Universidade do Minho

Escola de Engenharia

Alícia Vanessa Fontes Rodrigues

Phenotypic profiling of several strains of *Pseudomonas aeruginosa*: identification of potential virulence determinants

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Phenotypic profiling of several strains of *Pseudomonas aeruginosa*: identification of potential virulence determinants

Master Dissertation for Master's degree in Biomedical Engineering

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ABSTRACT

Numerous studies have described that bacteria display higher resistance to antimicrobials when growing in biofilm mode of growth, however the phenotypic and virulence factors changes associated with this increased resistance are still poorly understood.

The goal of this study was to enlarge the knowledge about the augmented biofilm-related resistance of *Pseudomonas aeruginosa* characterizing phenotypically *P. aeruginosa* biofilms formed by different strains in order to find whether these bacterial biofilms display a common profile. This bacterium is a common opportunistic pathogen that is often related with persistent infections that cause high rates of patient morbidity and mortality due to its intrinsic resistance to several antimicrobials and great adaptive resistance to environmental stimuli.

The experimental analysis consisted of a phenotypical examination comprising biofilm formation ability, susceptibility to antibiotics (ciprofloxacin and colistin), expression of several virulence factors (motility, toxin production, and slime production), and phenotypic switching. To best replicate real-world circumstances this work comprised the study of several *P. aeruginosa* strains: four reference strains (PAO1, ATCC 10145, CECT 111, and PA14) and three clinical isolates (PAI1, PAI2, and PAI3).

Biofilms are often related with augmented resistance to antimicrobial agents and are usually associated with chronic infections, which are difficult to eradicate. Therefore, in this study, antibiotic susceptibility was tested and related with *P. aeruginosa* strains biofilm-forming ability. Results showed that an improved biofilm-forming ability does not seem to be related with biofilm resistance since the seven *P. aeruginosa* strains showed identical resistance and susceptibility profiles against ciprofloxacin and colistin, respectively, with the exception of PAI3 which exhibited higher resistance to ciprofloxacin.

P. aeruginosa motility has been pointed out as a relevant factor for adhesion and biofilm development and also increased virulence. Thus, the swimming, swarming and twitching abilities of *P. aeruginosa* were evaluated. *P. aeruginosa* strains presented specific ability to move along surfaces. However, it was not found a clear relationship between higher motility and biofilm-forming ability and increased resistance.

Similarly, slime production also contributes to biofilm development and is often implied in increased biofilm-resistance. Considering the results obtained, it was not found a

evident relationship between slime production and both biofilm-forming ability and increased resistance.

The ability to produce and release extracellular toxins by *P. aeruginosa* is frequently related with higher levels of bacterial pathogenicity which can lead to severe consequences and even death. Among the several extracellular toxins, hemolysin and pyocyanin production were assessed in this study. Each *P. aeruginosa* strain displayed a different expression of these virulence factors. It was not possible to conclude if any *P. aeruginosa* strain was more virulent than other based only on these results.

Phenotypic switching is an usual adaptive mechanism used by *P. aeruginosa* to increase their diversity and overcome exposure to stress conditions and environmental changes. Because colony morphology variation is the most visible characteristic of phenotypic switching, both planktonic and biofilm colonies were observed in order to establish where there is a relationship between bacterial mode of growth and colony morphology and also biofilm resistance. It was observed that phenotypic switching occurred when the *P. aeruginosa* strains changed their way of growth to biofilm-form. The rising of small-colony morphotypes was recorded for some *P. aeruginosa* strains (PAO1, ATCC 10145, PA14, PAI1, and PAI3) when growing in biofilm-form, a morphotype usually associated with biofilm resistance. However it was not found a clear relationship with the rising of these small-colony variants and biofilm resistance and further studies are needed to better understand and establish the relationship of biofilm resistance and these morphotype variants.

This work contributed to a further understanding of *P. aeruginosa* biofilm characteristics, including resistance features, virulence factors expression and phenotypic variation. It was concluded that there is not a general *P. aeruginosa* biofilm profile. Although resistance and susceptibility profiles against ciprofloxacin and colistin, respectively, of all strains were very similar, all virulence factors evaluated showed to be variable characteristic among all strains tested. In addiction it was not verified direct correlations between all virulence factors studied. Because there was recorded a variability in the virulence factors studied among all strains, no strain was considered more virulent than other given strain.

Furthermore, results also show that there is an urgent necessity to reexamine the current clinical guidelines. These guidelines should regard phenotypic switching, intrinsic

responses of biofilms and virulence factors presented by *P. aeruginosa* biofilms, characteristics with therapeutical impact.

SUMÁRIO

Ao longo dos últimos anos, vários estudos têm descrito que as bactérias demonstram uma maior resistência quando crescem em biofilmes. No entanto, as mudanças fenotípicas e os factores de virulência que apresentam e que se encontram associados com o aumento desta resistência são ainda pobremente compreendidos.

O objectivo do presente estudo foi alargar o conhecimento acerca do aumento da resistência associada aos biofilmes de *Pseudomonas aeruginosa* e caracterizando fenotipicamente biofilmes formados por diferentes estirpes com o intuito de compreender se estes apresentavam um perfil de biofilme idêntico. *P. aeruginosa* é um agente patogénico oportunista muito comum que frequentemente se encontra associado a infecções persistentes que causam elevadas taxas de morbidade e mortalidade nos pacientes infectados devido à sua resistência intrínseca e à sua elevada resistência adaptativa a estímulos ambientais.

A análise experimental consistiu na caracterização fenotípica de biofilmes, nomeadamente, a capacidade de formação de biofilme, susceptibilidade a antibióticos (ciprofloxacina e colistina), a expressão de vários factores de virulência e variação fenotípica. Para melhor replicar as condições reais este trabalho incluiu o estudo de várias estirpes de *P. aeruginosa*: quatro estirpes de referência (PAO1, ATCC 10145, CECT 111 e PA14) e três isolados clínicos (PAI1, PAI2 e PAI3).

Os biofilmes encontram-se frequentemente associados com resistência aumentada a antimicrobianos e estão tipicamente relacionadas a infecções crónicas, muito difíceis de erradicar. Assim, neste trabalho, a susceptibilidade a dois antibióticos de uso clínico (ciprofloxacina e colistina) foi testada e relacionada com a habilidade de formação de biofilme das estirpes de *P. aeruginosa* de maneira a melhor compreender a resistência associada a biofilmes. No entanto, os resultados observados demonstraram que uma melhor habilidade de formação de biofilme não confere uma maior resistência a antibióticos, pois todas as estirpes de *P. aeruginosa* estudadas apresentaram perfis de susceptibilidade idênticos relativamente a ambos os antibióticos testados, com a excepção da estirpe PAI3 que revelou maior resistência à ciprofloxacina do que as restantes estirpes.

A motilidade da *P. aeruginosa* tem sido apontada como um factor relevante para adesão, desenvolvimento de biofilme e virulência aumentada desta bactéria. Três tipos de motilidade comummente exibidos pela *P. aeruginosa* foram avaliados neste trabalho.

Todas as estirpes de *P. aeruginosa* estudadas apresentaram habilidades específicas de se mover ao longo de superfícies. No entanto, não foi estabelecida uma relação clara entre uma maior motilidade e habilidade de formação de biofilme e resistência aumentada.

A produção de matriz ("*slime*") está também envolvida no desenvolvimento do biofilme e muitas vezes é implicada na resistência aumentada a antimicrobianos. Considerando os resultados obtidos, não foi encontrada evidência que relacione a produção de matriz com formação de biomassa de biofilme e um aumento da resistência.

A capacidade de produzir e libertar toxinas extracelulares pela *P. aeruginosa* é frequentemente relacionada com níveis mais elevados de patogenicidade que podem conduzir a graves consequências e ainda à morte do hospedeiro. Entre estas toxinas extracelulares foram estudadas as produções de hemolisina e piocianina. Cada estirpe de *P. aeruginosa* estudada demonstrou uma diferente expressão destes factores de virulência.

A variação fenotípica é um mecanismo adaptativo muito comum utilizado pela *P. aeruginosa* para aumentar a sua diversidade e ultrapassar a exposição a condições de *stress* e mudanças ambientais. Como a variação da morfologia de colónias é a característica da variação fenotípica mais visível, foram observadas colónias formadas por células planctónicas e de biofilme com o objectivo de estabelecer se há uma correlação entre o modo de crescimento bacteriano e variação da morfologia de colónias e, até, resistência associada a biofilmes. Foi observado que, de facto, a variação fenotípica ocorria quando as estirpes de *P. aeruginosa* cresciam em biofilme. Relativamente à resistência associada a biofilmes, foi registado o aparecimento de pequenas colónias (*small-colony variants*) em algumas estirpes *de P. aeruginosa* (PAO1, ATCC 10145, CECT 111, PA14, PAI1 e PAI3), um morfotipo usualmente relacionado com a resistência associada a biofilmes. No entanto, não foi encontrada a relação entre o aparecimento destes morfotipos com a resistência associada a biofilmes e são necessários estudos adicionais para melhor entender e estabelecer a relação entre a resistência associada a biofilmes e estes morfotipos.

Este trabalho contribuiu para uma melhor compreensão das características dos biofilmes formados pela *P. aeruginosa*, incluindo resistência, expressão de factores de virulência e variação fenotípica. Foi concluído que não existe um perfil de biofilme da *P. aeruginosa* universal. Apesar dos perfis de resistência e susceptibilidade contra a ciprofloxacina e a colistina, respectivamente, terem sido muito idênticos entre todas as

estirpes, todos os factores de virulência avaliados demonstraram ser características variáveis entre todas as estirpes estudadas. Para além disso, não foi verificada uma correlação directa entre todos os factores de virulência estudados. Devido à variabilidade encontrada na expressão dos diferentes factores de virulência estudados, nenhuma estirpe foi considerada mais virulenta do que outra.

Em adição, os resultados demonstram que há uma necessidade urgente de rever as directrizes clínicas actuais. Estas directrizes devem ter em conta a variação fenotípica, a resposta intrínseca dos biofilmes e os factores de virulência expressos pelos biofilmes de *P. aeruginosa*, características que têm maior impacto a nível terapêutico.

TABLE OF CONTENTS

ABBREVIATIONS AND ACRONYMS

Abbreviation/Acronym: term

BHI: Brain Heart Infusion
CA: Columbia Agar
CFU: Colony-forming Unit
CIP: Ciprofloxacin
COL: Colistin
CV: Crystal Violet
EUCAST: European Committee on Antimicrobial Susceptibility Testing
HCl: Hydrochloric acid
MBC: Minimum Bactericidal Concentration
MHB: Mueller-Hinton Broth
MIC: Minimum Inhibitory Concentration
OD: Optical Density
PLC: Phospholipase C
PLcHR: Hemolytic Phospholipase C
PLcN: Nonhemolytic Phospholipase C
sCV: small-colony Variant
TSA: Trypic Soy Agar
TSB: Trypic Soy Broth

LIST OF FIGURES

LIST OF TABLES

GENERAL OUTLINE

Until recent developments, acute infections were treated with antibiotics but these infections were not considered to involve biofilms[1] . Most of the infections that affect compromised hosts often comprise bacteria that are communal in the environment or are commensal with the human body such as *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*[1].

Biofilm-associated infections display some similar clinical characteristics. These infections are characterized by persistent inflammation and tissue damage[2]. Bacterial biofilms consist of a community of cells enclosed in an exopolysaccharide matrix and attached to solid inert surfaces, such as medical devices, or in dead or living tissues[3]. These biofilms can be composed by single or many species.

Infections associated with biofilms are often slow to manifest apparent symptoms[1] and are frequently related to hospital-acquired infections. When growing in biofilm forms bacterial cells can exhibit a 10-1000 times higher resistance to antimicrobial agents[3] and therefore biofilm-related infections are more difficult to eradicate. Given these evidences, it is clear that biofilms have a massive impact on medicine since they are associated with high patient morbidity and mortality and biofilm-based infections can increase costs linked with diagnostic and therapeutic practices. Therefore it is essential to rethink the current clinical guidelines and recognize the influence of biofilms in the treatment of infections.

P. aeruginosa is one of the major microorganisms involved in urinary, bloodstream, pulmonary, soft tissue, and surgical site infections[4] in compromised individuals as those in intensive care units. These infections are also associated with increase morbidity and mortality and therapeutic possibilities are nowadays becoming limited due to the increased emergence of resistant strains[5].

This bacterium pathogenicity relies on its ability to easily adapt to diverse environmental conditions, expression of several virulence factors, and intrinsic resistance to several antimicrobial agents. Moreover *P. aeruginosa* pathogenicity can be increased when this bacterium grows under stress conditions and/or in biofilm form.

Many processes for *P. aeruginosa* pathogenesis, antibiotic resistance and virulence, take place in the cell envelope and depend on components residing in the periplasmic space. Therefore, it is pivotal to analyze the phenotype of *P. aeruginosa* collected from different situations in order to identify some virulence and resistance factors of this

bacterium as well as characterize its biofilm-forming ability and recognize patterns of susceptibility to selected antibiotics.

One of the main goals of this work is to deeply characterize virulence and antibiotic resistance profiles of *P. aeruginosa* cells recovered from biofilm in order to determine common features. In order to accomplish this goal biofilm-forming ability, susceptibility, slime production, production of extracellular toxins (hemolysin and pyocyanin), motility, and phenotypic switching of *P. aeruginosa* will be assessed.

Ultimately, the impact of the *P. aeruginosa* characteristics studied on the therapy of infections caused by this bacteria will be discussed.

This thesis is organized into four chapters. The first chapter reviews some significant aspects of *P. aeruginosa* biofilm formation and virulence factors and resumes the state of the art understanding the antimicrobial resistance in biofilm cells. Chapter 2 summarizes the microorganisms, materials and methodology used in the experimental work. In Chapter 3 the results obtained in this study as well as the discussion of these results are presented. The final chapter involves the overall conclusions of the work presented in this thesis and suggests future research outline.

CHAPTER 1. THE INFLUENCE OF *PSEUDOMONAS AERUGINOSA* RESISTANCE AND VIRULENCE IN CLINICAL ENVIRONMENTS

This chapter resumes present knowledge on *P. aeruginosa* biofilm-associated virulence factors. Exceptional importance is given to biofilm resistance to antimicrobial agents, phenotypic variation, toxin production, and proteomic analysis of *P. aeruginosa*.

Bacteria growing in biofilm form are often the cause of chronic infections characterized by constant inflammation and severe tissue damage. Despite antibiotic treatment, biofilm-associated infections are difficult to eradicate and quite persistent causing high morbidity and mortality in compromised individuals. A vast number of bacterial species are known to cause these persistent infections (Table 1.1).

Table 1.1 Some human infections associated with biofilms and the microorganisms causing them. (adapted from Costerton et al.[1])

Infection or disease	Bacterial species
Dental Caries	Acidogenic Gram-positive cocci (e.g. *Streptococcus*)
Periodontitis	Gram-negative anaerobic oral bacteria
Otitis media	Nontypable strains of *Haemophilus influenzae*
Musculoskeletal infections	Gram-positive cocci (e.g. staphylococci)
Necrotizing fasciitis	Group A streptococci
Biliary tract infection	Enteric bacteria (e.g. *Escherichia coli*)
Osteomyelitis	Various bacterial and fungal species – often mixed
Bacterial prostatitis	*E. coli* and other Gram-negative bacteria
Native valve endocarditis	Viridans group streptococci
Cystic fibrosis pneumonia	*P. aeruginosa* and *Burkholderia cepacia*
Meloidosis	*Pseudomonas pseudomallei*
Nosocomial infections	
ICU pneumonia	Gram-negative rods
Sutures	*Staphylococcus epidermidis* and *S. aureus*
Exit sites	*S. epidermidis* and *S. aureus*
Arteriovenous shunts	*S. epidermidis* and *S. aureus*
Schleral buckles	Gram-positive cocci
Contact lenses	*P. aeruginosa* and Gram-positive cocci
Urinary catheter cystitis	*E. coli* and other Gram-negative rods
Peritoneal dialysis peritonitis	A variety of bacteria and fungi
Intrauterine device	*Actinomyces israelii* and many others
Endotracheal tubes	A variety of bacteria and fungi

Hickman catheters	*S. epidermidis* and *Candida albicans*
Central venous catheters	*S. epidermidis* and others
Mechanical heart valves	*S. aureus* and *S. epidermidis*
Vascular grafts	Gram-positive cocci
Biliary stent blockage	A variety of enteric bacteria and fungi
Orthopedic devices	*S. aureus* and *S. epidermidis*
Penile prostheses	*S. aureus* and *S. epidermidis*

Among these bacteria *P. aeruginosa* emerge as one of the microorganisms causing infection (Table 1.1). According to Hancock et al.[6], this bacterium is among one of the major opportunistic pathogens that origins hospital-acquired infections, causing up to 9-10% of nosocomial infections. An intrinsic resistance and a high adaptive flexibility are the key mechanisms that contribute to *P. aeruginosa* subsistence. Because several strains of *P. aeruginosa* are becoming resistant to the antimicrobials traditionally used in the treatment of the infections caused by it, it is very important to understand the mechanisms that allow *P. aeruginosa* to cause these infections in order to establish new clinical guidelines for the treatment and eradication of *P. aeruginosa* related infections.
For the reasons presented above, *P. aeruginosa* was selected for the development of this work among the several microorganisms that are known to cause biofilm-related persistent infections.

1.1 BACTERIAL BIOFILMS

Biofilms can be defined as organized communities of planktonic cells embedded in an extracellular exopolysaccharide matrix which is attached to an inert or living surface, such as medical devices[7, 8]. Biofilm formation is a complex process with a highly regulated development[9]. *P. aeruginosa* biofilm formation can be divided in five different stages (Figure 1.1): (i) reversible adhesion, (ii) irreversible attachment, (iii) microcolony formation, (iv) mature biofilm, and, ultimately, (v) dispersion[1, 10].

Biofilm formation and development is initiated once bacterial cells attach irreversibly to the surface. As bacterial cells start to replicate and produce extracellular components biofilm starts to increase its complexity hence formatting microcolonies (Figure 1.1 (iii)). Then biofilm differentiates and matures (Figure 1.1 (iv)), a stage characterized by its complex biofilm architecture[10]. The growth potential of the mature biofilm is restricted by numerous factors such as nutrient availability, nutrient perfusion within the biofilm, removal of waste products, and environmental physical and chemical conditions, such as pH, oxygen perfusion and osmolarity[11].

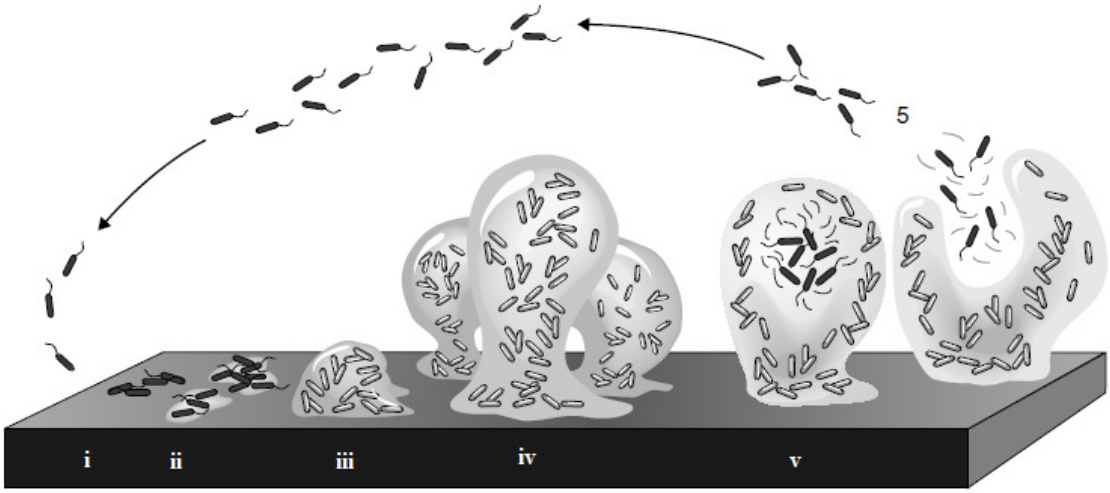

Figure 1.1 Stages of biofilm development. Stage (i) bacterial cells attach reversibly to the surface. Stage (ii) irreversal attachement. Stage (iii) microcolony formation. Stage (iv) maturation and diferentiation of the biofilm. Stage (v) dispersion of single motile cells from the cells. (adapted from Sauer et al[10])

As soon as the biofilm reaches a dynamic equilibrium and critical mass, some cells may detach from the biofilm and may colonize other surfaces (Figure 1.1 (v)). This stage occurs due to marked activity and cellular differentiation in the center of the mature biofilm causing bacterial cells to disperse from within the biofilm, a process called "seeding dispersal"[12]. This process involves enzymes which degrade the exopolysaccharide matrix and death of cell subpopulations within the biofilm[13, 14]. It has been hypothesized that cell death disturbs biofilm architecture and living bacteria profit from nutrients released from the dead ones, aiding the change of surviving bacteria to the motile dispersal phenotype.

All stages of biofilm development are controlled by bacterial population density gene expression regulated by quorum sense or cell-to-cell signaling molecules[15]. In *P. aeruginosa* two quorum sensing systems were identified: *lasR-lasI* and *rhlR-rhlI*. According to Davies et al.[16], both quorum sensing systems are related to biofilm differentiation, although they are not connected to the initial phases of biofilm

formation. Furthermore, Sauer et al.[12], suggested that physiologic changes as well as protein regulation alterations are also implied in biofilm development.

1.2 PATHOGENESIS OF *PSEUDOMONAS AERUGINOSA*

P. aeruginosa is a Gram-negative bacterium and, also, an opportunistic pathogen of plants, animals and humans[17, 18]. This bacterium is one of the leading cause of life-threatening infections such as dermatitis, chronic wounds, urinary tract infections, severe burn infections, ocular infections and respiratory tract infections[17,19]. Also it is often related with the high morbidity and mortality infections in cystic fibrosis patients and immune compromised individuals.

As an opportunistic pathogen, to initiate an infection, *P. aeruginosa* usually needs a substantial drop in the first line of the organism defenses[8]. This drop can have several backgrounds, such as alterations in the mechanisms of the immunologic system, a break in the mucosal or cutaneous barriers, or a disruption in the normal mucosal flora through the use of large spectrum antibiotics[20].

The pathogenicity of this bacterium is due to its ability to adapt phenotypically and to its high degree of genomic flexibility[18]. Moreover, this microorganism is able to augment its pathogenicity and virulence when exposed to stress conditions or when alternates its way of growth to biofilm. In the case of cystic fibrosis, the infected lungs provide a habitat where *P. aeruginosa* faces a diverse range of environmental barriers which cause morphologic modifications and forces its installation in specific niches[21]. When exposed to these conditions in the lungs, this bacterium assumes a mucoid form, this is, converts to a form that produces alginate exopolysaccharides at a large scale causing larger damages and a weak prognostic[22]. Therefore, similar to other bacteria species, such as *Pseudomonas tolaasi*[23], *P. aeruginosa* adapts its phenotype accordingly to regulatory environmental factors.

Besides phenotypic adaptation, *P. aeruginosa* is known to exhibit an intrinsic resistance to antimicrobials. This characteristic holds in clinical environment severe consequences for treatment procedures and infection control.

1.3 VIRULENCE FACTORS

This bacterium exhibits several virulence factors that are crucial to its pathogenesis. Production of extracellular enzymes and exopolysaccharides, low outer-membrane permeability, and motility are among some of the virulence factors produced by *P. aeruginosa*. Some of these aspects will have a special emphasis due to their importance in the upcoming results of this work.

1.3.1 Biofilm Resistance

P. aeruginosa antimicrobial resistance is due to a combination of several factors: this bacteria has an intrinsic resistance to antimicrobial agents due to a low outer-membrane permeability, it can become resistant trough mutations in its chromosomal genes responsible for the regulation of genes involved in resistance: it has a genetic ability to express a large repertoire of resistance mechanisms and, ultimately, it can acquire resistance genes throughout plasmids, bacteriophages and transposons[24].

When growing in biofilm form, bacteria benefit from a distinctive type of resistance, which is different from the standard antimicrobial resistance mechanisms in *P. aeruginosa*, referred above. Biofilms exhibit not only resistance to antimicrobial agents but also to components of the host immune system. As a result, diseases that involving biofilms are usually chronic and difficult to eradicate[15].

Bacterial biofilms can often exhibit a resistance up to 1000 times higher to antimicrobial agents than planktonic bacteria[25], suggesting that some mechanisms associated with antimicrobial resistance of biofilms can differ from those involved in antimicrobial resistance in planktonic cells[15]. Therefore, infections associated with biofilms are difficult to eradicate with antimicrobial treatment and susceptibility tests *in vitro* have shown a considerable resistance to killing.

Biofilm-associated resistance to antimicrobials (Figure 1.2) may be the outcome from several mechanisms: (i) bacterial cells grow very slowly due to oxygen and nutrient diminution within the biofilm; (ii) the physiology of the biofilm can be changed by intercellular signals, which can lead to the expression and overproduction of efflux pumps and augment biofilm resistance to a plurality of drugs; (iii) the negative charge

of the biofilm matrix neutralizes and damages antimicrobial agents, preventing them to reach the bacterial cells within the biofilm, this mechanism is denominated low outer-membrane permeability; (iv) the antimicrobial concentration diminishes from the periphery to the center of the biofilm, impeding antimicrobial agents to kill bacterial cells localized within the biofilm; (v) genetic diversity contributes to the survival of some cells; (vi) induction of the general stress response and activation of quorum-sensing systems can add to biofilm resistance; and (vii) the emergence of phenotypic and persister variants[15, 26].

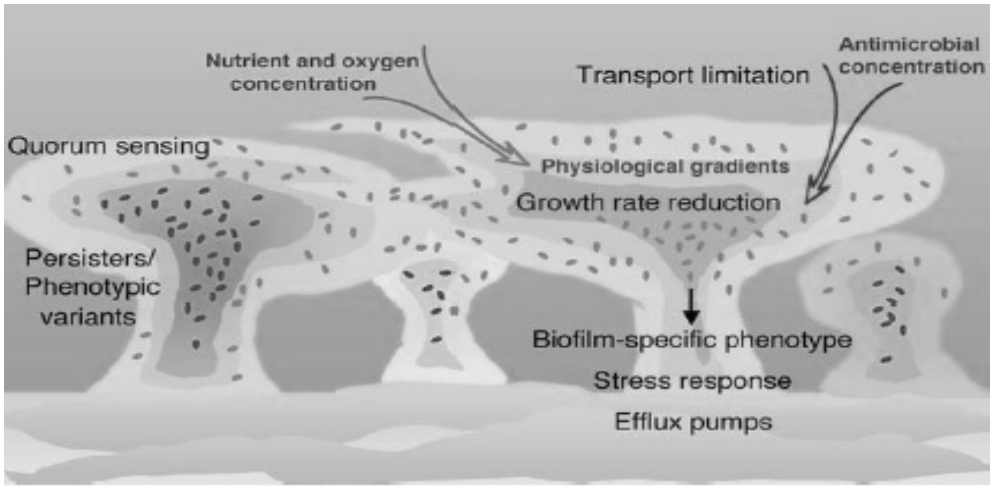

Figure 1.2 Numerous mechanisms comprised in *P. aeruginosa* biofilm-associated resistance. The increase of bacterial density within the biofilm (indicated by darkening colors) regulates gradients of nutrient and oxygen concentration (indicated by narrowing arrow). Reduced antimicrobial penetration is caused by the biofilm matrix (indicated by narrowing arrow). Resistance mechanisms may include the activation of quorum-sensing systems, general stress response and overexpression of efflux pumps. The emergence of biofilm-specific phenotypes, persisters and phenotypic variants is also a well-known resistance factor. (adapted from Drenkard et al[15])

Brooun et al.[27] demonstrated that, though most of the cells present in a biofilm of *P. aeruginosa* are effectively killed in low antibiotic concentrations, when raising the concentration of the antibiotic a small fraction of the cell population does not suffer death. Thus, the authors conclude that only a small fraction of the biofilm cells are related to its high resistance to antibiotics. Therefore, that study shows that, unlike planktonic cells where this small fraction of the cell population would be eliminated by the immune system, biofilm cells are protected by the matrix and this subpopulation of resistant cells can be responsible for the regrowth of the biofilm after antibiotic treatment. These cells are called persisters (Figure 1.3) and are not mutant[28].

In the same way, it is accepted that phenotypic variation of *P. aeruginosa* populations is intertwined with the resistance to high concentrations of antibiotics. Bacterial cells

undergo phenotypic, physiologic and metabolic alterations soon after the adhesion to a surface[15]. Drenkard et al.[29] suggested that the presence of phenotypic variants in biofilms can be partially responsible for great levels of resistance to antimicrobials in *P. aeruginosa* biofilms. In the same study, a protein that controls the conversion between resistant and susceptible bacterial forms was identified. Therefore, the authors conclude that *P. aeruginosa* is able to experience transitory phenotypic alterations associated with its ability to form biofilms which allow it to increase its resistance to antibiotics both *in vivo* and *in vitro*.

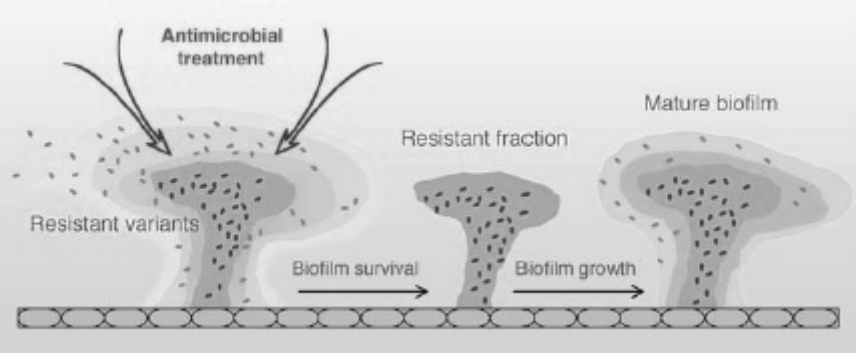

Figure 1.3 Schematic showing the resistance mechanism of persister/phenotypic variants. Antimicrobial treatment eradicates a great portion of biofilm susceptible population. A small fraction of resistant variants survives the antimicrobial treatment and is capable to re-start biofilme development once antimicrobial treatment is finished. (adapted from Drenkard et al[15])

Although these two types of variants described represent a small fraction of the bacterial population that are able to ensure the persistence of *P. aeruginosa* biofilms throughout several cycles of antimicrobial treatment, persister cells only survive to killing by antibiotics[30] while phenotypic variants grow normally in presence of high levels of antibiotics[29]. Consequently, it is believed that different mechanisms are involved in the susceptibility changes experimented by both these variants[15].

1.3.2 Motility

Motility can be considered one of the most interesting features exhibit by *P. aeruginosa*. This characteristic allows *P. aeruginosa* to move across different surfaces, permitting bacteria to establish pathogenic and symbiotic associations with the host[31]. Advantages of motility may include the aptitude to access ideal colonization niches within hosts, disperse in the environment, augment the nutrient uptake, and evade toxic substances released[31]. Bacterial motility can be classified into different types: (i) swimming,

which allows bacteria to move across aqueous surfaces and is a flagellum-mediated type of motility; (ii) swarming, refers to bacterial movement on semisolid medium and is, also, flagellum-mediated; and (iii) twitching, which is a surface motility mediated by type IV pili and allows bacteria to move on solid environments[32-34].

Motility is known to be involved in biofilm development. In a former study, O' Toole et al.[9] concluded that factors associated with bacterial surface, such as flagella and type IV pili, are essential to the *P. aeruginosa* biofilm development (Figure 1.4).

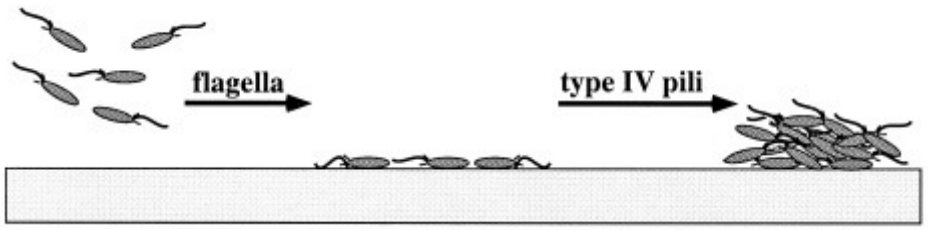

Figure 1.4 Model for the role of flagella and type IV pili in biofilm formation. Flagella-mediated motility is significant for the development of bacterial monolayer in the surface. Type IV pili are associated with initial attachment to the surface and microcolony formation. Flagella also seem to be connected to rapid movement of microcolonies which is imperative to protect the bacterial cells and bacteria migration. (adapted from O' Toole et al[9])

Prior to attachment *P. aeruginosa* cells swim across the surface in order to find the best colonization site. Therefore flagellum-mediated motility is important to the initial attachment to the surface and to initiate the biofilm formation[7]. Swarming is a rapid movement across the surface, allowing bacteria to migrate and disperse to other sites, also, it is thought to be involved in protecting the pathogens against the host macrophages and is associated with secretion of toxins[35]. Type IV pili are essential adhesins that promote initial attachment to a surface. In addition there are evidence that in *P. aeruginosa* biofilms they are associated with both initial attachment and development of microcolonies[34]. In first stages of adhesion, microcolonies can also move across the surfaces throughout swarming motility promoting interactions with the surface and with each other.

1.3.3 Slime Production

One of the virulence factors known to several bacteria species is slime production. Some *P. aeruginosa* strains are also slime producers[37]. In other species such as

staphylococci, slime production promotes its adherence to surfaces, facilitating biofilm development and survival[38].

As for *P. aeruginosa*, slime production seems to have a similar role as the ones described for different bacterial species, being essential in biofilm formation and resistance[39].

The term slime can be explained as extracellular material or the biofilm matrix. Most biofilms are only comprised in less than 10% of dry mass while the matrix accounts for the remaining 90%[40]. The matrix is constituted by a conglomeration of different types of biopolymers in which biofilm cells are embedded and these are responsible for biofilm consistency and adhesion to surfaces[40]. Biofilm matrix also comprises a mixture of DNA, fatty acids, and proteins[41]. Of all biopolymers, exopolysaccharides have a noted importance since they represent a major fraction of the matrix[40].

P. aeruginosa produces at least three distinct exopolysaccharides that contribute to biofilm formation and architecture: alginate, Pel, and Psl[40, 42]. Alginate is one of the extendedly studied exopolysaccharides and it is involved in microcolonies formation in mucoid biofilms and responsible for mechanical stability of mature biofilms[40, 42]. Non-mucoid strains produce Pel and Psl instead of alginate and both are involved in biofilm establishment.

1.3.4 Production of Extracellular Toxins

One of the virulence factors of *P. aeruginosa* is its ability to produce and release several extracellular toxins. These toxins include pigments, phytotoxic factor, phospholipases (hemolysins), proteolytic enzymes, enterotoxin, hydrocyanic acid, and exotoxin[36]. These extracellular toxins are often related to bacteria pathogenesis that can lead to severe consequences and even death of the host. Among those toxins, hemolysin and pyocyanin production will be described with more detail, due to their importance in the overall results of this work.

1.3.4.1 Hemolysin Production

Hemolysin is one of the earlier extracellular toxin released by *P. aeruginosa*. Hemolytic activity in many bacteria is considered an important virulence factor and often contributes to bacteria pathogenicity.

P. aeruginosa is known to produce two distinct types of phospholipases C (PLC's). One of them is hemolytic (PLcHR) and the other nonhemolytic (PLcN)[43]. Consequently it is implied that PLcN has no pathogenic significance, while the production of PLcHR is a noted virulence factor.

Hemolytic PLC has severe effects on the host. Several studies showed that clinical isolates from the lungs are able to produce phospholipase C and cystic fibrosis patients have circulating antibodies against PLC[44]. Also, PLcHR is capable of inducing leukotriene and thromboxane release from host cells, which can be an explanation for the inflammatory responses in *P. aeruginosa* infections[44].

In addition, the virulence associated with PLcHR seems to be connected to the suppression of neutrophil respiratory burst activity, suggesting that PLcHR is a significant toxin which facilitates pseudomonas survival in tissues despite the abundance of neutrophils[43].

1.3.4.2 Pyocyanin Production

Pyocyanin is a blue-green phenazine-derived pigment produced by several *P. aeruginosa* strains[45]. Production of this pigment is seen as one of the most significant virulence factors exhibited by this bacterium[46].

Pyocyanin exhibits a pro-oxidant propriety. Cellular respiratory inhibition is certainly the most significant toxic mechanism of pyocyanin against host cells[45]. Its ability to alter the redox cycle and increase oxidative stress appears to be the fundamental effects on host cells[45]. The increase of the oxidative stress will generate several consequences which depend on toxin concentration and time of exposure[46].

Lau et al.[47] reported that the production of pyocyanin by *P. aeruginosa* is critical in mice lung infections causing tissue damage and necrosis while the progression of the infection. In addition, Koley et al.[48] recently showed that when in biofilm pyocyanin

creates a redox potential gradient, called electroline, which increases iron availability, essential to biofilm development.

1.3.5 Phenotypic Switching

Bacteria are constantly confronted by environmental changes and adapting to these changes is vital to their survival. When these environmental alterations arise, a set of complex regulator mechanisms is activated allowing bacteria to survive. Often adaptation mechanisms include behavioral, physiological and genetic variations, as phenotypic variation[49].

Phenotypic switching or phase variation is considered to be a reversible switch between two phenotypic states. Usually it arises in a small fraction of biofilm population and is much more common than spontaneous mutations[49, 50]. Phenotypic switching is often used to increase population diversity and escape the host immunologic response[50].

Therefore, phase variation is translated in different expression of one or more genes resulting in two subpopulations: one subpopulation missing or having diminished level of phase variable gene(s) and the other expressing the gene completely[51]. A remarkable feature of phenotypic switching is the interchange between 'On' and 'Off' phenotypes. Meaning that bacteria can exhibit one of phenotypic states but keep the ability to switch to the other state[51] whenever different environment stimuli occur[49]. However, the event that results in this interchange is arbitrary because it is impossible to guess which bacterial cells will undergo the switch [51]. However several reports evidence that environmental signals and intercellular regulatory networks are involved on phenotypic switching mechanisms[49, 51, 52].

It is known that even in a homogeneous environment, isogenic bacterial population can display several phenotypes. This phenomena is called inherent phenotype heterogeneity and does not comprise changes in bacterial genes, it is a result of chemical reactions at DNA level, such as alterations in the rates of protein synthesis and degradation[49, 53].

One of the most visible characteristic of phenotypic switching is colony morphology variation[49]. Relationship between colony morphology and bacterial characteristics are yet to be understood. However colony morphology is very significant in the sense that its macroscopic observation can lead to which bacterial features were probably transformed and its connection with external stimuli[49]. In addiction, it can provide

information about the correlation between colony morphology, virulence, and antimicrobial resistance, which is valuable to plan new therapeutic approaches.

Different kinds of colony morphotypes have been identified, such as small colony variants (sCV). These sCV are believed to be responsible for the increased virulence and resistance in *P. aeruginosa* strains[54]. Small colony variants (sCV) have been involved in persistent and recurrent human infections[49], for example sCV have been isolated from the respiratory tract of patients infected with cystic fibrosis[54] and medical devices. For *P. aeruginosa* a sCV is defined as a colony with a diameter of 3 mm or less.

In bacteria that grow in biofilm, different colony morphotypes have also been identified and phenotypic heterogeneity within biofilms is considered one of the main reasons of biofilm resistance[49]. However, this is still a recent study field and is yet to be completely understood. Biofilm-colony diversity is affected by the stage of biofilm development and the proportion of each colony morphotypes is influenced by external factors, showing evidence that these morphotypes are highly connected to mechanisms in biofilm establishment and biofilm survival, increase tolerance to antimicrobials and other stress factors[49, 55, 56].

CHAPTER 2. MATERIALS AND METHODS

In this Chapter, the methods and techniques utilized in the present study are described.

2.1 BACTERIAL STRAINS

In the present study a total of seven strains of *P. aeruginosa* were used. Among these strains, four were reference strains (PAO1, ATCC 10145, CECT 111, and PA14) and three clinical isolates (PAI1, PAI2, and PAI3). All strains were routinely cultured on Trypic Soy Broth (TSB, 30 g/L, Liofilchem) or Trypic Soy Agar (TSA; agar, 15 g/L, Liofilchem; TSB, 30 g/L, Liofilchem) and incubated at 37°C. Bacteria were preserved in criovials at -80 ± 2°C. Before each experiment, bacteria were grown on TSA plates for 24h at 37°C.

2.2 BIOFILM FORMATION

The ability of *P. aeruginosa* to form biofilm was performed as previously described[7, 57]. *P. aeruginosa* strains were grown overnight on TSB at 37°C under agitation (120 rpm). Cell suspension was diluted in order to obtain the final concentration of 1×10^7 CFU/mL. Lastly, the 96-well microtitre plates were inoculated with 200 µL of the adjusted cellular suspension per well. After inoculation, plates were incubated aerobically at 37°C for 24 h under agitation (120 rpm).

After biofilm formation, the content of the plates was discarded and they were washed twice with sterile water in order to remove weakly attached cells and cell products which were in suspension. To remove biofilm-cells from the biofilm matrix, plates were subjected to an ultrasonic bath for 6 minutes.

2.3 QUANTIFICATION OF BIOFILM FORMATION

P. aeruginosa strains biofilm formation was quantified by crystal violet (CV)[57]. Biofilms in 96-well microtitre plates were washed twice with sterile distilled water to remove unattached or weakly attached bacteria. Afterwards 200 µL *per* well of methanol were added and plates were allowed to stand for 15 minutes in order to fix the biofilm-cells to the walls of the well. Then, methanol was discarded and plates were left to dry for 5 minutes at room temperature. Biofilms were stained with 200 µL of pure CV () and plates were incubated at room temperature for approximately 5 minutes. Then, plates were rinsed thoroughly and repeatedly with tap water. Finally, the amount

of biofilm formed was quantified by solubilization of the CV in 200 µL of acetic acid (33% v/v). The optical density (OD) was measured at 570 nm using a microtiter plate reader (Tecan (Sunrise-Basic Tecan), Austria).

2.4 QUANTIFICATION OF THE NUMBER OF VIABLE BIOFILM-CELLS

The number of viable cells obtained from biofilms was inferred through colony-forming unit (CFU). Cell suspensions obtained from biofilm removal were serial diluted, plated on TSA, and incubated overnight at 37 °C.

2.5 SUSCEPTIBILITY TESTING

Susceptibility of *P. aeruginosa* biofilm-cells was evaluated determining the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). The antibiotics ciprofloxacin and colistin were used and purchased from Fluka and Sigma, respectively. Ciprofloxacin and colistin were employed in the current study because they are both used in the treatment of *P. aeruginosa* infections.

The MIC values were determined according to standard European Committee on Antimicrobial Susceptibility Testing (EUCAST) through broth microdilution method[58]. The optical density at 640 nm of bacterial suspension was measured in order to adjust the cellular concentration to 10×10^5 CFU/mL. To maintain viable cell number concentration plates were inoculated within 30 minutes of standardizing the bacterial suspension. Working antibiotic solutions (diluted antibiotic in broth) were dispensed into plates at 50 µL per well with double of the desired final concentration. To each well containing 50 µL of diluted antibiotic agent in broth a volume of 50 µL of inoculum suspension was added. One of the wells only contained 100 µL of Mueller-Hinton (MHB, 22 g/L, Fluka) broth (antibiotic-free medium) to work as a negative control. Therefore, the final inoculum concentration was 5×10^5 CFU/mL. Plates were incubated overnight at 37°C under agitation (120 rpm).

For determination of MBC values, 10 µL were removed from the wells of the microdilution trays after incubation and were plated in TSA plates and incubated at 37 °C for 24 h.

2.6 Motility Tests

Optical density of the biofilm-cell suspension was measured at 640 nm so that the final concentration would be 1×10^8 CFU/mL. Swimming, swarming and twitching motility assays were performed in TSA plates with agar concentration of 0.3 %, 0.5% and 1.5% (w/v), respectively. Plates were inoculated with 1 µL of cellular suspension and then incubated at 37 °C for 24 h. After incubation the circular turbid zone formed by the bacterial cells migrating away from the point of inoculation was measured in millimeters.

2.7 Slime Production Assays

To determine whether *P. aeruginosa* cells collected from biofilms produced slime, Brain Heart Infusion (BHI, 37 g/L, Liofilchem) with Congo Red (0.8 g/L, Sigma) plates were used. Afterwards, optical density at 640 nm was measured in order to adjust the bacterial suspension to the final concentration of 1×10^8 CFU/mL. Then, BHI plates were inoculated with 1 µL of inoculum and incubated overnight at 37 °C.
The strong slime producers usually result in really dark colonies (almost black) with a dry crystalline consistency whereas the negative result was showed by almost pink colonies. Sometimes these pink colonies revealed darkening centers and these colonies indicated an intermediate result.

2.8 Extracellular Toxins Production

2.8.1 Hemolysis Assays

Resembling the previous assay, hemolytic activity of cells derived from *P. aeruginosa* biofilms was assessed using Columbia Agar (CA; agar, 15 g/L, Liofilmchem; Columbia broth, 43 g/L, Liofilchem) with sheep blood plates. Optical density at 640 nm was then measured to regulate the biofilm-cell suspension to the final concentration 1×10^8 CFU/mL. Lastly, CA with blood plates were inoculated with 1 µL of cellular suspension and incubated overnight at 37 °C.

2.8.2 Pyocyanin Quantification

Pyocyanin levels were determined using a previously described[59] quantitative chemical assay. Bacteria of *P. aeruginosa* planktonic and biofilm cultures were pelleted at 13000 rpm for 10 minutes. Then, 750 µL of cell-free supernatants containing pyocyanin were collected and mixed with the same amount of chloroform. Samples were mildly vortexed and centrifuged at 13000 rpm for 1 minute. Afterwards the inorganic phase was removed and 750 µL of 0.2M HCl (Panreac) was added to the organic phase. Samples undergone a brief vortexing and were centrifuged at 13000 rpm for 1 minute. After centrifugation the pink layer was collected and its optical density was measured at 520 nm. Ultimately, the pyocyanin concentration was determined multiplying the OD_{520} values with17.072 and results were expressed in µg/mL.

2.9 PHENOTYPIC CHARACTERIZATION OF BIOFILM-CELLS AND PLANKTONIC CELLS

To assess biofilm population diversity, bacteria derived from biofilms were serial diluted and plated on TSA and grown at 37 °C for 48 h. Colonies were observed by directly placing the petri plates under a magnifying glass (Olympus SZ-CTV) and photographed with a CCD camera (AVC, D5CE; Sony, Tokio, Japan). Each colony morphology identified was classified using ten parameters: form, margin, surface, texture, sheath, opacity, elevation, size, color and diameter (Appendix A). A phenotypic variant was considered when it differed in at least one of the referred morphological parameters.

2.10 STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism, version 5.0. Statistical significance values of the groups' means of biofilm quantification, number of viable biofilm-cells, swimming, swarming, twitching and biofilm and planktonic pyocyanin were evaluated using one-way ANOVA. Following comparisons were performed using Turkey's test. Statistical analyses performed were considered significant when $p < 0.05$.

CHAPTER 3. RESULTS AND DISCUSSION

This Chapter displays the experimental results, observations, and remarks about these results.

In this study seven *P. aeruginosa* strains were tested. Among these four were reference strains (PAO1, CECT 111, ATCC 10145, and PA14) and three were clinical isolates (PAI1, PAI2 and PAI3). Ability to form biofilm, expression of several virulence factors, and antibiotic susceptibility were evaluated for all of the strains.

3.1 Quantification of Biofilm Formation

The biofilm formation ability of *P. aeruginosa* strains is shown in Figure 3.1.

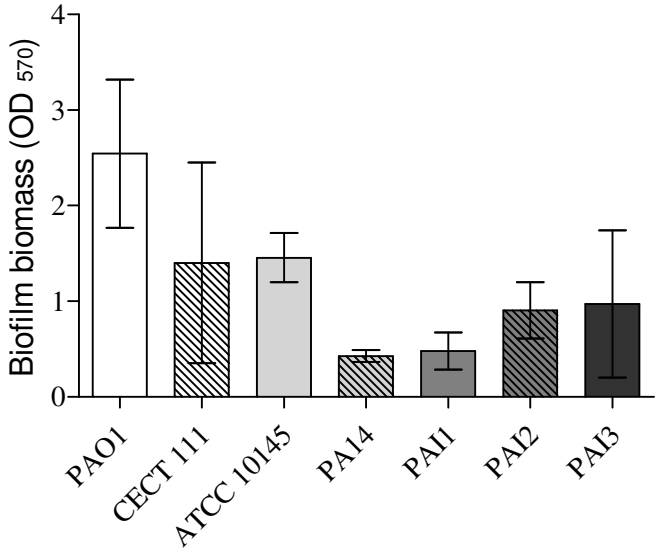

Figure 3.1 Biofilm mass (OD_{570}) of *P. aeruginosa* strains used in this work. Bars represent the average of four independent repeats ± SD.

It can be observed that PAO1 was the *P. aeruginosa* strain that formed the larger amount of biofilm biomass ($p < 0.05$), in contrast with PA14 that formed the lowest quantity of biofilm biomass ($p < 0.05$). Although is noted that PAO1 strain produced a larger biofilm biomass, differences between the quantity of biofilm biomass formed by this strain and CECT 111 and ATCC 10145 strains were not statistically significant ($p > 0.05$). In fact, only the differences of biofilm biomass produced by PAO1 strain and PA14, PAI1, PAI2, and PAI3 strains were found to be statistically significant ($p < 0.05$).

Concerning the amount of viable cells recovered from biofilms (Figure 3.2), it was noted that all *P. aeruginosa* strains formed biofilms with equal number of cells ($p > 0.05$).

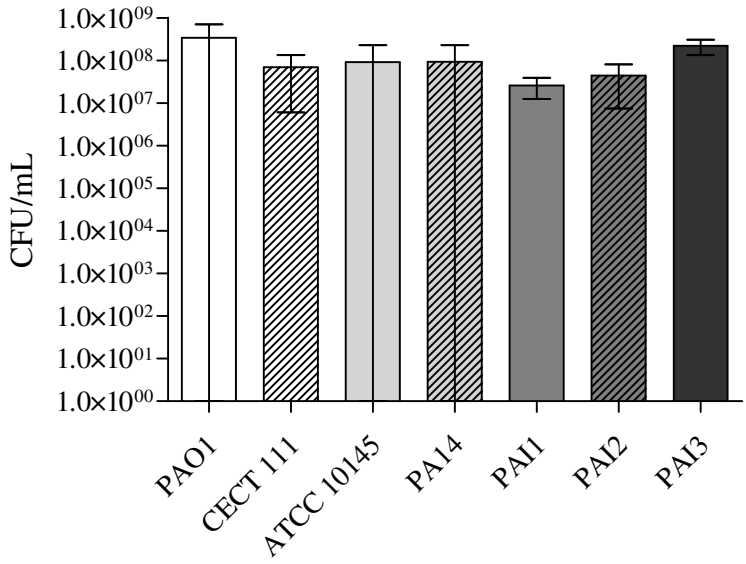

Figure 3.2 Number of viable biofilm-cells of *P. aeruginosa* strains used in this work. Bars represent the average of three independent repeats ± SD.

Taking into account data related to biofilm biomass quantification and number of viable cell, it is hypothesized that the amount of viable cells does not seem to influence the amount of biofilm biomass that each *P. aeruginosa* strain is able to form.

It is well known that biofilms are the favored mode of bacterial growth in nature and infectious diseases[60]. Biofilms are three-dimensional structures in which bacteria are imbedded in a polysaccharide matrix, protein, and DNA[60]. Thus, it is understandable that the amount of viable cells is just one of the many components that may contribute to the biofilm formation ability of *P. aeruginosa*. In this study, evidence that the amount of viable cells does not seem to be a relevant factor for the formation of biofilm was shown. Although all *P. aeruginosa* strains had similar amounts of viable cells, their biofilm formation ability was quite different among some strains. Therefore, the ability to form biofilms by *P. aeruginosa* must comprise other mechanisms, such as the cells capability to form the matrix components.

It is important to add that the crystal violet assay offers information about the adhesion of *P. aeruginosa* strains to a non-biological material. Therefore it can provide information about their adhesion onto medical devices, such as catheters, in which this bacterium can form biofilm leading to nosocomial and chronic infections[39]. It can be

concluded that PAO1 is the *P. aeruginosa* strain which represents higher risk to cause a nosocomial and chronic infection. This fact emphasizes that the ability to form biofilm is only one of the factors involved in the development of a *P. aeruginosa* infection however when dealing with these infections this is only one of the virulence factors to be considered among several that can be exhibit by *P. aeruginosa*.

3.2 VIRULENCE CHARACTERIZATION OF BIOFILM-CELLS

Biofilm-cells were subjected to an extensive phenotypic study in order to compare the expression of virulence factors of *P. aeruginosa*. Virulence factor expression is determinant for the success of *P. aeruginosa* survival in human environment. The display of these virulence factors allows *P. aeruginosa* to adapt to several host environments and to colonize different kinds of niches, being extremely important to the pathogenesis of this bacterium. Among the virulence factors exhibited by *P. aeruginosa* some can be enhanced like low outer-membrane permeability, motility and production of exopolysaccharides and extracellular enzymes. Some of these factors will be thoroughly studied in this work.

3.2.1 Susceptibility testing of biofilm-cells

Currently, the range of traditionally used antibiotics against pseudomonal infections includes ticarcillin, aminoglycosides, ceftazidime, carbapenems (with the exception of ertapenem), ureidopenicillins, aztreonam, cefepime, ciprofloxacin and levofloxacin[61].
In the present study, two antibiotics were chosen to perform the susceptibility tests: ciprofloxacin, an empirical therapy for *P. aeruginosa* infections, and colistin, a last-resort therapeutic option for such infections due to its severe toxicity[61].
Ciprofloxacin belongs to the fluoroquilonone drug class[6]. Ciprofloxacin is known to be particularly effective against gram-negative bacteria. Its action mechanism includes the inhibition of DNA replication and possibly transcription [62].
Colistin belongs to the polymyxins class[6]. Polymixins are positive charged and cyclic peptides antibiotics resultant from several species of *Paenibacillus polymyxa*. This antibiotic class action mechanism comprises the disruption of the cellular membrane resulting in outflow of intracellular components.

MICs and MBCs were determined concerning the two antibiotics of clinical use (Table 3.1). *P. aeruginosa* strains were classified into two classes according to the EUCAST clinical breakpoints: susceptible (S) if MIC was lower than the clinical breakpoint and resistant (R) if MIC is found to be higher than the clinical breakpoint.

Table 3.1 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of ciprofloxacin (CIP) and colistin (COL) against *P. aeruginosa* strains. S stands for susceptible strain and R stands for resistant strain according to the EUCAST clinical breakpoints. EUCAST clinical breakpoints (mg/L): (i) ciprofloxacin: S ≤ 0.5 and R > 1; (ii) colistin: S ≤ 4 and R > 4.

Strain	MIC (mg/L)				MBC (mg/L)	
	COL		CIP		COL	CIP
PAO1	2	S	4	R	16	8
CECT 111	2	S	4	R	16	8
ATCC 10145	2	S	4	R	8	8
PA14	2	S	4	R	16	8
PAI1	2	S	4	R	>16	16
PAI2	2	S	4	R	8	>16
PAI3	2	S	8	R	>16	>16

All *P. aeruginosa* strains were found to be susceptible to colistin and resistant to ciprofloxacin. MIC value was found to be highest for ciprofloxacin (4 and 8 mg/L) and lowest for colistin (2 mg/L). All strains showed the same level of susceptibility against colistin. However PAI3 strain was found to be the most resistant strain among those used in this study (8 mg/L) against ciprofloxacin. Therefore, and since colistin susceptibility was the same for all strains studied, this strain was considered the most resistant *P. aeruginosa* strain studied.

Concerning the MBC values, the lowest and highest values registered was equal for both antibiotics 8 mg/L and >16 mg/L, respectively. Against colistin, PAI1 and PAI3 strains were the *P. aeruginosa* strains that showed highest MBC values (Table 3.1). On the other hand, against ciprofloxacin, PAI2 and PAI3 were the strains that registered the highest MBC values (Table 3.1). Although some differences were noted, it can be said

that *P. aeruginosa* strains have similar MBC values because these values had only a four-fold maximum difference between them.

Given these results, it can be said that the administration of ciprofloxacin to control and eradicate *P. aeruginosa* biofilm-cells is actually ineffective. Therefore, it is important to replace the use of ciprofloxacin as an empirical therapy for *P. aeruginosa* infections.

It is also essential to remark that although pseudomonal infections seem very similar, the bacterial strain causing them can exhibit quite different antibiotic susceptibilities. Therefore, prescribing the same antibiotic treatments to patients revealing analogous symptoms may not be the correct methodology[61]. Sampling the local of infection in order to identify the pathogenic agent can be one of the solutions for this problem. However, a delay on the therapeutic treatment increases patient mortality hence antibiotic therapy should not wait for the sampling results.

Due to their action mechanism, polymyxins are sheltered from cross-resistance with other antimicrobial agents and protected from the rapid selection of resistance[61]. Therefore it is understandable that all *P. aeruginosa* strains show susceptibility against colistin. Hence, using colistin as a therapeutic treatment for *P. aeruginosa* related infections can be more effective than using ciprofloxacin. However to verify this hypothesis *in vivo* testing should be performed. Also, colistin features high toxicity including nephrotoxicity and neurotoxicity[61] and its prescription should only be an option when other therapeutic alternatives have run out.

3.2.2 Motility Tests

It is known that *P. aeruginosa* exhibits different types of motility when growing in environments with different compositions and viscosity[32]. The most characterized in literature are swimming in aqueous medium, swarming on semisolid environments and twitching on solid surfaces[32]. These three types of motility were evaluated for all seven strains of *P. aeruginosa* used (Figures 3.3, 3.4, and 3.5). All *P. aeruginosa* strains tested shown to possess all the three different forms of motility.

3.2.2.1 Swimming

Swimming allows bacteria to move in liquid surfaces and is mediated by flagellum. Also swimming motility is one of the most important factors to the initial attachment of *P. aeruginosa* cells to surfaces upon biofilm mode of growth[19]. Therefore, swimming motility is greatly essential for the effective development of biofilms.

The results of this experiments are shown in Figure 3.3 and were found to be statistically significant ($p < 0.05$) and all *P. aeruginosa* strains presented the ability to swim.

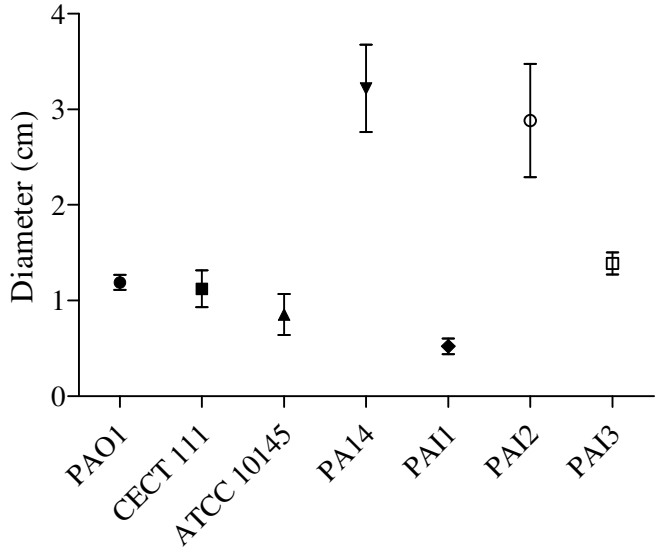

Figure 3.3 Diameter of the swimming migration zone formed by *P. aeruginosa* strains. Symbols represent the average of three independent repeats ± SD.

Because swimming motility is involved in the initial attachment of *P. aeruginosa* cells to surfaces, it was expected that the *P. aeruginosa* strains found to form higher amounts of biofilm biomass (Figure 3.1) would be the ones that registered greater swimming migration zones as well. However, evidence found in this study contradicts this hypothesis. Actually, PA14 strain and the clinical isolates PAI2 showed larger swimming migration zone diameters (Figure 3.3) in comparison with the remaining strains studied ($p < 0.05$). However, these strains formed lower amounts of biofilm biomass (Figure 3.1). In comparison, it was observed that *P. aeruginosa* strains that formed larger amounts of biofilm (Figure 3.1) showed smaller swimming migration zone diameters (Figure 3.3) like PAO1, CECT 111, and ATCC 10145.

These findings led to the hypothesis that, although swimming motility has a huge role in developing biofilms, its importance must be crucial only at an initial stage of biofilm formation and not throughout all phases of biofilm formation. Therefore, it can be said that that swimming translates the adhesion ability of the cells instead of their biofilm formation ability. In fact, swimming motility assays were performed with biofilm-derived cells recovered from biofilms after 24 hours of biofilm formation and not in an initial stage. To better understand the role of swimming motility in *P. aeruginosa* biofilm development, swimming motility assays should be executed in an early stage of biofilm formation or even in planktonic cells.

3.2.2.2 Swarming

Swarming is flagella-mediated type of motility and is defined as a quick and coordinated multicellular movement across a semi-solid surface[33]. The results of these experiments are presented in Figure 3.4.

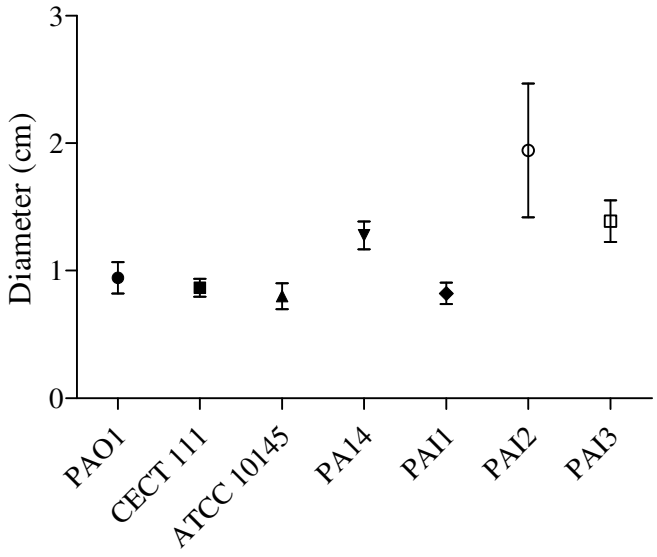

Figure 3.4 Diameter of the swarming migration zone formed by *P. aeruginosa* strains. Symbols represent the average of three independent repeats ± SD.

Concerning swarming motility, PA14, PAI2, and PAI3 ($p < 0.05$) were the *P. aeruginosa* strains that showed swarming rings with the larger diameters while the remaining strains formed the smaller rings (Figure 3.4). The differences between the diameters of PAO1, CECT 111, ATCC 10145, and PAI1 strains swarming migration zone were not found to be significant ($p > 0.05$).

Swarming is a type of social motility characterized of some degree of cellular differentiation and regulation through cell-to-cell communication usually named "social phenomena"[33]. Swarming allows rapid colonization of surfaces by highly differentiated swarm cells. Swarming motility has been associated with resistance to several antimicrobials[63]. According to Lai et al[33] this multiresistant characteristic of swarm cells is also applicable to *P. aeruginosa*.

Given this evidence, it was expected that strains that formed larger swarming migration zones (Figure 3.4) would be more resistant to the antibiotics used in this study. However, based on the results this hypothesis was not confirmed since all *P. aeruginosa* strains showed very similar resistance profiles against ciprofloxacin and equal susceptibility profiles against colistin. And, for instance, PAI3 strain considered the most resistant against ciprofloxacin (Table 3.1) was not the strain that developed greater swarming migration zones.

As swarming-associated resistance is a transient resistance this means that this kind of resistance is unstable and can be lost[33] and, also, swarm cells develop a resistant phenotype as a result of cell differentiation and not in response to antibiotic stress[33]. This can explain why the strains that developed greater swarming zones were not the ones found to be more resistant to both antibiotics tested. To better understand the relationship between resistance and swarming-cells susceptibility tests should be performed with swarming-cells instead of biofilm-cells.

3.2.2.3 Twitching

Twitching motility is mediated by type IV pili, it consists in a slow bacteria movement and plays an important role in the development of the biofilm architecture[34].

The results of this experiments are shown in Figure 3.5, these were found to be statistically significant ($p < 0.05$) and all *P. aeruginosa* strains tested displayed the ability to twitch.

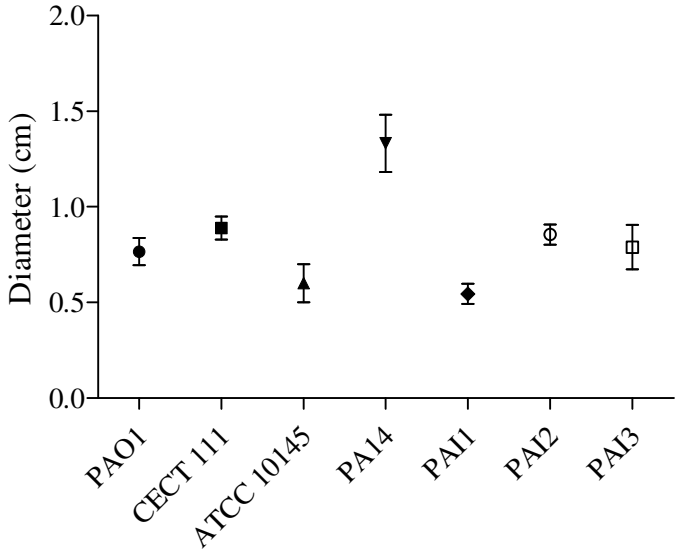

Figure 3.5 Diameter of the twitching migration zone formed by *P. aeruginosa* strains used in this study. Symbols represent the average of three independent repeats ± SD.

According to Figure 3.5, PA14 strain showed larger twitching migration zones. The three other reference strains (PAO1, CECT 111, and ATCC 10145) showed the smallest of the migration zone diameters as well as one of the clinical isolates (PAI1). Although some differences were noted, besides PA14 strain, and agreeing to Figure 3.5, all *P. aeruginosa* strains showed close migration zone diameters values.

Type IV pili are thought to be implicated in a direct role in stabilizing interactions with the abiotic surface and/or cell-to-cell interactions necessary to form a microcolony[9]. Taking into account these facts it is understandable that twitching motility contributes to biofilm formation and strains which exhibit the larger twitching migration zone diameters were expected to be great biofilm-forming strains since cell-to-cell and/or cell-to-surface interactions would be more cohesive. In this study, evidence show the opposite, PA14 strain showed the larger twitching migration zone diameter but it was the *P. aeruginosa* strain that formed the least amount of biofilm biomass (Figure 3.1) while strains with greater biofilm-forming ability, such as PAO1, CECT 111, and ATCC 10145 (Figure 3.1), displayed smaller twitching migration zone diameters. These results suggest the presence of unidentified and additional adhesions that promote cell-to-surface interactions besides type IV pili.

3.2.3 Slime and Extracellular Toxins Production

3.2.3.1 Slime Production Assays

One of the virulence features known to several bacteria species, including *P. aeruginosa*, is slime production[37]. Slime production promotes its adherence to surfaces, facilitating biofilm development and survival[38,39].

To evaluate slime production of cells obtained from biofilms of all *P. aeruginosa* strains, a qualitative method that consists in spreading biofilm-cells in BHI agar plates was used. The results obtained are outlined in Table 3.2.

Table 3.2 Classification of slime and hemolysin production of P. *aeruginosa* strains.

Strain	Slime Production
PAO1	Negative
CECT 111	Negative
ATCC 10145	Negative
PA14	Moderate
PAI1	Strong
PAI2	Moderate
PAI3	Moderate

P. aeruginosa strains were classified into three different categories according to the intensity of the red color of the *P. aeruginosa* colonies obtained on BHI agar plates: “negative” if the color of the colonies was the same as the medium, “moderate” if the color of the colonies was slightly darker than the medium, and “strong” if the color of the colonies was much darker than the medium (Figure 3.6).

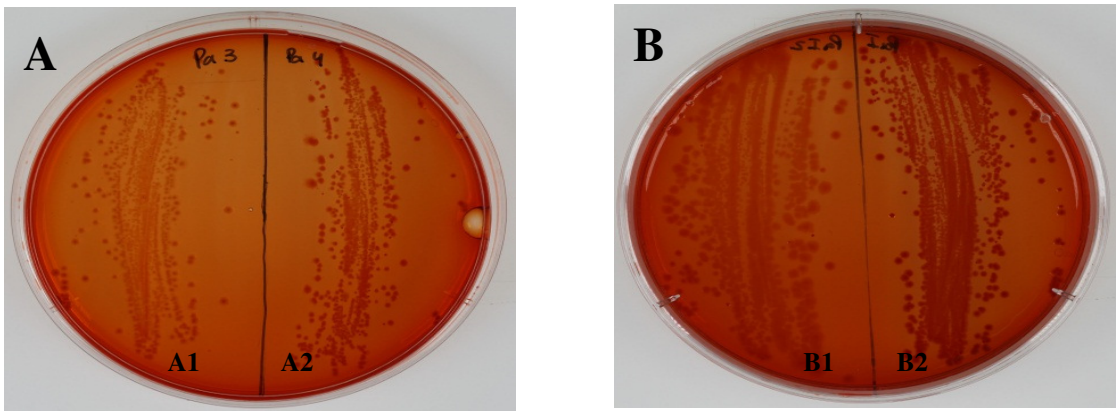

Figure 3.6 Slime Production Assay. **A:** BHI agar plate demonstrating the difference between negative (A1) and moderate (A2) slime production. **B:** BHI agar plate representing the difference between moderate (B1) and strong (B2) slime production.

Using these criteria, the slime production of three of the strains PAO1, CECT 111, and ATCC 10145 was considered negative, two clinical isolates (PAI2 and PAI3) and strain PA14 demonstrated to have moderate slime production and, finally, only one strain PAI1 showed a strong slime production (Table 3.2).

It has been reported that *P. aeruginosa* strains are slime producers. In a study aiming to evaluate biofilm production by uropathogens isolated from patients with urinary infections, Niveditha et al.[37] isolated among fifty strains two *P. aeruginosa* strains and both strains were slime producers.

According to Freeman et al.[38], the production of slime of coagulase negative staphylococci may benefit their adherence to biopolymers and also be accountable for reduced therapeutic response and relapse. The authors also stated that the production of slime may be associated with inhibition of the immune response. The production of slime by *P. aeruginosa* has a similar role to the one described by Freeman et al.[38]. It is known that this bacterium produces a great amount of exopolysaccharides and these besides being associated with biofilm formation are also implied in protecting this species against the complement system, mucociliary, phagocytosis and reduced antimicrobial activity since it makes the penetration in the bacterium demanding[39].

Although it is thought that strains that produce larger slime amounts are less susceptible to antibiotic stress, given that in this study all *P. aeruginosa* strains showed identical resistance profile against ciprofloxacin and an equal susceptibility profile against colistin, it is tempting to infer that slime production does not seem to be implied as a resistance mechanism or this hypothesis is not applicable in the case of ciprofloxacin and colistin.

Also, it was expected that the strains with a stronger ability of slime production were the ones capable of producing greater amounts of biofilm biomass. Based on the results referring to quantification of biofilm biomass (Figure 3.1), it is correct to conclude that the slime production ability does not have such a linear connection with biofilm-forming ability. For instance, PAO1, CECT 111, and ATCC 10145 were the strains considered able to form greater amounts of biofilm biomass (Figure 3.1) and were their slime production ability was considered negative (Table 3.2).

Several researchers use this method as a technique to quantify bacteria biofilm-forming ability. In a study comparing three different methods for detection of biofilm formation in Staphylococci clinical isolates, two of them being the CV and the BHI with Congo Red, Mathur el al.[64] found that the BHI with Congo Red method showed a very poor correlation with the two other methods. In the same study the authors also conclude that the BHI with Congo Red had a low sensitivity and accuracy[64]. In the present study, discrepancies between the two methods were also found. Based on the data obtained, it is not recommended to use the BHI with Congo Red method to evaluate the biofilm-forming ability of *P. aeruginosa*. Opposing the CV method, the BHI with Congo Red is a qualitative and visual technique, which depends on individual interpretation. However it provides truthful and useful information about the amount of slime produced while bacteria form the biofilm and is a simple and reproducible method.

3.2.3.2 Hemolysis Assays

Because hemolysins produced by several bacteria are recognized to contribute to their virulence, the hemolytic activity of the *P. aeruginosa* strains was also characterized in this study. Results obtained are presented in Table 3.3.

It is known that *P. aeruginosa* produces two different kinds of phospholipases C (PLC's): one being hemolytic (PLcHR) and the other nonhemolytic (PLcN)[43]. Therefore, while PLcN has no pathogenic significance, PLcHR is accepted as one of the most important virulence factors that can be expressed by *P. aeruginosa*[43].

Table 3.3 Classification of hemolysin production of P. *aeruginosa* strains.

Strain	Hemolysin Production
PAO1	Positive
CECT 111	Negative
ATCC 10145	Positive
PA14	Positive
PAI1	Positive
PAI2	Positive
PAI3	Positive

To determine the hemolytic activity of *P. aeruginosa* cells derived from biofilms were spread onto Columbia agar plates with blood and results were recorded as following: "negative" if the culture medium maintained its appearance and "positive" if the culture medium around the *P. aeruginosa* colonies became translucent, indicating lysis of the blood cells present in the culture medium (Figure 3.7). According to data obtained (Table 3.3) all strains with the exception of reference strain CECT 111 exhibited hemolytic activity.

These results showed that probably only CECT 111 strain is unable to produce PLcHR. All the other strains express one of the most virulent aspects of *P. aeruginosa*.

Hemolytic PLC was associated with increased vascular permeability, organ damage, and even death when injected into mice according with previous studies[65]. Also, several studies reported that PLC's have proinflammatory activity *in vitro* and *in vivo*[65].

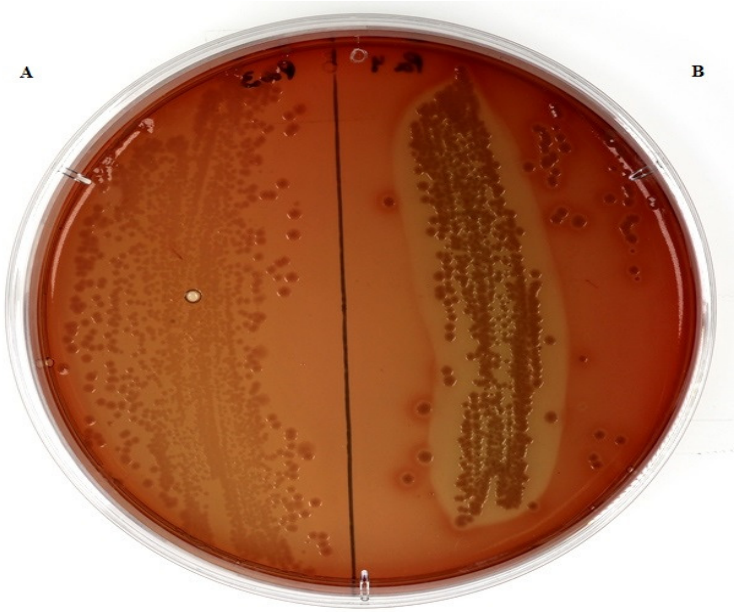

Figure 3.7 Hemolysis Assay. Blood agar plate demonstrating the nonhemolytic activity of PlcN (A) and hemolytic activity of PlcHR (B).

Therefore, considering only these results, all *P. aeruginosa* strains can be considered more virulent than CECT 111 strain when involved in infections since the hemolytic activity showed by these strains can lead to devastating consequences to the host's immune system and even to organ damage and death.

3.2.3.3 Pyocyanin Quantification

It is known that some *P. aeruginosa* strains are able to produce a blue-green pigment named pyocyanin. Pyocyanin is responsible for increasing oxidative stress and alter the redox cycle on host cells[45]. Also pyocyanin is able to disturb the cellular respiration and inhibit epidermal cell growth[45]. Therefore it is nowadays considered one of the most relevant virulence factors that a *P. aeruginosa* strain can exhibit.

In order to quantify the amount of pyocyanin produce by the *P. aeruginosa* strains, for both planktonic and biofilm cells, a chemical assay was performed and the amount of pyocyanin present in the supernatant was quantified.

The results of these experiments are presented in Figure 3.8 and Figure 3.9.

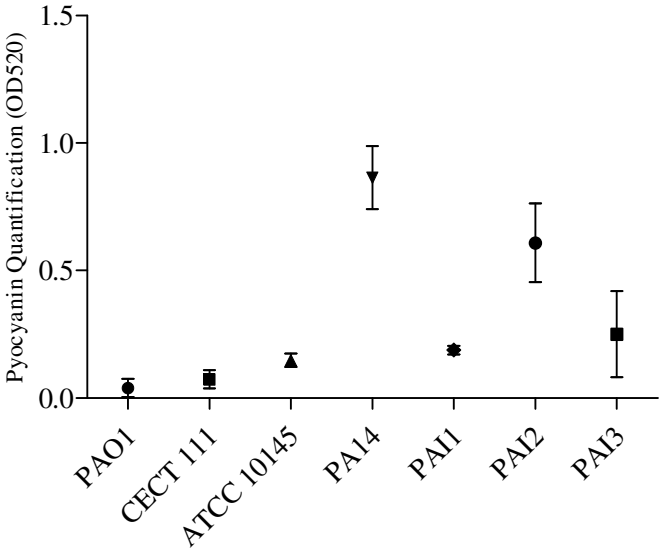

Figure 3.8 Amount of pyocyanin produced (OD_{520}) by *P. aeruginosa* planktonic cells. Bars represent the average of four independent repeats ± SD.

The results obtained for pyocyanin quantification for *P. aeruginosa* planktonic cells were found to be statistically significant ($p < 0.05$). For planktonic cells, the *P. aeruginosa* strain that produced a larger amount of pyocyanin was PA14 strain, followed by the clinical isolate PAI2 (Figure 3.8). The remaining strains showed similar amounts of production of this pigment ($p > 0.05$) and this amount was not considered significant.

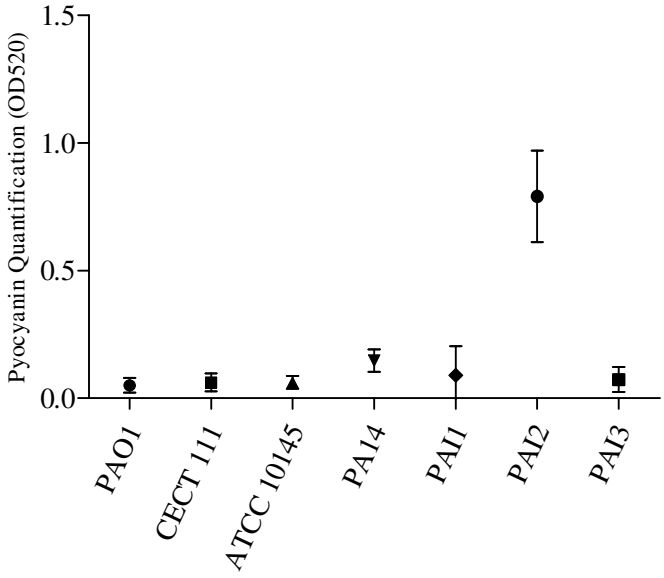

Figure 3.9 Amount of pyocyanin produced (OD_{520}) by *P. aeruginosa* biofilm cells. Bars represent the average of four independent repeats ± SD.

The results of pyocyanin quantification of biofilm-cells were considered significant ($p < 0.05$). Interestingly, for biofilm-cells, PAI2 strain produced a larger amount of pyocyanin (Figure 3.9). The remaining strains tested produced resembling amounts of pyocyanin (Figure 3.9) and these amounts were considered not significant ($p > 0.05$).

From these results, it is tempting to infer that *P. aeruginosa* way of growth influences the production of this pigment. As showed, the change to biofilm way of growth can augment or diminish the amount of pyocyanin produced. This evidence is noted for PA14 and PAI2 strains. The first produces a larger amount of pyocyanin when growing in planktonic form and this amount diminishes to irrelevant quantities when the growth is alternated to biofilm. As for the last strain, it is observed that the biofilm mode of growth can augment the pyocyanin production.

In a clinical context this evidence may be considered important. Since *P. aeruginosa* is known for its ample biofilm forming ability in infected hosts, is essential to evaluate pyocyanin production of *P. aeruginosa* strains biofilm-cells. In cystic fibrosis patients pyocyanin has a severe toxic effect as it induces neutrophil apoptosis and damages the neutrophil-mediated host defense[19]. In recent developments, several substances, as peptides and proteins, have been used as quorum sensing and virulence factor inhibitors[19, 66, 67]. These substances caused the reduction and inhibition of pyocyanin production and therefore are a possible solution for the treatment of *P. aeruginosa* infections involving pyocyanin producing strains.

3.2.4 Phenotypic switching

Phenotypic switching or variation is considered a reversible state between two or more phenotypes. It is a common mechanism used by bacteria to increase their diversity and overcome environmental changes and exposure to stress conditions, as such phenotypic switching can be described as an adaptive mechanism.

The most visible characteristic of phenotypic switching is colony morphology variation. Even though the connection between phenotypic switching and virulence and antimicrobial resistance is not completely understood the study of colony morphology can be a useful tool to understand which bacterial features were possibly changed and its association with external stimuli.

In this work, colony morphologies both from planktonic and biofilm-derived cells were recorded in order to establish whether bacteria mode of growth can affect colony morphology. For that, the number of colony morphotypes was registered and the results are shown in Figure 3.10 and Figure 3.11. It was also recorded the number of small-colony morphotypes, a colony phenotype commonly associated with bacterial resistance. The colony classification system used is described in Appendix A (Tables A.1 and A.2).

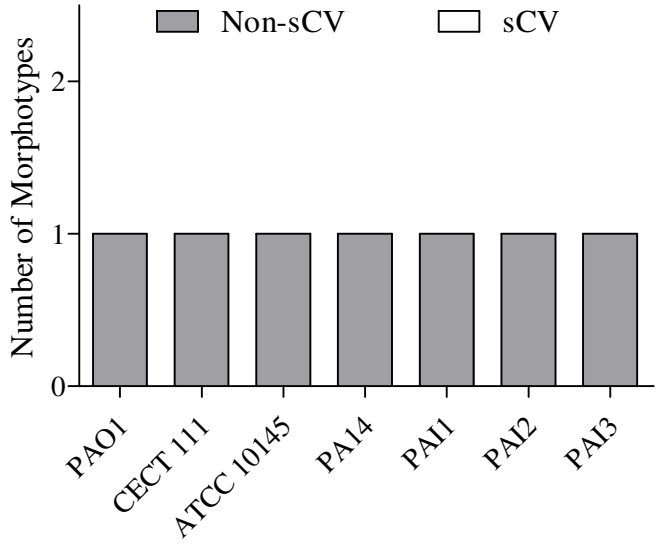

Figure 3.10 Number of colony morphotypes registered for *P. aeruginosa* planktonic cells. Non-sCV stands for non-small-colony variant strain and sCV stands for small-colony variant.

As showed in Figure 3.10 phenotypic variation was not registered for any *P. aeruginosa* planktonic-derived cells since every *P. aeruginosa* strain only exhibit one colony morphotype. The colony morphotypes recorded for planktonic cells are displayed in Appendix B (Figure B.1).

In contrast, for biofilm-derived cells (Figure 3.11), the phenotypic variation phenomenon was observed since these cells were able to form two or more distinct colony morphotypes, being this observed for every *P. aeruginosa* strain. The colony morphotypes documented for biofilm-derived cells are presented in Appendix C (Figure C.1).

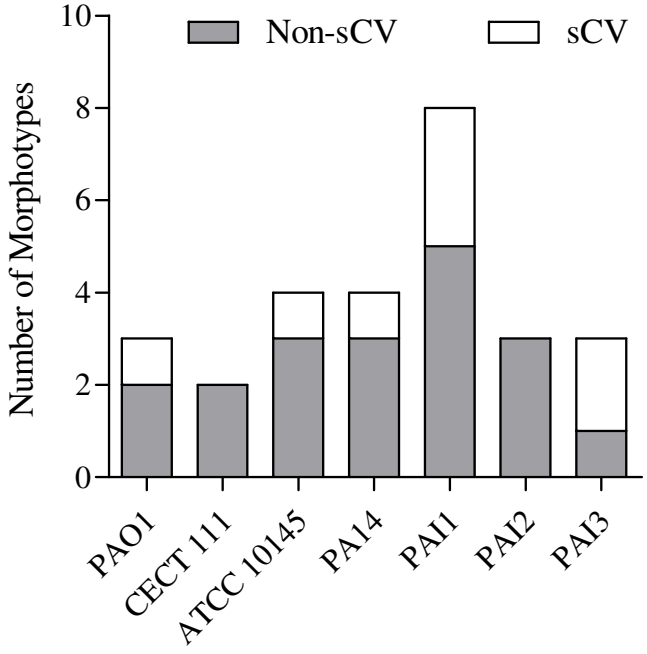

Figure 3.11 Number of different colony morphotypes recorded for *P. aeruginosa* biofilm-derived cells. Non-sCV stands for non-small-colony variant strain and sCV stands for small-colony variant.

When growing in biofilm-form, bacteria express a biofilm-specific phenotype, different from that assumed in the corresponding planktonic cells[49]. Changing the bacteria way of growth from planktonic to biofilm comprises a large number of phenotypic, physiological and metabolic differentiations[49]. These biofilm-associated phenotypic differentiations are often correlated with antimicrobial resistance, persistence and increased virulence.

Moreover, when growing in biofilm-form bacteria can display a phenotypic heterogeneity as a result of a variety of microniches with specific biological activities within biofilms[49]. Therefore, biofilm diversity can be translated in distinct colonies due to those sub-populations[49]. This colony heterogeneity was observed for biofilm-derived cells in every *P. aeruginosa* strain studied as opposed to planktonic cells of the same strains which exhibit only one type of colony morphology.

According to Stewart and Franklin[68], in a mature biofilm, three different physiological states can be expected: cells near the biofilm-bulk-fluid interface surrounded by an environment that contains both substrate and oxygen; cells in the intermediate zone surrounded by an environment that comprises only substrate; and cells in the deeper zone surrounded by an environment that does not contain oxygen or substrate. Cells near the biofilm-bulk-fluid interface can display similarities with the planktonic cells.

As so, some colonies formed by planktonic and biofilm-derived cells presented phenotypic similarities (Appendix B and C). These results lead to the conclusion that most of the cells recovered from *P. aeruginosa* biofilms were located in the more superficial layer of the biofilms. And because other colony phenotypes were observed it can be inferred that possibly biofilm-cells were also recovered from the remaining layers.

The observed biofilm-associated phenotypic heterogeneity found in all strains studied only come to reinforce the fact that *P. aeruginosa* biofilms exhibit a great adaptability since biofilm-derived cells were able to form more than one colony morphotype. Yet the mechanisms that make this phenotypic variation occur are not entirely understood. It is also important to refer that all *P. aeruginosa* strains gave rise to a different number of biofilm-associated morphotypes, being PAI1 the *P. aeruginosa* strain that displayed the higher number of distinct biofilm-associated morphotypes (Figure 3.11). These differences can be attributed to the different genetic background of all *P. aeruginosa* strains used.

Moreover phenotypic variation is also considered as a way to escape host immune mechanisms since it is related with several human pathogens. Since phenotypic switching affects host-pathogen interactions, it confers human pathogens the ability to evade innate and acquired immune defenses during colonization and infection[69]. Subpopulations created by phenotypic variation have the ability to exhibit different kinds of antigens and the host's immune system only reacts to those recognized. Thus, the immune system is incapable to successfully eliminate those pathogens[49]. This evading mechanism resides in the antigenic variation principle. Although if bacteria are able to switch off the expression of a determined antigen, the biological function associated with that antigen can be lost. So in order to maintain the biological function related with that antigen functional redundancy is essential. However, the frequency by which antigen variation arises is yet to be fully understood and studied[69].

Taking into account this concept of enhanced virulence it is correct to hypothesize that all *P. aeruginosa* strains studied when growing in biofilm-form display some type of evade mechanisms to the host's immune system once all strains exhibited more than one kind of colony morphotype (Figure 3.11). Once more, PAI1 strain can be highlighted since it was the *P. aeruginosa* strain that displayed a larger number of colony

morphotypes and, therefore, can be noted as the *P. aeruginosa* strain among those studied with the higher ability to elude the host's immune system.

Phenotypic variation can also lead to the development of microcolonies, denominated small-colony variants (sCV). The development of such colony morphotype is exhibited by several bacterial species, including *P. aeruginosa*. To be considered a sCV morphotype, *P. aeruginosa* colonies must have a diameter equal or smaller than 3 mm. Some of the *P. aeruginosa* strains studied displayed sCV (Figure 3.12) when growing in biofilm-form (Figure 3.11) but planktonic cells did not develop these microcolonies (Figure 3.10).

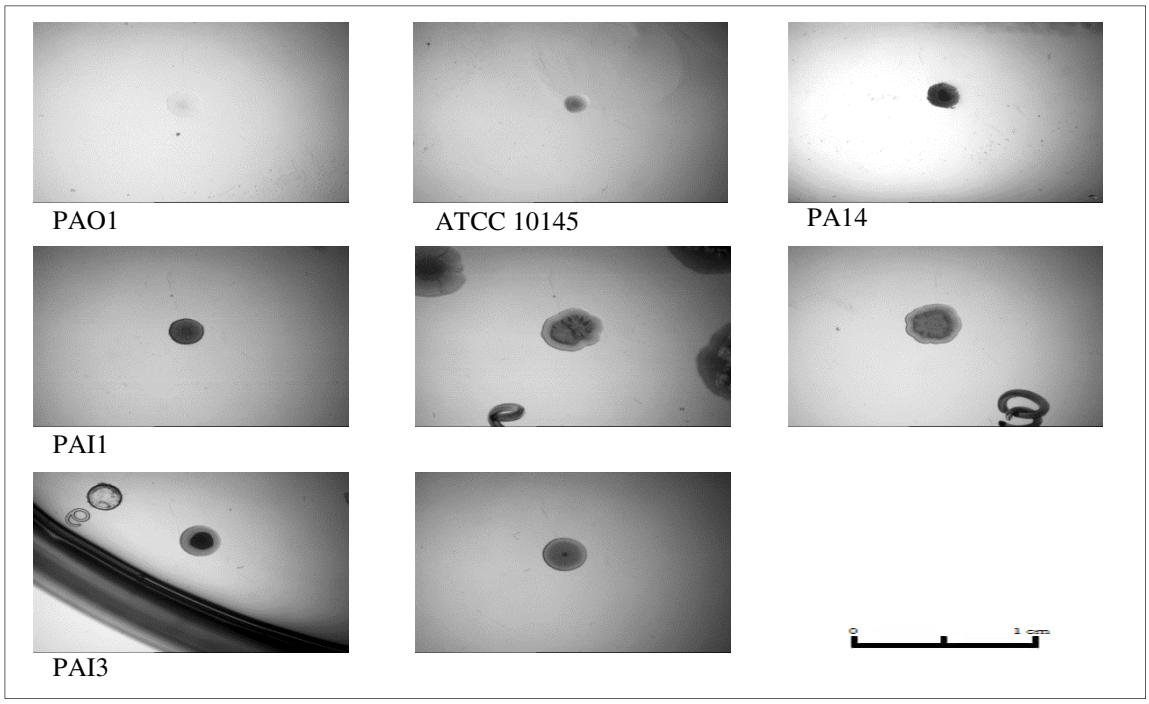

Figure 3.12 Small-colony variants (sCV) registered for *P. aeruginosa* biofilm-derived cells.

Besides enabling bacteria to escape the host's immune system, phenotypic switching also influences bacteria antimicrobial resistance. Currently standard therapeutic treatments and disinfection procedures are becoming less effective in eradicating human pathogens and, on the other hand, the antimicrobial over-using is augmenting the emergence of resistant bacteria[49]. Bacterial resistance comprises several types of mechanisms such as low outer-membrane permeability and efflux pumps, for example. However, in an earlier study Massey et al.[70] reported that *Staphylococcus aureus* exposure to gentamicin resulted in the emergence of sCV and in the absence of gentamicin these sCV were able to revert to normal phenotype.

In the light of these findings, it can be said that sCV morphotypes may be associated with antimicrobial resistance, causing bacterial persistence and pathogenicity. Therefore, the sCV morphotype is indeed associated with a better adaptive ability of bacteria to environmental changes and stress. Although planktonic cells did not exhibit sCV morphotype, this morphotype is not as exclusively a biofilm-specific phenotype, as demonstrated before[71]. Therefore, it is unclear the existence of a particular morphotype that could characterize exclusively a biofilm-associated phenotype.

In this work, when growing in biofilm-form, some *P. aeruginosa* strains, among those PAO1, ATCC 10145, PA14, PAI1, and PAI3, developed sCV morphotypes (Figure 3.12) and PAI1 strain was the one that developed a larger number of those morphotypes. Given these evidences, it was expected that these strains presented a higher resistance to the antibiotics used in this study. However this fact was not observed once all strains exhibit similar resistance both to ciprofloxacin and colistin. To further comprise the relation of antimicrobial resistance and sCV morphotypes it is necessary to isolate these colonies and expose them to antibiotic stress.

CHAPTER 4. CONCLUSION

In this final Chapter the main conclusions of this work are presented. Some future perspectives are also addressed.

Nowadays, in clinical settings the increased resistance of bacteria, including *P. aeruginosa*, leads to an augmented infection persistence and causes an increase of the morbidity and mortality associated with those infections.

In natural scenarios, bacteria grow in organized communities called biofilms. Within the biofilm community, bacteria assume distinct phenotypes which contribute to biofilm adaptation to different environments, stress conditions and resistance to several antimicrobials. Therefore it is important to understand if biofilms formed by different *P. aeruginosa* strains share some characteristics. However it was not verified a common *P. aeruginosa* biofilm profile. And there was not verified a direct relationship between all virulence factors studied. Due to this variability in the biofilm characteristics studied no strain was considered more virulent than other strain. For instance, since they already have been exposed to several stress conditions, it was expected that clinical isolates showed a greater virulence. However, because of the variability found that was not verified.

As shown in Chapter 3, the seven strains of *P. aeruginosa* studied have different biofilm-forming ability. The biofilm-forming ability can be related with increased virulence and resistance. However, in this study, a greater biofilm-forming aptitude did not translate in a higher resistance against the antibiotics used since the resistance profile against ciprofloxacin and the susceptibility profile against colistin were found to be very similar among all *P. aeruginosa* strains tested.

Biofilm-associated resistance in *P. aeruginosa* is still poorly understood and involves several complex mechanisms[72] and those mechanisms can be basically caused by: (i) the low outer-membrane permeability, which difficult the antimicrobial agents penetration within the biofilm; (ii) the limited gradients of oxygen and nutrients in the deeper layers of the biofilm, causing cells to grow slower; and (iii) the emergence of phenotypic and persister phenotypes. As referred above, in Chapter 3, all *P. aeruginosa* strains studied revealed similar resistance profile against ciprofloxacin and equal susceptibility profile against colistin, with the exception of PAI3 (Table 3.1) which revealed a higher resistance to ciprofloxacin. The same results showed that the use of ciprofloxacin in the treatment of *P. aeruginosa*-related infections is an ineffective and outdated therapeutic method since all *P. aeruginosa* strains were resistant to this antibiotic. Therefore, actual clinical guidelines should be reconsidered and the use of ciprofloxacin as an empirical therapy for *P. aeruginosa* infections should be replaced.

Moreover, the usage and misusage of antibiotics, such as ciprofloxacin, have been associated with increased resistance and infection persistence. For instance, colistin would be a great replacement of ciprofloxacin since all strains studied displayed to be susceptible to this antibiotic. However, due to its severe toxicity, colistin is considered a last resort antibiotic. In recent years, studies show that antibiotic combinatory therapies are indeed effective in killing *P. aeruginosa* isolates even under biofilm conditions and some of these combinatory therapies combine ciprofloxacin and colistin with other antibiotics and even with each other[73]. Moreover, due to the increased resistance caused by the use of antibiotics, recently agents that antagonize virulence factors have been developed, representing a potentially efficient approach to the treatment of infections caused by *P. aeruginosa*[4].

Besides biofilm-associated resistance, *P. aeruginosa* displays several virulence factors imperative to its pathogenesis. For instance, all *P. aeruginosa* strains studied have specific motility abilities. These particular motility abilities influence the biofilm way of life, in the sense that they are often related with increased virulence and possibly higher resistance. However, given this study results, there was not a clear link between the motility abilities studied, biofilm-forming ability, and increased resistance. To further understand these relations future works should be done in order to characterize proteomic and genetically the mechanisms comprised in the presence and loss of the organelles responsible for bacterial motility throughout the different stages of biofilm formation and under antibiotic stress.

Slime production ability is also a virulence factor that is often implied in increased biofilm-resistance and biofilm formation. Based on the results found in this work, there is not a connection between slime production, increased resistance and biofilm formation.

The ability of *P. aeruginosa* to produce extracellular toxins is also a very important virulence factor. These toxins often lead to serious consequences in patients and even to death. In this work hemolysin and pyocyanin production were evaluated. All *P. aeruginosa* strains revealed distinct production and release of the extracellular toxins studied. From the pathogenic point of view, hemolysin and pyocyanin production seem to be quite important, since they contribute to bacterial ability to avoid the host's immune system. Hemolytic activity of *P. aeruginosa* is translated in increased vascular permeability, organ damage, and even host's death, as for pyocyanin it has a relevant

role in pathogenesis of cystic fibrosis, causing neutrophil apoptosis and damaging the neutrophil-mediated host defense. Although the expression of these virulence factors was evaluated, it was concluded that all *P. aeruginosa* strain had different virulence profiles, meaning that was not any strain considered more virulent than other. Similarly to motility abilities of *P. aeruginosa*, future works could focus the proteomic profiling and genetic characterization of the apparatuses that lead to the production of these extracellular toxins in order to identify possible drug targets and the development of agents that are able to inhibit or reduce the severe effects caused by these toxins.

Lastly, biofilm development embodies a key strategy to bacterial survival. Phenotypic switching offers a source of bacterial diversity central to the survival in diverse and hostile environments since it presents several variants that are able to persist to different types of stress conditions. Also, phenotypic variation has been connected with increase virulence and bacterial resistance. For instance, the best evidence of this connection is the alteration of resistance and virulence profiles showed by sCV. However, the link between biofilms and biofilm-specific colony morphotypes is yet to be understood. Therefore, it is essential to comprehend how phenotypic switching contributes to antimicrobial resistance and infection persistence. Also, it is imperative to identify biofilm-associated colony morphologies and understand their role in biofilm mode of growth, chronic infections and increased bacterial resistance. In short, the understanding of the phenotypic switching phenomena is necessary to develop suitable disinfection and therapeutic protocols. The awareness of the phenotypic variation has a significant importance in clinical settings, since it can solve real issues, such as antimicrobial resistance and development of new therapeutic methodologies.

In summary, it is important to note that there is not an universal *P. aeruginosa* biofilm profile. Although resistance and susceptibility profiles against ciprofloxacin and colistin, respectively, were found to be identical for all strains tested, all virulence factors tested were found variable. And, based on the results obtained, it was not established an evident connection among those. Furthermore, as all *P. aeruginosa* strains showed resistance against ciprofloxacin, there is an urgent need to reconsider the use of this antibiotic as an empirical treatment against *P. aeruginosa* related infections. As such, actual clinical guidelines should be improved. These guidelines should regard phenotypic switching, intrinsic responses of biofilms to antibiotic and environmental

stress and virulence factors presented by *P. aeruginosa* biofilms, characteristics with the most potential therapeutic impact.

The information collected with this thesis contributes to a better understanding of *P. aeruginosa* biofilms characteristics. These findings can be the basis to new investigations that can lead to a further understanding of *P. aeruginosa* adaptive resistance and even to the improvement of strategies of biofilm control, eradication of already established biofilms, and prevention and control of *P. aeruginosa* related infections.

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APPENDIX

APPENDIX A – COLONY CLASSIFICATION SYSTEM

Table A.1 Morphological criteria of colony classification system

Class	Sub-class
Form	Circular Elliptical Erose Filamentous Irregular Rhizoid
Margin	Entire Filamentous Irregular Lobate Undulate
Surface	Heterogeneous Homogeneous
Texture	Concentric Rugose Smooth Striated Wrinkled
Sheath	Absent Present
Opacity	Iridescent Opaque Translucent Transparent
Elevation	Convex Crateriform Flat Pulvinate Raised Umbonate
Size	Large Small
Colour	White Yellow Yellowish Green
Diameter	

Table A.2 Morphological Concepts

Term	Concept	Synonymous
Elliptical	Form feature inhering in a colony virtue to present an oval configuration with two axes of symmetry, as produced by a conical section.	Elliptic, ellipsoid, ellipse-shaped, oval, ovoid
Erose	Form feature inhering in a colony by virtue of having an irregularly toothed shape	Jagged, toothed, notched, serrated, dentated
Filamentous	Form features inhering in a colony by virtue of having filamentous extensions at its edge	
Irregular	Morphological feature inhering in a colony by virtue of not having any distinguishable configuration	
Rhizoid	Form feature inhering in a colony by virtue of having extensions radiating from its center	Rhizoidal
Entire	Margin feature inhering in a colony by virtue of having a straight well-defined line border without irregularities or other projections	
Lobate	Margin feature inhering in a colony by virtue of having deeply undulating edges forming lobes	Lobular
Undulate	Margin feature inhering in a colony by virtue of having a sinuate and rippled margin	Rippled, sinuate, undulated, wavy, undulating, waved
Heterogeneous	Surface feature inhering in a colony by virtue of having more than one type of texture	
Homogeneous	Surface feature inhering in a colony by virtue of having only one type of texture	Uniform
Concentric	Texture feature inhering in a colony by virtue of having vertical irregularities in a configuration of a circumference or a circle	Concentrical
Rugose	Texture feature inhering in a colony by virtue of having small vertical irregularities	Rough, rugous
Smooth	Texture feature inhering in a colony by virtue of having a surface free of roughness or vertical irregularities	
Striated	Texture feature inhering in a colony by virtue of having vertical irregularities marked by narrow lines or grooves, usually parallel	
Wrinkled	Texture feature inhering in a colony by virtue of having large vertical irregularities	
(Sheath) Absence	Morphological quality inhering in a colony by virtue of not having a sheath around the colony	
(Sheath) Presence	Morphological quality inhering in a colony by virtue of having a sheath around the colony	

Iridescent	Optical quality inhering in a colony by virtue of changing colour as the angle of view changes	
Opaque	Optical quality inhering in a colony by virtue of not transmitting or reflecting light or radiant energy	
Translucent	Optical quality inhering in a colony by virtue of exhibiting low opacity	
Transparent	Optical quality inhering in a colony by virtue of lacking opacity	
Convex	Elevation feature inhering in a colony by virtue of the surface curves or bulges outward, like the exterior of a circle or sphere	
Crateriform	Elevation feature inhering in a colony by virtue of the surface becoming plane with a crater or a hold or a cavity	
Flat	Elevation feature inhering in a colony by virtue of the surface becoming more extended in a plane	Flattened, compressed
Pulvinate	Elevation feature inhering in a colony by virtue of the surface being shaped like a cushion or has a marked convex cushion-like form	
Raised	Elevation feature inhering in a colony by virtue of the surface becoming rounded out	Rounded out, embossed, lifted
Umbonate	Elevation feature inhering in a colony by virtue of the surface having a rounded knob or knoblike protuberance	

APPENDIX B – PLANKTONIC COLONY MORPHOTYPES

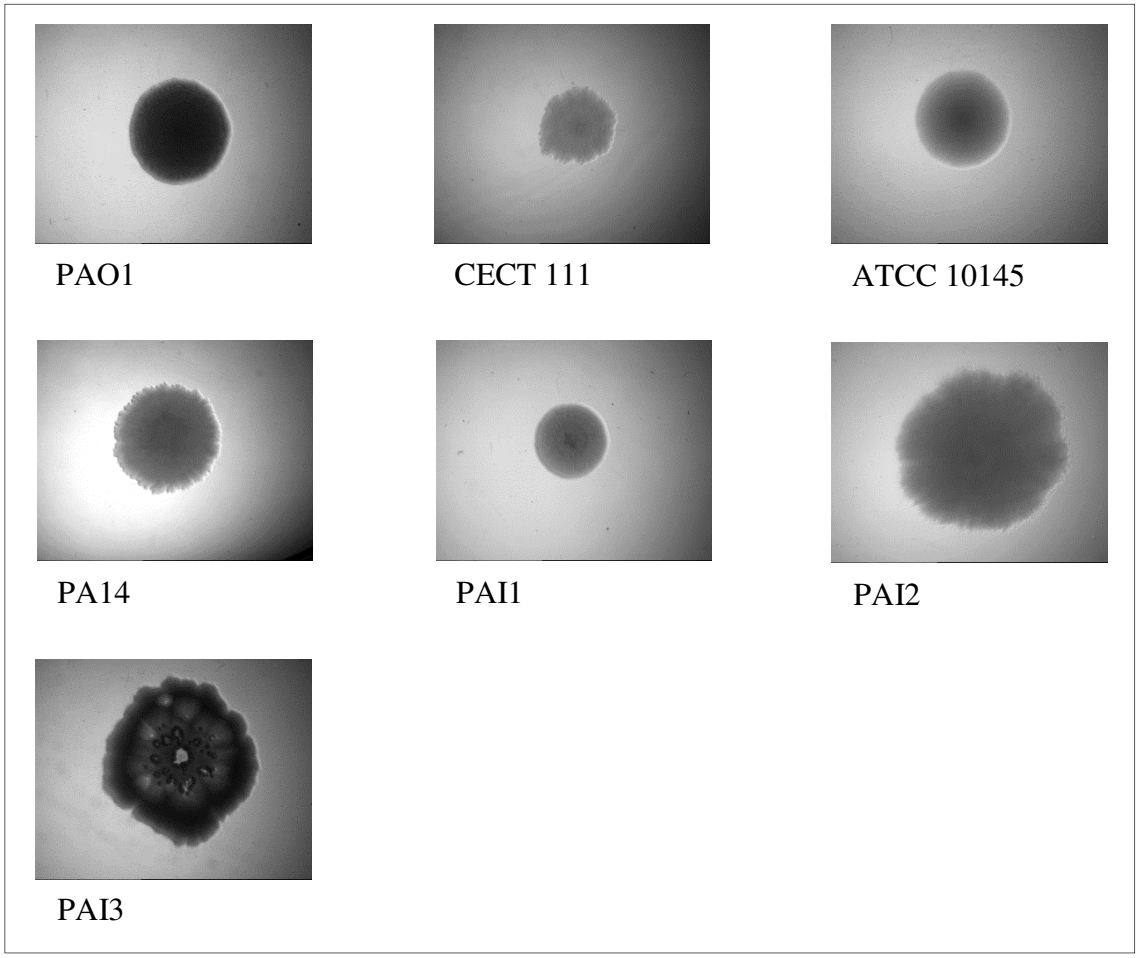

Figure B.1 Colony morphotypes registered for *P. aeruginosa* planktonic cells.

APPENDIX C – BIOFILM-DERIVED COLONY MORPHOTYPES

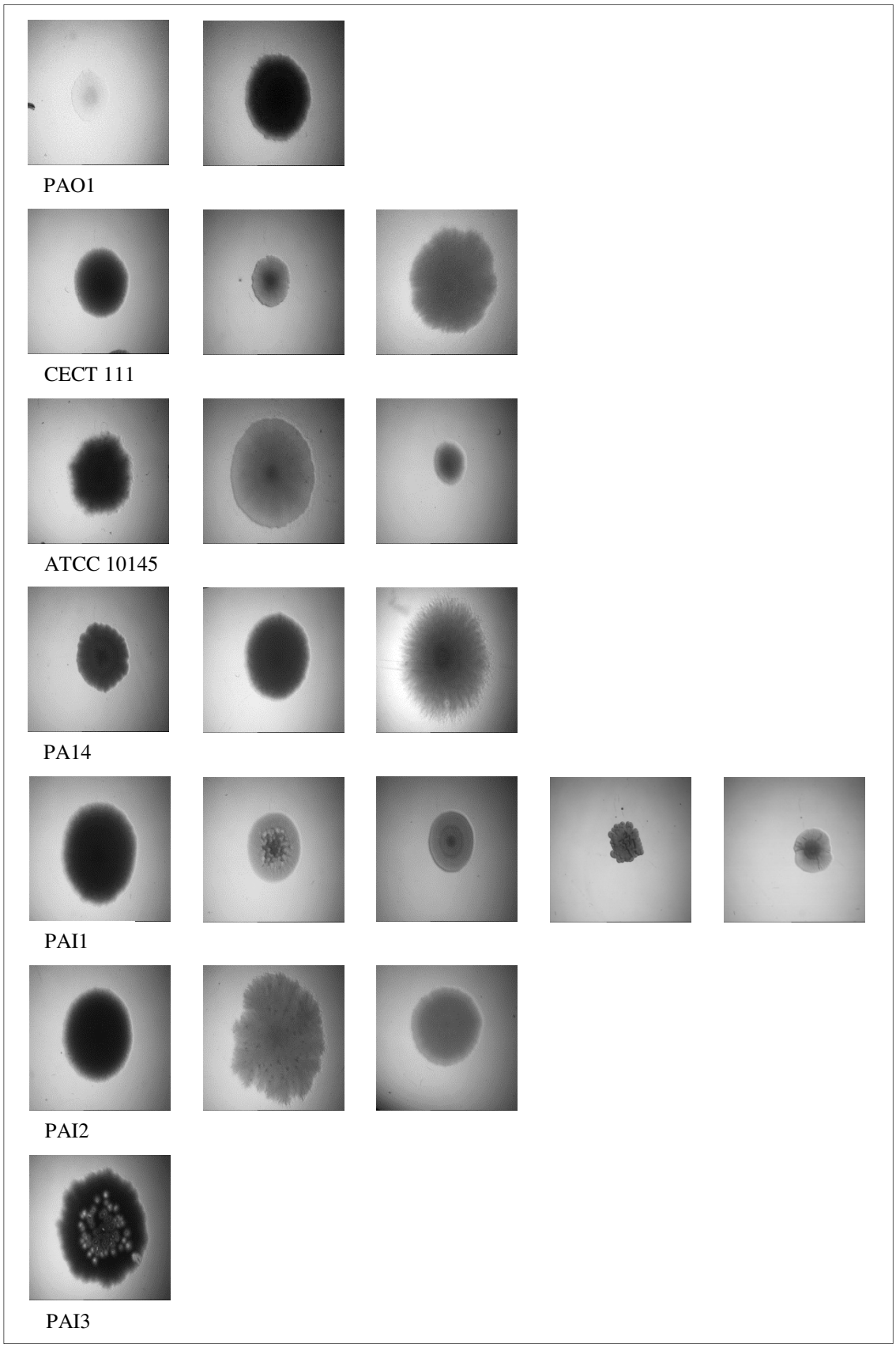

Figure C.1 Colony morphotypes registered for *P. aeruginosa* biofilm-derived cells.