Simões M., Vieira M.J., Pereira M. O.. Persisters: In the news Yesterday, on our labs Today–the world together for a safer Tomorrow. ESBG symposium and workshop: Bacterial adaptation mechanisms: Biofilms, hypermutability and antibiotic resistance, Palma de Mallorca, Spain, November 2007

CHAPTER 1

GENERAL INTRODUCTION

This chapter reviews current knowledge on *P. aeruginosa* virulence factors, particularly those related with biofilm development. Special emphasis is given on the sessile bacterial phenotypic response when biofilms are exposed to antimicrobial agents and on the proteomic characterization after stress exposure.

The genus Pseudomonas represents a diverse group of medically, environmentally and biotechnologically important bacteria. Pseudomonads are characterized by an enormous capacity to (i) adapt to diverse and challenging environments, (ii) degrade recalcitrant compounds and (iii) synthesize low-molecular-weight compounds[72].

Pseudomonas aeruginosa is a Gram-negative bacterium, nearly ubiquitous in nature and, in most environments, is quite innocuous. However, it can cause severe and life-threatening infections in cystic fibroses patients and individuals with a compromised immune system[24,41,70]. This bacterium is also able to survive in inanimate surfaces in hospitals for months[48] surviving to routine cleaning procedures[57].

1.1 Pathogenesis of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa exhibits intrinsic resistance to several antimicrobial agents. This resistance particularly in the clinic area holds serious consequences for infection control, treatment regimes, and disease progression. It has been recognized that resistance towards antimicrobials can augment when bacteria switch from the planktonic state to the biofilm mode of life.

Biofilms can form on abiotic surfaces, as medical implants, and during specific disease states, such as in the skin of burned patients and also in the lungs of cystic fibrosis patients, being extremely difficult to eliminate[74], leading thus to increased morbidity and mortality of affected individuals. In healthcare environment, it is suggested that biofilms are associated with both device-related infections (Table 1-1) and non device-related infections (Table 1-2). Biofilms are not only a reservoir of pathogenic bacteria that can detach, resume their planktonic state, and spread into the bloodstream of the patient or colonize new surfaces, but also a source of endotoxins that may enter the circulation system of the patient and cause systemic disorders[8].

Table 1-1 Device-related *P. aeruginosa* biofilm infections

Device	Infection	Ref
Contact Lens	Keratitis	26,52,61
Inner ear	Cochlear implant infection	10
Catheter	Intravascular catheter infection	49
Prosthetic valve	Endocarditis	30,73
Peritoneal dialysis catheter	Peritoneal dialysis catheter infection	80
Prosthetic joint	Prosthetic joint infection	86
Endoscope	Nosocomial outbreaks	47
Urinary stent	Urinary stent infection	34,86
Breast implant	Breast implant infection	63,86

Table 1-2 Non-device related *P. aeruginosa* biofilm infections

Organ	Infection	Ref
Skin	Folliculitis wound/ulcer infections; Burn infection	64
Ear	External otitis	52,92
Nasal Sinuses	Sinusitis	12
Urine Bladder	Urinary tract infection	26,52
Bones	Diabetic osteomyelitis in feet	51
Lungs/ bronchi	Ventilator associated pneumonia	52
	Endo bronchiolitis; Cystic fibrosis; Bronchiectasis	26
Blood	Sepsis	52,92

1.2 BACTERIAL RESISTANCE

Bacterial resistance to antimicrobials has been reported since 1950, particularly with the contamination of cationic biocide formulations[2,37]. Biocides are used in clinical settings to control the growth of pathogenic microorganisms or to eliminate them from inanimate objects, surfaces or intact skin. The role of environmental surfaces in spreading hospital-associated infections has not been clearly established. Even surfaces that do not come in contact with the patients may contribute to epidemic or endemic spread of *P. aeruginosa*[79,92].

The emergence of bacterial resistance is likely to be caused by either (i) a specific modification of a target site or (ii) by a by-pass of a metabolic process. The efficacy of biocides depends on a range of intrinsic and extrinsic factors. Intrinsic factors are characteristics of the biocidal agents and its application. Concentration and contact time are crucial parameters being the combination of these factors [named C(t)] that determines the result in terms of microbial reduction. The concentration of a biocide is considered the most important factor that affects its efficacy[77]. Extrinsic factors derive from the environmental conditions during the biocide application, like temperature, pH and the presence of organic material in the environment that may reduce biocide efficacy[18].

There are three types of bacterial resistance to biocides: (i) intrinsic (or innate) to the microorganism, (ii) acquired due to the forced mutations or through the acquisition of mobile genetic elements, usually plasmid mediated[66], and (iii) adapted resistance that results from the continuous exposure to a biocide[81].

The most described intrinsic resistance mechanism is the change in the permeability of the cell envelope or "permeability barrier". It limits the amount of biocide that enters in the cell, thus decreasing the effective biocide concentration[15,28]. Lipopolysaccharides (LPS)[57], as well as other outer membrane components, including proteins, fatty acids and phospholipids, account also for the reduced biocide efficacy. In the case of quaternary ammonium compounds (QAC), that are

positively charged, the charge of the cell surface also plays an important role in resistance, since it may annul QAC efficacy[13]. Another important mechanism of intrinsic resistance is the presence of efflux-pumps. These proteinaceous transporters localized in the cytoplasmic membrane are responsible for the intracellular decrease of biocide concentration and other toxic compounds[87]. The secretion of enzymes can be also responsible for bacterial resistance since enzymes can transform biocides making them innocuous to bacteria. This mechanism is specially reported for resistance mechanisms to heavy metals and parabens[17].

Concerning acquired bacterial resistance, mutation and the acquisition of resistance determinants are the most frequent described mechanisms[75]. The acquisition of resistant genes has particular importance as it can confer cross-resistance to other antimicrobials[7,16].

The induction of bacterial resistance mechanisms following exposure to a low concentration of a biocide[54] involves the over-expression of efflux-pumps[39,71] and the over-expression of multigene[27] and protein systems[32]. These mechanisms are part of the stress-response system in bacteria and have as consequence, for example a decrease in growth rate[1], altered gene[27] and protein expression[32], and altered exopolysaccharide production[29].

Traditional antibiotic resistance usually involves inactivation of the antibiotic, modification of targets, and exclusion of the antibiotic[25]. The diverse antibiotic molecules used during antibiotherapy of bacterial infections may be classified according to their mechanism of action: (i) alteration of cell envelope (*e.g.*: β-lactams); (ii) inhibition of protein synthesis (*e.g.*: aminoglycosides); (iii) inhibition of nucleic acid synthesis (*e.g.*: quinolones) and (iv) inhibition of a metabolic pathway (e.g.: sulfamides). Some bacterial species are innately resistant to one class of antibiotics due to their envelope that limits the antibiotic penetration, or due to the presence of a low level of efflux-pump systems that decrease intracellular antibiotic concentration[59].

An ongoing and increasing concern is that bacteria are becoming increasingly resistant to antibiotics. Initially susceptible, bacteria become resistant under the selective pressure exerted by the misuse of antibiotics. The various antibiotic resistance mechanisms adopted by bacteria may be classified in: mechanical barrier (altering the required intracellular dose of antibiotic), enzymatic barrier (expression of a detoxifying enzyme that modifies the antibiotic), target protection barrier (mutation or expression of a molecule impairing the antibiotic recognition and activity)[23].

Research indicates that biocides and antibiotics may have some similar and common interactions and target sites with bacteria, which might express shared resistance mechanisms to both antimicrobials[66]. Biocides tend to act concurrently on multiple sites within the microorganism, and thus resistance is often mediated by non-specific means. Among the similarities, it can be mentioned: (i) the penetration/uptake through bacterial envelope by passive diffusion, (ii) the effect on the membrane integrity and morphology, (iii) the effect on diverse key metabolic

pathways. A key question is whether the indiscriminate use of biocides facilitates the selection of antibiotic resistant bacteria.

1.3 Pseudomonas aeruginosa BIOFILM FORMATION

Biofilms have been described as structured communities of bacterial cells enclosed in a self-produced polymeric matrix and adhered to an inert or living surface[60]. The development of a biofilm occurs via a series of well-defined stages which include: (i) translocation to the surface and initial and reversible adhesion of cells; (ii) irreversible attachment; (iii) microcolony formation; (iv) maturation and differentiation of the biofilm; and (v) dispersal of single cells or microcolonies from the biofilm (Fig.1-1).

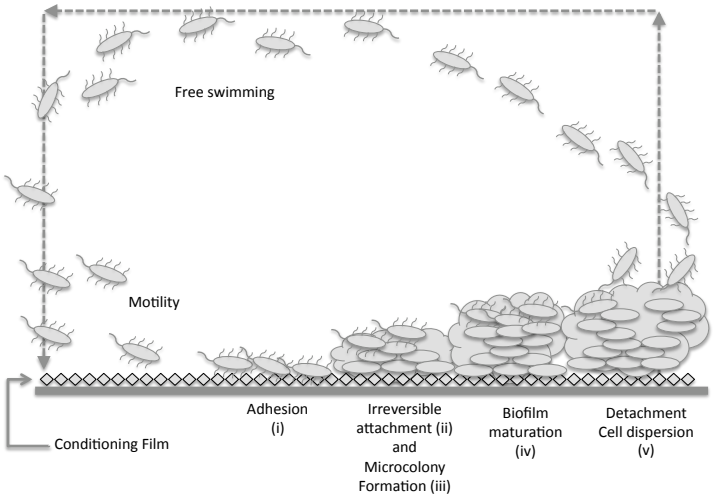

Fig.1-1 The biofilm life cycle (i) Translocation to the surface and initial and reversible adhesion of cells, (ii) Irreversible attachment, iii) Microcolony formation, (iv) Maturation and differentiation of the biofilm, and (v) Dispersal of single cells from the biofilm.

Once bacteria have irreversibly attached to a surface [Fig.1-1 (ii)], as described above, the process of biofilm development begins. The overall density and complexity of the biofilm increases as surface-bound organisms begin to actively replicate and to generate extracellular components, giving rise to the formation of microcolonies[67] [Fig.1-1 (iii)]. The bacterial biofilm maturation [Fig.1-1 (iv)] can be limited by the availability of nutrients in the surrounding environment, the perfusion of those nutrients to cells within the biofilm, and the removal of waste. Biofilms typically consist of differentiated mushroom- and pillar-like structures of cells embedded in the matrix, which are separated by water-filled channels that allow convective flows to transport nutrients and oxygen from the interface to the interior parts of the biofilm, and to remove metabolic wastes[62]. Cells within biofilms account just for a small fraction of the total biofilm volume (typically, 5–35%)[25], the higher fraction being composed by the extracellular matrix that creates a protective environment against external threats[25].

As the biofilm reaches a critical mass, and a dynamic equilibrium is attained, some portions begin to detach from the biofilm and to colonize other surfaces or sites [Fig.1-1 (v)]. Biofilm

development, maturation, and breakdown are regulated at the level of population density-dependent gene expression controlled by cell-to-cell signalling molecules such as acylated homoserine lactones[4,22]. The process of cell dispersion within biofilms has been termed "seeding dispersal" in order to differentiate it from the process of erosion, which is the passive removal of cells from the biofilm by fluid shear. Such process, besides involving enzymes that degrade the extracellular polysaccharide matrix, is also caused by death and lysis of subpopulations of cells within the biofilm. In *P. aeruginosa* biofilms, the molecular mechanisms of lysis remain to be fully elucidated, as symbiotic filamentous phage may play a role in cell killing within biofilms. It has been proposed that autolysis impacts on dispersal processes in biofilms by disrupting the biofilm architecture. The surviving cells in the biofilm benefit from the nutrients released by dead bacteria, which facilitates the conversion of surviving cells to the motile dispersal phenotype. It was also suggested that lysis is linked to activation of the phage of *P. aeruginosa*.

As the bacterial cells adapt to growth in hydrated surface-associated biofilm communities, they express phenotypic traits that are often distinct from those that are expressed during planktonic growth[83].

1.4 *PSEUDOMONAS AERUGINOSA* VIRULENCE FACTORS

This opportunistic pathogen produces a number of unique virulence factors that make it particularly skilled in infecting specific host tissues[56]. The success of *P. aeruginosa* lies in its flexible physiology, its extensive genetic regulatory networks[35], ability to secret enzymes and exopolysaccharides, as well as in the control of the permeability of its outer membrane[43,84]. Moreover, its aptitude to move from one environment to another, as well as its capacity to adhere to multiple surfaces, and form biofilms are a few types of virulence factors that have been implicated in the pathogenicity of *P. aeruginosa* (Fig.1-2). These aspects of *P. aeruginosa* virulence will have a special focus in this work.

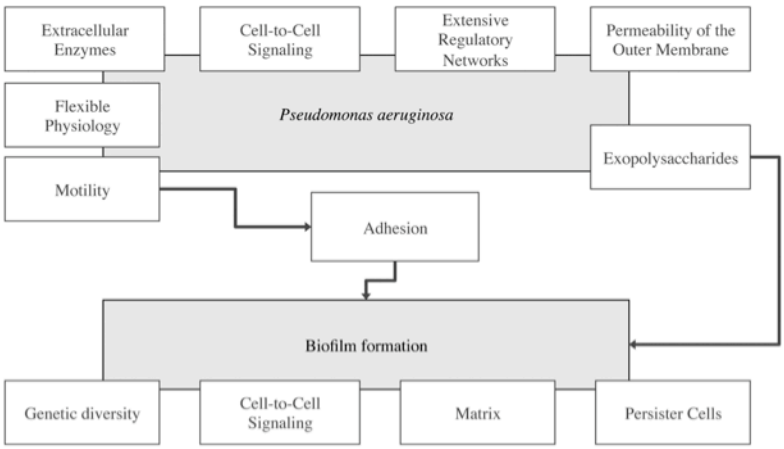

Fig.1-2 Schematic diagram of *P. aeruginosa* virulence factors

1.4.1 Motility

Motility is one of the most remarkable features of *P. aeruginosa* life on surfaces. Its roles include increased efficiency of nutrient uptake, evasion of toxic substances, ability to translocate to preferred hosts and to access to optimal colonization sites within them, as well as dispersal in the environment[46]. Bacterial movement has been classified into different types: Swimming is a flagellum-mediated type of motility that allows bacteria to move on liquid surfaces; Swarming motility is operationally defined as a rapid multicellular movement of bacteria across a surface, powered by rotating flagella[46]; and Twitching is a surface motility powered by the extension and retraction of type IV pili, which confers slow cell movement[46].

Motility is central in the biofilm development process since planktonic cells use this trait to make the initial contacts with a surface, and to spread across the surface[20]. Before attachment *P. aeruginosa* swims along the surface almost like scanning for an appropriate location for initial contact[60]. Flagella-mediated motility is also essential to bring the cell within close proximity of the surface and to overcome repulsive forces between bacterium and the surface. Type IV pili are responsible for a form of surface-associated movement known as twitching motility that plays an important role in bacterial adhesion[68]. Furthermore, there are indications that cells move by twitching motility only when they are in contact with other cells, suggesting that this is a social network behaviour[60]. Flagella-mediated motility is important for the formation of a bacterial monolayer on the surfaces, whereas type IV pili appear to play a role in subsequent microcolony formation[62]. In the early stages of adhesion, microcolonies can also disperse and/or move as a unit across a surface, further emphasizing the dynamic and somewhat transient early interactions of bacteria with a surface and with each other[60].

1.4.2 Bacterial Adhesion

Adhesion of bacteria to human cells and surfaces of medical devices is an important step in the pathogenesis of infection[9]. The process of microbial adhesion to surfaces is largely dictated by a number of variables, including the species of bacteria, cell surface composition, nature and coating of surfaces, nutrient availability, hydrodynamics and cell-to-cell communication[33].

In its basic form, bacterial adhesion can be divided into two stages: the primary or docking stage and the secondary or locking phase[9,33]. An additional step in the adhesion process, surface conditioning, can be added to describe the interaction of the substratum with its environment[9,42]. Conditioning can occur mainly by: i) the deplored practice of “topping-up” of biocide solutions without subsequent washing with water; ii) the incorrect use of biocides and incorrect concentrations used; iii) the use of a biocide that is not appropriate for a certain type of surface; iv) pre-cleaning errors, for example, residue deposition of several products that can interfere with the activity of each other[76,82]. Once a surface has been conditioned, its properties become permanently

altered, so the affinity of an organism for a clean or a conditioned surface can vary greatly depending on the molecules in the conditioning film[40,58].

The primary adhesion between bacteria and surfaces is reversible and is controlled by a number of physicochemical variables that define the interaction between the bacterial cell surface and the adhesion surface[55]. First, the organism must be brought into close proximity of the surface, either randomly by a stream of fluid or via chemotaxis and motility. Once the organism reaches critical proximity to the surface the final determination of adhesion depends on the net sum of attractive or repulsive forces generated between the two entities. These forces include electrostatic and hydrophobic interactions, van der Waals forces, temperature, and hydrodynamic forces[14]. The second stage of adhesion is the anchoring or locking phase and employs molecularly mediated binding between specific adhesins and the surface. At this point, loosely bound organisms strengthen the adhesion process by producing exopolysaccharides and/or receptor-specific ligands located on pili, fimbriae, and fibrillae, or both. At the end of the second stage, adhesion becomes irreversible in the absence of physical or chemical intervention, and the organism is attached firmly to the surface[9].

1.4.3 Biofilm resistance

Bacteria that attach to a surface and grow as a biofilm are protected from killing by host defences and antimicrobial agents. This type of resistance unique to biofilm-associated bacteria and distinct from conventional antimicrobial resistance, holds serious consequences for therapy of infections involving biofilms[74]. The resistance that bacteria exhibit when growing in biofilms is not only due to ‘‘classic’’ genetic mechanisms but is also determined by some particularities of biofilm growth.

A variety of potential mechanisms implicated in biofilm resistance are shown in Fig.1-3 [21,33,44]. Some of the referred biofilm resistance mechanisms will be described with more detail, due to their importance in the overall results presented in this work.

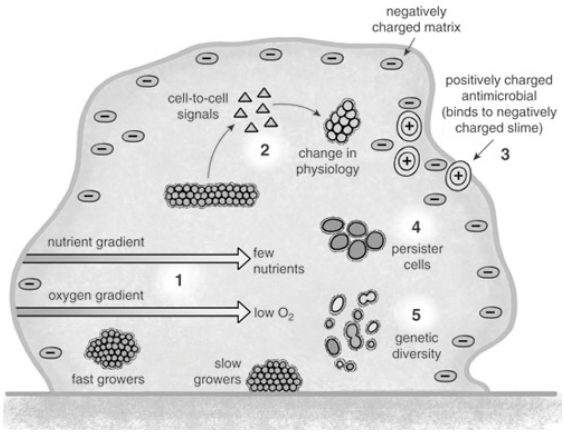

Fig.1-3 Mechanisms implicated in biofilm resistance to antimicrobial agents (adapted from Harrison *et al*.[44]).

Biofilm tolerance to antimicrobial products and antibiotics may result from: (i) slow microbial growth rates that are attributable to nutrient and oxygen depletion within biofilms; (ii) intercellular signals that can alter the physiology of the biofilm, causing members to produce molecular pumps that expel antibiotics from the cells and allow the community to tolerate the presence of the drug; (iii) the diffusion barrier promoted by the biofilm matrix that prevents antimicrobial products to reach their targets, being also responsible of the neutralization or degradation of the antimicrobial or antibiotic due to charge difference; (iv) the specialized populations of persister cells, that do not grow in the presence of an antibiotic, but neither do they die, so when the drug is removed, persisters can give rise to a normal bacterial colony; (v) population physiological and genetic diversity, that act as an "insurance policy", that improves the chance of surviving of some cells. Proteins involved in oxidative stress response, cell envelope synthesis, and EPS synthesis are up-regulated in biofilm-cells, indicating that these altered phenotypes might contribute to cell survival, persistence, and growth in a biofilm community. Moreover, biofilms can comprise more than one strain. Besides the ecological advantages, this trait can promote genetic changes during biofilm development, increasing species interactions[83], and also the chance for interspecies gene transfer and the consequent spread of virulence factors and antibiotic resistance[90].

According to Flemming and Wingender[36], in most biofilms, the microorganisms account for less than 10% of the dry mass, whereas the matrix can account for over 90% providing a complex microenvironment surrounding the biofilm-attached microorganisms[3] [Fig.1-3 (iii)]. Thus, matrix composition plays significant importance in the biofilm mode of life, acting as a “filter” to the chemical diffusion and the gradient of oxygen and nutrients to the cells, and managing also the outflowing of bacterial metabolites. One of the most important *P. aeruginosa* biofilm features is the production of extracellular polymeric substances (EPS) that makes up the matrix[38,91]. The EPS are mainly composed of alginate. Some biochemical studies revealed another two distinct carbohydrate-rich polymers that make significant contributions to *P. aeruginosa* biofilm structure: a glucose-rich polymer (PEL), and a mannose-rich polymer (PSL)[11,38]. Moreover, matrix also contains, in less amount, proteins, and nucleic acids[78] and a variety of cell appendages that, together with EPS, confer biofilms their structure but also the ideal surrounding to protect biofilm-entrapped cells and to establish changes with the environment.

Bacteria that grow within biofilms express phenotypic traits that are often distinct from those that are expressed during planktonic growth [Fig.1-3 (v)]. Physiological adaptation that bacteria experience following attachment and growth on surfaces include the production of extracellular organelles, such as pili and flagella, altered polysaccharide production and even cell morphology[83]. The environmental conditions to which biofilms are exposed or the metabolic activities of bacteria within biofilms result in microscale heterogeneities in the chemical and physical parameters of the

biofilm, resulting in unique environmental niches that confer biofilm-entrapped cells heterogeneity[31].

Although adaptation to the local environment explain much of the biological heterogeneity in biofilms, it is likely that other mechanisms also contribute to the phenotypic differentiation[83]. Mutation or recombination of the original population as well as stochastic gene expression and changes at specific genetic loci might also generate genetic variants in biofilms. These variants have been detected primarily as changes in the colony morphology of subpopulations of cells. Examples of differences observed in colony morphology include the small rough or wrinkly variants of *P. aeruginosa*. These biofilm variants hyperexpress *psl* and *pel* two of the previously referred EPS gene clusters secreted by *P. aeruginosa*[83]. It has been proposed that genetic change and diversification of bacteria within a biofilm community act as an insurance policy against antimicrobial challenges or environmental changes, with the diversified population being more robust than a single parent strain. Some studies have also suggested that phenotypic variation also generates cells more able to adhere to surfaces[53].

According to del Pozo *et al*.[25], biofilm antimicrobial treatment may act as selector of phenotypic traits, leading to the eradication of most of the susceptible population. As antimicrobial agents target rapidly growing cells, the small fraction of slow or non-growing bacteria [Fig.1-3 (i)] may differentiate into a protected phenotypic state. These cells referred as persisters [Fig.1-3 (iv)], are present in the deep layers of biofilms, surviving the antimicrobial action and being able to reconstitute the biofilm following the environmental pressure release by the antimicrobial therapy. These phenomena may explain in part, the resistance of bacterial biofilms to certain types of antimicrobials.

1.5 Proteomic approach to study biofilms

The term proteome was first used to identify the set of proteins encoded by the genome of *Mycoplasma genitalium*[88]. The study of proteome referred as Proteomics is now used to describe not only the proteins of any given cell, but also the set of all protein isoforms and modifications and the interaction between them[5].

The field of proteomics evolved rapidly during the last two decades due to the development of mass spectrometry techniques and also to the complete genome sequences of many microorganisms. Two-dimensional electrophoresis (2-DE) followed by image analysis allows the comparison of thousands of proteins from bacteria. Spot excision and subsequent peptide mass fingerprinting allows, in most cases, to positively identify proteins of interest and thus to perform studies to investigate protein expression change between bacteria growing in different conditions, different strains, treated or exposed to chemicals including antimicrobial drugs[50]. 2-DE has

significant advantages when compared with 1D as it provides high-resolution protein separation and for the downstream identification of the observed protein markers.

Many important fundamental studies on *P. aeruginosa* biofilms were performed with this gel-based technique, especially those related with the differences observed between planktonic and biofilm mode of growth[19,85]. During some stages of biofilm development, as much as 50% of the proteome can be differentially produced compared with the same cells growing in planktonic culture[65].

Microorganisms within the biofilms can also respond differently to environmental conditions when compared with planktonic counterparts, including: altering gene-expression patterns or physiological activities to adapt to a particular location within the biofilm; enriching for populations of a particular phenotype that are best adapted to a particular condition; and selecting for fitter mutant strains that can better survive under a given condition. Natural variants within biofilm subpopulations can also increase fitness by providing tolerance to antimicrobial challenges and resilience to changing environmental conditions[83].

Proteomic characterization of membrane proteins is of extreme importance, since proteins of this sub-cellular location are part of the interface between the outside and the inside of the cell. As such, they are implicated in key cellular functions such as small molecules transport, cell-cell and/or cell-pathogen and/or cell substrate recognition and interaction, cell communication and signalling, with all the possible modifications in cell functionality that can arise subsequently to the activity of these membrane proteins[69].

Bacteria are able to adapt to environmental stresses such as the presence of antimicrobial molecules and, as consequence, resistance may increase with increasing exposure to antimicrobials[45]. One of the most impressive mechanisms of bacterial adaptation is growth as part of a biofilm community. Biofilm formation is an important aspect, in bacterial diseases, especially in those device-related. When biofilms are identified as the cause of disease, treatment becomes tremendously difficult since bacteria within the biofilm demonstrate important features, being the adaptive response to antimicrobial stresses more effective than the corresponding planktonic populations[21]. *P. aeruginosa* is frequently studied because it is one of the major bacteria that cause hospital outbreaks[89] and exhibits notorious tolerance to antimicrobial agents, namely in the sessile mode of growth. Although some of the *P. aeruginosa* virulence factors have been well described and characterized, there is a scarce knowledge regarding phenotypic traits of biofilms after biocide pressure, as well as the possible link to cross-resistance to antibiotics. Emerging bacterial resistance against different types of antimicrobials lead to concerns regarding their prudent use, the correct definition of cleaning procedures in all areas and the identification of the major contributors to resistance. Moreover, it is also very important to understand how the selection and

the dissemination of biocide resistant pathogens occurs in real scenarios in order to develop new ways to control the increase of healthcare associated diseases[6]. The characterization of the biofilm population concerning its phenotypic switching, as well as the proteomic changes undergone after antimicrobial exposure, is of upmost importance, since little is known about this type of bacterial response.

Thus, this work aimed to bring insights into *P. aeruginosa* biofilms focusing on the interplay of different virulence factors, especially with regard to the role of motility in *P. aeruginosa* biofilm formation (Chapter 3.1), persister cell selection (Chapter 3.2) as well as the bacterial adaptive resistance to antimicrobials (Chapter 4), and the proteome characterization of *P. aeruginosa* after stress exposure (Chapter 5).

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CHAPTER 2

METHODOLOGY

The methods and techniques used in the present work are described in this Chapter. The rationale beyond its employment is also discussed.

2.1 Microorganisms and culture conditions

2.1.1 Microorganisms

Different *Pseudomonas aeruginosa* strains were used throughout this work:

- The type strain purchased from the American Type Culture Collection, *Pseudomonas aeruginosa* ATCC 10145.

- *Pseudomonas aeruginosa* surface isolated from a biofilm formed in a medical device (gastrointestinal endoscope) in a Hospital in France, this strain was kindly provided by Thierry Jouenne from University of Rouen, France.

- *P. aeruginosa* clinical isolated strains, I93488, I92986, I97824, C80117, U147016, these strains were kindly provided by Dra. Alberta Faustino from S. Marcos Hospital in Braga.

- The type strain *Escherichia coli* K12 substrain MG 1655, kindly provided by Rafael Costa from Centre of Biological Engineering of the University of Minho.

2.1.2 Bacteria preservation

Bacteria were preserved at – 80 ± 2˚C in 10% glycerol stocks. Prior to each experiment, bacterial cells were grown on Tryptic Soy Agar (TSA, Merck) plates for 24 h, at 37˚C.

2.1.3 Culture medium

To prepare the bacterial suspensions, one colony of each bacterium was collected from the TSA plates and grown in Tryptic Soy Broth (TSB, autoclaved at 121ºC for 20 min) for 24 h at 37°C, in a horizontal shaker (120 rpm). Subsequently, bacteria were washed twice with ultrapure sterilized water (UP). Standardized cell suspensions were prepared in TSB at a cell density of 1×10^7 cfu/mL, unless otherwise stated.

2.1.4 Antibacterial and antibiotic agents

Benzalkonium chloride (BC), a quaternary ammonium compound, widely used in clinical disinfectant formulations, was purchased from Calbiochem (Merck Biosciences).

Ciprofloxacin (CIP), a broad-spectrum antimicrobial from the fluoroquinolone drug class and Gentamicin (GM), an aminoglycoside, were purchased from Fluka. Both antibiotics are used clinically to treat chronic *P. aeruginosa* infections.

2.2 PLANKTONIC STUDIES

2.2.1 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC of BC of *P. aeruginosa* and *E. coli* were determined by the microdilution method according to Clinical and Laboratory Standards Institute (formerly NCCLS)[11].

Briefly, 96-well round-bottom polystyrene (PS) microtiter plates (Orange) with a total well capacity of 300 µL were used. In each well, 50 µL of fresh TSB with increasing concentrations of BC were added to 50 µL of each bacterial inoculum (containing 1 x 10^5 cfu/mL). The culture-plates were incubated at 37°C for 24 h in an orbital shaker at 120 rpm. The highest concentration of BC that did not promote growth was recorded as the MIC.

MBC determination was performed by transferring 10 µL of culture from each well without visible growth into TSA plates. The lowest antimicrobial concentration that yielded no colony growth after 24 h at 37°C was documented as the MBC.

2.2.2 Development of planktonic bacteria adaptive resistance

BC adaptive resistance was induced in *P. aeruginosa* ATCC 10145 to further evaluate the role of this feature on cell surface physicochemical properties, early adhesion and biofilm formation ability.

Adaptive resistance was induced by subculturing *P. aeruginosa* in TSB supplemented with increasing concentrations of BC, based on the adaptive procedures described by Mangalappalli-Illathu and Korber[8] and Loughlin *et al.*[7] (Fig.2-1). Five millilitres of an overnight culture (1 x 10^7 cfu/mL) were added to flasks containing 45 mL of TSB supplemented with BC at the final concentrations of 0.5, 0.9, 1.3 and 2.0 mM. Cultures were then incubated at 37ºC for 48 h on a horizontal shaker (120 rpm). Bacterial growth was monitored by optical density measurement at 640 nm (OD_{640}). Every two days, 5 mL of the bacterial culture, supplemented with the highest BC concentration that showed growth, were used to inoculate 45 mL of TSB containing BC in a final concentration 0.2 mM higher than the one that exhibited growth. At the end of the third growth cycle in increased BC solutions, no significant bacterial load was observed. Bacteria were then subcultured in the presence of the maximum BC concentration that allowed growth for another three complete cycles. At the end of the adaptation process, the purity of the cultures was checked by spreading aliquots on to Pseudomonas spp. selective agar (Cetrimide agar base, DIFCO).

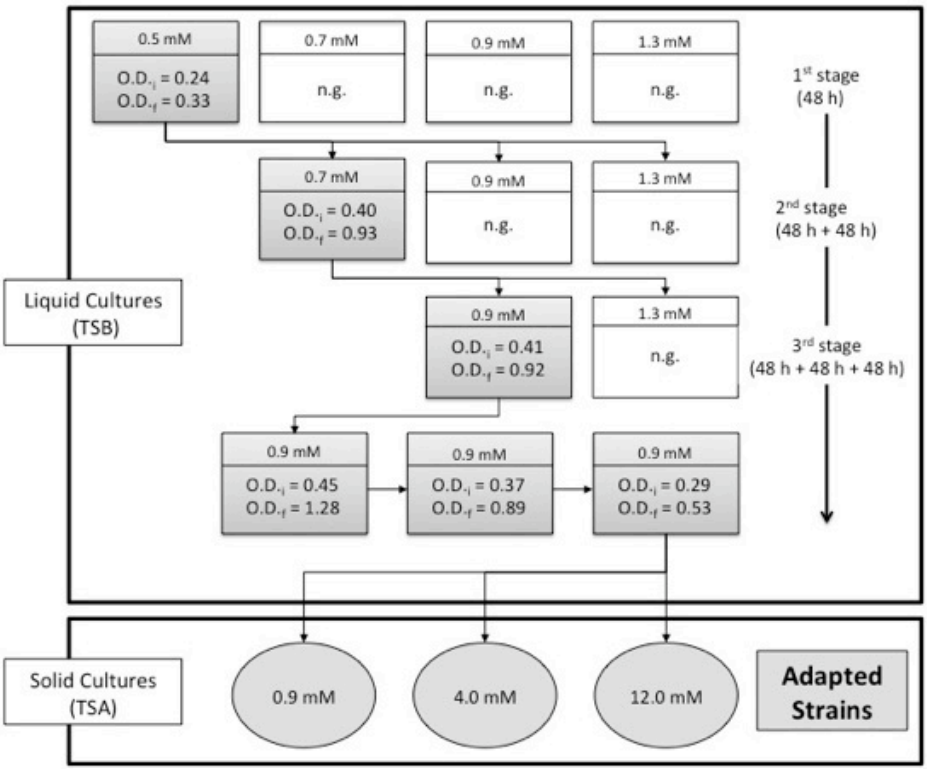

Fig.2-1 Summary scheme of the adaptation process. 0.5 to 12.0 mM refers to benzalkonium chloride concentration (mM); ODi and ODf are respectively the growth of bacteria observed by absorbance reading at 640 nm (OD_{640}) in the beginning and the end of each stage of adaptation. n.g. represents planktonic culture where no growth was observed.

The stability of the BC-adapted strain was later determined by continuous subculture every 24 h for 10 passages in TSA and subsequent evaluation of the adapted strain ability to maintain their growth in TSA supplemented with BC. To preserve the BC-adapted strains, petri dishes were prepared with TSA supplemented with BC at a final concentration of 0.9 mM, 4.0 mM and 12.0 mM. Bacteria from the step-wise training were preserved in the TSA supplemented with BC, being the plates incubated at 37ºC for 24 h.

2.2.3 Determination of the bacterial growth kinetics

In order to determine the potential adaptive resistance of the BC-adapted *P. aeruginosa* was checked by bacterial growth in the presence of increasing concentrations of BC.

Microtiter polystyrene (PS) 96-well round tissue culture plate (Orange) containing 100 µL of TSB supplemented with BC in a range of concentrations from 0.3 to 3.0 mM, were inoculated with 100 µL of *P. aeruginosa* ATCC 10145 and BC-adapted *P. aeruginosa* aliquots, prepared by adjusting overnight cultures to 1 x 10^7 cfu/mL in TSB. Plates were incubated aerobically at 37ºC on a horizontal shaker (120 rpm). Each plate included positive control wells comprising 100 µL of TSB and 100 µL of each strain aliquots. Negative controls were also performed comprising 100 µL of BC solution at each concentration tested, and 100 µL of TSB.

The OD_{640} of each well content was recorded using an automated plate reader (Bio-Tek Synergy HT, Isaza) as a measure of bacterial growth. The average OD_{640} and the standard deviation from eight different wells were determined for each time point and each condition tested. This experiment was performed in three independent assays.

2.2.4 Bacterial motility assessment

To evaluate the motility ability of each bacterial strain, swimming, swarming and twitching assays were performed. Bacteria were inoculated with a needle into the bottom of Luria Broth (LB) agar medium (1.5, 0.3 or 0.6% agar for, respectively, twitching, swimming or swarming tests, average inoculation depth of 3 mm). Plates were incubated at 37ºC for 24 h and for each *P. aeruginosa* strain, five colonies were examined regarding the diameter of the migration zone as well as the border shape. Images were taken on an Olympus SZ 40 stereo microscope (Olympus, Tokyo). All the images were digitized and saved using a CCD AVC D5CE Sony grey scale video camera (Sony, Tokyo) and a DT 3155 Data Translation frame grabber (Data Translation, Marlboro) with 768 x 576 pixel size in 8 bits (256 grey levels) by Image Pro Plus (Media Cybernetics, Silver Spring, MD) software package.

2.3 Adhesion Assays

2.3.1 Surfaces conditioning

In order to examine the effect of BC residues in the thermodynamic prediction of adhesion, early bacterial adhesion and biofilm formation, several PS surfaces of different sizes, depending on the type of experience, were previously conditioned through random deposition of BC residues.

Prior to surface conditioning, the coupons and the adhesion plates were cleaned by 50% maximum amplitude sonication using an ultrasonic processor (Cole-Parmer Instruments), for 3 min, in a commercial detergent solution (Sonasol Pril, Henkel Ibérica S. A.). Coupons and plates were rinsed with UP sterilized water, subsequently immersed in ethanol at 96% (v/v) for 10 s and then rinsed three times with UP sterilized water. For conditioning purposes, coupons or plates were left in contact with a 0.9 mM BC solution for 1 h. Non-conditioned surfaces were achieved by the contact of the surfaces with UP sterilized water, for the same period of time.

For biofilm studies, microtiter plates of 96-wells, with flat and clear bottoms, were conditioned by filing the wells with 200 µL of a 0.9 mM BC solution for 1 h. Wells filled with 200 µL of UP sterilized water were considered non-conditioned wells or clean surfaces.

All the conditioned and non-conditioned surfaces were air dried at room temperature for 30 min before being used in each experience.

2.3.2 Physicochemical characterization of surfaces and cells

Several studies dealing with relations between physicochemical surface properties of bacterial cells and substrata have been reported in the literature[9]. These studies are of great significance, since thermodynamic properties play a key role in the initial bacterial adhesion to surfaces[3]. In order to investigate the physicochemical properties of *P. aeruginosa* reference strain, and BC-adapted cells and PS surface, the contact angles (Fig.2-2) and hydrophobicity were determined for both bacteria and clean and BC-conditioned surfaces, following the van Oss *et al*. approach[17].

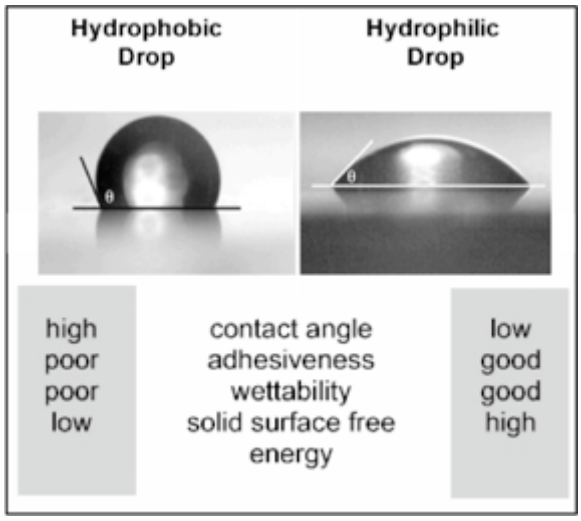

Fig.2-2 Schematic diagram of the relation between the surface contact angle and wetting of the surface (adapted from http://pixes.eu and http://www.harrickplasma.com (March, 2011)).

The free energy of interaction was calculated through the surface tension parameters of the interacting entities, according to:

$$\Delta G_{iwi}^{TOT} = -2\left(\sqrt{\gamma_i^{AB}} - \sqrt{\gamma_w^{AB}}\right)^2 + 4\left(\sqrt{\gamma_i^+\gamma_w^-} + \sqrt{\gamma_i^-\gamma_w^+} - \sqrt{\gamma_i^+\gamma_i^-} - \sqrt{\gamma_w^+\gamma_w^-}\right) \quad (1)$$

where γ^{AB} accounts for the Lifshitz-van der Waals component of the surface free energy and γ^+ and γ^- are the electron acceptor and electron donor parameters.

Bacterial cells were grown in TSB at 37°C under agitation (120 rpm) until reaching the stationary phase of growth. Cells were harvested by centrifugation (5 000 x *g* for 10 min, at 4°C), washed three times in UP sterilized water and resuspended in 200 mL of UP water in order to achieve the bacterial concentration of 1 x 10^9 cfu/mL. To obtain a thick lawn of cells, a volume of 20 mL of the cell suspension was filtered through a 0.45 μm cellulose membrane filter, previously washed with 10 mL of sterile distilled water. The lawn of cells was then air-dried for at least 3.5 h, until the so-call "dried-plateau" was obtained.

The surface tension of strains and materials was determined using the sessile drop contact angle method. Contact angles were standardized using as reference liquids: water, formamide and α-bromonaphthalene (Sigma). The determination of contact angles was performed automatically using a model OCA 15 Plus (Dataphisics) video based optical contact angle measure instrument that allows image acquisition and data analysis. At least 25 determinations for each liquid and for each bacteria and material were performed. This experiment was repeated in three different assays.

2.3.3 Parallel Plate Flow Chamber (PPFC)

A parallel plate flow chamber (PPFC) (Fig.2-3) and image analysis system were employed to study early-stage bacterial adhesion and detachment, as described by Sjollema *et al*.[13]. The PPFC consists of a nickel-coated frame measuring 16 x 8 x 1.8 cm. Teflon spacers were placed between the PS plates, to separate them by 0.06 cm [Fig.2-3 (a)].

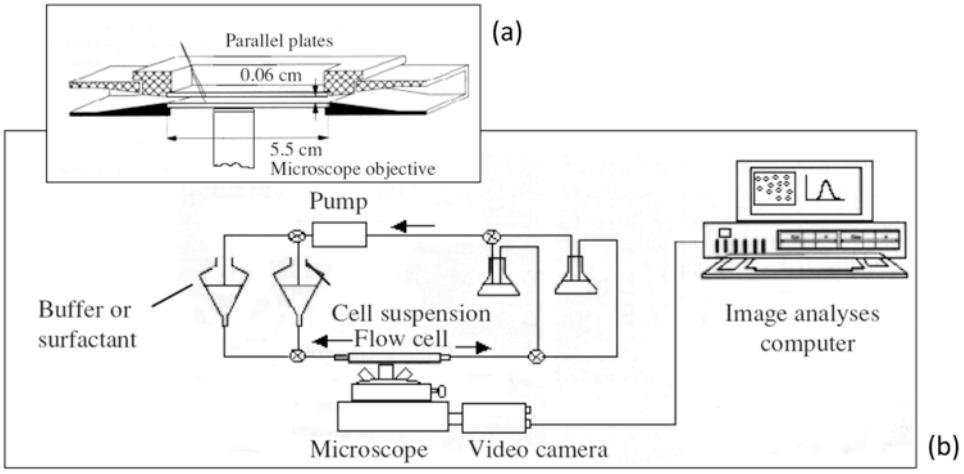

Fig.2-3 Representative scheme of the parallel plate flow chamber system (adapted from Azeredo *et al*. [1])

The PFFC device was mounted in a phase contrast inverted microscope (Diaphot 300; Nikon) equipped with a 40 x ultra long working distance objective [Fig.2-3 (b)]. The images were acquired in a CCD camera (AVC, D5CE; Sony, Japan) connected to the microscope and coupled to an image analyser (Image Proplus 4.5; Media Cybernetics).

Prior to each experiment, all tubes and the flow chamber were filled with sterile PBS, taking care to remove air bubbles from the system. To assess the rate of wild-type and BC-adapted *P. aeruginosa* attachment to clean and BC-conditioned PS surfaces, bacterial suspensions were circulated through the PPFC at 0.020 mL/s for 30 min. Then, PBS was circulated at the same flow through the equipment to remove unattached and weakly adhering cells, thus allowing the evaluation of the bacterial cells detachment.

2.4 BIOFILMS

2.4.1 Biofilm Formation

2.4.1.1 Biofilm inoculum

For biofilm formation, cells within the bacterial suspension were harvested by centrifugation and washed twice with ultrapure (UP) sterilized water. Standardized cell suspensions were prepared in TSB at a cell density of 1 x 10^7 cfu/mL. In order to develop mixed species biofilms, 50% of cell suspensions of each bacterium were used.

2.4.1.2 High-throughput devices

Tissue culture plates with flat bottoms, of 6, 24 and 96 wells were used throughout this work. These high-throughput devices allow the formation of high number of biofilms with similar characteristics, making them the ideal surfaces to test antimicrobial products effects in mature biofilms.

2.4.1.3 Biofilm Development

The methodology used to grow the bacterial biofilms was based on the microtiter plate test developed by Stepanovic *et al*.[15]. Two hundred microliters per well of cell suspensions were transferred to sterile 96-well flat-bottom tissue culture plates (Orange). All the plates were incubated aerobically on a horizontal shaker (120 rpm), at 37˚C.

2.4.2 Biofilm adaptation

To induce the adaptation of the biofilm-embedded cells, biofilms were developed for 24 h in 96-well flat-bottom tissue culture plates. For biofilm adaptation, the wells were re-filed with fresh TSB or TSB containing BC in a final concentration of 0.9 mM. The process of supernatant removal and media filling was repeated for five subsequent days, every 24 h.

For proteomics studies, biofilms were developed during 24 h in TSB in 6-well flat-bottom tissue culture plates being after adapted to 0.9 mM (320 mg/L) of BC or 6.0 mg/L of CIP during 12 days.

2.4.3 Biofilm Treatment

After being developed, biofilms were subjected subsequently to treatment with the same antimicrobial product. For that, the content of each well was removed and washed once with 200 µL of UP sterilized water, being the well-attached biofilms subsequently treated with 200 µL of 1.0 mM of BC for 30 min. The BC concentration was similar to the one used for biofilm adaptation due to the fact that the concentration of 0.9 mM is already a high concentration when compared with the normally in-use BC concentration in cleaning products [1.0% (w/v)][6]. This procedure had the major purpose of eliminating more susceptible and outward biofilm-cells. Non-treated wells were filled with 200 µL of UP sterilized water for the same period of time. After that,

the content of each well was removed and biofilms were washed twice with 200 µL with UP sterilized water and reserved for subsequent analysis.

2.4.4 Biofilm analyses methods

2.4.4.1 Scanning electron microscopy (SEM) observations

Prior to SEM observations, the wells-attached washed biofilms were gradually dehydrated in an absolute ethanol (Merck) series (15 min each in 10, 25, 40, 50, 70, 80, 90 and 100% v/v). The biofilms were kept in a dessicator until the walls of the wells were cut and coated with gold. The examination of the surface structural conformation of the biofilms was performed with a Leo scanning electron microscope (Cambridge). SEM observations were documented through the acquisition of representative microphotographs.

2.4.4.2 Biofilm mass

Biofilm biomass was quantified by crystal violet (CV) staining method adapted from Stepanovic *et al.*[15]. For that, the plates containing the washed biofilms were left to air dry for 30 min, and 200 µL of 98% methanol were transferred to each well in order to fix the remaining attached bacteria, for 15 min. Afterwards, the plates were emptied and left to air dry again. The fixed bacteria were stained with 200 µL of 1% (w/v) CV (Gram colour-staining; Merck) per well, for 5 min. After the staining step, the plates were washed with running tap water and air dried for approximately 20 min and, at last, 200 µL of 33% (v/v) of acetic acid (Merck) were added to each well in order to solubilise the CV bound to the adherent bacteria. The quantitative analysis of biofilm production was performed through the measurement of optical density at 570 nm (OD_{570}) in each well using a microtiter plate reader, being the biofilm mass presented as OD_{570}. Control experiments to avoid false results were also performed in order to determine whether the tested media and the plate material could adsorb CV and interfere with biomass quantification. When the optical density was higher than 1.0 the sample was diluted with 33% (v/v) of acetic acid. For each condition tested, 16 different wells were used to perform biofilm analysis and the experiment was repeated in three independent assays.

2.4.4.3 Biofilm activity

Biofilm activity determination was evaluated with 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide sodium salt (XTT) colorimetric method as described by Stevens and Olsen[16], with some modifications.

Bacteria, when metabolically active, reduce XTT to a water-soluble orange formazan that diffuses from the cells and thus does not require solvent extract prior to its quantification. Since the formazan product is water soluble, it is easily quantified by spectrophotometry analysis. Therefore, the quantity of formazan produced is used as a measure of the total respiratory activity of the

bacteria. This latter point is important in biofilm research because the XTT assay allows the study of intact biofilms without disruption biofilm structure[5].

After biofilm growth and washing procedures, 200 µL of a combined solution of XTT (Sigma) and phenazine methosulfate (PMS) (Sigma) were added to each well in order to obtain a final concentration of 150 mg/L of XTT and 10 mg/L of PMS. After that, plates were incubated at 37°C for 3 h, at 120 rpm, in the dark. Biofilm activity was determined through the measurement of the optical density at 490 nm (OD_{490}) in each well using a microtiter plate reader, being biofilm activity presented as OD_{490}. Control tests, using culture medium and empty wells, were also carried out in order to avoid misleading results. For each condition tested, 16 different wells were used to perform biofilm analysis and the experiment was repeated in three independent assays.

2.4.4.4 Biofilm entrapped cells

Bacterial suspensions of the biofilms to be used in cfu determination and also in protein and polysaccharide quantification were prepared as described hereafter. Two hundred microliters of UP sterilized water were added to each well, being the wells-attached washed biofilms removed by ultrasonic bath in a Sonicor SC-52 (Sonicor Instruments) operating at 50 kHz, during 6 min (these parameters were previously optimized in order to promote the complete removal of all the biofilm-attached cells without lysis). Afterwards, bacterial suspensions of each 5 wells per condition were collected, gently vortexed for 2 min to disrupt possible cell aggregates, and reserved for later analysis.

In order to determine the number of cfu, biofilm suspensions removed by sonication were serially diluted. After plating the serial dilution on TSA, plates were incubated at 37°C for 24 h prior to enumeration.

2.4.4.5 Proteins and Polysaccharides Content

The total biofilm content of proteins of biofilms was determined using the BCA Protein Assay Kit (Pierce), with bovine serum albumin as a protein standard, and the optical density (OD) values recorded at 740 nm. The total polysaccharides content was determined by the phenol-sulphuric acid method described by Dubois *et al.*[4] with glucose as standard, and the OD values were recorded at 490 nm. This biochemical characterization was performed in three independent experiments.

2.5 PROTEOMICS

2.5.1 Preparation and analysis of planktonic outer membrane protein (OMP) extracts

For planktonic proteomic studies, crude outer membrane extracts were prepared from bacterial pellets following the spheroplast procedure described by Mizuno and Kageyama[10].

For protein extraction, standardized cell suspensions were prepared in a minimal salt medium (MSM medium, pH 7.5)[19]. The bacterial cell concentration of each suspension was estimated by OD_{640} referred to a calibration curve[2]. The bacterial planktonic cultures were prepared in 800 mL of MSM medium by adjusting the final cell concentration to 1 x 10^7 cfu/mL and were let to grow at 37°C for 20 h, at 150 rpm. Bacterial cultures were harvested for 15 min at 3 500 x *g* and washed twice with 20% (w/v) sucrose solution. Cells were suspended in a digestion solution with the following composition: 9 mL 2.0 M sucrose solution; 10 mL 0.1 M Tris-HCl pH 7.8, at 25 °C; 0.8 mL 1% (w/v) Na-EDTA, pH 7.0; 1.8 mL 0.5% (w/v) lysozime. The mixture was incubated for 1.5 h at 37ºC in the presence of DNAse and RNAse (5 mg/L; Sigma). Spheroplasts were collected by centrifugation (20 min at 10 000 x *g*) and outer membranes were then pelleted (120 000 x *g* for 1 h at 4°C) and resuspended in 1 mL of sterile UP water. The protein amount was measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

2.5.2 Preparation and analysis of biofilm-entrapped cells outer membrane protein (OMP) extracts

For biofilm proteome analyses, OMP from biofilm-entrapped cells and matrix proteins were extracted. Bacterial cultures were harvested for 15 min at 3500 x *g* and cells were suspended in one millilitre of CHAPS. Cells were then disrupted by sonication (Ultrasonic Processor, Cole-Parmer, USA) using 10 W power for 6 x 10 s on ice. Unbroken cells and cell debris were removed from the resulting suspension by centrifugation at 7 000 x *g* for 10 min at 4°C. The supernatant was then centrifuged at 100 000 x *g* for 1 h at 4°C, the formed pellet representing the total membrane fraction. The separation of the bacterial membranes was adapted from that described by Winder *et al*.[20]. Membrane proteins were incubated in 25 mL of 2% (w/v) sodium lauryl sarcosinate solution at room temperature for 1 h, followed by centrifugation at 100 000 x *g* for 1 h at 4°C. The resulting pellet, which represents the outer membrane fraction, was resuspended in one millilitre of UP water. The protein amount was measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

2.5.3 Two-dimensional gel electrophoresis

Outer Membrane Protein patterns were analysed by two-dimensional gel electrophoresis (2-DE). Two hundred micrograms of proteins were added to isoelectric focusing (IEF) buffer (final volume, 300 µL)[18]. The first-dimension gel separation was carried out with Immobiline Dry Strips L (pH 4–7, Amersham Pharmacia Biotech). The second dimension was obtained by SDS–PAGE

using a 12.5% (w/v) polyacrylamide resolving gel (width 16 cm, length 20 cm, thickness 0.75 mm). After migration, proteins were visualized by silver nitrate staining[12].

2.5.4 Gel analysis

Spot quantification was achieved by computing scanning densitometry (ProXPRESS 2D, PerkinElmer Sciex). Gels were analysed using the Progenesis Samespot (Nonlinear Dynamics) software. For each experimental condition, three 2-DE gels were matched together to form a reference image. The two reference gels were then matched together so that the same spot in different gels had the same number. Protein spots from the two bacterial populations were considered to display significant quantitative differences if they fulfilled the following criteria: p values ≤ 0.05 (t-test); detection threshold, average volume ≥ 20 (n = 3); differential tolerance, fold change ≥ 2^{14}. It was also considered the q value (q ≤ 0.05) to guarantee that no false positives were erroneously analysed.

2.5.5 Protein identification

Spots were excised from the polyacrylamide gel and sliced into small pieces with an automatic spot cutter (ProXCISION). Selected spots complied with the following criteria: volume varying with the incubation condition and displaying a high (average) value with a low coefficient of variation. Gel plugs were then dried using a SpeedVac centrifuge. Trypsin digestion was performed using an automatic digester (MultiPROBE II, PerkinElmer Sciex). After lyophilisation, the peptide extracts were resuspended in 10 μL of 0.2% formic acid/5% acetonitrile.

Peptides were enriched and separated using a lab-on-a-chip technology (Agilent) and fragmented using an on-line XCT mass spectrometer (Agilent). The fragmentation data were interpreted using the Data Analysis program (version 3.4, Bruker Daltonic).

2.5.6 Bioinformatic tools for subcellular location

For protein identification, MS/MS peak lists were extracted and compared to the NCBInr protein database restricted to *P. aeruginosa* (February 2, 2007, version 4, 1,342,017 residues, 4243 sequences), using the MASCOT Daemon (version 2.1.3) search engine. All searches were performed with no fixed modification and allowed for carbamidomethylation, oxidation and a maximum of one missed trypsin cleavage. MS/MS spectra were searched with a mass tolerance of 1.6 Da for precursor ions and 0.8 Da for fragment ions, respectively. If a protein was characterized by 2 peptides with a fragmentation profile score higher than 25 the protein was validated. When one of the criteria was not met, peptides were systematically checked and/or interpreted manually to confirm or cancel the MASCOT suggestion.

For identified proteins, and in particular for unknown proteins, the prediction of their location within the bacterial cell was obtained from the genome annotation of *P. aeruginosa* (accessible at http://www.pseudomonas.com/).

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CHAPTER 3

LEARNING WITH *PSEUDOMONAS AERUGINOSA* CLINICAL ISOLATES

CHAPTER 3.1

MOTILITY, BIOFILM FORMING ABILITY AND TOLERANCE DEVELOPMENT OF *PSEUDOMONAS AERUGINOSA* CLINICAL ISOLATES

Pseudomonas aeruginosa is an opportunistic pathogen, widely investigated for its high incidence in clinical environments. Motility over surfaces is a survival strategy that contributes to bacterial ability to colonize different surfaces and to develop biofilms.

This work aimed to compare the virulence behaviour of a series of five *P. aeruginosa* clinical isolates, namely the motility phenotype and to assess whether this bacterial trait could be related to biofilm formation ability and biofilm resistance to benzalkonium chloride (BC) and ciprofloxacin (CIP).

Biofilm tolerance was assessed by the determination of the bactericidal effect of 1x, 4x and 16x the MIC of each antimicrobial in biofilm development and survival.

Results showed that one of the most motile isolated strains was the stronger biofilm former. Biofilms formed by the less motile strain, that exhibited reduced biofilm mass, activity and number of cells, showed however increased resistance to BC and CIP. Although high flagellated bacteria may have advantages amongst the other strains in terms of colonization, data suggest that the presence of appendages has no relation with biofilm resistance to BC and CIP. Biofilm tolerance was observed for all the clinical isolates, as higher values of biofilm mass and activity were observed after biofilm exposure to 16x MIC of BC and CIP in comparison with the reference strain.

This study suggests that the presence of isolates can confer biofilms greater phenotypic variability that may contribute to the easiness of biofilm adaptation to external antimicrobial pressures and subsequent recalcitrance to antimicrobial treatments. This work also reinforces the need of determining the biofilm-forming ability and the sensitiveness to in-use antimicrobials of each clinical bacterial isolate not only in planktonic cultures, but also in the biofilm mode of growth, following standardized operational procedures.

3.1.1 INTRODUCTION

Pseudomonas aeruginosa, an opportunistic high flagellated pathogenic bacterium, has been widely investigated for its association with a ample variety of nosocomial infections and high incidence in clinical surfaces[8]. The pathogenesis of *P. aeruginosa* is due to the production of a vast diversity of virulence factors including secreted factors (such as lipopolysaccharides and flagella)[22], as well as the ability to form biofilms[20]. *P. aeruginosa* is capable of three types of motility: twitching, swimming and swarming. Twitching is mediated by type IV pili on solid substrates, whilst swimming and swarming are mediated by the flagellum in aqueous environments[7]. Cellular appendages, including motility structures, like flagella, and adhesive structures like type IV pili and other nonpilus adhesins, are important for attachment to abiotic surfaces and for the establishment of infection in the epithelial cells. Various studies[13,16,21] have addressed bacterial motility both as means of initiating contact with surfaces and in biofilm formation and maturation. Previous studies related with bacteria attachment and early-stage of biofilm formation have demonstrated that flagellar-based swimming and type IV pili-dependent twitching are important for bacterial adhesion. The most noted pathogenic ability of *P. aeruginosa* is the colonization of patients and contamination of medical devices through the development of strong biofilms, which are often hard to eradicate with conventional strategies. Biofilms can be defined as structured communities of microorganisms encased in a self-produced polymeric matrix attached to inert or biotic surfaces[5]. The biofilm-growing cells acquire physiological characteristics that differentiate them from planktonic cells, being in part responsible for the increased resistance towards antimicrobial treatment[6]. When *P. aeruginosa* is found in clinical settings, efforts should be made to substantiate its clinical relevance, namely determining its pathogenicity and the most effective antimicrobials for its complete eradication. In fact, it has been noticed notable differences in virulence traits and degrees of innate resistance between the majorities of *P. aeruginosa* clinical isolates. Those efforts must also encompass strategies to avoid the establishment and recalcitrance of further bacterial adhesion and biofilm development[2].

The aim of this work is to characterize and compare clinical isolates of *P. aeruginosa* regarding their motility, biofilm-forming ability and to determine whether biofilms might have augmented tolerance towards benzalkonium chloride (BC) and ciprofloxacin (CIP).

3.1.2 MATERIAL AND METHODS

Strains and culture conditions

P. aeruginosa ATCC 10145 and *P. aeruginosa* clinical isolates (catalogued as I93488, I92986, I97824, C80117 and U147016) were used in this work. Bacteria were preserved and cultured as described in sub-chapter 2.1.

Antibacterial Agents

Benzalkonium Chloride (BC) was used throughout this work.

Bacterial motility

To evaluate the motility ability of each bacterial strain, swimming, swarming and twitching assays were performed as described in sub-chapter 2.2.4.

Biofilms

The methodology used to grow the bacterial biofilms was based on the microtiter plate test developed by Stepanovic *et al*.[18], as described in sub-chapter 2.4.1.3

Biofilm Treatment

The wells-attached biofilms were subsequently treated with 200 µL of 1280 mg/L (8x MIC) of BC or 1 mg/L (8x MIC) of CIP for 30 min. Non-treated wells were filled with 200 µL of UP sterilized water for the same period of time. After that, the content of each well was removed and biofilms were washed with 200 µL of UP sterilized water and reserved for subsequent analyses (see below).

BC and CIP bactericidal activity in biofilms

The determination of bactericidal effect of BC and CIP in biofilm development was based on the procedure described by Riera *et al*.[17]. In order to determine the concentrations to be evaluated, the MIC and the minimum bactericidal concentration (MBC) of the reference strain were determined as described in sub-chapter 2.2.1.

Biofilms were formed for 24 h as previously described and incubated for additional 24 h in TSB at 0x, 1x, 4x and 16x the MIC of BC and CIP. Afterwards, biofilms were washed twice with UP sterilized water and characterized as described below.

Biofilm mass

The biomass of *P. aeruginosa* biofilms was quantified by the crystal violet (CV) staining method adapted from Stepanovic *et al*.[18] according to the procedure described in sub-chapter 2.4.4.2.

Biofilm activity

Biofilm activity was determined with the 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide sodium salt (XTT) colorimetric method by Stevens and Olsen[19] as described in sub-chapter 2.4.4.3.

Biofilm cell enumeration

The enumeration of *P. aeruginosa* biofilm cells was performed by colony counts following the procedure described in sub-chapter 2.4.4.4.

Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 4.0 software for Macintosh. Normality of data distribution was tested by the Kolmogorov-Smirnov method. Statistical significance values of the groups' means of biofilm biomass, biofilm activity and cell number were evaluated using a one-way analysis of variance. The statistical analyses performed were considered significant when $p < 0.05$.

3.1.3 RESULTS

Bacterial motility

In order to evaluate the motility ability of the isolated strains, swimming, swarming and twitching assays were performed (Fig.3.1-1). Macroscopic observations of the colony pattern in the LB media, with specific density, revealed that the clinical isolates exhibit different motility structures and/or colony appearance.

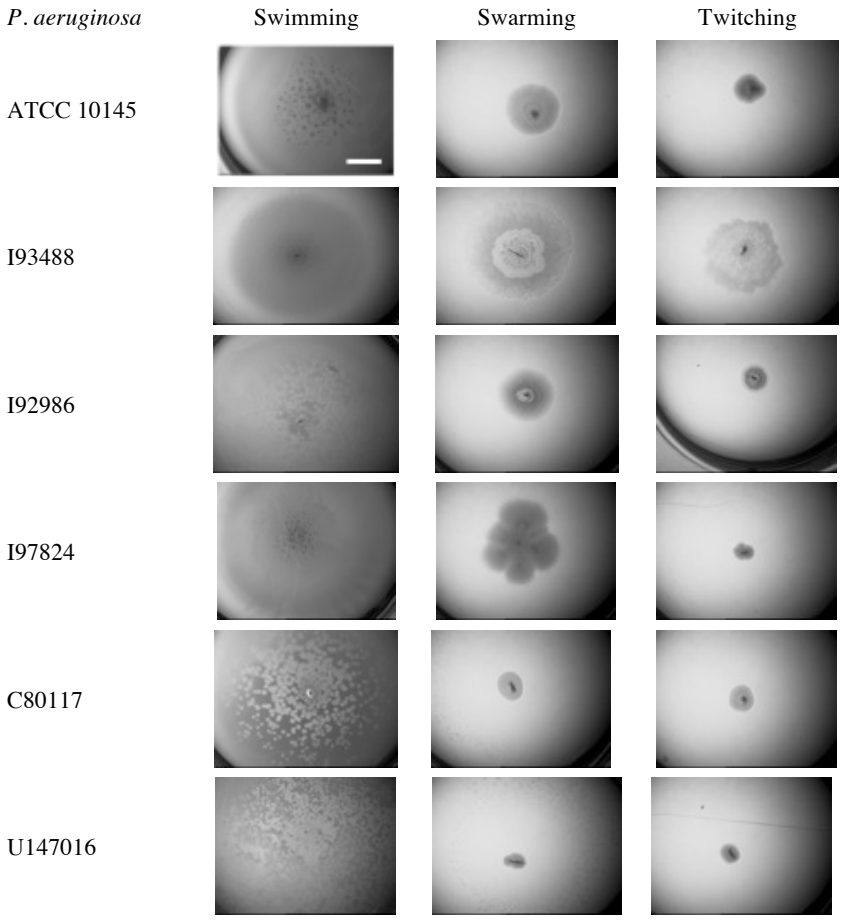

Fig.3.1-1 Macroscopic observations of the motility assays of *P. aeruginosa* ATCC 10145 and clinical isolates I93488, I92986, I97824, C80117 and U147016. Bar represents 0.5 cm.

Concerning swimming ability (Fig.3.1-1), all the tested strains exhibited similar swimming patterns through a relatively low-viscosity environment, as the diameter of the migration zones produced by the isolates were not significantly different ($p>0.05$). In terms of swarming motility, the reference strain and the clinical isolates I93488 and I97824 showed swarming rings with the largest diameter among the strains evaluated while C80117 and U147016 formed the smallest colonies (Fig.3.1-1). Regarding twitching motility, the I93488 strain revealed to be the strain able to develop colonies with the largest diameter and more irregular borders (Fig.3.1-1, Twitching).

Biofilm phenotype

Biofilm phenotypic characterization shows that, in general, the 48-h-old biofilms have higher values of biomass, activity and number of cells than those observed for 24-h-old biofilms (Fig.3.1-2 A). The exceptions were the reference strain that exhibited similar values of biofilm activity at both periods of biofilm development, and the clinical isolate I93488 that showed less number of cells entrapped in the older biofilms.

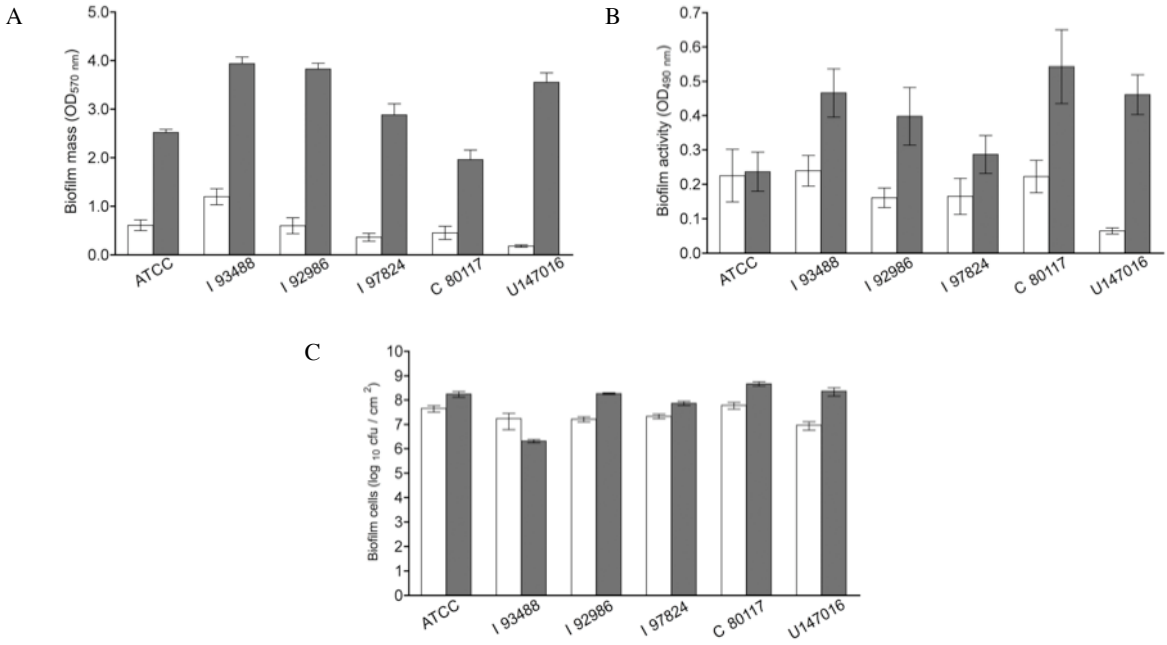

Fig.3.1-2 Biofilm mass (OD_{570}) (A), metabolic activity (OD_{490}) (B) and number of cultivable cells (C) of 24-h-old (withe) and 48-h-old (grey) biofilms of *P. aeruginosa* ATCC 10145 and clinical isolated strains I93488, I92986, I97824, C80117 and U147016. Bars represent the average of three independent repeats ± SD.

After 24 h of biofilm growth, *P. aeruginosa* isolated strain I93488 exhibited the highest biofilm-formation ability ($p<0.05$) (Fig.3.1-2 a) compared with the other clinical isolates and the reference strain (Fig.3.1-2). Conversely, *P. aeruginosa* U147016 is the strain that formed biofilms with less

biomass ($p<0.05$) and lower respiratory activity ($p<0.05$). However, after 48 h of biofilm growth, this isolated strain displayed biofilms with superior activity and number of cells when compared with the reference strain. All other strains exhibited similar biofilm respiratory activity after 24 h of growth ($p>0.05$). After 48 h of biofilm development, the clinical isolate C80117 was the strain that developed biofilms with higher respiratory activity (Fig.3.1-2b). The number of biofilm-entrapped cells was similar for all the strains and for both periods of biofilm development (Fig.3.1-2c).

These results indicate that all the strains are good biofilm producers, although the clinical isolates stand out relatively to the reference strain when biofilm respiratory activity is analysed.

Bactericidal effect of BC and CIP in biofilm development

In order to assess the antimicrobial effects of BC and CIP during biofilm development, 24-h-old biofilms were allowed to growth for additional 24 h in the presence of 0x, 1x, 4x and 16x the MIC of each antimicrobial agent. The MIC of BC and CIP for the *P. aeruginosa* reference strain was respectively 160 and 0.125 mg/L. The results regarding the evaluation of the bactericidal activity of BC and CIP in biofilm development are shown in Fig.3.1-3 and Fig.3.1-4.

The presence of 160 mg/L of BC (1x the MIC value) during biofilm development reduced the biofilm mass for all the strains, with the exception of the reference strain and the clinical isolate C80117 (Fig.3.1-3 A). However, the application of the highest BC concentrations did not reduce biofilm biomass in comparison with the control biofilms. In general, biofilm treatment with 4x MIC of BC (640 mg/L) increased the biofilm mass, as indicated by the increase in the OD_{570} values when compared to the untreated controls (0x MIC). Similar behaviour was observed for biofilms developed in the presence of the highest BC dose tested, 2560 mg/L (16x MIC of BC). Data shown in Fig.3.1-3 A suggests that under this concentration treatment, all the strains produced biofilms with higher biomass than the corresponding untreated controls, developed only in TSB for 48 h ($p<0.05$). Moreover, at 2560 mg/L of BC, with the exception of C80117 and U147016 strains, biofilms developed by the clinical isolates presented more biomass than the biofilms developed by the reference strain. Regarding biofilm respiratory activity data (Fig.3.1-3 B), in general, the 48-h-old biofilms showed a reduced activity, this reduction being proportional to the BC concentration used to growth the *P. aeruginosa* biofilms.

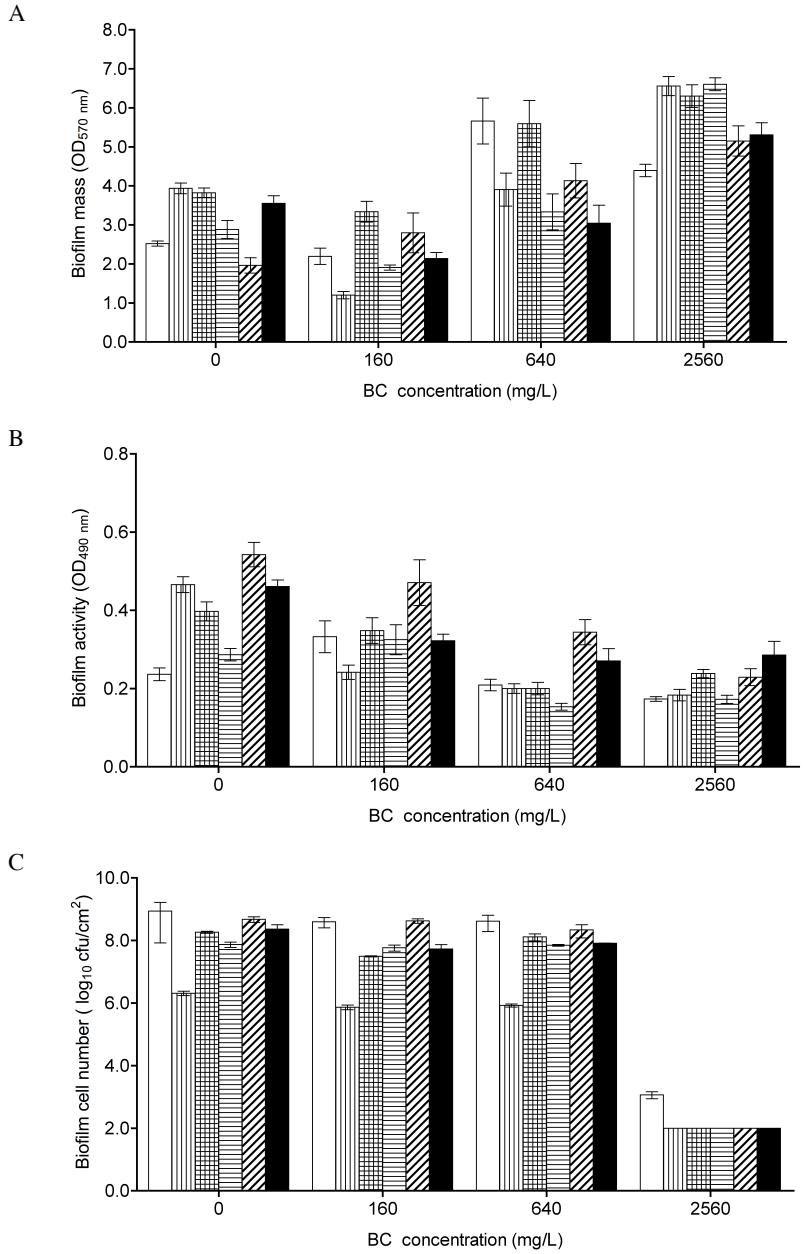

Fig.3.1-3 Biofilm mass ($OD_{570\ nm}$) (A), metabolic activity ($OD_{490\ nm}$) (B) and number of cultivable cells (C) of 24h-biofilms developed for additional 24h in the presence of 0x, 1x, 4x and 16x the MIC of *P. aeruginosa* ATCC 10145 to BC (160 mg/L). Biofilms were formed by ATCC 10145(□) and isolated strains I93488 (▥), I92986(▦), I97824 (▤), C80117 (▨), U147016(■). Bars represent the average of three independent repeats ± SD.

The presence of 640 mg/L of BC during biofilm growth caused a high reduction in the respiratory activity of the biofilms developed by the *P. aeruginosa* isolates when compared with control biofilms ($p<0.05$). The increase in the BC concentration, from 640 mg/L (4x MIC) to 2560 mg/L (16 x MIC) of BC did not promote higher activity reduction than that observed at 4 x MIC ($p>0.05$). However, in terms of culturable cells, the results obtained reveal that 2560 mg/L BC (16x MIC) (Fig.3.1-3 C) promoted a reduction of approximately 8 $\log_{10}/cm^2$ in the number of biofilm-entrapped cells for all the strains.

Concerning the development of biofilms in the presence of CIP (Fig.3.1-4), in general, it was observed a dose-dependent biomass reduction for all the strains (Fig.3.1-4 A), with the exception of biofilms developed by the U147016 strain. In fact, this clinical isolate developed biofilms with similar values of biomass despite the CIP dose assayed (from 0 to 2 mg/L) ($p>0.05$) (Fig.3.1-4 A).

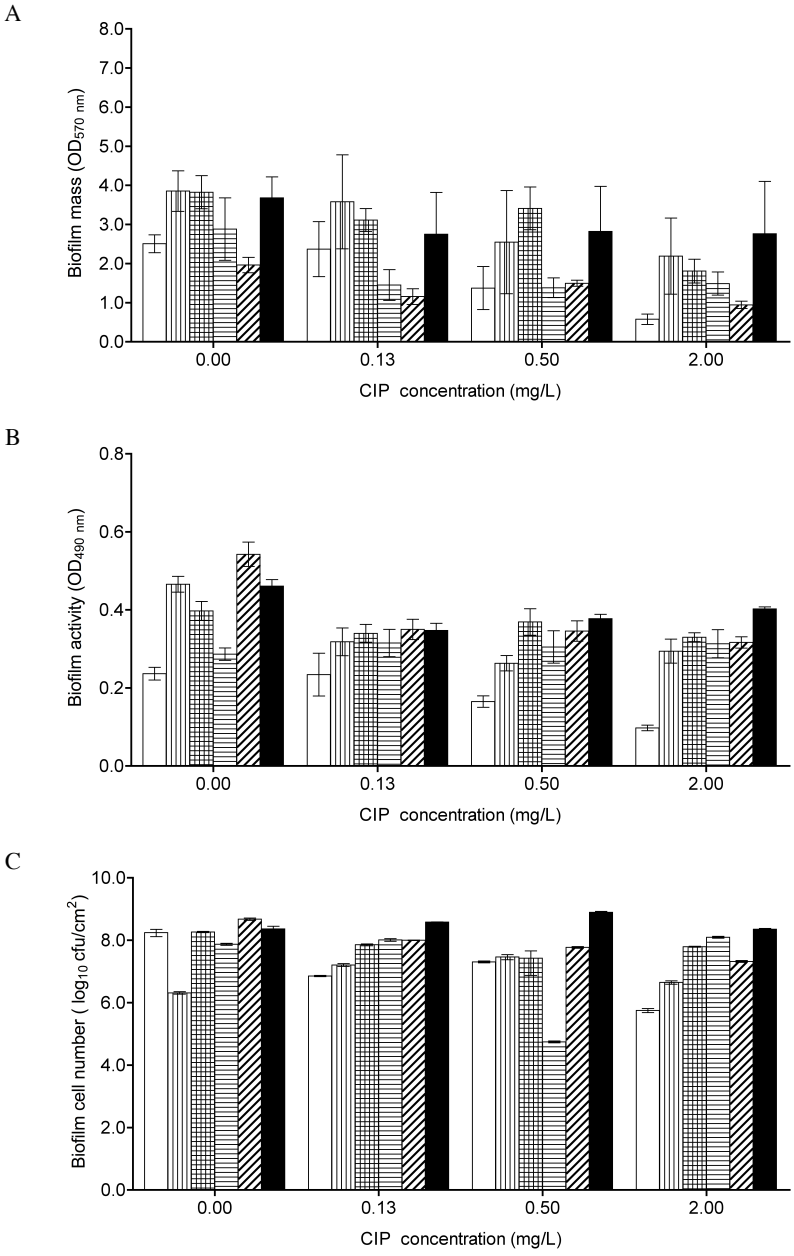

Fig.3.1-4 Biofilm mass ($OD_{570\ nm}$) (A), metabolic activity ($OD_{490\ nm}$) (B) and number of cultivable cells (C) of 24h-biofilms developed for additional 24h in the presence of 0x, 1x, 4x and 16x the MIC of *P. aeruginosa* ATCC 10145 to CIP (0.13 mg/L). Biofilms were formed by ATCC 10145(□) and isolated strains I93488 (▥), I92986(▦), I97824 (▤), C80117 (▨), U147016(■). Bars represent the average of three independent repeats ± SD.

The presence of the higher CIP concentration tested (2.0 mg/L) during biofilm development did not promote any considerable decrease in biofilm cells respiratory activity when compared with the biofilms developed in the absence of CIP, for all the strains, except for the reference strain ($p<0.01$). At the higher CIP concentration tested (2.0 mg/L) all the biofilms developed by clinical

isolates showed higher values of activity when compared with those developed by the reference strain ($p<0.001$).

Concerning the effect of CIP on the biofilm-entrapped cells (Fig.3.1-4 C), it was observed a reduction of about 3 $\log_{10}/cm^2$ in the number of I97824 culturable cells after biofilms exposure to 0.5 mg/L of CIP (4x MIC) in comparison with the untreated controls. The highest CIP concentration, 2.0 mg/L (16x MIC), disturbed *P. aeruginosa* ATCC 10145 biofilms, promoting a reduction in the number of cells of about 2 $\log_{10}/cm^2$ (Fig.3.1-4 C). The use of 2.0 mg/mL of CIP on biofilm development by the clinical isolates did not cause any significant reduction in comparison with the biofilms developed in the absence of CIP (Fig.3.1-4 C).

3.1.4 Discussion

Biofilms are sessile communities of microorganisms adhered to a surface and enclosed in an extracellular matrix. In most environments, bacteria are thought to reside predominantly in biofilms rather than in a planktonic or free swimming state[4]. *P. aeruginosa* is capable of three types of motility. Specifically, twitching motility is mediated by type IV pili on solid substrates, whilst swimming and swarming motility are mediated by the flagellum in aqueous environments[7]. During biofilm formation, flagella or flagella-mediated motility are important for the establishment of the bacterial monolayer and to bring the cell within close proximity to the surface, overcoming repulsive forces between the bacterium and the surface[7]. Furthermore, type IV pili appear to play a role in the stabilization of the interactions between the abiotic surface and/or in the cell-to-cell interactions required to form a microcolony, therefore contributing for biofilm formation[16].

In this study, it was aimed to inspect the virulence traits of five *P. aeruginosa* clinical isolates regarding their motility phenotype and to assess whether this bacterial trait could be directly related to biofilm formation ability and biofilm resistance to a frequently used antimicrobial product (BC) and to an antibiotic commonly prescribed to treat *P. aeruginosa* infections (CIP).

Results presented in Fig.3.1-1 (Swarming) reveal that the strain I93488 showed the highest swarming phenotype. Swarming is a type of social motility that allows rapid colonization of surfaces by highly differentiated swarm cells[12,14]. It has been also pointed out that swarmer cells are more resistant to several types of antimicrobials[11,14]. As swarming is a type of collective locomotion, the concept of a unique resistant phenotype when cells are organized as a society is emerging and has been named as a "social phenomena"[14]. Moreover, *P. aeruginosa* I93488 was also the strain that formed colonies with highest diameter in the twitching assays (Fig.3.1-1). As referred previously biofilm formation has been linked to twitching motility[14] and in accordance,

this strain exhibited the thickest biofilms after 24 h of growth and, together with the clinical isolate I92986, also at 48 h of biofilm development (Fig.3.1-2).

Biofilms formed by *P. aeruginosa* I93488 strain were also the ones that suffered less mass reduction after treatment with both BC (Fig.3.1-3) and CIP (Fig.3.1-4). To expand previous knowledge, the BC and CIP bactericidal effects on 24-h-old biofilms formed by *P. aeruginosa* clinical isolates were evaluated and compared. For that, the 24-h-old biofilms were allowed to growth for more 24 h in the presence of 1x, 4x and 16x of MIC of each antimicrobial agent determined for the *P. aeruginosa* ATCC 10145. Results showed that all the strains were able to develop biofilms when antimicrobial pressure exists. In fact, the increase in BC and CIP concentrations to values of 4x the MIC (Fig.3.1-3 and Fig.3.1-4) was not able to completely eradicate *P. aeruginosa* biofilms. The biofilms formed by the isolated strains revealed high tolerance to CIP, even at 16x the MIC (Fig.3.1-4). Despite the fact that BC showed ability to significantly reduce the number of cultivable biofilm-entrapped cells, the complete eradication of biofilms was not achieved as total reduction of biofilm mass or activity was not attained (Fig.3.1-3). These data are in accordance with those reported by Delissalde[8], that pointed out that *P. aeruginosa* clinical strains were more capable to form biofilms than reference strains, displaying resistant phenotypes.

The overall results also show that the isolate U147016, a strain that was characterized as non-motile, developed biofilms with less mass, activity and number of cells after 24h of biofilm formation (Fig.3.1-2). However, at 48 h of biofilm growth, this strain formed biofilms with the higher values of mass, activity and number of cells. In fact, no significant adverse effect was observed on biomass or activity when biofilms formed by U147016 were after developed in the presence of high-doses of antimicrobial products (Fig.3.1-3 and Fig.3.1-4).

Bacterial appendages are not only related with locomotion and biofilm formation, but also play a role in pathogenesis[3,16]. Clinical isolates, frequently exposed to stress conditions in a hospital environment, can suffer a selection process that favours more motile and/or more pathogenic strains[9,10]. As the number of isolated strains was not representative, no robust conclusion can be anticipated. However, with the strains tested, it can be stated that flagella and pili IV are in the origin of biofilm development, and in some cases, may be related to BC and CIP resistance. However, it was not found any relationship between the denser biofilms formed by the motile strains and higher biofilm resistance of *P. aeruginosa* clinical isolates biofilms.

Results also revealed that, high-doses (16x MIC) of BC and CIP applied in biofilms during 24 h, instead of eradicating the cells may contribute to biofilm-entrapped cells persistence, which may be on the basis of biofilm subsistence in natural environments. Even if the reduction of cultivable cells within the biofilm was almost total, especially after treatment with the higher concentration of BC tested, the reduction of biofilm mass and activity was not accomplished. When the level of

antimicrobial drops the small fraction of biofilm-entrapped cells that remain alive and protected within the biofilm matrix[1] are able to multiply and repopulate the biofilm allowing the bacteria to persist attached to the surface[15]. This fact may be on the basis of biofilm persistence in hospital environments where bacteria are permanently exposed to antimicrobial products. In general, the BC action was superior to CIP against 24-h-old biofilms, but none of the products revealed to be completely effective. A better understanding of the mechanism of action of BC and CIP, and the resulting response elicited by *P. aeruginosa* clinical isolates to these antimicrobial agents will facilitate its effective utilization for *P. aeruginosa* biofilm control in critical environments.

In real scenarios *P. aeruginosa* surface isolates can confer greater phenotypic variability to biofilms that may contribute to the easiness of adaptation to external antimicrobial pressures and subsequent biofilm persistence after antimicrobial treatments and cleaning practices. This study underlines that the bacterial sensitiveness to antimicrobials should be tested not only in planktonic cultures, but also with standardized operational procedures to evaluate biofilm formation ability as well as biofilm resistance to in-use antimicrobial products.

3.1.5 References

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CHAPTER 3.2

ANTIMICROBIAL PRESSURE OF CIPROFLOXACIN AND GENTAMICIN ON BIOFILM DEVELOPMENT BY AN ENDOSCOPE ISOLATED *PSEUDOMONAS AERUGINOSA* – ROLE OF PERSISTER CELLS ON BIOFILM RECOVERY

This work aims at characterizing and comparing endoscope biofilm-isolated (PAI) and wild-type P. aeruginosa (PA) initial adhesion, biofilm formation ability and sensitivity to antibiotics. Additionally, the recovery ability of the biofilm-growing bacteria subjected to intermittent antibiotic pressure with ciprofloxacin (CIP) and gentamicin (GM), as well as the development of resistance towards antibiotics and a disinfectant, benzalkonium chloride (BC), were also determined.

Data showed that both strains have high ability to adhere to surfaces and are good biofilm producers. However, PAI was able to adhere in higher extent and to form thicker biofilms. The capacity of both strains to develop biofilms was greatly impaired in the presence of CIP and GM. However, sanitization was not complete allowing always the recovery of the *P. aeruginosa* biofilms following the intermittent cycles of antibiotic pressure. This cycle of biofilm decrease/biofilm recovery may be due to the selection of persister cells able to survive the antibiotic selective pressure, ensuring biofilm survival. The environmental pressure exerted by CIP and GM did not developed *P. aeruginosa* resistance to the same antibiotics or cross-resistance towards BC. However, data highlighted that none of the antimicrobials led to complete eradication of the biofilms, allowing the recovery of the remaining adhered population. This feature may lead to biofilm recalcitrance, reinforcing bacterial attachment and the re-colonization of other accessible sites by persister cells.

3.2.1 INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogenic bacterium[7] widely investigated for its high incidence and extraordinary ability to form strong biofilms in clinical equipment, medical devices and wounds[5,19]. This microorganism is commonly associated with nosocomial infections and is a leading cause of severe and life-threatening infections, specially in immunosuppressed hosts[29]. *P. aeruginosa* is one of the most common microorganisms transferred by bronchoscopes, being the most frequent in gastrointestinal endoscopy[4]. Flexible endoscopes undergo repeated rounds of patient-use and reprocessing. Studies related to endoscope contamination have reported the presence of biofilms on the inner surface of endoscope channels[10,21], highlighting the importance of effective measures for cleaning and disinfection in endoscope reprocessing. Biofilm removal is a crucial step to prevent lapses in reprocessing being thus of clinical relevance in endoscopy[4,27]. Biofilms represent a reservoir of pathogenic bacteria that can detach, resume their planktonic state, and contaminate new surfaces and patients. Moreover, microbial biofilms are notorious for their high level of resistance towards antibiotic and biocide treatments[13]. Bacteria within biofilms can easily live in the presence of high antibiotic concentrations similar to the ones that are prescribed during the course of therapies[9,36]. Biofilm resistance mechanisms involve not only the reaction-diffusion limitation of antimicrobial access to the biofilm-entrapped bacteria[14,41], but also the expression of spatially heterogeneous, less susceptible phenotypes, caused either by growth as a biofilm *per se*[12] or through the expression of high cell density[6], or starvation phenotypes[11]. Antibiotics and biocides are frequently used in hospitals with the purpose to control the growth of, or to kill bacteria in, respectively, infection control and sanitation. The use of certain active substances in biocides in various settings may contribute to the increased occurrence of antibiotic resistant bacteria. Cross-resistance between biocides and antibiotics and between different antibiotics has been reported previously[25], and there are several studies suggesting that if two antimicrobial compounds have similar mechanisms of action, they may also share resistance mechanisms[35]. It has also been demonstrated that highly antibiotic-resistant clinical isolates of Gram-negative bacteria are generally more resistant to disinfectants[22]. Although there is much concern regarding the risks of antibiotic resistance induced by the use of and resistance to biocides, there is a lack of studies evaluating the performance of disinfectants after bacterial exposure to antibiotics. Studies related with the use of sessile bacteria to assess the efficacy of antibiotics and biocides are even lesser, even though biofilm formation is an important aspect of many bacterial diseases. These biofilm tests should also include bacteria isolated from real scenarios as they can present genetic diversity and thus possess distinct virulence factors. The pathogenesis of *P. aeruginosa* is attributed to the production of several cell-associated and extracellular virulence factors that arise under certain environmental conditions[42].

In the present work, the phenotype (early-stage adhesion, biofilm formation and sensitivity to antimicrobials) of *P. aeruginosa* isolated from a biofilm formed on an endoscope was determined and compared with the wild-type. Furthermore, this study was also undertaken to determine whether exposure of *P. aeruginosa* biofilms to intermittent cycles of antibiotic chemotherapy [ciprofloxacin (CIP) and gentamicin (GM)] could lead to regrowth and potential resistance and cross-resistance towards BC, CIP and GM.

3.2.2 METHODS

Test organisms and culture conditions

Pseudomonas aeruginosa (ATCC 10145) (PA) and *Pseudomonas aeruginosa* isolated (PAI) from a biofilm formed in a medical device (gastrointestinal endoscope) were used throughout this work. Bacterial suspensions were prepared as described in sub-chapter 2.1.3.

Antibacterial and antibiotic agents

Ciprofloxacin (CIP), Gentamicin (GM), and Benzalkonium chloride (BC), were used. The antibiotics concentrations used in the present work were determined using, as reference the 3x MBC[1] of each product for the reference strain. So, for biofilm disturbance cycles and biofilm treatment, a concentration of 3 mg/L of CIP and 10 mg/L of GM were used. Concerning BC, the MBC concentration of 320 mg/L was used.

Early bacterial adhesion

In order to determine the adhesion ability of both strains, a parallel plate flow chamber (PPFC) and image analysis system were used to study the early-stage bacterial adhesion and detachment, as described by Sjollema *et al*.[38] according with the procedure described in sub-chapter 2.3.3.

The initial increase in the number of adhering microorganisms with time was expressed as the so-called initial deposition rate [j0, cells/(cm^2 s)], *i.e*., the number of adhering microorganisms per unit area and time. The number of adhering microorganisms after 30 min of bacterial suspension flow [n_{30}, cells/(cm s)] as well as the number of microorganisms after PBS passage [n_{60}, cells/(cm s)] were also determined. The rate of detachment (%) denotes the percentage of *P. aeruginosa* cells that were detached upon the passage of PBS trough the flow chamber.

Biofilm formation

The biofilm formation ability of PA and PAI on polystyrene (PS) was inspected along time (24, 48, 72, and 96 h) using the microtiter plate test developed by Stepanovic *et al*.[39] as described in sub-chapter 2.4.1. After 24 h of biofilm growth, the supernatant containing planktonic cells and media was removed. The wells were re-filed with fresh TSB and this process of supernatant removal and media filling was repeated for every 24 h until 96 h of biofilm formation.

After 24, 48, 72, and 96 h of growth, biofilms were characterized in terms of biomass, respiratory activity and number of viable biofilm-entrapped cells.

Biofilm disturbance and recovery

To assess whether the presence of antibiotics could interfere with the establishment of biofilms by both strains on PS surfaces, biofilms were allowed to form for 24 h in the presence of both antibiotics. To ascertain the post-antibiotic effects (PAE) of CIP and GM on *P. aeruginosa*, those challenged biofilms were later subjected to intermittent cycles of antibiotic chemotherapy with CIP and GM. Biofilms were formed in microtiter plates with cell suspensions of both strains at a final concentration of 1.0 x 10^7 cfu/mL prepared in TSB containing CIP or GM in a final concentration of respectively, 3.0 mg/L or 10 mg/L. Each cycle of antibiotic treatment was followed by a recovery period of 24 h, where biofilms were developed in 200 µL of fresh TSB. After 24, 48, 72, and 96 h the content of each well was removed, biofilms were washed and phenotypically characterized.

Biofilm susceptibility

The 96-h-old biofilms untreated and submitted to the intermittent antibiotic chemotherapy with CIP and GM were inspected regarding their susceptibility towards the same antibiotics as well as the potential occurrence of cross resistance towards the disinfectant BC. In order to determine the biofilm response after antibiotic therapy, biofilms were treated with 200 µL per well of 360 mg/L of BC, 3 mg/L of CIP or 10 mg/L of GM for 30 min. Non-treated wells were filled with 200 µL of UP sterilized water. After that, the content of each well was removed and biofilms were washed with 200 µL with ultrapure sterilized water (UP) being reserved for posterior analysis.

Biofilm analyses

Biofilm mass, activity and number of cells

Biomass of *P. aeruginosa* biofilms was quantified by crystal violet (CV) staining method adapted from Stepanovic *et al*.[39] described in sub-chapter 2.4.4.2. Biofilm activity was determined with 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) colorimetric method colorimetric method as described by Stevens and Olsen[40], and referred in sub-chapter 2.4.4.3.The number of viable biofilm-entrapped bacteria from biofilm suspensions was determined according with the procedure described in sub-chapter 2.4.4.4.

Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 4.0 software for Macintosh. Normality of data distribution was tested by the Kolmogorov-Smirnov method. Statistical significance values of the groups' means of biofilm mass, biofilm activity and cell number were evaluated using a one-way analysis of variance. Subsequent comparisons were performed using Tukey's *post-hoc* test. Two-way analysis of variance with Bonferroni post-hoc test were used to compare means of biofilms obtained after 96 h, and after CIP and GM regrowth cycles after treatment. The statistical analyses performed were considered significant when $p < 0.05$.

3.2.3 RESULTS

Attachment and detachment monitoring

The results obtained with early bacterial adhesion assay (Table 3.2-1) revealed that both strains had high ability to adhere to surfaces, but the rate of cell deposition (j_0) of the isolated strain was higher than that of PA ($p<0.05$). After 30 min of adhesion, the number of adhered cells (n_{30}) is about twice for the isolated strain ($p<0.05$). Also, the number of PAI cells that remained attached in the PS surface (n_{60}) was the double of PA cells ($p<0.05$), being the percentage of detachment of PA cells after PBS passage of about 20%.

Table 3.2-1 Initial deposition rate (j0), number of adhered cells, (n30 min), number of adhered cells after PBS passage (n60 min), and percentage of detachment determined through the parallel plate flow chamber. Values are means ± SD for three measurements.

	j_0 (10^3 cells/(cm^2 s))	$n_{30\ min}$ (10^7 cells/cm^2)	$n_{60\ min}$ (10^7 cells/cm^2)	Detachment (%)
P. aeruginosa ATCC 10145	3.9 ± 1.2	4.56 ± 0.8	3.62 ± 0.5	20.6
P. aeruginosa isolated strain	6.3 ± 2.0	7.49 ± 1.4	7.34 ± 1.2	2.0

Biofilm formation

In order to examine the biofilm formation ability of both PAI and PA strains, the biofilm phenotype was characterized in terms of mass, activity and number of cells after 24, 48, 72 and 96 h of growth (Fig.3.2-1).

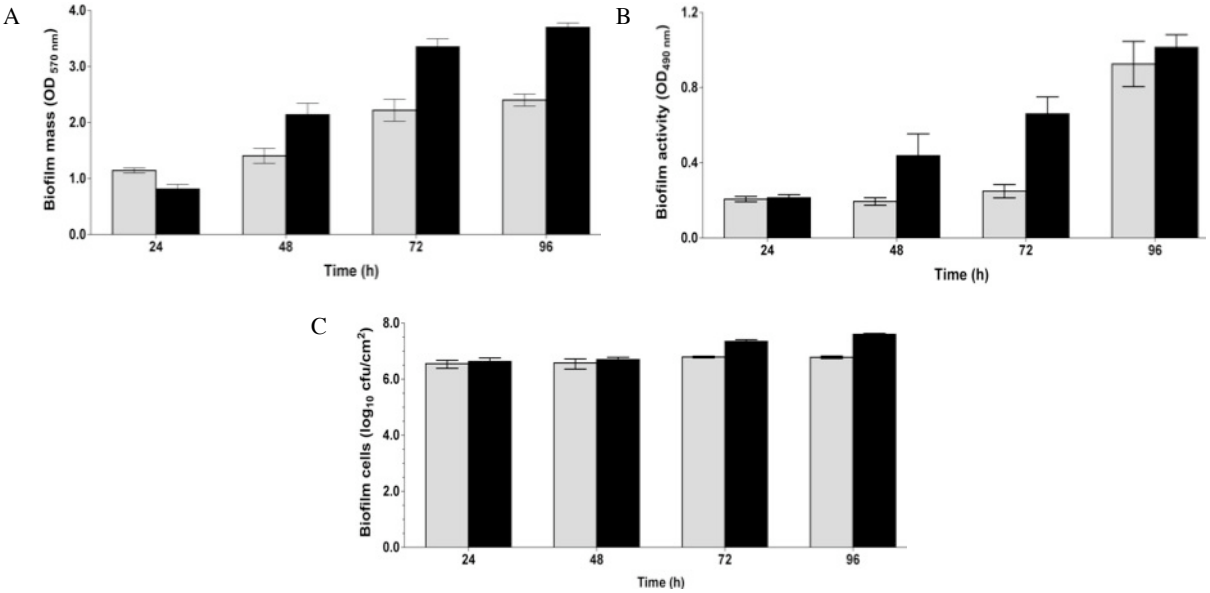

Fig.3.2-1 Biofilm mass (OD_{570}) (A), metabolic activity (OD_{490}) (B) and number of biofilm-entrapped cells (C) of *P. aeruginosa* ATCC (grey) and *P. aeruginosa* isolated strain (black) biofilms. Biofilms where grown in TSB and characterized at 24, 48, 72 and 96 h. Bars represent the average of 3 independent repeats ± SD.

In general, data showed that mass and activity of biofilms increased along time, whereas the number of biofilm-entrapped cells was approximately in the same magnitude, for all the time periods of biofilm formation. Comparing both strains, Fig.3.2-1 shows that, in general, PAI gave rise to biofilms with more mass than PA ($p<0.05$) and activity ($p<0.05$). The number of biofilm cells (Fig.3.2-1C), was identical to those quantified for PA biofilms, except for 72-h-old and 96-h-old biofilms in which there was an increase in the number of cells ($p<0.001$). These results indicate that both strains are good biofilm producers although the isolate stands out relatively to the collection. The presence of CIP and GM in the first 24 h of biofilm development clearly hampered the establishment of biofilms by both strains on PS surfaces (Fig.3.2-2).

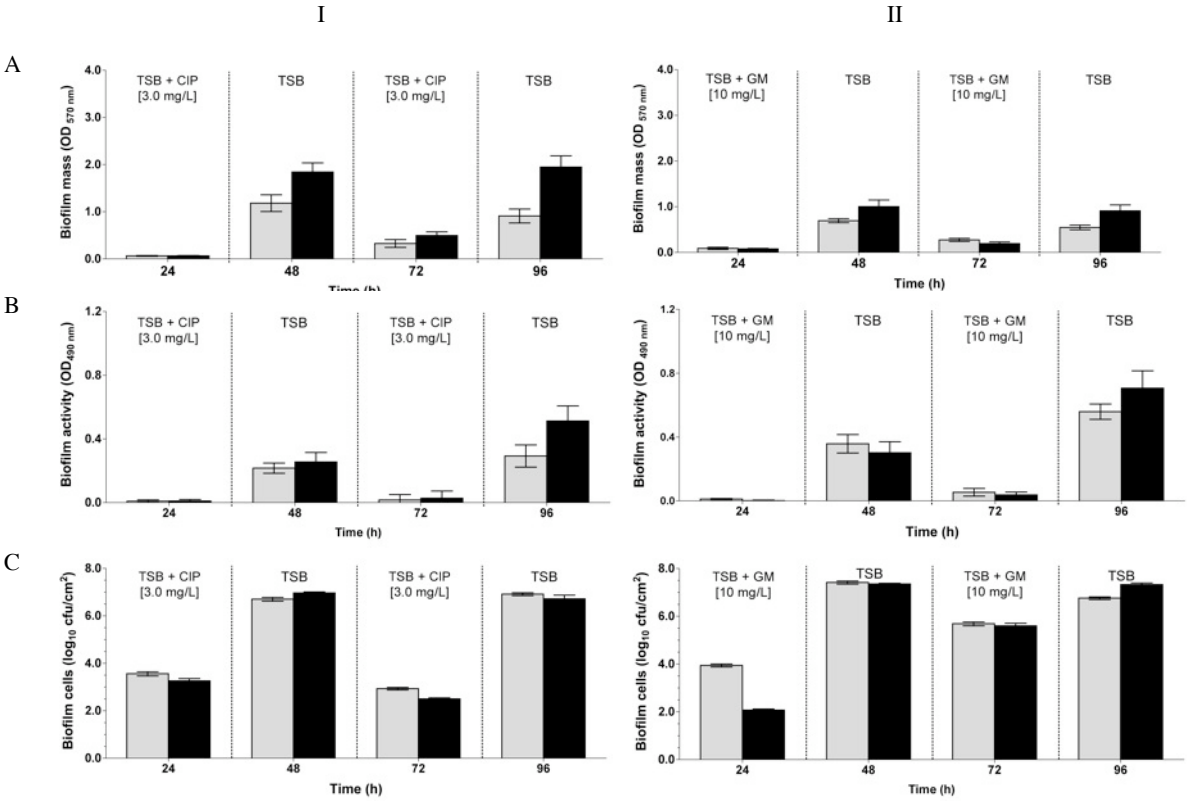

Fig.3.2-2 Biofilm mass (OD_{570}) (A), metabolic activity (OD_{490}) (B), and number of cultivable cells (C), of *P. aeruginosa* ATCC (grey) and *P. aeruginosa* isolated strain (black) biofilms. Biofilms where grown in 24 h cycles in TSB (I) + 3 mg/mL CIP or (II) in TSB + 10 mg/mL GM and TSB during 96 h. Bars represent the average of 3 independent repeats ± SD.

In fact, the phenotype of PA and PAI biofilms grown for 24 h under antibiotic pressure was characterized by a large decrease in biofilm mass and activity (about 95%) and a reduction of about 4 log in the number of viable biofilm-entrapped cells. However, it must be emphasized that a considerable number of cells remained viable on the surfaces. These data revealed that CIP and GM have a significant *in vitro* anti-biofilm formation activity, this effect being similar for both strains.

After a recovery period of 24 h, where growth occurred in absence of antibiotics, those remaining less dense biofilms recovered its levels of biomass, activity and number of cells (Fig.3.2-2).

Biofilm recovery post CIP pressure (Fig.3.2-2I) gave rise to PAI biofilms with higher values of biomass ($p<0.001$), activity and viable cells ($p<0.01$), when compared with PA biofilms. This trend was similar to that observed in the 48-h-old biofilms formed without any stress factor (Fig.3.2-1). Regarding the post-GM effect (Fig.3.2-2II), the superiority of the biofilms formed by the isolated strain is no longer evident as PAI biofilms only showed higher biomass ($p<0.001$).

The second cycle of biofilm growth under antibiotics pressure clearly reduced the mass, activity and number of biofilm-encased cells, for both strains (Fig.3.2-2). However, these reductions were lower than those obtained after the first cycle of antibiotic treatment, mainly when GM was used. These results showed that both antibiotics have ability to disturb established *P. aeruginosa* biofilms causing its removal and inactivation. Nevertheless, as Fig.3.2-2 shows, this sanitation was not total, allowing biofilm regrowth during the second recovery period. In fact, the resulting 96-h-old biofilms recuperated again its levels of biomass and activity, although they are far from those observed in biofilms developed by both strains in the absence of antibiotic stress (Fig.3.2-1). The numbers of viable biofilm-cells were also restored reaching however values in the same order of magnitude of those determined in the 96-h-old biofilms formed in TSB. Comparing the behaviour of both strains, in general, PAI biofilms showed higher biomass, activity and number of cells than the biofilms formed by the reference strain.

The post-antibiotic effects observed after the second cycle of antimicrobial treatment is similar to that observed after the first biofilm growth under antibiotic pressure, except for biofilm activity (Fig.3.2-2). In fact, the activity of the 96-h-old biofilms was higher than those observed after the first 24-h recovery period, specially for biofilms grown under GM pressure ($p<0.001$) and for those developed by PAI ($p<0.001$) (Fig.3.2-2B).

The overall results highlighted that both antibiotics have good anti-biofilm characteristics and ability to remove and inactivate established *P. aeruginosa* biofilms. Nevertheless, sanitization was not complete allowing the resumption of the biofilms immediately following antibiotic pressure.

The susceptibility of the 96-h-old biofilms, subjected to the intermittent cycles of antibiotic pressure, towards antibiotics (GM and CIP) and biocide (BC) treatment can be observed in Fig.3.2-3.

In the range of conditions tested, the 96-h-old biofilms formed by both strains in TSB were practically tolerant to the action of antibiotics and susceptible to the toxic effect of BC. In fact, only treatment with BC of PA and PAI biofilms promoted a significant reduction of biomass (Fig.3.2-3A)($p<0.001$), respiratory activity (Fig.3.2-3B)($p<0.001$), and number of viable cells (Fig.3.2-3C)($p<0.001$).

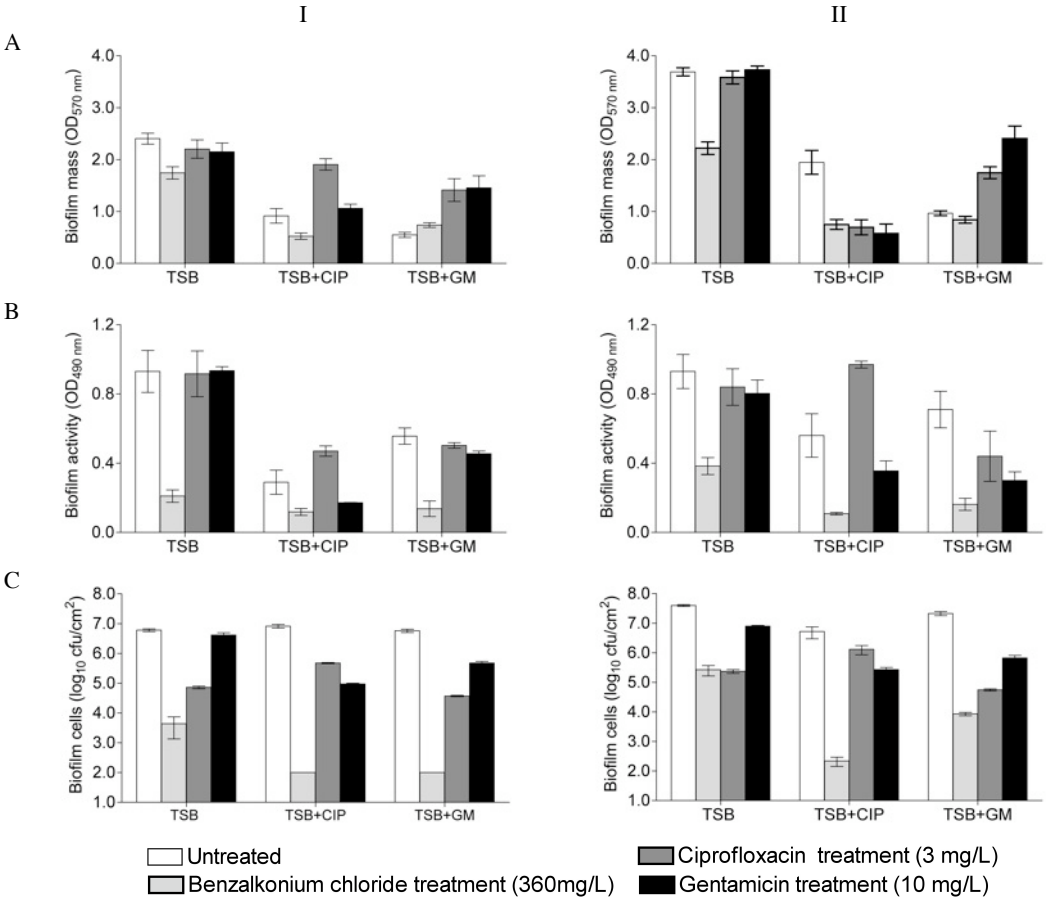

Fig.3.2-3 Biofilm mass (OD_{570}) (A), metabolic activity (OD_{490}) (B) and number of cultivable cells (C) of 96 hours biofilms of *P. aeruginosa* ATCC (I) and *P. aeruginosa* isolated strain biofilms (II). Biofilms were developed in TSB, CIP and GM therapy. Normal Biofilms (control, white) and treated with BC (light grey), CIP (dark grey), and GM (black). Bars represent the average of 3 independent repeats ± SD.

The 96-h-old biofilms formed by both the ATCC and the isolated strain under intermittent cycles of CIP or GM pressure were also clearly disturbed by the action of BC. It appears that antibiotic pressure during biofilm growth gave rise to biofilms more susceptible to the antimicrobial action of benzalkonium chloride.

The response of PA biofilms towards CIP and GM treatment depended on the antibiotic used. The PA biofilms challenged by CIP pressure (Fig.3.2-3I) are practically indifferent to the posterior aggression with the same antibiotic, as the values of biofilm mass (Fig.3.2-3I-A) and activity (Fig.3.2-3I-B) were higher than those observed in biofilms without treatment ($p<0.01$), although there was a reduction in the number of viable biofilm-cells ($p<0.001$) (Fig.3.2-3I-C). The response of these biofilms to the action of GM was somewhat different, as GM caused the reduction of biofilm metabolic activity and number of biofilm-entrapped cells.

The 96-h-old biofilms formed under GM intermittent pressure appeared to be more tolerant towards both antibiotics, as the aggression of CIP and GM for 30 min only decreased the number of viable biofilm-cells ($p<0.001$) (Fig.3.2-3I-C).

Regarding PAI biofilms (Fig.3.2-3II), those developed under CIP pressure were more sensitive to the action of GM than CIP, since only significant reductions in biofilm mass ($p<0.001$) (Fig.3.2-3II -A), respiratory activity ($p<0.001$) (Fig.3.2-3II-B), and number of viable cells ($p<0.001$) (Fig.3.2-3II-C), were observed after GM treatment. The action of CIP and GM against the 96-h-old PAI biofilms, previously grown under GM pressure, were quite similar as both antibiotics increased the biomass accumulated on the PS surfaces (Fig.3.2-3II-A)($p<0.05$) and slight decreased the biofilm activity (Fig.3.2-3II-B) ($p<0.05$) and the number of the biofilm-cells (Fig.3.2-3II-C)($p<0.001$).

Based on Fig.3.2-3, it can be stated that none of the conditions led to complete sanitation of the biofilms, being, in general, the action of the antimicrobials more effective in bacteria inactivation than in biofilm removal. Data also highlighted that cross-resistance between antibiotics and the biocide did not occur.

3.2.4 Discussion

Adhesion and biofilm formation are two important aspects of many bacterial diseases, especially those related with medical devices[34], as flexible endoscopes. When biofilms are identified as the main cause of infection, treatment becomes very difficult since bacteria within biofilms adopt special features that confer them increased resistance to antimicrobial agents[13]. This resistance usually makes sessile microorganism more difficult to kill and remove from surfaces than planktonic counterparts. Furthermore, in many cases incomplete removal of the biofilm allows it to quickly return to its equilibrium state.

In this work, some phenotypic characteristics (early-stage adhesion, biofilm formation ability and sensitivity to different antimicrobials) of *P. aeruginosa* isolated from an endoscope were inspected.

The ability to adhere of the isolated strain was superior to that of wild-type strain as j_0 and n_{30} are around two-fold higher than those observed for the reference strain. Besides revealing higher rate of adhesion (j_0), the isolated strain also showed higher number of cells adhered to the surface after 30 min of contact (Table 3.2-1). Furthermore, the strength of adhesion of this bacterium on PS surfaces was stronger as PBS circulation failed on cell detachment. In fact, for the reference strain the detachment of cells was around 20%, while for the isolated strain, no significant cell detachment was observed. Knowing that PAI was obtained from a real biofilm formed on an endoscope, it is conceivable to speculate that the isolated strain has been exposed to mechanical and chemical stress conditions, namely during endoscope reprocessing. So, as a survival strategy, this isolated strain may have acquired phenotypic and physicochemical changes that allowed it to adhere easily on PS and with superior strength. In fact, other authors[2] reported that organisms isolated from any given niche, medical, environmental, or industrial, have different mechanisms of

adhesion and retention, mainly due to changes in their structural components, such pili, fimbriae, and adhesive surface proteins that have adapted differently over time through selective pressures. Furthermore, exposure to antimicrobials may as well induce changes in cell surface hydrophobicity and surface charge that can alter bacterial adhesion properties[24].

The prominence of PAI was also visible in terms of biofilm formation ability, as it has developed biofilms with more biomass, respiratory activity and number of cells than PA (Fig.3.2-1). This feature together with the greatest capacity to adhere allow to speculate that this isolated strain is more pathogenic than ATCC strain, as bacterial attachment and biofilm formation are considered important virulence factors of bacterial pathogens[31,42]. In fact, the formation of thick biofilms gives bacteria, amongst other advantages, protection from external aggressions, as host defences and antimicrobials, due to the lypopolysaccharides that constitute the EPS matrix[8]. This EPS matrix acts also as a diffusion barrier that can reduce antimicrobial efficacy by diminishing its penetration into the deeper layers of the biofilm. Protected within this niche, bacteria can detach, proliferate and furthermore disseminate in large amounts making possible the spread of pathogens[16].

Although the isolated strain develops thicker biofilms, it must be referred that luckily the capacity of both strains to develop biofilms was greatly impaired in the presence of antibiotics. The concentration of antibiotics used to cause antimicrobial stress (3 mg/L of CIP and 10 mg/L of GM) was similar to those referred in literature[33] to have high bactericidal activity (16 x MIC or 3 x MBC), however it must be highlighted that a substantial number of viable cells remained adhered on PS surfaces. CIP is known for being initially very effective against *P. aeruginosa*. GM is also used to control *P. aeruginosa* growth and has been described in several works as a potential antibiotic to treat biofilm associated infections[20,26,28]. However, the diminished biofilm-forming capacity shown by PA and PAI under CIP and GM pressure may be related with other action than bactericidal activity. Biofilm formation by *P. aeruginosa* is hypothesized to follow a developmental pattern involving essentially four steps[18]: surface attachment, irreversible attachment, microcolony formation and differentiation into a mature population encased in a polymeric matrix. The presence of the antibiotics during biofilm formation may have interfered in the transition from reversible bindings to stable and irreversible interactions[32], affecting the transition from microcolonies to biofilms and thus delaying the mature biofilm development[15]. With the cessation of the antibiotic pressure, those less dense biofilms resumed their developmental process and gave rise to thicker biofilms, being again the PAI biofilms superior to those formed by the wild-type strain.

During endoscopy procedure, the external environment surrounding the medical device provides optimal conditions for microbial adhesion and biofilm growth[10,21]. If the disinfection procedures implemented during endoscopy reprocessing are not fully effective, biofilms may form and persist. These biofilms can later release bacterial cells that can spread to other locations, contaminating

new surfaces and infecting the patients that underwent endoscopy. A vicious circle of biofilm growth, antimicrobial treatment, partial killing or inhibition of some susceptible population and regrowth of resilient cells can thus be created. This vicious circle was clearly observed in Fig.3.2-2, when biofilms obtained after the first period of PAE were submitted to a new cycle of antibiotic pressure being observed again, a decrease in their density and activity. The number of biofilm-entrapped cells has also decreased but a substantial number of cells remained viable and adhered, mainly when GM was used. This latter event may be related with GM difficulties to diffuse across the biofilm matrix[17], limiting thus its access to the biofilm-growing cells. To augment its efficacy it has been recommended in-use concentrations of GM higher than those used for other antibiotics. Conversely, ciprofloxacin is known for its ability to penetrate rapidly[43]. Based on these data, it can be concluded that both antibiotics have great ability to disrupt established biofilms but poor capacity to completely inhibit biofilm-growing bacteria. Furthermore, when the level of antibiotics dropped, in the PAE periods, the population of adhered cells was able to multiply and to repopulate the biofilm, as observed during the second recovery period when the remaining cells recovered biofilm mass and metabolism.

This cycle of biofilm decrease/biofilm recovery may be explained by the existence, within the entire *P. aeruginosa* biofilm population, of a subpopulation of dormant cells that survive antibiotic treatment ensuring population survival. These cells that do not grow in the presence of an antibiotic, but neither do they die, are known as persister cells[23]. This subpopulation is recognized as "drug-tolerant" as they remain metabolically inactive in stressful conditions, but they can resort to normal growth rates and susceptibility in the absence of antibiotic. The data gathered in this study allows speculating that biofilm development in the presence of antibiotics can be problematic as antimicrobial pressure can select persister cells and encourage bacterial adhesion and biofilm development. This resilient sessile population, that is normally able to sustain an antimicrobial attack, can account for the prevalence of biofilm-associated infections and for recalcitrance of surface contamination[23,30].

The environmental pressure exerted by CIP and GM did not contribute to the development of *P. aeruginosa* tolerance to the same antibiotics. In fact, the 96-h-old biofilms developed only in TSB are practically tolerant to the toxic action of CIP and GM, however those biofilms formed under antibiotic selective pressure are to a certain extent susceptible to the same antibiotic attack. The sensitivity to the antibiotics of the biofilms formed by the isolated strain is similar to that displayed by the biofilms developed by the reference strain. Based on this evidence, it can be referred that the environmental stresses to which the isolated *P. aeruginosa* have been submitted during endoscope reprocessing did not caused the development of a resistant phenotype towards the antibiotics studied. It is accepted that antimicrobial selective pressure may result not only in selection of persister cells but also in the development of cross-resistance towards other

antimicrobials. In this study, data showed that BC was quite effective against *P. aeruginosa* biofilms, despite not having caused complete sanitation. All *P. aeruginosa* 96-h-old biofilms developed or not under antibiotic pressure were similarly susceptible to BC attack (Fig.3.2-3). These data highlighted that biofilm-growing bacteria subjected to CIP and GM pressure did not exhibited cross-resistance to benzalkonium chloride. The use of this cationic surfactant is not advised in endoscope washing procedures, but this product is still used in clinical practice for surface disinfection, antisepsis preservation and cleaning[37].

The exposure of cells within the biofilm to antibiotics pressure did not further promoted antimicrobial resistance to any of the antimicrobials tested (Fig.3.2-3). However, it must also be referred that none of the conditions caused complete sanitation of the biofilms, being, in general, the action of the antimicrobials more effective in bacteria inactivation than in biofilm removal. This fact is of upmost importance as cells may then detach from the remaining biofilms and disseminate infection elsewhere.

The antimicrobial treatment of bacterial biofilms may lead to eradication of most of the susceptible or metabolically active population but again, the small fraction of persister cells or bacteria in the deeper biofilm layers that are just exposed to sub-inhibitory concentrations can survive and be able to reconstitute the biofilm after discontinuation of antimicrobial therapy[8].

Since biofilms do not develop or mature in stress conditions but rather maintain a remaining adhered population and are exceptionally complex to eradicate, are considered recalcitrant[17]. The persister cells give rise to a new diverse biofilm community with high genetic variability. This "new" biofilm is not as persistent as the cells that where in its foundation, being on the contrary as sensitive to external aggressions as a biofilm developed in normal conditions[17]. The persister cell role in biofilm survival will undoubtedly drive the effort to understand the mechanisms of their remarkable recalcitrance[3].

In this study, it was showed that the isolated endoscope strain possesses the ability to adhere in higher extent than the wild-type strain, developing after, thicker biofilms. Its increased ability to adhere may be due to its previous stress exposure to cleaning agents and disinfection procedures. Moreover, biofilm development in the presence of high doses of antibiotics might lead to the eradication of the most part of the biofilm population, selecting just a small fraction of persister cells, which can survive being able to reconstitute the biofilms following discontinuation of antibiotic therapy. This may represent an increased risk of infection to patients, requiring careful surveillance. As persister cells, that survive within the biofilm after treatments, can develop new biofilms and re-colonize other accessible sites, the anti-biofilm efficacy of a cleaning agent or antibiotic should not be just related with the reduction of biofilm mass or number of cells, but its success depends largely of its ability to kill all biofilm-cells, promoting the complete biofilm eradication from surfaces.

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CHAPTER 4

PSEUDOMONAS AERUGINOSA ADAPTIVE RESISTANCE

CHAPTER 4.1

A STUDY ON THE EFFECTS OF ANTIMICROBIAL RESIDUES ON EARLY ADHESION AND BIOFILM FORMATION BY WILD-TYPE AND BC-ADAPTED STRAINS OF *PSEUDOMONAS AERUGINOSA*

Antimicrobial residue deposition can change the physicochemical properties of bacteria and surfaces and thus promote or impair bacterial adhesion. This study focuses on benzalkonium chloride (BC) deposition on polystyrene (PS) surfaces and the influence of this conditioning film in PS physicochemical properties and on early adhesion and biofilm formation of *Pseudomonas aeruginosa* wild-type and its laboratory BC-adapted strain.

BC-adapted strain easily acquired ability to grow in BC, exhibiting also physicochemical changes in surface. The existence of residues on PS surfaces altered its hydrophobicity and favoured adhesion as determined by the free energy and early adhesion characterization. Adapted bacteria revealed higher ability to adhere and to develop biofilms especially on BC-conditioned surfaces, which thereby could enhance its resistance to sanitation.

These findings highlight the importance of investigations concerning the antimicrobial deposition effect after cleaning procedures, which may encourage bacterial adhesion, especially of bacteria that have been exposed to chemical stresses.

4.1.1 INTRODUCTION

Pseudomonas aeruginosa is an important opportunistic Gram-negative human pathogen[14,16]. This environmental bacterium is capable of living planktonically or in abiotic and biotic surface-associated populations known as biofilms[14,15]. Biofilms are sessile microbial communities encased in matrices mainly composed of extracellular polymeric substances (EPS)[14] that act as a scaffold, holding biofilm-cells together[12] and protecting bacteria from external agressions[4].

Biofilm formation is a developmental process that involves essentially four main stages: i) bacterial deposition on a surface and irreversible adhesion, ii) formation of microcolonies, iii) maturation and iv) cell dispersion to the planktonic stage[9]. During the initial stage, the adhesive properties of bacterial cells cause irreversible attachment to a colonisable surface[12], this process being mediated by biological, physical and chemical factors[9]. The attachment process is usually preceded by the formation of a conditioning film on a surface as a result of interactions between the substratum and the surrounding environment[1,8]. Surface conditioning can also occur, for example, after cleaning procedures, with the unintentional deposition of biocides residues on the surfaces. This unintentional surface conditioning can occur mainly by: i) the deplored practice of "topping-up" of biocide solutions without subsequent washing with water, ii) the incorrect use of biocides and incorrect concentrations, iii) the use of a biocide that is not appropriate for a certain type of surface and iv) pre-cleaning errors, for example, the residues of several products can interfere with the activity of each other[20,23]. Once a surface has been conditioned, its properties become permanently altered, so the affinity of an organism for a native or a conditioned surface can vary greatly depending on the molecules in the conditioning film[7,18]. After adhesion, biofilm formation takes place by auto-aggregation of the attached cells and formation of microcolonies[9]. Once a biofilm is established and matured, EPS are produced[12] creating a three-dimensional structure within the biofilm community[9]. The last stage in biofilm formation involves cell detachment, which is an active dispersion of cells from the biofilm matrix[9].

Evidence suggest that adhesion to surfaces and subsequent biofilm development are linked to the persistence and virulence of *P. aeruginosa*[12], contributing to the increased resistance of this bacterium to antimicrobial agents[14,21]. Altered cell phenotypes with different sensitivities to biocides can be the result of three types of bacterial resistance: i) intrinsic (or innate) to the microorganism, ii) adapted resistance that results from the continuous exposure to certain environmental conditions, such as antimicrobial compounds, or due to epigenetic phenomena like persistence and iii) acquired due to the forced mutations or through the acquisition of mobile genetic elements[11]. *P. aeruginosa* has long been regarded as an organism that easily adapts to quaternary ammonium compounds (QACs)[10]. QACs are bacteriostatic at low concentrations and bactericidal at high concentrations. Thus, at low concentrations, this agent may favour the development of adaptive resistance. Various mechanisms have been reported to play, either singly

or synergistically, roles in bacterial adaptive responses to antimicrobials. These adaptive mechanisms can include alterations: i) in outer membrane proteins, ii) antimicrobial uptake, iii) cell surface charge and hydrophobicity, and iv) fatty acid content of the cytoplasmatic membrane[13]. However, microorganisms may have multiple, possibly interconnected, adaptive mechanisms depending on the nature of the antimicrobial agent.

The aim of this work was to study the role of surface conditioning with benzalkonium chloride (BC) on early-stage adhesion and the biofilm formation ability of two *P. aeruginosa* strains (wild-type and a laboratory BC-adapted strain). The physicochemical properties of clean and BC-conditioned polystyrene surfaces (PS) and of non-adapted and BC-adapted *P. aeruginosa* strains were also evaluated.

4.1.2 Materials and Methods

Strain and culture conditions

P. aeruginosa (ATCC 10145) and BC-adapted *P. aeruginosa* were used throughout this work

Antibacterial Agent

Benzalkonium chloride (BC) was used.

Induction of BC adaptive resistance in *P. aeruginosa*

BC adaptive resistance was laboratory induced in *P. aeruginosa* wild-type strain as described in sub-chapter 2.2.2 to further evaluate the role of this step on the physicochemical properties of the cell surface, early adhesion and biofilm forming ability.

Growth kinetics

The potential adaptive resistance of the BC-adapted *P. aeruginosa* was checked by bacterial growth in the presence of increasing concentrations of BC, as described in sub-chapter 2.2.3.

Surfaces conditioning

In order to examine the effect of BC residues in the thermodynamic prediction of adhesion, and on early bacterial adhesion and biofilm formation, several PS surfaces of different sizes, were conditioned through random deposition of BC residues, as described in sub-chapter 2.3.1.

Physicochemical characterization of surfaces and cells

In order to investigate the physicochemical properties for *P. aeruginosa* wild-type and BC-adapted cells and PS surface, the contact angles and hydrophobicity were determined for both bacteria and clean and BC-conditioned surfaces, following the van Oss *et al.* approach[27], according to the procedure described in sub-chapter 2.3.2.

Early bacterial adhesion

A parallel plate flow chamber (PPFC) and an image analysis system were employed to study early-stage bacterial adhesion and detachment, as described by Sjollema *et al*.[22] according to procedure described in sub-chapter 2.3.3.

Biofilm formation

The biofilm forming ability of wild-type and BC-adapted *P. aeruginosa* on clean and BC-conditioned surfaces was assessed using the microtiter plate test developed by Stepanovic *et al*.[24] as described in sub-chapter 2.4.1. Periodically, *i.e*., after 2, 4, 6 and 24 h of biofilm growth, the content of each well was removed and the biofilms were washed twice with 200 µL of sterilized UP water being reserved for analysis. Each experiment was performed in five independent assays.

Biofilm analyses

Biofilm mass of both *P. aeruginosa* strains was quantified by the crystal violet (CV) staining method, adapted from Stepanovic *et al*.[24]. Biofilm activity was evaluated using the 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide sodium salt (XTT) colorimetric method, as described by Stevens and Olsen[25]. Both procedures are described in detail in sub-chapter 2.4.4.2 and 2.4.4.3.

Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 4.0 software for Macintosh. Normality of data distribution was tested by the Kolmogorov-Smirnov method. Statistical significance values of the groups' means of i) contact angles, ii) biofilm mass, and iii) biofilm activity were evaluated using a one-way analysis of variance. Subsequent comparisons were performed using Tukey's *post-hoc* test. The statistical analyses performed were considered significant when $p<0.05$.

4.1.3 RESULTS

Growth kinetics

In order to assess the growth behaviour of *P. aeruginosa* wild-type and BC-adapted strains, and to establish the minimum inhibitory concentration (MIC), bacterial growth in the presence of several concentrations of BC was followed over time. Fig.4.1-1 shows that in the absence of BC both strains exhibited similarly patterns of growth. However, in the presence of BC significant differences in the bacterial growth curves were detected. Fig.4.1-1 A shows that complete growth inhibition was only detected for the wild-type strain, at a BC concentration > 1.5 mM. However,

concentrations between 0.8 mM and 1.5 mM of BC disturbed the growth pattern of *P. aeruginosa* wild-type cells. Even the lower concentration tested (0.3 mM) promoted the extension of the latency phase in comparison with growth in the absence of BC (Fig.4.1-1 A).

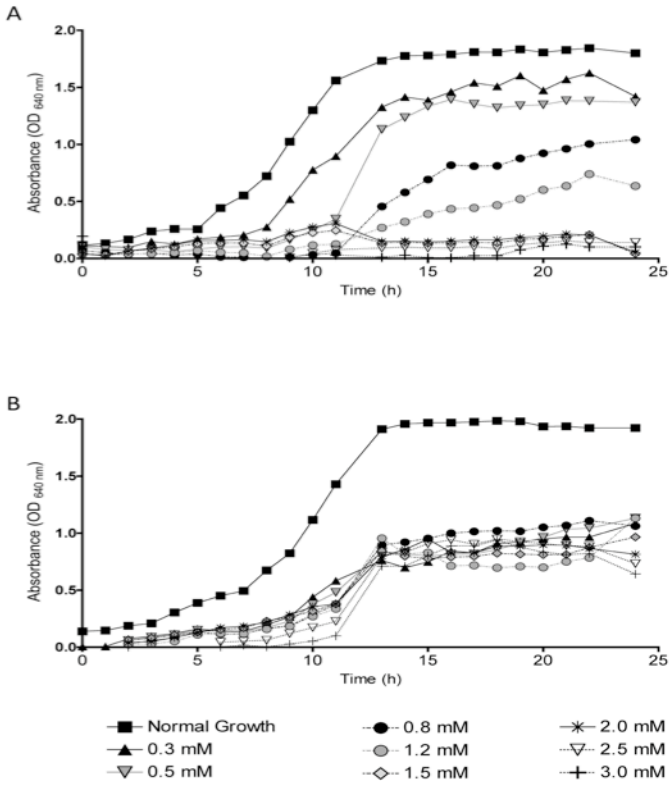

Fig.4.1-1 Growth kinetics of *P. aeruginosa* wild-type strain (A) and BC-adapted *P. aeruginosa* (B), in the absence (■) and presence of different concentrations of benzalkonium chloride (BC): (▲) 0.3 mM, (▼) 0.5 mM, (●) 0.8 mM, (●) 1.2 mM, (◆) 1.5 mM, (∗) 2.0 mM, (▽) 2.5 mM, (+) 3.0 mM.

The BC-adapted strain exhibited growth patterns in the presence of BC significantly different from those of *P. aeruginosa* wild-type (Fig.4.1-1 B). The adapted strain was able to survive and grow in the presence of all the concentrations tested, although OD_{640} values were lower than the observed in the absence of BC. However, an increase in the lag phase was observed for all the BC concentrations tested.

These data indicated that the laboratory adaption process of *P. aeruginosa* to BC gave rise to a strain with significant physiological changes, mainly in response to antimicrobial stress induced by BC.

Prediction of adhesion

The evaluation of the hydrophobicity parameters for the wild-type and BC-adapted strains and the clean and BC-conditioned surfaces are shown in Table 4.1-1.

Table 4.1-1 Values of contact angles (°) with water (θ_W), formamide (θ_F), α-bromonaphtalene (θ_B), surface tension parameters (mJ/m^2), and free energy of interaction (ΔG_{iwi}^{TOT}) (mJ/m^2), of the bacteria and the surface (i), when immersed in water (w). Values are means ± SD.

	Contact angle (°)			Surface tension parameters (mJ/m^2)			Free energy of interaction (mJ/m^2)
	θ_W	θ_F	θ_B	γ_i^{AB}	γ_i^+	γ_i^-	ΔG_{iwi}^{TOT}
P. aeruginosa wild-type	29.0 ± 5.3	26.3 ± 2.1	78.0 ± 3.0	16.2	10.0	44.1	11.2
P. aeruginosa adapted strain	30.7 ± 4.0	30.9 ± 2.6	58.4 ± 4.8	25.8	3.6	46.6	22.1
Clean surface	90.9 ± 4.0	73.3 ± 3.5	26.4 ± 1.0	40.0	0.7	4.4	-55.2
BC-conditioned surface	103.0±14.3	69.9 ± 9.5	29.5 ± 8.6	40.0	0.2	0.1	-94.2

The results indicate that the contact angle formed by the cell layer and water (θ_w) was similar for both strains ($p>0.05$) and lower than 65 °. These data mean that the *P. aeruginosa* wild-type and its adapted strain can be classified, from a thermodynamic point of view, as hydrophilic. Concerning the free energy of interaction of both strains (ΔG_{iwi}^{TOT}), calculated through Equation (1), an increase of this parameter for the BC-adapted strain was observed (Table 4.1-1).

PS surfaces can be considered hydrophobic since the water contact angle values (θ_w) of the material were higher than 65 °. When surfaces were conditioned with BC residues, their thermodynamic properties were altered. This is clearly shown in terms of the free energy of interaction (ΔG_{iwi}^{TOT}) since the difference between clean and BC-conditioned surfaces is about 40 mJ/m^2 (Table 4.1-1). This means that, theoretically, the affinity of an organism for the BC-conditioned surface is superior when compared to the clean surface.

The physicochemical parameters of each adhesion entity (bacteria and surface) allowed the determination of the thermodynamic relation between both entities, namely the free energy of adhesion (Table 4.1-2).

Table 4.1-2 Free energy of adhesion (ΔG_{bsb}^{TOT}), between the bacteria (b) and the surfaces (s)

	Free energy of adhesion (mJ/m^2) (ΔG_{bsb}^{TOT})	
	Clean Surface	BC-Conditioned Surface
P. aeruginosa wild-type	4.4	-4.2
P. aeruginosa adapted strain	-5.0	-15.1

These data (Table 4.1-2) suggest that, when the surfaces are not conditioned with BC, the initial adhesion is favoured for the BC-adapted strain *vs* wild-type, as indicated by the more negative value of free energy of interaction. Table 4.1-2 also highlights that the conditioning of the surfaces with BC residues thermodynamically favoured the initial adhesion of both strains, since reductions in the free energy of interaction from 4.0 to -4.2 mJ/m^2, for the wild-type strain, and from -5 to -15 mJ/m^2, for the BC-adapted strain, were observed.

Early stage bacterial adhesion

The PPFC assays revealed that the initial adhesion patterns differ according to the strain and the conditioning of the surface (Fig.4.1-2). The early adhesion profile of the BC-adapted *P. aeruginosa* revealed that this strain had a slower start in its adhesion to PS (up to around 350 s), which was followed by a period of higher cellular adhesion in comparison with the wild-type strain. After the adhesion period (1800 s), the number of adapted-cells adhered to clean and BC-conditioned PS was higher than for the wild-type strain ($p<0.05$).

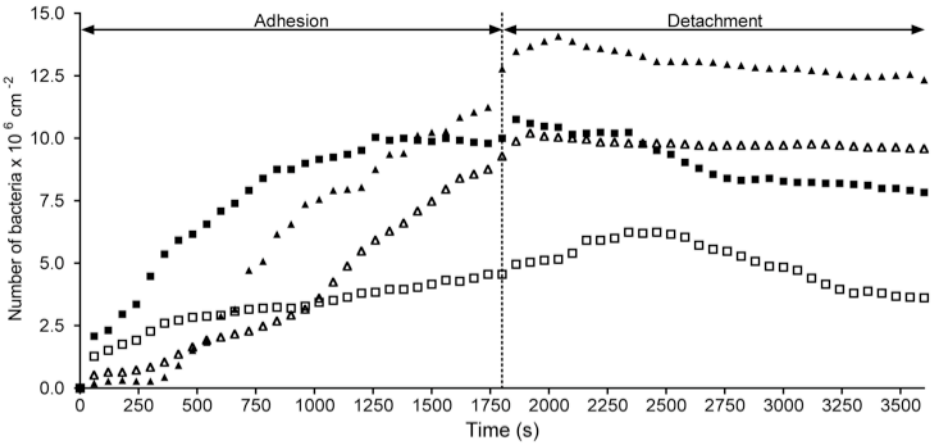

Fig.4.1-2 Adhesion profiles of *P. aeruginosa* wild-type (□■) BC-adapted strain (△▲) on clean (open symbols) and BC-conditioned (filled symbols) surfaces.

Concerning the influence of surface conditioning in this assay, the BC deposition on the PS surface enhanced bacterial adhesion, for both strains tested (Fig.4.1-2). The number of adhered bacteria was higher on the conditioned surfaces, this being more evident for the wild-type strain.

The detachment stage (circulation of PBS between 1800 s and 3600 s) (Fig.4.1-2), revealed that the BC-adapted strain was more able to establish a firm adhesion on the surface, since the number of adapted-cells adhered to PS was higher than the number of wild-type adhered bacteria, irrespective of which surface was used.

Concerning the influence of the surface conditioning in this assay, the BC residue deposition on the PS surface enhanced bacterial adhesion, for both strains tested (Fig.4.1-2). In fact, the number of adhered bacteria was higher in the conditioned surface, being this more evident for the wild-type strain.

Biofilm formation

The evolution of the biomass and activity of biofilms formed by the wild-type and BC-adapted *P. aeruginosa* over time on clean and BC-conditioned surfaces are shown in Fig.4.1-3

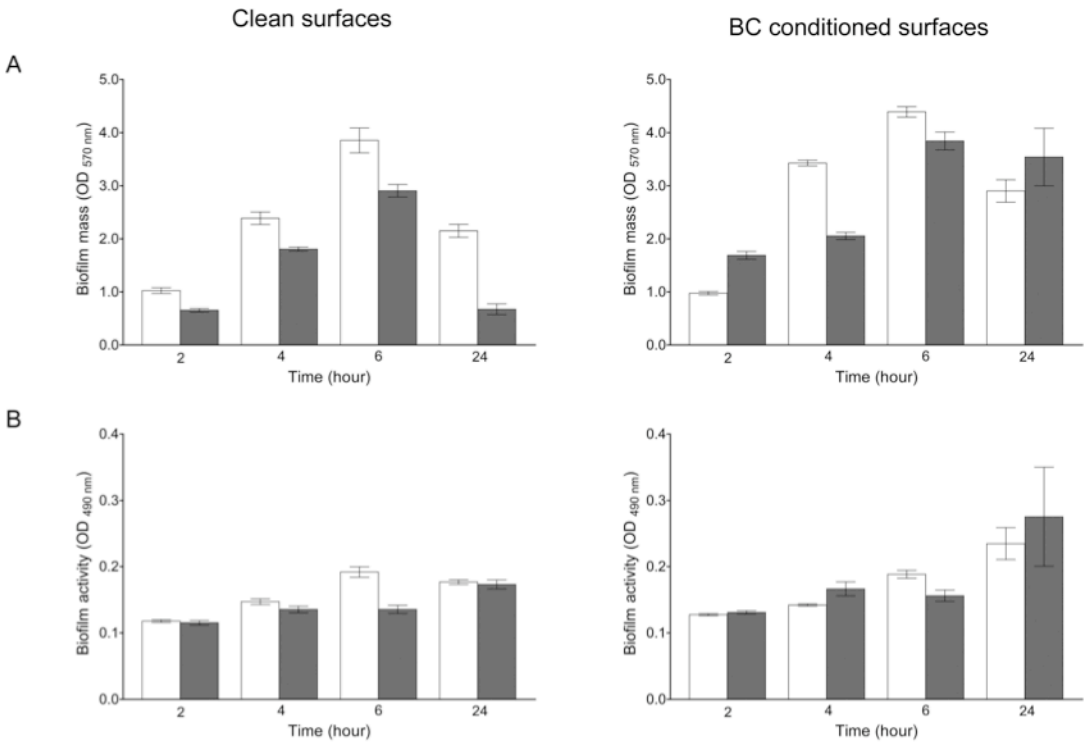

Fig.4.1-3 Biofilm mass (OD_{570}) (A), and metabolic activity (OD_{490}) (B) of biofilms developed for 2, 4, 6 and 24 h, in clean and BC-conditioned surfaces by *P. aeruginosa* wild-type (white), and BC-adapted strain (grey). Bars are mean (± SD) of eight replicates obtained on five independent experiments.

Fig.4.1-3 A shows that, in general, the amount of biofilm mass accumulated on the surfaces increases until the sixth hour of biofilm formation. On clean surfaces, the wild-type strain developed thicker biofilms than those formed by the BC-adapted strain. Concerning the influence of BC residues deposition on biofilm development, higher biomass accumulation was observed for biofilms developed by both strains on BC-conditioned surfaces when compared with biofilms developed on clean surfaces ($p<0.01$).

Data related with biofilm activity (Fig.4.1-3B) show that, for both surfaces, no significant differences were observed between strains ($p>0.05$). Regarding the effect of BC residues on biofilm activity, the presence of the conditioning film, only influenced biofilm formation at 24 h. At this time point, biofilms developed on the conditioned surfaces revealed higher values of biofilm activity than biofilms developed on the clean surfaces ($p<0.01$).

4.1. 4 Discussion

Antimicrobial residues deposition can alter surface physicochemical properties and thus interfere with bacterial adhesion. In this work, PS surfaces were conditioned with a BC solution and the changes on the surface physicochemical properties, as well as their implication in adhesion ability and biofilm formation by *P. aeruginosa* wild-type and BC-adapted strain were investigated.

Moreover, the surface hydrophobicity of *P. aeruginosa* wild-type and its laboratory-adapted strain was also assessed.

The differences observed in the growth curves of the BC-adapted strain indicated that this strain acquired traits of adaptive resistance, since none of the BC concentrations tested were able to prevent bacterial growth (Fig.4.1-1 B). The continuous exposure of *P. aeruginosa* to BC gave the strain the ability to persist and grow at high BC concentrations. Table 4.1-1 shows that BC-adaption induced changes in bacterial surface hydrophobicity and that the deposition of BC residues modified the PS surfaces giving rise to surfaces more prone to promoting adhesion. The analysis of the free energy of adhesion between strains and surfaces (Table 4.1-2) suggested that, in theory, adhesion of both strains was favoured on conditioned surfaces, the strength of adhesion being higher for the BC-adapted strain when compared with the wild-type. These data reinforce the conclusion that the thermodynamic properties play a key role in initial bacterial adhesion to surfaces, as indicated by previous work[3]. Based on these results, it can be inferred that adhesion depends most on the properties of the conditioning film rather than on the surface itself[1,19].

Concerning early adhesion, deposition of BC residues on the adhesion surfaces promoted an increase in the number of adhered bacteria (Fig.4.1-2). Similar features were also noticed by other authors that observed a higher number of adhered bacteria when surfaces were conditioned with blood[18], gold[2] and biosurfactants[17]. Another study[26] reported that BC-impregnated catheters exhibited reduced microbial colonization by several microorganisms, suggesting that the BC conditioning film may prevent colonization. In the present study, the experimental conditions used to promote the surface conditioning (t=30 min and 0.9 mM of BC) were clearly not enough to change its properties so they become less prone to bacterial adhesion. Therefore, *P. aeruginosa* adhesion on the modified surfaces could not be impaired, especially when the bacterium was previously adapted to BC. It is thought that BC acts on the properties of cell wall as well as on the outer membrane of Gram-negative bacteria. According to Gilbert and Moore[6], BC molecules are positively charged and bind strongly to bacterial surface, changing their native negative charge. In this study, as bacteria grew during the step-wise adaptation process in the presence of increasing BC concentrations, changes in the cell wall or of the outer membrane of *P. aeruginosa* cells could have occurred and may explain the alteration in the free energy of interaction of the BC-adapted bacteria (Table 4.1-1) and, subsequently, the free energy of adhesion to the PS surfaces (Table 4.1-2).

The formation of a conditioning film and the adhesion of bacteria to a surface are the first two steps of the biofilm developmental cycle[5]. To seek the role of these events in the sequential steps of biofilm formation by *P. aeruginosa* wild-type and its adapted strain, in this study, biofilms were developed for different time periods (2, 4, 6 and 24 h) (Fig.4.1-3). For all the periods of biofilm development and with the preconditioning conditions tested, the presence of residues on the

surfaces favoured biofilm phenotype since for both strains it promoted biofilm mass accumulation (Fig.4.1-3 A). Other authors[19], when studying the ability of *P. fluorescens* to adhere to silicone rubber, also observed an increase in biomass accumulation on BC conditioned surfaces. The preconditioning of the silicone surfaces was also carried out for 30 min[19]. In the same study, the biofilm biomass increased gradually with the increase in the BC concentration used in the preconditioning, 0.5 mM being the highest concentration tested.

The *P. aeruginosa* adapted strain was shown to have more ability to adhere regardless of whether the surfaces were preconditioned or not. The adsorption of BC residues on PS surfaces seems to favour biomass accumulation by this strain when compared with biofilms formed by the wild-type strain (Fig.4.1-3). The study of the BC-adapted strain was performed in order to highlight the fact that special care should be taken with antimicrobial and disinfection experiments that use only reference strains. Bacteria, in real situations, are permanently exposed to external factors and thus, may exhibit different phenotypic traits that can alter their behaviour and response to the action of antimicrobials.

This study gives new insights into the significance of the conditioning film, not only in early adhesion events, but also in the development of biofilms, even when the effect of the antimicrobial was thought to be masked by the surrounding environmental conditions.

In conclusion, the results show that deposition of BC residues can support changes in the thermodynamic properties of surfaces and enhance bacterial adhesion and even favour biofilm formation, mainly by the BC-adapted bacteria. These features may explain the increased resistance and virulence often observed in *P. aeruginosa* strains in real-life scenarios. Therefore, in order to avoid bacterial outbreaks and disinfection practice failures, special care should be taken with the design of effective sanitation programs and antimicrobial residues accumulation should be avoided.

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CHAPTER 4.2

ADAPTIVE RESPONSE OF SINGLE AND BINARY *PSEUDOMONAS AERUGINOSA* AND *ESCHERICHIA COLI* BIOFILMS TO BENZALKONIUM CHLORIDE

The main goal of this work was to examine whether the continuous exposure of single and binary *Pseudomonas aeruginosa* and *Escherichia coli* biofilms to sub-lethal benzalkonium chloride (BC) doses can induce adaptive response of bacteria.

Biofilms were formed during 24 h and then put continuously in contact with BC for more five days. The six-day-old adapted biofilms were then submitted to BC challenge, characterized and inspected by Scanning Electron Microscopy (SEM).

Both single and binary adapted biofilms have clearly more biomass, polysaccharides and proteins and less activity even though the number of cells was identical. After BC treatment, adapted biofilms maintained their mass and activity. SEM examination revealed that those adapted biofilms had a slimier and denser matrix that became thicker after BC treatment.

Continuous exposure of bacteria to antimicrobials can lead to development of biofilms encompassing more virulent and tolerant bacteria. This adaptive resistance can be the result of a phenotypic adaptation, a genetic acquired resistance or both.

Instead of eradicating biofilms and kill microorganisms, the use of a disinfectant can favour biofilm formation and tolerance. This must be a genuine concern as it can happen in clinical environments, where the use of antimicrobials is unavoidable.

4.2.1 INTRODUCTION

Pseudomonas aeruginosa and *Escherichia coli* are two of the most important human associated pathogens and commensal bacteria[24] that can contaminate inanimate surfaces during months, specially when attached and developing biofilms[10]. Both bacteria show an increase in pathogenicity due to their motility structures (type IV pili in *P. aeruginosa* and curli in *E. coli*) and matrix production through extracellular polymeric substances (EPS) secretion. Previous works[11,12,16,33] showed that EPS synthesis (alginate for *P. aeruginosa* and colanic acid in *E. coli*) is induced upon attachment of the bacteria to a surface.

Usually, the procedures for surface cleaning involve the use of detergents followed by application of disinfectants that reduce the viability of microorganisms[49]. Antimicrobials, as quaternary ammonium compounds (QAC), have been continuously used in hospitals and healthcare facilities, and have significantly contributed to maintain sanitary conditions and to prevent hospital-acquired infections. Despite their valuable properties, concerns have been raised about the widespread and irrational use of disinfectants that could fail the eradication of the biofilm-associated microorganisms and serve to select disinfectant-insusceptible microbes among hospital-acquired pathogens[8,22,34,41].

Physiologic or phenotypic adaptation resulting in "antimicrobial tolerance" has been attributed to biofilms[17,42]. Biofilm tolerance to disinfectants may result from (i) slow microbial growth rates that are attributable to nutrient depletion within biofilms, (ii) binding of the biocide to the EPS, (iii) neutralization or degradation of the biocide, as well as (iv) the expression of biofilm-specific phenotypes[10,42,47]. Evidence suggest that proteins involved in oxidative stress response, cell envelope synthesis, and EPS synthesis are up-regulated in biofilms, indicating that these altered phenotypes might contribute to cell survival, persistence, and growth in a biofilm community[32,39].

Moreover, biofilms usually comprise more than one microbial species, and can harbour strains with less ability to develop a biofilm, contributing to their survival and persistence. It has been reported that two bacteria present in an *in vitro* binary biofilm may live synergistically and enhance each other survival to antimicrobial treatments compared with the corresponding single species biofilms[28]. An important aspect of the study of the interactions in multispecies biofilms is to evaluate whether individual species, or the bacterial consortium, gain any fitness advantages compared to single-species biofilms. A fitness advantage in this context is defined as the ability of the organism or biofilm to persist or grow in a given environment or under a particular environmental stress. Multiple species biofilms may be thicker and more stable than the single ones, which may influence their susceptibility to disinfectants[6].

The aim of this work was to examine whether exposure of *P. aeruginosa* and *E. coli* to benzalkonium chloride (BC) during single and binary biofilm formation, could induce an adaptive response in bacteria by evaluating the biofilms behaviour after treatment with the same agent. BC

is a well-known QAC that has been widely used as surface disinfectant, antiseptic and preservative in medical arenas. Considering the intense use of BC as an antimicrobial agent, it is important to increase the knowledge about the effects of external chemical pressure and polimicrobial growth on the susceptibility of the sessile target organisms to this QAC.

4.2.2 Materials and Methods

Strain and culture conditions

Pseudomonas aeruginosa ATCC 10145 and *Escherichia coli* K12 substrain MG 1655 were used.

Antibacterial Agent

Benzalkonium Chloride (BC) was used throughout this work.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The BC MIC and MBC of *P. aeruginosa* and *E. coli* were determined by the microdilution method according to Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards)[35], as described in sub-chapter 2.2.1.

Biofilm development and adaptation

The methodology used to grow the bacterial biofilms was based on the microtiter plate test developed by Stepanovic *et al*.[45] as described in sub-chapter 2.4.1.3.

After 24 h of biofilm growth, the supernatant containing planktonic cells and medium was removed. The wells were re-filed with fresh TSB or TSB containing BC in a final concentration of 0.9 mM to mimic external BC chemical pressure in order to induce the adaptation of the biofilm-embedded cells. This process of supernatant removal and media filling was repeated for five subsequent days, every 24 h. The six-day-old biofilms formed in TSB supplemented with BC are hereafter referred as adapted biofilms while the biofilms formed only in TSB will be referred as normal biofilms.

Biofilm treatment with BC

After being developed in the absence and presence of BC for six days (during the adaptation process), biofilms were subjected subsequently to sudden attack with the same antimicrobial product. For that, the liquid content of each well was removed and washed once with 200 µL of UP sterilized water, being the well-attached biofilms (biofilms formed by *P. aeruginosa* and *E. coli* in the inner surfaces of each well of the microtiter plates) subsequently treated with 200 µL of 1.0 mM of BC for 30 min. This procedure had the major purpose of eliminating more susceptible and outward biofilm-cells. Non-treated wells were filled with 200 µL of UP sterilized water for the same period of time. After that, the content of each well was removed and biofilms were washed twice with 200 µL with UP sterilized water and reserved for subsequent analysis (see below).

Bacterial suspensions of the single and binary *P. aeruginosa* and *E. coli* non- and adapted biofilms to be used in protein and polysaccharide quantification and cfu determination were prepared as described hereafter. Two-hundred microliters of UP sterilized water were added to each well, being the wells-attached biofilms removed by ultrasonic bath in a Sonicor SC-52 (Sonicor Instruments, Copaique, NY, USA) operating at 50 kHz, during 6 min (these parameters were previously optimized in order to promote the complete removal of all the biofilm-attached cells without lysis). Afterwards, the single and mixed bacterial suspensions of each five wells per condition were collected, gently vortexed for 2 min[3,23,50] to disrupt possible cell aggregates, and reserved for later analysis.

Biofilm analyses

Biofilm mass determination

Biomass of *P. aeruginosa, E. coli* and binary biofilms were quantified by crystal violet (CV) staining method adapted from Stepanovic *et al*.[45] according to the procedure described in sub-chapter 2.4.4.2.

Biofilm activity determination

Biofilm activity determination was evaluated with 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide sodium salt (XTT) colorimetric method as described by Stevens and Olsen[46], as described in 2.4.4.3.

Biofilm cell enumeration

Untreated and BC treated axenic and binary biofilm suspensions removed by sonication were serially diluted and the cfu determined according to the procedure described in sub-chapter 2.4.4.4.

Proteins and polysaccharide quantification

The total content of proteins and the total polysaccharides content of the single and mixed suspensions from normal and adapted biofilms, untreated and treated with BC, were determined according to the procedure described in sub-chapter 2.4.4.5.

Scanning electron microscopy (SEM) observations

The wells-attached washed biofilms were observed through SEM according to the procedure described in sub-chapter 2.4.4.1.

Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 4.0 software for Macintosh. Normality of data distribution was tested by the Kolmogorov-Smirnov method. Statistical significance values of the groups' means of biofilm mass, biofilm activity, cell number, protein and polysaccharide content were evaluated using a one-way analysis of variance. Subsequent comparisons were performed using Tukey's *post-hoc* test. The statistical analyses performed were considered significant when $p < 0.05$.

4.2.3 RESULTS

Determination of MIC and MBC

The MIC and MBC of planktonic *P. aeruginosa* and *E. coli* are shown in Table 4.2-1. *P. aeruginosa* revealed the highest MIC and MBC values when compared with those obtained for *E. coli*. To promote a selective BC pressure on the growth of both strains, a BC concentration of 0.9 mM was chosen for the biofilm adaptation process.

Table 4.2-1 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of BC against *P. aeruginosa* and *E. coli*

	MIC (mM)	MBC (mM)
P. aeruginosa	0.45	0.90
E. coli	0.23	0.23

Biofilm analyses

Biofilm mass

In order to determine the biofilm formation ability of *P. aeruginosa*, *E. coli* and its binary combination, in the absence (normal biofilms) and in the presence of 0.9 mM of BC (adapted biofilms), the total biomass was determined. Fig.4.2-1 A shows that the six-day-old biofilms formed by single species and by the combination of both species in TSB had similar biomass values. The posterior treatment with 1.0 mM of BC did not alter the biofilm mass accumulated on the surfaces ($p>0.05$).

It was also observed that both single and binary six-day-old adapted biofilms have clearly more biomass ($p<0.001$), about five times more, than the normal biofilms formed in the absence of BC. Fig.4.2-1 A also reveals that *E. coli* adapted biofilms exhibited more mass than those of *P. aeruginosa* ($p<0.01$) and than binary biofilms ($p<0.05$). Furthermore, posterior BC treatment seems to reduce the mass of *P. aeruginosa* adapted biofilms ($p<0.05$) and to increase the biomass of the binary biofilms ($p<0.001$).

Biofilm activity

The metabolic activity of the viable biofilm-entrapped cells is presented in Fig.4.2-1 B. *P. aeruginosa* cells of normal biofilms revealed an increased activity in comparison with that of adapted biofilm-entrapped bacteria ($p<0.001$). Data also showed that both single and binary adapted biofilms have less activity than those formed in the absence of BC ($p<0.01$). Among the adapted biofilms, binary biofilms presented the highest cell activity and *P. aeruginosa* biofilms the lowest ($p<0.001$). Concerning biofilm treatment with BC, the activity of the biofilm-associated cells was significantly reduced ($p<0.001$) in normal biofilms. Furthermore the six-days old *P. aeruginosa* biofilms developed in the presence of BC, when challenged with the same product,

revealed even more activity than the biofilms without treatment ($p<0.001$). Adapted binary biofilms and *E. coli* biofilms did not exhibit major changes in their activity upon QAC treatment ($p>0.05$).

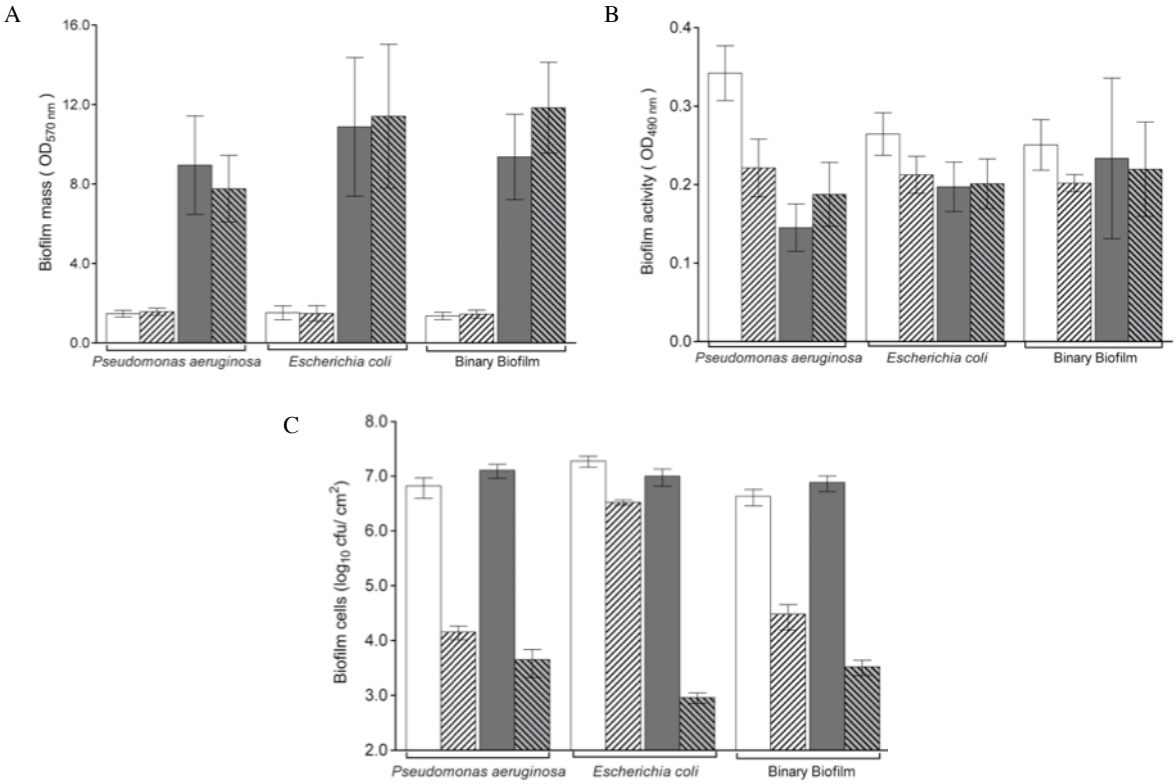

Fig.4.2-1 Biofilm mass (OD_{570}) (A), metabolic activity (OD_{490}) (B) and number of cultivable cells (C) of single and binary six-day-old *P. aeruginosa* and *E. coli* biofilms developed in TSB (white bars) and TSB supplemented with 0.9 mM of BC (grey bars). Biofilms were after treated with 1.0 mM BC (striped bars), non-treated biofilms (solid bars). Bars represent the average of 3 independent repeats ± SD.

Biofilm-entrapped cells

Concerning the number of biofilm-associated cells, after six days of biofilm growth (Fig.4.2-1 C), it was observed that the adaptation to BC, both for single and binary biofilms, seemed not to have influence on the number of total cells. In fact, the total number of cells entrapped in the biofilms was similar (approximately 10^7 cfu/cm^2), whether adapted or not to BC. Furthermore, BC treatment of single *P. aeruginosa* and binary normal biofilms led to a 3 log reduction ($p<0.001$) in cell number whereas *E. coli* biofilm-entrapped cells just suffered a reduction of about 1 log.

The effect of BC treatment on adapted biofilms was similar to the one observed in the normal biofilms as it also led to a decrease of about 3 log ($p<0.001$) in the number of biofilm-entrapped cells.

Biochemical biofilm characterization

As the data showed that adaptation to BC seems to promote an increase in biomass values (Fig.4.2-1 A), it was considered pertinent to inspect the biochemical features of all biofilms.

According to Fig.4.2-2, *P. aeruginosa* normal biofilms exhibit more protein content when compared to the other biofilms (*E. coli* and binary) ($p<0.001$). However, when the biofilms were adapted to BC, *E. coli* biofilms were those that revealed the highest protein content ($p<0.001$). The presence of BC during biofilm development led to biofilms characterized by 4-fold higher protein content, for *P. aeruginosa* and binary biofilms, and 8-fold higher for *E. coli* biofilms.

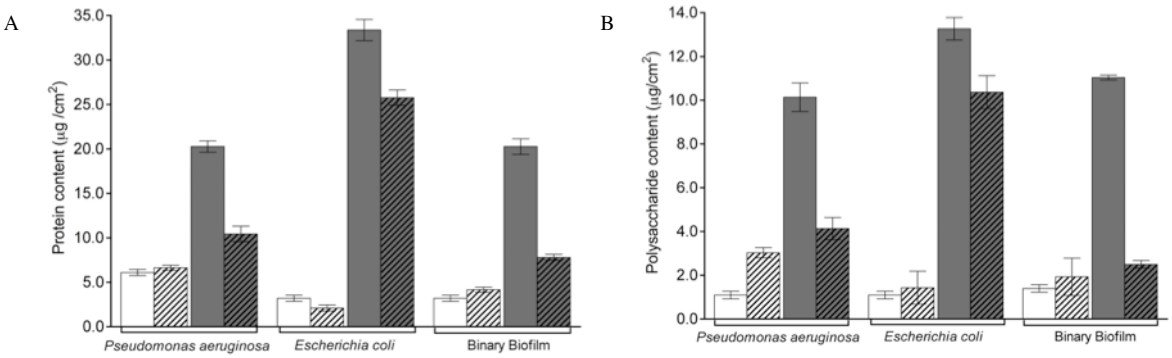

Fig.4.2-2 Biochemical biofilm characterization. Values of protein (A) and polysaccharide (B) content for of single and binary six-day-old *P. aeruginosa* and *E. coli* biofilms developed in TSB (white bars) and TSB supplemented with 0.9 mM of BC (grey bars). Biofilms were after treated with 1.0 mM BC (striped bars), non-treated biofilms (solid bars). Bars represent the average of 3 independent repeats ± SD.

After BC challenge, normal biofilms did not show great variability in their protein content (Fig.4.2-2 A). Concerning BC-adapted biofilms, the treatment with 1.0 mM of BC promoted a reduction of about 50% of the protein content for *P. aeruginosa* and binary biofilms while for *E. coli* biofilms this reduction was just about 20 %.

The amount of polysaccharides in normal biofilms was similar for all the strains (Fig.4.2-2 B). The presence of BC during biofilm development led to a significant increase of polysaccharide content, being about 10 times superior in *P. aeruginosa* and binary biofilms, and about 13 times superior in the case of *E. coli*.

The BC treatment promoted an augment in polysaccharide content of *P. aeruginosa* biofilms ($p<0.01$) while no significant change was observed for *E. coli* and binary biofilms ($p>0.05$), when developed only in TSB. The BC challenge after biofilm adaptation to the same product promoted a significant decrease in the polysaccharide content, similar to that obtained for protein content.

SEM observations

The influence of BC, on biofilm superficial structure and morphology was assessed by SEM inspection (Fig.4.2-3). SEM observations revealed a morphological alteration induced by the

presence of BC during biofilm formation. The images of the BC-adapted biofilms surface seem to indicate a larger amount of EPS, especially for *P. aeruginosa* and *E. coli* single-species biofilm.

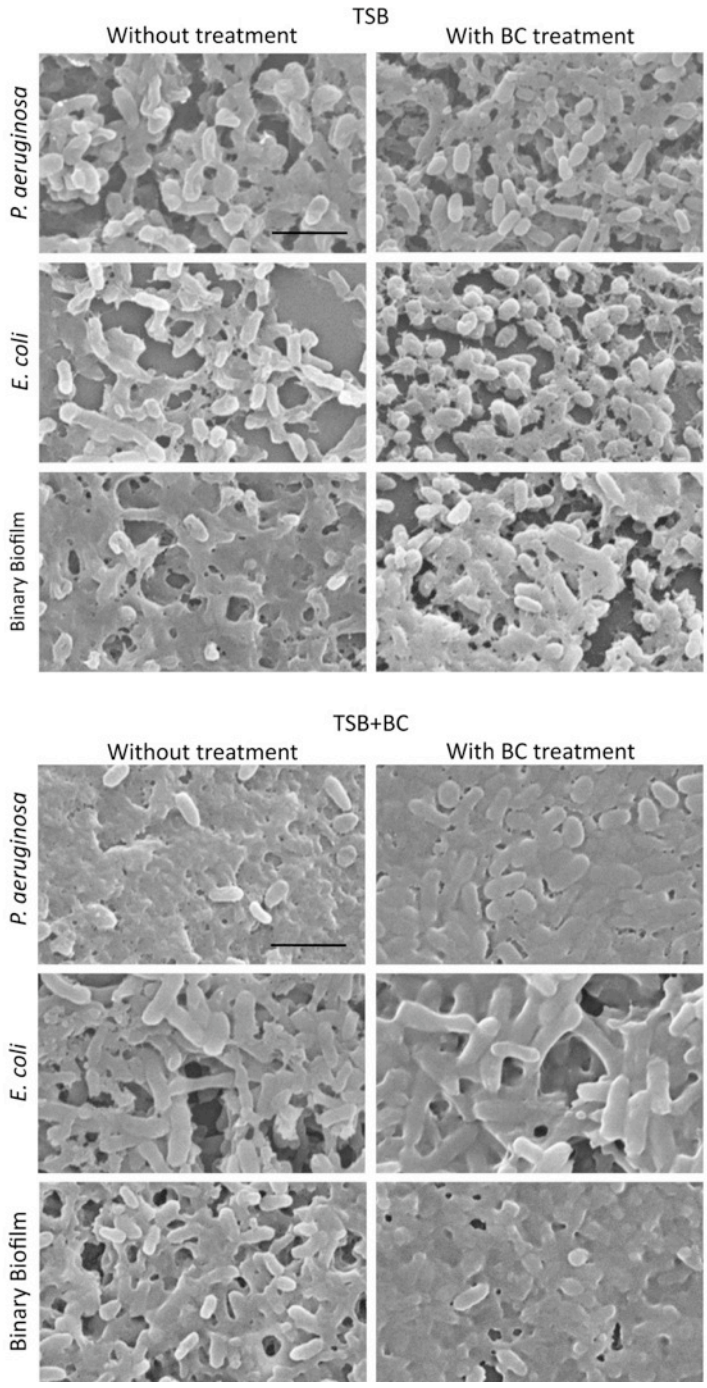

Fig.4.2-3 SEM analysis of single and binary six-day-old *P. aeruginosa* and *E. coli* biofilms developed in TSB and TSB supplemented with 0.9 mM of BC (TSB + BC) without and with 1.0 mM BC treatment. X 10 000 magnification, bar=2.0 µm.

The BC treatment after biofilm formation has different effects depending on the biofilms being adapted to BC or not. For biofilms formed in TSB, BC appears to cause some damages in the EPS matrix protecting biofilms. This matrix disruption is well noticed in *E. coli* and binary biofilms.

For BC-adapted biofilms, the images suggest that the subsequent BC challenge strengthens the existing matrix, giving the biofilm-entrapped cells an interconnected structure.

4.2.4 Discussion

P. aeruginosa and *E. coli* are two human pathogens and commensal organisms often related with foreign body infections, mainly due to their ability to form biofilms. It is widely held that a biofilm represents a highly selective micro-niche where pathogens may also have high mutation rates or easily exchange genetic material, thus facilitating the development of antimicrobial drug resistance[36].

The continuous and, sometimes, inappropriate use of antimicrobials in several settings (households, hospitals and other institutions), together with the increased use of antibiotics to cure everyday diseases has been on the basis of selection of resistant bacteria. Moreover, the well-known biofilm microniche properties and their intrinsic resistance to antimicrobial products due to their specific features, namely, EPS matrix protective barrier, nutrient and oxygen gradients, cell-cell signalling, persister cells and genetic diversity, play a key-role in this easily acquired resistance. The planktonic bacterial adaptation to BC is a well documented subject[1,4,21,29] and, recently, there have been some studies reporting biofilm adaptation to BC sub-MIC[7,31,32,40,43]. From the analyses of the latter studies, it was observed that the BC concentrations used for biofilm adaptation are not always the same, albeit being always lower than the MIC concentration determined. The adaptation method used to induce an adaptive tolerance of the biofilm-entrapped cells is not standardized, varying among authors, microorganisms and studies.

In the present work, the adaptation of the *P. aeruginosa* and *E. coli* bacteria embedded in sessile life-style was attained at a BC concentration of 0.9 mM. It was not our objective to characterize which phenomenon (recalcitrance or resistance) was involved, but to study the biofilm phenotype after exposure to chemical stress conditions. The BC concentration used is higher than the ones used to adapt *Salmonela* (0.29 mM)[31,32] within biofilms and *Listeria* planktonic cells (0.012 mM)[40]. Despite being the highest MBC determined for the most resistant strain (*P. aeruginosa*), the concentration of 0.9 mM was used to promote the BC pressure. Results showed that the presence of this BC concentration in the liquid environment surrounding biofilms did not impair their growth or caused cell death within the biofilms, since no reduction of the absorbance related with biomass (Fig.4.2-2 A) or decrease in cell number (Fig.4.2-2 C) was observed. These data are not surprising since it is widely accepted that cells entrapped in biofilms easily gain tolerance to antimicrobials. Normally, this insusceptibility is overcome with the use of supra-minimum inhibitory concentrations (supra-MIC) to ensure sanitation. The unexpected result of this study is the significant increase of biofilm mass due to BC pressure (5-fold higher) regardless the strain or the number of strains that generate the biofilm (Fig.4.2-1 A). These results show how the inappropriate use of an antimicrobial can favour bacteria attachment and biofilm formation,

instead of eradicating biofilms and kill microorganisms. In fact, in real situations, this can be a genuine concern as it can happen in clinical settings or apparatus, where the use of antimicrobials is unavoidable, as reported by Lee *et al*.[26] in liquid reservoirs where antimicrobials are kept.

The BC-adapted biofilms showed an increase in biofilm mass (Fig.4.2-1 A) that was corroborated by its biochemical characterization (Fig.4.2-2) since the biofilms formed in TSB supplemented with BC revealed a protein and polysaccharide content higher than the normal biofilms. Since extracellular polysaccharides and proteins are two of the major matrix components[19,30], our data suggest that BC-adapted biofilms present a well-defined and strong EPS matrix.

SEM pictures (Fig.4.2-3) confirmed this feature as it was well noticed a denser outward EPS matrix evolving bacteria, being this especially evident in *P. aeruginosa* and *E. coli* single biofilms. Based on these characteristics, it can be speculated that the presence of BC during biofilm formation may give rise to adapted biofilms with a stronger and cohesive structural conformation. EPS are a complex and extremely important component of biofilms, providing architectural structure and mechanical stability to the attached bacteria[2]. From the point of view of disinfection, this feature can represent an additional nuisance since biofilm matrix acts as shield to external stresses, limiting, for instance, the diffusion of antimicrobials. According to Branda *et al*.[5], small changes in the environmental conditions may promote dramatic changes in biofilm architectures, being the environment where biofilms are developed responsible for the matrix composition. So, it can be concluded that the presence of BC in the liquid interface surrounding the single and binary biofilms during their establishment can be the environmental factor that changes biofilm surface structure, making the biofilm-associated bacteria possibly more tolerant to antimicrobials.

In attempting to expand the understanding of biofilm cell physiology, the response of *P. aeruginosa* and *E. coli* entrapped in single and binary biofilms to BC treatment was examined. The BC concentration used for the subsequent attack of the established six-days-old biofilms was similar to that used for biofilm adaptation due to the fact 0.9 mM is already a high concentration when compared with the normally in-use BC concentration in cleaning products [1.0% (w/v)][25].

Lindsay *et al*.[28] referred that the growth of *Pseudomonas* spp with other strains might be beneficial as the EPS produced by *Pseudomonas* spp may confer some protection to the binary biofilm and thus protect it against sanitizer treatment[28,37]. In the present study, the data did not corroborate those studies, as the binary growth of biofilm does not seem to give any advantage or disadvantage concerning the response to the presence of BC during biofilm development or the BC challenge.

The *E. coli* biofilms formed in normal conditions, *i.e*. in the absence of BC, seem to show less sensitivity to BC attack (Fig.4.2-1 C) presenting also more unwavering protein and polysaccharide content (Fig.4.2-2). Actually, a careful observation of the values obtained with the biochemical characterization of *E. coli* biofilms reveals that after BC treatment there is just a small reduction in these contents. In this case, the EPS matrix seems to be a crucial structural parameter for *E. coli*

biofilm stability and architecture providing a refuge for bacterial community[5]. The limited effectiveness of antimicrobials to eradicate the microorganisms of biofilm infections may be related to non-mutational or physiological conditions that allow survival, like those related to stationary-phase physiology, low oxygen and nutrient penetration, low penetration of antimicrobials or antibiotics and slow growth. This particular phenotype confers biofilms tolerance or recalcitrance[15,20,27].

P. aeruginosa is known to produce mainly alginate during EPS excretion[2,9] and there are studies referring alginate overproduction during nitrogen limitation[13] and after *P. aeruginosa* membrane perturbation induced by ethanol[14]. *E. coli* matrix is composed mainly of colanic acid[11,16,38,44,48,51]. Pringent-Combaret *et al.*[38] reported that the copious amount of appendages present at the cell surface (such as curli in *E. coli* and type IV pili in *P. aeruginosa*) and the various exopolymers excreted by bacteria can also concentrate ionic molecules from the biofilm phase as the biofilm develops. These different cell appendages and matrix main components together with the possible different response of each strain to BC stress can be the reason for the different biofilm behaviour observed and it can also explain the ability of BC treatment to disrupt matrices.

The maintenance of the number of entrapped-cells of BC-adapted biofilms (Fig.4.2-1 C) can be due to the protective effect of the cohesive matrix observed in SEM (Fig.4.2-3). In this study, *E. coli* biofilm-entrapped cells probably have undergone some kind of phenotypic change due to the fact of being part of a biofilm. In fact, the entrapped-cells of adapted biofilms appeared to be more sensitive to BC than the cells of normal biofilms. It can also be due to the difficulty of detecting persister cells by culturability methods[27]. According to a study of Mangalappalli-Illathu[31], the regrowth of *Salmonella enterica* serovar Enteritidis after BC exposure was due to a small percentage of survivor cells that, after BC stress relief, were able to recover and multiply, being these survivors the sign of the so called persistence and regrowth of biofilms.

When considering the antimicrobial effect of disinfectants, it is not merely the killing of bacteria that is of interest. Removal of biofilm from the surfaces is also important as residual sessile cells may facilitate rapid regrowth of new biofilms[18]. So, in conclusion, it appears that *E. coli* and *P. aeruginosa* entrapped in single and mixed biofilms may develop adaptive resistance to BC, noticeable by the over secretion of matrix components during biofilm development in presence of that antimicrobial product. Overall results clearly point out that BC had poor antimicrobial efficacy against *E. coli* and *P. aeruginosa* biofilms development and control.

Knowing that the amount of matrix is one of the most important contributes to the virulence of biofilms, the use of BC as an antimicrobial agent should be avoided and special care should be taken when dealing with inappropriate doses of this antibacterial agent. The suitable concentration of antimicrobial should always be supported by experimental assays. Otherwise, biofilm establishment and bacteria virulence might be favoured, contributing, in this way, to the increased

prevalence of surface contamination and biofilm-associated infections. The data of this study did not allow the determination of which phenomenon (phenotypic adaptation, genetic acquired resistance or both) was responsible for the adaptation of biofilms to sub-lethal QAC exposure. Therefore, future work will be required to unravel the mechanism(s) by which the biofilm-entrapped bacteria respond to environmental pressures. This can be achieved by the determination of genetic and proteomic regulation of EPS production under BC stress.

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CHAPTER 5

PROTEOMIC STUDIES OF *PSEUDOMONAS AERUGINOSA* OUTER MEMBRANE PROTEINS

CHAPTER 5.1

PSEUDOMONAS AERUGINOSA PLANKTONIC ADAPTATION TO BENZALKONIUM CHLORIDE: A PROTEOMIC APPROACH

This study aimed to assess the outer membrane modifications of planktonic *Pseudomonas aeruginosa* ATCC 10145 after continuous exposure of bacteria to increasing doses of benzalkonium chloride (BC). The proteome of two laboratory-adapted strains to two BC concentrations (0.9 and 12 mM) was characterized.

Proteomic studies revealed that adaptation select some changes in the membrane proteome, these alterations being BC concentration dependent. BC adapted cells differentially expressed seven outer membrane proteins. OprF and two hypothetical proteins (PA1041 and PA2800), OprE3, OprL and OprI and OprG. Some of these proteins are porins and have key roles in multidrug efflux pumps, being related with antibiotic resistance. Other are lipoproteins that are essential for cellular processes being also involved in *P. aeruginosa* mucoidity.

The modification of the outer membrane proteins profile of the laboratory-adapted *P. aeruginosa* emphasizes that simple exposure to antimicrobial stress endorses differential expression of some key proteins related with membrane permeability function that may thus have impact in bacteria pathogenicity.

5.1.1 Introduction

Many processes essential for *P. aeruginosa* pathogenesis, *e.g.*, nutrient uptake, antimicrobial resistance and virulence, take place in the cell envelope and depend on components residing in the periplasmic space[16].

P. aeruginosa is often intrinsically resistant to antimicrobials, especially because this bacterium has low-permeability membrane barriers[29]. Industry is permanently trying to find agents that can be effective in bacteria eradication and able to be extremely successful by acting in less specific resistance mechanisms like the permeability barriers and multidrug active efflux[29].

Antimicrobial chemical agents are widely employed to reduce microbial levels on inanimate objects and may occasionally act as chemosterilants, some being employed as high-level disinfectants[41]. It has been reported in literature that the widespread use of biocides and disinfectants in hospitals, and to a lesser extent at home, could act as a selective pressure for antimicrobial-resistant bacteria[41]. Some authors[20,28] refer to hospital sinks, where disinfectants and detergents are discarded, as one of the main environmental sources of several *P. aeruginosa* resistant strains responsible for outbreaks in clinical settings. Antimicrobial agents can cause significant membrane damage, namely disorganization[25] or rupture in gram-negative bacteria[42]. Benzalkonium chloride (BC) has been shown to cause changes in the outer membrane permeability and can lead to bacterial resistance to that or to other antimicrobial agents[1].

Due to their interfacial position in cells, membrane proteins are central to cell life because they are in the interface between the outside and the inside of the cell[36], playing important roles in various cellular processes including signal transduction, metabolites and ions transport and cell adhesion[45]. Membrane proteins are very important for pharmacological action because they represent potential targets for vaccine development [44]. The membrane is also a protective barrier and constitutes more than half of all known drug targets[49].

One of the factors that can contribute to the understanding of the parameters involved in sanitation failure is to establish whether the repeated contact of bacteria with a chemical can select changes in its proteomic traits. So, the purpose of this study was to examine the outer membrane (OM) proteome modifications promoted by the adaptation of *P. aeruginosa* to BC and to correlate them with the increased tolerance showed by those strains.

5.1.2 Material and Methods

Strains and culture conditions and antimicrobials

P. aeruginosa (ATCC 10145) and adapted to 0.9 mM and 12 mM of benzalkonium chloride (BC) (obtained as described in sub-chapter 2.2.2) strains were used throughout this work.

Proteomic analysis

Preparation and analysis of outer membrane protein (OMP) extracts

Crude OMP extracts were prepared from bacterial pellets following the spheroplast procedure described by Mizuno and Kageyama[26] in sub-chapter 2.5.1.

Two-dimensional gel electrophoresis

OMP patterns were analysed by two-dimensional gel electrophoresis (2-DE) as described in sub-chapter 2.5.2.

Gel analysis and protein identification

Spot quantification was achieved by computing scanning densitometry and spots were excised from the polyacrylamide gel and identified as described in sub-chapters 2.5.3 and 2.5.4 respectively.

Bioinformatic tools for subcellular location

For the identified proteins, and in particular for unknown proteins, the prediction of their location within the bacterial cell was obtained from the genome annotation of *P. aeruginosa* (accessible at http://www.pseudomonas.com/).

5.1.3 Results and Discussion

Antimicrobial adaptation

The stability of the BC-adapted cells (A0.9 and A12 strains) was determined after three transfers in TSB medium. After the de-adaptation process, the adapted strains lost their adaptive resistance, as they were unable to growth in TSA supplemented with BC after 10 passages in TSA. Joynson *et al*.[18] referred that despite being easily attained, the adaptive resistance can be lost if bacteria are sub-cultured in the absence of the selection pressure. This seems to be a characteristic of bacterial adaptation achieved by continuous exposure. This feature was also reported by Gilleland *et al*.[12] who confirmed the instability of *P. aeruginosa* resistance as the strains readily revert to susceptible during several transfers in the absence of antibiotic. So, to keep the bacterial tolerance, A0.9 and A12 *P. aeruginosa* cultures were maintained in TSA supplemented with 0.9 and 12.0 mM of BC, as referred in the Materials and Methods section.

OMP patterns

As BC is one if the antimicrobials which action occurs mainly at the bacterial outer membrane level[47], this work investigated the changes induced by BC on the OMP pattern of *P. aeruginosa*. However, membrane proteins are notorious complex to analyse via 2-DE due to difficulties in extracting them from the membrane and their inherently hydrophobic nature[27]. The hydrophobicity of these proteins makes them also difficult to solubilise for isoelectric focusing (IEF). Moreover, they often precipitate at their p*I* (isolelectric point) in the IPG strip, reducing the transfer to the

second-dimension gel (2-D). The incorporation of the zwitterionic detergent ASB-14 in the 2-D sample solution prior to 2-DE has previously been shown to improve the solubility of the membrane proteins compared with sample solutions containing CHAPS or SB 3-10[4]. In this study, ASB-14 was added to IEF buffer in order to increase the solubility of the proteins.

In the present study, some proteins were found as mass isoforms (represented by * in Table 5.1-1). The isoforms exhibited different apparent molecular masses and p*I* values, witness of posttranslational modifications[19]. Thus, the major constitutive porin OprF was found as three isoforms, confirming previous observation[45]. It was also the case of OprL and OprG. The range of the membrane proteome alterations following adaptation is very low (about 11 %) *i.e.* 22 on 206 spots which were discriminated on 2-DE gels and BC concentration dependent.

Protein identification showed some of these spots correspond to cytosolic proteins, *e.g.* HemB, Ndk, PyrB, SucC, Tsf, PA4352 and PA 5339 (Table 5.1-1). The presence of these proteins in the bacterial samples pointed out cytoplasmic contaminations.

Only four OMP (PA2800, OprF, OprL and OprI) exhibited different amounts in 0.9 mM BC-adapted cells when compared with non-adapted organism, whereas seven OMP (PA2800, OprF, OprL, OprI, PA1041, OprG and OprE3) were differentially expressed in 12 mM adapted organisms (Table 5.1-1). This observation is surprising considering the antimicrobial action of BC which is thought to involve a general perturbation of lipid bilayer membranes as the outer-membrane of Gram-negative bacteria[47], and the strategic role of the outer membrane in the cell adaptation to environmental stresses. BC binds firmly to anionic sites found on the membrane surface, causing the loss of osmoregulatory capacity as well as the leak of potassium ions and protons[11]. Therefore, in this study it was expected to find a higher number of membrane proteins with different expression in the adapted bacteria when compared with the OM profile of the reference strain.

Table 5.1-1 Identification of the selected OMP whose amount was differentially expressed in *P. aeruginosa* cells adapted to 0.9 and 12 mM benzalkonium chloride strain when compared with the reference strain

Locus tag	Protein	No. of matching peptides		Location [a]	Gene name	Gene ID	Behaviour in adapted strains [b]	
		A0.9	A12				A0.9	A12
PA3190	Putative binding protein component of ABC sugar transporter	**8**	**3**	P	*gltB*	882901	-	-
PA0766	Serine protease MucD precursor	5	5	P	*mucD*	879207	+	+
PA3655	Elongation factor Ts	12	**6**	C	*tsf*	5358722	-	-
PA3988	Hypothetical protein PA3988	14	**18**	U	-	878911	-	+
PA2800	Hypothetical protein PA2800	11	**8***	**OM**	*vacJ*	879842	+	-
PA5339	Hypothetical protein PA5339	5	**6**	C	-	877913	+	+
PA2951	Electron transfer flavoprotein alpha-subunit	22	**10**	U	*etfA*	882932	+	+
PA4671	50S ribosomal protein L25/general stress protein Ctc	**9**	-	C	-	881395	+	=
PA4495	Hypothetical protein PA4495	7	-	U	-	881066	+	=
PA1777	Major porin and structural outer membrane porin OprF precursor	**17***	**17***	**OM**	*oprF*	878442	+	+
PA0973	Peptidoglycan associated lipoprotein OprL precursor	**11***	**7**	**OM**	*oprL*	882991	-	-
PA2853	Outer membrane lipoprotein OprI precursor	**3**	7	**OM**	*oprI*	879851	-	-
PA5489	Thiol:disulfide interchange protein DsbA precursor	6	**5**	P	*dsbA*	877731	+	+
PA1041	Hypothetical protein PA1041	-	**10**	**OM**	-	881756	=	+

PA4385	GroEl	-	**5**	C	*groEL*	881348	=	-
PA1588	Succinyl-CoA synthase beta-subunit	**7**	-	C	*sucC*	882016	-	=
PA3807	Nucleoside diphosphate kinase	**7**	-	C	*ndk*	879892	+	=
PA0402	Aspartate transcarbamylase	**7**	-	C	*pyrB*	878267	+	
PA5243	Delta-aminolevulinic acid dehydratase	-	**9**	C	*hemB*	879701	=	+
PA4067	Outer membrane protein OprG precursor	-	**13***	**OM**	*oprG*	879793	=	-
PA2760	Probable outer membrane protein precursor (OprE3)	-	**9**	**OM**	-	882719	=	-

[a] OM, outer membrane; P. periplasmic; EC, extracellular; C, cytoplasmic; U, unknown

[b] Symbols represent differential expression of proteins in adapted strains compared with reference strain: (-) down regulated , (+) up regulated, (=) no change

* Indicates proteins that are identified in more than one spot

Localisation prediction, gene code and function according to genome annotation, PSORTdb 2.0 and www.pseudomonas.com

Matching peptides in bold indicate a statistically significant difference between *Pseudomonas aeruginosa* ATCC 10145 and the adapted strains ($p < 0.05$)

OprF was up-regulated in both laboratory-adapted bacteria. This OM protein is non-covalently linked to peptidoglycan and it is involved in the maintenance of *P. aeruginosa* cell shape, having homology to another structural OMP, the OmpA from *Escherichia* coli[51]. It has been shown that the N-terminal domains of OprF from *Pseudomonas* strains are able to form ion channels in planar lipid bilayers similar to those induced by the OmpA N-terminal transmembrane domain[43]. Besides that, OprF is also a major environmental sensor[13,50], with an important role in adaptation to hyperosmotic conditions[13]. However, the involvement of OprF in antibiotic resistance remains controversial[2]. It has been suggested that the loss of this protein may be involved in the multiple-antibiotic resistance phenotype[33,34]. According with Jouenne *et al.*[17], this protein loss also occurs when bacteria evolve from planktonic to biofilm mode of life, reflecting a tendency of the microorganism to limit the exchanges with the extracellular medium. In this study, the increase in the expression of this protein might be related with an increase of exchanges within the cell and the medium containing BC.

Bacteria adapted to the higher BC concentration accumulated the probable OMP PA10141 (Table 5.1-1) whose function is unknown. OprL and OprI were down-regulated in adapted *P. aeruginosa* for the two tested BC concentrations (Table 5.1-1). Lipoproteins, like OprL and OprI have been shown to play a role in many fundamental cellular processes and in the pathogenesis of several bacterial infections[38]. In *P. aeruginosa*, microarray analysis has revealed that there is a prominent induction of lipoprotein-encoding genes during mucoid conversion[9,38]. OprL is a peptidoglycan-associated protein with strong immunogenicity and a key-protein for the identification of *P. aeruginosa* required to maintain OM integrity[3,23] and cell morphology[22,39,40]

OprI is a structural lipoprotein, essentially studied for its low variability between *P. aeruginosa* strains, which makes it useful as a phylogenetic marker[7], as a potential vaccine[37] and as an epidemic detection marker in the case of cystic fibrosis[35]. As an integral OM component, OprI plays a role in cell shape and membrane fluidity maintenance[13]. According to Linares *et al.*[21], the exposure of *P. aeruginosa* to tobramycin and ciprofloxacin also promoted the down-regulation of the OprI protein.

The underexpression of such important proteins in adapted cells can be a sign of disruption of the OM and loss of morphology after antimicrobial stress. Moreover, it might reflect some changes in *P. aeruginosa* mucoidity.

Bacteria adapted to 12 mM of BC also under-expressed OprG, OprE3 and the probable OMP PA2760 with unknown function[46] (Table 5.1-1). These OMP are frequently described as involved in antibiotic resistant and act as porins playing an important role in multidrug efflux[32]. OprG expression is highly dependent on growth conditions[14], including temperature and medium osmolarity[13] and magnesium[10] and iron[53] concentration. Additionally, this protein has been suggested to be involved on modifications of the lipopolysaccharide layer[10] in *P. aeruginosa*.

There is some controversy regarding the involvement of OprG protein expression in bacterial resistance to antimicrobial agents. Loughlin *et al*.[24] evaluated the effect of BC in *P. aeruginosa* protein patterns and had observed an accumulation of OprG. In contrast, more recent studies[5,32] showed that *P. aeruginosa* tolerance to kanamycin and tetracycline was associated with the down-regulation of OprG[32].

OprE3 is a hypothetical protein with homology to the *E. coli* protein, b0681 that function as a porin of the outer membrane. The accumulation of OprE3, an anaerobically induced porin[52], by biofilm *P. aeruginosa* cells has been reported[48].

Surprisingly, the conserved hypothetical protein PA2800, whose location is predicted in the outer membrane, was accumulated by the 0.9 mM BC-adapted cells, but down-regulated by the 12 mM BC-adapted ones (Table 5.1-1). The function of this protein is yet unknown.

GroEL has been localized in the membrane and periplasm[6,31] and in extracellular medium[30]. So, although this protein is predominantly cytoplasmic, it can be found in different cellular extracts. In fact, proteins belonging to the chaperon family, such as GroEL, have already been described as associated with the OM or even extracellular secretions[1,8]. Other studies indicated that this protein might be involved in the folding of membrane-associated proteins[52] and in cell adherence[15].

Results showed that few OMP exhibited changes in their expression, suggesting that bacterial adaptation to BC do not mobilize outer membrane systems while its antimicrobial action mainly acts at the bacterial outer membrane level. In order to achieve better knowledge concerning bacterial adaptive resistance to BC and to other QAC, future proteomic characterization of subproteomes such as OM proteins, as well as the complete proteome analysis of *P. aeruginosa* would be of interest.

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CHAPTER 5.2

PROTEOMIC VIEW OF BIOFILM-ADAPTED *PSEUDOMONAS AERUGINOSA* TO BENZALKONIUM CHLORIDE AND CIPROFLOXACIN

Adaptive resistance to antimicrobials has been widely reported in planktonic state and characterized in terms of phenotypic traits and proteomic modifications. Concerning biofilm adaptation and subsequent complex and specific biofilm resistance mechanisms, these are not fully understood. This work aimed to examine whether exposure of *Pseudomonas aeruginosa* biofilms to benzalkonium chloride (BC) and ciprofloxacin (CIP) during a laboratory adaptation process could induce any proteomic alteration in the outer membrane (OM) of the biofilm-growing cells. The understanding of the process on the basis of adaptive response of biofilms will help in gaining insights concerning the resistance mechanisms and the increased virulence of *P. aeruginosa* when growing in a biofilm.

Biofilms were formed for 24 h being after submitted to the presence of 320 mg/L of BC and 6.0 mg/L of CIP, during 12 days. Sessile cells were separated from the matrix and the OM proteins patterns compared. Biofilm proteome analyses showed that *P. aeruginosa* adaptation to BC and CIP changed the expression of six proteins. Regarding outer membrane proteins, the biofilm exposure to both antimicrobials generated common down-regulation of only one OM protein: the putative tail sheath protein. The type 4 fimbrial biogenesis OM protein PilQ precursor was over-expressed only by biofilm cells submitted to BC. These alterations of the OM protein pattern probably contribute to exacerbate biofilm cells resistance. Since some phage proteins were found differentially expressed in adapted biofilms, more research concerning biofilm development in the presence and absence of antimicrobials is required to determine the localization and function of phage-related proteins.

Some insights in the proteomic characterization were achieved, allowing the identification of several proteins that can be related with the increased virulence of *P. aeruginosa* and that can support future studies in this area. Results also show that the exposure to different products can promote the differentially expression of OM proteins, revealing a possible common response to stress supporting the phenomena of cross-resistance. However, it was not possible to describe completely the resistance mechanism, at the membrane level, responsible for bacterial adaptation and ability to growth in the presence of antimicrobials.

5.2.1 INTRODUCTION

The Gram-negative bacterium *Pseudomonas aeruginosa* is a major cause of infection in hospitalized, burned, immunocompromised, and cystic fibrosis patients and is recurrently considered as a resistant organism[14] and frequently develops resistance to antimicrobial agents during antimicrobial chemotherapy[10]. *P. aeruginosa* resistance is often associated with its high ability to attach to a surface, embedding itself in a matrix of exopolymeric substances[9], containing also proteins, and nucleic acids[31], establishing a biofilm. Bacteria embedded in biofilms are much more resistant to antimicrobials when compared with planktonic-growing cells[22], being this complex increased tolerance not yet fully understood. However, several mechanisms have been recognized as playing major roles in biofilm resistance: the slow microbial growth rates; the intercellular signals that alter the biofilm physiology, causing bacteria to produce molecular pumps that expel antibiotics from the cells and allow the community to tolerate the presence of the drug; the diffusion barrier promoted by the biofilm matrix that prevents antimicrobial products to reach their targets, being also responsible of the neutralization or degradation of the antimicrobial and the specialized populations of persister cells. Some antimicrobial agents act by causing membrane damage, affecting membrane organization[23] or rupture of biological membranes in gram-negative bacteria[30]. Also, the different expression of efflux pumps[33] [13], porins [7] and other proteins at the membrane level[32] is related with the intracellular concentration of antimicrobials [5] and thus mediate resistance. Antimicrobial agents used to control biofilms, are usually applied in high doses and during long periods of time in order to achieve better efficiency. This procedure, when not fully efficient, tends to kill only external bacterial layers of biofilms, exposing the inner-entrapped biofilm cells to gradually reduced concentrations of the antimicrobial products. When biofilms that are not eradicated when these procedures are applied, the enclosed protected bacterial cells persist and instigate biofilm regrowth. This type of adaptive resistance to antimicrobials is a way of antimicrobial stress response and has been widely reported in planktonic growth and studied through phenotypic characterization and proteomic analyses. Concerning biofilm adaptation, the increased resistance and the resistance mechanisms at the membrane level of biofilm-entrapped cells to antimicrobial exposure has been scarcely studied.

This work aimed to characterize the proteomic changes, induced at the membrane level, of *P. aeruginosa* cells that subsist in biofilms exposed to benzalkonium chloride (BC) and ciprofloxacin (CIP). These proteomic changes can help in the characterization of resistance mechanisms associated with biofilm adaptation and can allow the establishment of common proteome changes after the action of different classes of antimicrobials, which can help in the clarification of the cross-resistance mechanism to antibiotics that is known to occur after antimicrobial action.

5.2.2 Material and Methods

Strain and culture conditions

Pseudomonas aeruginosa (ATCC 10145) was used throughout this work.

Biofilm development and adaptation

The methodology used to grow the bacterial biofilms was based on the microtiter plate test developed by Stepanovic *et al*.[35] as described in sub-chapter 2.4.1.3. Biofilms were adapted to 320 mg/L (0.9 mM) of BC and 6.0 mg/L of CIP, as described in sub-chapter 2.4.2 during 12 days. After that, the content of each well was removed and biofilms were washed twice with 2 mL with UP water. Non-adapted biofilms were developed in wells containing 2 mL of TSB for the same period of time. The biofilms were scraped using a sterile rubber scraper, until the wells were visually clear, and the biofilms were resuspended in UP water and vortexed vigorously for 2 min.

Proteomic analysis

Preparation and analysis of outer membrane protein (OMP) extracts

Bacterial cultures and matrix were separated and bacterial membranes were extracted according to the method described by Winder *et al*.[39] as described in sub-chapter 2.5.2.

Two-dimensional gel electrophoresis

OM protein patterns were analysed by two-dimensional gel electrophoresis (2-DE) as described in sub-chapter 2.5.2.

Gel analysis and Protein identification

Spot quantification was achieved by computing scanning densitometry and spots were excised from the polyacrylamide gel and identified as described in sub-chapters 2.5.3 and 2.5.4, respectively.

Bioinformatic tools for subcellular location

For identified proteins, and in particular for unknown proteins, the prediction of their location within the bacterial cell was obtained from the genome annotation of *P. aeruginosa* (accessible at http://www.pseudomonas.com).

5.2.3 Results and discussion

Some reports refer that the widespread use of biocides and disinfectants in hospitals, and to a lesser extent at home, could act as selective pressure for antimicrobial-resistant bacteria[29]. The present study aimed to investigate the stress response of sessile bacteria when exposed to two different antimicrobials, BC (a biocide) and CIP (an antibiotic). As the cell envelope plays a key role in bacterial responses to environmental stresses[1,20,21,34], the proteomic approach was focused on OMP profile alterations induced by antimicrobials exposure. It has been shown that permeability changes are frequently caused by BC exposure and can lead to bacterial resistance to this or to

other antimicrobial agent[6]. High doses of antimicrobials were used in order to cause damage in cells and also to ensure the selection of *P. aeruginosa* biofilm cells that were able to survive upon a long exposure to these antimicrobial agents.

Proteomic studies on biofilms normally comprise the characterization of the alterations of the protein patterns between planktonic and sessile organisms and during the different developmental biofilm stage[8,26,37]. In this study, the proteomic characterization of the OM of the biofilm-growing cells challenged for 12 days with supra-minimum inhibitory concentrations of two antimicrobials was assessed. The main difficulty of this task was related with the reduced number of bacteria that persist within the stressed biofilm and, in consequence the reduced concentration of OM proteins obtained after the extraction procedure. Additionally, the separation of bacterial cells from the biofilm matrix may also have led to sample contamination. This difficulty became even higher when biofilms were developed under the chemical stress since more extracellular polymeric substances are secreted by bacteria in order to defend the surviving cells from the aggression[22]. Consequently, an increase in samples contaminants was observed.

The protein expression of the biofilm-growing bacteria challenged by BC and CIP is listed in Table 5.2-1 and shown in Fig.5.2-2. The experimental OMP characterization discriminated nearly 600 proteins with $p<0.05$ and fold>2 (Fig.5.2-1). However, and due to the small number of gels that could be analysed for each condition, it was also considered the q value ($q \leq 0.05$) to guarantee that no false positives were mistakenly analysed. The examination of the OM profiles of BC- and CIP-adapted cells showed changes in the expression of a small number of proteins (Fig.5.2-2). The name of the altered proteins is given in Table 5.2-1

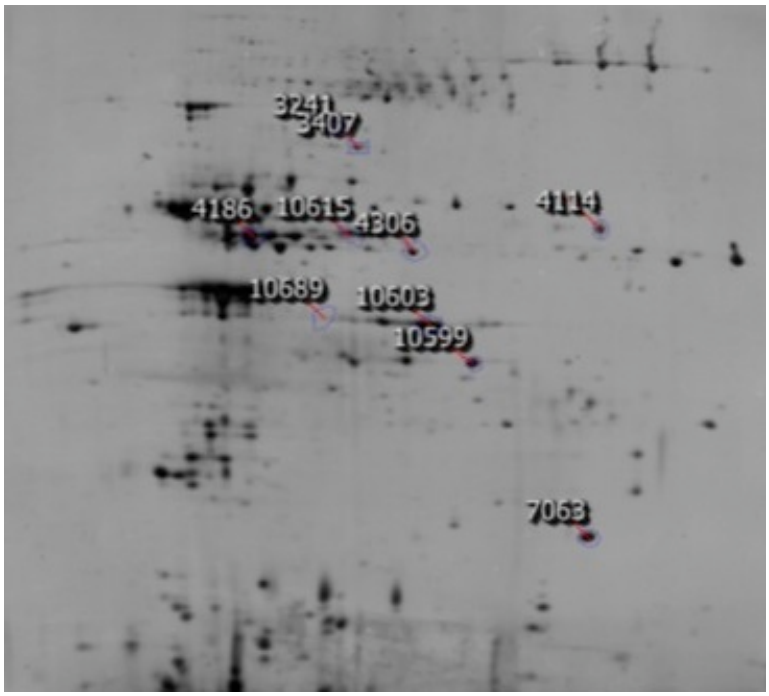

Fig.5.2-1 Silver-stained experimental 2-DE map obtained with an OM extract of *P. aeruginosa* biofilm cells (protein loading 200 µg).

Table 5.2-1 Identification of selected proteins differentially expressed in *P. aeruginosa* biofilm-cells adapted to BC and CIP when compared with reference strain

Protein number [a]	Protein	Subcellular Localization [b]	Number of matching peptides	Accession number	Normalized volumes mean (SD) [c]				
					TSB	BC		CIP	
3241	Chain A, Crystal Structure Of The Asymmetric Chaperonin Complex GroelGROES(ADP)7	C	10	38491472	5.79 (0.047)	5.05 (0.25)	-	5.32 (0.16)	-
3407	GroEL	C	6	6225121	6.16 (0.063)	5.62 (0.063)	-	6.00 (0.21)	-
4114	GroEL	C	3	6225123	6.40 (0.032)	5.51 (0.21)	-	6.08 (0.22)	-
4186	Putative tail sheath protein	OM	3	148807411	6.98 (0.099)	6.61 (0.18)	-	6.39 (0.066)	-
4306	Putative tail sheath protein	OM	1	148807411	6.52 (0.18)	5.75 (0.14)	-	6.47 (0.19)	-
7063	Hypothetical protein PA0537	U	9	15595734	6.66 (0.06)	5.68 (0.057)	-	6.35 (0.16)	-
10599	Predicted major capsid protein	U	7	148807393	6.6 (0.012)	5.4 (0.56)	-	5.25 (0.21)	-
10603	Predicted major capsid protein	U	10	148807393	6.93 (0.20)	5.84 (0.13)	-	5.81 (0.49)	-
10615	Probable Bacteriophage protein	C	1	15595819	6.35 (0.26)	5.73 (0.32)	-	6.69 (0.12)	+
10689	Type IV fimbrial biogenesis outer membrane protein PilQ precursor	OM	8	254244078	6.0 (0.18)	6.6 (0.14)	+	5.59 (0.35)	-

[a] Spot numbers refer to those in Fig.5.2- 1.
[b] Localisation prediction according to genome annotation, PSORTdb 2.0 and www.pseudomonas.com
[c] Symbols represent differential expression of proteins in adapted strains compared with reference strain: (-) down regulated , (+) up regulated

The analysis of the biofilm cells proteome showed that sessile *P. aeruginosa* adaptation to both BC and CIP endorsed changes in ten OMPs (Table 5.2-1) (i.e., 1.6% of the membranome), when compared with the proteome of the non-adapted biofilm counterparts. These data suggest that antimicrobials induced small alterations in the OMP patterns of the biofilm adapted organisms. This may be the consequence of the accumulation of stress proteins by biofilm-growing cells even in the absence of antimicrobials. Indeed, it has been shown that some OMP involved in the adaptation process were overexpressed by biofilms organisms, probably due to alterations in the environmental conditions prevailing within biofilms[8].

Down-regulated proteins

Biofilm exposure to both antimicrobials generated common down-regulation of four main proteins: GroEL (and the chain A crystal structure of the asymmetric chaperonin complex GroelGROES), predicted major capsid protein, putative tail sheath protein, and a hypothetical protein PA0537 revealing a possible similar stress response (Table 5.2-1).

GroEL was found in several gels spots (3241, 3407, 4114), as well as putative tail sheath protein (4186, 4306) (Table 5.2-1). A different position in a 2-DE gel (Fig.5.2-1) is the result of a different chemical structure of the protein, with each covalent chemical modification leading to a "new" protein species[16]. Thus these spots may correspond to different protein species with posttranslational modifications[16]. GroEL has been localized in several sites of the membrane, cytoplasm and periplasm[27]. So, although this protein is predominantly cytoplasmic, it can be found in different cellular extracts. In fact, proteins belonging to the chaperon family, such as GroEL have already been described as associated with the OM or even extracellular secretions[14,17]. Other studies indicated that this protein facilitates the folding process of membrane associated proteins[11], as well as the adhesion of bacteria to tissues[12]. This protein is downregulated in bacteria from both adapted biofilms, the amount decrease being larger in BC than in the CIP adapted sessil *P. aeruginosa*. The downregulation of this chaperon protein in the presence of antimicrobials is quite unpredicted in the light of its role in bacterial adaptation[36], since antimicrobials act at the outer membrane level, being expected higher folding of proteins related with this bacterial compartment. The major capsid protein and putative tail sheath proteins, that were also under-expressed in BC and CIP adapted cells (Fig.5.2-2, Table 5.2-1), have unknown function(s) and are encoded by genes that are part of a previously described genomic island (GI), PAGI-6[2]. This GI is an example of a prophage that has undergone multiple recombination and deletion events resulting in *P. aeruginosa* altered virulence[19]. The putative tail sheath protein has been also associated with membrane vesicles (MV) produced by pathogenic bacteria and known to contain active toxins and other virulence factors[3]. In the literature there is no consistent information regarding the expression of this proteins in biofilms. However, the fact that *P. aeruginosa* biofilm cells

underexpress this protein under stress caused by antimicrobials suggests the loss of biofilm membrane vesicles during the adaptation process. In the literature there is no consistent information regarding the expression of this proteins in biofilms. The underexpression of such important proteins might be a reflect of the ability of *P. aeruginosa* biofilms to adapt and inhabit extreme environments[2]. A hypothetical protein, PA0537, was also underexpressed in biofilm-growing cells exposed to antimicrobial pressure (Fig.5.2-2; Table 5.2-1).). The function of this protein is still unknown.

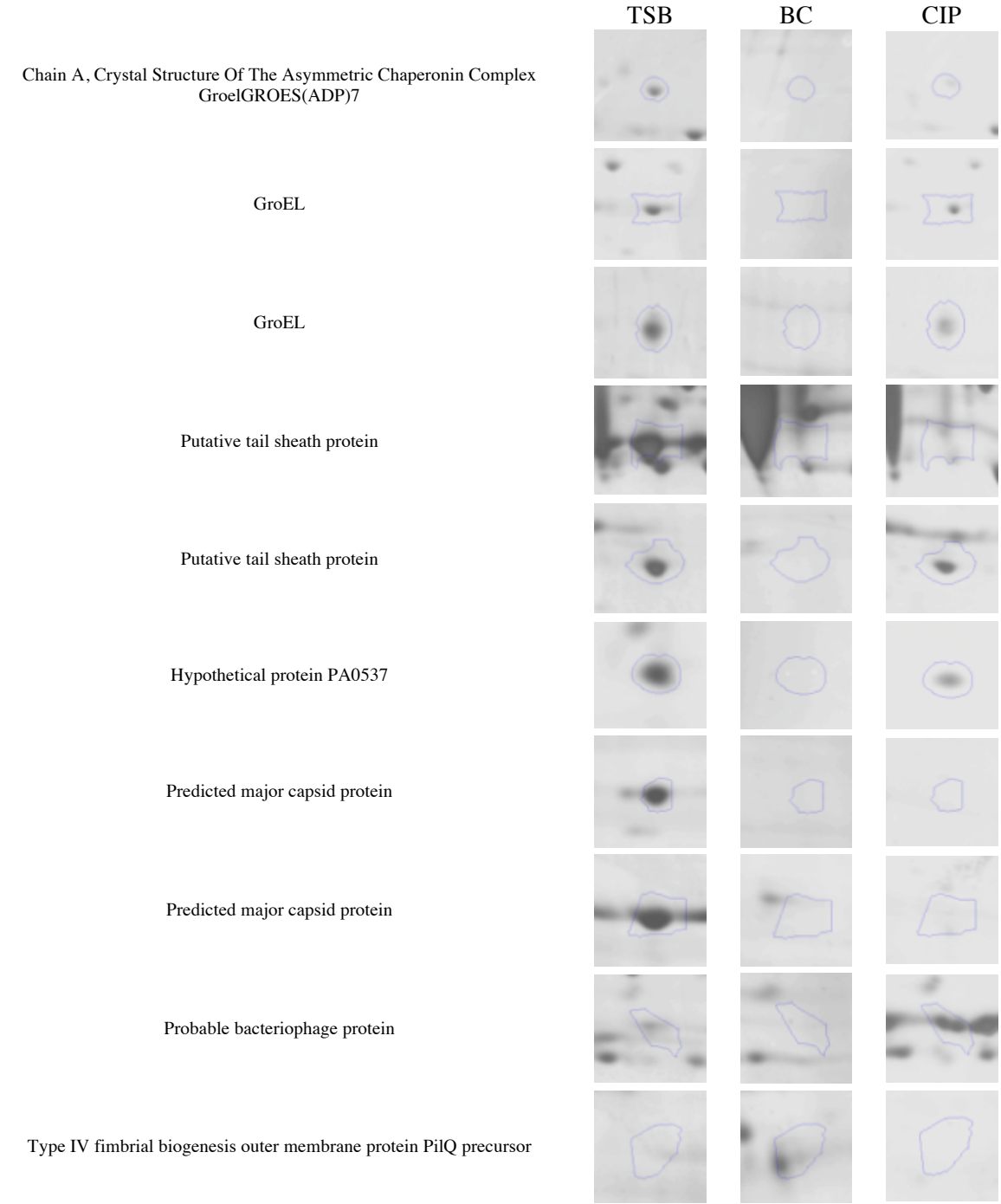

Fig.5.2-2 Comparison of the behaviour of selected proteins in *P. aeruginosa* biofilm-cells of grown in TSB and adapted to BC and CIP.

Differentially expressed proteins

The type 4 fimbrial biogenesis outer membrane protein PilQ precursor was over-expressed only in biofilms exposed to BC (Fig.5.2-2, Table 5.2-1), while the probable bacteriophage protein was overexpressed in biofilms exposed to CIP (Fig.5.2-2, Table 5.2-1).

Bacteriophage protein is, as the putative tail sheath protein, a MV protein of *P. aeruginosa*. Membrane vesicles are naturally released from the bacterial outer surface and are constituted of OM proteins, lipopolysaccharides and periplasmic components. *P. aeruginosa* MV are a component of the matrix of the *P. aeruginosa* mature biofilm and contain some virulence factors, including proteases, phospholipase C, alkaline phosphatase, and antibacterial factors[24].

The PilQ protein is involved in type IV pilus biogenesis[25], being essential for *P. aeruginosa* pilus formation and thus it can affect motility and adhesion[25]. This protein was overexpressed in BC-adapted biofilm-cells although it was underexpressed in CIP-adapted biofilm-entrapped cells (Fig.5.2-2, Table 5.2-1). Nde *et al.*[25] reported the downregulation of the gene PilQ after *P. aeruginosa* exposure for 60 min to chlorhexidine diacetate (CHX). According to this author, the mechanism of action of CHX in *P. aeruginosa* involves changes in outer membrane permeability. Although BC and CHX have similar action at the membrane level, in this work bacteria were grown as biofilms that were after exposed to antimicrobials while in the study of Nde *et al.*[25], planktonic bacteria were grown in CHX. This different mode of growth can be on the basis of the contrary expression of this protein after antimicrobial exposure.

Several studies have suggested that the most highly activated genes in sessile bacteria are those of temperate bacteriophages[15,38]. These take part in the diversification and phenotypic variation, including the production of small colony variants phenotypes[28]. The fact that biofilms were developed under antimicrobial stress could regulate proteins related with phages that may be in the origin of increased population diversity found within biofilms[4]. The phenotypic diversity amongst a biofilm population can intensify virulence factors like adhesion, hydrophobicity, swimming and twitching motilities[18] increasing also the ability of a biofilm to survive under stress conditions.

A proteomic approach was here used to characterize the alterations of the outer membrane proteome of *P. aeruginosa* biofilm cells after BC and CIP adaptation. When bacteria are within biofilms and exposed to chemical stress, the regulation of OM proteins expression can contribute to the increase of bacterial virulence and biofilm antimicrobial resistance. Herein proteins involved in pili biogenesis, vesicle formation, pathogenicity islands and some phage related proteins were identified in BC and CIP adapted *P. aeruginosa* biofilms.

The number of differentially expressed proteins obtained after OM extraction of biofilm cells adapted to BC and CIP was not enough to clearly describe the resistance mechanism associated with this *in vitro* adaptive response. However, this work gave some insights that can support future studies in this area since it was observed the differentially expression of some OM proteins after

exposure to both antimicrobials, revealing a possible link between the response of bacteria within biofilms when exposed to external stresses. Further proteome studies will provide more comprehensive information on protein variations and reveal antibacterial resistance mechanisms, allowing the identification of candidate target proteins for novel antibiotics and defining some possible cross-resistance mechanisms associated with both antimicrobial products.

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CHAPTER 6

CONCLUDING REMARKS AND FUTURE PRESPECTIVES

In this Chapter, the major conclusions of the present thesis are addressed. Suggestions for future work are also proposed.

In clinical settings, bacteria with increased resistance may represent an augmented risk, as it can lead to infection outbreaks due to unexpected disinfection failures[9], being also directly involved in the increase of morbidity and mortality caused by infection diseases[1].

In real scenarios and within the bacterial biofilm community, multiple types of bacterial isolates are undoubtedly present conferring greater phenotypic variability that contributes to biofilm adaptation to external antimicrobial pressures and subsequent biofilm persistence to antimicrobial treatments. As shown in Chapter 3.1, clinical isolates possess particular motility ability, thus influencing the biofilm mode of life. Since *P. aeruginosa* appendages can be related with higher bacterial virulence and probably subsequent increased resistance, future studies could be performed in order to genetically characterize the presence and loss of appendages in bacteria during the different stages of biofilm formation, and under different antimicrobial treatments. Moreover, due to the reappearance of motility appendages after loss (*e.g.*, when bacteria disperse from biofilms to planktonic state) together with their function in adhesion to surfaces and adhesion to host tissues, it would be of great interest to determine the role of those appendages in biofilm formation.

Antimicrobial resistance of *P. aeruginosa* biofilms appears to be complex and multifactorial and essentially instigated by: (i) the diffusional difficulty of the antimicrobial agents to penetrate the biofilm, (ii) the slow growing cells at the deeper layers of the biofilm, owing to limited oxygen and nutrients, as well as the (iii) presence of a great phenotypic variability of cells, being some of them persister cells[6], that neither dye nor reproduce themselves in stressful conditions, but become active when stress is relieved. Persister cell formation and the reason why this particular phenotype shows increased resistance remain unknown, but their selection may occur via multiple pathways and be encouraged by growth conditions and environmental stresses[15]. Results obtained in Chapter 3.2, highlighted the importance of evaluating the biofilm capacity to regrowth after exposure to antimicrobial products. The existence of this type of cycle of dead followed by cell regrowth, together with the selection of cells that did not died, mostly due to protection conferred by the biofilm niche, may be responsible for contamination outbreaks when external pressure is released. Moreover persister cells that survive can be more resistant to antimicrobials leading to the selection of resistant strains. In light of these findings, it may be interesting to continue this work, by evaluating the resistance of biofilm cells that are released from the biofilm during the several steps of the regrowth cycle, with special care in analysing also phenotypic characteristics of the cells that are able to survive until the last pressure is applied. In a recent study[6], the selection of this type of slow-growing or non-dividing cells is shown to be a result of toxin-antitoxin (TA) system that gives bacteria the possibility to “turn off” cell death. Studies concerning persister cell selection and TA characterization would also be an attractive challenge.

Various mechanisms have been reported to play, either singly or synergistically, roles in bacterial

adaptive responses to antimicrobials. The presence of low antimicrobial concentrations can be one of the reasons that support bacterial adaptive resistance (Chapter 4). Microorganisms may have multiple, possibly interconnected, adaptive mechanisms depending on the nature of the antimicrobial agent. These adaptive mechanisms can include: i) alterations in outer membrane proteins, ii) antimicrobial uptake, iii) cell surface charge and hydrophobicity, (iv) and fatty acid content of the cytoplasmatic membrane[7]. Despite the existence of several novel anti-pseudomonal agents (*e.g.*: cationic antimicrobial compounds, efflux pump inhibitors and phage therapy), there will always be a lack of effective solutions to control biofilm formation and even to eradicate it. Preventive measures in areas like antimicrobial stewardship, resistance surveillance and infection control should be applied in order to control biofilm formation[12]. Surface coating or conditioning of intravascular catheter (IVC) is one of the most recent advances used to impair microbial adhesion, as suggested by a recently published work of Zhang *et al.*[18]. This author also highlights the importance of balancing the cost-benefit of surface conditioning since despite conditioning with antimicrobials or antibiotics could reduce the risk of IVC-related infection and biofilm formation, they can also increase the risk of emergence of antimicrobial resistance. Concerning the BC residue deposition as a conditioning film in PS surfaces (Chapter 4.1), it is shown that the existence of residues on the surfaces alters hydrophobicity and modifies the surface properties of both entities (bacteria and surface) favouring bacterial adhesion. These findings suggest that the unintentional deposition of residual antimicrobials on surfaces after cleaning procedures should be carefully studied as it may encourage bacterial adhesion, especially of bacteria that have been previously exposed to chemical stresses. In order to completely characterize the potential application of BC conditioning on PS surfaces, it would be interesting to study: (i) what is the ideal period of contact between the biocide and the surface (t) and the best concentration (C), determining the most advantageous C(t) relation; (ii) the biocide concentration at the surface after conditioning; (iii) the use of other types of surfaces, as well as other microorganism or combination of microorganisms. The answers obtained can give positive insights regarding the possible and positive use of this or other biocides as agents that impair adhesion in other types of surfaces and areas where biofilm control is required.

The studies presented in Chapter 4.2 highlighted that two different bacterial species could live synergistically in a biofilm, giving rise to a new distinct biofilm population with different behaviour when facing antimicrobial products action. The continuous exposure of these bacterial consortia to antimicrobials leads to the development of biofilms encompassing more virulent and tolerant bacteria. Furthermore the existence within a biofilm of multiple bacterial species can facilitate gene transfer and also the occurrence of plasmid dispersal by conjugation, due to the higher population density, which might stimulate biofilm development and increase biofilm stability[10]. After BC biofilm adaptation more cohesive biofilms were observed being the augment of the matrix amount the greater difference in adapted *vs* normal biofilms. This study raised some

issues that would be interesting to address: (i) to characterize the matrix structure and composition, namely the polysaccharides, proteins and eDNA. Since *P. aeruginosa* during EPS production, secretes mainly alginate[13] and *E. coli* matrix is composed largely of colanic acid[19], molecular studies, based on these genes expression would be important to identify the secreted factors in the several stages of binary biofilm development as well as to identify possible target genes of interest to develop new antimicrobial agents, (ii) it would be also interesting to elucidate the contribution of each individual microorganism along the biofilm development time, as well as the biofilm resistance in several time-periods. This will allow a deeper characterization of the symbiotic relationship between those microorganisms as well as the contribution for resistance of each microorganism as being part of a binary biofilm, (iii) when analysing experiments with mixed biofilms, it urges also the need to study other types of microorganism combination that can take part in the biofilm consortia as well as the possible solution to eradicate these polymicrobial biofilms. In fact, in real situation microorganisms associate themselves in mixed-species biofilms, as in device-related infections[14], chronic otitis media[3], cystic fibrosis and also in chronic wounds[11] so, it would be interesting to study multiple bacterial species combinations, that mimic real biofilms.

Pseudomonas aeruginosa adaptive resistance is a phenomenon that is simply to attain by bacterial exposure to increasing concentration of antimicrobial agents, as the works of this thesis confirmed. However, adapted bacteria are unstable and tend to revert to their susceptible phenotypes, being the molecular mechanisms that contribute to these phenotypic changes unclear[17]. The outer membrane protein profile of the BC-adapted strain was studied in order to elucidate the extraordinary versatility and mechanism of adaptation of *P. aeruginosa* (Chapter 5.1). Results showed that *P. aeruginosa* adaptation to BC does not seem to endorse proteomic changes at membrane level. Future works should comprise technique improvements, in order to increase the knowledge in adapted bacteria selection and outer membrane protein separation. Moreover, adaptation to other biocides can also be studied and adapted *P. aeruginosa* total proteome must be studied. The proteomic characterization of adapted biofilms to biocides (BC) and antibiotics (CIP) was studied in the scope of this thesis (Chapter 5.2), since very little information is available on the cross resistance of sessile bacteria to antibiotics and biocides. Although proteomic studies (Chapter 5) began to dissect *P. aeruginosa* adaptive resistance mechanisms, further studies should be addressed in order to understand how the protein functions relates with adaptive resistance and how the regulatory network permits them to be expressed when they are needed. Moreover, and due to the difficulties felt during protein extraction and also in obtaining enough outer membrane protein concentration, mainly due to the small amount of biofilm that can be produced and adapted *in vitro*, the already available protein extraction techniques should be improved and customized for biofilms. Furthermore, studies regarding biofilm matrix proteome should also be performed as they may answer some important questions related with biofilms increased resistance after

antimicrobial exposure. In the case of sample preparation of matrix proteins, as this type of sample shows high content of contaminants, such as polysaccharides and eDNA, special care should be taken in order to obtain a clean sample without loss of protein amount representativeness. Moreover, it would be interesting to understand what are the proteomic changes induced at membrane level in isolated strains, wild-type bacteria as well as bacteria adapted to the products used during medical devices cleaning procedures, in order to understand if the resistance mechanism, as well as the natural selection phenomena is similar for each type of microorganism.

In summary, the information resulting from this thesis contributes to a better understanding of the phenotypic changes in *P. aeruginosa* cells when exposed to antimicrobial agents, especially when established in biofilms. It is though to have contributed to the increase of the knowledge on *P. aeruginosa* clinical isolates behaviour when exposed to stress (Chapter 3), on specific changes induced by biofilm adaptation, namely in polysaccharides content (Chapter 4), and cell surface hydrophobicity (Chapter 4), as well as in outer membrane protein profiles (Chapter 5). These findings can be the start to new investigations that can lead to a better understanding of bacteria adaptive resistance and to the development of new strategies that can be applied in biofilm control, to impair its formation and to eradicate already established biofilms.

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