Universidade do Minho
Escola de Engenharia

Raquel Alexandra Palas Vaz

Pseudomonas aeruginosa diversification during infection development in cystic fibrosis lungs

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UMinho|2015

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Master Dissertation for Master's degree in
Biomedical Engineering

Supervisor:
Professora Doutora Maria Olívia Pereira
Co - Supervisor:
Doutora Ana Henriques

outubro de 2015

DECLARAÇÃO

Nome:

Raquel Alexandra Palas Vaz

Endereço eletrónico: raquel.vaz.787@gmail.com Telefone: 915289438

Número do Bilhete de Identidade: 14180183

Título da dissertação:

Pseudomonas aeruginosa diversification during infection development in cystic fibrosis lungs

Orientador(es):

Professora Doutora Maria Olívia Pereira

Doutora Ana Henriques

Ano de conclusão: 2015

Designação do Mestrado:

Mestrado Integrado em Engenharia Biomédica

Ramo de Engenharia Clínica

Universidade do Minho, 30 / 10 / 2015

Assinatura:

AGRADECIMENTOS

Foram várias as pessoas que direta ou indiretamente contribuíram para a realização deste trabalho e sem as quais não teria conseguido atingir todos os objetivos. Esta secção é dedicada a todas elas, como forma de agradecimento.

Antes de tudo, gostaria de agradecer à Professora Olívia Pereira a oportunidade para realizar esta dissertação e toda a orientação e apoio durante a realização da mesma.

À Ana Margarida, um especial **OBRIGADO** por todo o apoio que me prestou durante este ano. Obrigada por todos os ensinamentos, paciência, disponibilidade e conselhos. Foi sem dúvida uma ajuda essencial.

À Doutora Ana Henriques agradeço pela ajuda incansável, por toda a disponibilidade e simpatia, durante toda a realização desta dissertação. Muito obrigada pela paciência e por tudo o que me ensinaste. Foi um verdadeiro prazer trabalhar contigo.

A todos os colegas do LIBRO e do grupo MOP, um sincero obrigada por toda a ajuda e apoio durante o meu trabalho no laboratório.

A todos os meus colegas de curso, um **MUITO OBRIGADA** por toda a amizade e por estes 5 anos incríveis. Foram sem dúvida anos repletos de bons e maus momentos, que nunca vou esquecer. Quero agradecer de forma especial aos que me acompanharam mais de perto durante este último ano e sem os quais teria sido tudo mais difícil. Não posso, também, deixar de agradecer em especial às minhas companheiras de casa por todo o apoio, por todos os bons momentos e por nunca me deixarem desistir.

À minha irmã, um grande obrigado por ser sempre um modelo a seguir e por todo o apoio e incentivo que sempre me deu.

Ao David, agradeço todo o amor, compreensão e apoio ao longo destes anos. Um obrigada será pouco para agradecer que tudo o que fazes por mim, e toda paciência nos meus "maus" momentos. És, sem dúvida, uma inspiração.

E por último, e não menos importante, aos meus Pais, por todo esforço e apoio, quer financeiro quer moral, que sempre me proporcionaram. Sem os vossos conselhos, paciência e ajuda a ultrapassar os maus momentos, nunca teria conseguido. Vocês serão sempre a minha inspiração e razão de tentar sempre fazer mais e melhor. Por isso e por todas as razões, dedico-vos esta dissertação.

ABSTRACT

Cystic Fibrosis (CF) is a genetic disorder in which a defect in the cystic fibrosis conductance regulator (CTFR) occurs. This disorder affects many organs, the airways being one of the most affected. Patients with CF lung disease are very susceptible to chronic infections by various pathogens, one of them being *Pseudomonas aeruginosa*. This pathogen has a high ability of adaptation to the harsh environment found in CF lungs, characterized by inflammatory defences, antibiotic treatments, oxygen restriction, and poor availability of nutrients, among others. It is well established that once *P. aeruginosa* infection is installed in the lungs, it is almost impossible to eradicate. *P. aeruginosa* adaptation is achieved by some sophisticated mechanisms, as resistance to antibiotics, hypermutability, biofilm mode of growth, differential expression of virulence factors, and according to the stage of infection in the lungs and other genotypic and phenotypic changes. With the onset of chronic CF, *P. aeruginosa* diversifies into different morphotypes, with the emergence of mucoid phenotype and small colony variants (SCV) that are known to persist after continuous antibiotic treatment. *P. aeruginosa* adaptation under microaerophilic and anaerobic conditions was assessed in terms of growth kinetics, colony morphology, mutants frequency, pyocyanin production, antibiotic susceptibility, sub-population characterization and expression of mexA and 16S genes. Results showed, in both microaerophilic (10% CO_2 and 5 % O_2) and anaerobic environment, similar growth profiles and pyocyanin production. The emergence of intermediate susceptibility profiles by different strains for Ticarcilin/clavulanic acid, ciprofloxacin and imipenem was observed, as well as hypermutability, with anaerobic conditions. Phenotypic diversification was also observed, under all conditions, particularly in microaerophilic conditions with 5 % O_2, with the appearance of morphotypes not identified before. Sub-populations characterization shown similar characteristics between morphotypes, with one morphotype standing out in terms of biofilm formation and antibiotic susceptibility. Results obtained with the regard of the effects of the decrease in oxygen tension on gene expression showed high levels of contamination of the RNA samples collected through the study to assess differential expression of mexA gene at different oxygen concentration. Also, it was found that both pair of primers used (16S and mexA) were not adequate for the tested strain, which translate in a lack of specificity and efficiency. Given the results of characterization of growth and phenotypic characteristics, is possible to conclude that oxygen depletion has no significant effect on *P. aeruginosa* growth, affecting, however, the phenotypic characteristics and antibiotic susceptibility profiles. Oxygen depletion increases the

presence of intermediate resistant profiles, and strong mutator phenotypes, as observed in chronic infections. Microaerophilic environment seems to be a turning point of the stage of infection, as some distinctive characteristics were observed, as higher colony diversification, appearance of new sub-populations and slight decrease in antibiotic resistance profiles. In future studies of expression of mexAB-OprM efflux pumps, a more suited, or more than one, reference genes should be selected, as well as specific design of mexA primers for the strains used will be required. It is also recommended the treatment of RNA samples with acid phenol:chlorophorm to reduce possible DNA contaminations.

SUMÁRIO

A Fibrose Quística (FQ) é uma doença genética que ocorre quando existe um defeito no *cystic fibrosis conductance regulator gene* (CFTR). Esta doença afeta vários órgãos, sendo as vias respiratórias as mais afetadas. Doentes com fibrose quística são muito suscetíveis a infeções provocadas por variados agentes patogénicos, tal como a *Pseudomonas aeruginosa*. Este agente patogénico possui uma grande capacidade de se adaptar ao exigente ambiente presente em pulmões de doente com fibrose quística, caracterizado pela presença de agentes inflamatórios, tratamentos com antibióticos, restrição de oxigénio e baixa disponibilidade de nutrientes, entre outros. É facto aceite que uma vez que a infeção crónica pela *P. aeruginosa* se encontre estabelecida, é praticamente impossível a sua erradicação. O processo de adaptação da *P. aeruginosa* é conseguido através de sofisticados mecanismos de resistência a antibióticos, hipermutabilidade, formação de biofilmes, expressão diversificada de fatores de virulência, de acordo com o estágio de infeção, entre outras alterações fenotípicas e fenotípicas. Com o estabelecimento de infeção crónica, a *P. aeruginosa* sofre uma diversificação em vários tipos morfologias (ou morfotipos), com o surgimento de um fenótipo mucoide e de *small colony variants* (SCV), conhecidas por persistir mesmo depois de continuamente sujeitas a tratamentos com antibióticos. Durante este trabalho, a adaptação da *P. aeruginosa* a condições de microaerofilia e anaerobiose, foi estudada relativamente à cinética de crescimento, morfologia de colónias, frequência de mutantes na população, produção de piocianina, suscetibilidade a antibióticos, caracterização de subpopulações e expressão genética do gene *mexA*. Os resultados obtidos demonstraram que, em ambos os ambientes de microaerofilia testados (com 10% CO_2 e 5 % O_2), bem como em anaerobiose, houve um crescimento semelhante das estirpes de *P. aeruginosa*, bem como uma constante produção de piocianina. Foi verificado o aparecimento de perfis intermédios de resistência, em diferentes estirpes, relativamente ao antibiótico TTC (Ticarcilina/ ácido clavulânico), CIP (ciprofloxacina) e IMI (imipenem), bem como o surgimento de hipermutabilidade com a exposição a condições anaeróbicas. Diversificação fenotípica foi também observada em todas as condições testadas, mas particularmente em 5 % O_2, onde se verificou o aparecimento de morfotipos não observados anteriormente. Os diferentes morfotipos observados neste estudo, apresentam características semelhantes entre si, havendo no entanto um morfotipo (MT25) que se destacou no que diz respeito à formação de biofilme e suscetibilidade a antibióticos. Os resultados relativos ao efeito do decréscimo da concentração de oxigénio na expressão genética, mostraram um nível considerável de contaminação das amostras de ARN recolhidas

durante o estudo da expressão diferencial do gene *mexA* em diferentes concentrações de oxigénio. Para além disso, concluiu-se que os *primers* usados neste estudo (*mexA* e 16S) não eram adequados pata a estirpe testada, o que se traduziu numa falta de especificidade e eficiência. Relativamente aos dados obtidos, é possível afirmar que a restrição de oxigénio não provoca um efeito significativo no crescimento da *P. aeruginosa.* Afetou, no entanto, as características fenotípicas e os perfis de suscetibilidade a antibióticos observados. A diminuição da disponibilidade de oxigénio provocou o aumento de perfis intermédios de resistência e o aparecimento de fenótipos hipermutantes, tal como verificado em infeções crónicas pela *P. aeruginosa*. A presença de condições de microaerofilia apresenta características que podem ser interpretadas como um ponto de evolução do estágio de infeção, dado que foram verificadas características distintas nesta condição, como uma maior diversificação de colónias, o aparecimento de novos morfotipos e um ligeiro decréscimo dos perfis de resistência a antibióticos observados.

Em estudos a ser realizados futuramente, relativamente à expressão do operão *mexAB* é recomendado a seleção de um ou mais genes de referência, mais adequados ao estudo, bem como a o desenho dos primers para o gene *mexA*, específicos para a estirpe em uso. É também recomendado o tratamento das amostras de ARN obtidas com ácido fenol:clorofórmio, de modo a reduzir possíveis contaminações com ADN.

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AK: Amikacin

ASL: Airway Surface Liquid

ASM: Artificial Sputum Medium

ATM: Aztreonam

CAZ: Ceftazidime

CF: Cystic Fibrosis

CFU: Colony Forming Unit

CIP: Ciprofloxacin

CN: Gentamicin

DNA: Deoxyribonucleic acid

FEP: Cefepime

HCl: Hydrochloric acid

HCO_3^-; Hydrogen Carbonate

IMI: Imipenem

NaCl: Sodium Chloride

NRT: No Reverse transcriptase

NTC: No Template Control

PRL: Piperacillin

RIF: Rifampicin

RNA: Ribonucleic Acid

TOB: Tobramycin

TSA: Tryptic Soy Agar

TTC: Ticarcillin / Clavulanic acid

SCV: Small Colony Variants

General Outline

Cystic Fibrosis is considered one of the most common lethal genetic diseases in affecting Caucasian populations is caused by a defect on the cystic fibrosis conductance regulator (CFTR) gene. CFTR defective expression is characterized by abnormal salt and water transport across epithelial surfaces, since CFTR functions mainly as a chloride channel in exocrine epithelia. For this reason, CF affects many organs, with effects on the respiratory tract, gastrointestinal and hepato-biliary systems, reproductive system and sweat glands being the most reported manifestations [1].

In spite of the diverse manifestations, CF lung disease is the most common and the one with higher associated morbidity and mortality. Over 80% of patients with CF lung disease succumb to the multiple and persistent infections that characterize this disease, becoming, for that reason, the most documented CF manifestation [2][3].

CF pulmonary disease is characterized by the production of dehydrated and hyperviscous mucus in the lungs that obstruct the airways, impairs the mucociliary clearance and enhances the inflammatory response by neutrophil recruitment [4]–[6] . All these effects together translate in bacterial infections that begin at a young age, intermittent at first, but developing into chronic infection later in life. *Staphylococcus aureus* and *Haemophilus influenzae* are the most prevalence in younger patients, although by adolescence *Pseudomonas aeruginosa* infections already affect 80% of CF patients [7][8]. Colonization by this pathogen in CF lung disease is associated with increased morbidity and mortality, since after periods of intermittent acute infections, *P. aeruginosa* has the ability to persist in CF airways [8][9] . It is widely known that after chronic *P. aeruginosa* infections occurrence in CF lungs, eradication is almost impossible. For that reason urges the need to understand the mechanisms underlying the process of *P. aeruginosa* persistence in CF lungs and the pursuit of options of effective treatment and early eradication of this pathogen.

Pseudomonas aeruginosa, a Gram negative bacteria commonly found in soils, aqueous environments, plants and animals, is one of the most prevalent pathogens responsible for nosocomial infections [10] . Due to *P. aeruginosa*'s large genome and genetic flexibility, a rapid adaptation to the CF airways environment occurs, assisted by the good ability to form biofilm within the airways[11]. In order to respond to the selective pressure, imposed by persistent antibiotic treatment and the harsh CF environment with oxygen limitation*, P. aeruginosa* can suffer a series of genetic and phenotypic alterations, such as the development to mucoid phenotype and loss of virulence factors, in order to survive. Therefore, the assessment of the development and

diversification of *P. aeruginosa* under oxygen limitation is essential for understand the influence of CF environment in *P. aeruginosa* adaptive process.

One of the main goals of this study is the characterization of *P. aeruginosa* development under a simulated environment of CF airways. Therefore, growth kinetics, antibiotic susceptibility, pyocyanin production, mutation frequency, genetic expression of genes described as responsible for persistence, colony morphology diversification and phenotypic switching, under several steep oxygen-limitation conditions, will be assessed.

Finally, the importance and understanding of the adaptive *P. aeruginosa* characteristics observed from acute to chronic infection in CF airways will be discussed.

This dissertation is structured into four chapters. In Chapter 1 the basis of CF disease and the principal *P. aeruginosa* characteristics are reviewed and the most important aspects of *P. aeruginosa* development from acute to chronic infection in CF airways are described. Chapter 2 describes the microorganisms, materials and methodology used in the experimental work. The third Chapter includes the results obtained during this study, as well as the discussion of these results. In Chapter 4, the overall conclusion of this work and future research suggestions are presented.

1. Cystic Fibrosis disease and relevance of Pseudomonas aeruginosa infection development and diversification

In this chapter the basic mechanisms and consequences of cystic fibrosis disease focusing on lung disease. The importance of Pseudomonas aeruginosa infections in CF lung disease is also addressed, focusing on the development of chronic infection and consequent *P. aeruginosa* diversification.

1.1 Cystic Fibrosis: a genetic disease

Cystic Fibrosis (CF) is a genetic disorder on chromosome 7, in which a defect in the cystic fibrosis conductance regulator (CFTR) occurs [12]. It is one of the most frequent autosomal recessive disease, inherited and lethal, among the Caucasian population and affects many organs, such as pancreas, small intestine, liver, reproductive system, and most commonly the airways and sweat glands [12][13]. Currently, more than one thousand mutations in CFTR gene that codify this disease have been identified [13]. However, the ΔF508del mutation the most common, being present in 70% of the reported cases of CF around the world [2][6][14][15].

The respiratory complications associated with CF are the most characterized in the literature due to the high rate of morbidity and mortality that they represent, thanks to various episodes of extreme and uncontrolled inflammation of the airways, assisted by chronic infection by several microorganisms (such as Pseudomonas aeruginosa, Staphylococcus aureus, among others) present in CF patients [2]. Some of the most significant symptoms of this disease are the presence of viscid mucus in the airways and recurrent/chronic respiratory infections, and increased electrolyte concentration in sweat. The primary test for CF diagnosis is based on the measurement of the electrolyte concentration in the patient sweat [3][13].

The CFTR is a cAMP-regulated chloride channel protein, present in many types of cells as epithelial cells and PMNs, comprising two membrane-spanning regions and a cytoplasmic regulatory R domain and is important as a Cl^- channel, regulation of ion transport (Na^+ and HCO_3^-), pH regulation and innate immunity [2][6]. Of all organs that can be affected by CFTR deficiency, as stated before, the most commonly affected are the respiratory, the gastrointestinal and reproductive tracts. Respiratory complications account for a 90% cause of dead in CF patients [3][16].

It has been suggested that CFTR regulates the level of hydration of the surface fluid in the airways by regulation of Na^+ and Cl^- transport [15]. Therefore, a mutation occurring in CFTR causes a malfunction in this chloride channel and ion transport, resulting in lower volume of the paraciliary fluid in the lungs and, consequently, in poor mucociliary clearance of inhaled microbes [6]. Altogether, results in abnormal electrolyte composition, as mentioned before, that translates into augmented viscose and dehydrated mucus that causes entrapment of bacteria in airway secretions and, consequently, the establishment of chronic infections [6][17].

Some studies also suggest that proteases secreted by inflammatory cells can intensify the defect in ion transport in CF [5].

The airways epithelium is known to be water permeable and so, the hydration of the ASL is determined by the quantity of salt present on the airway surface. Deficiencies in Cl^- secretion and Na+ absorption, by ENaC, were associated with dehydrated ASL [18].

Several studies aimed at understanding how CF ion transport defect and consequent lung disease are related demonstrate that ASL is composed by two layers: the mucus layer, that entraps pathogens and other inhaled particles; and a periciliary layer, responsible for lubrication of airway surface to promote mucus clearance [19][20]. In non-CF airways, epithelia are capable of regulating ASL volume by responding to signals that adjust Na^+ adsorption and Cl^- secretion, adjusting ASL height to the length of the cilia (Figure 1.1). In CF airways, this regulation fails due to deficient Cl^- secretion, causing a reduced volume of ASL. This lower volume of ASL affect the periciliary layer, collapsing the cilia onto airway surface and reducing the mucus transport rate.

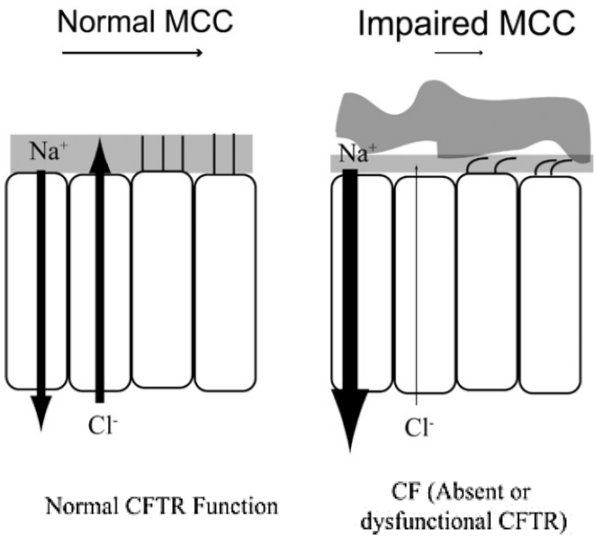


Figure 1.1- The dysfunction of CFTR results in hyperabsorption of sodium from the ASL that is not observed with functional CFTR. This hyperabsorption leads to a higher absorption of water and, consequently, to depletion of ASL. The clear mucociliary clearance, observed in normal CFTR function, is impaired in CF airways and mucus retention occurs [12].

Studies with βENaC-Tg mice showed that the entrapment and accumulation of particles and pathogens due to mucociliary malfunction in CF airways activates the release of pro inflammatory chemokines from inflammatory and epithelial cells, causing airway inflammation [13][21].

CFTR also mediates epithelial HCO_3^- secretion and pH regulation of mucosal surfaces. Therefore, a dysfunction in CFTR causes a reduced secretion of $HCO3^-$ and, consequently lower pH in ASL, which can be related to the reduced CL^- dependent fluid secretion and to the formation of viscous and dehydrated mucus that contribute to mucociliary impairment and mucus plugging [22][23].

pH also plays an important role in the airways. Lower pH in CF airways may contribute to reduced antimicrobial activity of ASL and reduced bacterial elimination in CF airways [5][18][22].

All of the CFTR mutation consequences create a prosperous environment in CF airways for infection establishment. For that reason, in addition to being a genetic condition, CF also is characterized as an infection disease [3]. CF patients are often colonized by various organisms since infancy which results in damage of epithelial surfaces since young age[3]. The bacterial species most frequently associated with CF airways infections include both common human pathogens, as *Staphylococcus aureus*, and opportunistic pathogens, as *Pseudomonas aeruginosa* [8]. Indeed of all bacteria that can cause infection in CF lungs, *P. aeruginosa* and *S. aureus* are the most prevalent [25]. For many years, *P. aeruginosa* has been reported as the overall dominant pathogen responsible for infection in CF airways, but latest reports state that *S. aureus* as the most prevalent pathogen [23][24]. Despite this data*, P. aeruginosa* continues to be the most prevalent pathogen in adult CF patients, while *S. aureus* and *Haemophilus influenzae* affect mostly younger patients [9]. Despite *P. aeruginosa's* decrease of prevalence due to the adoption of eradication strategies in initial stages acquisition, this pathogen continues to be the principal cause of chronic infection and worse prognosis in CF patients [25]–[27].

1.2 *Pseudomonas aeruginosa*: an opportunistic pathogen

Pseudomonas aeruginosa is a pathogenic and opportunistic bacterium, Gram-negative, known for its ability to thrive and adapt to various environments and cause severe infections in susceptible individuals, as pneumonia, bacteraemia, chronic airway infections, among other [26][27]. This bacterium is responsible for about 10 % of all nosocomial infections, and is in this environment that the majority of CF patients acquire this pathogen, causing in 80-100% of the cases, chronic infections, almost impossible to eradicate after installed in CF lungs [28][31].

P. aeruginosa strains can be found in multiple environments, from environmental habitats, as in soil, seawater and fresh water, and animal or human hosts [10]. It is known for its large genome, which encodes for several quorum sensing molecules, virulence factors, metabolic diversity, intrinsic antibiotic resistance and great capability to form biofilms, contributing for a good adaptability to the environment. [32][33]. These features have a significant importance in *P. aeruginosa* capability to avoid eradication by the immune system and persist and evade from phagocytic clearance, when an infection is established [4].

All of these mechanisms are known to be present in patients with CF lung disease. It is described in the literature that *P. aeruginosa* colonizes the lungs, intermittently, in earlier stages of infection and, after a short period of time, establishes chronic infections due to the capacity to adapt to the CF airways [36]. In order to better adapt and to establish chronic infection in CF airways, *P. aeruginosa* suffers a wide range of phenotypic and genotypic changes, becoming less inflammatory and less cytotoxic and more suitable to persist in that environment [37].

1.3 *P. aeruginosa* infection development in CF airways

Acute infection of the CF airways by *P. aeruginosa* take place at early ages of patients and the acquisition of this bacterium can be from the environment or from healthcare settings (nosocomial infection) [37]. At this stage, *P. aeruginosa* still presents wild-type characteristics, being non mucoid and susceptible to antibiotic treatments, and having almost all virulence factors expressed, such as pyocyanin, type III secretion system, several proteases, lipases, phospholipases and rhamnolipids [38]. These characteristics, together with the lower severity of lung disease when compared to cases in the adult age, make this stage the best for *P. aeruginosa* eradication. Studies show that aggressive treatment at this point of infection have good results in slowing down the progression of chronic infection [39]–[41].

However, this eradication is only temporary and recolonization by *P. aeruginosa* occurs either from the same source of the initial colonization or from reservoirs within the patient (paranasal sinuses), which contributes to posterior chronic infection [7][37][40].

The process of infection covers, essentially, 5 stages, which are 1) colonization and bacterial adherence of the epithelium mediated by type IV pili, 2) local invasion and 3) systemic dissemination.

Along all this stages, *P. aeruginosa* suffers a series of changes related to the development of infection over colonization to acute and chronic infection. In order to understand all the changes in *P. aeruginosa*, it is import to understand how the infection process occurs in CF airways. [43]

It all begins with a simple breath of air, containing multiple particulates and microorganisms from the environment. In a healthy lung, the epithelium of the airways (first line of defence) acts against infectious agents and are able to remove efficiently the threat, by the action of immune cells , such as dendritic cells, T cells, macrophages and neutrophils. The epithelium is composed predominantly of ciliated cells which rhythmically beat their cilia in one direction to push particles

and microorganisms out of the lungs. It is the ASL that forms a mucous layer in the epithelium, which allows the transport of these particles to the throat, so that can be expelled (mucociliary clearance) [44].

It has been suggested that in consequence of the CFTR mutation, CF lungs produce a high amount of thickened ASL, due to the dehydration of the mucus layer that coats all the epithelial surface. As a consequence, the mucociliary clearance, as well the immune response, is impaired and so, particles and microorganisms are not efficiently cleared, which causes a hyperactive inflammatory response, deterioration the lung function [37]. The viscid mucus creates a thigh mucin mesh that restricts the motility of *P. aeruginosa* and other bacteria that lead to the accumulation of bacteria in the mucus, and later to biofilm formation [19]. Beside this, the mesh also limits the ability of neutrophils to penetrate the mucus and capture bacteria [19]. The sputum has, therefore, a unique composition, with mucin, lipids, proteins, amino acids, ions and DNA in high concentrations, the perfect composition for bacterial development [45]. Overall, the viscid CF lungs secretions are a protective environment for bacteria, which enables them to escape antibiotic treatment and immune cells, favouring colonization and persistence [19][43].

Some studies have suggested that the layer of mucus present in the CF airways lumen is anaerobic, occurring, in that condition, the formation of well-structured and resistant *P. aeruginosa* biofilms.[47] In CF lungs, *P. aeruginosa* develops under anaerobic conditions and builds a different type biofilms. Instead of attachment of cells to a solid surface, in CF lungs cells adhere to each other and to the sputum components, especially to mucin forming microcolonies. [45]

With the progression of the disease, strains rich in alginate overproduction (mucoid) thrive, forming biofilms resistant to antibiotic treatment and phagocytosis. The best characterized mechanism of pseudomonas conversion to mucoid, is the mutation in mucA gene. The lack of MucA leads the extracytoplasmic α factor AlgT(U) to transcribe genes involved in alginate biosynthesis, that leads to alginate overproduction [47].

In CF airways, *P. aeruginosa* persist due to accumulation of point mutations, beneficial for the adaptation to the environment. Those mutations occur in the regulators of alginate biosynthesis and virulence genes involved in the LPS modification, motility, in quorum sensing regulation, in multidrug-efflux pumps, among others [40].

CF airways are known to be a very complex environment, in which bacteria needs to adapt in order to establish infection. Oxygen distribution, nutrient availability, the interactions between various

microorganisms, immune system and antibiotic treatments are some of many factors that enhance the CF airways complexity [48].

One of the most important factor recognized is oxygen distribution in the airways. Various studies, in which oxygen tension in CF airways has been measured, indicated that oxygen is depleted in this environment, suggesting that *P. aeruginosa* grow anaerobically within the CF airways mucus. However, has also been reported that *P. aeruginosa* prefers microaerophilic growth as major mode of growth in CF airways [46][47].

As previously described, in CF airways there is an accumulation of viscid and dehydrated mucus. When air is supplied to the airways, the oxygen dissolves in the mucus layer, creating a gradient along the depth of the layer. Thereby, at the surface of the mucus layer, oxygen is abundant and aerobic growth can be archived. Below the surface of the mucus, oxygen concentration begins to lower and microaerophilic growth is possible. The oxygen concentration continues to diminish and, at higher depths, is almost none, and anaerobic growth begins [47][48].

Contrary of what initially was expected, *P. aeruginosa* does not infect the epithelial cells, it infects only the mucus layer, never reaching the cells. Initially, when *P. aeruginosa* reaches the CF airways, it adheres to the mucus layer, and penetrates the mucus, since *P. aeruginosa* is still motile. Here, as described before, oxygen concentration is lower, and microaerophilic growth occurs, leading *P. aeruginosa* to suffer some changes in order to better adapt to these conditions [48][52]

One of the changes that occur in *P. aeruginosa* affects the alginate production. In response to hypoxia stress (lower oxygen concentration), *P. aeruginosa* starts to overproduce alginate, switching from a non-mucoid to a mucoid phenotype. This change is significant to the formation of biofilm like aggregates that characterize proliferation of *P. aeruginosa* in CF airways [48][50].

With the onset of chronic infection, *P. aeruginosa* suffers a series of phenotypic and genotypic alteration in the context of CF lung disease. These alterations all occur in order to make P. aeruginosa more persistent and adapted to the CF airways, reason why P. aeruginosa infections are almost impossible to eradicate.

1.3.1 Small colony variants: a persistent form of colony

Small colony variants differ from wild-type phenotype in multiples ways, as small colony size, reduced growth rate, pigmentation and haemolysis, altered expression of virulence factors, autotrophy, antibiotic resistance, among other characteristics [54].

Studies focused on small colony variants (SCV) have shown that these colonies have increased antibiotic resistance and enhanced biofilm formation, morphotypes with reversion to wild-type, impaired motility, slow growth and auto-aggregative behaviour. SCV are also known to be associated with chronic infections and poorer lung function, since they persist in the airways and become almost impossible of eradicate [31][39].

In *P. aeruginosa*, as in other Gram-negative bacteria, cyclic-di-GMP is known to be associated with the promotion of biofilm lifestyle and SCV presence, by positively regulate the production of secreted polysaccharides, as Pel and Psl, and downregulating motility [52][53].

As reported before, SCV are associated with the establishment of chronic infection, and that is partly due to SCV capacity to escape phagocytosis by macrophages. Evidence has been found suggesting that the exopolysaccharide overproduction protects each cell from phagocytosis, helped by SCV cell aggregation characteristics. This feature is also important to establishment of chronic infection since *P. aeruginosa* SCVs are exceedingly adherent, hindering their removal from the CF airways [57]. Increased antibiotic resistance is also associated with small colony variants. Many studies reported that antibiotic resistance of *P. aeruginosa* SCVs to aminoglycosides, quinolones, colistin and β-lactams is higher when compared with wild-type phenotype, which enhances even more the establishment of chronic infections [58].

1.3.2 Contribution of pyocyanin production to CF lung infections

Pyocyanin, or 5-methyl-1-hydroxyphenazine, is a nitrogen-containing heterocyclic compound, known as phenazine, and is synthesized by several *Pseudomonas* species and other bacteria. This compound has a blue colour and is a redox-active phenazine produced by *P. aeruginosa* [40][56]. Pyocyanin has been detected in the mucus of CF patients infected with *P. aeruginosa*, sometimes visually by the coloration of patients sputum samples, and has been proven that, in some infection models, has important role on the success of establishment of acute or chronic infections in lungs [57][58]. It plays a secondary role as a metabolite which is involved in several important biological activities, with toxic effects relevant to CF. Pyocyanin can, among other activities, easily interact with and penetrate cytoplasmic membranes, raising the concentrations of cytosolic calcium in airway epithelial cells and redirects electrons, leading to the generation of ROS which damage the cells, interfering with cellular respiration [40][59]. The generation of ROS decreased the concentration of intracellular cAMP, affecting the immune response by increasing neutrophil apoptosis [63]. Pyocyanin also subverts the lysosomal pathways and the macrophage capacity of

collecting apoptotic cells. Ciliary clearance of inhaled particles, stuck in the viscid mucus, is also affected by pyocyanin and its degradation products, by slowing the movement of the cilia and, in some cases, paralysing them. This specific effect is thought to be beneficial for *P. aeruginosa* in the initial stages of infection. Pyocyanin has also a role in the establishment of chronic infection. It prevents an effective T cell response against *P. aeruginosa* and can also prevent the activation of monocytes and macrophages [64]. Pyocyanin also contributes to the adaptability of *P. aeruginosa* to the CF environment. It acts as an alternative electron acceptor, maintaining the redox balance in low oxygen environments, as present in CF airways [61][40][53].

1.3.3 Motility impairment in chronic infections

Motility has been recognised as one of the most important features of *P. aeruginosa* contributing to its pathogenicity [65]. The presence of this characteristic has many beneficial aspects: allows *P. aeruginosa* to move across different surfaces to access perfect niches for colonization and to search for increased nutrient availability, to escape to toxic substances and host immune defences, among other aspects [62][63]. Swimming, swarming and twitching are the three types of motility observed in *P. aeruginosa*, among other bacterial species. Swimming and swarming are mediated by flagellum, and while the first allows bacteria to move through aqueous surfaces, swarming allows the movement of bacteria on semi-solid surfaces. On the other hand, twitching is mediated by type IV pilli and is responsible for movement on solid surfaces [63][64].

Bacterial motility of flagellum is achieved by rotational torque of a helical filament of several flagellin subunits that act as propellers. This process is possible due to a motor complex which acts as a molecular switch and regulates the way of rotation [68]. With the onset of chronic infection by *P. aeruginosa* in CF airways, this bacteria suffers a series of phenotypic and genotypic alterations, loss of motility being one of these. It has been proposed that the expression of flagellum is essential to success of nonopsonic phagocytosis. Studies shown that for macrophages to be able internalize bacteria by phagocytosis, a ligand was necessary to trigger that response, the flagellum being that trigger [69]. Given that, through the development of infection is believed that *P. aeruginosa* suffers a series of genetic alterations in the genes that codify the motor complex that impairs the bacteria swimming capability [65][66]. This loss of motility modifies the immune response since there is no trigger to phagocytosis, and bacteria effectively evade the immune system. Thereby, *P. aeruginosa* acquires a phenotypic advantage that improves the development of chronic infection. With *P. aeruginosa* growth in biofilm microcolonies, in CF lungs, motility cease to be an essential

mechanism for bacterial survival [70]. Despite motility being a decisive factor for biofilm formation and bacterial survival during acute infection, later on infection development, when microcolonies are already the lead form of *P. aeruginosa* growth, bacteria show slower growth and metabolic activity, and this characteristic is no longer essential [71][72].

1.3.4 Hypermutability as adaptive mechanism

P. aeruginosa has a genome composed by a conserved set of genes and many accessory genes that impair and allow the fast adaptation to the host, reason why hypermutability has been considered one of the hallmarks of chronic airway infections by *P. aeruginosa* [4][67]. Hypermutable bacteria have an increased random and spontaneous mutation rate due to defects in genes involved in DNA-repair or error-avoidance system [74]. One example is the defect of the mismatch-repair system (MMR), as in *mutS* or *mutL* genes that increase the rate of mutation up to a 1000 fold, being these the most frequent mutations [69][70]. Defects on this system promote the rise of mutation rates, genetic diversification and the change in expression of regulator of numerous virulence factors [4]. A MMR system also increase the rate of homologous recombination, enhancing the horizontal gene transfer of exogenous DNA [75]. *P. aeruginosa* is an example of bacterial population with high incidence of hypermutable strains in patients with CF. Studies have reported an increase of hypermutable isolates since the early colonization to chronic colonization of *P. aeruginosa* (from 0% to 65 % of hypermutable isolates). This mechanisms is thought to be important in the establishment of chronic infection since it provides a selective advantage for bacteria to thrive in challenging and changing environment, as in CF airways. Hypermutation has been proved to be beneficial to *P. aeruginosa* development, since it's linked to the selection of beneficial characteristics to chronic infection, as verified for antimicrobial resistance, reduced virulence and metabolic adaptation to growth under microaerophilic or anaerobic conditions [69][70].

Some example of these mutations, that occur quite frequently, are inactivation mutations of lasR transcriptional quorum-sensing regulator, mutation leading to alginate hyperproduction (in mucA and algU), virulence genes as motility (rpoN), multidrug-efflux pump (mexA), mutator phenotypes (mutS) [69][71].

1.3.5 Mechanisms of antibiotic susceptibility

One major characteristic associated with *P. aeruginosa* chronic infection in CF patients is the ability to resist to antibiotic treatment. Some studies have demonstrated that early chronic antibiotic therapy, at initial colonization by *P. aeruginosa*, can delay, or even eradicate the establishment of chronic infection. However, after several years of intermittent *P. aeruginosa* infection, in most cases, chronic infection sets in and from that point, it's almost impossible to eradicate [78].

The *P. aeruginosa* resistance to antibiotics has been vastly reviewed by many authors, and has been demonstrated that are many factors contributing to *P. aeruginosa* resistance.[43][76][80] In most cases, this resistance is a result of not only one factor, but the join action of several of these factors, selected by the environment in which the infection is developing. One of the most important factors is the prolonged and frequent antibiotic treatments that CF patients are subjected [41]. This continued exposure to antibiotic drives a selection of antibiotic-resistant *P. aeruginosa* strains within the CF airways [41]. It has also been shown that *P. aeruginosa* can penetrate into the thick mucus within CF airways, being exposed to steep gradients of oxygen, decreasing from top to bottom of the mucus layer. [48] Therefore, *P. aeruginosa* grows under both microaerophilic and anaerobic, reducing the efficacy of most antibiotics used. In addition to the environment and the prolonged antibiotic exposure, *P. aeruginosa* is also possess several genetic mechanisms of drug resistance and biofilm-specific resistance mechanisms [80][72].

There is a great variety of antibiotics used to treat *P. aeruginosa* infections in several clinical settings, including CF patients, belonging to several antibiotic classes. The class of β-lactams is frequently used to treat *P. aeruginosa* infections, including cephalosporins as ceftazidime, carbapenems as imipenem and monobactams as aztreonam. Other major classes of antibiotics also frequent in the treatment of *P. aeruginosa* infections are aminoglycosides as gentamicin and amikacin, quinolones as ciprofloxacin, and polymixins as colistin [79][81][82].

There are several *P. aeruginosa* strains identified as multi-resistant to antibiotics, due to *P. aeruginosa* ability to rapidly adapt to the environment and acquire a variability of mutations and mechanisms that led to resistance. *P. aeruginosa*, being a Gram negative bacteria, possess intrinsic mechanisms involving the semi permeable outer membrane that restrain the rate of antibiotics that enter the cell [43]. The outer membrane, composed of polyanionic lipopolysaccharide (LPS) acts as a barrier to the penetration of antibiotics through the water-filled porin channels present in the membrane. So, hydrophilic antibiotics as β-lactams and quinolones pass slowly through the membrane, which makes *P. aeruginosa* more resistant [43]. Other factor

contributing to intrinsic resistance are the efflux-pumps, as multi-drug efflux systems as MexAB- OprM, MexCD-OprJ, MexEF-OprN and MExXY-OprM systems, that given the slow uptake of antibiotics though the membrane, pump efficiently the molecules out of the cell.[79][83][84] MexAB-OprM is reported as the better exporter of carbapenems (with exception of imipenem), and was been linked to resistance to penicillins and aztreonam, fluoroquinolones such as ciprofloxacin [83][84]. Also the presence of periplasmatic β-lactamses as AmpC in *P. aeruginosa* take advantage of the impaired influx of antibiotics, by degrading them as they pass through, and increasing P. aeruginosa resistance [79][80].

Acquired resistance by antibiotic exposure can also occur with *P. aeruginosa*. This resistance can be achieved by selection of chromosomal gene mutations and acquisition of resistance genes through plasmids, transposons and integrons, and can also enhance intrinsic *P. aeruginosa* resistance mechanisms [79][85]. One example is the resistance to most β-lactams, specifically carbapenems by the acquisition of plasmid –encoded carbapenemases by *P. aeruginosa*. Another route known to let to carbapenems resistance is through mutations ta lead to reduced production of the OprD specific outer membrane porin channel, or even its loss [86][87].

It has been shown that the conditions in which bacteria, including *P. aeruginosa*, develop can have an effect on the resistance to antibiotics. The growth conditions, environmental stimuli and physical or chemical stresses can trigger a series of regulatory events in bacteria that led to adaptive resistance. Some conditions known to affect this type of resistance include antibiotics, pH, anaerobiosis, polyamines, nutrient deficiencies, biofilm formation, swarming motility, among many others [80].

In the context of CF disease, antibiotic resistance within *P. aeruginosa* biofilms is important to assess. All types of resistance (intrinsic, acquired and adaptive) are thought to be involved in *P. aeruginosa* resistance in biofilms [35]. The intrinsic characteristics of biofilms are known to contribute to antibiotic resistance, as the metabolic heterogeneity of sub-populations within the biofilms, the low oxygen availability in robust biofilms, the extracellular matrix and the variant expression of multiple gene networks [88][71]. Acquired resistance of biofilms can be due to horizontal gene transfer between the different populations within biofilms and due to high concentrations of β-lactamases in highly structured biofilms[79]. However, adaptive resistance is known to be very important in *P. aeruginosa* antibiotic resistance in biofilms. Several factors of the biofilm mode of growth play an important role to this type of resistance, including the low metabolic state of the cells deep in the biofilm, the high amount of extracellular enzymes responsible for

antibiotic degradation in the biofilm matrix and adaptive genetic changes associated with the biofilm mode of growth[72][71]. It has been described that the accumulation of these adaptive genetic changes can be responsible for the emergence of acquired resistance. The low availability of oxygen influences directly the antibiotic activity within biofilms. In the anaerobic or microaerophilic environment the energy dependant uptake of antibiotics is decreased and, also, the changes in gene expression are triggered [79][43].

2. MATERIALS AND METHODS

In the present chapter, techniques and methods used throughout this study are described.

2.1 Bacterial Strains

In the present study three strains of *P. aeruginosa* were used, PA01, ATCC 39324 and U147016-1. Two of the strains, ATCC 39324 and PA01 are from collection, being the first isolated from the sputum of a cystic fibrosis patient and PA01 from a wound infection. U147016-1-1 is a clinical isolate from a urinary infection isolated in hospital settings. Bacteria were preserved in criovials at – 80 °C, and before each experiment, were grown on Trypic Soy Agar (TSA) plates for 24h at 37 °C. All three strains were cultured for the assays on Trypic Soy Broth (TSB; 30 g/L, Liofilchem), Trypic Soy Agar (TSA; TSB, 30 g/L, Liofilchem, agar, 15 g/L, Liofilchem) or Artificial Sputum Medium (ASM) and incubated at 37 °C. Bacteria were preserved in criovials at – 80 °C, and before each experiment, were grown on TSA plates for 24h at 37 °C.

2.2 Study design

Three different essays were performed in order to understand the contribution of oxygen depletion in the phenotype of the three strains of *P. aeruginosa* in study during infection simulation. The infection simulation was performed for 10 days, performing checkpoint analysis at day 3, day 5, day 7 and day 10 of CF infection simulation, in order characterize the *P. aeruginosa* population. All environments tested were design to introduce to *P. aeruginosa* strains a steep oxygen gradient, as can be observed in CF lungs in the development of chronic infection. The design of the infection in that three environments is represented in Figures 2.1, 2.2 and 2.3.

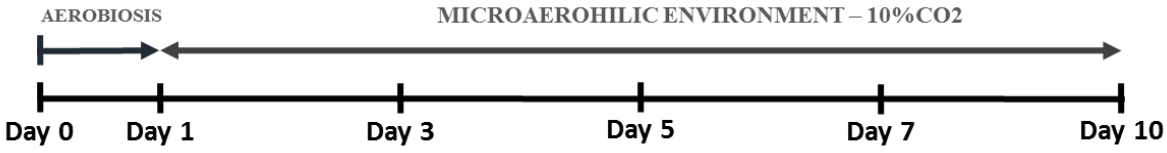


Figure 2.1 - Design of the microaerophilic environment with 10 % CO_2

In this environment, conditions observed in acute CF infection were mimicked. Therefore, initially all *P. aeruginosa* strains were grown under aerobic conditions and, after 24h of growth, oxygen concentration was slightly decreased. This oxygen depletion was achieved through the increase of carbon dioxide (CO_2) up to 10 %, and persisted until the end of the simulation CF infection period at day 10.

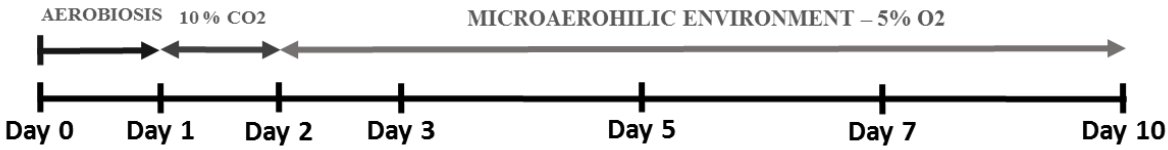


Figure 2.2 - Design of the microaerophilic environment with 5 % O_2

In order to study more advanced stages of CF infection, a new microaerophilic environment was studied. In this environment, the first two day were similar to the previous conditions (Figure 2.1), in which *P. aeruginosa* growth were initially performed on aerobic conditions for 24h, followed by a decrease in oxygen availability with 10 % CO2, for another 24h. An additional steep of oxygen depletion was inserted, from day 2 until the end of the simulation of CF infection, with 5 % O_2. This percentage was achieved by direct decrease in oxygen in a Heracell 150 O_2 Incubator (Heraeus).

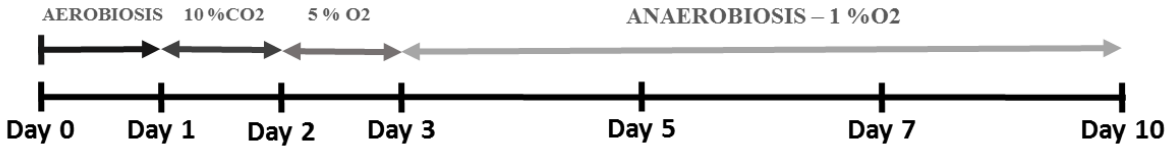


Figure 2.3- Design of the anaerobic environment with 1% O_2..

Late stages of CF lungs infection (chronic infection) are characterized by low presence of oxygen, typical anaerobic conditions. Therefore, to better understand this *P. aeruginosa* chronic infection development, a new environment was studied in which oxygen availability was gradually decreased to anaerobic conditions. As can be seen in Figure 2.3, the three first days of CF infection simulation were similar to the previous microaerophilic environment (5 % O_2). Initially, aerobic conditions were present, for 24h, followed by microaerophilic conditions with 10 % CO_2 (from day 1 to day 2) and 5 % O_2 (from day 2 to day 3). From that point, oxygen concentration was directly decreased to anaerobiosis (1 % O_2) with anaerobiosis bags (AnaeroGen™ 2.5L; Thermo Scientific).

2.3 Artificial Sputum Medium

To better mimic the conditions present in CF lungs, all *P. aeruginosa* strains were grown on Artificial Sputum Medium (ASM). The ASM medium used in this study was composed by 5 g/L of mucin from porcine stomach (Sigma-Aldrich), 4 g/L of deoxyribonucleic acid (DNA; low molecular weight from salmon sperm, Sigma-Aldrich), 5.9 mg/L of diethylenetriaminepentaacetic acid (DTPA, Sigma-Aldrich), 5 g/L of Sodium Chloride (J.T.Baker), 2.2 g/L of Potassium Chloride (Fisher Chemicals), 1.81 g/L of Tris-Base (AppliChem), 5 g/L of Casamino acids (AMRESCO), 5 ml/L of Egg yolk emulsion (Fluka Analitical), and with pH adjusted to 7.0 [45]. All the components were

added under constant stirring to achieve complete dissolution of all the components. pH was adjusted after the addition of casamino acids, with Tris-Base. Before the addition of the Egg yolk emulsion, the medium was sterilized at 121°C for 15 minutes. When the medium was at room temperature, Egg yolk emulsion was added without compromising the sterile conditions of the medium, and was stored at 4°C up to one month.

2.4 Growth Conditions

Before each assay, *P. aeruginosa* strains were grown overnight on TSB at 37 °C with agitation (120 rpm; Shaker & Incubator NB-205Q, N-BIOTEK). Each cell suspension of *P. aeruginosa* strain was washed twice with sterile water, and then diluted in order to obtain a final concentration of 4×10^{9} CFU/ml. This concentration is used in order to obtain a final concentration of $1x10^{8}$ in the CF infection simulation plate, in which 5 µl of cell suspension are added to 2 ml of ASM.
To finish, 24-well microtitre plates (Orange Scientific) were filled with 2ml of ASM and inoculated with 5 µl of the adjusted cell suspension, in a schematic similar to Fig.1.

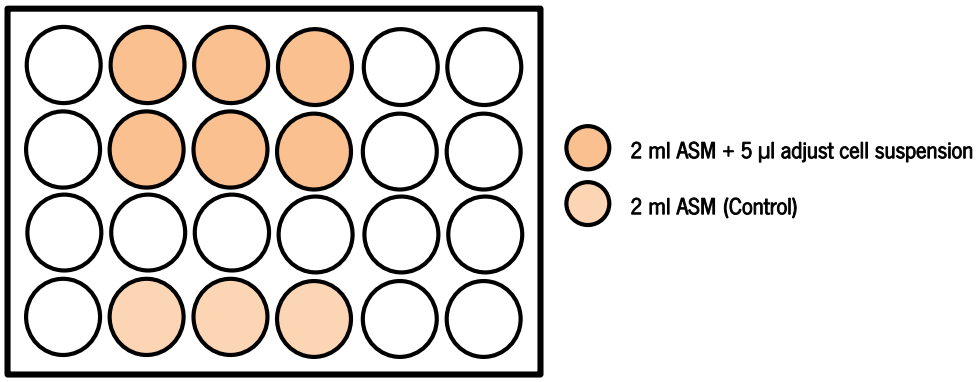


Figure 2.4 – Scheme of the microtitre plate used to simulate CF infection.

The plates described in figure 2, were then incubated, in each one of the three environments described in the previous topic, in figure 1, at 37 °C. A minimum of 3 biologically different assays were performed for each strain, under all conditions tested, using in each assay 3 technical replicates.

2.5 Phenotypic characterization of *P. aeruginosa* population

To fully characterize the total *P. aeruginosa* population present in the CF infection simulation plates, several tests were performed. Given that, growth kinetics of all *P. aeruginosa* was assessed in every checkpoint day, was well was pyocyanin production, mutation frequency, antibiotic susceptibility

profiles and colony morphology. With base on colony morphology assessment of PA01, U147016- 1 and ATCC 39324 for all environments tested, subpopulations observed within this study that were not identified in previous studies, were further characterized in terms of biofilm formation, motility, antibiotic susceptibility, TSA reversion, haemolysin production and growth on minimal medium.

2.5.1 Growth kinetics

The growth kinetics of PA01, U147016-1 and ATCC 39324 was assessed at day 3, 5, 7 and 10. In order to do so, the CFU (colony forming units) method was used. The samples taken at each checkpoint were serially diluted in the proportion1:10 in each individual dilution, until 10^{-6}. After that, 10 µl of the dilutions (-4), (-5) and (-6) were spread in TSA plates. After 24h, CFU were counted and corrected after 48h of growth.

2.5.2 Pyocianin Quantification

The production of pyocyanin was determined using an adjusted method previously described in the literature. Briefly, *P. aeruginosa* cultures were centrifuged at 15,000 rpm for 10 minutes, in order to obtain cell-free supernatants that contains pyocyanin. To 700 µl of the cell-free supernatant equal volume of chloroform was added, slightly vortexed and centrifuged again for 1 minute. After this step, the inorganic phase obtained was removed and, to the organic phase 700 µl of 0,2M HCL was added. Afterwards, samples were vortexed and centrifuged (ScanSpeed 1730R, LABOGENE) for 1 minute at 15000 rpm. Finally, the pink layer obtained was collected and its optical density at 520 nm measured. Triplicates of each condition and each strain were performed.

2.5.3 Mutation Detection

To determine the mutation rate of the stains being studied, Tripyc Soy Agar (TSA; TSB, 30 g/L, Liofilchem, agar, 15 g/L, Liofilchem) and TSA supplemented with 300 µg/ml of rifampicin (Sigma-Aldrich) plates were used. The samples were vortexed in order to obtain homogeneous cell suspensions. Afterwards, using a CFU method, 3 drops of cell suspension were added and drained thought the solid media plates, with and without rifampicin. The plates were then incubated at 37 °C for 48h and the number of colonies in the plates were counted after 24 and 48h of bacterial growth. The mutation rate was determined using the following equation:

$$[1] \quad \text{Frequency}(\text{mutants per cell}) = \frac{[\text{median of number of mutant cells}]}{[\text{median of total number of cells}]}$$

2.5.4 Susceptibility Testing

P. aeruginosa susceptibility was determined through the disc diffusion method [89], for ten different antibiotics (Antibiotic Disc, Liofilchem) : Amikacin (AK), Aztreonam (ATM), Cefepime (FEP), Ceftazidime (CAZ), Ciprofloxacin (CIP), Gentamicin (CN), Imipenem (IMI), Piperacillin (PRL), Ticarcillin/Clavulanic acid (TTC) and Tobramycin (TOB). To assess the susceptibility profile of all three strains of *P. aeruginosa*, Mueller Hinton Agar (MHA; MHB, 21 g/L, Liofilchem; agar, 15g/L, Liofilchem) plates were used. Before the execution of the procedure, the antibiotic discs were taken from the freezer in order to prevent condensation on the discs that could affect long-term stability. Afterwards, the samples were centrifuged at 15,000g for 10 minutes, in order to obtain the pellet containing all the biomass. The pellet were then ressuspended in 1 ml of sterile water, and vortexed until the suspension was homogeneous. Then, the cell suspension concentration was adjusted to $1x10^8$ CFU/m L by OD measurement, and all the surface of the MHA plates was inoculated with the cell suspension. Finally, each antibiotic disc was place in one plate and, when the discs were adhered to the agar, they were incubated at 37 °C for 18-21h. After incubation, the diameter of inhibition halo was measured and classified according to the Performance Standards for Antimicrobial Susceptibility Testing [89]. Three replicates of each measure were performed (for each strain and condition). The susceptibility profiles observed for, at least 2 of 3 measurements, was considered as the profile present for the strain in question, under the condition tested.

2.5.5 Colony morphology assessment

To assess the morphologic diversity existent among the cultures of all strains during the different assays, cell suspensions were diluted and inoculated on TSA plates by CFU method and incubated at 37°C for 48h. After that, colonies were observed to identify some parameters such as form, margin, surface, texture sheath, opacity, elevation, size, colour and diameter. To fully identify all the colony morphologies present for each sample, the plates containing the colonies were directly observed under a magnifying glass (Olympus SZ-CTV) and photographed with a CCD camera (AVC, D5CE; Sony, Tokio, Japan) to identify more morphologic parameters. The colony classification system used for this characterization can be found in Appendix II. Colonies with similar characteristics as classified with a morphotype code and represented in the schemes bellow. To classify a morphotype as present at each checkpoint, it had to be observed in two of three replicates, or three in five replicates. Is important to note that the morphotype code is only a representation, in order to better group colonies with the same characteristics.

2.6 Sub-populations phenotypic characterization

New subpopulations, or morphotypes, identified in colony morphology assays were characterized in order to better understand the morphological differences in each new morphotypes when compared with *P. aeruginosa* entire population. As "new" were classified the morphotypes not previously identified in a similar study with aerobic conditions (data unpublished).

2.6.1 Motility assays

Colonies identified as a new morphotype were harvest and suspended in sterile water and the suspension concentration was adjusted to $1x10^8$ CFU /ml (optical density measured at 640 nm). Swimming, swarming and twitching motility assays were performed in TSA plates with different agar concentrations: 0.3 % (w/v) for swimming, 0.5 % (w/v) for swarming and 1.5 % (w/v) twitching . Plates were inoculated with 1 µl of cellular suspension and incubated at 37 °C for 24h. The turbid zone formed by the migration of bacterial cells away from the inoculation point for each plate and agar concentration was directly observed and measured with a ruler, in millimetres.

2.6.2 Susceptibility Testing

To determine the susceptibility profile of each new morphotype, colonies of the morphotype in study were harvest, suspended and optical density was measured at 640 nm to adjust concentration to $1x10^8$ CFU/ml. After that, the protocol described previously in section 2.5 was performed.

2.6.3 Biofilm formation and quantification

Cell suspension of the colonies in study were obtained by harvesting them and resuspending them in TSB, adjusting the concentration to $1x10^8$ CFU /ml. Afterwards, 96-well microtiters (Orange Scientific) were inoculated with 200 µl of cell suspension and incubated aerobically and under agitation (120 rpm), at 37 °C for 24h. The quantification of biofilm formation was performed by crystal violet (CV) method.

After incubation, the content of the wells was discarded and washed twice with sterile water to remove poorly attached cells and 200 µl of methanol were added to each well in order to fix the biofilm. After 15 minutes, the methanol was discarded and plates were left to air dry, at room temperature, for 5 minutes. To stain the biofilms, 200 µl of pure CV (PRO-LAB Diagnostics) were added to each of the plates and incubated for 5 minutes at room temperature. After incubation,

the plates were rinsed repeatedly with tap water to remove all of the CV excess in the walls. To quantify the biofilm formed, 200 µl of acetic acid (Fisher Chemical) (33% v/v) were added to the plates and optical density was measured at 570 nm using a microtiter plate reader (Synergy HT II, Biotek).

2.6.4 Haemolysin Production

Hemolytic activity was assessed using Columbia Agar (CA; Columbia Broth, 43 g/L, Liofilchem; agar, 15g/L, Liofilchem) with sheep blood (50 ml/L; PROBIOLÓGICA) plates. Cell suspension concentration was adjusted to $1X10^8$ CFU/ml, by measurement of optical density at 640 nm. Plates were inoculated with 1µl of cell suspension and incubated at 37 °C for 24h.

2.6.5 Growth in Minimal Medium

The assessment of auxotrophic capacity of the morphotypes was evaluated using M9 Agar (M9; M9 Broth, 10.5 g/L, AMRESCO; agar, 15g/L, Liofilchem), with or without the addition of casamino acids (Casamino acids, 2.0 g/L, AMRESCO). Initially, the concentration of cell suspension was adjusted to $1X10^8$ CFU /ml. After that, 10 ml of cell suspension were added to a M9 plate with amino acids and to another plate of M9 without amino acids. The plate of M9 with the addition of amino acids was used as positive control. The cellular growth on each plate was observed after 48h of incubation at 37 °C and auxotrophy was assessed. Morphotypes that grew on plates without the addition of amino acids were considered auxotrophic, when compared to the control.

2.6.6 TSA reversion

The colonies related to the morphotype in study, were ressuspended in sterile water and concentration was adjusted $1x10^8$ CFU /ml. After that, the cell suspension was serial diluted and plated on TSA for 48h at 37 °C. The colonies were observed directly on the petri plate, by placing it under a magnifying glass (Olympus SZ-CTV) and photographed with a CCD camera (AVC, D5CE; Sony, Tokyo, Japan). After observation of the colonies, the reversion to wild-type of the morphotype in study was assessed and documented.

2.7 Gene expression assays

Several studies have shown an increased expression of the efflux pumps MexXY and MexAB-oprM in small colony variants, which is linked to resistance to several antibiotics by this type o colonies [90]. Given that, in order to study the expression of these antibiotic resistance mechanisms,

especially of MexAB-OprM efflux-pump, genetic expression assays were performed on SCV colonies from *P. aeruginosa* U147016-1.

2.7.1 RNA extraction

The total amount of RNA from *P. aeruginosa* SCV colonies was obtained (from at least 7 colonies), using GRS Total Kit (Grisp, Portugal). The protocol for the extraction and the reagents were provided in the Kit. Briefly, a minimum of 7 colonies were transferred to a 1.5 ml RNAse-Free eppendorff tube with ultra-pure water and by centrifuged at 15,000 g for 1 minute. The supernatant was completely removed and 200 µl of Buffer BL and Lysozyme mixture (10 mg/ml; previously prepared) was added and incubated for 10 minutes at room temperature. After another tube inversion, 200 µl of Buffer B1 and 3 µl of β-mercaptoethanol were added to the lysate, incubated for a further 5 minutes followed by centrifugation for 2 minutes at 15,000 g. The supernatant was then transferred to a new 1.5 ml RNAse-Free eppendorf tube, 700 µl of 70 % ethanol was added and the lysate was mixed by vigorous shaking. To a RNA mini spin column 500 µl of the sample mixture was added, centrifuged for 1 minute at 15,000 g and the flow-through discarded. This procedure was repeated for the rest of sample. After this step, the RNA mini spin column was placed in a new collection tube and a DNAse I treatment was performed by addition of 100 µl of DNAse I mixture (200U/µl) to the centre of the spin column, followed by incubation for 10 minutes at room temperature. Then, 400 µl of Wash Buffer 1 was added to the spin column, centrifuged for a minute at 15,000 g, followed by the addition of 600 µl of Wash Buffer 2 and another centrifugation at 15,000 g for 1 minute. The follow-through was discarded and one more centrifugation was performed for 3 minutes at 15,000 g, to dry the matrix of the column. To finalize the process, the spin column was transferred to a new 1.5 ml RNAse-Free micro centrifuge tube, 50 µl of RNAse-Free water were added to the centre of the column and incubated at room temperature for 2 minutes, followed by centrifugation at 15,000 g for 1 minute to elute the purified RNA . The RNA obtained was stored at -20 °C for further use.

2.7.2 RNA quantification

Prior to cDNA synthesis, RNA obtained previously in section 2.7.2 was quantified. To accomplish that, Thermo Scientific Nanodrop™ 1000 Spectrophotometer was used. Before the measurement of RNA concentration, one blank sample was analysed in order to place the initial concentration to 0 ng/µl. After that, 1 µl of the sample on the Nanodrop plate to quantify, selecting the right wavelength to RNA measurement.

2.7.3 cDNA synthesis

For the synthesis of cDNA from the RNA obtained by the previous step, the GRS RT-PCR Kit from Grisp was used. The protocol followed was provided in the kit. Before starting the protocol for cDNA synthesis, was performed a quantification of RNA obtained in the previous step (Thermo Scientific NanoDrop™ 1000 Spectrophotometer), For each reaction, two 1.5 µl microtubes were marked and chilled on ice for 5 minutes, one for the sample and other for RT-minus control. After that, the volume corresponding to 2.5 µg of RNA sample was used, with 1 µl of random hexamer primer and a volume of up to 13.4 µl water up were added to the microtubes. Then, the microtubes were briefly centrifuged and incubated at 70 °C in a thermo cycler (BioRad) for 5 minutes. After incubation, the microtubes were placed on ice for 1 minute, followed by a brief centrifugation, and placed again on the ice. To each microtube the following components were added: 4 µl of First-stand Buffer(5x), 1 µl of dNTP Mix, 0.6 µl of RNAse Inhibitor and 1 µl of M-MLV RT. To the RT- minus control RNAse-Free water was added instead of M-MLV RT. The mixture was inverted several times for mixture and incubated at 37 °C for 60 minutes in the thermo cycler (MJ Mini, Personal Thermal Cycler, BioRad). To finish the reaction, the microtubes were incubated at 70 °C for 5 minutes. The cDNA was after stored at -20 °C for further use.

2.7.4 DNAse I treatment

Additional DNAse I treatment was performed to the RNA samples in order to diminish possible contamination [91]. For this purpose, to 45 µl of RNA obtained in the extraction, 0.5 µl of DNAse I (2000U/mg; Grisp) and 5 µl of Reaction buffer (10x; Grisp) were added. The mixture was thoroughly mixed by pipetting up and down and posteriorly incubated at 37 °C for 30 minutes. After incubation, 5 µl of 25 mM EDTA (50 mM; Thermo Scientific) added, and mixed again by pipetting, followed by incubation at 65 °C for 10 minutes, to inactivate the enzyme. After that, RNA quantification was performed, as described in Section 2.7.2, followed by cDNA synthesis (Section 2.7.3).

2.7.5 Acid Phenol:Choroform genomic DNA removal

Initially, RNA sample volume was adjusted to 200 µl with nuclease-free water. After that step, 200 µl of the lower phase of acid phenol:chloroform solution was added to the initial sample tube, and mixed by inverting the tube 5 times and incubated at room temperature for 3 minutes. After that, the tubes were centrifuged for 5 minutes at 16,000 g at 4 °C, and the upper phase of the

centrifuged mixture was transferred to a new 1.5 ml RNAse-free eppendorf. The next step was the addition of 0.1 volume of sodium acetate (3M) followed by the addition of 3 volumes of ice-cold 100% ethanol, the solution was mixed by inversion of the tubes and incubation at -80 °C for one hour. To recover the RNA, the samples were centrifuged at 16,000g and 4 °C, for 30 minutes and the supernatant was removed. Two washing steps were implemented, by the addition of 800 µl of ice-cold 70% ethanol, and a spin to the tube was performed to remove residual ethanol. After that step the pellet was let to air-dry. The dried pellet was then resuspended in 50 ml nuclease-free water and left to sit for 2 minutes. The RNA samples were immediately placed on ice and RNA quantification (Section 2.7.2) and cDNA synthesis (Section 2.7.3) were performed afterwards.

2.7.6 qPCR

In order to perform quantitative Polymerase Chain Reaction (qPCR) KAPA SYBR® FAST qPCR Kit Master Mix (2x) Universal (KAPABIOSYSTEMS) was used. Prior to each reaction, a qPCR master mix for each primers set (16S and mexA) was made, with 5 µl of 2X KAPA SYBR® FAST qPCR Master Mix, 0.4 µl primers mix (Table 2.1), 0.2 µl 5X ROX high/low buffer and 3.9 µl PCR-grade water, to a total volume of 10 µl per reaction. A primer mix was used in order to avoid error by several pipetting, being the mix composed by same quantities of forward and reverse primers in a concentration of 10 µM.

Table 2.1 - Primers used for qPCR method, in this study

Gene		Sequence (5' to3')	Reference
MexA	Forward	CAG GCC GTG AGC AAG CAG	[92]
	Reverse	CCT TCG TGT AGC GCA GGT	
16S	Forward	CTC AGA CAC AGG TGC TGC	
	Reverse	CAC CGG CAG TCT CCT TAG	

On the first qPCR run the quality of the samples was determined by testing the NRT (no reverse transcriptase; a RNA sample is used to evaluate DNA contamination of the extracted RNA) controls and unknown DNA samples for the housekeeping gene 16S. the protocol used was the following: 1 cycle of enzyme activation for 3 minutes at 95 °C followed by 39 cycles of denaturation also at 95 °C for 10 seconds, annealing at 60 °C for 30 seconds and extension at 72 °C for 30 seconds. A second qPCR run was performed to test the primers efficiency for serial dilution and to choose the temperature with best Cq from both primers, already with the knowledge that the best efficiency

of 16S was obtained at 60 °C. Therefore several cDNA dilutions were tested being the ratios used 1:10, 1:20, 1:50, 1:100, 1:1000 and 1:10000. The efficiency of all dilutions tested was performed to three different temperature: 58 °C, 60 °C and 62 °C.

In order to ensure the specificity of primer annealing in every qPCR, a melt curve was performed from 72°C to 95 °C, 1 °C at a time and 5 seconds per °C in all qPCR protocols.

2.8 Statistical Analysis

Statistical analysis was performed using GraphPad Prism, version 6.01. Statistical significance values of the means of growth kinetics, pyocyanin quantification, motility and biofilm formation, were assessed by one-way ANOVA test. Further analysis was performed by Tukey's multiple comparisons test.

All tests were performed with a 95% confidence level.

3. RESULTS AND DISCUSSION

In this chapter the impact of oxygen depletion in *P. aeruginosa* clonal diversification was evaluated using three distinct strains, PA01, U147016 and ATCC 39324. As so, *P. aeruginosa* strains experience two microaerophilic environments (10 % CO_2 and 5 % O_2) and an anaerobic environment (1 % O_2) when grown in ASM for 10 days. After that, the overall *P. aeruginosa* populations were phenotypically characterized, as well as their sub-populations. Sub-populations were identified by the detection of colony morphology variation. Phenotypic characterization of the whole population included growth kinetics, mutation rate, pyocyanin production and antibiotic susceptibility.

3.1 Growth Kinetics

The growth profiles of *P. aeruginosa* strains grown in ASM with microaerophilic environments (10 % CO_2 and 5 % O_2) and an anaerobic environment (1 % O_2) are shown in Figure 3.1.

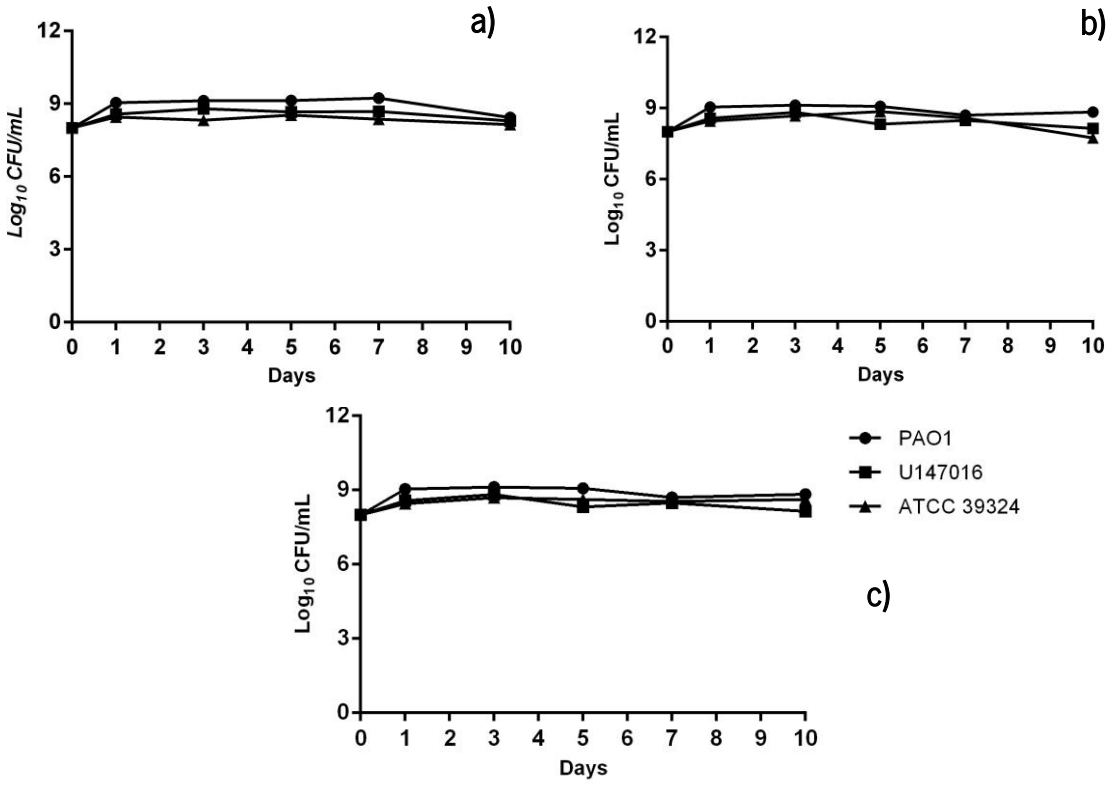


Figure 3.1- Growth kinetics of PA01, U14 7016-1 and ATCC 39324 under the different conditions tested. a) Microaerophilic environment with 10 % CO_2; b) Microaerophilic environment with 5 % O_2; and c) anaerobic environment with approximately 1 % O_2.

It can be observed that *P. aeruginosa* strains appear to have similar growth profiles in three different conditions tested, however some statistically significant variations ($p<0.05$) occurred during *P. aeruginosa* strains on all tested environments. In Appendix I, schemes with the statistically significant differences of growth observed for each strains in all environments, can be observed. Under both microaerophilic environments, with 10 % CO_2 and 5 % O_2, ATCC 39234 show a stable behaviour throughout the infection period ($p>0.05$). PA01 and U147016-1 shown, however similar behaviour with significant differences of growth in all days, when compared with day 10 of infection period ($p<0.05$), as can be observed in Appendix I. PA01 growth in microaerophilic conditions with 5 % O_2 present significant variations ($p<0.05$) in the intermediate days of infection, after day 5 of CF infection simulation. At the first and last days of infection, however, the PA01 growth profile is stable ($p>0.05$). The variations observed in the intermediate days of infection can be explained as the period of adaptation of PA01 population to the oxygen

availability conditions, being perfectly adjusted by the end of the infection period. Observing the growth profile of U147016-1 with 5 % O_2, statistical significant variations can be observed throughout the CF infection simulation period ($p<0.05$). It can be assumed that U147016-1 growth did not stabilize in this condition, as observed for PA01. Regarding anaerobic growth *of P. aeruginosa* populations, PA01, U147016-1 and ATCC 39324, all show similar behaviours during the infection period. All strains show significant variations during growth in anaerobic conditions ($p<0.05$), stabilizing growth in the end of the CF infection simulation period ($p>0.05$).

Even with these differences, by direct analysis of all growth profiles, we can conclude that tested *P. aeruginosa* strains show similar growth values despite the decreasing oxygen concentration to which all strains have been exposed. The variation in growth observed throughout the tested environments can be due to the process of adaptation by the strains to the oxygen concentration present. Given that, it can be hypothesized that *P. aeruginosa* is possesses the physiological tools needed to grow under limited oxygen availability.

Worlitzsch et al. were the first to describe the 'steep hypoxic gradients' within CF airway mucus, with several posterior studies supporting this information. [50], [51], [71] It has been demonstrated that, with chronic infection development, oxygen tension within the airways is highly reduced and the airway mucus can represent a microaerophilic or even an anaerobic environment. For that reason, different conditions with decreasing oxygen availability were studied. The results obtained were in accordance with other studies previously performed [50]. *P. aeruginosa* has been described to prefer aerobic respiration as "highest-energy-yielding process" for growth [93]. However, other researchers have also shown a preference of *P. aeruginosa* to grow under microaerophilic conditions, specially strains colonizing CF lungs [50][53]. In fact, it has been proven that *P. aeruginosa* has the capacity to secret compounds that actively generate microaerophilic environments. *P aeruginosa* is also capable of growing under anaerobic conditions, using in that case nitrate or nitrite respiration [50][46][49]. Therefore, the observed growth behaviour of *P. aeruginosa* under the conditions tested, aerobic, microaerophilic and anaerobic environments, is in accordance with the results found in the literature.

3.2 *P. aeruginosa* population diversity: colony morphology variation

Phenotypic switching is a common mechanism used by bacteria to increase population diversity to better adapt to a specific niche, to better face environmental fluctuations and/or stressful conditions. Through phenotypic switching, bacteria are able to reversibly change their virulence factors expression pattern and antimicrobial resistance profiles [94]. This commutable behaviour represents an enormous advantage to survive in harsh environments as CF lungs.

Colony morphology variation is the most observable characteristic associated with phenotypic switching and it was clearly observed in CF lung through isolation of mucoid morphotypes and SCV [45]. Moreover, distinct colony morphologies have been isolated from acute and chronic infections, for instance in chronic infections SCV and mucoid morphotypes are frequently isolated in contrast to acute infections. Therefore, it was hypothesized whether the monitoring of colony morphology variation within different environments with limited oxygen concentration could be useful to better understand the changes in *P. aeruginosa* infection in CF lungs.

As so, colony morphology of PA01, U147016 and ATCC 39324 populations grown in ASM and exposure to different microaerophilic and anaerobic environments was assessed throughout the infection simulation period. In Figure 3.2 is presented the colony morphology evolution of the three stains exposure to microaerophilic conditions established increasing CO_2 concentration to 10 %. Colony morphology was assessed at day 3, 5, 7 and 10, accordingly to the observed characteristics, represented in appendix II. All morphotypes observed in this study and correspondent characteristics can be observed in appendix III and IV.

Colony morphology was not assessed to day 1 of infection simulation since a previous study already performed this characterization for aerobiosis (unpublished data), condition present at day 1 of all tested environments.

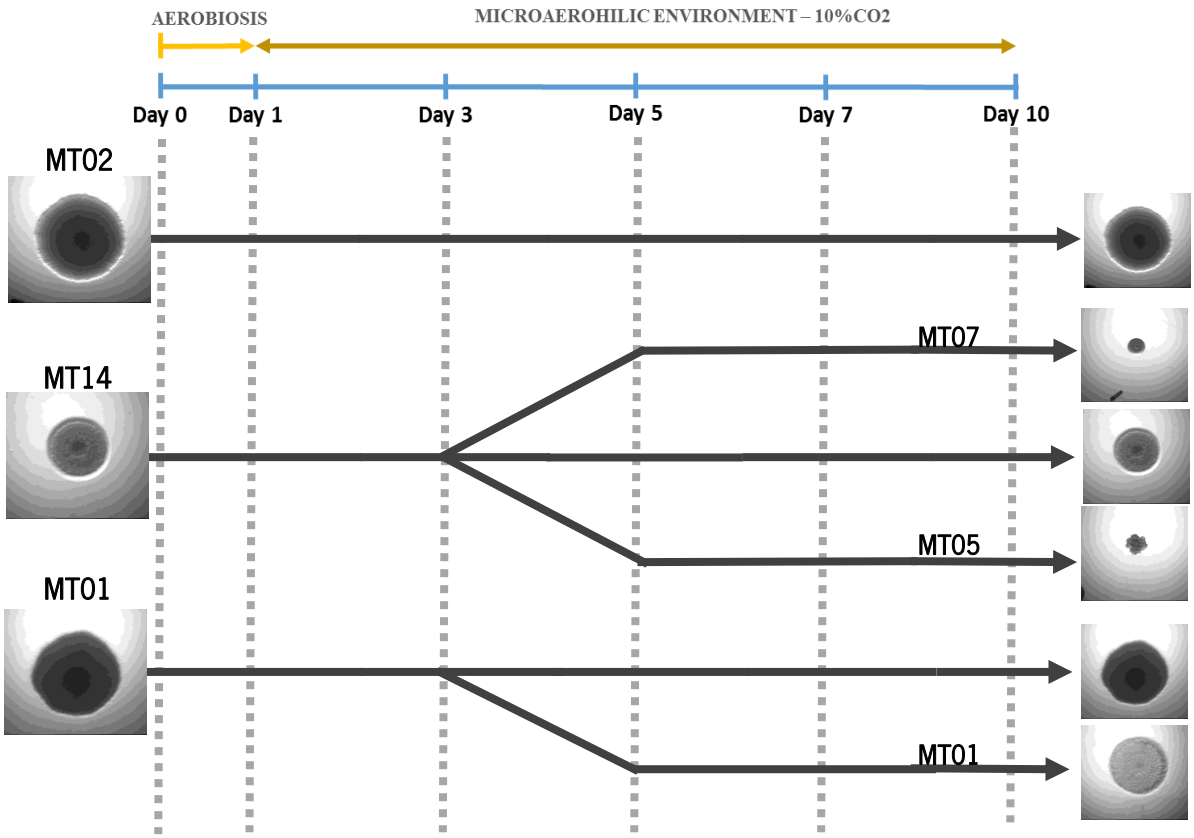


Figure 3.2 - **Colony morphology diversification during 10 days of infection simulation, under microaerophilic environment established increasing CO_2 concentration to 10 %.** For each *P. aeruginosa* strain, population diversity assay were initiated with colonization of ASM (day 0) with the respective wild morphotype bacteria. Each bifurcation represents the appearance of a new colony morphotype and the day in which that variation was observed. Full lines indicate that one morphotype was observed continuously during the infection period. Dashed lines are present when one morphotype was not observed. Colony diversification of MT01, MT14 and MT01 represent, respectively, the PA01, U147016-1 and ATCC 39324 colony morphology variations.

Analysing the colony morphology evolution of the three *P. aeruginosa* strains, it can be observed that the wild–morphotypes used for initial colonization of ASM, MT02 for PA01, MT14 for U147016 and MT01 for ATCC 39324, persisted during all infection period. PA01 strain was the only one that did not diversify in contrast to U14 and ATCC strains. Two different morphotypes were observed in U14 population at day 3, being two of them SCV (MT05 and MT07). In ATCC 39324 population, only one new morphotype, MT12, was observed also at day 3.

Interestingly, the variation in colony morphology of U147016-1 and ATCC 39324 was observed after the alteration in oxygen availability from aerobiose to the microaerophilic environment. Therefore, the emergence of new colony morphotypes could be an indication of an oxygen-induced adaptations. To evaluate this hypothesis, these new morphotypes were phenotypically characterized (see in section 3.6), in order to verify whether in effect *P. aeruginosa* undergone adaptation.

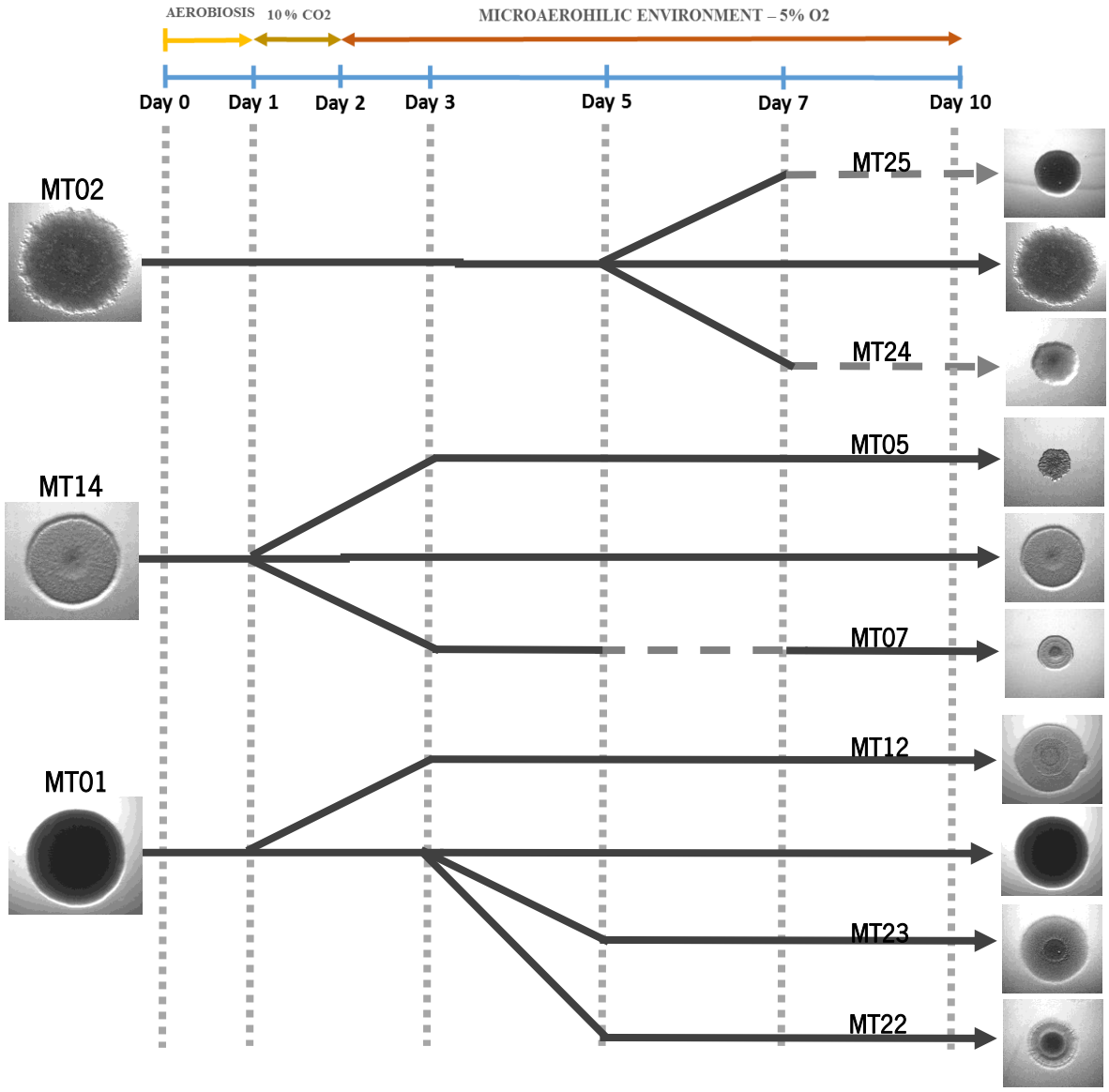


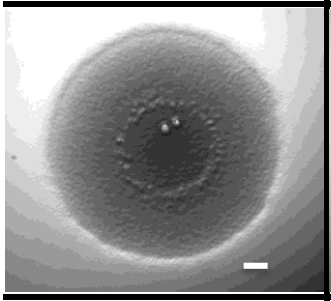
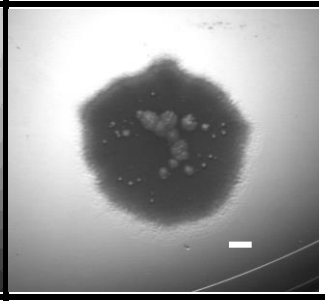
Figure 3.3 - **Colony morphology diversification during 10 days of infection simulation, under microaerophilic environment established by direct decrease in O_2 concentration to 5 %.** For each *P. aeruginosa* strain, population diversity assay were initiated with colonization of ASM (day 0) with the respective wild morphotype bacteria. Each bifurcation represents the appearance of a new colony morphotype and the day in which that variation was observed. Full lines indicate that one morphotype was observed continuously during the infection period. Dashed lines are present when one morphotype was not observed. Colony diversification of MT01, MT14 and MT01 represent, respectively, the PA01, U147016-1 and ATCC 39324 colony morphology variations.

Under microaerophilic conditions with 5 % O_2, variations in colony morphology were observed for all strains used, as can be observed in Figure 3.3. PA01 only initiated in colony diversification, after day 5 of infection simulation, 3 days after being exposed to the new microaerophilic environment. The initial morphotype, MT02, was present during the entire assay and, at this point, two new morphotypes were observed, MT24 and MT25, only at day 5. This was the first observation of these two morphotypes, in comparison with previous studies (unpublished data), which were the

target of more extensively characterization during this study, in terms of motility, biofilm formation ability, TSA reversion, growth in minimal medium, haemolysin production and antibiotic susceptibility. The results obtained for the morphotype characterization, by the colony morphology classification system (Appendix I), can be found in Appendix II.

U147016-1 showed, under these conditions (5 % O_2), similar development as observed under microaerophilic environment with 10 % CO_2. The only difference was the transient "disappearance" of the small colony MT07 at checkpoint of day 5 only. Regarding ATCC 39324, a great diversification was observed, since day 3 of infection. The wild-morphotype, MT01, and MT12 were present continuously during the infection simulation, as verified in the previous microaerophilic environment studied. In addition to MT01 and MT12, two new morphotypes were observed, MT22 and MT23. These two morphotypes were further characterized as previously described. However, in the process of classification of MT23, a problem in the classification system was found. Apparently, by visualization of the colonies under magnifying glass, MT23 seemed not to have been identified before. However, by the colony morphology classification system, this "new morphotype" corresponded to a morphotype identified in previous studies, as can be observed in Table 3.1.

Table 3.1 – Comparison of the morphological criteria of colony morphology classification system, between the previously identified morphotype, MT03, and the new morphotype found

New morphotype		MT 03	
			
Form	Circular	**Form**	Circular
Margin	Undulate	**Margin**	Undulate
Surface	Homogeneous	**Surface**	Homogeneous
Texture	Rough	**Texture**	Rough
Sheath	Present	**Sheath**	Present
Opacity	Opaque	**Opacity**	Opaque
Elevation	Flat	**Elevation**	Flat
Consistency	Dry	**Consistency**	Dry
Size	Large	**Size**	Large
Colour	Yellow	**Colour**	Yellow

Analysing the images of both colonies, a clear difference can be noted, and becomes impossible to identify these two colonies under the same morphotype code. Given that, an alteration in the classification system is needed, in order to resolve this problem. The solution found was the addition of a new criteria that can efficiently distinguish both colonies, as the presence or absence of surface elements. It can be observed that MT03 possess surface elements, whereas the new morphotype does not. So, with the addition of this new characteristic, the two colonies become different and the new code, MT23, can be attributed to the new morphotype. As a consequence of this, the colony morphology classification system now includes a new criteria, surface elements, having a total 11 different characteristics used for the classification of different colony morphologies. MT22 and MT 23, as a new morphotypes observed, were further characterized, as described before, being the results at Section 3.6.

The colony diversification of all *P. aeruginosa* population was more extensive in this environmnent (5 % O_2) when compared with microaephilic conditions with 10 % CO_2. At this point, the conclusion that oxygen depletion is a driving force for *P. aeruginosa* in CF simulated infection can be made. The observation of morphotypes not observed in previous CF infection simulation studies in aerobiosis (data unpublished) reinforces this conclusion and show a good ability of *P. aeruginosa* population to adapt to adverse conditions.

Since a greater diversity was found for 5 % O_2 it can be expected that this adaptive mechanism can be observed for other characteristics discussed later on this study, essentially for the characterization of the new morphologies found.

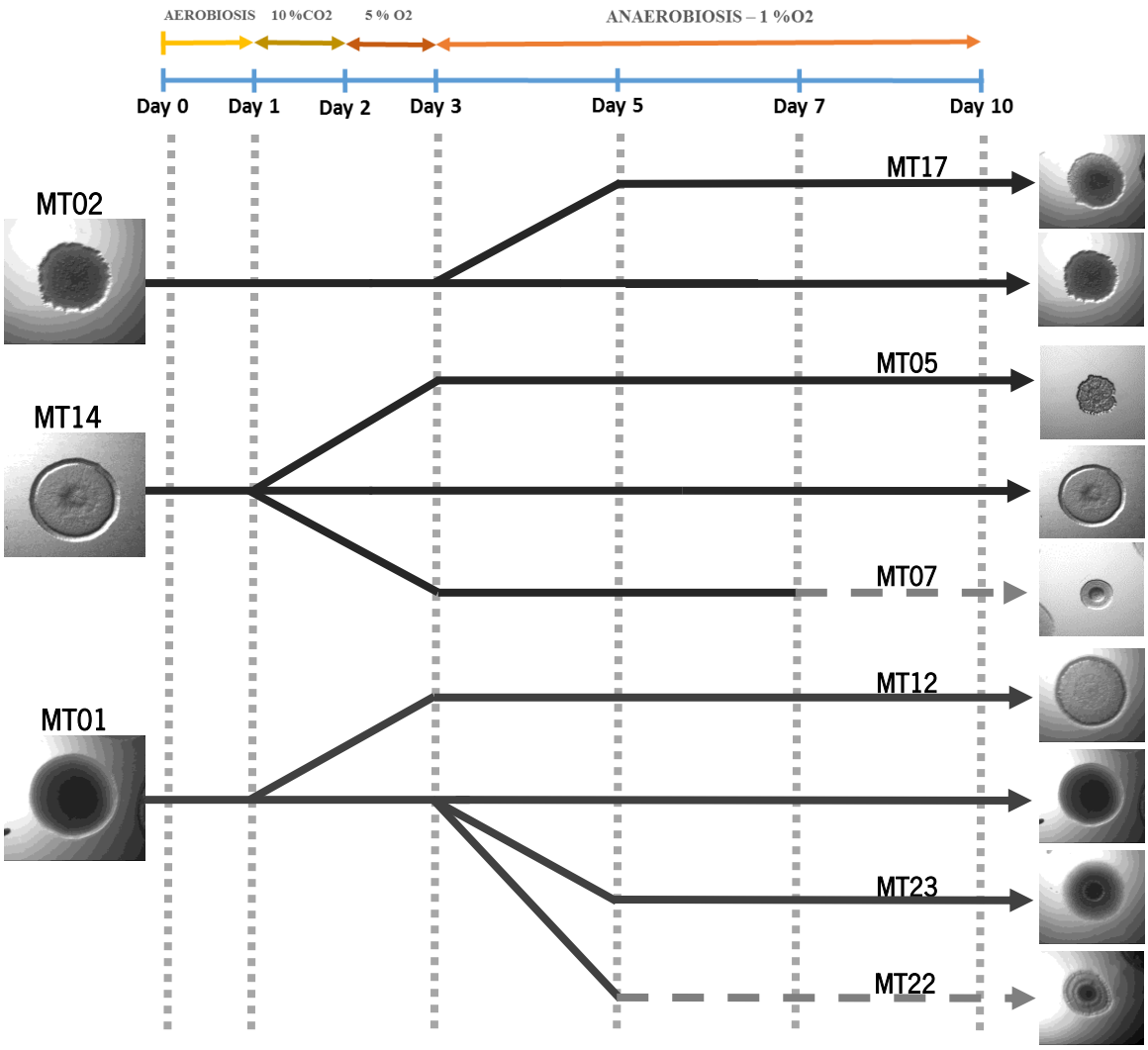


Figure 3.4 - **Colony morphology diversification during 10 days of infection simulation, under microaerophilic environment established by direct decrease in O_2 concentration to 1 %.** For each *P. aeruginosa* strain, population diversity assay were initiated with colonization of ASM (day 0) with the respective wild morphotype bacteria. Each bifurcation represents the appearance of a new colony morphotype and the day in which that variation was observed. Full lines indicate that one morphotype was observed continuously during the infection period. Dashed lines are present when one morphotype was not observed. Colony diversification of MT02, MT14 and MT01 represent, respectively, the PA01, U147016-1 and ATCC 39324 colony morphology variations.

The last studied condition was an anaerobic environment, with approximately 1 % O_2. The oxygen depletion was gradually achieved, intercalating all preciously studied environments, beginning with an aerobic environment, followed by microaerophilic environment with 10 % CO_2 and a microaerophilic environment with 5 % O_2, reaching, at day 4, the anaerobic conditions. This gradual reduction of oxygen availability has the purpose of better simulate the reported oxygen gradient present in CF lungs, leading to a better simulation of the real conditions associated in CF lung disease.

Under this environment, PA01 presents a different evolution profile when compared with the previous environment. As can be observed in figure 3.4, MT02, that persists until the end of CF

infection simulation period suffer only one diversification at checkpoint on day 5, with the observation of MT17. It is important to note that this evolution occurred after day three, the same day when oxygen availability was reduced to an anaerobic condition.

P. aeruginosa U147016-1 population shown a similar behaviour as observed for microaerophilic conditions with 5 % O_2, as MT14 and MF05 (diversification observed at day 5) persisted throughout the CF infection simulation period. MT07, observed after day 5, disappear from the population at checkpoint on day 10.

At this checkpoint, 3 different morphotypes were observed, which had been previously observed on the other conditions, indicating that the oxygen depletion to anaerobic had little or no significant effect on the colony morphology variation for U147016-1.

For ATCC 39324, the colony morphology profile described in previous microaerophilic environment persisted, with the continuous observation of MT01, MT12 and MT23. MT22, detected under microaerophilic environment with 5 % O_2, persisted on anaerobic conditions only until checkpoint at day 5. It is possible that until day 5 of infection, the harsh oxygen limitation was not an effective selective pressure for this morphotype, since that condition was only enforced at day 3 of infection. It is possible that the anaerobic environment was still not stable and a higher oxygen concentration was still present, reason why MT22 would still be present in the population, since it was identified under 5 % O_2 growth conditions.

All strains tested presented distinctive behaviours regarding the adaptation to oxygen depletion. PA01, under 10 % CO_2 showed no colony diversification during the infection period simulation, persisting only the initial wild-type morphotype. With the decrease in oxygen availability, a unique diversification was observed, not being observed again, including in anaerobic conditions, in which a morphotype previously observed and characterized (see Appendix III and IV) emerged in the population. It is possible to conclude that some selective pressure was present under 5 % O_2 that caused the morphologic diversification under this environment. Studies suggest *P. aeruginosa* prefers microaerophilic conditions of grow to aerobic condition [95][96]. In this environments *P. aeruginosa* is still capable to accomplish aerobic respiration with oxygen as terminal electron acceptor [48]. However, this oxygen concentration is in the threshold of oxygen concentration, between the classification as microaerophilic and anaerobic environments [95][96]. Therefore, is possible that also anaerobic respiration with nitrite as final electron acceptor may occur, as an initial adaptation to anaerobic conditions [48]. This turning point between modes of growth could enhance biological diversity as a mode of *P. aeruginosa* be adapted to both environments.

In all conditions tested, U1479016-1 exhibited the most regular evolution of the three strains. Under both microaerophilic environments and anaerobiosis, this strains showed the same three different morphotypes, MT14, the initial colony morphology, MT05 and MT07, both small colony variants. A conclusion that can be reached from these results is that oxygen depletion has no significant interference in diversification of colony morphology of U147016-1. To support this conclusion, results from previous studies with aerobic growth of this strain (unpublished data) showed similar colony variants as observed in this study, proving that oxygen reduction is not trigger of morphologic diversification to this strain.

The third strain used, ATCC 39324, displayed a mucoid phenotype through all CF infection period simulation (MT01), under all conditions tested, as well as a dry and transparent morphotype, MT 12, observed and characterized in previous studies. In addition to that, two new morphotypes were found, MT22 and MT23, that had not been previously observed or characterized, emerged in microaerophilic environment with 5 % O_2 and were also observed in anaerobic conditions. The first observation of these new colony morphologies, MT22 and MT23, was under 5 % O_2 and they were also observed under anaerobic conditions. Despite MT22 being identified in the anaerobic environment, it disappear after two days of exposure to this condition. It can concluded that MF22 didn't adapt to the new environment imposed, and was eliminated from the population.

CF lungs have been documented for the high heterogeneity present for various characteristics, as nutrient availability, oxygen distribution, interactions with other organisms, biofilm mode of growth, antibiotic treatment, among others [48]. Therefore, colony heterogeneity results in sub-populations due to different niches in the environment. The heterogeneity observed during this study reinforce the idea that oxygen depletion is a driving force of diversification for *P. aeruginosa*, being this bacteria perfectly capable of adapt and thrive in harsh environments. The greater diversity observed in microaerophilic environment with 5 % O_2 can be interpreted as an indication of switch to chronic infection.

3.3 Mutators frequency

In harsh environments such as chronic CF lung infections, it has been described that hypermutability is a beneficial characteristic for the selection of adapted niche specialists and survival of pathogens [97]. From acute to chronic infection in CF lungs, the emergence of hypermutators have been widely reported in literature [73][78]. In this study, mutation frequency was assessed through the infection simulation period, in order to understand the impact of oxygen depletion in *P. aeruginosa* strains. At each checkpoint, strains were classified based on mutation frequency, *f*, calculated as described in section 2.5.3. Strains were considered hypomutable when $f<7x10^{-9}$; normomutable when $7x10^{-9}<f<2 \times 10^{-7}$; weak mutators when $2x10^{-7}<f<1x10^{-6}$ and strong mutators when $f \geq 1x10^{-6}$.

The results obtained concerning the exposure of *P. aeruginosa* strains to microaerophilic conditions, established increasing CO_2 concentration to 10 %, revealed that the presence of mutators seemed to be not the driven force for *P. aeruginosa* population diversity (Table 3.2).

Table 3.2 - Mutation frequency of *P. aeruginosa* strains tested during microaerophilic environment with 10 % CO_2

	Mutation frequency (mutants per cell)					
	PA01		U147016-1		ATCC 39324	
Day 1	$< 5 \times 10^{-11}$	HM	$< 5 \times 10^{-11}$	HM	$< 5 \times 10^{-11}$	HM
Day 3	$8{,}75 \times 10^{-8}$	NM	$6{,}29 \times 10^{-8}$	NM	$2{,}78 \times 10^{-7}$	WM
Day 5	$< 5 \times 10^{-11}$	HM	$< 5 \times 10^{-11}$	HM	$8{,}57 \times 10^{-7}$	WM
Day 7	$5{,}66 \times 10^{-8}$	NM	$4{,}48 \times 10^{-8}$	NM	$4{,}17 \times 10^{-7}$	WM
Day 10	$5{,}56 \times 10^{-8}$	NM	$< 5 \times 10^{-11}$	HM	$< 5 \times 10^{-11}$	HM

*HM – hypomutable; NM- normomutable; WM – weak mutator; SM – strong mutator

Indeed, some variations in mutation frequency for the three strains were observed. Mutation frequency of PA01 fluctuates from hypomutable (at days 1 and 3) to normomutable on the remaining days of infection period. U147016 showed a similar behaviour to PA01, the only difference being at day 10, where it presents a hypomutable phenotype. Higher mutation frequencies were observed for ATCC 39324, in comparison to the other two strains. On the first and last day of infection simulation, hypomutable phenotype was observed. However, at days 3, 5 and 7, ATCC 39324 presented weak mutator phenotype. Despite all the referred fluctuations in mutation frequencies, the three *P. aeruginosa* did not achieve frequency rates higher enough for hypermutability to be consider a driven force of the observed *P. aeruginosa* diversification in this microaerophilic environment.

Interestingly, slightly differences were observed in mutation rates of *P. aeruginosa* population exposure to microaerophilic environment with 5% O_2 (Table 3.3)

Table 3.3 -Mutation frequency of *P. aeruginosa* strains tested during microaerophilic environment with 5 % O_2

	Mutation frequency (mutants per cell)					
	PA01		U147016-1		ATCC 39324	
Day 1	$< 5 \times 10^{-11}$	HM	$< 5 \times 10^{-11}$	HM	$< 5 \times 10^{-11}$	HM
Day 3	$8{,}03 \times 10^{-7}$	WM	$2{,}90 \times 10^{-5}$	SM	$1{,}54 \times 10^{-7}$	NM
Day 5	$5{,}67 \times 10^{-7}$	WM	$7{,}41 \times 10^{-8}$	NM	$3{,}64 \times 10^{-7}$	WM
Day 7	$1{,}23 \times 10^{-7}$	NM	$7{,}12 \times 10^{-8}$	NM	$1{,}69 \times 10^{-7}$	NM
Day 10	$3{,}70 \times 10^{-7}$	WM	$< 5 \times 10^{-11}$	HM	$< 5 \times 10^{-11}$	HM

*HM – hypomutable; NM- normomutable; WM – weak mutator; SM – strong mutator

PA01 showed an evolution from hypomutable phenotype at day 1 of infection to weak mutator at days 3, 5 and 10. At day 7, normomutable phenotype was recovered. A strong mutator phenotype was observed for U147016 at day 3, not being observed on the remaining days. At day 1 and day 10, U147016 was hypomutable and, at days 5 and 7, a normomutable phenotype was observed. Regarding ATCC 39324, a hypomutable phenotype at day 1 evolved to normomutable at day 3 and to weak mutator at day 5. From that day, a decrease in mutation frequency occurred, with a normomutable phenotype observed at day 7 and, that change to hypomutable ate day 10.

As observed for 10% CO_2, overall fluctuations in mutation frequency for incubation with 5 % O_2 did not achieved rates high enough to induce significant diversification in *P. aeruginosa* population. However, as one strong mutation phenotype was observed for U147016-1, even if transient, it can be sufficient to raise a possibility of an alteration in *P. aeruginosa* population at day 3 of this microaerophilic environment.

In anaerobic conditions, however, U147016-1 show a significant increase in mutation frequency when compared with the previous microaerophilic environments, as can be seen in Table 3.4.

Table 3.4 - Mutation frequency of *P. aeruginosa* strains tested during microaerophilic environment with 1 % O_2

	Mutation frequency (mutants per cell)					
	PA01		U147016-1		ATCC 39324	
Day 1	$< 5 \times 10^{-11}$	HM	$< 5 \times 10^{-11}$	HM	$< 5 \times 10^{-11}$	HM
Day 3	$8{,}03 \times 10^{-7}$	WM	$2{,}90 \times 10^{-5}$	SM	$1{,}54 \times 10^{-7}$	NM
Day 5	$6{,}94 \times 10^{-8}$	NM	$8{,}37 \times 10^{-6}$	SM	$3{,}55 \times 10^{-7}$	WM
Day 7	$8{,}89 \times 10^{-8}$	NM	$6{,}82 \times 10^{-6}$	SM	$6{,}73 \times 10^{-7}$	WM
Day 10	$4{,}76 \times 10^{-8}$	NM	$8{,}89 \times 10^{-5}$	SM	$1{,}40 \times 10^{-7}$	NM

*HM – hypomutable; NM- normomutable; WM – weak mutator; SM – strong mutator

With an anaerobic environment, mutation frequency of PA01 changed from hypomutable to weak mutator, from day 1 to day 3, as can be observed in Table 3.4. From day 5 until the end of the assay, a normomutable phenotype was observed. U147016-1 shown a high mutation frequency under this conditions, with strong mutator phenotype through the infection period, with the exception on day 1, were it was hypomutable. Mutation frequency of ATCC 39324 increased since day 1, from hypomutable to normomutable at day 3, and weak mutator at day 5 and 7. At day 10, mutation frequency decreased again to normomutable phenotype. In this condition, we can expect that hypermutability to be a driven force of diversification in U147016-1 population, since a strong mutator phenotype persisted during all infection simulation period. Despite that, the other two tested *P. aeruginosa* strains show a similar behaviour as observed for 10 % CO_2 and 5 % O_2, in which mutation frequency rate.

Analysis of the overall results of mutation frequency during these experiments of all strains tested, shows an increase of mutation frequencies with the decrease of oxygen availability. This general behaviour is consistent with results obtained in other studies which have reported an increase in mutator strains with the development of chronic infection and growth under microaerophilic/anaerobic environments [74]. Indeed, results show the appearance of strong mutation phenotype for U147016-1 under anaerobic conditions, confirming the influence of oxygen availability in the emergence of mutator phenotypes [98]. However some discrepancies can be observed, since there is no linear evolution of mutator phenotypes through each condition tested. It was expected that, under the same oxygen conditions, mutation frequencies of the same strain would show a pattern. However, this behaviour is not observed for all conditions. In the first condition tested, microaerophilic environment with 10 % CO_2, both PA01 and U147016-1 mutation frequencies fluctuated between hypomutable and normomutable through all checkpoints. The same was observed for ATCC 39324, which after three checkpoints with weak mutator phenotype,

shown a loss of mutation frequency on the last day of infection simulation (hypomutable). This alteration was not expected since, the same conditions were present since day 1 until day 10 (see figure 1). However it is possible that at the end of the infection period in that oxygen concentration, ATCC 39324 did not need the mutator phenotype to be perfectly adapted to the environment, since it was isolated from the sputum of a CF patient. Therefore, it was better adapted to the conditions tested in this study than PA01 and U14701-1, strains isolated from very distinctive environments. Under the second condition tested (Table 3.3), none of the three strains maintained the same mutation phenotype under the same oxygen concentration. Only at the anaerobic environment tested, a stable mutation frequency was observed, with PA01 as normomutable, U147016-1 as strong mutator. ATCC shown the same behaviour as in the previous conditions and at the last day of infection period, mutation frequency evolved from weak mutator to normomutable.

This variation of mutation frequency can be caused by the oxygen fluctuations applied to the plates of infection simulation in each checkpoint day. In order to collect samples necessary to the testes effectuated during this study, the plates were exposed to normal concentrations of oxygen, for a short period of time, which can be sufficient to induce variations on the mutation frequencies. As checkpoints were performed almost every couple of days, oxygen concentrations were difficult to maintain stable.

Some studies hypothesize that in CF airways hypermutation might be "short-lived" in certain niches, with the imposition of strong selective pressures over the infection development, which avoids hypermutability domination [31]. Besides this, hypermutability itself can be only temporary, in accordance with the DNA repair capacity of *P. aeruginosa*. The selection pressure, imposed by nutrient availability, oxygen depletion, reactive oxygen species, among other, can saturate the capacity of DNA repair, leading to the rise of temporary mutator phenotype, partially explaining the heterogeneity of mutation frequencies observed during this study [25][29].

3.4 Pyocyanin production

The capacity *P. aeruginosa* to produce a blue-green pigment named pyocyanin is widely known. This pigment is frequently detected in mucus samples from CF lungs and it has been associated with the succeeded establishment of acute and chronic infections in the lungs.[60], [61] Some studies have demonstrated that pyocyanin has an effect on microbial community development and control redox homeostasis in *P. aeruginosa*, revealing its contribution to survival of cells during

oxidant limitation. [99] Therefore, the determination of pyocyanin production was included in this study as it has an important role in *P. aeruginosa* infection development in CF lungs.

Throughout this study, pyocyanin production was measured by a chemical assay, quantifying its amount present in the ASM. The results obtained for the quantification of pyocyanin production by PA01, U147016 and ATCC 39324 for the three environments tested are presented in Figure 3.6.

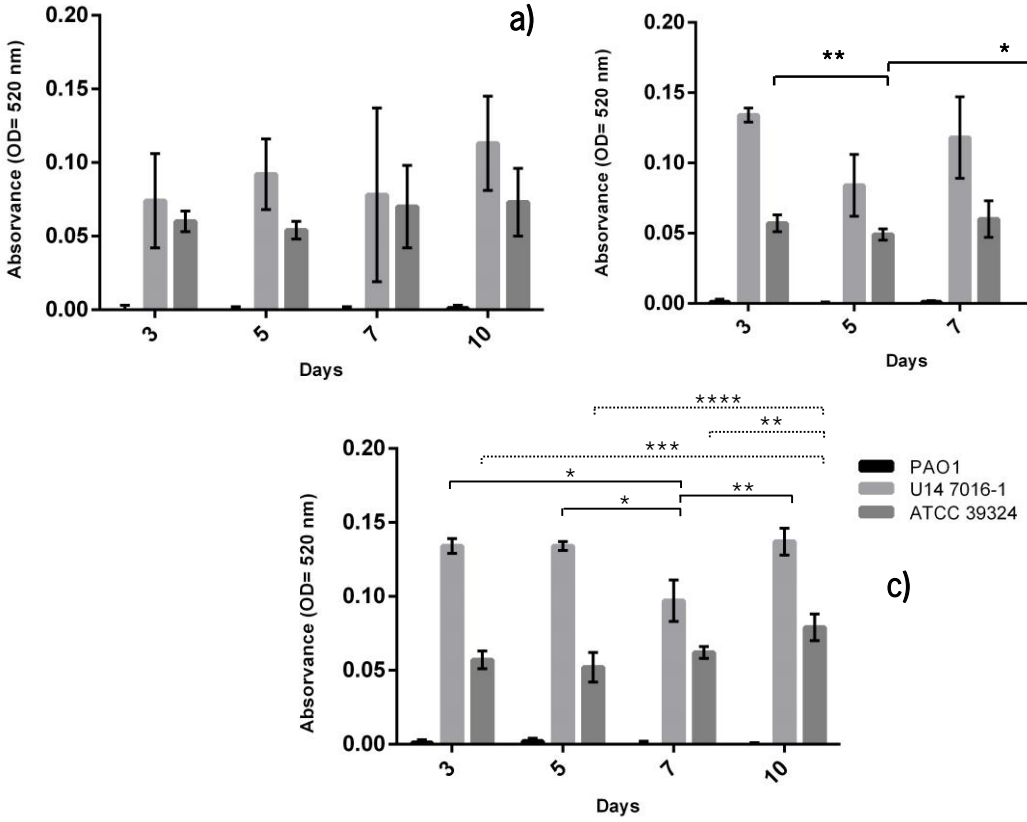


Figure 3.5 – The pyocyanin produced (OD_{520}) by *P. aeruginosa strains*, in all conditions tested: a) Microaerophilic environment with 10 % CO_2 ; b) Microaerophilic environment with 5 % O_2; c) Anaerobic environment with approximately 1 % O_2. Bars represent the average of three independent repeats ± SD (* - p<0.05; ** - p<0,01; ***- p<0,001; ****- <0,0001).

From the results obtained, represented in Figure 3.5, it was possible to verify for all oxygen concentrations tested that U147016 had the higher production of pyocyanin, followed by ATCC 39324 strain and PA01 presented a residual pyocyanin production. Pyocyanin production (by all strains) under microaerophilic conditions (with 10 % CO_2), was stable throughout the entire CF infection simulation period (p>0.05). The same was observed for microaerophilic conditions with 5 % O_2 for ATCC 39324, with no significant variation on pyocyanin production between all checkpoint days. At this condition, variations on pyocyanin production of U147016-1 were found, as can be observed on Figure 3.5, variations that persisted and become more pronounced in anaerobic conditions, essentially at day 7 of CF infection simulation. Under the anaerobic

conditions tested, significant differences ($p<0.05$) of pyocyanin production by ATCC 39324 were observed at day 10. It can be concluded that some stimuli has occured during microaerophilic conditions with 5 % O_2 and anaerobiosis, that caused the significant variation in pyocyanin production through the CF infection period. A possible explanation for this variation is the exposure of the *P. aeruginosa* population to oxygen at the checkpoint days. Despite this exposure had been reduce to the minimal possible, it is possible that had an interference in the values obtained. For that reason, the variations observed may not translate into significant biological variations in the simulation performed in this study.

The general consensus regarding CF chronic infection by *P. aeruginosa* is that with the development of infection from the acute to chronic stages, these bacteria lose some of its virulence factors, in order to establish a long-term presence in CF airways [10]. Under aerobic conditions, pyocyanin production and other virulence factors are regulated in a cell density-dependant manner (quorum sensing) [77]. There are several mechanism responsible for impairment of the quorum sensing mechanisms, such as oxygen restriction, reason why under the anaerobic conditions observed in chronic infected patients, *P. aeruginosa* become avirulent [79][80]. With this knowledge, is was expected that under diminished oxygen availability conditions, pyocyanin production by *P. aeruginosa* was impaired [79][80]. This behaviour was not observed in this study, as variations in pyocyanin production under microaerophilic and anaerobic environments, were not significant (Figure 3.5) in the three conditions tested.

Despite the unexpected values for *P. aeruginosa* development under hypoxia, this can be explained by the *P. aeruginosa* load during the infection periods tested. Studies by Hunter *et al*. [100] demonstrated that *in vivo*, levels of pyocyanin production are not determined by the bacterium ability to produce this toxin, but instead by the total load of bacteria within the sputum. Comparing both results of growth kinetics and pyocyanin production, both have similar behaviour, which can be in accordance with previous studies.

3.5 Antibiotic susceptibility of the population

The antibiotic susceptibility of the whole *P. aeruginosa* populations grown in ASM were tested using the disc diffusion method, in accordance with the Performance Standards for Antimicrobial Susceptibility Testing.[89] Ten different antibiotics were tested, belonging to different classes of antibiotics: penicillin (PRL), β-lactams (TTC), cephems (CAZ, FEP), monobactams (ATM), carbapenems (IMI), aminoglycosydes (CN, TOB, AK) and fluoroquinolones (CIP). The antibiotic

susceptibility was assessed for all conditions, the two microaerophilic environments (established by increasing CO_2 concentration to 10% and decreasing O_2 concentration to 5%) and anaerobic environment (1% O_2 concentration) and assayed over the *in vitro* infection model used. The results obtained for each conditions, with 10 % CO_2, 5 % O_2 and 1 % O_2, are represented in Tables 3.5, 3.6 and 3.7, respectively.

Table 3.5 - Antibiotic susceptibility of PA01, U14 7016-1 and ATCC 39324 under microaerophilic environment under 10 % CO_2, through infection period

		AK	ATM	CN	CAZ	TOB	FEP	IMI	TTC	CIP	PRL
PA01	Day 3	S	S	S	S	S	S	S	S	S	S
	Day 5	S	S	S	S	S	S	I	S	S	S
	Day 7	S	S	S	S	S	S	S	S	S	S
	Day 10	S	S	S	S	S	S	I	S	S	S
U147016-1	Day 3	S	S	S	S	S	S	I	I	S	S
	Day 5	S	S	S	S	S	S	S	S	I	S
	Day 7	S	S	S	S	S	S	S	S	I	S
	Day 10	S	S	S	S	S	S	S	I	I	S
ATCC 39324	Day 3	S	S	S	S	S	S	S	S	S	S
	Day 5	S	S	S	S	S	S	S	S	S	S
	Day 7	S	S	S	S	S	S	S	S	S	S
	Day 10	S	S	S	S	S	S	S	S	S	S

Table 3.6 - Antibiotic susceptibility of PA01, U14 7016-1 and ATCC 39324 under microaerophilic environment under 5 % O_2, through infection period

		AK	ATM	CN	CAZ	TOB	FEP	IMI	TTC	CIP	PRL
PA01	Day 3	S	S	S	S	S	S	S	S	S	S
	Day 5	S	S	S	S	S	S	I	S	S	S
	Day 7	S	S	S	S	S	S	S	S	S	S
	Day 10	S	S	S	S	S	S	I	S	S	S
U147016-1	Day 3	S	S	S	S	S	S	S	I	I	S
	Day 5	S	S	S	S	S	S	S	S	I	S
	Day 7	S	S	S	S	S	S	S	S	I	S
	Day 10	S	S	S	S	S	S	S	S	I	S
ATCC 39324	Day 3	S	S	S	S	S	S	S	S	S	S
	Day 5	S	S	S	S	S	S	S	S	S	S
	Day 7	S	S	S	S	S	S	S	S	S	S
	Day 10	S	S	S	S	S	S	S	S	S	S

Table 3.7 - Antibiotic susceptibility of PA01, U14 7016-1 m and ATCC 39324 under anaerobic environment under 1 % O_2, through infection period

		AK	ATM	CN	CAZ	TOB	FEP	IMI	TTC	CIP	PRL
PA01	Day 3	S	S	S	S	S	S	S	S	S	S
	Day 5	S	S	S	S	S	S	I	S	S	S
	Day 7	S	S	S	S	S	S	I	S	S	S
	Day 10	S	S	S	S	S	S	I	S	S	S
U147016-1	Day 3	S	S	S	S	S	S	S	I	I	S
	Day 5	S	S	S	S	S	S	S	I	R	S
	Day 7	S	S	S	S	S	S	S	I	I	S
	Day 10	S	S	S	S	S	S	S	I	I	S
ATCC 39324	Day 3	S	S	S	S	S	S	S	S	S	S
	Day 5	S	S	S	S	S	S	S	S	S	S
	Day 7	S	S	S	S	S	S	S	S	S	S
	Day 10	S	S	S	S	S	S	S	S	S	S

Observing susceptibility profiles of ATCC under all different conditions tested, it can be concluded that this strain suffered no alteration on antibiotic susceptibility profile with the alteration of oxygen availability. In both microaerophilic and anaerobic environments, ATCC 29324 was susceptible to all antibiotics tested. The same behaviour was not observed for PA01 and U147016-1. Though all infection simulation period and under the decreasing oxygen availability in the simulated environments, U147016-1 was susceptible to seven of ten tested antibiotics and has changed its sensitive to TTC to intermediate resistant, at day 3 under microaerophilic environment with 10 % CO_2. This change in susceptibility for this antibiotic was not found on any of the other environments. Regarding the β-lactam tested (TTC), under microaerophilic environment with 10 % CO_2, intermediate susceptibility was observed at day 3 and day 10 of infection simulation period. Under 5 % O_2, the same intermediate susceptibility profile was only verified at day 3. However, examining table 3.7, with the results of antibiotic susceptibility under anaerobiosis, intermediate susceptibility profile to this antibiotic was found on all checkpoint days. Results, concerning the susceptibility profiles of U147016-1 to ciprofloxacin (fluoroquinolone, CIP), show a variation on susceptibility profile when compared with the other strains. Under microaerophilic conditions, with 10 % CO_2, U147016-1 has an intermediate susceptibility to CIP though all checkpoint days, with the exception of day 3. Similar behaviour is observed for the microaerophilic environment with 5 % O_2 and for the anaerobic environment. In the first conditions intermediate susceptibility was also found on day 3 of the infection period, and persisted until the end. Under anaerobic conditions, resistance of U147016-1 to ciprofloxacin was observed at day 5 of the infection period, being intermediate susceptible to this antibiotic though the remaining days of the simulation. It is important to note

that all replicates of U147016-1, at day 5, were resistant to CIP. This strain was isolated from an urinary infection in an hospital setting and an antibiogram was performed to this strain for seven antibiotics: imipenem, ciprofloxacin, ceftazidime, gentamicin, amikacin, tazobactam and tobramycin (unpublished data). The results shown a resistance profile of this strain only to ciprofloxacin (unpublished data), reason why it can be concluded that the resistant profile observed in this study for ciprofloxacin is not due to the conditions and CF infection simulation, but to the intrinsic resistance of the strain.

Analysing the results of PA01, it can be observed that this strain is susceptible to 9 of 10 antibiotics used, under all conditions tested during this study. The exception was IMI (carbapenem). In the first condition studied, microaerophilic environment with 10 % CO_2, intermediate susceptibility was found on checkpoint at day 5 and 10, being susceptible on the remaining days. At 5 % O_2, no alterations were found, being PA01 susceptible to IMI though the infection period. However, under anaerobiosis, intermediate susceptibility was stable and emerged again, on checkpoints at day 5, 7 and 10.

Overall, under both microaerophilic and anaerobic environments, alterations in the susceptibility profiles were found for three of the 10 antibiotics tested: TTC, CIP and IMI.

Several resistance mechanisms have been described for *P. aeruginosa*, mainly in the heterogeneous environment present in cystic fibrosis lungs. Oxygen depletion, biofilm mode of growth, elevated expression of efflux-pumps and hypermutation are some of the mechanisms observed in this context, which can be responsible to the emergence of resistance in *P. aeruginosa*. [79], [80]

Anaerobic conditions, as observed in CF airways, are known to diminish the efficacy of most antimicrobials used to manage *P. aeruginosa* infections. In oxygen-limited environments, *P. aeruginosa* grow in biofilms and, therefore, have reduced metabolic activity and slower growth. [79] Since many antimicrobials target active cells, it is expected that under low oxygen availability, as used in this study, some alterations in susceptible profiles can be observed. The results obtained in this study show, indeed, a more stable presence of intermediate resistance to some antibiotics in anaerobiosis. However some intermediate resistance profiles were already observed in both microaerophilic conditions tested, those profiles were intermittent throughout the infection period. The variation observed in the microaerophilic environment (mainly at 10 % CO_2) can be due to variations in O_2 availability that can be present in the checkpoints days (due to the removal of the plates form the incubator).

Moreover, in the anaerobic environment, one resistant profile was observed, for U147016-1, only in one day of the infection period. Despite this can be considered a not normal result, it can be explained by the mutation frequency assessed for this strain, since U14716-1 presented a strong mutator phenotype under anaerobic conditions. Hypermutability, a common characteristic in CF lung infections, has an important impact in antimicrobial resistance. It has been shown that mutator strains have higher rates of antibiotic resistance than non-mutator strains. [74] Therefore, it is possible that at day 5, under the anaerobic conditions, mutants resistant to CIP were selected as better adapted to the conditions. However, with the maintenance of the anaerobic environment, those mutants were no long the fittest to survive in that conditions, and were eliminated from the population.

3.6 Phenotypic characterisation of new colony morphotypes-associated bacteria

Four new colony morphotypes were identified during this study: MT24 and MT25 from PA01 strain and MT22 and MT23 from ATCC strain. These morphotypes are referred as "new" since were not observed in any previous study of *P. aeruginosa* diversification in aerobic conditions (unpublished data). Biofilm formation, motility, antibiotic susceptibility, haemolysin production, colony reversion and growth on minimal medium were assessed for each new morphotype.

3.6.1 Biofilm formation

Biofilm formation is considered one of the most important adaptation mechanism of *P. aeruginosa* in CF infections, allowing evasion of the inflammatory defences and antibiotic therapy [49]. Therefore, the biofilm formation ability of the morphotypes was performed and represented in Figure 3.6, to understand the impact of this virulence factor in *P. aeruginosa* adaptive process.

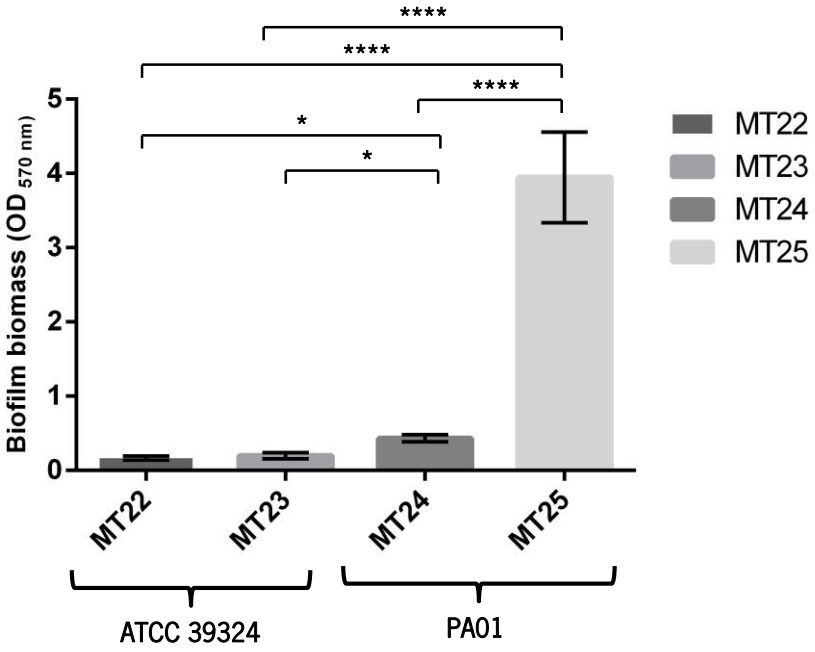


Figure 3.6 - Biofilm formation ability of all four new morphotypes, MT22, MT23, MT24 and MT25. Bars represent the average of three independent assays ± SD (* - p<0.05; ** - p<0.01; ***- p<0.001; ****- <0.0001).

Analysing Figure 3.6., it can be observed that MT 25 is the morphotype most able to form biofilm. MT22, MT23 and MT24 present low biofilm formation, and can be classified as poor biofilm producers. As can be seen in Figure 3.6, differences in biofilm formation are significant when we compare the results of ATCC 39324 morphotypes with PA01 morphotypes ($p<0.05$). The same is observed for comparison of both PA01 morphotypes. Overall, morphotypes of the same strain have similar abilities of biofilm formation, even displaying different colony morphology phenotypes. Regarding the general characteristics of colony morphologies of both PA01 morphotypes, in appendix II and III, it can be observed that MT24 can be classified as a small colony variant (size ≤ 3mm). [90] Although MT25 has superior size than MT25, is on the limit of classification as small colony. SCV have been reported has good biofilm producers, which can explain the results obtained for MT24 and MT25, when compared with other colony morphologies [102]. Since biofilm formation is seen as an adaptive mechanism to the CF lung environment, conclusions can be made regarding MT25, since this morphotype show much higher biofilm formation than the other morphotypes analysed. Microcolonies formation by *P. aeruginosa* in CF airways is been identified as the predominant mode of growth in chronic infection, conferring to *P. aeruginosa* protection against oxidative stress, antibiotic treatment and phagocytic activity [45][46]. Therefore, biofilm formation is an adaptive characteristic responsible for *P. aeruginosa* persistence in chronic infections. So, it can be assumed that better biofilm producers such as MT25 provide to *P. aeruginosa* population an advantage to persist under oxygen depletion and possibly to establish

chronic infection. Although this morphotype appears to be an adaptation to the microaerophilic conditions, it does not represent a relevant feature to chronic infection. In conditions typically found in the context of chronic infection (anaerobic conditions), this morphotype is no longer identified. This morphotype cease to give an advantage to population, and is eliminated.

3.6.2 Motility tests

P. aeruginosa is known to exhibit different types of motility, in accordance with the environment in which is inserted. [103] Swarming, swimming and twitching are the most well characterized types of motility of this bacteria, allowing movement in aqueous environments (swimming), in semisolid medium (swarming) and on solid surfaces (twitching) [103].

Swimming

Swimming allows bacteria to move across liquid surfaces and is mediated by flagellum, and is one of the most important mechanisms of attachment of *P. aeruginosa* cells to surfaces in biofilm mode of growth. [104] Results of the swimming motility characterization of the four morphotypes are shown in Figure 3.7.

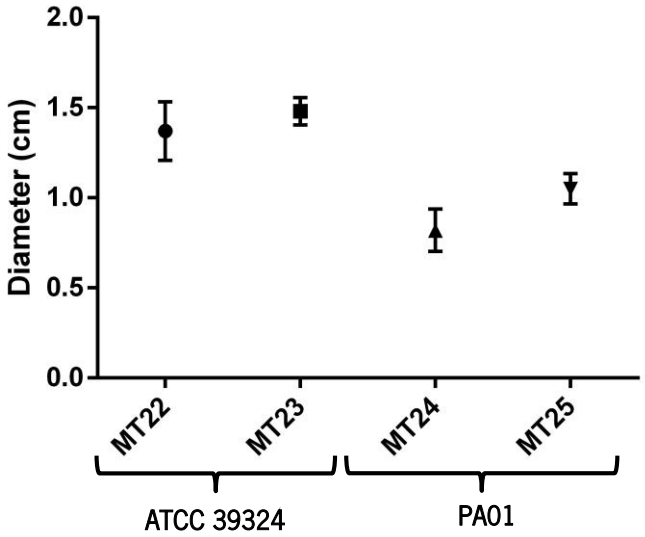


Figure 3.7 - Diameter of the swimming migration zone formed by *P. aeruginosa* PA01 and ATCC morphotypes found during this study. Symbols represent the average of three independent assays ± SD

Observing Figure 3.7, it can be said that ATCC 39324 new morphotypes are the ones with larger motility ring diameters, when compared with MT24 and MT25, PA01 morphotypes. This can be due to the fact of ATCC 39324 be a CF isolate and could be already adapted to the growth medium (ASM). However, differences are only significant between both ATCC 39324 morphotypes and MT24 ($p<0.05$). As said before, swimming motility is important in biofilm mode of growth.

Therefore, is expected that bacteria with better ability to form biofilm also present higher swimming migration zones. However, results show an opposite behaviour. MT25, the morphotype with higher biofilm forming ability, shows, as well lower swimming migration zones than MT22 and MT23, poorer biofilm producers. Nevertheless, SCV colonies have also been reported as possessing impaired swimming motilities, in association with the higher biofilm production described in Section 3.6.1. Due to the auto aggregative nature of these type of colonies, which allow bacteria to attach to each other and form biofilm microcolonies, they are described as one of the most important virulence factors associated with CF lungs. Swimming motility is necessary only to the initial attachment of cells to the surface, in this case, the mucus layer within CF lungs [57]. After this initial step, swimming motility cease to be the principal mechanism responsible to biofilm formation, which explain the fact that better biofilm producers do not show better swimming ability.

Swarming

Swarming motility, also flagellum-mediated motility, is responsible for multicellular movement across semi-solid surfaces [105]. This type of motility can be assessed in vitro in TSA plates with 0.5 % of agar. The results obtained during this study are shown in Figure 3.8.

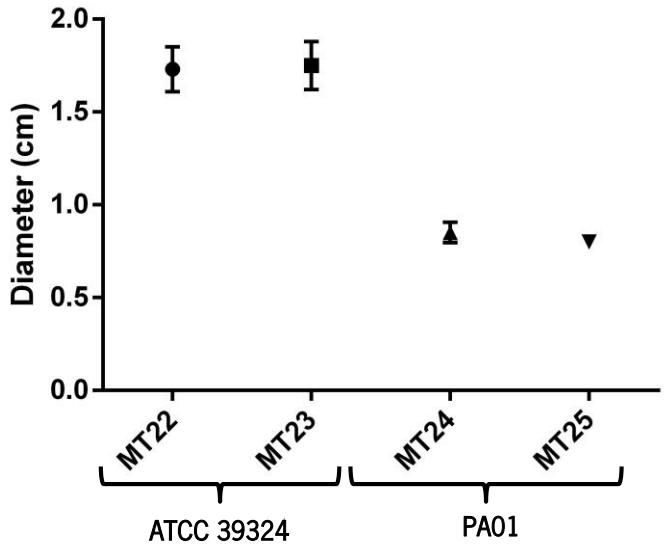


Figure 3.8– Diameter of the swarming migration zone formed by *P. aeruginosa* PA01 and ATCC morphotypes. Symbols represent the average of three independent assays ± SD

Regarding the four new morphotypes in study, MT22 and MT23 present higher swarming rings than MT24 and MT25 ($p<0.05$). Therefore, ATCC morphotypes (MT22 and MT23) show higher swarming ability than PA01 morphotypes (MT24 and MT25). One conclusion for these observations

is that morphotypes of the same strain, show similar swarming migration zones ($p<0.05$), behaviour also observed for biofilm formation and swimming motility.

Swarming motility and antibiotic resistance have been associated in some studies, and this behaviour can be applicable to *P. aeruginosa* [88][90]. Despite no correlation between antibiotic resistance profiles and swarming motility were performed, it could be expected that strains or morphotypes with larger swarming migration zones showed an increased resistance to the antibiotics tested in this characterization. However, this behaviour was not observed in this study. First, the morphotypes with higher migration zones, MT22 and MT23 are susceptible to all antibiotics tested. Second, the only difference in susceptibility was observed MT25 for imipenem, as analysed in section3.6.3. This morphotype, as well as MT24 show lower swarming ability.

This type of resistance is known to be transient, which means that can be unstable and easily lost [105] which can explain the results obtained.

Twitching

Twitching motility is mediated by type 4 pili and play an important role in biofilm development and structure, with an important role in microcolonies formation, allowing as well the movement of bacteria under solid surfaces [70]. In figure 3.9, results obtained for twitching motility ability for all morphotypes, are presented.

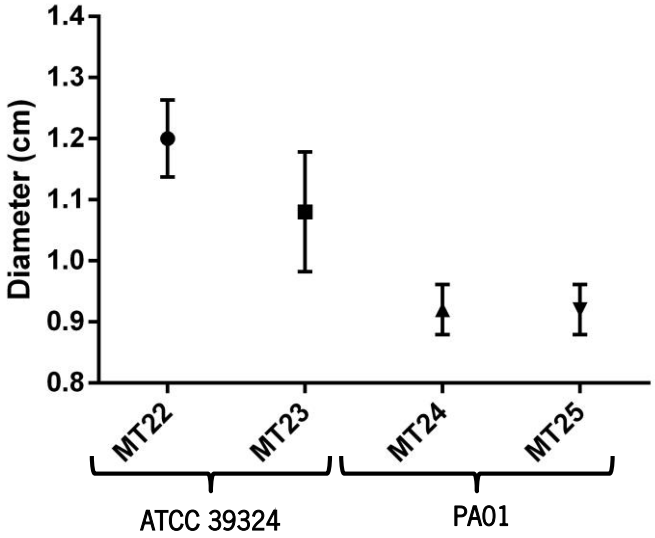


Figure 3.9 - Diameter of the twitching migration zone formed by *P. aeruginosa* PA01 and ATCC morphotypes. Symbols represent the average of three independent assays ± SD.

According to Figure 3.9, MT22 showed the larger twitching migration zone, followed by MT23 ($p<0.05$). MT24 and MT25 exhibit similar twitching migration zones ($p>0.05$), with values significantly lower that MT22 and MT23 ($p<0.05$). Twitching motility did not follow the previous

behaviour observed for both swimming and swarming motility, in which morphotypes from the same trains showed similar motility values. ATCC morphotypes vary significantly in values twitching motility ability, although PA01 morphotypes show similar twitching motilities.

As said before, twitching is mediated by type IV pili which, in turn are associated with interaction stabilization of cell-to-cell interactions, necessary to microcolonies formation, as observed in CF lungs [70]. Once more, an association can be made, between biofilm formation and twitching motility. Ideally, higher biofilm producers should have higher twitching migration zones, which is not observed in this study (see Figures 3.6 and 3.9). It can be assumed that, in this study, this mechanism was not responsible for the high ability of MT25 to form biofilm and that some additional yet unidentified mechanisms were present, being responsible for the observed behaviour. It can also be concluded that morphotypes of ATCC 39324 have better adapted motility in the medium used in this study due to the fact of this strain be a CF isolate.

3.6.3 Antibiotic Susceptibility

Antibiotic susceptibility of MT22, MT23, MT24 and MT25 for all antibiotic previously tested, can be observed in table 3.8.

Table 3.8 - Antibiotic susceptibility of all new morphotypes

	MT22	MT23	MT24	MT25
AK	S	S	S	S
ATM	S	S	S	S
CN	S	S	S	S
CAZ	S	S	S	S
TOB	S	S	S	S
FEP	S	S	S	S
IMI	S	S	S	I
TTC	S	S	S	S
CIP	S	S	S	S
PRL	S	S	S	S

According to table 3.8, all morphotypes characterized presented a susceptible profile for all tested antibiotics, with exception to MT25. This morphotype exhibit intermediate resistance against imipenem. Since these morphotypes and were only identified in microaerophilic conditions with 5 % O_2, we can compare the results for antibiotic susceptibility of the population, shown in Section 3.5 (Table 3.7) with the new isolated morphotypes (Table 3.8). Analysing the results for ATCC morphotypes, MT22 and MT23, we can conclude that these morphotypes follow the susceptibility

profiles of the general population (also susceptible for the ten antibiotics tested). The same behaviour is observed for MT24, PA01 morphotype, since in this environment was susceptible to all antibiotics as well. On the other hand, MT25 presented intermediate susceptibility profile for imipenem, which was not observed for PA01. We can assume that, although this morphotype displays a different susceptibility pattern for imipenem, this profile is not dominant in the population. This difference can be analysed, however, as a prediction of an emergent resistance of PA01 to imipenem caused by the lower oxygen availability in the environment. Supporting this conclusion is the intermediate resistance profile observed for PA01 in the anaerobic environment, as can be observed in Table 3.7. This new morphotype was, possibly, a partial adaptation of PA01 to the microaerophilic environment that fully develop under anaerobic conditions.

3.6.4 Colony morphology reversion

Colony morphology reversion is the ability of colonies to maintain their morphological traits in several and consecutive spreading in solid media. Reversion is important to infer whether colony alterations are reversible adaptations or irreversible mutations occurred [58][106]. To evaluate this characteristic, colonies of each morphotype were plated in TSA and observed after 48 h of growth, being the results shown in Table 3.9.

Table 3.9 – Colony morphology reversion on TSA plates of all morphotypes characterized

	TSA Reversion
MT22	Positive
MT23	Positive
MT24	Negative
MT25	Negative

As can be seen in Table 3.9, both ATCC 39324 morphotypes tested, MT22 and MT23, were positive for reversion, meaning that reverted to wild-morphotype after sub-culturing. MT24 and MT25 (PA01 morphotypes) exhibited the same morphotype as when were identified. Therefore, it can assumed that ATCC 39324 morphotypes represent reversible adaptations to the microaerophilic environment, while PA01 morphotypes are a product of irreversible mutations being with increased stability in the population. With this in mind, it could be possible that irreversible morphotypes result from a stronger adaptation to the conditions and, therefore are more susceptible to disappear in case of alterations in the environment, whereas reversible morphotypes have a stronger ability to overcome environmental changes and survive in the

population. This hypothesis seems to be true according to the results obtained. Analysing the colony morphology variants observed for PA01 and ATCC 39324 in anaerobic conditions, it can be seen that both PA01 morphotypes (irreversible) disappear from the population, while ATCC 39324 (reversible morphotypes) persisted. A conclusion of this observations is that reversion can be a measure of adaptive ability of the morphotypes to new conditions.

3.6.5 Characterization of haemolysin production and growth on minimal medium

In order to better characterize the new morphotypes found in this study, the presence of other virulence factors was evaluated. As such, hemolytic ability and growth on minimal medium was assessed for all morphotypes.

Auxotrophs are colony variants that are dependent on the addition of specific amino acids to the growth medium in order to sustain growth, while variants capable of growth on minimal medium are designated as prototroph [107]. The results obtained in this study showed that none of the four characterized morphotypes were auxotrophic, which can be said that these morphotypes were prototrophic. Auxotrophic variants are known to emerge with the development of chronic infection, coexisting with their prototrophic precursors [10]. With the decrease of oxygen availability, that characterizes chronic infection, some auxotrophic variants should appear among the *P. aeruginosa* population. Therefore, as the new morphotypes represent an adaptation of *P. aeruginosa* to the microaerophilic environment, it could be expected that auxotrophy characterized these morphotypes. That behaviour was not observed, which means that perhaps auxotrophy does not represent an advantage for *P. aeruginosa's* survival under microaerophilic conditions.

Hemolysins are accepted as major contributor to virulence ability of several bacteria, including *P. aeruginosa*. For this reason, haemolytic activity of the new morphotypes observed, was assessed.

P. aeruginosa produce two different kinds of phospolipases C: an haemolytic one (PLcRH) an one without haemolytic activity (PLcN), with PLcHR being recognised as one of the most important virulence factor of this bacteria in normal aerobic conditions [108][109] .The determination of haemolytic activity of each morphotype was performed by the visualization of the colonies on Columbia blood agar plates. If the medium surrounding the colonies was translucent (lysis of blood cells), the morphotype was considered hemolytic (positive). Otherwise, colonies were considered non hemolytic (negative). The results obtained showed that all morphotypes characterized in this studied have no hemolytic activity. It is possible that, due to adaptation to oxygen limitation, these

strains loss some virulence ability and were unable to produce PLcHR. The same way that *P. aeruginosa* can acquire characteristics that helps adaptation to stressful environments, this bacteria can lose that same characteristics when the environment changes.

3.7 Genetic expression of MexAB-OprM efflux pump

Multidrug efflux-pumps have been recognized as one of the most common mechanisms responsible for antibiotic resistance to most antibiotics in bacteria [110]. Several types of multidrug-efflux pumps have been described in literature for *P. aeruginosa*, such as MexXY-OprM, MexCD-OprJ, MexEF-OprN, among others [79].

In gram negative bacteria, as *P. aeruginosa* MexAB-OprM efflux pump is the one with higher levels of expression, being responsible for the discharge of most lipophilic and ambiphilic antibiotics out of bacteria [110].

The understanding of the expression of these efflux-pumps in context of acute and chronic infections has acquired a great importance, as it helps to understand to what extent the conditions present in chronic infections enhance the expression of these pumps with the onset of chronic infection by *P. aeruginosa*. With that goal, we aimed to analyse genetic expression of mexA gene in typical acute infection environments, such as the microaerophilic environment with 10 % CO_2, and in chronic infection conditions, usually hypoxic environments (1 % O_2). The overall aim was the comparison of *mexA* expression in microaerophilic and anaerobic conditions, with that of aerobiosis, in order to better understand the evolution this mechanism with the development of infection. Initially, to test this hypothesis, SCV colonies from strain U147016-1 were recovered, as these types of colonies have been described to possess an important role in the persistence of chronic *P. aeruginosa* infections in CF lungs. In order to do this, RNA was extracted at the beginning of CF infection simulation (day 0) and at the end (day 10), for aerobic (control), microaerophilic (10 % CO_2) and anaerobic (1 % O_2) conditions. The results obtained for initial RNA extraction are represented in Table 3.10.

Table 3.10 - RNA quantification values obtained after different DNA removal treatments. (* - samples dilution 1:10)

Sample		RNA Yield (ng/µL)		
		RNA extraction (first DNAse I treatment)	Posterior DNAse I treatment	DNA removal by Acid Phenol:Cloroform
10 % CO_2	Day 0	768,8 651,5	805,6 620,2	4,3 10,8
	Day 10	81,1* 250,9	176,1 392,7	0 185,6
1 % O_2	Day 0	651,2 743,2	720,3 839,3	283,6 128,4
	Day 10	422,4 48,5*	453,8 1034,8	68,1 27,4
21 % O_2	Day 0	412,0 205,9	366,9 224,3	20,3 6,8
	Day 10	71,1* 71,1*	745,7 748,6	289,5 41,6

Initially, DNAse I treatment was performed during the RNA extraction protocol, in the extraction column, as indicated in the extraction kit protocol. The following step was the assessment of the quality of samples, to understand if contamination with DNA was present in RNA samples. At this step, all samples obtained were tested, and comparison between NRT (no-reverse transcriptase control) and unknown cDNA samples was performed to better analyse the level of contamination of samples, a schematic of the qPCR plate design can be seen in Figure 3.10.

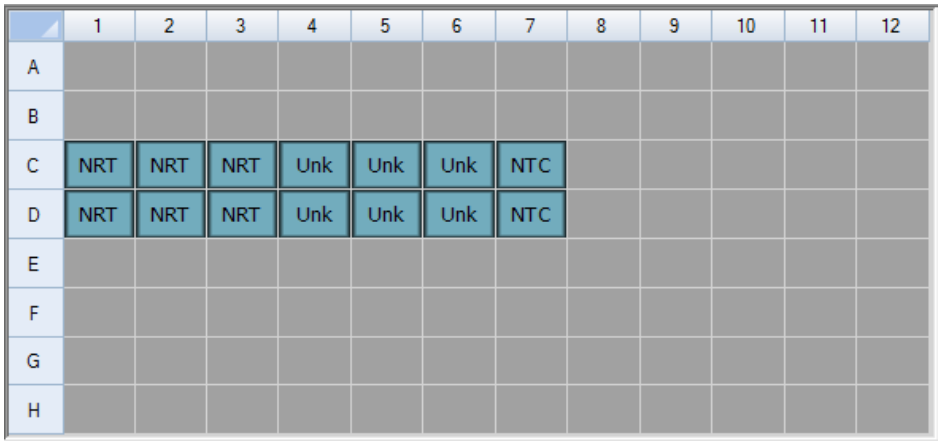


Figure 3.10 - Schematic respresentation of the PCR plates used for qPCR analysis of quality of RNA samples. NRT- no reverse transcriptase control; Unk- cDNA samples; NTC – no template control.

The results obtained for the PCR run for all samples are represented at Figure 3.11.

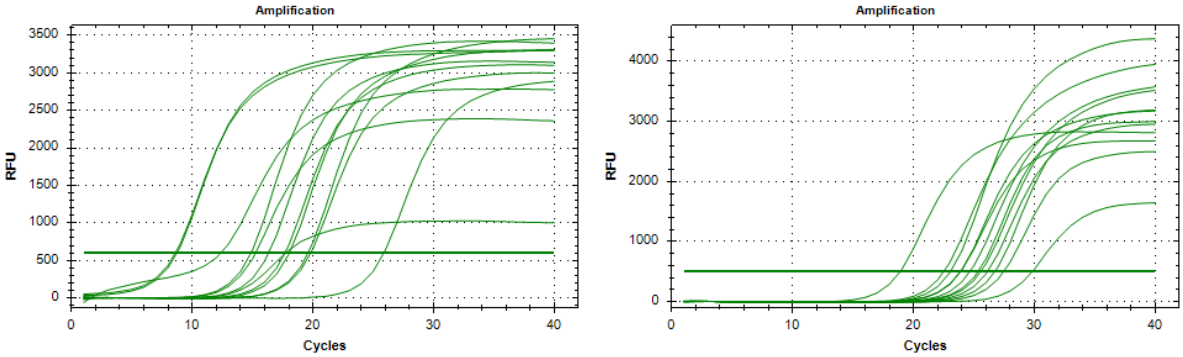


Figure 3.11 – Amplification curves obtained for NRT and cDNA samples, obtained after double treatment with DNAse I. Duplicates of each type of samples were obtained in all extraction procedures. Results obtained for the initial samples are represented on the left image, being the results of duplicate samples represented in the image on the right.

Analysing the results obtained, a high amount of DNA contamination can be observed in all samples. The expected behaviour was that NRT samples had amplification many cycles after cDNA samples (or none at all), since only DNA is amplified. The results show that all samples have amplification at similar cycles for the duplicate samples (both the NRT and the cDNA samples), almost all samples showing amplifications at least after 20 cycles. Observing the results for the first samples, a high variability is observed, with the majority of samples being amplified between 10 and 20 cycles. It can conclude that all samples show high levels of DNA contamination, reason why posterior DNAse I treatments were performed. Comparing both groups of samples, it is visible that despite all samples still being contaminated, the duplicate group showed better results than the first samples – with a shift of the curves towards the right side, and higher Ct values - and for that reason, posterior tests were performed using only the duplicate samples.

As the results obtained were far from being what was desired, and an additional DNAse I treatment was performed, as well as a new RNA quantification (Figure 3.10, right hand side figure) and verification of the quality of the samples after the treatment. To minimize possible influences in the results obtained, new primer dilution were performed with RNAse free water, as well as new SYBR Green Master mix. It was expected that the level of contamination of the samples was reduced with this treatment, hypothesis not confirmed by the results (see Figure 3.12).

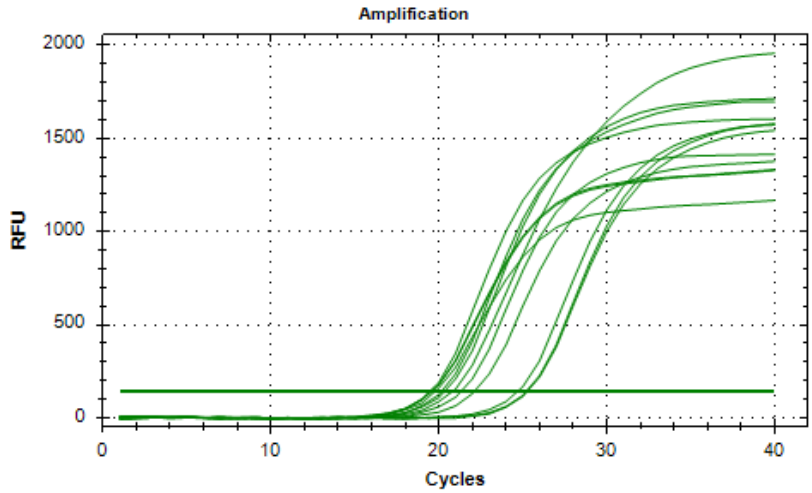


Figure 3.12 – Amplification curve of NRT and cDNA samples performed after the second DNAse I treatment.

Analysing the results of the second RNA quantification, comparing with the first quantification values, show a high variation between the two measurements, as can be seen in Table 3.10. While some of the RNA values decreased with the second DNAse I treatment, other values increase, leading to the conclusion that this treatment might not be very effective. It was expected a reduction in nucleic acid concentration because the DNA contaminating the samples should be eliminated, allowing more accurate and normally lower values of RNA concentration. With the new verification of the samples quality, this was confirmed, since the results still showed a high concentration of genomic DNA present in the samples. Despite the fact that the most discrepant results were "eliminated" (samples with amplification before cycle 20), both NRT and cDNA samples have amplifications after similar number of cycles, as observed before, in Figure 3.11.

To attempt a more thorough RNA purification, the samples were treated with an acid phenol:chloroform purification protocol followed by new RNA quantification and quality verification by qPCR. The RNA quantification of the samples after the DNA removal with phenol: chloroform (Table 3.10) showed a marked decrease in the values of RNA concentration in the majority of samples, which may be an indication that this treatment was more effective than the protocols previously used. With a new NRT analysis, DNA contamination was still verified in all samples, since both NRT and cDNA samples continue to be amplified after similar number of cycles, as can be observed in Figure 3.13.

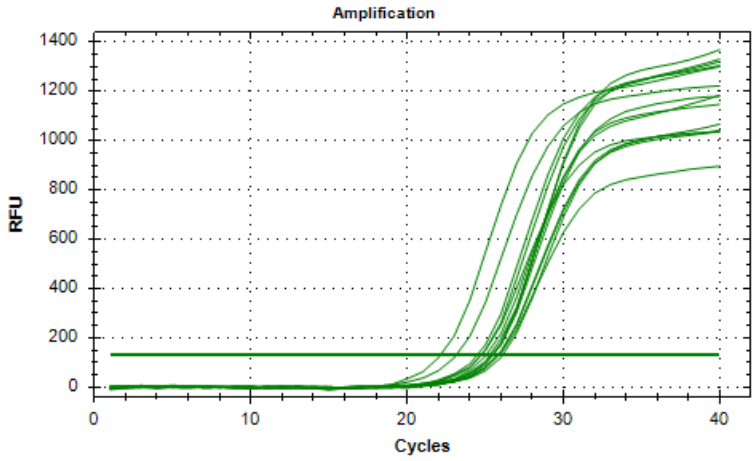


Figure 3.13 – Amplification curves obtained for samples treated with acid phenol:chlorophorm.

Although DNA contamination is still present in RNA samples, the results after 3rd RNA purification show that all samples higher Ct values, which can be considered an indication that the last treatment performed was more efficient than the previous treatments. Another problem that can be associated with these results is the real concentration of cDNA used in the qPCR analysis might not have been ideal. As the total concentration of RNA recovered from the last purification treatment was greatly reduced in almost all samples, it is possible that cDNA synthesis was not as efficient (although attempts were made to homogenize the quantities of RNA used for cDNA synthesis in each reaction), which could interfere with the qPCR assays that followed.

It is necessary, as well, to take into account the possible contamination of PCR reagents with genomic DNA. As in example, contamination of bacterium-derived polymerases is quite common, which largely affects the high sensitivity of PCR procedure and results [91][111]. As can be observed in Figure 3.10, NTC (no template control) samples were added to the qPCR run to discard possible contamination of the reagents. The observation was that also PCR reagents were contaminated, since there were no real distinction between NTC controls and the other samples tested, and as fresh dilution of primers were prepared to attempt to eliminate this source of contamination.

Despite the less than positive results of RNA purification, the efficiency of the primers selected for this study was determined to define their usefulness in future expression assays, testing six dilutions, at three annealing temperatures (62 °C, 60 °C and 58 °C). The results are shown in

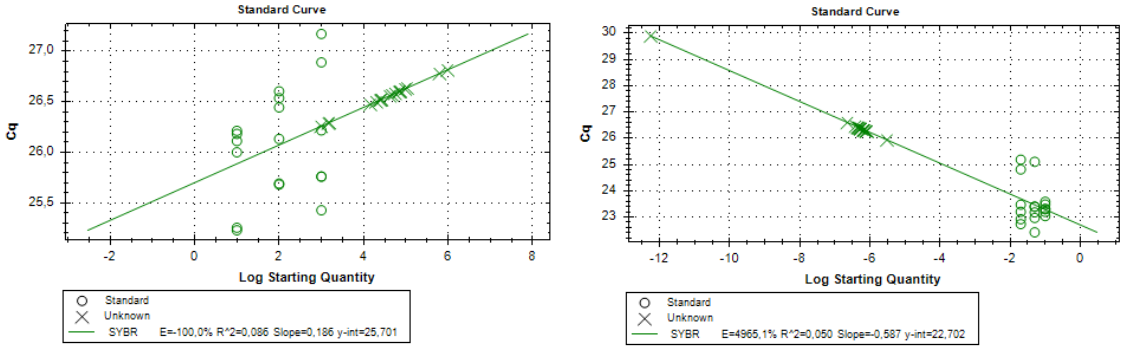


Figure 3.14 – Primer efficiency of 16S and mexA for different tested dilution: 1:10, 1:20 and 1:50 in the right image and 1:100; 1:1000 and 1:10000 on the left.

Figure 3.14.

The efficiency results obtained were proved not real. Analysing both images of Figure 3.14 it is possible to observe opposite behaviours for the two sets of dilutions. A more detailed observation of the slope values of both images, value necessary to calculate the efficiency of the primers, it becomes impossible to assess the efficiency of the primers for the dilution 1:100, 1:1000 and 1:10000, and the remaining dilutions still resulted in efficiency values which were unrealistic (400 %, for example).

The conclusion that can be made from the analysis of the results obtained is that the primers used are not the appropriate for this study. Several reasons can be responsible for this result in primers efficiency. The primers used in this study, 16S and mexA, were designed and specific for *P. aeruginosa* PA01 (wound isolate) and CF isolated strains respectively. As the objective was the study of mexA expression in SCV, U147016-1 as it was the strain that produced more SCV in the environmental conditions studied. However, U147016-1 is a strain isolated from an urinary infection, which can explained the lack of efficiency of both primers for this strain. It was hoped the the 16S gene, being considered a house keeping gene whose expression remained constant, could still be useful in this assay, despite the diverse background of the strain in which genome it was design, the results obtained in this study proved the opposite.

CF environment has been described for its complexity and presence of several selective pressures and continues stressful conditions [48]. All this characteristics induce several genotypic and phenotypic changes in *P. aeruginosa*, has been described throughout this study. For that reason, the use of primers designed to study bacteria in CF environments using strains isolated from

different sources proved not to be effective. Specificity is a key characteristic to study genetic expression of bacteria exposed to particularly with regards to the sources of strains tested.

The choice of housekeeping gene to be used in this study might, also proved to be a challenge. The references in literature to the best housekeeping gene for *P. aeruginosa* in CF infection context are very divergent. There are many studies using 16S as *P. aeruginosa* housekeeping gene, since a highly conserved bacterial gene throughout many bacterial species, reason why was used in this study [111][112]. However, a simple change in the source of the isolate use as a target showed that, although it could be efficient for PA01 (the strain used to design the primers) it was not specific for *P. aeruginosa* isolated from a different settings. Nonetheless, even if a CF isolate was used in this study, there was no guarantee that the primers would work, since the conditions tested could enhance the inherent hypermutability of *P. aeruginosa*. The ideal would be the verification of PCR products of the colonies exposed to the different oxygen concentrations tested.

With the great adaptation that *P. aeruginosa* suffers in CF environments, the use of this reference gene in particular, or even the use of just one housekeeping gene might not be the most effective approach. Indeed, there are some studies that already used different and more than one housekeeping gene, indicating that this hypothesis is more appropriated [113][114][115]. Studies performed by analysis of sputum samples of CF patients found two reference genes, PA2875 and PA3340, that shown a steady expression on both planktonic growth and biofilm formation [113]. *rspL* gene has also been used in many studies of MexAB-OprM efflux pump in *P. aeruginosa* [114][115]. *proC* and *rpoD* genes have been demonstrated as a good pair of reference genes to be used in comparative studies of *P. aeruginosa* resistance genes expression [116].

4. CONCLUSIONS

In this final chapter the main conclusions of the present work are presented. Future studies perspectives and recommendations are also addressed.

CF lung disease is characterized by continuous airway infections since young age of patients that develop to chronic infection later in life, principally by *Pseudomonas aeruginosa* [3]. The colonization by this pathogen is associated with increased morbidity and mortality, since after *P. aeruginosa* infection is established, is almost impossible to eradicate [8].

The understanding of the infection persistence mechanisms in CF lungs assumes great importance, in order to allow the identification of *P. aeruginosa* diversification patterns and identification of specific characteristics that allow early eradication of infection. To simulate and test different stages of infection, environments with decreasing oxygen concentration were applied to different *P. aeruginosa* strains, culminating with anaerobiosis as simulation of chronic infection. During this study, the simulation of CF infection by *P. aeruginosa* strains shown a great variability of the characteristics observed. However no major alteration was found in *P. aeruginosa* infection development on the different conditions tested, as well as a direct relationship between differences in distinct characteristics. Despite ASM be a complex medium that attempts to mimic the also complex CF airway environment, *P. aeruginosa* strains were already exposed to demanding growth conditions, since were isolated from different types of infection, reason why the results obtained did not show as much variability as expected, as well as more similar development was it is observed in CF sputum isolates.

As shown in Chapter 3, the three *P. aeruginosa* strains have similar and stable growth profiles between each other and in the different decreasing oxygen environments, despite some variation observed. Since *P. aeruginosa* populations were exposed to normal oxygen concentration in each checkpoint, this variations could possibly be attributed to this. It has also been reported before the ability of *P. aeruginosa* to grow in microaerophilic and anaerobic environments, which can be the reason for the stable and similar growth profiles observed.

Phenotypic diversification is one of the most common mechanisms used by bacteria to increase population diversity and persistence in different niches. This diversification is most readily traceable in colony morphology variation, which allows us to understand the induced differences of various conditions, as observed in acute and chronic infection. Assessment of colony morphology diversification in all tested environments, showed an increase of diversification with lower oxygen concentrations, however this behaviour is not linear. In microaerophilic conditions with 5 % O_2 a more significant diversification was detected, with the observation of four “new morphotypes” that were not observed in previous environments. However, not all of this “new” morphotypes persisted in anaerobic conditions, and diversification decreased. It could be that a sub-population well

adapted to already stressful environments were not able to persist in extreme conditions. Therefore, microaerophilic conditions seem to be a driving force of diversification, which can be an indication of the transition to chronic infection.

Hypermutability has also been described as a beneficial characteristic for *P. aeruginosa* adaptation to different niches, and the emergence of hypermutators in CF infection bacteria has been reported. Hypermutability can be responsible for antibiotic resistance of *P. aeruginosa* sub-populations, has well as for the appearance of diverse types of sub-populations, as mucoid colonies or SCV. Of all conditions tested, mutation frequencies were only relevant for the anaerobic environment, with the emergence of strong mutator phenotype in to U147016-1. Despite this strain has not be isolated in a CF infection context, the emergence of a mutator phenotype is in accordance with the expected behaviour in CF chronic infection simulation in this study. However these results do not show a direct relation with phenotypic switching observed in colony morphology, as it was expected, could be responsible for the intermediate resistance profile of this strain to TTC.

Pyocyanin is frequently detected in mucus samples from lungs of CF patients, and is associated with the success of establishment of chronic infection in CF lungs. In order to establish long-term presence in CF airways, *P. aeruginosa* adapt in order to loss some of its virulence factors, as pyocyanin. However this behaviour was expect in this study, it was not observed. The cause of this observation can be the high growth observed for *P. aeruginosa* strains in all conditions tested. The levels of pyocyanin production detected are not only a measure of *P. aeruginosa* ability to produce pyocyanin, but also due to total load of bacteria in the sputum [100]. Therefore, pyocyanin production in this study cannot be associated with development of chronic infection. This hypothesis could be tested using an inferior inoculum concentration in the infection simulation, which may allow a better understanding of *P. aeruginosa* ability to produce pyocyanin in the development of CF chronic disease.

The principal characteristic associated with chronic infection in CF lungs is the emergence of antibiotic resistant sub-populations. In chronic stages of infection, biofilm mode of growth is observed, as well as diminished oxygen availability, all reasons in favour of impaired efficacy of antimicrobials. The expected behaviour was the observation of decreased susceptibilities with the decrease of oxygen availability. In microaerophilic conditions with 5 % O_2 this was not observed. As well as observed in colony morphology assessment, in 5 % O_2 a distinctive behaviour was observed, being in this case a slight decrease in antibiotic intermediate resistant profiles. This can also be an

indication that some turning point was reached, since in anaerobic conditions intermediate resistant profiles arose and even a resistant profile was observed.

As referred before, during colony morphology assessment in microaerophilic environment with 5 % O_2, four "new" colony morphologies were observed. These morphotypes were characterized for expression of common virulence factor as well as for antibiotic susceptibility profiles and motility. Results shown that overall all morphotypes presented similar characteristics. One morphotype, MT25, stand out from the rest, since shown much higher biofilm formation ability and an intermediate resistant profile to imipenem. Despite this characteristics, associated with chronic infection, this morphotype was not observed in anaerobiosis.

Overall, the subpopulations that persisted in anaerobic conditions showed wider characteristics, being susceptible to all antibiotics tested and showing reversible behaviour. Therefore, we can conclude that more adapted characteristics of sub-populations to a distinctive niche indicate a decrease in the fitness to adapt to new conditions.

Analysing all the results obtained in this study, it is perceptible that microaerophilic conditions with 5 % O_2 presented some interesting behaviour that should not be ignored. In this environment were found distinctive behaviour that can indicate a switch in the state of *P. aeruginosa* infection. In order to confirm this hypothesis, further studies should be performed, with more extensive characterization of *P. aeruginosa* diversification in this condition.

Efflux pump expression study performed showed that specificity is a key characteristic to study genetic expression of bacteria exposed to similar conditions. I was found that specific primer design is of paramount importance for a successful qPCR assay of gene expression in *P. aeruginosa*, as primers design based on the genome information of a specific strain may not be suitable for other strains isolated from different environments, as observed in this study.

To enhance the knowledge of *P. aeruginosa* development in the steep oxygen conditions tested in this study, more CF infection isolated strains should have been used, to understand *P. aeruginosa* adaptive mechanisms and to determine if more strains follow the patterns observed in this study.

As infection by other bacteria are emerging in context of CF lung disease, as *Bulkoderia cepacia* and *Staphylococcus aureus*, it becomes important to understand how those emerging develop in this conditions, in association with *P. aeruginosa*. This type of study can be very useful, as a better characterization of these mechanisms and interaction between different bacteria can help the development of new therapeutic strategies and allow the early eradication of infections in CF lung disease.

Further studies should be performed on the molecular aspects of the expression of genes associated with antibiotic resistance and bacterial persistence in *in vitro* CF models such as the one proposed in this study regarding mexAB-OprM expression in steep oxygen gradients. In order to make the process more efficient, *P. aeruginosa* strains isolated from CF sputum samples should be used, as well as primers specifically design for the stains in use. To overcome the problems observed with RNA samples contamination, various extraction kits should be tested in order to choose the one with most efficient extraction and with lower sample contamination with DNA. To ensure that RNA samples used are good, genomic DNA removal by acid phenol:chlorophom is recommended to be performed as it has been showed in this work to be more efficient.

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APPENDIX I – GROWTH KINETICS STATISTICAL ANALYSIS

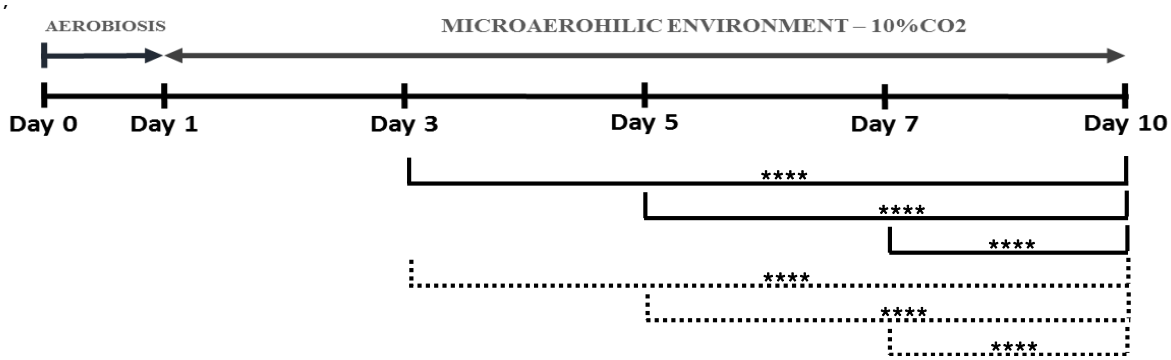


Figure AI.1 - Schematic of the significance levels observed between the different checkpoint days, for PA01, U147016-1 and ATCC in microaerophilic environment with 10 % CO_2. Represented with full lines is PA01 statistical significance and, with dot-line is represented U147016-1 significance. (* - p<0.05; ** - p<0,01; ***- p<0,001; ****- <0,0001).

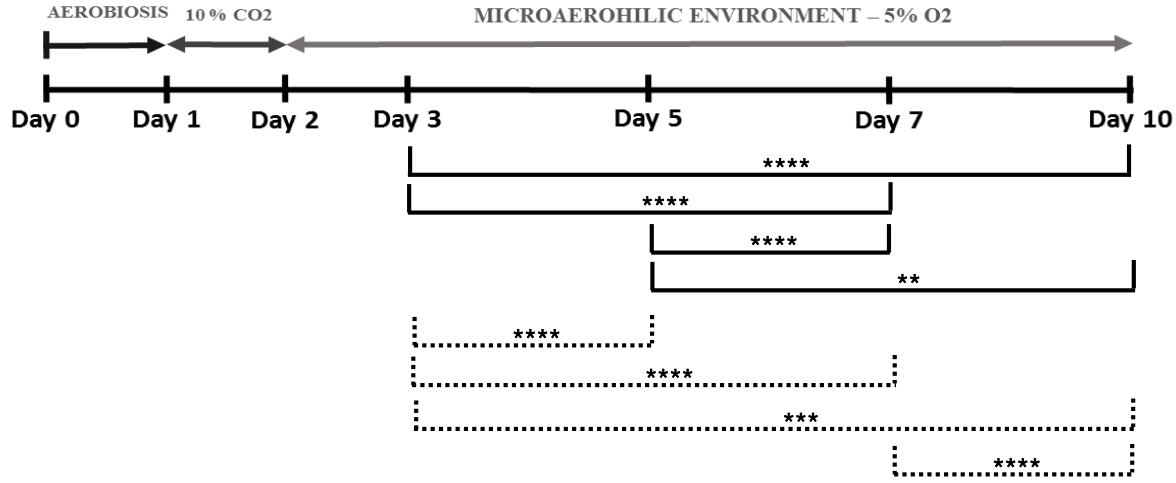


Figure AI.2 - Schematic of the significance levels observed between the different checkpoint days, for PA01, U147016-1 and ATCC in microaerophilic environment with 5 % O_2. Represented with full lines is PA01 statistical significance and, with dot-line is represented U147016-1 significance. (* - p<0.05; ** - p<0,01; ***- p<0,001; ****- <0,0001).

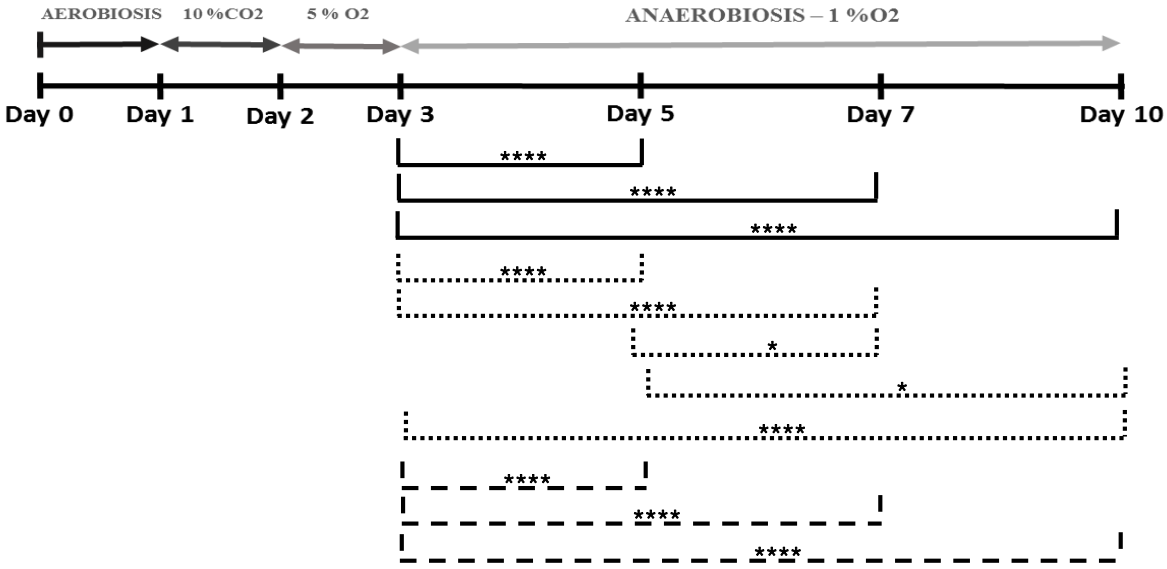


Figure AI.3 - Schematic of the significance levels observed between the different checkpoint days, for PA01, U147016-1 and ATCC in microaerophilic environment with 5 % O_2. Represented with full lines is PA01 statistical significance, with dot-line U147016-1, and with dashed line, ATCC 39324 significance. (* - p<0.05; ** - p<0,01; ***- p<0,001; ****- <0,0001).

APPENDIX II – COLONY MORPHOLOGY CLASSIFICATION SYSTEM

Table AII.1 - Morphological criteria of colony classification system

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

Table AII.2 - Morphological Concepts

Term	Concept	Synonymous
Moist	Form feature inhering in a colony by virtue to present a configuration of a circumference or a circle due to any point of the edge be equidistant from the center	Round
Elliptical	Form feature inhering in a colony by virtue to present a 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 feature 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, undulating, waved, wavy
Heterogeneous	Surface feature inhering in a colony by virtue of having more than one type of texture	

Term	Concept	Synonymous
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	

Term	Concept	Synonymous
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	
Brittle	Consistency quality inhering in a colony by virtue of the shattering easily	
Dry	Consistency quality inhering in a colony by virtue of not being covered by a liquid	
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	

Term	Concept	Synonymous
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	
Brittle	Consistency quality inhering in a colony by virtue of the shattering easily	
Dry	Consistency quality inhering in a colony by virtue of not being covered by a liquid	

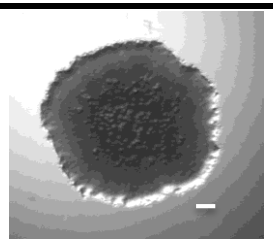
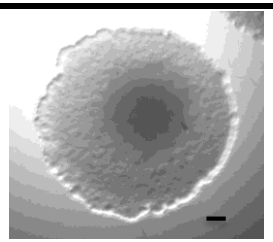
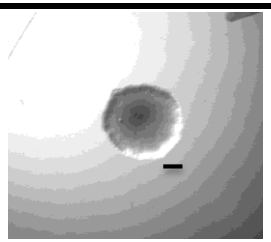
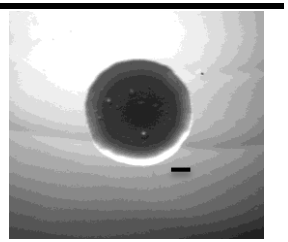
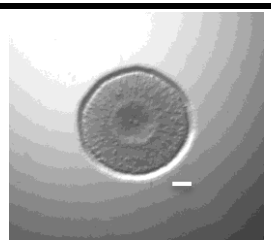
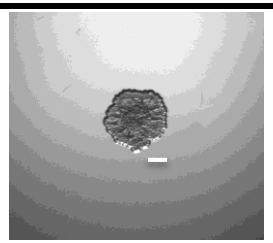
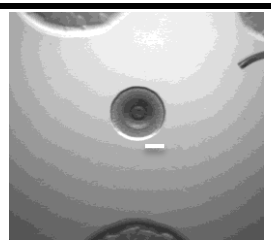
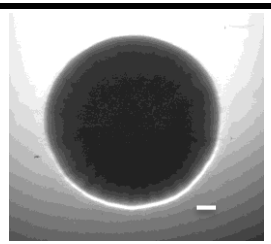
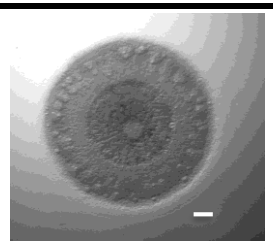
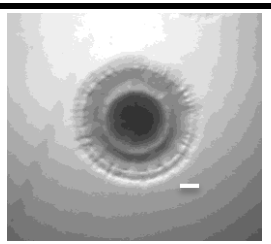
APPENDIX III – COLONY MORPHOLOGICAL CHARACTERIZATION

Table AIII.1 -Colony morphological characterization of *P. aeruginosa* morphotypes observed in this study. Characterization was performed based on colony morphology classification system

	Form	Margin	Surface	Texture	Sheath	Opacity	Elevation	Consistency	Size	Colour	Surface Elements
PA01											
MT02	Circular	Undulate	Homogeneous	Rugose	Present	Opaque	Flat	Dry	Large	Yellow	None
MT17	Circular	Undulate	Homogeneous	Rugose	Present	Opaque	Flat	Moist	Large	Yellow	None
MT24	Circular	Undulate	Homogeneous	Smooth	Present	Opaque	Flat	Dry	Small	Yellow	None
MT25	Circular	Undulate	Homogeneous	Smooth	Absent	Opaque	Flat	Dry	Small	Yellow	None
U147016-1											
MT14	Circular	Entire	Homogeneous	Rough	Present	Iridescent	Flat	Dry	Large	Green	None
MT05	Irregular	Undulate	Homogeneous	Rough	Absent	Opaque	Flat	Dry	Small	Green	None
MT07	Circular	Entire	Homogeneous	Rough	Absent	Opaque	Flat	Dry	Small	Green	None
ATCC 39324											
MT01	Circular	Undulate	Homogeneous	Smooth	Present	Opaque	Raised	Mucoid	Large	Yellow	None
MT12	Irregular	Undulate	Homogeneous	Rough	Present	Transparent	Flat	Dry	Large	Brown	Present
MT23	Circular	Undulate	Homogeneous	Rugose	Present	Opaque	Flat	Dry	Large	Yellow	None
MT22	Circular	Undulate	Homogeneous	Smooth	Present	Opaque/Transparent	Flat	Dry/Mucoid	Large	Yellow	None

APPENDIX IV – COLONY MORPHOTYPES

Table AIV.1 – *P. aeruginosa* colony morphotypes observed during the present study

PA01			
MT02 	MT17 	MT24 	MT25 
U147016-1			
MT14 	MT05 	MT07 	
ATCC 39324			
MT01 	MT12 	MT22 	MT23