Universidade do Minho
Escola de Engenharia

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Insights into bacterial colony morphology evolution and diversification during Infection development in cystic fibrosis

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Tese de Doutoramento em Engenharia Biomédica

Trabalho efetuado sob orientação da
Professora Doutora Maria Olívia Pereira

março de 2016

STATEMENT OF INTEGRITY

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration.
I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

University of Minho, March 2016

Full name: Ana Margarida Rodrigues de Sousa

Signature: Ana Margarida Rodrigues de Sousa

"Traço sozinho, no meu cubículo de engenheiro, o plano,
Firmo o projecto, aqui isolado
Remoto até de quem eu sou"

Álvaro de Campos, *in* Poesia de Fernando Pessoa

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SCOPE OF THE THESIS

The majority of patients with cystic fibrosis (CF) suffer from chronic infections of *Pseudomonas aeruginosa* that are hardly eradicated from lungs. Its long persistence is associated with sophisticated mechanisms of adaptation, including biofilm formation, resistance to antibiotics, hypermutability and customized virulence factors expression. One of the hallmarks of *P. aeruginosa* adaptation to CF lungs is its diversification in an extensive number of phenotypes specially adapted to CF environment, called as clonal diversification, ensuring bacteria survival and infection persistence. By this reason, *P. aeruginosa* infections in CF disease are associated with poor patient outcomes.

The gold standard of CF disease management is the eradication of *P. aeruginosa* from airways as soon as detected in order to avoid or, at least, to retard the establishment of chronic infections preserving lung function. To achieve a successful eradication, it is need understanding the adaptation mechanisms used by *P. aeruginosa* for antimicrobial agents target them. Otherwise, re-infection and antibiotic resistance episodes will continue occur. A lot of studies have been conducted over years about the genomic and phenotypic adaptations undergone by *P. aeruginosa* during chronic infections, in contrast to the less significant amount of studies concerning *P. aeruginosa* early adaptations. However, the early adaptations could be the key to considerably reduce the mortality and morbidity rates. Understanding the *P. aeruginosa* early adaptations in CF lungs it will be possible to design effective antimicrobial strategies and so to prevent antibiotic resistance, re-infection episodes and progression to chronic stages. As so, this project was designed and developed to respond to the central research question: which are the early adaptations undergone by *P. aeruginosa* after CF lungs colonization. *P. aeruginosa* during its residence in CF lungs can experience several environmental stresses and this question is quite complex and difficult to answer without considering several clinical scenarios. Therefore, this central research question was decomposed in several smaller research questions: what are the early adaptations undergone by *P. aeruginosa* 1) grown in CF lungs without antibiotic treatment; 2) grown in CF lungs and exposure to an early antibiotic treatment; 3) grown in CF lungs and exposure to a delayed antibiotic treatment; 4) are there disease markers indicators of *P. aeruginosa* early adaptation to CF lungs. The answers to these questions will introduce great improvements in CF disease management, in particular, helping to design an effective antimicrobial strategy to early eradicate *P. aeruginosa* infections and thus to prevent chronic infections development.

OBJECTIVES OF THE THESIS

Microbiological studies have been focused on to determine the mechanisms underlying *P. aeruginosa* persistence and the development of chronic infections. In fact, antibiotic therapies are currently adequate according the gained information about chronic infections, but episodes of antibiotic and multidrug resistance and re-infection continue occurring. Therefore, attentions must be directed to early *P. aeruginosa* adaptation in order to block bacteria evolution and adaptation to cystic fibrosis (CF) lungs and consequently to avoid the development of chronic infections.

Isolates of *P. aeruginosa* with differing colony morphology characteristics have been cultured from CF patients and considered a sign of clonal diversification. Some morphological traits, including colony size, texture and consistency, are indeed indicators of virulence factors expression, antibiotic resistance and even of the infection severity. Therefore, it was detected an opportunity to macroscopically monitor the clonal diversification of *P. aeruginosa* inside CF environment. The main goal of this PhD thesis was determine the evolutionary pathway followed by *P. aeruginosa* after CF lungs colonization in order to to find out the adaptation mechanisms underlying *P. aeruginosa* early adaptation and their driven forces. Moreover, it was aimed to verify the existence of disease markers of *P. aeruginosa* adaptations. Biomarkers discovery could have great impact on early *P. aeruginosa* infection diagnosis. To achieve these goals, eight key aims were established to draw the early *P. aeruginosa* adaptation:

1) Standardize the experimental parameters that impact on colony morphology characterisation.
2) Organize the vocabulary and update the concepts used to describe colony morphologies.
3) Explore a molecular method in the detection and identification of *P. aeruginosa* colony morphology variants;
4) Identify the specific colony-biofilm variants to further detect biofilm growth in CF environment;
5) Identify the early adaptations undergone by *P. aeruginosa* and the driven forces;
6) Determine the impact of antibiotic treatment timings and antibiotic concentrations in early *P. aeruginosa* eradication and the response of population against the different treatments;
7) Identify the biomarkers associated with early *P. aeruginosa* adaptations.
8) Construct a public database fully dedicate to bacterial colony morphologies and their phenotypic-associated data.

OUTLINE OF THE THESIS

The present thesis includes research work at Centre of Biological Engineering, University of Minho, Braga, Portugal and at BIOSCOPE group, University of Vigo, Ourense, Spain, under the supervision of Doctor Maria Olívia Pereira and Doctor Jose Luis Capelo, respectively.

This thesis is organized into five chapters. **Chapter 1** is a general introduction of cystic fibrosis (CF) airway disease and their microbial-associated infections. A particular description of the impact of *P. aeruginosa* infections in the context of CF, the in-use antibiotic therapies to eradicate these infections and the strategies used by *P. aeruginosa* to ensure its long-persistence in CF lungs is provided. In **Chapter 2**, some improvements introduced in colony morphology characterisation method at experimental procedure and lexicon levels are reported in order to overcome some limitations of this method. **Chapter 3** focuses on the study of *P. aeruginosa* biofilm populations, in particular, it is reported the diversity and functionality of the sub-populations detected inside biofilms. In **Chapter 4** some important research questions are answered: which are the early adaptation undergone by *P. aeruginosa* after CF lungs colonisation, the impact of antibiotic treatments on *P. aeruginosa* adaptation and the identification of early CF disease biomarkers. **Chapter 5** describes the MorphoCol, a new ontology-based knowledgebase for the standardised, consistent and machine-interpretable description of the morphology of colonies formed by human pathogenic bacteria. The last chapter, **Chapter 6**, contains the major conclusions of this thesis and some suggestions of relevant research lines that should be addressed in near future in order to construct the overall picture of *P. aeruginosa* adaptation in CF lungs since colonization to the development of chronic infections.

ABSTRACT

Pseudomonas aeruginosa infections are the major cause of high morbidity and mortality in cystic fibrosis (CF) patients. Despite the long and aggressive antibiotic treatments, *P. aeruginosa* still persists causing chronic infections. Its long-persistence is due to sophisticated mechanisms of adaptation, including clonal diversification into specialized CF-adapted phenotypes. While CF community awaits the development of effective therapies targeting conductance regulator mutations, the gold standard of CF disease management is the eradication of *P. aeruginosa* from CF lungs as soon as detected. Early eradication of *P. aeruginosa* avoids or, at least, retards the development of chronic infections preserving lung function. But to achieve a successful eradication, it is need understanding the early adaptations mechanisms used by *P. aeruginosa* for antimicrobial agents target them. Up to now these mechanisms are unclear. Being so, this project aimed to determine the early adaptations undergone by *P. aeruginosa* after CF lungs colonization and their driven forces in several clinical scenarios.

Alterations in colony morphology are one of the indicators of clonal diversification of bacteria in CF lungs and are also associated with different virulence factors expression and antibiotic resistance. Therefore, it was take the advantage of this macroscopic feature of *P. aeruginosa* to monitor its evolution in CF environment after *in vitro* initial colonization. Despite its potential, colony morphology characterisation method exhibit has some restrictions, such as the unclear impact of the experimental parameters in colony morphogenesis and detection of colony diversity, variability of vocabulary and inconsistent concepts about morphological traits and the time to return results. Therefore, before analysing *P. aeruginosa* clonal diversification in CF environment, it was attempted to introduce some improvements on this method related to standardisation of the experimental parameters and vocabulary and speed up colony analysis in three different studies. The results of the first study demonstrated that all experimental parameters analysed, including colony growth time, colony density *per* plate, culture media, bacterial mode of growth and bacterial genetic background influenced colony morphogenesis and the detection of bacterial diversity. Therefore, a set of guidelines was created and proposed to clinical community in order to standardise the experimental parameters. Further, a morphological classification system to unambiguously characterise and describe bacterial colonies was constructed based on literature. The system was deeply tested and demonstrated to be accurate for bacterial colonies characterisation. The third study, it was aimed to introduce some quickness and high-throughput features to colony morphology characterisation verifying whether MALDI-TOF MS could distinguished morphotypes. MALDI-TOF MS colony classification did not totally match with manual colony characterisation, leading to conclude that MALDI-TOF MS provided additional information about colony variants not visible at naked eye. MALDI-TOF MS cannot thus “replace” colony morphology method but rather complement it.

The data obtained in the first study demonstrated that colony morphology characterisation could be used as a reliable method to detect biofilm-derived colony morphotypes. So, it was tried to find out a colony morphology variant characteristic of biofilm lifestyle, which could be used as a marker of *P. aeruginosa* biofilm growth and the development of chronic infections. The results revealed that biofilm population diversity was highly strain-dependent, hindering the unambiguous establishment of a colony variant marker of *P. aeruginosa* biofilms. Nonetheless, small colony variants (SCV) demonstrated to be a strong hint of biofilm growth.

In order to investigate the early adaptations undergone by *P. aeruginosa* after CF lungs colonisation, bacteria grow in artificial sputum medium during ten days in the presence and absence of ciprofloxacin, an antibiotic often used in CF context. Data revealed that substantial phenotypic diversity is likely to be present in *P. aeruginosa* populations shortly after CF lungs colonization. Ciprofloxacin concentrations at this simulated early stage of disease demonstrated to play dose-dependent role. Inhibitory concentrations of ciprofloxacin, established based on the MIC of biofilm cell, were effective in bacterial eradication, in contrast to the sub-inhibitory doses that triggered *P. aeruginosa* diversification into new “fitter” variants. Impaired swimming motility was one of the first signs of adaptation noticed in all *P. aeruginosa* clonal variants, either in the presence or absence of ciprofloxacin. It was speculated that this adaptation was triggered by the environmental CF conditions due to the absence of mutators. Being so, impaired swimming motility was considered a potential disease marker for early *P. aeruginosa* adaptation and infection development. It was also found that some colony morphology traits, including sheath, size and colour, could be useful indicators of antimicrobial resistance towards some antibiotics and of some virulence factors expression.

During the development of this project the comparison of morphological traits and phenotypic data among colony morphotypes was a hard task. So, to easily perform this kind of analyses it was created a new

ontology-based tool for the description of colony morphologies formed by bacteria, called MorphoCol. MorphoCol establishes and standardises in consistent way the minimum information necessary to describe colony morphotypes and also the phenotypic data related to the colony-forming bacteria. In near future, this knowledgebase will transform the findings of this project and upcoming results related to colony morphology variation into valuable information for clinical decision making.
The overall data will have great impact on CF disease management because relevant improvements were introduced in the colony morphology characterisation method that will strengthen clinical diagnosis. Clonal diversification in CF lungs and other host sites could be now better monitored and accurately described. Moreover, the identification of a putative CF disease marker of early *P. aeruginosa* adaptation and infection development will also assist in the design of tailored effective antimicrobial therapies.

RESUMO

Pseudomonas aeruginosa é a maior causa das elevadas taxas de morbidez e mortalidade associada a pacientes com fibrose cística (FC). Apesar dos longos e agressivos tratamentos com antibióticos, a *P. aeruginosa* persiste originando infecções crónicas. A sua longa persistência deve-se a sofisticados mecanismos de adaptação, designadamente a diversificação clonal em fenótipos altamente adaptados às condições de FC. Enquanto a comunidade médica aguarda pelo desenvolvimento de terapias eficazes direcionadas ao regulador mutacional de condutância, o *standard* terapêutico seguido é a erradicação da *P. aeruginosa* logo que detetada. A erradicação atempada da *P. aeruginosa* evita ou, pelo menos retarda, o desenvolvimento de infecções crónicas, preservando assim a função respiratória. Contudo, para obter a erradicação bacteriana é necessário perceber os mecanismos iniciais de adaptação da *P. aeruginosa* para que os agentes antimicrobianos os possam bloquear. Assim sendo, este projeto pretendeu determinar quais as adaptações iniciais da *P. aeruginosa* após colonização das vias respiratórias de pacientes com FC e as suas respetivas forças impulsionadoras para diversas situações clínicas.

As alterações na morfologia de colónia é um dos indicadores de diversificação clonal das bactérias em ambiente de FC. Estas alterações estão igualmente associadas à expressão diferencial de factores de virulência e à resistência a antibióticos. Assim, utilizou-se esta característica macroscópica da *P. aeruginosa* para monitorizar a sua evolução e adaptação em ambiente FC. Apesar do seu potencial, a caracterização de morfologia de colónias apresenta algumas restrições, tais como o impacto incerto dos parâmetros experimentais na morfogénese das colónias e na detecção de diversidade de colónias, a variabilidade de vocabulário e conceitos inconsistentes sobre os traços morfológicos, e o tempo de obtenção de resultados. Assim, antes da análise da diversidade clonal da *P. aeruginosa* em ambiente FC, tentou-se introduzir alguns melhoramentos neste método em três diferentes estudos, nomeadamente na estandardização dos parâmetros experimentais e vocabulário, bem como acelerar a obtenção de resultados. Os resultados do primeiro estudo demonstraram que todos os parâmetros experimentais analisados (tempo de crescimento das colónias, densidade de colónias por placa de cultura, meio de cultura, modo de crescimento bacteriano e os antecedentes genéticos) influenciaram a morfogénese das colónias e na detecção de diversidade bacteriana. Assim sendo, foi elaborado e proposto um conjunto de linhas de orientação para estandardização dos parâmetros experimentais. Posteriormente, foi construído um sistema de classificação para inequivocamente caracterizar a morfologia de colónias com base na literatura. Este sistema foi amplamente testado e demonstrou-se adequado para a caracterização de colónias bacterianas. No terceiro estudo tentou-se introduzir alguma celeridade e aumento do débito de resultados verificando se MALDI-TOF MS seria capaz de distinguir os morfótipos. Esta técnica forneceu uma classificação dos morfótipos ligeiramente diferente comparativamente à classificação manual. Tal, leva a concluir que MALDI-TOF MS fornece informação adicional sobre os morfótipos que não foi observável macroscopicamente. Assim, o MALDI-TOF MS não pode assim "substituir" o método de caracterização manual de morfologia de colónias mas pode ser um método complementar.

Os dados obtidos com o primeiro estudo demonstraram que a caracterização de morfologia de colónias pode ser usado na detecção de morfótipos derivados de biofilmes. Assim, tentou-se identificar um variante morfológico característico do crescimento em biofilmes que possa ser posteriormente usado para detectar este modo de crescimento e, consequentemente o desenvolvimento de infecções crónicas. Os resultados revelaram que diversidade população dos biofilmes foi dependente da estirpe, dificultando assim a identificação do variante morfológico característico de biofilme. Ainda assim, verificou-se que as *small colony variants* (SCV) são um forte indicador do crescimento séssil.

De forma a investigar as adaptações iniciais da *P. aeruginosa* após colonização de vias respiratórias com FC, as bactérias foram crescidas em muco artificial (MA) na presença e ausência de ciprofloxacina durante 10 dias. Os resultados deste estudo revelaram que é provável a existência de diversidade fenotípica logo após a colonização das vias respiratórias. As diferentes concentrações de ciprofloxacina testadas demonstraram desempenhar uma ação dose-dependente. Concentrações inibitórias, estabelecidas com base na MIC de células de biofilme, foram eficazes na erradicação da *P. aeruginosa* em MA, contrariamente às concentrações sub-inibitórias. Estas concentrações "acionaram" a diversificação fenotípica em variantes melhor adaptados ao ambiente FC. A limitada capacidade de *swimming* foi um dos sinais de adaptação inicial da *P. aeruginosa* verificada em todos os variantes fenótipicos. Foi assim especulado que esta adaptação foi promovida pelas condições típicas de FC devido à ausência de *mutators*. Como tal, a capacidade limitada de *swimming* foi considerada um potencial marcador dos estágios iniciais do desenvolvimento de infecções em FC. Foi igualmente

verificado que alguns traço morfológicos de colónias, nomeadamente a bainha, tamanho e cor, foram indicadores de alterações na expressão de fatores de virulência e resistência a antibióticos.
Durante o desenvolvimento deste projeto a comparação de traços morfológicos das colónias, bem como de dados fenótipos foi uma tarefa árdua. Neste sentido, foi desenvolvida uma base de dados, designada por MorphoCol, para a descrição de morfologia de colónias formadas por bactérias. MorphoCol estabelece e uniformiza a informação mínima necessária de uma forma estruturada e consistente. Num futuro próximo, esta base de dados transformará os resultados deste projeto e outros subsequentes em informação relevante para a tomada de decisão clínica.
O conjunto de resultados obtidos neste projeto terá um impacto considerável na gestão clínica da FC devido aos consideráveis melhoramentos introduzidos no método de caracterização de colónias pois fortalecerão o diagnóstico clínico. A diversificação clonal nas vias respiratórias de FC e nos outros locais pode ser assim melhor monitorizada e descrita adequadamente. Para além disso, a identificação de possíveis marcadores para estágios iniciais de adaptação de *P. aeruginosa* em FC ajudarão na definição de terapias antimicrobianas eficazes.

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ABBREVIATION LIST

%	percent
ºC	Degree Celsius
ACN	Acetonitrile
AHL	N-acylhomoserine lactones
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
Bcc	*Burkholderia cepacia complex*
CET	Cetrimide agar
CF	Cystic fibrosis
CFTR	Cystic fibrosis conductance regulator
CFU/mL	Colony forming units *per* millilitre
CFU	Colony forming units
Cl^-	Chloride ion
cm	Centimetre
Da	Dalton
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ELITE	Early Inhaled Tobramycin for Eradication
EPIC	Early *Pseudomonas* Infection Control
g/L	Gram *per* litre
g	Gram
g	G-force
h	Hour(s)
HCO_3^-	bicarbonate
kV	Kilovolt
L	Litre
LPS	Lipopolysaccharides
m/z	Mass-to-charge ratio
M	Molar
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time of flight combined with mass spectrometry
mg	Milligram
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MIC	Minimum inhibitory concentration
min	Minute
mL	Millilitre
mm	Millimetre
mM	Millimolar
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
MT	Morphotype
Na^+	Sodium ion
NO_3^-	Nitrate
NO_2^-	Nitrite
nm	Nanometre
ns	Nanosecond
O_2	Oxygen
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIA	Pseudomonas isolation agar
ppm	Parts per million
pqs	Pseudomonas quinole signal

QS	Quorum sensing
ROS	Reactive oxygen species
rpm	Rotations per minute
SA	Sinapinic acid
SEM	Standard deviation of the mean
SCV	Small colony variants
TFA	Trifluoroacetic acid
TIS	Tobramycin inhalation solution
Tris-HCl	Trizma hydrochloride
TSA	Tryptic soy agar
TSB	Tryptic soy broth
T3SS	Type III secretion systems
v/v	Volume to volume
w/v	Weight to volume
WM	Wild-morphotype
WT	Wild-type
α-CHCA	α-cyano-4hydroxycinnamic acid
μg	Miligram
μL	mililitre
μm s^{-1}	Micrometer per second

SCIENTIFIC OUTPUT

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POSTERS IN INTERNATIONAL CONFERENCES:

Sousa, A.M, Pereira M. Emergence of mucoid and small colony variants of *P. aeruginosa* at early infection development in cystic fibrosis. 24th ECCMID, 10-13 May 2014, Barcelona, Spain.

Sousa, A. M., Pereira M.O., Lourenço A. Mining clinically relevant signatures from colony morphology images. 24th ECCMID, 10-13 May 2014, Barcelona, Spain.

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Chapter 1

Introduction

1.1. Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive disease caused by a defect in the cystic fibrosis conductance regulator (CFTR) gene located in humans on chromosome 7. The CFTR depletion or malfunctioning affects mainly lungs, digestive and reproductive systems, but also secretory glands, such as the endocrine and sweat glands [1, 2]. CF is thus a multi-system disorder causing several complications on the human body, but its effects on lungs are the best studied so far due to the severe symptoms that patients suffer and the high mortality rate associated to poor lung function [3, 4].

It is generally accepted that CFTR acts as a channel that pumps chloride from the intracellular to extracellular space through the membrane of the epithelial cells that produce sputum (Figure 1.1A). Several hypotheses have been formulated attempting to explain the relationship between CFTR deficiency and sputum accumulation. It has been considered that the transport of chloride partially controls water movement and consequently influences the production of thin and flowing sputum, fundamental to maintain the lungs protected [1, 5]. The CFTR lack causes, thus, a defective chloride secretion creating an osmotic gradient that, consequently, provokes water hyper-reabsorption and abnormal thick and sticky sputum (Figure 1.1B) [1, 6]. This sputum with altered pH interferes, reducing or even inhibiting, the activity of epithelial antimicrobial molecules of the innate immune system and the ciliary function, both crucial for homeostasis [2].

Other functions are also associated with CTFR including inhibition of sodium absorption, which loss causes excessive sodium (and water) absorption, regulation of HCO_3^- and some proteins transport through epithelial cell membranes [6, 7]. The relevance of the latter mechanisms in CF airways is unclear, however it is believed that reduced chloride secretion or sodium hyper-absorption can occur. Both mechanisms lead to airway-surface-liquid depletion and sputum viscosity increase causing impaired cilia beats and accumulation of thick dehydrated airway sputum, which profoundly accounts for the typical symptoms suffered by CF patients [7, 8].

Sputum retention leads to infection and consequently to inflammation, and this circle perpetuates itself since inflammatory products, such as elastase released by neutrophils, stimulate sputum secretion and breakdown [6, 9]. In addition, CFTR mutation leads to dysregulation of various components of the innate immune system. In effect, CFTR has been considered a relevant receptor for internalization and further elimination of some bacteria, for instance *Pseudomonas aeruginosa*. Thus, its absence compromised the immune response [2, 10]. The defective mucociliary transport and the compromised immune defences predispose CF patients to the establishment of recurrent bronchopulmonary infections.

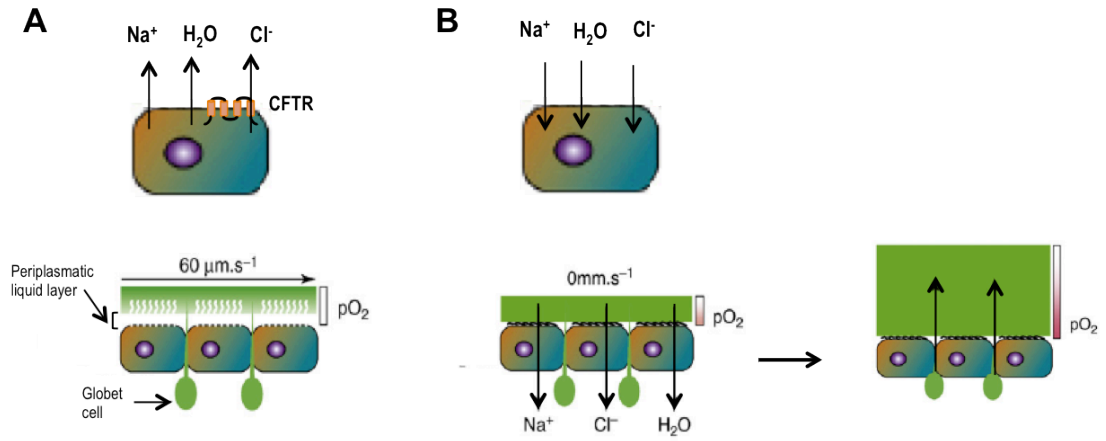

Figure 1.1. Schematic model of transport of ions in normal and CF epithelia. (A) Normal expression of CFTR maintains the constant depth of periciliary liquid layer on top of the airway epithelia leading to efficient mucociliary clearance, at a rate of approximately 60 μm s^{-1} (denoted by vector), by the ciliated cells. A normal rate of epithelial O_2 consumption produces no O_2 gradients within this thin airway surface liquid (denoted by white bar); (B) In the absence of CFTR, water, Na^+ and Cl^- are reabsorbed, removing the periciliary liquid layer. The globet cells underneath the epithelia hypersecreted sputum that becomes highly thickened due to the depletion of the periciliary liquid layer. This thick sputum adheres to the epithelium and reduce or stop sputum clearance (0 μm s^{-1}). The ineffective mucociliary clearance and raised O_2 consumption by CF epithelia result in hypoxic gradients within the sputum masses. The colour bars to the right of each diagram represent the O_2 pressure, with increasing red colour presenting a progressively more anaerobic environment (Adapted from Hassett *et al.* (2009) [11] and Worlitzsch *et al.* (2002) [12]).

1.2. Bacterial infections

Bacterial infections pose a serious challenge in the care of CF patients. The accumulated sputum has nutrients being, thus, a good environment for microbial colonization [13, 14]. CF lungs are infected with a complex microbial flora, mainly composed by bacteria, provoking acute and chronic infections that result in decline of the lung function, respiratory failure and premature death of patients [15, 16]. Once bacterial infections are established, their eradication by antibiotic treatment is hardly achieved [10, 15].

For many decades, the microbiome of CF airways has been determined by culture methods focusing on the most commonly isolated organisms including *P. aeruginosa*, *Staphylococcus aureus* and *Haemophilus influenzae*. Advances in microbial diagnosis techniques have revealed that CF lungs are an environment much more richer than previously estimated and, most important, that many other opportunistic organisms are associated with the evolution of the airway disease in CF individuals [17, 18]. Over the last decades, culture independent approaches, such as sequencing of bacterial 16S rRNA gene, have been identified more than 100 distinct genera, including commensal genera, gram negative such as *Burkholderia cepacia* complex (mostly *B. multivorans* and *B. cenocepacia)*, *Stenotrophomonas maltophilia*, *Achromobacter* spp., and *Inquilinus limosus*; non-tuberculous mycobacteria; and fungal organisms, such as *Aspergillus fumigatus* [17, 19, 20]. Viruses have been also detected, although their specific role in CF lung disease is still unclear [21, 22].

The diversity of the CF microbiome varies over time. During childhood microbial diversity reaches its peak falling during the second decade of life when it is more frequently dominated by *P. aeruginosa* [23, 24, 25]. *S. aureus* and non-typeable *H. influenza* are prevalent in infections occurring during childhood and

then colonization by *P. aeruginosa* progressively increases as CF patients grow older [19, 26, 27]. It has been proposed that *S. aureus* may somewhat prepare the CF environment to promote *P. aeruginosa* colonization and, by this reason, *S. aureus* pre-colonization has been considered a risk factor [2, 28]. The end-stage (or the chronic stage) of CF airway disease is generally associated with reduced community diversity and dominance by a few antibiotic resistant pathogens such as *P. aeruginosa* and *B. cepacia* complex (Bcc) [29]. Among the panoply of opportunistic bacteria that may colonize and infect CF lungs, *P. aeruginosa* is considered the most significant as it has evidently been linked to worsening of the lung and respiratory status [30].

It is now clear that the different bacterial species coexisting in CF airways establish interactions, contributing to the pathogenesis of the disease and in driving a response to therapy [16, 31]. Despite the documentation about this coexistence, very little is known about the interactions among species and their impact on severity and progression of infections. More studies are needed to understand how these microbial interactions are establish, how they affect the ecological balance of multispecies communities and the progression of disease.

1.2.1. Pseudomonas aeruginosa

As aforementioned, the microbial community resident in CF lungs is known to be complex and it has considerably changed mainly due to alterations in antibiotic regimens. Nevertheless, *P. aeruginosa* is still the most common pathogen isolated from CF sputum [5, 15, 32].

P. aeruginosa is a versatile microorganism, ubiquitously distributed in different environments, including terrestrial, aquatic, animal, human and plant [33]. It is a Gram-negative opportunist pathogen in hospitalized or immune-compromised patients causing infections such as pneumonia, burn, wound, urinary tract and gastrointestinal infections, otitis media and keratitis [32, 34, 35]. Its versatility arises from its largest genome with nearly 6000 genes that encloses, for instance, genes associated with diverse metabolic pathways, virulence factors, transport, efflux and chemotaxis conferring to *P. aeruginosa* great adaptive ability [33, 34]. This human pathogen is intrinsically resistant to a wide range of antibiotics and frequently displays intrinsic multidrug resistance [36, 37]. Moreover, this bacterium is able to coordinate metabolic pathways, optimize nutritional and reproductive potential according to the surrounding conditions and resources and, thus, it can survive, grow and cause infection in different environments [34, 38].

The presence of *P. aeruginosa* in CF airways is highly associated with poor lung function, morbidity and mortality of patients. The longer a patient remains free of colonization with *P. aeruginosa*, the longer his life expectancy will be. The incidence of *P. aeruginosa* infections varies widely among CF centers but there is common increase of prevalence with age, being more prevalent in adults [5, 32]. In the last years, *P. aeruginosa* was detected in 20-25 % of infants, 25-50 % of children aged 2-10 years and almost 80 % of young adults [19, 26, 27].

Despite the inflammatory response and the long-term and intensive antibiotic treatments, infections caused by *P. aeruginosa* persist in CF lungs. Once entering in CF airways, this bacterium is virtually impossible to eradicate due to its remarkable genome plasticity that allows rapid adaptation to the stressful CF environment [5, 39, 40]. After *P. aeruginosa* colonization, patients may suffer of successive episodes of re-colonization until resulting in a chronic infection that can persist from years to decades or even never been eradicated [40, 41].

Several factors can influence the *P. aeruginosa* infection course in CF airways and, unfortunately, there is limited knowledge about the characteristics of this microorganism that have impact on the severity of infection. Until now, it is just known that during CF infection development *P. aeruginosa* switches from an acute environmental virulent pathogen, characteristic from early infection stages, to a CF-adapted pathogen typical from chronic infection stages [9, 38, 42]. This switching is one of the most striking adaptive mechanisms used by *P. aeruginosa* to survive and grow in such complex environment as CF lungs. CF environment is a complex ecosystem due to the different conditions that bacteria may found, for instance, different oxygen concentrations, nutrient availability, other microbial species, oxidative stress, antibiotic treatments and immune defences. *P. aeruginosa* is able to generate progressively multiple phenotypes highly adapted to changing CF environment and their emergence and coexistence is the key factor for persisting in CF lungs for several decades or even an entire life [42]. This intense diversification of the bacterial population is named of clonal diversification and supports the 'Insurance Hypothesis' which states that (more) diversity within a population maintains or enhances its functioning against a wide range of adverse environmental fluctuations [43]. *P. aeruginosa* clonal diversification provides thus subpopulations resistant to the range of different CF stresses and able to colonize a variety of CF airways niches [44, 45]. The role of each subpopulation generated by *P. aeruginosa* in CF airway is discussed in section 1.5.

1.3. Antibiotic treatments

Some treatment progresses were made in order to extend the life expectancy of CF patients, however it remains very reduced, around 38 years, mainly because of bacterial infections [26, 27]. In the last decades, new therapies have emerged based on the knowledge of CFTR dysfunction and airway CF microbiome, such as targeting CFTR replacement, stimulation of alternative chloride channels, inhibition of sodium absorption and airway rehydration, in order to avoid sputum accumulation and, consequently, the establishment of bacterial infections [6, 7, 8, 46]. None of those strategies has sufficient potential to stop CF infections development so far.

The best strategy is certainly to prevent infection as late as possible mainly from acquisition of *P. aeruginosa*. The preventive procedures may include avoiding person-to-person contact and contacting with contaminated surfaces, which may lead to transmission and acquisition of microorganisms. As *P. aeruginosa* may survive in abiotic (or inanimate) surfaces for months [47, 48], cleaning and disinfection of the clinical settings, including rooms, clinical equipment and medical devices are thus important. Health

workers should practice hand hygiene and during hospitalization CF patients should be isolated to prevent transmission and acquisition of pathogens in particular *P. aeruginosa* and multi-resistant strains [48].

Although the prophylactic efforts, infections can still occur and the actual and more effective approach to fight CF-associated infections relies on antimicrobial treatment. Currently, there is not a consensual antimicrobial treatment to eradicate bacterial infections from CF lungs as treatments vary among clinics, countries and even continents [49, 50]. Numerous strategies have been used varying in route of antibiotic administration (systemic, oral, inhaled antibiotics or route combination), classes of antibiotics and treatment duration (reviewed by Schelstraete *et al.* (2013) [41]). Inhaled antibiotics, mainly aminoglycosides, have high successful rates in bacteria eradication, in particular against *P. aeruginosa*, due to the direct delivery of high-dose of antibiotic to the bronchial lumen space with limited systemic toxicity. A tobramycin inhalation solution has been used to treat long-term and chronic bacterial infections with significant benefits for lung function delaying re-infection and reducing mortality [51, 52, 53]. Other inhaled antibiotics have been studied and have demonstrated efficacy against microbial infections, including colistin, gentamicin, ceftazidime, cephaloridine, aztreonam lysine, taurolidine, and a gentamicin/carbenicillin combination [51].

It is very common the treatment of CF-associated infections with combination of oral and inhaled therapies using two antibiotics with distinct modes of action. The quinolones, in particular ciprofloxacin and levofloxacin, are the most used as oral agents combined with inhaled tobramycin, aztreonam or colistin. Oral and intravenous antibiotics have also attracted interest, including the intravenous regimes of anti-pseudomonal antibiotics β-lactams (pipercillin or ticarcillin), third- and fourth-generation cephalosporins (ceftazidime and cefepime), carbapenems (meropenem or imipenem), or monobactams (aztreonam), combined with an oral aminoglycoside (amikacin, gentamicin or tobramycin) [49, 54, 55, 56].

Even so, treatment of bacterial infections in CF lungs remains a challenge. Multiple factors may disturb the antibiotic action, including the nature of the drug, the microbial population resident in CF airways, host characteristics, patient adherence to the treatment and the timing of antibiotic treatment [50, 57]. CF environment conditions represent as well a challenge to treat bacterial infections. Mucin and extracellular deoxyribonucleic acid (DNA), which are present in sputum, can interfere in antibiotic action. Biofilms are as well of special concern as the extracellular matrices restrict seriously the action of the majority of the antimicrobial agents (discussed later in section 1.4). Due to these limitations, other antibiotics have been introduced and used as alternative agents, such as inhaled amikacin, aerosolised levofloxacin (both have completed phase 3 trial) and the combination of fosfomycin and tobramycin (in phase 2 clinical trial) [4, 46, 49, 50].

Regardless the antibiotic agent used, it is consensual that soon as infection is detected, an antibiotic treatment must be initiated. Some studies suggest a window of opportunity for successful eradication of 12 weeks since primary detection of infection, however this period is not well defined [58, 59]. At this stage, it is important to verify if patients are colonised by *P. aeruginosa*. If not, it is of upmost importance to delay the acquisition of *P. aeruginosa* since it is associated with worsening patient prognosis. Anti-pseudomonal antibiotic treatments could still be administrated to prevent *P. aeruginosa* acquisition but a prospective 3-

year study demonstrated no differences in *P. aeruginosa* rate acquisition between a control and treatment group with oral ciprofloxacin and inhaled colistin [60]. Therefore, the current status of antibiotic treatments does not recommend the prophylactic treatment with antipseudomonal antibiotics to prevent *P. aeruginosa* acquisition in CF individuals [41, 50, 61]. For the cases of *P. aeruginosa* early colonization, it is recommended aggressive antibiotic treatments at early infection stage in order to eradicate bacteria from lungs and thus retard the establishment of chronic infection preserving lung function [62, 63, 64]. The antibiotic treatment administrated to these CF patients early colonised with *P. aeruginosa* typically follows the scheme represented in Figure 1.2.

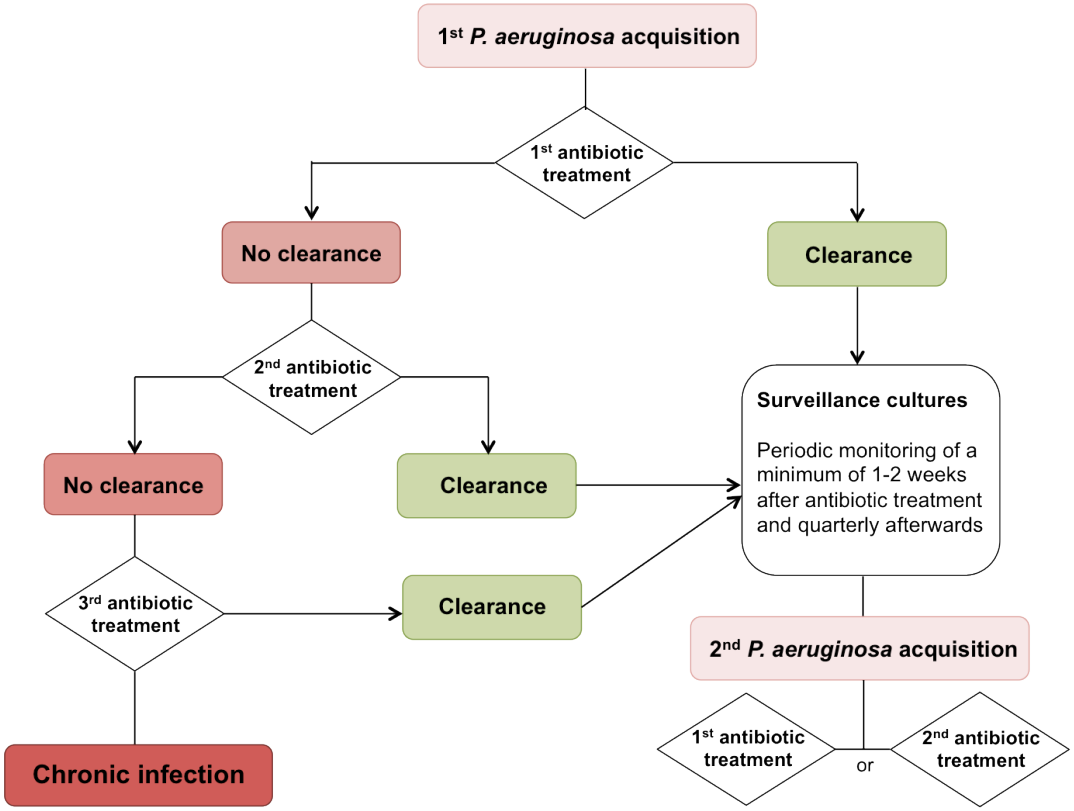

Figure 1.2. Antibiotic treatment scheme after first detection (Adapted from Doring *et al.* (2012) [50]).

In general, it is prescribed a 3-week antibiotic treatment. In cases of treatment failure, the treatment is prolonged for more 28 days, however, there is no consensual treatment for *P. aeruginosa* infections [41, 50, 63]. In attempt to standardize early antibiotic treatments, it was performed two large multicentre studies, the Early *Pseudomonas* Infection Control (EPIC) program in the United States and the Early Inhaled Tobramycin for Eradication (ELITE) study in Europe. The EPIC trial consisted in randomized patients in four different antibiotic eradication strategies during 18 months: 1) tobramycin inhalation solution (TIS) combined with oral ciprofloxacin every 3 months; 2) TIS combined with oral placebo every 3 months; 3) TIS combined with oral ciprofloxacin only when quarterly respiratory cultures were found

positive for *P aeruginosa*; 4) TIS combined with oral placebo only when quarterly respiratory cultures were found positive for *P aeruginosa.* The results published by the EPIC trial demonstrated that all patients groups exhibited similar successful eradication rates [65].

The ELITE study treated patients as well with twice-daily inhaled tobramycin 300 mg for 28 and 56 days and similar results were obtained for both patients groups. 93 % of patients were *P. aeruginosa*-free after one month and 63 % of patients were *P. aeruginosa*-free after 2 years. [53]. From both studies it was concluded that adding ciprofloxacin to antibiotic regimes did not produce benefits in *P. aeruginosa* eradication rates. However, the Copenhagen CF center performed a long study during 20 years in which CF patients were treated with inhaled colistin and intravenous ciprofloxacin for 3 months. 80 % of the treated patients were *P. aeruginosa*-free for up 15 years and the bacterial isolates recovered showed reduced antibiotic resistance [58, 66]. Other European CF center performed a similar study and obtained the same results [62]. Although these controversial results, the main clinical practice is the combination of inhaled colistin and oral ciprofloxacin for 3-week to 3-month, which has demonstrated to be a successful approach [66]. As inhaled colistin for 3 months with inhaled tobramycin for 1 month revealed results quite similar to the colistin/ciprofloxacin treatment, colistin/tobramycin is also a common treatment in hospitals [67].

The current European status of antibiotic eradication treatments does not recommend a particular strategy due to the limited number of comparative treatment studies available [50].

Despite early antibiotic treatments have demonstrated high rate of success, there are some patients for whom early treatments fail (Figure 1.2). In those cases, *i.e.* in the presence of persistent infections, it is crucial the maintenance of the therapy for extended periods of time [50]. After a successful eradication, re-infection episodes can occur mainly due to the original site and source of infection be the sinonasal cavities that inhaled antibiotics can not reach [68, 69].

Finally, culture negativity following an antibiotic treatment does not absolutely mean that bacteria were eradicated. Culture negativity may mean that bacterial load is in fact significantly reduced but some pathogens may persist in CF lungs. Moreover, some pathogens may be metabolic inactive or unculturable and, thus undetectable by culturing methods [41, 50]. Therefore, patients who had a successful eradication of bacteria from CF lungs, as well as those who had never been infected, should be routinely monitored to detect as early as possible the infection in order to treat it with high success rates.

1.4. Clonal diversification of *P. aeruginosa* in cystic fibrosis lungs

The long-term persistence of *P. aeruginosa* infections in CF lung is associated with clonal diversification, or expansion, into specialized phenotypes (Figure 1.3). Driven by the challenging selective pressures imposed by the typical CF conditions, e.g. interspecies competition, deficient oxygen availability, biofilm growth, the immune system action, oxidative stress and antibiotic treatment, *P. aeruginosa* progressively generates phenotypes specially adapted to CF airways conditions [5, 10, 70, 71].

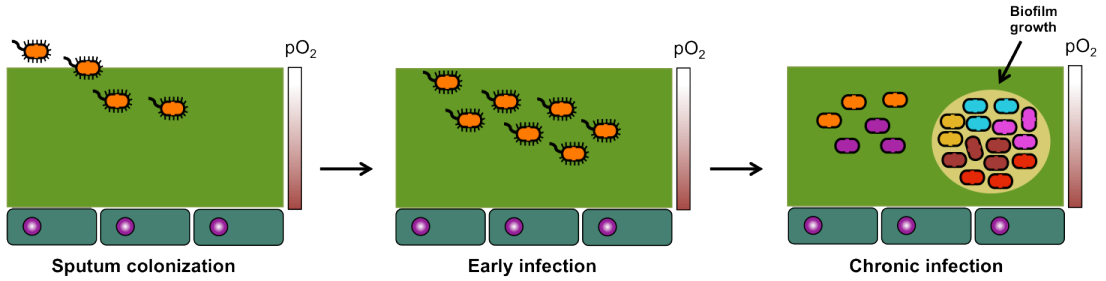

Figure 1.3. Time course of *P. aeruginosa* infection development. (a) Sputum colonization stage - *P. aeruginosa* equipped with full virulence factors enter in CF sputum; (b) Early infection stage – *P. aeruginosa,* which exhibit the environmental or wild-phenotypes species characteristics, starts its adaptation to CF environmental conditions; (c) Chronic infection stage – *P. aeruginosa* is full adapted to CF environment. At this stage, there is high phenotypic and genotypic diversity and formation of biofilms.

The CF selection forces are evident when clinical isolates of *P. aeruginosa* are frequently mucoid. Indeed mucoid variants are rarely isolated from non-CF environments supporting the existence of specific CF selective pressure [5, 40]. By this reason, *P. aeruginosa* conversion from non- to mucoid form is considered the hallmark of CF airway.

However, other phenotypic features of clonal variants adapted to CF airways have been frequently observed, including small colony variants (SCV), non-pigmented variants, increased antibiotic resistance, excellent ability to form biofilms, increased mutation frequencies, altered metabolic pathways and attenuated virulence potential [6, 39, 71]. The repeated occurrence of these particular phenotypic features and/or gene expression in chronic isolates sampled from different patients and clinical settings suggests the existence of a parallel evolution of *P. aeruginosa* in CF airways [72, 73].

Biofilm formation

The biofilm lifestyle represents a reservoir of high phenotypic diversity and it is considered one of the most important adaptive mechanisms of *P. aeruginosa* within CF sputum (Figure 1.3) [11, 39, 74]. Biofilms are microbial communities bound to a surface and/or to each other encased in a self-produced matrix composed of exopolysaccharides, proteins and DNA [75, 76, 77]. Worlitzsch *et al.* (2002) [12] have shown that in the conductive zone, region from the trachea to the terminal bronchiolus, *P. aeruginosa* grows mainly in biofilms, whereas very few bacteria are localized at the epithelial surface of the bronchi. Other studies have provided as well evidence of biofilm growth through inspection of sputum samples and lung tissue sections [39, 74, 78]. There are evidences that biofilm strategy is used as well by *Burkholderia* spp., *A. xylosoxidans* and *Stenotrophomonas* species [79].

Nowadays, biofilms are recognized as an important issue in human disease management due to their remarkable resistance achieving 10 to 1000-fold higher tolerance to antimicrobial agents than corresponding planktonic bacteria [77, 80]. This biofilm resistance has multifactorial nature resulting from the combination of several mechanisms, including restricted penetration of antimicrobials through the exopolysaccharide matrix, slow growth of bacteria within biofilms caused by nutrient and oxygen

restriction and accumulated metabolic wastes, and quorum-sensing (QS) molecules [81, 82, 83, 84]. The limited penetration of antibiotics and immune defences through the exopolysaccharide matrix is definitely one of the major contributors for their ineffective action and thus *P. aeruginosa* persistence. Alginate is the major component of CF biofilm matrix providing structure and protection to bacteria from the stressful environmental conditions of CF lungs. Augmented levels of alginate are generally observed in CF patients chronically infected and it is associated with poor prognosis because alginate triggers a significant antibody response [85, 86].

Airway CF biofilms are genetic, proteomic and physiologic different of surface-attached biofilms formed on, for instance, indwelling devices (catheters, prostheses, pacemakers, stents) and medical and clinical equipment. Instead of the direct surface colonization, clearly observed in device-associated infections, bacteria in the CF lungs preferentially form multicellular clusters or macrocolonies within the sputum and not on the epithelium surface of the bronchi and non-respiratory bronchioles as initially supposed [12, 74, 87, 88]. Additionally, the environment in which CF-associated biofilms are formed is considered to be microaerophilic or anaerobic. Bacteria enter and colonize CF sputum, consume oxygen *via* respiration and generate steep oxygen gradients within the sputum [10, 11, 88]. The limited oxygen availability to anaerobic environments in CF sputum was confirmed by direct *in situ* oxygen measurements using a microelectrode [12]. The oxygen-limited and anaerobic growth conditions significantly increase antibiotic resistance of biofilm-forming bacteria [89].

Until now, it is not clear when bacteria after CF airway colonization switch to sessile lifestyle, but it is known that biofilm formation enables bacteria to successfully establish chronic infections. Presumably, *P. aeruginosa* form biofilms in response to stressful conditions including microaerobiosis and/or antibiotic treatments [40, 90].

The great variability or heterogeneity of phenotypes included and developed within biofilms is certainly one of the major contributors for sessile bacteria recalcitrance that it is not observed in planktonic state [44, 91, 92]. To switch from planktonic to biofilm mode of growth, bacteria undergo a number of complex physiological, metabolic and phenotypic differentiations. For instance, biofilm-growing bacteria undertake specific changes in protein regulation, especially those related with proteins involved in resistance to oxidative damage, exopolysaccharide production, phospholipid synthesis and membrane transport [93, 94, 95]. Global gene expression analyses of mature *P. aeruginosa* biofilms have revealed 1 % of differential gene expression between the planktonic and biofilm mode of growth, with 0.5 % of the genes being activated and about 0.5 % being repressed [96]. Among the transcription factors, repression of flagellar and pili genes and stress response regulator genes, such as *rpoS*, hyperexpression of genes for ribosomal proteins and metabolism and transport functions were the most identified.

Gene expression varies during biofilm development, which means that there are stage-specific temporal and spatial gene expression patterns leading to temporal and spatial phenotypic diversity. This is particular relevant concerning the resistance of mature biofilms to antimicrobial treatment. The biofilm-specific phenotype can trigger mechanisms responsible for antimicrobial resistance and persistence and consequently enhance pathogenicity. *P. aeruginosa* genome sequencing has revealed that a mature

biofilm can express several cluster genes encoding efflux pump involved in resistance to some antibiotics [96].

Within biofilms, various heterogeneous environments exist as a result of the distinct levels of nutrients and oxygen availability and accumulated metabolic wastes that bacteria have to face and adapt in a process similar to CF airway adaptation [97]. This diversification profits the whole biofilm population with diverse abilities to face environmental challenges as long as bacteria coordinate with each other. Bacterial cooperation and differentiation are facilitated through the production and perception of QS small signalling molecules called autoinducers. QS is a cell-cell communication system used by bacteria to regulate gene expression in response to fluctuations in cell-population density and it has being reported to play a role in early and later stages of biofilm development [98, 99]. QS allows bacteria to "sense the quorum", which means the relative density of the population, and coordinate their behaviour according to that sense of "quorum". Bacteria produce and secreted small molecules (the QS signal) that when are sufficiently high the QS signal binds to an intracellular receptor that activates (or represses) a sub-set of genes [98, 100, 101]. Because biofilms are in general high dense amount of bacteria, QS play a key role in gene and protein expression during biofilm formation [98, 102, 103]. *P. aeruginosa* has at least three distinct QS systems, termed *las*, *rhl* and *Pseudomonas* quinolone signal (*pqs*) [101, 104, 105]. The lack of *las* QS system allowed the formation of biofilms, however does not allow them to achieve the mature stage. The *rhl* QS system has been reported as active in the early stages of biofilm development and its blockage may prevent biofilm formation [93, 106]. The interbacterial communication is mediated by two types of molecules, N-acylhomoserine lactones (AHL) and 4-quinolones, allowing bacteria to percept their density and regulates their gene expression properly. For instance, up-regulating genes encoding virulence factors, such as those related to the production of enzymes or toxins, optimize the metabolic and behavioural activities of bacteria within the community [107, 108]. The role of QS is also evidenced in interspecies communication where the secreted AHL is perceived by other adjacent microbial species that may respond to establish new biofilms [109]. There are evidences of cross-communication mediated by QS between *P. aeruginosa* and Bcc within biofilms [110].

Biofilm heterogeneity is also reflected in distinct antibiotic susceptibility profiles. Due to the different biofilm cell physiological states, biofilms have typically a top-to-bottom decreasing susceptibility profile. Antibiotics are effective against the cells located in the top of the biofilm, generally in active state, in contrast to the middle and bottom zones where cells are intermediate resistant or resistant to antibiotics. Even when antibiotics reach the middle and/or bottom biofilm zones, the majority of them has no activity against dormant cells (cells with reduced or even absence of metabolic activity) and, thus, are unsuccessful in biofilm eradication [83, 97, 111].

Planktonic *P. aeruginosa* cells are also found in CF sputum [74]. Due to alterations in CF environment, such as pH and oxygen and nutrients availability, biofilm-cells dispersion may occur [112]. The dispersal of biofilm population provides to *P. aeruginosa* an opportunity to colonize new zones or niches in CF sputum and, thus, perpetuating infection. In fact, dispersal events can be responsible for the acute exacerbations observed in chronic infections [90, 92, 113].

Hypermutability

The whole adaptation process to CF airways can be accelerated by the emergence of mutator phenotypes (or hypermutable phenotypes) which have high mutation rates up to 1000–fold than non mutator phenotypes [114, 115, 116]. In extreme selective conditions, as those occurring in CF airways, this sophisticated mechanism improves the microevolution of *P. aeruginosa* accelerating its intraclonal diversification. The emergence of phenotypic variants and mutators can be intrinsic, relying on mutations (or recombinations) caused by defects on one of the several DNA repair or error avoidance systems, combined or not with extrinsic or environmental factors, such as competition for different niches in a spatially heterogeneous environment as CF airways, and/or selection that favours mutants better "fitter" to CF airways [115, 117, 118]. Mutators can also be stimulated by environmental factors as the presence of reactive oxygen species (ROS) generated from inflammatory responses [119]. ROS can trigger the generation of phenotypic variants damaging DNA and cause mutations in bacteria. Further, sub-inhibitory or sub-lethal concentrations of antibiotics can induce mutations and recombinations and, consequently, supporting the emergence of phenotypic variants and mutators [70, 115, 120]. The genes mainly affected are the antimutator genes *mutS*, *mutL*, and *uvrD* but it can be observed defects as well in the genes *mutT*, *mutM*, and *mutY* [114, 121, 122].

The amount of mutators in biofilms is significantly higher than in planktonic state. This condition may explain the enhanced antibiotic resistance, and frequently multidrug resistance of biofilm-associated bacteria, and the high genetic and phenotypic diversity observed within biofilms [123, 124].

The generation of several clonal variants represents a huge biological advantage because it prepares *P. aeruginosa* population for extreme and unpredictable stresses ('Insurance Hypothesis') supporting the long-term survival of this pathogen [43, 44]. Mutators achieve more quickly CF adaptation due to the expression of virulence traits, antibiotic resistance, increased ability to form biofilms and alternative metabolic functions representing thus a serious clinical problem [115, 125, 126]. In effect, mutators can increase the transcription of genes involved in the metabolism of fatty acids and amino acids crucial for obtaining energy in CF ecological niches where aerobic respiration is not possible [127].

Although all the biological advantages, mutators may have a reduced ability to survive in other distinct environments indicating they can reach high levels of habitat- or niche-specialization spending its biological fitness [40, 115]. During infection development non- and mutators coexist in CF airways, however mutators prevail at chronic stage, which may be indicative that they have in fact adaptive advantage.

Through the combined action of these sources of clonal diversification, including phenotypic switching, biofilms and hypermutability, *P. aeruginosa* may achieve impressive levels of diversification, adaptation and evolution promoting the persistence of the bacterial populations in CF airways. Therefore, these sources should be intensively studied in order to understand the underlying mechanisms to further block them and combat the recalcitrant infections.

1.5. *P. aeruginosa* evolution and adaptation during infection development

The regular sampling of CF sputum has allowed performing a detailed characterization of *P. aeruginosa* over infection development through transcriptomic, metabolomics and proteomic techniques. Therefore, it is now possible to start drawing an evolutionary trajectory of *P. aeruginosa* within CF airways.

During infection development, genotypes and phenotypes differ markedly from those that initially colonized CF airways (Figure 1.4). Microbiological studies have reported changes in *P. aeruginosa* phenotypic and genetic traits, relevant in the context of bacterial pathogenesis, and different antibiotic resistance patterns along infection development, as well as after antibiotic treatments [128, 129, 130]. Moreover, similar evolution and adaptation profiles were observed in distinct clonal lineages of CF-adapted strains suggesting that in fact there is a similar selective pressure in CF airways. This evolution and adaptation processes lead to the generation of several phenotypes varying in characteristics such as colony morphology with distinct consistency, size, texture and colour, inactivation of QS, hypermutation, loss of the O-antigen components of the lipopolysaccharide (LPS), loss of motility, resistance to antibiotics, changes in nutritional requirements and other virulence-associated traits [5, 70, 129, 130]. In fact, some of those factors have been considered the hallmark of CF disease and even can indicate the infection stage, such as the conversion of *P. aeruginosa* to mucoid phenotype, loss of motility and the emergence of SCV, characteristics indicative of chronic infection. However, many other characteristics have been described across all phenotypes isolated so far and it is unknown their role in bacterial adaptation and persistence and in infection progression.

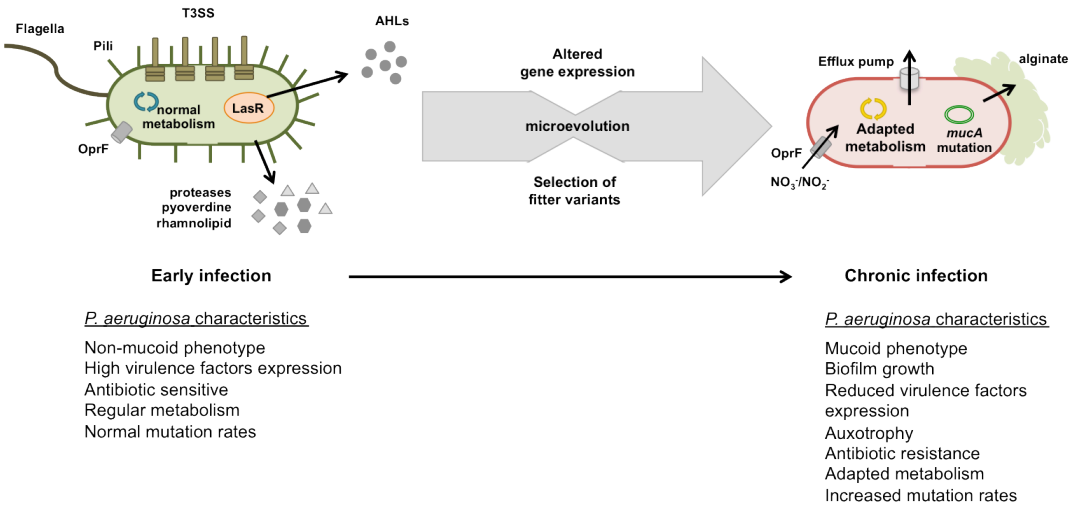

Figure 1.4. Representation of *P. aeruginosa* microevolution during infection in CF airways. At early stage of infection, *P. aeruginosa* is full equipped with cell-associated virulence factors, including flagella, pili, type III secretion systems (T3SS) and secreted virulence factors (e.g., proteases, pyoverdine and rhamnolipid) and exhibit antibiotic sensitivity. At the chronic stage of infection, *P. aeruginosa* is fully adapted to CF environment and exhibits a variety of adaptations, including overproduction of alginate, loss of the implicated virulence factors for initial infection establishment, resistance to antibiotics (expression of efflux pumps) and adapted metabolism. This microevolution occurs by the repeated interplay of mutation and selection.

Antibiotics have provided significant control of bacterial infections in CF airways, however the occurrence of antibiotic resistance and the lack of new drugs or therapeutic strategies make imperative the identification of alternative targets for treatment. For understanding the mechanisms underlying bacterial adaptation to CF environment and the resistance to antibiotic treatments, it is need an overall picture of the actual knowledge about *P. aeruginosa* populations resident in CF lungs.

The compilation of the phenotypic traits exhibited by bacteria according to the infection stage is a hard task due to the lack of agreement on the definitions of early, intermediate and chronic colonization and infection stages. Next, the evolution, adaptation and diversification profiles of *P. aeruginosa* are reviewed and compared agreed by the "European Consensus" that just considers two infection stages, early and chronic stages, according to the presence of *P. aeruginosa* be lower or higher than 6 months, respectively [131].

1.5.1. Early infection stage

Most CF patients acquired pathogens mainly from their surrounding environment, especially from clinical settings where patients remain for long periods of time. Therefore, early CF isolates exhibited identical microbial characteristics of their environmental or wild-phenotypes species (Figure 1.4) [38, 132, 133]. At the first colonization of CF airways, *P. aeruginosa* have to regulate properly its gene expression to quickly adapt to this challenging environment, including host immune defences, antibiotics and different substrate composition.

The bacterial characteristics among acute CF isolates significantly vary, however there is a trend towards high virulence potential and cytotoxicity and lower frequency of mutators strains [60]. The expression of virulence factors, including cell-associated and secreted virulence factors, is considered to be fundamental at early stage for the success of infection establishment. These virulence factors include, for instance, i) the increased production of pyoverdine, hemolysin and phospholipase C, ii) the augmented production of rhamnolipid regulated by QS, which helps biofilm formation that protects cells against oxidative stress, decreases liquid surface tension, due to its biosurfactant feature, and facilitates the access to nutrients within biofilms; iii) the increased production of total protease seemed to promote mucoidy essential for long-term bacterial persistence; iv) swimming and twitching motilities; and iv) the expression of the T3SS that augments cell cytotoxicity potential and facilitates infection development [40, 132, 133, 134, 135, 136].

Typically, *P. aeruginosa* exhibit a non-mucoid phenotype, sensibility to antibiotics and have low bacterial density in lungs, in contrast to chronic infections [41, 133]. Acute CF isolates produce AHL suggesting that QS circuit plays a role for *P. aeruginosa* pathogenesis at this stage of infection. Afterwards, QS seems be no longer needed and *lasR* mutants are frequently isolated. Mutator strains are not prevalent at this stage because they are not efficient to establish a primary infection [137, 138].

At the early stage, eradication is still possible whether an antibiotic treatment was started as soon as possible. Otherwise, approximately 20 % of those first *P. aeruginosa* colonisations could become directly

chronic infections and may persist up to the end of patient life [41, 133]. Following *P. aeruginosa* eradication, it is common a new acquisition event with a different genotype or a re-colonization with the same genotype. Re-colonization with the same genotype may occur due to the persistence of the environmental source or due to the colonization of the upper airways, such as the paranasal sinuses [68, 139]. Upper airways can function as reservoirs of pathogens and interchange of *P. aeruginosa* can be possible. A recent study revealed that biofilms in fact can be found in sinuses of CF patients with intermittent lung colonization [140].

Colonisation of the CF airways with mucoid strains is associated with an accelerated rate of decline in pulmonary function, however, there are some evidences that early acquisition of mucoid strains could be successfully achieved [41, 141].

In summary, although the virulence potential of early CF isolates is higher than chronic isolates, they exhibited increased antibiotic sensitivity. Therefore, *P. aeruginosa* early detection and eradication are currently the main goal to avoid infection progression to chronic stage. Early infections are intensively treated with antimicrobial therapy resulting in the majority of the cases in *P. aeruginosa* eradication, at least temporal eradication [41]. In cases of antimicrobial therapy failure, infection can shortly evolve to chronic infection. Identification of the CF patients who may evolve to chronic infections based on the acute bacterial characteristics is still not possible because of factors related to host-pathogen and pathogen-pathogen interactions may play a role that is unknown so far [60, 142].

1.5.2. Chronic infection stage

The continuous and selective pressure over the population leads to the emergence of diverse phenotypic and genetic variants specially adapted to CF airways. It has been observed among chronic *P. aeruginosa* isolates alterations in colony morphology, namely the conversion to the mucoid morphotype, to SCV and non-pigmented variants, changes in surface antigens, reduce virulence potential, increased antibiotic resistance, overproduction of exopolysaccharides and modulation of microaerobic and anaerobic metabolic pathways (Figure 1.4). These alterations suggest a survival strategy to save or, at least, to reduce energy costs with virulence factors expression in favour of the use of alternative metabolic pathways crucial at this stage.

The repeated occurrence of the described phenotypic features in chronic isolates of *P. aeruginosa* indicates that they may be a result of parallel evolution, which means that related microorganisms develop the same adaptive features in identical but independent environments [72]. Several studies have profiled isolates of *P. aeruginosa* in attempt to find the common route towards the chronic phenotype and the mechanisms underlying such route. Among them, longitudinal studies using transcriptomic approaches have provided relevant information about the genetic changes undergo by *P. aeruginosa* and allowed comparing the expression of specific set of genes among patients in different periods of time. It has been identified gene expression changes in multidrug efflux pumps and regulators of QS and alginate biosynthesis, being these two latter the hotspots of mutations [38, 72, 143, 144].

In the scope of genomic evolution in chronic CF lung infection, it should be highlighted the work performed by the Copenhagen and Hanover clinics. They have regularly collected *P. aeruginosa* from all their CF patients in the 1970s and 1980s and performed the genome sequencing of all the isolates. They started their investigation with the most prevalent clones, the C and PA14, and observed that both clones convert their phenotypes becoming deficient in the LPS O-antigen, with impaired motility and decreased siderophores secretion, as well as in other virulence factors expression, and remaining non-mucoid [145]. The isolates later collected just exhibited impaired competitive growth. These cases demonstrated that the evolutionary transition might be through additive effects of various mutations. However, a single loss-of-function mutation can induce dramatic changes in *P. aeruginosa* as those observed through the mucoid variant due to the pleiotropic effects of *mucA* mutation [5, 38].

Mucoid colony morphology results from alginate overproduction, absence of flagellin and pilin and expression of other virulence factors. Within the mucoid form, *P. aeruginosa* is more difficult to eradicate because its highly resistance to antibiotics and to the action of host immune defences, for instance, to phagocytosis mediated by macrophages and neutrophils and to antibodies oponization [6, 11, 146]. Alginate promotes *P. aeruginosa* encapsulation and biofilm formation protecting sessile bacteria from the action of ROS, antibiotics and host immune defences persisting in CF lungs [39, 40]. In particular, alginate has been shown to retard the penetration of aminoglycosides antibiotics, for instance tobramycin, through *P. aeruginosa* biofilms achieving a minimum inhibitory concentration (MIC) 50 times higher than the planktonic bacteria [14, 147]. The presence of biofilms is thus a key factor for the persistence of infection in CF airways [148]. Biofilm cell differentiation contributes to the generation of higher diversity that consequently increases the ability of *P. aeruginosa* to colonize new niches in CF airways, and thus perpetuating infection [44, 90, 92, 113].

Because mucoid *P. aeruginosa* raise a vigorous antibody response, its presence contributes to tissue damages, decreased lung function and a decline in health [85, 86]. The genetic mechanisms underlying *P. aeruginosa* transition to the mucoid form have been intensively studied and conversion is mainly caused by mutational inactivation of the *mucA* gene and more rarely of *mucB* or *mucD* genes [149, 150]. *mucA* gene encodes a cytoplasmatic membrane bound protein that acts as anti-σ-factor, σ^{22}, limiting the expression of the *algD* operon required for alginate synthesis. MucA binds to AlgT (also termed AlgU) that negatively controls the transcription of the *algD* gene. Inactivation of *mucA* results in upregulation of AlgT and production of alginate [5, 151, 152]. It is believed that the constant oxidative stress found in the CF airways induces the *mucA* gene mutations [39, 153]. In fact, σ^{22} can also activate the transcription of several other genes related to virulence factors expression and to stress response, including heat shock, osmotic and oxidative stress [38, 154]. Additionally, it can repress the expression of T3SS genes through activation of AlgU that in turn activates the regulatory genes *algP*, *algQ*, *algB*, and *algR*. AlgR, a global regulator, affects the expression of multiple genes including T3SS [155]. This suggests an impressive coordination of two high-cost energy systems in order to bacteria persist in CF airways.

Although mucoid phenotype is very successful at chronic infection stage, non- and mucoid phenotypes can coexist [74]. Non-mucoid isolates can occur due to persistence of *P. aeruginosa* wild-type or re-

conversion of mucoid phenotypes (revertants). Mucoid phenotypes can revert to non-mucoid form in the absence of *in vitro* selective pressure or through secondary mutations. Non-mucoid phenotypes can carry as well *mucA* mutation suggesting that mutation occurred when selective pressure occurs and when it vanished secondary mutation takes place [38, 156]. This suggests that the production of alginate represents high-energy costs and thus its unstable feature. At this stage, non-mucoid phenotypes revertants have its alginate production at minimal levels [104].

Several other mutations may appear in CF chronic isolates, mainly in genes associated with acute toxicity, for instance lipopolysaccharide biosynthesis, twitching motility, regulation of exotoxin A, pyoverdine synthesis, and QS factors [143]. Another *P. aeruginosa* variant frequently isolated from chronic CF lung infections and clinically relevant is the SCV, so designated because of their small-colony size, typically 1-3 mm after 24-48 h of growth on agar media [157]. SCV are normally hyperpiliated, hyperadherent, excellent biofilm formers and exhibit autoaggregative behaviour and increased twitching motility [158, 159, 160, 161]. In addition, SCV display augmented resistance to several classes of antibiotics, notably to aminoglycosides, contributing to *P. aeruginosa* persistence in CF airways and poorer lung function. SCV are generally selected after prolonged antibiotic treatments [157, 162]. In contrast with mucoid phenotype, the mutations that arise in SCV appear to be very diverse and a challenge for understanding the underlying molecular mechanisms [163]. This phenotype may arise from the increased expression of the *pel* and *psl* polysaccharide gene loci and elevated intracellular c-di-GMP levels that enhance the ability to form biofilms, motility and the expression of the T3SS persisting thus more efficiently in the CF airways [161, 163]. Until now, SCV were mostly studied regarding *S. aureus* but currently it has been equally assumed that *P. aeruginosa* SCV is as well a cause for infection persistence [77, 164, 165, 166].

Other colony morphologies have been isolated from CF airways typically exhibiting rough texture due to alteration of the lipid A moiety of LPS. Those variants contain few, short, or no O side chains and exhibit augmented proinflammatory activity [40]. Alterations in the Toll-like receptor ligand reduce the affinity of *P. aeruginosa* lipid A for polymyxin and for cationic antimicrobial peptides improving *P. aeruginosa* persistence in CF lungs [167, 168].

In chronic infections, *P. aeruginosa* lives in biofilm-growth mode as abovementioned in which cells downregulate flagellum and type IV pili since they are no longer needed to move across sputum and along epithelial cell surfaces [99]. Nonpiliation may arise from mutations of *pilB,* encoding an ATPase needed for the extension and retraction of pili, or defects in *pilQ* gene, required to extrude the pilus through the bacterial outer membrane [169]. Lacking flagella (e.g. *fliC* mutant), *P. aeruginosa* isolates are hardly phagocytosed by alveolar macrophages and neutrophils helping bacteria to evade the host immune defences and allowing its persistence in CF airways [40, 104]. Nevertheless, the majority of CF isolates exhibited *rpoN* mutations that provoke the loss of both pili and flagella [170]. Biofilm phenotypes reduce thus their immune recognition factors leading to diminished immune detection of these antigens. Through sputum and lung samples, it was observed neutrophils surrounding *P. aeruginosa* biofilms and rarely it is observed neutrophils inside of biofilms [39, 74]. Moreover, it has been shown that the lysis of spent neutrophils add viscosity and volume to biofilms because of the release of DNA and protein [171, 172].

Chronic CF isolates show other attenuated virulence factors such as reduced production of AHL, proteases, phospholipase C, loss of pyoverdine, pyocyanin, and elastase and decreased cytotoxicity potential, due to the switch off the T3SS [173]. These alterations also reduce the efficacy of the immune system to recognize *P. aeruginosa* helping thus its persistence in CF airways [40, 104]. This decrease in virulence potential may be associated with some downregulated genes such as *oprG*, *lasB*, *rsaL* and *lecB* [174].

Chronic *P. aeruginosa* isolates are commonly *lasR* mutants. *lasR* gene encodes QS transcriptional regulator LasR and its downregulation may explain the reduced or absent production of AHL at this infection stage, the autolysis and the iridescent gloss of *P. aeruginosa* colonies, the growth advantage on amino acids and decreased virulence potential [143, 175]. In addition, *lasR* mutants can use nitrate (NO_3^-) and nitrite (NO_2^-) as the terminal acceptor of electrons allowing *P. aeruginosa* growth in anaerobic niches and confering resistance against ciprofloxacin and tobramycin. The loss of social and cooperative behaviour may confer an adaptive advantage since the production of QS signal molecules, such as N-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), N-butanoyl-L-homoserine lactone (C4-HSL), 2-heptyl-4(1H)-quinolone (C7-HHQ), and 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS), are costly. Avoiding these costs, *P. aeruginosa* can ensure its persistence for long-term [104, 129]. QS seems just contribute to *P. aeruginosa* pathogenesis at colonization or acute stages [129, 176].

To survive and adapt to CF airways, *P. aeruginosa* has as well to adapt its metabolic pathways. In fact, those metabolic alterations are also considered a marker of the chronic stage. The generation of energy is mainly based on oxidative substrate catabolism, however *P. aeruginosa* is able to use alternative electron acceptors. The carbon metabolism of *P. aeruginosa* is mediated by catabolite repression control, which determines the catabolism of substrates in a preferred order. Short-chain fatty acids, amino acids and polyamines are generally the preferred carbon sources and sugars the less favoured [177]. CF sputum contains high amount of mucin, DNA, lipids, amino acids and proteins that *P. aeruginosa* can uptake. Several studies have reported that peptides, amino acids and fatty acids belonging to host defences, such as prostaglandins and phosphatidylcholine, supports *P. aeruginosa* growth in CF airways [13, 127]. The increased availability of those components is highlighted by the frequent isolation of auxotrophic variants for different amino acids, however the adaptive advantage of those variants in CF airways is unclear so far [178, 179]. Arginine and methionine are the most common auxotrophisms detected among CF isolates [179, 180, 181, 182]. Auxotrophic variants may be more common than actually reported because those variants may be less cultivable *in vitro* and consequently underestimated.

Another nutritional key factor for *P. aeruginosa* survival and persistence in CF lungs is the acquisition of iron. This bacterium uses several strategies to obtain iron, including siderophore mediated uptake, heme and ferrous iron uptake [183, 184].

As aforementioned, the distinct oxygen availability in CF sputum represents a challenge for *P. aeruginosa* that undergo metabolic and physiologic changes with high impact on antibiotic treatments. Along chronic infection progress, *P. aeruginosa* can face aerobic, microaerophilic and anaerobic zones within the CF sputum and different enzymes, transporters and regulators for different metabolic pathways are up-

regulated to achieve this adaptation and grow in these environments [60]. *P. aeruginosa* preferentially uses oxygen as terminal electron acceptor to obtain maximum energy. Under anaerobic conditions, *P. aeruginosa* can obtain energy to grow from denitrification or fermentation of arginine [11, 127]. Denitrification or anaerobic respiration allows detoxification of NO generated during infection development. The outer membrane protein OprF represents a crucial factor in anaerobic metabolism since it allows the permeation of the ions NO_3^-/NO_2^- fundamental to perform denitrification [11, 88, 127]. In niches where oxygen and N-oxides are unavailable but amino acids are in high amounts, *P. aeruginosa* can use fermentation of arginine converting it into ornithine [127]. In cases of arginine limitation, *P. aeruginosa* can still convert pyruvate into acetate and thus obtain energy. By this way, anaerobic biofilms can be formed and support *P. aeruginosa* survival, growth and persistence in CF airways. Anaerobic environments increase *P. aeruginosa* antibiotic tolerance and the robustness of biofilms through the increased production of alginate, typically via mutation in *algT*/*algU* [89, 185, 186, 187]. Consequently, CF mucoid strains, that are alginate producers, are selected at this chronic stage.

Despite all these findings about the metabolic pathways used by *P. aeruginosa* during chronic infections, it is still scarce the information about the regulation and the mechanisms underlying each metabolic pathway and the specific effects on virulence, antibiotic resistance and persistence in CF lungs. Certainly, the understanding of those mechanisms could help to new therapeutic solutions arise.

The presence of mutators within the populations is characteristic of chronic infections and considered a virulence determinant of *P. aeruginosa* often associated with parallel occurrence of subpopulations with distinct phenotypic characteristics [114]. Mutators ensure *P. aeruginosa* survival against various CF stress conditions and other unpredictable stress factors being, moreover, a key factor in the development of multi-antimicrobial resistance [121]. At chronic stage, hypermutability increases also due to the presence of biofilms in which the frequency of mutators is higher than the free-living mode of growth [45, 123].

Chronic infections are usually punctuated with worsening of symptoms (acute pulmonary exacerbations), in which *P. aeruginosa* may regain the increased levels of acute virulence of early stages, suggesting that the expression of some virulence factors can be reversible [113, 188]. It is believed that biofilm cells dispersion events are one of the underlying mechanisms of a pulmonary exacerbations [92, 113, 189]. Because the definition of exacerbation is under debate, some authors have proposed other implications such as altered antibiotic treatment [190] or alterations of bacterial load or the emergence of a virulent subpopulation [181, 191, 192]. The first hypothesis seems to be supported by other studies that demonstrated increased levels of *P. aeruginosa* exoenzyme S, exotoxin A, elastase, and alkaline protease during exacerbations episodes [193, 194]. In addition, high-doses of antibiotics are used to treat exacerbations decreasing the bacterial load and resulting in attenuation of the symptoms [188, 193, 195]. Besides improving lung function, antibiotic treatment is used to extend the period of time between acute exacerbations [50]. Because *P. aeruginosa* is the most common bacteria isolated from CF patients with exacerbations, in general, the choice of the antibiotic agent is an antipseudomonal agent [3]. So far, there

is not a successful antimicrobial regime able to totally eradicate *P. aeruginosa* in chronic infections, but it can be achieved one or two log reduction of bacterial load [57].

Despite the intensive and long antibiotic treatment, chronic infections of *P. aeruginosa* are rarely eradicated due to the occurrence of antibiotic resistance. It is frequently observed β-lactam-resistant *P. aeruginosa* phenotypes [196], as well as ciprofloxacin [197], colistin [198] and tobramycin resistant phenotypes and even multi-drug resistance [199]. The main reasons for such increased antibiotic resistance is the biofilm-growth style, the presence of mutators and increased production of resistance factors, for instance, the antibiotic efflux pumps (e.g. MexCD-OprJ) and the production of β-lactamases [121, 200, 201, 202, 203, 204, 205]. *P. aeruginosa* is equipped with a two-component regulatory system ParR-ParS that regulates resistance against, at least, four classes of antibiotics throughout activation of the efflux pump MexXY-OprM and modification of LPS operon and downregulation of oprD porin expression [59]. Mutations in *cbrA* gene coding a sensor kinase of the cbrAB two-component regulatory system conferred resistance against polymyxin B, ciprofloxacin and tobramycin. In addition, it enhances the ability to form biofilms [206]. CF isolates can accumulate mutations in antibiotic resistance genes that combined with biofilm antibiotic resistance mechanisms results in an impressive expression and activity against antibiotics.

In summary, the exhibition of certain characteristics, including alginate overproduction (mucoid phenotype), slow growth (SCV), alternative metabolic pathways, antibiotic resistance and loss of virulence factors expression, is currently considered a chronic phenotypic profile and the end-point result of *P. aeruginosa* evolution in CF airways. *P. aeruginosa* clearly adopts a strategy aiming to reduce its energy costs in favour of activation of other biological pathways that ensure its long-term persistence. The actual evolutionary "model" of *P. aeruginosa* within CF airways consists in an initial and rapid adaptation period dominated by positive selection and adaptive mutations, followed by a period with minor phenotypic changes dominated by negative selection and fewer adaptive mutations [5]. This evolutionary process ends with an advent of a lineage of highly adapted bacteria with impressive ability to persist in CF lungs for long-term. Despite the assumption of parallel evolution to CF-well adapted phenotypes and the limited number of adaptive features, it is important to highlight that the actual evolutionary route towards a common profile among different patients is still not well understood. In fact, genomic and transcriptomic studies have just begun tracking *P. aeruginosa* evolution.

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Chapter 2

Advances in detection of bacterial diversity using colony morphology characterisation

2

Advances in detection of bacterial diversity using colony morphology characterisation

Abstract

Colony morphology may be an indicator of clonal diversification of bacteria in CF lungs which alterations in the colony aspect may reflect increased pathogenic potential. Despite the relevance of using colony morphological characterisation to inspect the virulence and antimicrobial resistance of pathogens, there is great variability in experimental conditions, morphological criteria and vocabulary used to characterise bacterial colonies, which hampers accurate clinical diagnosis.

This chapter includes three distinct and complementary works that aimed to introduce some improvements in the colony morphology characterisation method in order to enhanced clinical diagnosis. First, it was intended to intensively study the impact of some experimental conditions, including colony growth time, number of colonies *per* plate, solid culture medium, bacterial mode of growth and genetic background on colony morphogenesis and on the detection of bacterial diversity. The results demonstrated that all parameters studied had impact on colony differentiation and on the detection of bacterial diversity. These results allowed to clearly understanding that morphologies just should be compared in identical plating conditions, otherwise the detected colony differentiation could be caused by *in vitro* conditions rather than bacterial adaptations in human host. Based on the results obtained, it was suggested some guidelines that should be followed in order to obtain the most accurate results regarding colony characterisation. The second study intended to construct a morphological characterisation system for bacterial colonies. The great variability of vocabulary, concepts and morphological criteria currently used by authors prompted the construction of a system that might assist clinical and research laboratories to perform accurately colony morphology characterisation. It is believed that this system will facilitate the comparison of morphologies across samples, strains, species, experiments and laboratories that significantly will advance the knowledge about infection and improved antimicrobial stewardship. In the last study, it was aimed to introduce speed and high-throughput to colony morphology characterisation method exploring MALDI-TOF MS as a tool for bacterial colony morphotyping. MALDI-TOF MS provided distinct colony classification in comparison with manual morphotyping. The differences between these two methods could arise from molecular alterations not perceptible by macroscopic observation of colony morphologies. These results may suggest that MALDI-TOF MS could, in some extent, be used as a significant complementary tool for colony morphotyping.

2.1. Introduction

A timely diagnosis is the most effective approach to prevent or control bacterial infections in CF disease. To perform a successful diagnosis it is required an accurate (sensitive and specific) and fast identification of the infecting pathogens [1]. The current issue on clinical diagnosis is the ability of generating not just taxonomic results, but also providing information about the pathogenic potential of the bacteria isolated from CF patients [2]. According to bacterial identification and characterisation, such as antibiotic susceptibility profile and virulence factors expression, the best antimicrobial approach is chosen to control or eradicate the bacterial infection. Therefore, rapid pathogen detection, identification and characterization are the gold standard for clinical diagnosis. Furthermore, bacterial identification and characterization is not only an issue of clinical diagnosis, but it also accounts for understanding the infection development and persistence within the host.

Over the years, important technological advances have occurred in bacterial identification that have provided a wide range of techniques to detect, identify and differentiate bacteria. Molecular methods, such as the enzyme-linked immunosorbent assay (ELISA), the polymerase chain reaction (PCR) and, the most promising one, matrix-assisted laser desorption/ionization time of flight combined with mass spectrometry (MALDI-TOF MS) have introduced improvements in bacterial identification as they contributed to speed up the analysis and the reduction of handling [1, 2]. Microbial infecting communities of CF lungs encompass a wide clonal heterogeneity that these recent methods are limited to detect as the bacterial identification and characterisation is performed based on detection of specific molecules. However, there are just few identified probable active molecules contributors to the activation of phenotypic diversification. These molecules are highly dependent on environmental stresses that bacteria has experienced, which seriously limit the ability to detect such increased molecular variability.

Isolation of specimens using bacteriological techniques, such as the culturing in non- and selective or differential media, is a routine procedure during clinical diagnosis [3]. Growing on agar surfaces, bacteria form colonies whose appearance helps the clinicians and researchers to identify genera or even species. This method is called as colony morphology characterisation and is defined by Moore; V.A. (1912) [4] as "The examination of plate cultures…determining the character of the different colonies, their action upon the medium, and the rapidity of their development...". Colony morphology characterisation relies thus on the description of the phenotypic traits exhibited not by an individual microorganism but by a group of microorganisms, the colonies. The colony observation is performed through naked eye examination or using a magnifying glass and characterised using morphological criteria, such as form, surface, size and colour.

Despite being described by several authors as an old-fashioned method [1, 2], colony morphology characterization is a useful diagnosis method. It can provide valuable insights into diversity of the infecting microbial population fundamental to design an effective antimicrobial treatment customized to a particular infecting population [5, 6, 7, 8, 9, 10]. Moreover, colony morphology characterization has undoubtedly advanced the knowledge about microbial adaptation, persistence and evolution concerning several human

infections and diseases. In CF airway disease *P. aeruginosa* adaptation was evident when it was spreading bacteria on agar plates and observed their different colony appearances over infection development, including iridescent colonies [11], mucoid colony variants [12, 13, 14], SCV and rough SCV [15, 16, 17, 18]. The different aspect of colonies can reflect differences in virulence [16, 19, 20, 21, 22], antimicrobial resistance [23, 24, 25, 26] and persistence [25, 27], features that impact the efficacy of antimicrobial agents and the response of bacteria towards them. This means that any modification in colony morphology traits, such as colour, opacity, size and texture, may be a sign of altered expression of one or more bacterial traits important to antimicrobial stewardship. The correlation between pathogenic features, such as antimicrobial resistance, virulence factors expression, persistence ability, and colony morphologies is just almost unknown though extremely important. The scant knowledge regarding those biological/morphological relationships is partly due to the fact that colony morphotyping has some inherent disadvantages of being a culture-based method, but also because of other barriers related to the lack of standard experimental conditions, universal morphological criteria, unequivocal and common concepts and terms to perform colony morphology examination. Therefore, conceptual and practical advances in this method are urgent, considering its importance in the generation of comprehensive knowledge on microbial adaptation, evolution and infection development and, most important, in helping to design effective antimicrobial treatments for bacterial eradication from CF lungs.

This chapter presents and discusses some improvements performed in the scope of the detection of bacterial diversity using the colony morphology characterisation method. It was aimed to overcome some technical limitations, in particular to reduce handling time, to establish more accurate colony morphology procedures and to standardise colony morphology data. Therefore, in a first stage, the influence of some important experimental parameters on colony morphology development and diversity detection was extensively studied. Afterwards, vocabulary, concepts and morphological criteria about bacterial colonies was compiled to construct, as far as it is known, the first renewed colony morphology characterisation system since the first publication of colony terms. Finally, the ability of the MALDI-TOF MS to discriminate distinct colony morphologies was assessed in order to verify the possibility of introducing some speed and high-throughput to colony morphology characterisation method.

2.2. Improvements on colony morphology identification towards bacterial profiling

2.2.1. Objective

Apart from obvious morphological differences among bacterial colonies that have been reported, the plating conditions in which evaluation has been performed were different among published works. This variety is especially significant in what concerns the medium used and the growth time allowed for colony development. The continuing reliance of clinical diagnosis on colony morphology characterisation demands understanding the role of plating conditions in colony morphogenesis and their impact in the detection of bacterial diversity. To obtain new insights about this issue, two *P. aeruginosa* strains (a reference strain and a clinical isolate) were used to perform a detailed evaluation of colony morphologies along time testing different colony growth times, number of colonies *per* plate, culture media and mode of growth, including planktonic and biofilm lifestyle. The goal was at determining the impact of these experimental conditions on each colony morphological features, including form, margin, texture, size and colour, in a systematic way.

2.2.2. Materials and Methods

Bacterial strains and culture conditions

P. aeruginosa ATCC 10145 and a clinical isolated from medical equipment (from now on referred as PAI1) were used throughout this study. Bacteria were routinely cultured on tryptic soy broth (TSB) or agar (TSA) medium at 37 ºC. All strains were preserved in criovials (Nalgene) at – 80 ± 2 ºC. Prior to each experiment, bacterial cells were grown on TSA plates for 24 h at 37 ºC. The use of a reference strain and a PAI1 ensured the different genetic background.

Planktonic cultures

Planktonic bacteria grew overnight in TSB at 37 ºC and 120 rpm. Cell suspension of each strain was washed twice in phosphate buffered saline solution (PBS) by centrifugation (9000 *g*, 5 min) and further serial diluted and plated on solid media.

Biofilm formation

Biofilms were developed as previously described [28]. Briefly, bacteria were grown overnight on TSB at 37 ºC in air conditions. Cell suspension of each strain was diluted in TSB to obtain 10^7 CFU/mL as final concentration. Afterwards, the bacterial suspension was transferred to a 96-well polystyrene microtiter plate where biofilms were developed aerobically on a horizontal shaker (120 rpm) at 37 ºC for 24 h. After

that, biofilms were sonicated into PBS and vortexed to homogenize. Finally biofilm-cells were serial diluted with PBS and spread plated on solid media.

Observation and classification of colony morphology.

To assess the impact of the solid media composition on colony morphology features, bacteria were serial diluted and plated on different solid media in air conditions, including TSA (15 g/L, Liofilchem), *Pseudomonas* isolation agar (PIA, 45 g/L, Fluka) and Cetrimide (CET, 45,5 g/L, Merck) plus 10 mL/L of glycerol, at 37 ºC. It was observed that the amount of solid medium in the plate had impact on colony morphogenesis. However, this factor was not included in this study and the amount of solid media could be variable. To evaluate the influence of the lifestyle of the bacteria on their colony morphology, bacteria coming from biofilms and planktonic cultures were used. Plates with different colony numbers were observed to infer about the role of colony density *per* plate on morphology differentiation. Finally, different times of growth were used to assess the effect of growth time: after 15, 24, 30, 45 and 50 h of incubation. Colonies were observed by directly placing the petri plates under a magnifying glass (Olympus SZ-CTV) and recorded with a CCD camera (AVC, D5CE; Sony, Tokio, Japan). The identification and characterisation of colony morphotypes were carried out using five parameters: colony size, form, colour, texture and margin, according to Table 2.1. A phenotypic variant was considered when it differed in, at least, one of the referred morphological parameters. All experiments were performed 5 times.

Table 2.1. Morphological features used to characterize *P. aeruginosa* colony morphologies.

Class	Sub-class
Colony Form	Circular Irregular
Colony Margin	Entire Irregular
Colony Texture	Smooth Rough Wrinkled
Colony Size[a]	Small Large
Colony Colour	White Brown Yellow Green

[a] colonies were considered small if presented diameter below 3 mm and large if presented diameter above 3 mm [15].

2.2.3. Results

To describe each colony, regarding the distinct morphological characteristics, the following terms were used as synonymous: colony morphology, colony type, colony variant and morphotype, all meaning a group of bacteria grown from a single cell on agar surface exhibiting a typical colonial pattern. It must be remarked for further studies that this definition does not exclude the possibility that different strains or species exhibit the same morphotype or that a strain or species exhibits more than one morphotype. All morphotypes presented were observed at least in 3 of the 5 replicates.

Effects of growth time on colony morphogenesis

Planktonic and biofilm bacteria were cultured on solid media plates and colonies were examined after 15, 24, 30, 45 and 50 h of growth at 37 ºC. These intervals of time allowed observing the sequential stages of colony morphological features during growth on the different solid media (data shown just addressing TSA plates, Figure 2.1, 2.2, 2.3 and 2.4). After 15 h of growth, heterogeneity among colony morphologies was only incipient. Between 15 and 45 h of growth, colonies changed their appearance (intermediate morphologies) could ending in totally different morphotypes. Similar 15 h colonies may still end in similar colonies as it happened with the planktonic *P. aeruginosa* ATCC (Figure 2.1) or ended in completely distinct colony morphotypes as occurred in the biofilm-variants of ATCC (Figure 2.3) and planktonic and biofilm variants of PAI1 (Figure 2.4A – 2.4H). An interesting result was that, independently of the strain or the type of morphotype, after 45 h, no morphological changes were observed, apart from the increase of the colony size, meaning that at 45 h the colony morphogenesis was complete and all morphological traits were well defined. This evidence was verified in all solid media tested (TSA, PIA and CET). Therefore, further analyses of colony morphology were performed considering the traits exhibited at 45 h of growth.

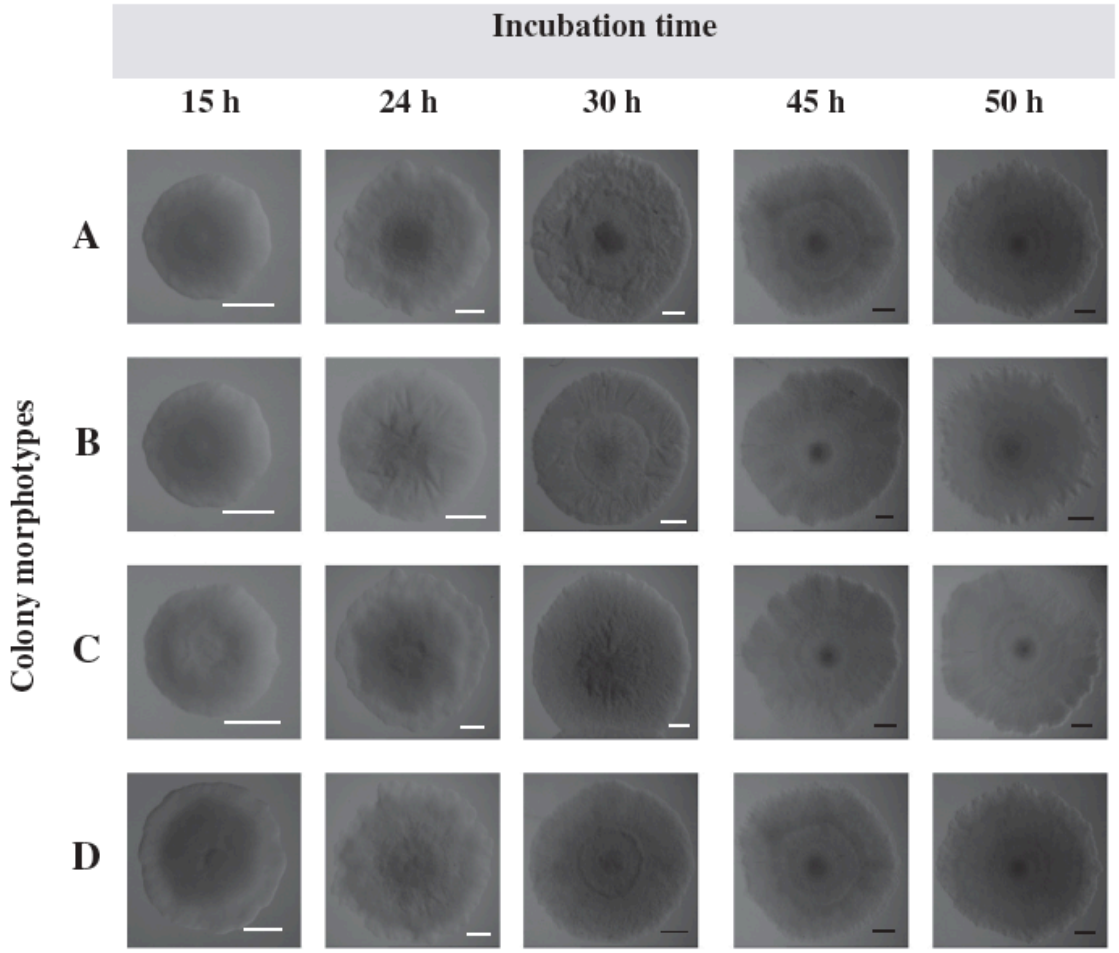

Figure 2.1. Morphological evolution of planktonic *P. aeruginosa* ATCC 10145 colonies observed on TSA. Rows A to D represent the colony morphology development of the variants identified after 15 h of growth. Morphotypes of the rows A and B at 15, 45 and 50 h of incubation were the same variant, however they showed distinct intermediate morphological stages over time (at 24 and 30 h). All morphotypes were observed at least 3 times of the 5 performed. White bars = 0,5 mm; black bars = 1 mm.

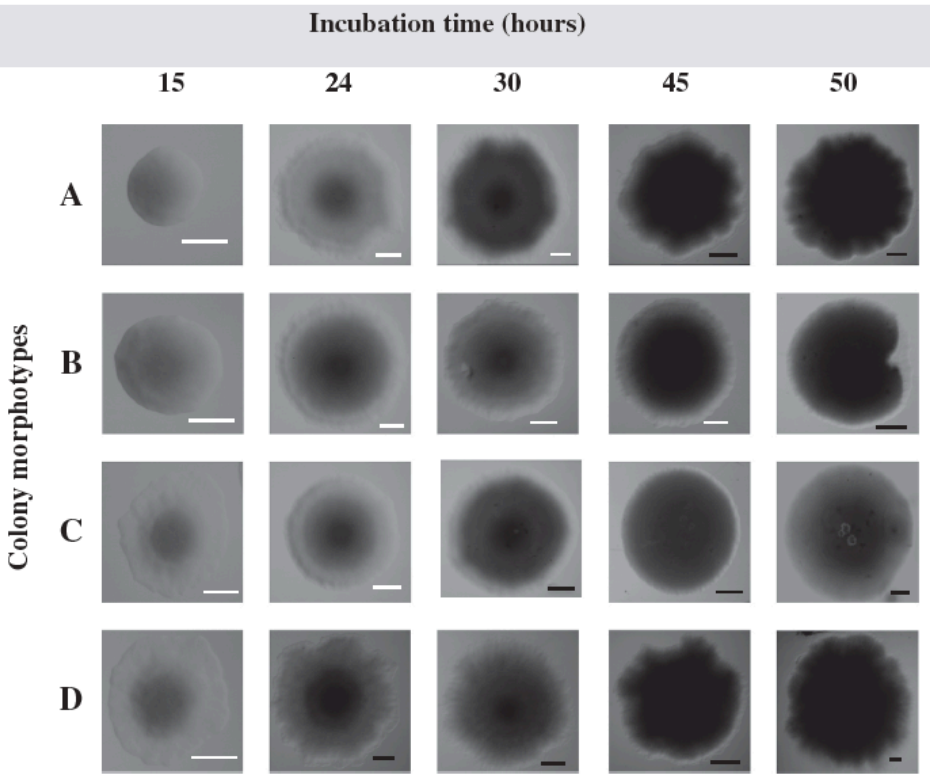

Figure 2.2. Morphological evolution of planktonic *P. aeruginosa* PAI1 colonies observed on TSA. Rows A to D represent the colony morphotypes development of the variants identified after 15 h of growth. Morphotypes of the rows A/B and C/D at 15 h of incubation were the same variant, but they showed distinct intermediate morphological stages (at 24 and 30 h) ending into different variants, B=C and A=D. All morphotypes were observed at least 3 times of the 5 performed. White bars = 0,5 mm; black bars = 1 mm.

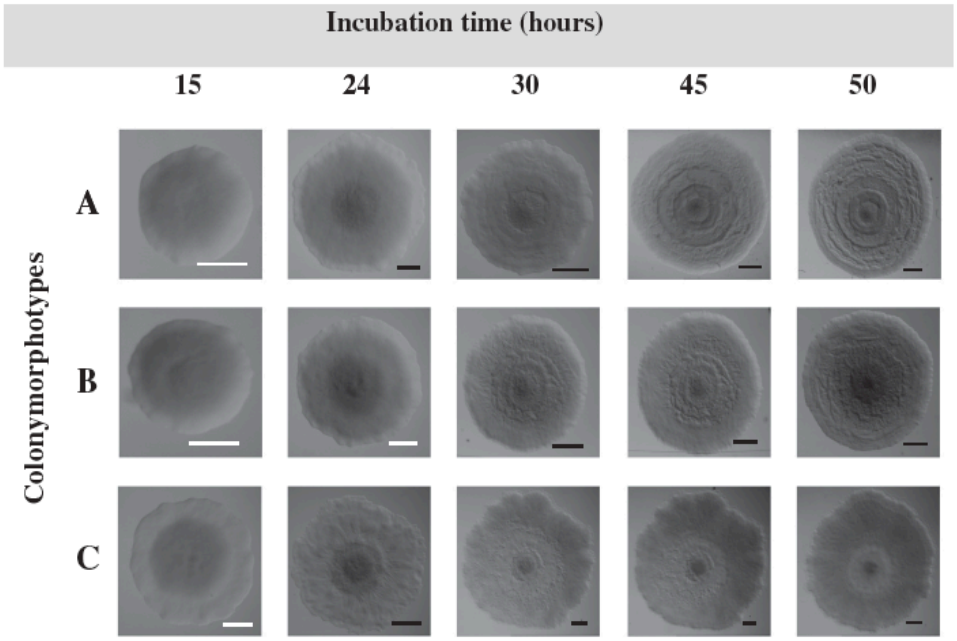

Figure 2.3. Morphological evolution of colonies derived from *P. aeruginosa* ATCC 10145 biofilms. Rows A to C represent the colony morphotypes development of the variants identified after 15 h of growth. Morphotypes of the rows A and B at 15 h of incubation were the same variant, but they showed over time distinct intermediate morphological stages (at 24 and 30 h) and ended as different variants (columns 45 and 50 h). All morphotypes were observed at least 3 times of the 5 performed. White bars = 0,5 mm; black bars = 1 mm.

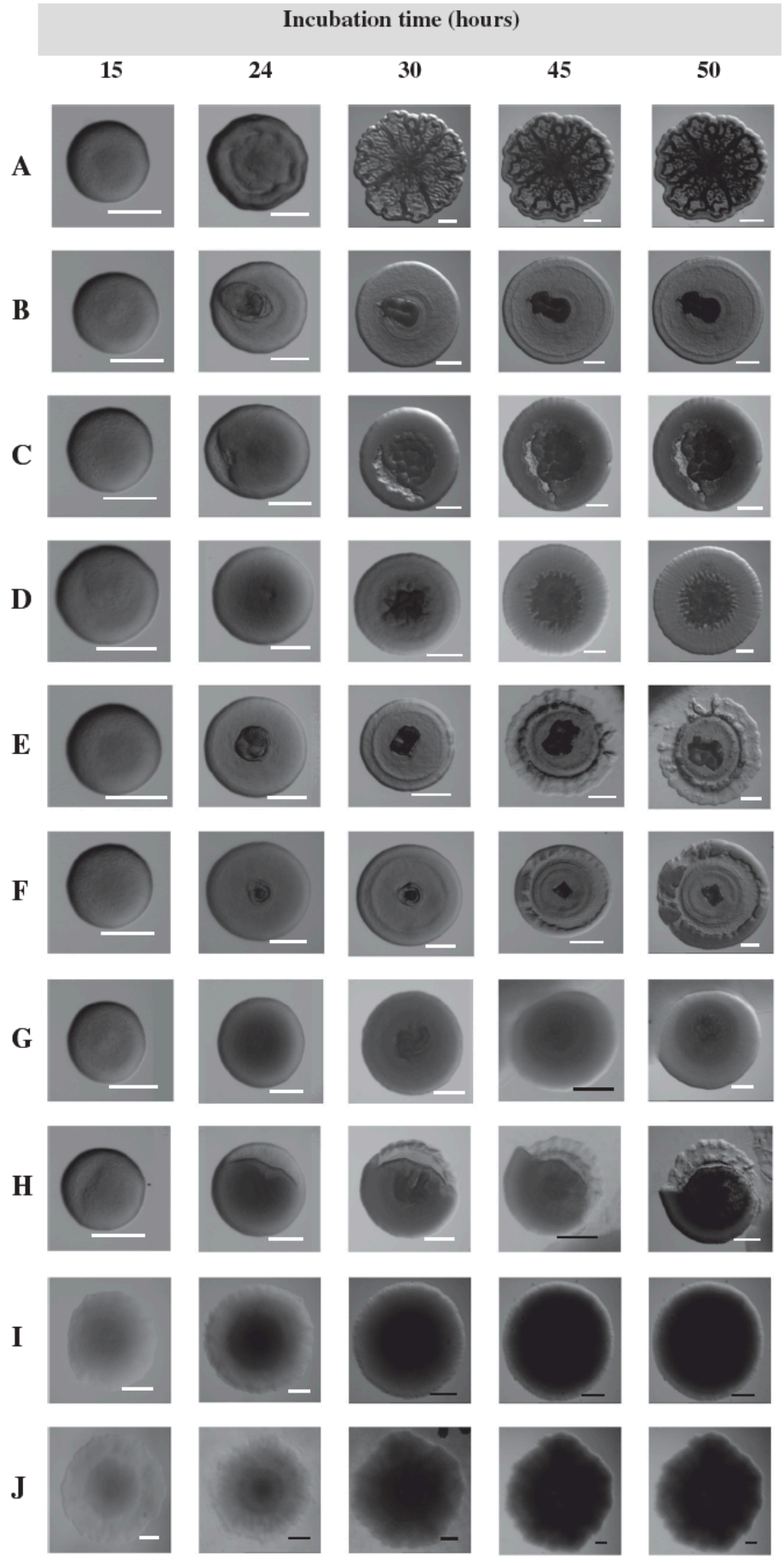
Incubation time (hours)
15
24
30
45
50
A
B
C
D
E
F
G
H
I
J

Figure 2.4. Morphological evolution colonies derived from *P. aeruginosa* PAI1 biofilms. Rows A to J represent the colony morphotypes development of the variants identified after 15 h of growth. Morphotypes of the rows A to H at 15 h of incubation were the same variant, but they showed distinct intermediate morphological stages (at 24 and 30 h) ending into different variants. At 45 h a total 10 morphotypes were identified: the rows A to H were considered 8 distinct SCV (diameter below 3 mm) and I and J were considered 2 distinct non-SCV. All morphotypes were observed at least 3 times of the 5 performed. White bars = 0,5 mm; black bars = 1 mm.

Effects of plate colony density on colony morphogenesis

During colony evaluation, it was observed that colonies growing close to each other altered their development (Figure 2.5). A colony density higher than 20 colonies *per* agar plate resulted in close neighbouring growth and thus in limited colony formation when compared with more distant or isolated colonies. For instance, distant colonies of planktonic *P. aeruginosa* PAI1 that exhibited circular form, undulate margin, rough texture and large size (Figure 2.5C), when grown in plates with 20 to 100 colonies exhibited circular form, irregular margin, rough texture and small size (Figure 2.5B) and in plates with more than 100 colonies exhibited undistinguished form and margin, rough texture and undetermined size (Figure 2.5A). To ensure unbiased data, morphology characterization was performed in plates with a maximum of 15 colonies.

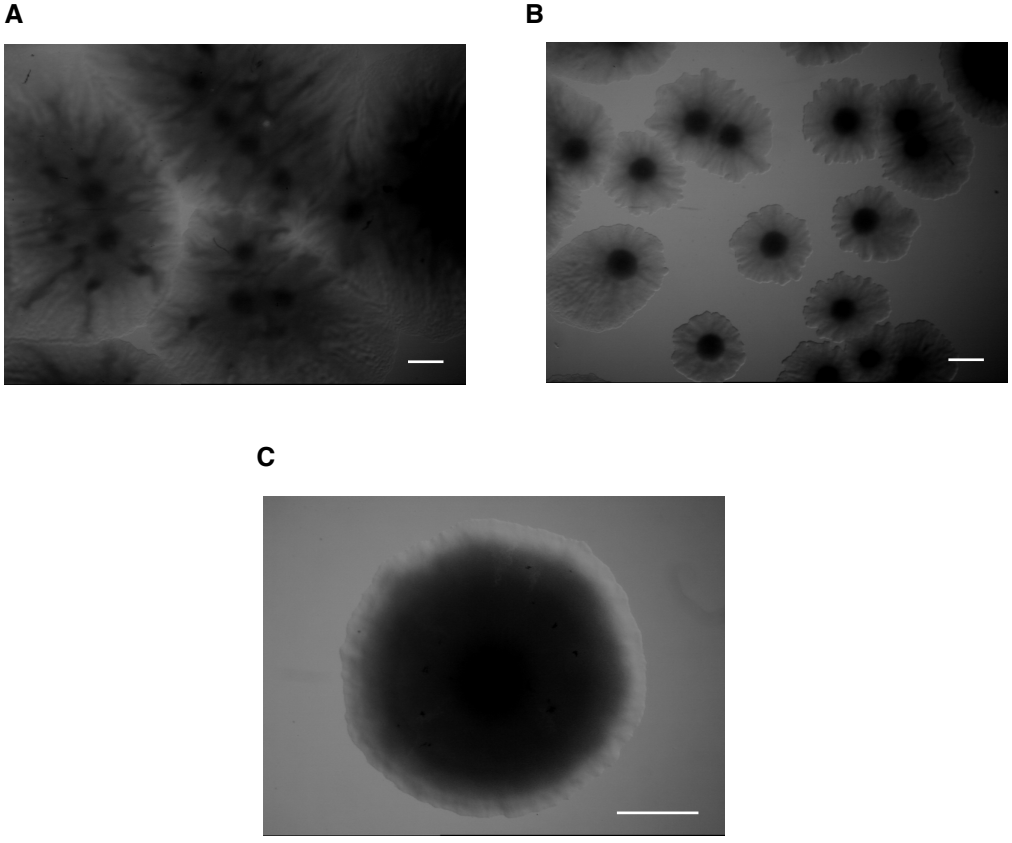

Figure 2.5. Colony morphologies of planktonic *P. aeruginosa* PAI1 after 30 h of growth on plates with: (a) more than 100 colonies; colonies exhibited undistinguished form and margin due to high colonies density on the plate, rough texture and undetermined size; (b) approximately 30 colonies; colonies exhibited circular form, irregular margin, rough texture and small size; (c) 20 or less colonies; colonies exhibited circular form, undulate margin, rough texture and large size, distinct morphology in contrast to (a) and (b) These experiments were performed 5 times. White bars = 1 mm.

Effects of culture medium composition on colony morphogenesis and diversity detection

Planktonic and biofilm-associated *P. aeruginosa* were plated onto different solid media (TSA, PIA and CET) in order to assess the role of nutritional composition of solid media on colony morphology definition and detection of bacterial diversity. The results showed that solid media clearly influenced colony morphogenesis and variants detection. Both *P. aeruginosa* strains, either from planktonic and biofilm cultures, when plated on TSA, PIA and CET exhibited distinct colony morphologies according the media used (Table 2.2). It was verified that the features margin, texture and colour were the most affected. The most relevant result was the high diversity of morphotypes detected in TSA in comparison with PIA and CET for both *P. aeruginosa* strains. This result had particular expression regarding morphotypes obtained from biofilms.

Impact of the mode of growth on colony morphogenesis and diversity

Based on the fact that biofilm formation is usually originated by planktonic cells, it was considered of utmost importance to identify the colony morphotypes of the initial population that originated the biofilms.

As TSA was the medium where higher colony diversity was observed, thus the data analysis was just focused on morphotypes observed on TSA. The most relevant result was the high diversity of morphotypes obtained from the biofilm cultures of both strains, namely the PAI1 that originated 5-fold more colony morphotypes when plated onto TSA than the reference strain. ATCC biofilms encompassed three distinct large and circular colony morphologies: a wrinkled and concentric variant with an entire margin (Figure 2.3A, column 45h); a rough colony variant with irregular margin (Figure 2.3B, column 45h); and the third variant (Figure 2.3C, column 45h) similar to the planktonic counterpart (Figure 2.1, column 45h). In contrast, the planktonic reference strain gave rise to a homogeneous population composed just by large and circular colonies, with a small marked centre, irregular margin and with a sheath (Figure 2.1, column 45 h). The surface showed to be predominantly rough with some small wrinkled zones. These colonies were yellow when plated on TSA.

Much higher diversity was detected from the biofilm-associated bacteria developed by the PAI1. Eight distinct SCV, colonies with less than 3 mm of diameter (Figure 2.4A – H, column 45h), and two larger colony variants were observed (Figure 2.4I and 2.4J, column 45h). In turn, the planktonic PAI1 originated a heterogeneous population just composed by two large yellowish colony variants: smooth with entire margins (Figure 2.2B and 2.2C, column 45h), and by smooth variants with irregular margins (Figure 2.2A and 2.2D, column 45h). The observation of new colony variants in the case of biofilm-associated bacteria, suggests that a fraction of the planktonic cells changed phenotypically when growing as a biofilm.

Table 2.2. Characteristics of the distinct colony morphologies generated by the reference and the clinical strain of *P. aeruginosa* on different solid media (TSA, CET and PIA) grew as planktonic cultures and biofilms.

		Media	Form	Margin	Texture[a]	Size[b]	Colour
Planktonic	*P. aeruginosa* ATCC 10145						
	Morphotype I	TSA	circular	irregular	rough and wrinkled	large	yellow
	Morphotype II	PIA	circular	irregular	wrinkled and rough	large	green
	Morphotype III	CET	circular	irregular	rough	large	green
	P. aeruginosa PAI1						
	Morphotype IV	TSA	circular	entire	smooth	large	yellow
	Morphotype V	TSA	circular	irregular	smooth	large	yellow
	Morphotype III	PIA	circular	irregular	rough	large	green
	Morphotype VI	CET	irregular	irregular	rough and smooth	large	green
Biofilm	*P. aeruginosa* ATCC 10145						
	Morphotype I	TSA	circular	irregular	rough and wrinkled	large	yellow
	Morphotype VII	TSA	circular	entire	wrinkled	large	yellow
	Morphotype VIII	TSA	circular	irregular	rough	large	yellow
	Morphotype II	PIA	circular	irregular	wrinkled and rough	large	green
	Morphotype IX	PIA	circular	entire	wrinkled and rough	small	green
	Morphotype III	CET	circular	irregular	rough	large	green
	Morphotype X	CET	circular	entire	rough	small	green
	P. aeruginosa PAI1						
	Morphotype IV	TSA	circular	entire	smooth	large	yellow
	Morphotype V	TSA	circular	irregular	smooth	large	yellow
	Morphotype XI	TSA	irregular	irregular	wrinkled	small	yellow
	Morphotype XII	TSA	circular	entire	Rough and wrinkled	small	yellow
	Morphotype XIII	TSA	circular	entire	smooth and wrinkled	small	yellow
	Morphotype XIV	TSA	circular	irregular	smooth and wrinkled	small	yellow
	Morphotype XV	TSA	circular	irregular	wrinkled	small	yellow
	Morphotype XVI	TSA	circular	irregular	rough and wrinkled	small	yellow
	Morphotype XVII	TSA	circular	entire	smooth	small	yellow
	Morphotype XVIII	TSA	circular	entire and irregular	smooth and wrinkled	small	yellow
	Morphotype III	PIA	circular	irregular	rough	large	green
	Morphotype IX	PIA	circular	entire	wrinkled and rough	small	green
	Morphotype VI	CET	irregular	irregular	rough and smooth	large	green
	Morphotype X	CET	circular	entire	rough	small	green

[a] texture should be described from out to inside; morphotypes with same types of texture but in different zones was considered as distinct colony variants.
[b] colonies were considered small if presented diameter below 3 mm and large if presented diameter above 3 mm.

Effect of genetic background on colony morphogenesis

Despite belonging to the same species, the genetic background exerted significant influence on the definition of colony traits as showed on Table 2.2. Colonies of both strains in the same experimental conditions shared some characteristics and differed in others. For instance, planktonic cultures resulted mostly in colonies that shared the large dimension and the circular form, but were clearly different in other traits, such as the texture and the margin. Another interesting result was the different ability of the two strains to generate colony variants: the PAI1 originating 2- and 5-fold more colony morphotypes- planktonic and -biofilm associated when plated onto TSA than the reference strain.

2.2.4. Discussion

The recognition of typical colony morphologies is crucial for clinical diagnosis. Scientific and clinical laboratories frequently use the colony morphology displayed by bacteria on agar media as an auxiliary means to identify bacterial species because of their different and specific growth patterns. Despite the technological advances, colony morphology characterisation is still used for taxonomic purposes and, most important, to obtain information about the microbiological effect of the antibiotic courses, in particular, to determine the occurrence of phenotypic selection [2, 7, 31].

Colony morphology characterisation is gaining attention because it is thought that changes in morphology could be the expression of bacterial adaptation to different environments and thus hampering the bacteria identification based on morphological traits. Distinct colony morphotypes may arise and their erroneous identification and/or characterization may significantly influence the clinical diagnosis.

The actual interpretation of the function of colony morphotypes isolated from CF samples or identified *in vitro* experiments is mainly based on results comparison among reports. A detailed analysis of methodological procedures of these reports revealed that experimental conditions are highly variable among reports. For instance, *P. aeruginosa* colonies have been characterized and compared after being grown on different agar media, and also with different medium supplements, as well as after different growth times [16, 29, 30]. This has also happened with other species such as *S. aureus* [31, 32], *Streptococcus pneumoniae* [33, 34] and *Enterococcus faecalis* [9, 35]. The gain of knowledge about the mechanisms involved in bacterial adaptation and survival in CF context and in other infections and diseases, such as clonal diversification, biofilm resistance, bacterial persistence and other biological processes may be compromised since the influence of experimental conditions in colony morphology definition and detection is still unknown. The present study showed the impact of experimental parameters, including colony growth time, number of colonies *per* plate, solid medium, bacterial lifestyle and genetic background on morphological features of *P. aeruginosa* colonies.

Regarding the bacterial lifestyle, it seemed important to study the impact of the planktonic and biofilm mode of growth on colony morphology. Biofilm formation is a relevant strategy used by *P. aeruginosa* to introduce bacterial diversity in order to face the stressful CF conditions [36, 37, 38]. The switch of mode of growth from planktonic to biofilm implies cellular alterations and it was hypothesised that they could be

observed in colony morphology variation. Biofilms encompass a wide range of microniches with specific biological activities that may somewhat translate the well-known biofilm heterogeneity. Stewart and Franklin (2008) [39] reported that in a mature biofilm at least three distinct physiological states can be anticipated: cells near the biofilm-bulk-fluid or in the more superficial layer, presenting similarities with planktonic cells; cells in the middle zone; and cells in the deeper zone. In fact, the present results demonstrated the potential of colony morphology characterization to discriminate the biofilm population diversity since several colony morphotypes were isolated from biofilms (Figure 2.3 and 2.4).

The monitoring of the colony development over time demonstrated that, in case of *P. aeruginosa* colonies, morphological characterization should just be performed after, at least, 45 h of colony growth. Characteristic morphological features remained unchanged only after 45 h of growth for all tested solid media (Figure 2.1, 2.2, 2.3 and 2.4), and by this reason the assessment of *P. aeruginosa* colonies with less than 45 h of growth led to inaccurate characterizations and further misinterpretations. Several studies addressed observations of *P. aeruginosa* colonies with less than 45 h, which may raise the question whether the authors would achieve the same conclusions observing older colonies [30, 40]. The diversity of SCV observed and described in the present study (Figure 2.4A – 2.4H) supports, as well, the need of colony characterization just when colonies reached the definitive and unchanged state. In literature, no studies reported high SCV heterogeneity besides the rough SCV possibly due to limited colony growth time allowed [40, 41, 42]. The series of SCV presented in this study may play a relevant role on biofilms resistance or persistence against environmental stressors and antimicrobial agents. However, this role is unknown until now, possibly due to the improper colony characterization.

Based on the results disclosed in the present study, a previous study of colony development before the main biological study is recommended in order to prevent the possible loss of data about colony morphology. In alternative to a prior colony development study, prolonged time of growth should be allowed and several subsequent colony observations should be performed until verification of unchangeable traits (with exception of size). This last approach is most adequate for hospital laboratories that generally do not have time for prior studies. Both approaches would allow determining the duration of complete colony morphogenesis and no morphotypes would be overlooked and mischaracterized. The detection of variants with slower growth rates is, for instance, particularly important for patients who are receiving antibiotic treatment. Morphotypes as SCV may be not detected due to their slow growth and/or overgrown and higher prevalence of other morphotypes as mucoid variant that can conceal SCV. Inter- and intra-species diversity of CF populations can be thus overlooked which represent a serious clinical issue.

Another relevant factor when CF or other clinical samples are processing and plating in solid media, is the bacterial concentration of the sample. It is imperative the serial dilution of samples prior to plating on non- and selective media to facilitate detection and quantification of bacteria. Several reports exhibit images of colonies with various colonies surrounding each other [17, 43, 44]. The present data showed that distance between colonies, an issue frequently ignored, is important. Neighbouring colonies might limit or alter morphogenesis possibly due to competition for nutritional resources or bacterial signalling and

communication. Be'er *et al.* (2009) [45] reported that sibling colonies decelerate or even stop their growth when facing each other and stated that the production and perception of small signalling molecules may influence colony morphogenesis, gene expression and cell differentiation. Therefore, for colony morphology characterisation intermediate and low concentrations are preferred for obtaining properly separated colonies.

The solid media used to grow bacterial colonies is also a condition of great discrepancy among reports. Regarding *P. aeruginosa* colonies, media such as TSA, LB agar, PIA and blood agar have been used to observe colony morphology variation [16, 29, 46]. Selective media such as PIA and CET are very useful when clinicians suspect of *P. aeruginosa* infection. The selective media inhibit the growth of non-*Pseudomonas* pathogens and facilitate the recovery of *P. aeruginosa* if it is present in the CF sample. Currently, there are insufficient evidences to recommend a particular type of medium to plate CF samples for better *P. aeruginosa* detection. Therefore, it is recommended to plate CF samples in both non- and selective media [47]. In this study, both non- and selective media were used.

It is well known that solid media composition influence fungal colony morphogenesis [48], but concerning bacterial colonies, it was just demonstrated some colony morphology dependency on nutritional and agar concentration in the scope of colony pattern modelling studies [49, 50, 51]. More recently, it was reported that mucoid morphotype detection might be variable among solid media [52]. However, the real impact of solid medium variations on other morphological characteristics, including size, form, colour, texture and margin, has not been explored. The preservation of morphological features among solid media is critical in clinical diagnosis. The inconsistent detection of certain morphotypes such as SCV or mucoid variant makes the diagnosis unsure, leading to inaccurate antimicrobial treatment that may cause antimicrobial resistance in bacteria and contribute for infection persistence. Thus, the question of whether the detection of colony morphotypes is affected by the solid media used or not, i.e., dependent of nutritional composition of media, is of critical importance. Results obtained showed that the composition of the solid media used to plate *P. aeruginosa*, either coming from planktonic or biofilm cultures, is relevant to colony morphology definition. The effect of medium composition in colony patterns was clearly evident in traits as margin, colour and texture (Table 2.2). In addition, the present results evidenced that bacteria spread onto non-selective medium as TSA generate more colony morphology diversity, in contrast with *Pseudomonas* selective agar such as PIA and CET. For instance, the expression of SCV morphotype from PAI1 biofilm-cells on TSA were higher (eight SCV were detected), in contrast with just one morphotype on PIA and CET. This is maybe explained by the presence of irgasan in PIA and nalidixic acid and cetrimide in CET that may inhibit the growth of some colony variants [53]. The perception of colony morphologies dependence of nutrients concentration challenges the traditional morphology-based methods to detect bacterial diversity and may affect the actual performance of clinical diagnosis culture dependent approaches. The results here obtained provided data for international centres such as those existent for CF disease to modify their guidelines, and expressly recommended to plate CF samples in both non- and selective media for detection diversity purposes. Furthermore, research laboratories should as well

consider which media is more adequate to obtain results, in particular, if they are testing hypothesis against other published studies. Comparisons should be performed in similar conditions.

Considering all the issues previously discussed, a set of guidelines was proposed for authors, clinicians and technicians to implement when performing colony morphology characterization and further comparing results (Table 2.3). Scientific research and clinical diagnosis are the most benefited with similarity of the experimental procedures of the colony morphology method. This will lead to better comprehension of bacterial adaptation and evolution, purposes of fundamental science, with the ultimate goal of predicting antimicrobial resistance, expression of virulence factors and persistence ability based on morphological traits, relevant for supporting clinical diagnosis on bacterial profiling.

Table 2.3. Guidelines to accurately perform and compare results among bacterial colony morphologies observations.

General considerations	For studies that are testing hypothesis against others studies, the conditions of colony observations should be as similar as possible, including solid medium, colony growth time and sample concentration.
Solid media	Select the solid media that most alike the human sites from which bacteria were isolated in order to improve detection of bacterial diversity.
Height of solid media	The height of solid media *per* plate should be standardized and not less than 0,5 cm (approximately 15 mL in 90 cm plates).
Colony growth time	A prior study should be performed before colony morphology evaluation; colony growth time is established for the time from which all morphological traits are constant over the time (with exception of size). For hospital laboratories, it should be allowed prolonged time of colonial growth and performed several subsequent colony observations until verification of unchangeable traits (with exception of size).
Colony density per plate	Colony morphology observation should not be performed using plates with increased number of colonies; there is not a general threshold because colony size is highly depending of the bacterial species. For instance, *P. aeruginosa* colonies are typically large and observation should be performed in plates with less than 15 colonies; in contrast, *S. aureus* colonies are typically minor, and observation can be performed in plates with 30 to 40 colonies.
Bacterial strain	Some reports and manuals indicate typical morphological patterns for bacterial species, however different genetic background and biological phenomena as phenotypic switching may alter the considerate typical patterns of a bacterial species. Therefore, previous guidelines should be always performed.
Bacterial diversity detection	To avoid losing information about diversity, clinical samples should be plated into non-selective media. Plating in selective media should be performed only for detection of bacterial species in a sample.

2.3. Morphotyping of bacterial colonies using a renewed characterisation system

2.3.1. Objectives

Colony morphology is considered a method with great potential to detect bacterial diversity and to predict some bacterial features such as antimicrobial resistance and expression of virulence factors. Although the number of publications about this method has shown a substantial increase *per* year, the knowledge about the correlation between bacterial features and colony morphologies is inconsistent and unstructured. The main rationale of this work is the great variability of vocabulary, concepts and morphological criteria used by authors to describe colony morphologies. This inconsistency prompted the construction of a renewed universal characterisation system for bacterial colonies that might assist clinical and research laboratories to perform accurately colony morphology characterization. With the general use of this system, it would be possible to achieve a common lexicon for bacterial identification and characterization by colony morphology. Colony descriptions will become explicit, reliable and unambiguous and information about infecting populations will be easily shared among researchers, laboratories/centres, and clinicians. Through the interchange of information, clinical diagnosis may gain substantial and relevant knowledge about the infecting populations and disclose their mechanisms of adaptation and persistence and, this way, could prevent, control or even eradicate bacterial infections.

2.3.2. Materials and Methods

Revision of literature

The process adopted to construct a characterisation system consisted the extraction and learning of terms and concepts used by authors in their publications (Figure 2.6). The process of document retrieval encompassed the screening of books, encyclopaedias and research papers. The research papers were compiled through PubMed keyword-based searches, combining terms related to morphological descriptions and bacterial colonies. Most content was harvested from the ‘Materials & Methods’ and ‘Results & Discussion” sections, including the captions of figures and tables.

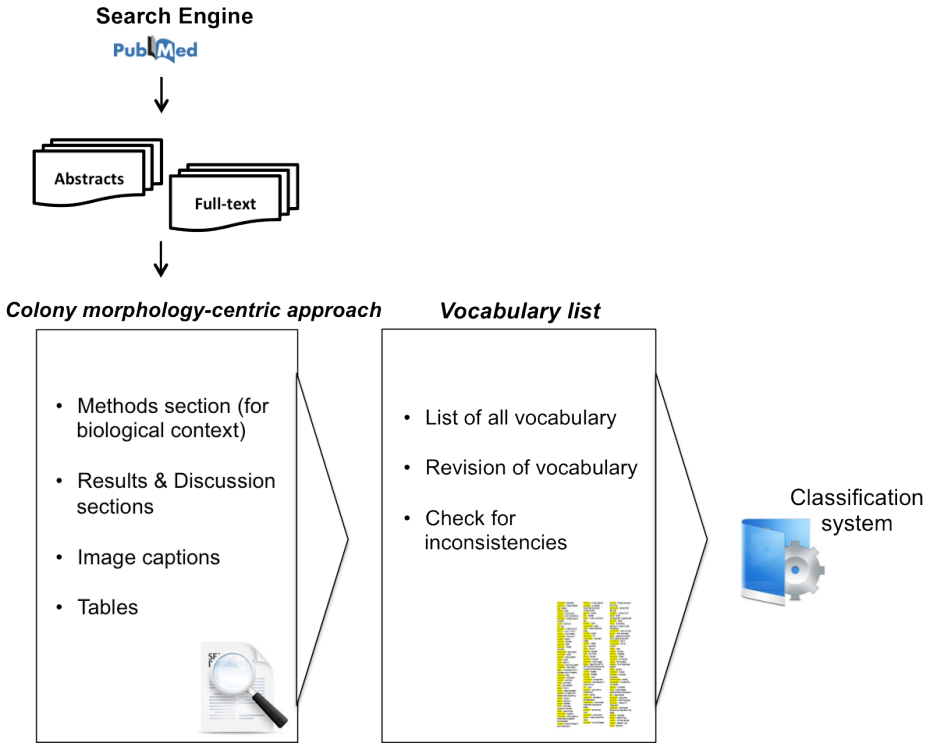

Figure 2.6. An overview of the literature revision for terms and concepts collection. The documents were retrieved via PubMed and analysed in detailed. Typically, colony morphology vocabulary was contained on "Methods & Materials" and "Results" sections and captions of figures. After vocabulary collection and listing, terms were organized in a structured way creating a renewed classification system for colony characterisation.

Bacterial strains

To test the constructed system based on vocabulary learning from literature for characterization of bacterial colony morphologies, in-house experiments were performed, including several bacterial species and strains to ensure colony variability. A total of 30 strains were used in this study belonging to 8 bacterial species as described in Table 2.4, including *P. aeruginosa*, *S. aureus*, *Escherichia coli*, *S. pneumonie*, *Klebsiella oxylota*, *Klebsiella pneumonie, Dolosigranulum pigrum* and *Proteus mirabilis*. Bacteria were routinely cultured on TSB or TSA medium at 37 ºC. All strains were preserved in criovials (Nalgene) at – 80 ± 2 ºC. Prior to each experiment, bacterial cells were grown on TSA plates for 24 h at 37 ºC.

Planktonic cultures

Planktonic bacteria grew overnight in TSB at 37 ºC, 120 rpm. Cell suspension of each strain was washed twice in sterile water by centrifugation (9000 *g*, 5 min) and, further serial diluted and plated on several solid media.

Table 2.4. List of the bacterial species and strains used in this study.

Species	Strain	Description
P. aeruginosa		
	ATCC 10145	Reference strain
	ATCC 39324	Reference strain
	PA01	Reference strain
	PA14	Reference strain
	CECT 111	Reference strain
	PAI1	Superficial isolated
	PAI2	Clinical isolated
	PAI3	Clinical isolated
S. aureus	ATCC 25293	Reference strain
	SAI1	Medical device isolated
	SAI2	Clinical isolated
	SAI3	Clinical isolated
	SAI4	Clinical isolated
	SAI5	Clinical isolated
	SAI6	Clinical isolated
	SAI7	Clinical isolated
	SAI8	Clinical isolated
	SAI9	Clinical isolated
	SAI10	Clinical isolated
	SAI11	Clinical isolated
	SAI12	Clinical isolated
	SAI13	Clinical isolated
E. coli	K12 MG1655	Reference strain
	rpoS mutant	Mutant strain
	bolA mutant	Mutant strain
S. pneumonie	SPI1	Clinical isolated
Klebsiella oxylota	ATCC 13182	Reference strain
Klebsiella pneumonie	KPI1	Clinical isolated
Dolosigranulum	CIP 104051	Reference strain
Proteus mirabilis	SGSC 414	Reference strain

Biofilm formation

Biofilms were development as previously described [28]. Briefly, bacteria grew overnight on TSB at 37 ºC, 120 rpm. Cell suspension of each strain was diluted in TSB to obtain 10^7 CFU/mL as final concentration. Afterwards, bacterial suspensions were transferred to 96-well polystyrene microtiter plate where biofilms were developed aerobically on a horizontal shaker (120 rpm) at 37 ºC for 24 h. After that, biofilms were scrapped into sterile water, homogenized and biofilm-cells were serial diluted with sterile water and spread plated on several solid media.

Colony observation

To assess colony morphology, bacteria were serial diluted and plated on different solid media, including TSA, CET, PIA, Muller Hinton agar (MHA), MacConkey agar, *Klebsiella* Selective Agar and Mannitol. After several hours (different intervals of time were tested) at 37 ºC, colonies were observed by directly placing petri plates on magnifying glass (Olympus SZ-CTV) and photographed in a CCD camera (AVC, D5CE; Sony, Tokio, Japan).

2.3.3. Results

An extensive search in literature, including research papers, books and encyclopaedias, transversal to all knowledge areas was performed in order to gather terms used to describe colony morphologies formed by bacteria. More than one hundred terms related to colony morphology characterization were compiled. More than reviewing, it was attempted to organize the terms and construct a characterisation system based on those findings. Literature revision was filtered out, considering the exclusion criteria below:

- terms with no clear definition (for example, “normal colony”; “atypical morphology”, “irregular shaped”, and “normal size”);
- terms referring to characteristics of bacteria-forming colony, i.e. characteristics of the bacteria that form the colony rather than the morphological features of the colony (for example, “rod-shaped bacteria”);
- derived terms (for example, “semi-fluffy”, “semi-dry”, “degree of colour”, “non-mucoid”, “slightly rhizoid”, “marginally convoluted”);
- infrequent terms, i.e. those apparently used by only one author or research group.

Then, the remaining terms were checked for definition inconsistencies and term synonyms. Typically, the most used term was chosen as the main descriptor of the morphological feature and the other related terms, but with identical meaning, were associated as synonyms to provide flexibility to the system. Based on these criteria, a first characterisation system was constructed (Figure 2.7). Seven categories, including form, margin, surface, elevation, opacity, size and colour composed the system. All seven categories comprised sub-categories that represented the detailed features of a colony. For instance, *smooth*, *rough*, *wrinkled*, *brittle*, *moist*, *viscous*, *dry* and *mucoid* are the sub-categories of *surface*.

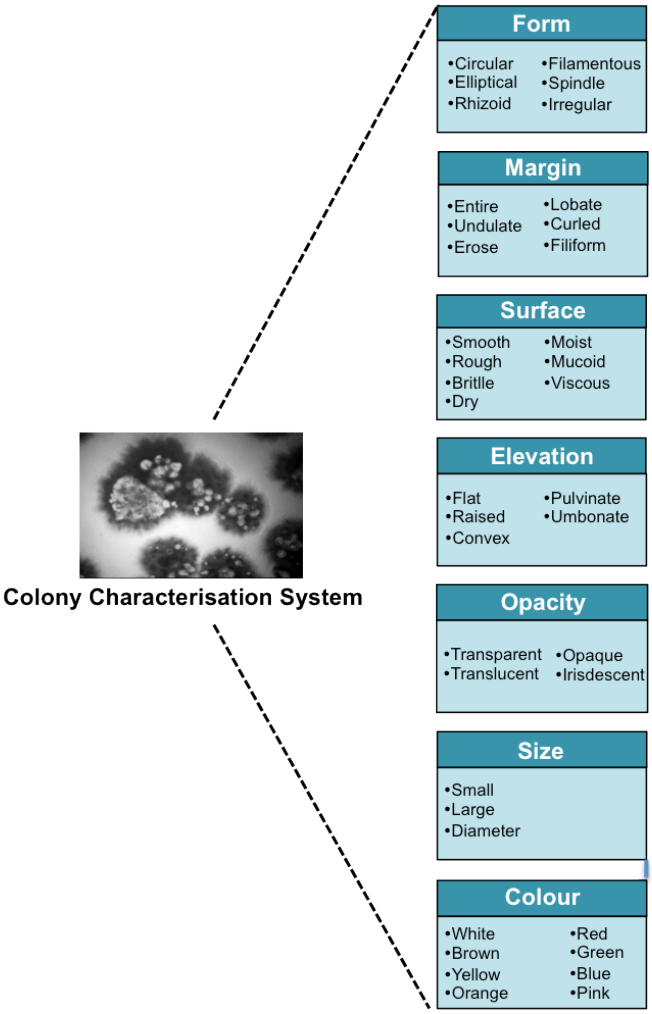

Figure 2.7. The literature-based system of the colony morphology characterisation. The system is composed by 7 categories (form, margin, surface, elevation, opacity, size and colour) and 39 sub-categories, which are the detailed features of the main categories.

Testing of the colony morphology characterisation system

For the purpose of testing the feasibility of the system, several experiments were performed using different bacterial species in a total of 30 strains. All bacterial strains were grown in planktonic and biofilms state under different conditions: exposure to disinfectants, antibiotic treatments, oxygen limitation (anaerobiosis, microaerophilic), osmotic stress, inter- and intraspecies competition and combination of stresses. In addition, bacteria were collected, plated and grown under several conditions: distinct agar media, time of colony growth and oxygen availability (anaerobiosis, microaerophilic, aerobiosis). The diversity of bacterial strains, two modes of growth, panoply of stress factors and distinct plating conditions ensured *a priori* increased colony morphology diversity to intensively test the system constructed.

Hundreds of colony morphologies were analysed and characterized. The vast colony morphologies obtained allowed noticing that the system based on the actual criteria was valid but pointed out some constrains. First, some *P. aeruginosa* colonies exhibit an enveloping part or structure after the margin that surrounds the colony (Figure 2.8A), which was not included in the system. This part of the colony was named as ‘sheath’. Because of the fact that some colonies exhibited sheath and others did not, it seemed

that this differential feature should be described and included in the system Second, colonies can exhibit more than one type of surface. For instance, *P. aeruginosa* colonies can exhibit surfaces with smooth and wrinkled zones (Figure 2.8B), smooth and rough zones (Figure 2.8C), or rough and wrinkled zones (Figure 2.8D). These types of morphologies with more than one type of surface were not predicted in the system and should be well identified. Third, the large number of colonies examined allowed to take notice another relevant issue about colony surface, the difference between wrinkled and rough surfaces. It was observed that some colonies could present greater vertical irregularities (Figure 2.8B) and other exhibited smaller vertical irregularities (Figure 2.8C). As the dimension of the irregularities was different, it should be described as a distinct feature. In fact, there are two terms to describe colony surface irregularities, *wrinkled* and *rough*, but they are frequently used as synonymous and rarely as a differential feature and thus the system considered them as the same characteristic [16, 40]. However, the system should differentiate these two types of irregularities to better typing colonies. Fourth, still regarding colony surfaces, features as dry or mucoid, included in *surface* category, were not characterized through simple observation as the other surface features (smooth, rough and wrinkled). Brittle, moist, viscous, dry and mucoid features were evaluated when colonies were taken off from agar media. By this reason, the inclusion of different kind of features in the same category did not seem adequate. Fifth, the definition of size was not adequate for all species. The presence of SCV was detected in several biofilm populations, however they exhibited a wide range of diameters according to the bacterial species. For instance, *P. aeruginosa* and *K. pneumonie* SCV exhibited typically diameters above 3 mm (Figure 2.8C and E, respectively), in contrast to *S. aureus* SCV that typically exhibited diameters above 1-2 mm (Figure 2.8F). Therefore, the system was improper, concerning colony size description.

To enhance the descriptive abilities of the characterisation system, the initial structure was extended to a total of 10 main categories and 45 sub-categories (Figure 2.9), and some definitions of categories and sub-categories improved (Table 2.5 and Table S2.1 in Supplemental Material, respectively). The following subsections introduce all terms, explaining the semantics adopted by the system and discussing the semantic inconsistencies and ambiguities found in literature.

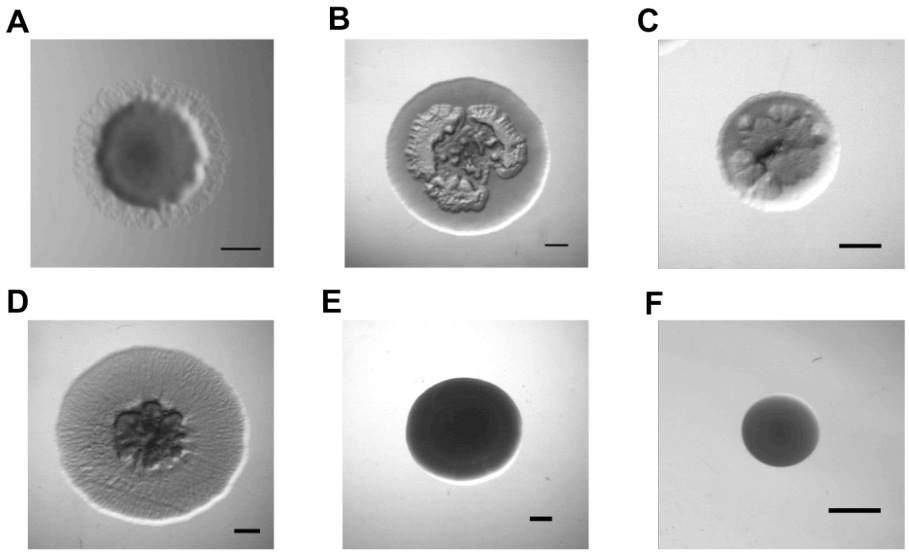

Figure 2.8. Colony morphologies of (A) to (D) *P. aeruginosa*; (E) *K. pneumonie*; and (F) *S. aureus* that exhibited traits not predicted by the first version of the colony morphology characterisation system. Black bars = 1 mm

Table 2.5. Definitions of the terms that composed the categories of the last version of the colony morphology characterisation system.

Category name	Definition
Form	The external appearance or configuration of the clearly defined area of a colony.
Margin	A limit zone or area immediately adjacent to a colony.
Sheath	A structure after the margin that surrounds a colony.
Type of Surface	A morphological feature related to the superficial or outer appearance of a colony having one or more type of textures.
Texture	Appearance or physical structure or composition or organization of morphological features or irregularities present on a colony surface.
Consistency	Degree of coherence, density, firmness or viscosity of a colony.
Elevation	Appearance or configuration of a colony above the agar surface and observed from lateral perspective or side view.
Opacity	An optical quality of colony based on the amount of visible light transmitted through the colony mass.
Size	Physical dimension, extension or magnitude of a colony including sheath, if present.
Colour	Chromatic appearance of a colony.

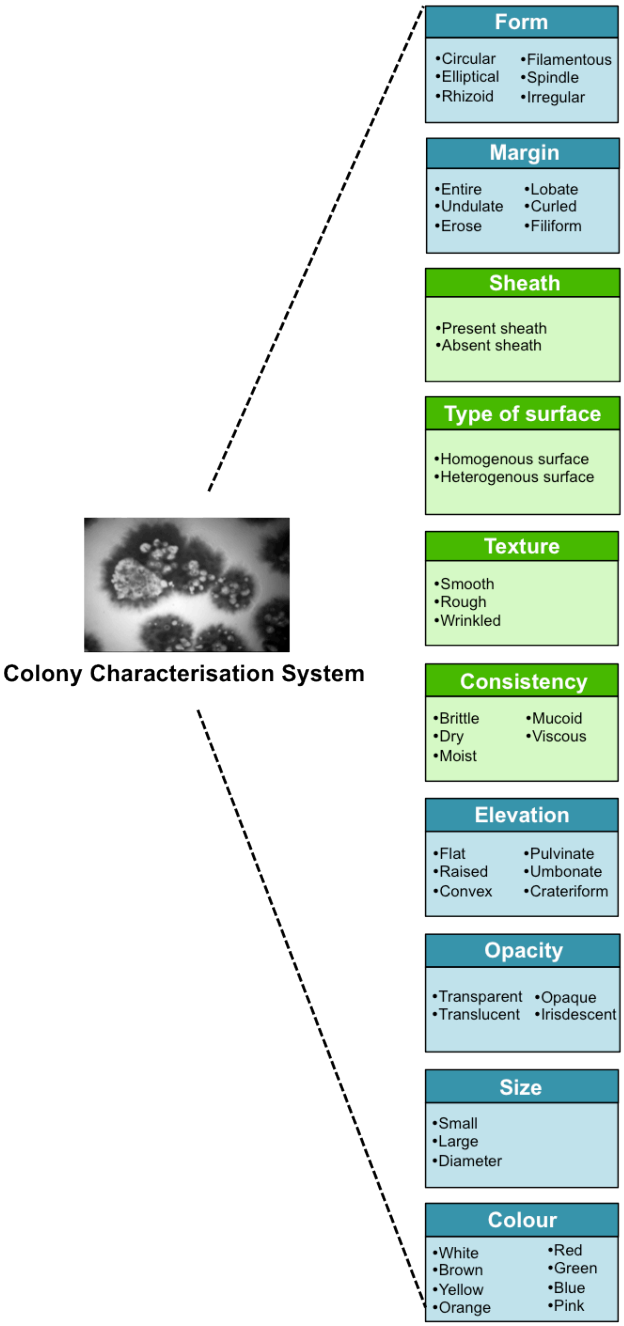

Figure 2.9. Final structure of the colony morphology characterisation system. The system is composed by 10 categories (form, margin, sheath, type of surface, texture, consistency, elevation, opacity, size and colour) and 45 sub-categories, which are the detailed features of the main categories (for instance brittle, dry, moist, mucoid and viscous). Green boxes represent the new categories introduced in this version of the system.

Form

The term *form* is commonly used to describe the whole configuration of a colony. For example, "…overall surface shape (convex, crater, lobulated, radial, radioumbilicated, radio-umbonated, peaked, rugose, segmented-rugose, segmented-umbilicated, umbilicated, umbilicated with irregular edge, umbilicated with heaped-up irregular edge, or umbonated)." [54], or to differentiate colonies with certain characteristics, such as "…two distinct colony morphology variants that switched between a transparent form, facilitating adherence and carriage, and an opaque form that poorly adhered…" [33]. So, in the characterisation system *form* is concerned to the geometrical configuration of the colony, for instance circular, elliptical, rhizoid, filamentous, fusiform and irregular.

Margin

The description of the margin of the colony, also referred to as edge or border, is typically based on the characteristics of the colony circumference. For example, "Colony type 2 was hard, more orange in colour, non-rhizoid or only slightly rhizoid, and had irregular edges and convex growth form. Colony type 3 had round edges, and smooth, yellowish appearance." [20], " 'fried egg' SCVs, with translucent edges surrounding a smaller elevated…" [55], "other colonies (approximately 60 % of the total) were smaller with somewhat rough edges" [56]. In the characterisation system, margin represents the configuration of the limiting border of the colony, including entire, undulate, erose, lobulated, filamentous.

Sheath

The description of *sheath* is not common in colony morphology observation. However, this structure is often present in *P. aeruginosa* colonies, which are clinically recurrent, and it is quite variable (Figure 2.7). Based on the results obtained, this may be a valuable element in the description of colony differentiation. So, it was included in the characterisation system. The descriptors of *sheath* include sheath present, sheath absent.

Type of Surface

As abovementioned, some bacterial species can form colonies with more than one type of texture at the surface (Figure 2.7B, 2.7C and 2.7D). As such, a new category, named 'type of surface', was added to the system to describe how many types of textures or irregularities were exhibited by the colony surface. Notably *homogeneous surface* when a surface has just one type of texture and *heterogeneous surface* when the surface presents more than one type of texture.

Texture

Colony texture, also referred to as surface or roughness, is a very common feature in morphological descriptions. Its importance arises from experimental evidence that rough colonies are often associated with key pathogenic phenomena, such as augmented virulence potential [16, 57].

In literature, it was found examples of general texture descriptions applied to colony morphology such as: "PA14 lasR mutant formed a flat, smooth colony as compared to the wrinkled wild-type phenotype" [57], "the ST variant formed colonies that were smaller and had a rough, wrinkled, and dry surface appearance compared to the smooth, larger WT colonies" [40] and "… type I (wrinkled, purple, dry and irregular giant cristae; *n* = 8), II (wrinkled, purple, dry and volcanolike; *n* = 6), (…) V (smooth, pale, mucoid and raised circles; *n* = 3), VI (smooth, pale, mucoid and even glistening; *n* = 2)…" [58]. This feature may also be used to describe the form of the colonies. For instance, "… overall surface shape (convex, crater, lobulated, radial, radio-umbilicated, radio-umbonated, peaked, rugose, segmented-rugose, segmented-umbilicated, umbilicated, umbilicated with irregular edge, umbilicated with heaped-up irregular edge, or umbonated)" [54].

The contents of the previous category *surface* were separated since they are assessed in different ways. The features *smooth*, *wrinkled* and *rough* are indeed surface features and thus remain in the category. However, as they particularly characterise the texture of the surface, the category was renamed as *texture*. Concerning the other elements of the category *surface* (Brittle, moist, viscous, dry and mucoid), they are not surface but consistency features and, thus, were moved to a new category (*see* consistency section). Then, the *texture* in this system denotes the presence or absence of irregularities on the colony surface.

In addition, often enough, the types of texture *rough* and *wrinkled* are used as synonymous or as discriminating characteristics [16, 40, 59]. The characterisation system addresses this conceptual ambiguity by defining the terms *wrinkled* and *rough* in accordance to the interpretation provided by the results here obtained.. When irregularities are present, their form is depicted as *wrinkled* or *rough* if the vertical irregularities are large or small, respectively. A surface showing no irregularities is described as *smooth*.

Elevation

Typically, the elevation describes the form of growth of the colony observed from a side perspective. For example, "Colony type 1 was rhizoid and flat with yellow centre. Colony type 2 was hard, more orange in color, non-rhizoid or only slightly rhizoid, and had irregular edges and convex growth form." [20]. In the characterisation system, *elevation* and *form* were differentiated, that is, one corresponds to the side perspective of colony form and the other describes the overall geometric colony configuration, respectively. The descriptors of elevations include flat, raised, convex, pulvinate, and umbonate.

Consistency

Consistency is frequently described as a surface property. For instance, "… characterisation of two *B. cenocepacia* sequential isolates displaying different morphotypes (mucoid vs non-mucoid) isolated from a CF patient…" [60], "… *P. aeruginosa* PDO300 showed a nonmucoid colony morphology…" [30] and "… type I (wrinkled, purple, dry and irregular giant cristae; *n* = 8) (…) IV (wrinkled, pale, semi-dry and volcanolike; *n* = 2), V (smooth, pale, mucoid and raised circles; *n* = 3)…" [58]. Similarly to what happens with the *texture*, *consistency* is often described as a form or surface characteristic [54]. The examination of consistency is distinct of form and texture. It is needed to take off colonies from the agar plate to describe their consistency in contrast from form and texture whose description is just based on observation. By these reasons, in the characterisation system, *form*, *texture* and *consistency* were separated and *consistency* was defined as density, firmness, or viscosity. Colonies can be brittle, moist, viscous, dry and mucoid.

Opacity

Opacity has been described as an important colony feature in different pathogenic morphotypes. For instance, in the case of *S. pneumonie* colonies "…two distinct colony morphology variants that switched between a transparent form, facilitating adherence and carriage, and an opaque form that poorly adhered…" [33] and in the case of *Pseudomonas fluorescens* colonies, "… The original isolate formed thick opaque colonies…", "… After three days of growth in liquid KB medium, flat and translucent colonies were found…" [21]. Therefore, opacity was included in the characterisation system and defined as a characteristic of the colony to absorb visible light.

Size

The feature *size* is a well-recognised trait in colony characterisation. For instance: "… strain 43895OR is also similar to the rdar strains of *S. enterica* serovar Typhimurium in characteristics such as colony size…", "… *E. coli* strain 43895OR forms a larger colony…" [26], "… Temperature-induced SCVs were <1 mm in colony size …" [61] and "… Analysing the persisting bacteria revealed a high phenotypic diversity, showing normal, small and very small colonies…" [18].

The significance of the size description arises from the association between the appearance of small colonies and increased antibiotic resistance [55, 62]. However, it has conceptual gaps. For instance, Haussler and colleagues (1999) [15] considered *P. aeruginosa* SCV those that have 1-3 mm of diameter. However, several other authors study *P. aeruginosa* SCV without any clear assumption about the diameter range of the colony [16, 63]. Additionally, this definition of size is highly taxon-dependent as abovementioned. For instance, the dimension of a small colony of *P. aeruginosa*, in general defined as below 3 mm, does not correspond to the dimension of a *small* colony of *S. aureus*, typically defined as below 1-2 mm. Therefore, in the characterisation system the sub-categories of *size* were defined in a

taxon-independent manner such that the small denotes a colony dimension nearly one-tenth less than the size of the wild-type colony and the *large* denotes colonies exceeding this threshold.

Colony size can be reported as well in a quantitative manner. Diameter measurements are also frequently used to characterise the size of the colony. For instance, "… The following features of colony morphology were recorded: colony size (measured in mm by ruler)…" [33] and "… cells of the rdar strains were reported to reach colony diameters of 6 cm…" [26]. The inclusion of diameter into the system, the unique quantitative class, was due to limitations of qualitative size characterization. The characterization of a colony as *small* or *large* is dependent of the wild-morphotype species. The real dimension of a colony must be included and recorded numerically for unequivocal perception of small or large dimensions. In the characterisation system, *diameter* was thus included as a sub-category of *size* and defined as the distance between two equidistant points of the margin or sheath.

Colour

The chromogenesis or colour exhibited by bacterial colonies is very important when describing clinical morphotypes because it is associated with the differential production of various pigments [64, 65]. Some examples of colour description applied to colony morphology are: "… certain strains of *Salmonella* that displayed a red and dry colony phenotype…"; "… the white variants do not revert back to a dry, red phenotype..." [26], "… StNMSm (straw-colored, nonmucoid, and smooth) morphotypes exhibited decreased frequency of pyocyanin overproduction…" [64] and "Colony type 3 had round edges, and smooth, yellowish appearance. Type 4 colonies were white or light yellow, smooth and spreading on the agar with irregular shape." [20]. In the characterisation system, colour was included and defined as a composite chromatic quality composed of hue, saturation and intensity parts. A diverse and automatic *pallete* of colours could be incorporated into colour category, but for now it was chosen to focus on the colours that have already been documented in the literature. Therefore, characterisation system includes these "basic" colours, notably white, black, grey, brown, yellow, red, orange, green, blue, pink and violet.

2.3.4. Discussion

Colony morphology variation or "dissociative behaviour", firstly described in 1964 by Zierdt *et. al* [66], is able to detect phenotypic diversity of bacterial populations that other methods do not easily identify. Through colony morphology observation, it was possible to verify, for instance, the occurrence of clonal diversification of bacteria in CF lungs crucial to understanding the existence of microevolutionary pathway [67, 68]. Despite its importance and potential, colony morphology characterization has many limitations. In addition to the restrictions inherent to being a culturing-dependent method, colony morphology characterization presents high variability in its performance, despite having a simple procedure. The vocabulary, concepts and morphological criteria used by authors to characterize and describe the colony features are indeed one of the most important handicaps to achieve an accurate typing of bacteria. All of the work related to colony characterisation has been performed without common

lexicon for colony description, classification or even designation. Such conceptual variability makes comparisons among colony morphology data inadequate. Vocabulary and criteria used by community need to be universal, consistent, unambiguous to effectively represent, analyse and, most important, to interchange data among experiments, species, clinical samples, patients, infections or diseases.

The first attempt to obtain a systematization of vocabulary about bacterial growth was in 1901 when Chester [69] compiled several criteria and terms that can be used to characterize the different types of bacterial cultures. Some of those criteria and terms are now old-fashioned and inaccurate, considering current knowledge about bacterial diversification in terms of colony morphology patterns. Later, David Bergey modified the manual proposed by Chester and it remains today the major treatise since its first publication in 1926 [70]. Bergey extensively described the typical morphological patterns of colonies formed by the several bacterial species identified so far. However, Bergey did not state which criteria were chosen to characterize those bacterial colonies. Meanwhile, several different terms and criteria have emerged and have been used to characterise colonies. As so, a renewed and universal characterisation system for bacterial colonies is required. Such system will have great impact in several scientific areas, since colony morphology characterization has been used for different purposes in panoply of scientific areas and, certainly, will impact the clinical diagnosis. The novelty of this study was the organisation of the current terms in a structured way never performed before.

After a careful and extensive study of all terms and criteria used since early ages to characterise bacterial colonies, an organised system was constructed. The literature-based system was tested with in-house colony morphologies and, although it has revealed to be valid, it has exhibited some limitations. According the detected limitations, the system was improved. In the end, the colony characterisation system indicated as the main morphological criteria that should be used to describe colony morphologies: form, margin, sheath, type of surface, texture, elevation, consistency, opacity, size and colour (Figure 2.5). Each of these categories has its own vocabulary, the sub-categories, allowing thus to achieve an accurate description of the colony morphology. Most of the terms and definitions that composed this system are widely used by the microbiological community to characterize and differentiate colony variants.

This system provides to scientific and clinical communities a set of minimum information about colony morphologies that should be contained on descriptions, reflecting the most essential aspects of colonies. It should be emphasised that colonies described using a common lexicon are more easily compared across species, strains, patients, experiments, laboratories, infections or diseases.

More than the organisation of the terms, this system aimed at standardising the conceptual definitions of each term (main categories and sub-categories), included in the system. This characterisation system attempted to capture the precise meaning of each term, avoiding variability, heterogeneity and ambiguity. Therefore, each term had an associated concept to avoid dubious understandings so that authors might use them unmistakably. The general understanding of the meaning and use of terms and vocabularies is essential to standardize colony morphology descriptions and to facilitate the comparisons between reports and knowledge advance about phenotypic plasticity, bacterial adaptation and evolution.

This characterisation system also included alternative terms or synonymous for categories or sub-categories. Terms, either main or sub-categories, are not obligatory. Authors can use any term of their preference although with the commitment that term means which is stipulated in the system. The inclusion synonymous makes the system flexible. It is important not to break with current concepts abruptly and impose a radical “culture” change of colony terms and criteria. The clinicians and researches adherence to the system is desirable.

This classification system of bacterial colony morphologies does not represent a static entity. The characterization of colony morphologies is a challenging task because bacteria are unpredictable organisms always changing to overcome stressor factors, such as host environment and/or fluctuations, immune defences and antimicrobial actions. Therefore, morphological criteria, vocabulary and concepts of this system should be continuously upgraded. Suggestions of new or alternative terms, as well as concepts are very welcome.

2.4. A new approach to bacterial colony morphotyping by matrix-assisted laser desorption ionization time of flight-based mass spectrometry

2.4.1. Objectives

Over the years automated methods for bacterial identification and characterization were continuously demanded, attempting to respond to the clinical needs of fast and reliable diagnosis. Colony morphology characterisation can take days to return results because bacteria need to grow, which imposes significant delay to action in situations where timely intervention is urgently needed to reduce associated costs. MALDI-TOF MS is considered a robust and reliable tool for bacterial taxonomy identification including species and strain differentiation. To date MALDI-TOF MS ability to discriminate at colony morphology level has not been assessed yet. To enable faster response of colony morphology characterisation method demanded by clinical diagnosis, the present study aimed at assessing MALDI-TOF MS as a tool for colony morphology differentiation. To this end, a standardized sample preparation protocol for bacteria and a MS data analysis taken with a MALDI-based method elaborated for rapid colony morphology profiling was used as proof-of concept with two main human pathogenic bacteria, *P. aeruginosa* and *S. aureus*.

2.4.2. Materials and Methods

Materials and reagents

All culture media used in this study were obtained from Liofilchem Diagnostic. The following reagents were used to perform MALDI-TOF MS analysis: trifluoroacetic acid (TFA) 99 % (v/v) was from Riedel-de-Haen (Seelze, Germany), α-cyano-4hydroxycinnamic acid (α-CHCA) and sinapinic acid (SA) both puriss were from Fluka (Buchs, Switzerland). Milli-Q water was used throughout the experiments. All the chemicals used were of analytical-reagent grade, unless otherwise specified. All materials were used without further purification.

Bacterial strains and culture conditions

The strains tested were *P. aeruginosa* ATCC 10145 and *S. aureus* ATCC 25293. These bacteria were routinely cultured on TSB or TSA medium at 37 ºC. All strains were preserved in criovials (Nalgene) at –80 ± 2 ºC. Prior to each experiment, bacterial cells were grown on solid media for 24 h at 37 ºC. *S. aureus* was included in this study to verify whether the possible MALDI-TOF MS ability to discriminate colony morphologies was not exclusive to one bacterial species.

Biofilm formation

P. aeruginosa ATCC 10145 and *S. aureus* ATCC 25293 were allowed to form 24-h-old biofilms as described previously [28]. Bacteria inocula were grown overnight on TSB at 37 ºC in air conditions, and then diluted in fresh TSB to obtain suspended cultures with 10^7 CFU/mL as final concentration. Afterwards, each bacterial suspension was transferred to 6-well polystyrene plate, where biofilms were developed aerobically on a horizontal shaker (120 rpm) at 37 ºC for 24 h. Then, biofilms were scrapped into sterile water, homogenized; being the biofilm-cells serially diluted with sterile ultrapure water and spread on solid media plates.

Observation and classification of colony morphology

To assess colony morphology, bacteria from planktonic and biofilm cultures were allowed to grow on solid plates during 72 h at 37 ºC. This step was aimed at obtaining complete and unchangeable morphotypes of both species. Colony morphologies were observed by directly placing the petri plates on a magnifying glass (Olympus SZ-CTV) and photographed in a CCD camera (AVC, D5CE; Sony, Tokio, Japan). Colony morphotyping was based on the proposed characterisation system of bacterial colonies (*see* section 2.3). Therefore, colonies were described using key morphological features, including form, margin, type of surface, texture, size, sheath, elevation, opacity, consistency and colour [54, 58, 71]. In addition, a quantitative parameter, the diameter, expressed in millimetres, was also included in the characterisation.

Bacterial preparation for MALDI-TOF MS

The different colonies of each bacterium were transferred from solid media to the extraction tube with a plastic loop. *P. aeruginosa* cells were re-suspended in ultrapure water and treated following a previous described protocol with some minor modifications as follows [72]. Cells were washed with 0,1 % (v/v) of TFA, re-suspended in 200 μL of chloroform-methanol (1:1) and vortexed for 1 min. The cell suspension was centrifuged at 9000 *g* for 8 min and the resulting pellet re-suspended in 15 μL of 0,1 % (v/v) of TFA. Different matrix solutions were daily prepared with α-CHCA and SA as follows: 10 mg of α-CHCA were dissolved in 50 % (v/v) of acetonitrile (ACN) and ultrapure water containing 0,1 % (v/v) of TFA; 20 mg of SA were dissolved in 30 % (v/v) of ACN and ultrapure water containing 0,3 % (v/v) of TFA. Then, each of the aforementioned matrices were mixed with the cellular sample (1:1), and the mixture was vortexed for 30 s. Each mixture (cell sample and matrix solution) was spotted on the MALDI target plate and allowed to dry at room temperature.

The treatment of *S. aureus* cells was performed according to Carbonnelle *et al.* [73] with some alterations. 1:1 (v/v) matrix to sample ratios were investigated by changing the matrix composition to assess the best matrix for this bacteria. The bacteria were first spotted into the MALDI plate and then the bacteria were covered by the matrix. The mixture was allowed to dry at room temperature.

MALDI-TOF MS analysis

An Ultraflex II MALDI-TOF/TOF instrument equipped with a nitrogen laser radiating at 337 nm from Bruker Daltonics was used to acquire bacteria mass spectra.

Measurements were done using the reflector positive ion mode, with a 20 kV accelerating voltage, 75.1 % grid voltage, 0.002 % guide wire, and a delay time of 140 ns. Mass spectra were taken in the *m/z* range of 2000-20000 and obtained in different regions of the same sample, based on the acceptance criteria of 1000 laser shots *per* spot. External calibrations were performed with a Protein Calibration Standard I (Bruker Daltonics).

Mass spectra were processed with FlexAnalysis® software (Version 2.4), subjected to baseline correction, noise filtration, normalized to the base peak, smoothed using Gauss algorithm and analysed considering the mass interval of 2000-20000 Da (due to the good reproducibility of spectra profile at that range). In order to avoid the presence of noisy peaks, each sample was spotted five times in the MALDI MS, the resulting spectra were aligned allowing an *m/z* error of 750 ppm, and those peaks that did not appear in, at least, three of the five spectra were discarded.

Clustering Analysis

An agglomerative hierarchical clustering was employed to analyse the individual spectrum and peak similarities. The clustering was constructed using a custom implementation of the Unweighted Pair Group Method with Arithmetic Mean [74] algorithm with Hamming distance [75] as the distance metric. It was decided to choose this distance metric instead of the more common Euclidean distance since it was not used the peak intensity information. Therefore, the clustering was built taking into account solely the presence or the absence of the peaks in each spectrum. The clustering results were presented as heat maps using the Java Treeview software [76].

The peak lists used in the clustering analysis were filtered applying a Chi-squared test of independence corrected using Williams' Correction, and selecting those peaks with a p-value under 0.05. That is, only the most discriminative peaks were used.

2.4.3. Results and Discussion

S. aureus and *P. aeruginosa* are the most prevalent pathogens in CF lungs in child and adults, respectively [77]. One of the major concerns of the medical community related to those pathogens is the increasing resistance displayed by bacteria to antibiotics over infection development in CF lungs [78, 79, 80]. Resistance seems to be potentiated when bacteria switch from planktonic or free-living state to sessile lifestyle and start growing as biofilms that are more resistant and persistent to antimicrobial stressors than their planktonic counterparts [81]. Therefore, it seemed important to include in this study colony morphotypes developed by both planktonic and biofilm-associated bacteria.

The success of the treatment to eradicate those infections is highly dependent on the speed and precision of pathogen identification and characterisation. Microbial diagnosis demands thus rapid, sensitive, specific, easy to perform (not labour intensive) and high-throughput methods for bacterial identification and characterization [1]. MALDI-TOF MS approaches are able to perform species identification and even strain differentiation based on molecular signatures in a quick and simple way [82]. Direct bacterial profiling by whole bacteria analysis is one of the most attractive approaches of MALDI-TOF MS, particularly in bacterial identification and differentiation. The direct deposition of "intact" bacteria mixed with matrix on the MALDI plate simplifies the laboratory procedures, thus minimising the costs and time taken in preparing the samples, without accuracy losses [83].

The fast and reliable results obtained by MALDI-TOF MS coupled with its simple procedure seemed an excellent method to combine with colony morphology characterisation. The high-throughput feature of MALDI-TOF MS could accelerate the rate of generating data on colony morphology discrimination and therefore morphotypes could be anticipated without culturing steps. In near future, morphotypes discrimination could be based on matching against constructed libraries with characteristic spectra/peaks for each morphotype. Researchers and clinicians could be able to automatically identify the morphotype-associated bacteria and even the biological features of the bacteria (antibiotic resistance profiles and virulence factors expression). This will greatly impact the cuurent clinical diagnosis.

MALDI-TOF MS analysis optimization

For colony morphotypes fingerprint and further comparison of spectra, reproducibility plays a crucial role. As this study aimed intra-strain differentiation, high quality and reproducibility of spectra have even more significance. A total of 4 morphotypes for each species were studied and for each morphotype a total of three to six biological replicates were used. The range of replicates was variable because of distinct prevalence of each morphotype in planktonic and biofilm cultures. Each biological replicate was spotted into the MALDI plate five times.

Spectra reproducibility is also highly dependent on the sample preparation method used and, for that reason, an extensively search about sample pre-treatments was performed. Liu *et al.* (2007) [72] reported an extensive study of sample preparation methods for identification of Gram-negative and Gram-positive bacteria ensuring high quality and reproducibility of spectra obtained. Due to being simpler and faster, this method was chosen to test in this study. Following this optimized method, to clean bacteria from contaminants originated by the culture medium present at the bacterial surface and that would eventually cause interfering m/z peaks in the MALDI spectrum, a solution of chloroform and methanol was used [72]. After the cleaning procedure, the bacteria were pelleted by centrifugation and then the pellet was re-suspended in 0.1 % (v/v) of TFA. Before MALDI analysis, an equal volume of sample and matrix, α-CHCA or SA, was mixed. The mixture was then vortexed and 1 μL of this solution was spotted into the MALDI target in five different spots.

Influence of the type of matrix

The matrix used to aid in the ionization process is of main concern in MALDI analysis. The most used matrices are α-CHCA, SA, 2,5-dihydroxybenzoic acid (DHB), or ferulic acid [84]. Currently, there is no consensus about the most adequate matrix or which matrices should be used for a particular sample or analyte. In general, α-CHCA is used in those cases where the analytes are mainly peptides with molecular masses below 10 KDa, whilst SA is used for those cases where the analytes are high mass peptides or proteins, above 10 KDa [84, 85]. DHB is considered a universal matrix for MALDI analyses although several studies have reported no peptide/protein signals due to low homogeneity of sample/matrix mixture, lower sensitivity, and difficulties in ionization of analytes of high-mass range. DHB is usualy used to study oligosaccharides, glycopeptides, and glycoproteins. Ferulic acid is usually used for ionization of analytes with higher molecular weigh up to 70 kDa. Several studies have reported good resolution of signal and reproducibility among shots [73, 84].

Evidences have demonstrated that the use of different matrices causes significant alterations in MALDI spectra [84]. In order to avoid the loss of potential significant information needed to obtain accurate fingerprints of morphotypes, this study included the two most used matrices, α-CHCA and SA. Figure 2.10 shows spectra of different morphotypes of *P. aeruginosa* using both matrixes. From the results obtained it may be concluded that the spectrum obtained with α-CHCA had more *m/z* signals with higher intensity that the ones observed in the spectrum obtained with SA. In other words the best matrix for *P. aeruginosa* was anticipated to be α-CHCA, which was later confirmed by statistical assay. The richer is the spectrum in signals, the better classification is attained, as there is a higher chance to find differences between morphotypes.

Although the method mentioned above had provided excellent spectra for the *P. aeruginosa,* it failed to obtain *m/z* signals for *S. aureus*. It was hypothesised that this was due to the low mass of bacteria transferred to the MALDI plate, since the size of the *S. aureus* colonies were considerably lower than *P. aeruginosa* colonies, and differences in the size and in the surface topologies of both types of bacteria. The cell walls of Gram-positive bacteria are usually more difficult to analyse using MALDI-TOF MS than the cell walls of Gram-negative bacteria which results in fewer peaks and lower peak intensities [86]. In addition, colonies should be not grown for no more than 48 h because the excessive cultivation time can weaken and reduce the detection of characteristic peaks by MALDI-TOF MS [3]. In this study, colonies were allowed to grow during 72 h to achieve complete colony morphogenesis, which may have affected the obtainment of MALDI-TOF MS spectra. Consequently, for the case of *S. aureus* a different approach was attempted. First, the cleaning step was avoided, as it was observed that some bacteria were lost during this step. However, again no signal was obtained. So an additional change was made. The crystallization process was modified. It is well known that in many cases the crystallization process severely affects the MALDI spectrum [85]. According to Vaidyanathan *et al.* (2002) [87], the deposition method that produces the best spectra in terms of number of detected signals and signal-to-noise ratio was first deposited bacterial suspension on MALDI plate, let it dry and after overlaid with the matrix solution. Therefore, it was applied the described method for *S. aureus* colonies. First, the bacterial

solution was spotted, let to dry and, then, the matrix solution (α-CHCA or SA) was spotted over the dry sample. Once the matrix was also dry, the MALDI analysis was performed. With such modifications, good spectra were obtained using either α-CHCA or SA, as shown in Figure 2.11. As a matter of fact, the spectra were similar for both α-CHCA and SA, being difficult to distinguish through naked eye. So, for this case it was not possible to anticipate which matrix would provide the best results for classification purposes.

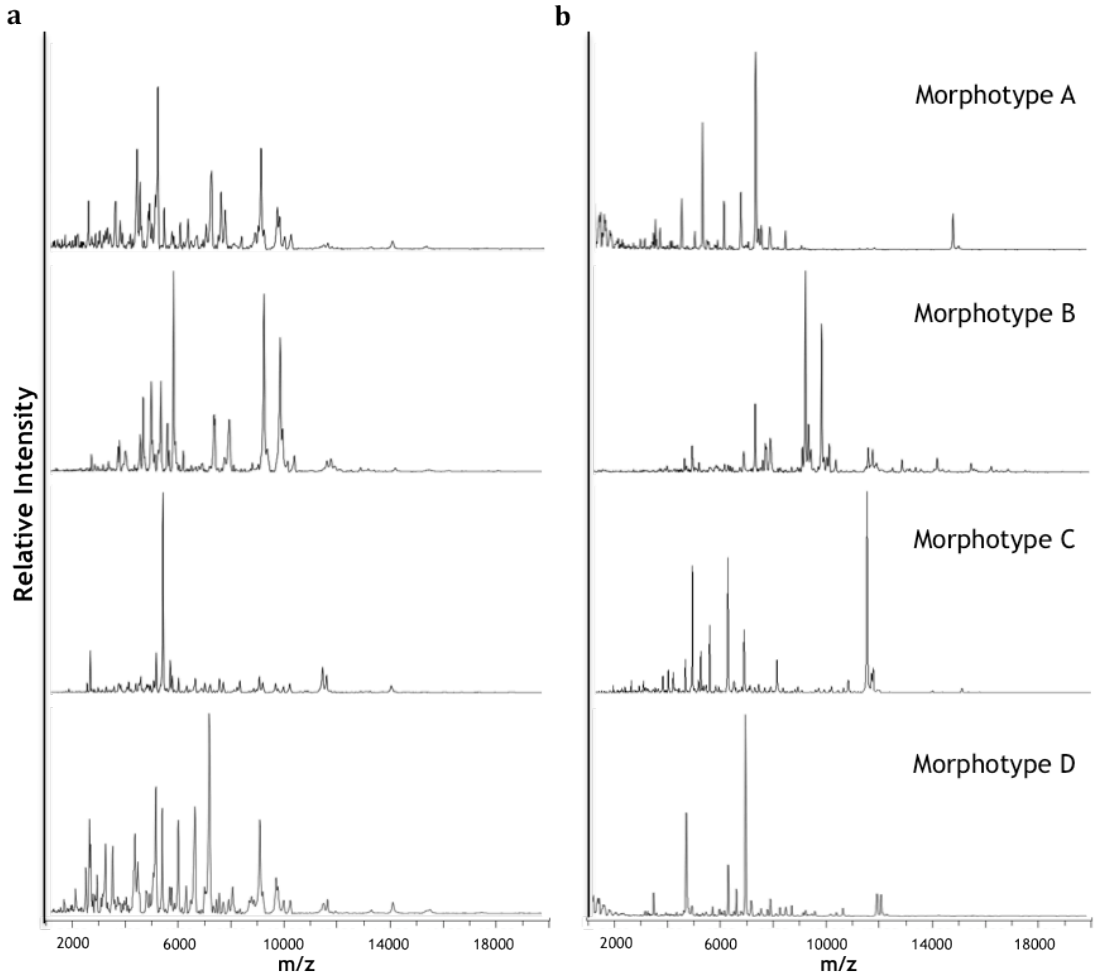

Figure 2.10. MALDI-TOF MS spectra of the different colony morphotypes of *P. aeruginosa* obtained using (a) α-CHCA and (b) SA matrices.

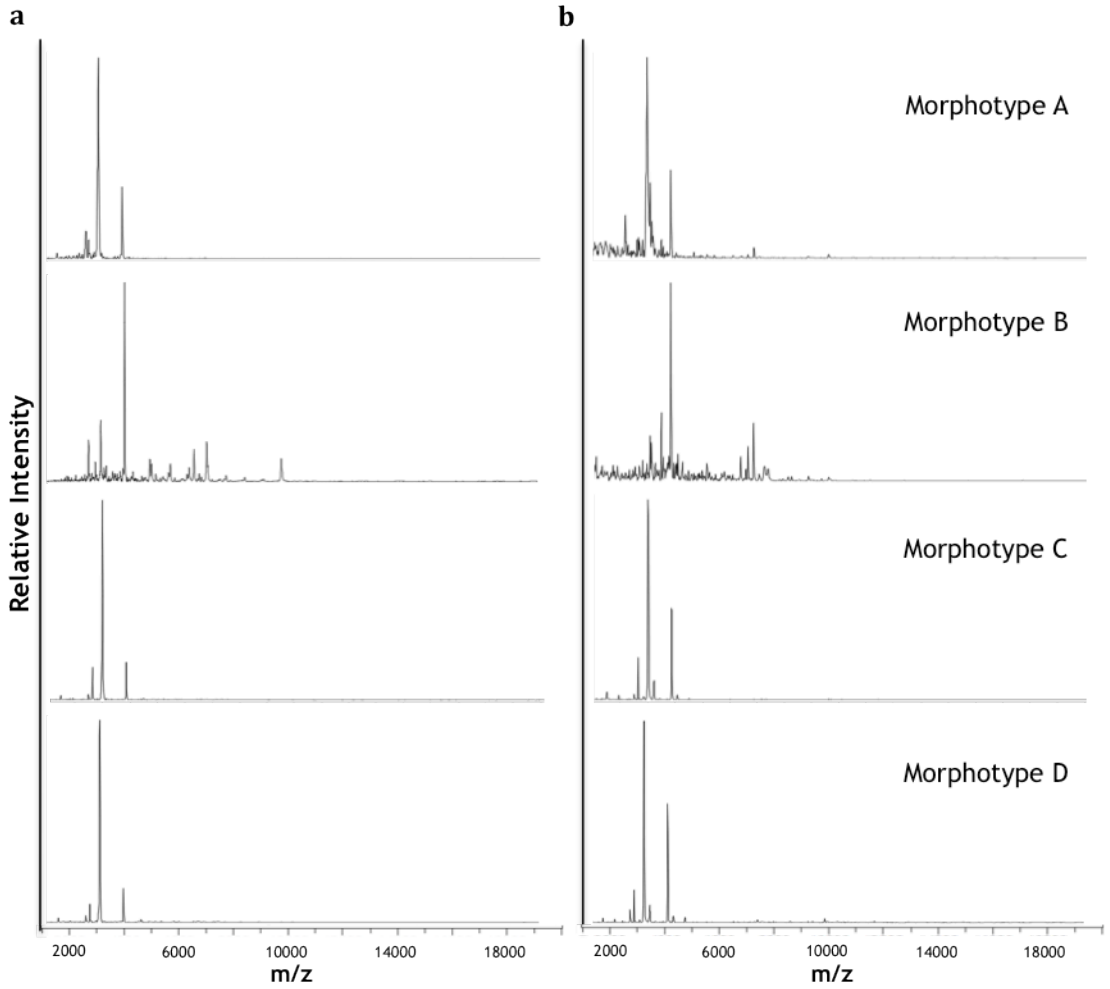

Figure 2.11. MALDI-TOF MS spectra of the different colony morphotypes of *S. aureus* obtained using (a) α-CHCA and (b) SA matrices.

Colony Morphology Classification

In this work, the new standardising morphological characterisation system proposed in section 2.3 was at the base of the colony annotation process. Morphological criteria used include form, margin, sheath, type of surface, texture, size, elevation, opacity, consistency and colour. Based on the aforementioned criteria four morphotypes of *P. aeruginosa* and four of *S. aureus* (Figure 2.12) were classified as described in Table 2.6. As may be seen, in terms of morphology, *P. aeruginosa* morphotype A differs from B, C and D in 3 of the 10 parameters investigated. B differs from C and D in 2 parameters, and C differs from D on one parameter. In other words, the classification seems as follows A>>>B, C, D; B>>C, D; and C>D, where each ">" means one parameter of difference.

For the case of *S. aureus*, the morphological classification was more troublesome. Non- and SCV morphotypes only differed in a maximum of two parameters (Figure 2.12 and Table 2.6). Thus, morphotypes A and B differs in two parameters as well as B and C. A and B differs from C and D in one parameter whilst C and D differs between them in one parameter. In other words, A>>B; B>>C; A>C,D; B>D; C>D.

In an attempt to classify those morphotypes in a new, fast and straightforward way, we used MALDI mass spectrometry-based fingerprinting, as described below.

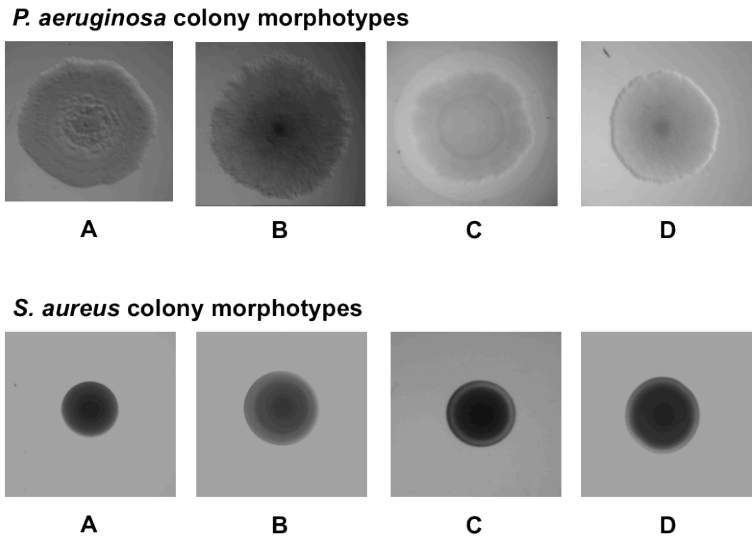

Figure 2.12. Colony morphotypes of *P. aeruginosa* and *S. aureus* analysed by MALDI-TOF MS.

Table 2.6. Comparison of the classification results obtained from morphological and MALDI-TOF MS categorization of *P. aeruginosa* and *S. aureus* colonies. Colonies were morphologically organized in four distinct groups according ten qualitative and one quantitative criteria.

Morphotype designation	Colony Morphotyping classification											MALDI-TOF MS classification
	Form	Margin	Type of Surface	Texture	Sheath	Opacity	Elevation	Consistency	Size	Colour	Diameter (mm)	
P. aeruginosa												
A	Circular	Undulate	Heterogeneous	Rough and wrinkled	Inexistent	Opaque	Flat	Dry	Large	Green	9	A = B
B	Circular	Lobulate	Homogeneous	Rough	Inexistent	Opaque	Flat	Dry	Large	Green	7	
C	Circular	Undulate	Homogeneous	Rough	Inexistent	Opaque	Flat	Dry	Large plus[a]	Green	10	C
D	Circular	Undulate	Homogeneous	Rough	Inexistent	Opaque	Flat	Dry	Large	Green	6	D
S. aureus												
A	Circular	Entire	Homogeneous	Smooth	Inexistent	Opaque	Flat	Dry	Large	Yellow	3	A = B
B	Circular	Entire	Homogeneous with 3 distinct zones	Smooth	Inexistent	Opaque	Flat	Dry	Large	Yellow	4	
C	Circular	Entire	Homogeneous with 2 distinct zones	Smooth	Inexistent	Opaque	Flat	Dry	Small	Yellow	2	C = D
D	Circular	Entire	Homogeneous with 3 distinct zones	Smooth	Inexistent	Opaque	Flat	Dry	Small	Yellow	2	

MALDI-TOF MS Classification

The spectra obtained, a total of 140, were clustered using the methodology described in section 2.4.2. For the *P. aeruginosa*, a cluster was done with the data taken from the spectra that were obtained using α-CHCA (Figure 2.13A). As it may be seen, MALDI clusters the morphotypes into three main groups, two single groups constituted by the morphotypes C and D and a group constituted by morphotypes A and B. Morphotype D is clearly separated from the other morphotypes, while morphotype C appears slightly mixed with some samples of morphotype B. Although samples of morphotype A are also mixed with samples of morphotype B, there is a clear separation between morphotype A and morphotypes C and D. The same result was attained with the spectra obtained with SA.

The characteristic peaks concerning the three groups (A/B, C and D) were compared with those reported in literature. It was found that this type of information is not abundant because the studies regarding species identification by MALDI-TOF MS are focused on specific parameters, such as the matching at genus and species level and non-matching percentages [8, 88, 89]. Furthermore, peaks reported by those studies correspond to conserved domains expressed by the whole species, which do not apply to the present study. Therefore, no common peaks were found between the list of characteristic peaks reported by strain differentiation studies by MALDI-TOF MS and the list of peaks reported by this study. Typically, peaks obtained from strain differentiation studies are characteristic of each bacterial strain, whilst the present study was focused on intra-strain level differentiation.

For *S. aureus,* the cluster done with the data taken from α-CHCA which is shown in Figure 2.13B reveals two main groups, one constituted by morphotypes A and B and another one formed by C and D. Although samples of morphotype D are grouped together, the number of peaks shared with samples of morphotype C indicates that both morphotypes are very similar. This group is more homogeneous than the groups A and B, where two samples (i.e. A_3_314 and B_2_60) were placed closer to the groups C and D than to their corresponding group. This classification was also obtained with SA although it was better attained with the α-CHCA, because for the case of SA one A and B morphotypes were mixed with the morphotypes C and D.

The comparison of the characteristic peaks obtained for the two *S. aureus* groups with those reported in literature revealed that the 2646 of *m/z* signal was already noticed. Edward-Jones *et al.* [90] pointed out a peak of 2647 *m/z* value as one of the characteristic peaks exhibited by methicillin-sensitive staphylococcal isolates. Such evidence suggests that C and D morphotype-associated bacteria may be susceptible to methicillin, in contrast to morphotypes A and B.

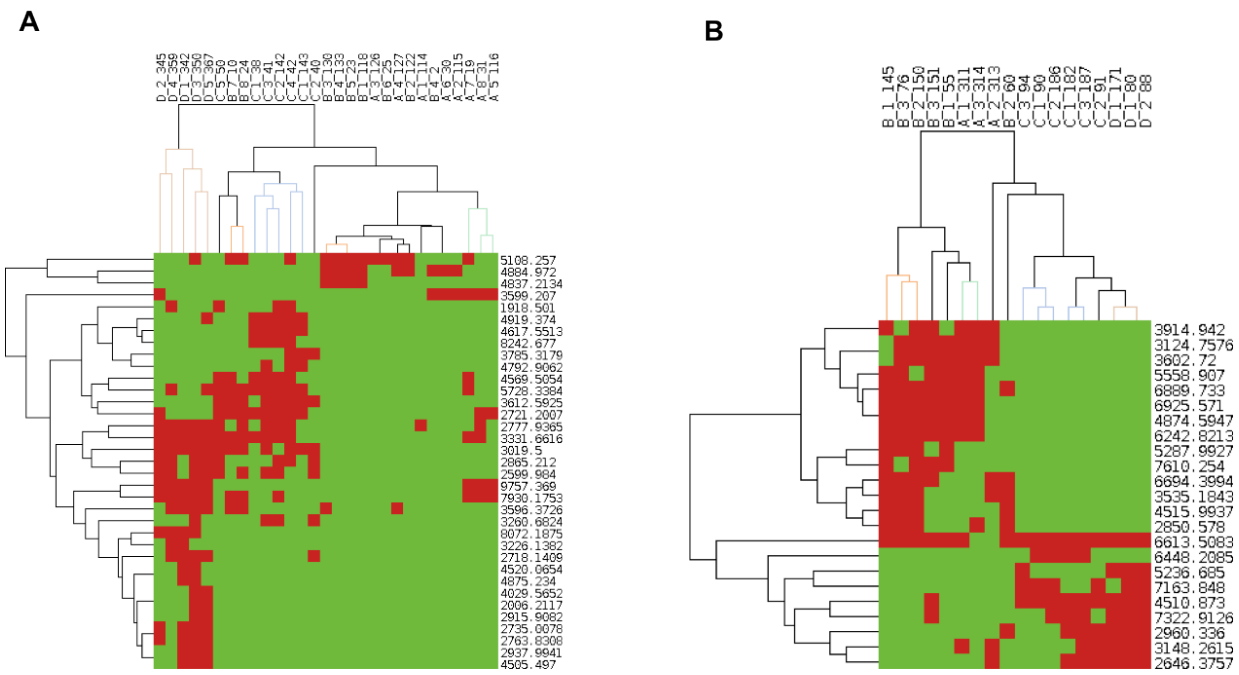

Figure 2.13. Heat map for the clustering of the (A) *P. aeruginosa* and (B) *S. aureus* spectra data generated using α-CHCA matrix. Vertical dendrogram shows the samples hierarchical clustering, while the horizontal dendrogram shows the m/z signals hierarchical clustering.

MALDI-TOF MS versus morphological classification of bacterial colonies

MALDI-TOF MS and conventional identification methods are both highly accurate for the identification and differentiation of isolated bacteria and yeast [89, 91, 92]. However, minor discrepancies between conventional and MALDI-TOF MS-based identification and differentiation results have been observed [86] and noticed in this study (Table 2.6). *P. aeruginosa* is oxidase positive, frequently mucoid, and produces green (pyoverdin) or blue-green (pyocyanin) pigments. These characteristics are present at least in 90% of CF isolates that MALDI-TOF MS has been shown to be able to accurately identify both non- and mucoid morphotypes of *P. aeruginosa* [93, 94]. However, *P. aeruginosa* morphotypes can exhibit differential antibiotic resistance profiles that MALDI-TOF MS can or not distinguish them. Furthermore, distinct *P. aeruginosa* morphotypes may express different cell-associated virulence factors that also can interfere with MALDI-TOF MS classification. These facts could explain the discrepancies between MALDI-TOF MS and colony morphology characterisation. For the case of *P. aeruginosa*, the morphological classification of colonies provides 4 phenotypes, whilst the MALDI classifies only into three phenotypes because morphotypes A and B seemed similar in terms of their MALDI fingerprintings. This group is different from the other two groups formed by C and D.

The morphological classification done for *S. aureus* gave rise to four phenotypes whilst the clustering done with the MALDI spectra classified the morphotypes into two groups: A, B and C, D. Several authors have reported the accurate ability of MALDI-TOF MS to identify *S. aureus* bacteria and even to discriminate isolates in methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *Staphylococcus aureus* (MSSA) [90, 95, 96]. In fact, this study detected a peak of 2647 *m/z* value that was pointed out as one of the characteristic peaks exhibited by MSSA [90]. Therefore, it was

hypothesized again that antibiotic susceptibility profile might be the cause of MALDI-TOF MS classification.

Although the progressive advances demonstrated in microbial diagnosis field, the ability of MALDI-TOF MS to identify SCV has not been reported. This study reported for the first time evidences that MALDI-TOF MS could discriminate colony morphotypes at least by their size. The two groups that MALDI-TOF MS classification originated were composed by non-SCV (A and B) and SCV (C and D). This ability of MALDI-TOF MS to distinct SCV is of great importance in clinical diagnosis. SCV could be detected from complex infecting populations more quickly. Even so, this ability to differentiate SCV should be more intensively studied with higher number of SCV samples, distinct SCV morphotypes and SCV of non-*S. aureus*.

In short, the discrepancies observed between MALDI-TOF MS and colony morphology methods for *P. aeruginosa* and *S. aureus* may arise due to the differential antibiotic patterns exhibited by the colony morphotype-associated bacteria. Indeed, a number of studies have reported that MALDI-TOF MS may distinguish antibiotic resistant and susceptible strains of the same species [90, 91, 95, 96, 97]. In addition, the expression of differential virulence factors may also contribute to discrepancies between both techniques. The hypotheses should be addressed in future works to disclose which criterion or criteria MALDI-TOF MS is using to group colony morphotypes.

2.5. Conclusions

An accurate identification and characterization of pathogens is crucial in disease management. The appropriateness and effectiveness of the microbial diagnosis method influence the choice of the antimicrobial agent to be used in the treatment of infection. Despite the great potential of colony morphology characterization in the context of clinical diagnosis, scarce improvements have been noticed in this method. This chapter aimed at improving the colony morphology characterisation method at different levels in order to achieve appropriate experimental procedures, more quickness in analysis and data standardisation, consistency and unambiguity.

Although colony morphology characterisation method has a simple procedure to perform and analyse the colonies, there is great variability among experimental conditions, including colony growth time, colony density *per* plate and solid media. In this chapter, the impact of those parameters and also the impact of bacterial lifestyle (planktonic or biofilm) and bacterial genetic background were intensively studied. The results obtained demonstrated that all parameters had impact on colony morphogenesis and on detection of bacterial diversity. These results are extremely important because for the first time it is known which colony traits are affected by each experimental parameter. For instance, colony size was a trait highly dependent on solid media, which is of serious concern in the context of clinical diagnosis. Diagnosis could be distinct according the experimental procedure used, in this case solid medium, which consequently affects the antimicrobial therapy chosen. Inadequate diagnosis may lead clinicians to prescribe an

inadequate antibiotic for an infecting population that could encompass morphotypes that were no detected in that solid medium.

Time of colony growth also revealed to be an important culturing condition because insufficient time of growth may cause overlook of some colony morphotypes, for instance morphotypes formed by bacteria with slower growth rates, or wrongly identify colonies that have not concluded their morphogenesis yet. Therefore, it is urgent to standardize the experimental parameters. In this logic, some guidelines were proposed that should be taken into account when performing colony morphology characterization. The general use of these guidelines will allow to accurately inferring about bacterial diversity of the infecting populations, a quite valuable input to clinical diagnosis, aiding the decision-making towards the selection of the most suitable antibiotic and supportive treatments.

In addition to the morphological traits exhibited by the colonies which may be significantly affected by the procedures taken to grow the bacteria, colonies have been described using a panoply of vocabulary and morphological criteria. It is very important to establish the minimum set of information to be part of the morphotype description and to employ harmonised vocabulary in that morphological description. The standardisation of the colony morphology data was considered crucial to make the resulting knowledge more explicit, ensure data reliability and promote data interchange. In effect, standardization augments the global value of results leading to great advance of science knowledge. This advance is not limited to scientific purposes, as clinical diagnosis interests might gain serious advantages in eradication or, at least, control of bacterial infections.

The second part of this chapter focuses on extensively reviewing and collecting all the terms used in literature and organising them in a structured way. However, a system just based on the vocabulary of the current descriptions has exhibited some constraints. Those constraints were overcome with few alterations in the characterisation system. At the end, the proposed system encompassed 10 morphological criteria (form, margin, sheath, type of surface, texture, elevation, consistency, opacity, size, colour). Each criterion included detailed features that authors should use to describe bacterial colony morphologies (e.g. circular, wrinkled, opaque, small, green).

A general use of this system leads to consistent and unambiguous descriptions of morphotypes and systematic comparison of morphotypes across experiments (and laboratories), species, diseases and clinical samples. Combining the standardisation of the experimental conditions with a general lexicon, the understanding about infection development, bacterial diversification, adaptation and evolutionary mechanisms may impressively advance.

Among the current microbial diagnosis techniques, colony morphology is the method that returns the results later, normally 1-3 days, and with low-throughput. Therefore, any modification on this method reducing the time of analysis and to obtain the results is very welcome. In the third part of this chapter, MALDI-TOF MS was explored as a tool for bacterial colony morphotyping. MALDI-TOF MS approaches have been used to perform species identification and even strain differentiation but its ability to discriminate at colony morphology level has not been assessed yet. From the results obtained it was

verified that MALDI-TOF MS provided a different classification of the morphotypes in contrast to colony morphotyping, suggesting that MALDI-TOF MS of intact bacteria could, in some extent, be used to complement the classical morphological characterisation. It was hypothesized that MALDI-TOF MS could be used as criterion of classification the antibiotic resistance profile. Future studies should urgently address this issue to find out whether this was in fact the criterion of MALDI-TOF MS classification. The discovery of that criterion will allow the fast categorization of colony morphotypes, which represents a huge advance in colony morphology characterisation. The method will take advantage of the fast and high-throughput MALDI-TOF MS technology to test large amounts and complex clinical samples simultaneously, without the need of culturing steps, acquiring information about the infecting population diversity at species, strain and intra-strain level.

2.6. References

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2.7. Supplemental Material

Table S2.1. Definitions of the terms that composed the sub-categories of the last version of the colony morphology characterisation system.

Sub-category name	Definition
Form	
Circular	The external appearance or configuration of a colony by having a form of a circumference or a circle due to any point of the edge be equidistant from the centre.
Elliptical	The external appearance or configuration of a colony by having a oval form with two axes of symmetry, as produced by a conical section.
Filamentous	The external appearance or configuration of the colony area by having a threadlike growth, i.e., extensions at the margin.
Rhizoid	The external appearance or configuration of the colony area by having extensions radiating from its centre.
Spindle	The external appearance or configuration of the colony area by having a long tapered rod that is wide in the middle and tapers at both ends.
Irregular	The external appearance or configuration of a colony by not having any distinguishable form.
Margin	
Entire	A margin quality of a colony by having a straight well-defined line border without toothing, irregularities or other projections.
Undulate	A margin quality of a colony by having a wavy indentation.
Lobate	A margin quality of a colony by having marked indentation-forming lobes.
Erose	A margin quality of a colony by having tooth-like indentation.
Curled	A margin quality of a colony by having parallel chains in undulate fashion on the border.
Filiform	A margin quality of a colony by having filaments.
Sheath	
Present sheath	The existence of a structure after the margin that surrounds a colony, the sheath.
Absent sheath	The lack of a structure after the margin that surrounds a colony, the sheath.
Type of Surface	
Homogeneous	A surface quality of a colony by having only one type of texture.
Heterogeneous	A surface quality of a colony by having more than one type of texture.
Texture	
Smooth	A texture quality of a colony by having a surface free of roughness or vertical irregularities.
Rough	A texture quality of a colony by having small vertical irregularities.
Wrinkled	A texture quality of a colony by having large vertical irregularities.

Table S2.1 (continuation)

Sub-category name	Definition
Consistency	
Brittle	A consistency quality of a colony by shattering easily when touched by a loop.
Dry	A consistency quality of a colony by not being covered by a liquid.
Moist	A consistency quality of a colony by having humidity, not completely dry.
Mucoid	A consistency quality of a colony by having consistency of mucus.
Viscous	A consistency quality of a colony by having viscosity.
Elevation	
Flat	An elevation quality of a colony by having no discernable raise, being thin spread over the agar surface.
Raised	An elevation quality of a colony by being slightly elevated.
Convex	An elevation quality of a colony by having a dome-like raise.
Pulvinate	An elevation quality of a colony by being marked convex or having a convex cushion-like raise form.
Umbonate	An elevation quality of a colony by having a rounded knob raised or knoblike protuberance.
Crateriform	An elevation quality of a colony by having a raise with a depression.
Opacity	
Transparent	An optical quality of the colony mass by full transmitting light.
Translucent	An optical characteristic of the colony mass by partial transmitting light.
Opaque	An optical quality of the colony mass by not transmitting light.
Iridescent	An optical quality of the colony mass by changing colour in reflected light as the angle of view changes.
Size	
Small	A size quality of a colony by having a physical dimension or extension or magnitude nearly one-tenth less than the size of the wild-type colony.
Large	A size quality of a colony by having a physical dimension or extension or magnitude higher than the size of the wild-type colony.
Diameter	A size quality which is equal to the length of any straight line segment from side to side through the centre of the circular colony or the average of the lengths of the longest and shortest line segments from side to side through the centre of the non circular colony.

Chapter 3

Understanding biofilm population diversity and functionality to prevent chronic infections development

3

Understanding biofilm population diversity and functionality to prevent chronic infections development

Abstract

The extensive *P. aeruginosa* diversification in CF-adapted phenotypes allows bacteria to persist in CF lungs for long periods of time causing chronic infections hardly eradicated by antibiotics. Biofilms are the main cause of phenotypic heterogeneity, antibiotic resistance and persistence of these infections. Therefore, the early detection of biofilm formation is of upmost importance in CF disease management. Colony morphology variation had proven to be a reliable method to detect biofilm-phenotypes representative of the existent sub-populations (Chapter 2), however it is unknown the typical *P. aeruginosa* colony-biofilm variants. Being, this study aimed at determining one or more colony morphology variants characteristic of biofilm growth to better detect biofilm formation in further studies. This study also aimed to investigate the functionality of the distinct sub-populations within *P. aeruginosa* biofilms in order to gain insights into biofilm recalcitrance mechanisms. The results revealed that biofilm population diversity was strain-dependent and a colony variant characteristic of *P. aeruginosa* biofilm growth was not found. Nevertheless, SCV seemed to be a strong hint of sessile lifestyle. This study highlighted as well that population diversity seemed to have no impact on augmented biofilm virulence potential and resistance to the antibiotic tested. In contrast, the absence of SCV within biofilm populations was associated with increase biofilm virulence potential, notably with increased amount of biofilm produced. This correlation was proven by robust statistical tests. In conclusion, the results obtained constituted a new perspective on *P. aeruginosa* biofilms, in particular on biofilm dynamics and functionally.

3.1. Introduction

The long-term persistence of *P. aeruginosa* infections in CF lungs is associated with clonal diversification into specialized phenotypes that causes chronic infections hardly eradicated [1, 2, 3, 4]. Biofilms are the underlying cause of chronic infections and they provide a reservoir of high phenotypic diversity [5, 6, 7]. This great variability of phenotypes is certainly one of the major contributors for sessile bacteria recalcitrance that it is not observed in planktonic state [8, 9, 10]. CF chronic isolates of *P. aeruginosa* display a number of characteristics indicative of sessile lifestyle, including overproduction of alginate, slow growth rate, loss of motility and antibiotic resistance [5, 11, 12]. Biofilm phenotypes could be also observed by a wide range of distinct colony morphologies as detected in Chapter 2, section 2.2. It is recurrent the isolation from biofilms colony morphologies such as mucoid variants, SCV and rough SCV [7, 13, 14, 15]. As these variants are rarely observed in planktonic state, the phenotypic switching for mucoid and SCV has been pointed out as biofilm mechanisms against antimicrobial agents and survival in stressful environment as CF lungs [16, 17, 18].

It is thought that bacteria mode of growth at the early stage of CF disease is essentially the planktonic state because early CF isolates exhibited identical microbial characteristics of their wild-phenotypes species. They displayed dry (non-mucoid) colony morphology, sensibility to antibiotics and have low bacterial density in lungs in contrast to chronic infections [19, 20, 21]. Until now, it is not clear what time bacteria after CF airway colonization switch to sessile lifestyle and develop chronic infections. This temporal window until initiation of biofilm formation is of upmost importance to CF diagnosis and disease management. Before biofilm formation, bacteria are more sensitive and infections are thus more easily eradicated. Mature *P. aeruginosa* biofilms could be generally never be eradicated mostly due to be multidrug resistant [22]. Early eradication avoids thus the biofilm formation and the establishment of chronic infections and consequently *P. aeruginosa* long-persistence in CF lungs.

Based on the previous findings that colony morphology variants of biofilm cells were different from the planktonic counterparts (Chapter 2, section 2.2), it was hypothesized that these variants could be markers of biofilm formation and growth. Using a colony biofilm marker, it would be easier identify biofilm formation and gain knowledge about the time and the driven forces of bacteria switching to biofilms in CF sputum. As so, this study used seven *P. aeruginosa* strains to study biofilm diversity and to determine the typical *P. aeruginosa* colony biofilm variants. Phenotypic heterogeneity is one of the major contributors to biofilm persistence in human host, but the role of each subpopulation in biofilm pathogenesis is poorly understood. In this study, it was also aimed to verify the function of population diversity on biofilm pathogenesis potential.

3.2. Materials and Methods

Bacterial strains and culture conditions

Seven *P. aeruginosa* strains were used throughout this work and they are listed in Table 3.1. Four strains were laboratory strains (commonly named as reference strains) originally isolated from clinical patients and environment locations but have been used for laboratory study for decades. These strains were ATCC 10145 (from now on named as ATCC), PA01, PA14 and CECT 111. PAI1 strain was isolated from an endoscope, PAI2 and PAI3 were isolated from infected patients with no record of CF disease. These seven strains were used due to their distinct genetic background that ensure biofilm variability, based on previous results obtained in Chapter 2.

Bacteria were routinely cultured on TSB or TSA at 37 ºC. All strains were preserved in criovials (Nalgene) at – 80 ± 2 ºC to minimise putative adaptation to the laboratory environment. Prior to each experiment, bacterial cells were grown on TSA plates for 24 h at 37 ºC.

Table 3.1. Description of the *P. aeruginosa* strains used in this study.

***P. aeruginosa* strain**	**Description**	**Reference**
ATCC 10145	Laboratory sequenced strain	[23]
PA01	Laboratory sequenced strain	[24]
PA14	Laboratory sequenced strain	[25, 26]
CECT 111	Reference strain	
PAI1	Isolate from an endoscope	[27]
PAI2	Clinical isolated from non-CF environment	[28]
PAI3	Clinical isolated from non-CF environment	[28]

Planktonic growth

Planktonic bacteria grew overnight in TSB at 37 ºC, 120 rpm. Cell suspension of each strain was washed twice in sterile water by centrifugation (9000 *g*, 5 min) and, further serial diluted and plated on TSA to perform colony morphology characterisation. The supernatants of cell suspensions were used to determine the pyocyanin produced.

Biofilm formation

Biofilms were developed as previously described by Stepanovic *et al.* (2000) [29]. Planktonic cell suspension of each strain was diluted in TSB to obtain 10^7 CFU/mL as final concentration. Afterwards, 200 μL of the bacterial suspension was transferred to a 96-well polystyrene microtiter plate where biofilms were developed aerobically on a horizontal shaker (120 rpm) at 37 ºC for 24 h. After that, biofilms were sonicated into sterile water to detach cells from biofilms, vortexed to homogenize and biofilm-cells were serial diluted with PBS and spread on TSA for viable and culturable cells counting and colony morphology characterisation. Other part of the biofilm cell suspension was used to determine the antibiotic susceptibility and virulence factors expression.

Counting the number of viable cells

The number of viable cells obtained from biofilm cell suspensions was determined through colony-forming unit (CFU). Cell suspensions were serial diluted, plated on TSA and incubated overnight at 37 °C. After that, the number of colonies were counted and verified after more 24 h of growth.

Observation and classification of colony morphology

To inspect colony morphology, biofilm-bacteria were allowed to grown on TSA plates during 48 h at 37 ºC. This step was aimed to obtain complete and unchangeable morphotypes according the results obtained in Chapter 2, section 2.2. Colony morphologies were observed by directly placing the petri plates on a magnifying glass (Olympus SZ-CTV) and photographed in a CCD camera (AVC, D5CE; Sony, Tokio, Japan). The morphological features of the colonies were annotated using morphological criteria previously stipulated in Chapter 2, section 2.3. In brief, colonies were evaluated according their form, margin, type of surface, texture, size, sheath, elevation, opacity, consistency and colour. A phenotypic variant was considered when it differed in at least one of the referred morphological parameters and it was considered a member of the population when detected at least 3 in 5 independent assays.

Antibiotic susceptibility assays

The antibiotic susceptibility profiles were constructed determining the minimum inhibitory concentration (MIC) by microdilution assays following the recommendations of the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards) [30]. MIC was assayed using biofilm cells treated with different concentrations of antibiotics, ranging 0,125 to 16 mg/L of ciprofloxacin (Fluka) and colistin (Sigma Aldrich), 0,125 to 64 mg/L of amikacin and tobramycin (Sigma Aldrich), in Mueller Hinton Broth (MHB) at 37 ºC, 120 rpm for 24 h. After antibiotic treatment, MIC was determined by the concentration of antibiotic required to inhibit 90 % of growth measuring OD at 640 nm on a microtiter plate colorimeter (Bio-Rek Synergy HT, Izasa). The following clinical breakpoints for resistance were used: amikacin, MIC ≥ 64 mg/L; ciprofloxacin, MIC ≥ 4 mg/L; colistin, MIC ≥ 8 mg/L; tobramycin, MIC ≥ 16 mg/L [30]. All tests were performed 5 times with 3 technical replicates.

Characterisation of virulence factors expression

- Total amount of biofilm biomass

The total amount of biofilm produced (cells and exopolysaccharide matrix) was evaluated through a quantitative assay of total using crystal violet (CV) adapted from Stepanovic *et al.* (2000) [29]. After biofilm formation during 24 h, the content of the plates was discarded and washed twice with sterile water in order to remove weakly attached cells and cell products that were in suspension. Afterwards 200 µL *per* well of methanol were added and plates were allowed to stand for 15 min in order to fix the biofilm-cells. Methanol was discarded and plates were left to dry at room temperature. Biofilms were stained with 200

µL of pure CV for approximately 5 min and were rinsed thoroughly and repeatedly with tap water. Finally, the amount of biofilm formed was quantified by solubilization of the CV in 200 µL of 33% (v/v) acetic acid. The optical density (O.D.) was measured at 590 nm using a microtiter plate reader (Bio-Rek Synergy HT, Izasa).

- Motility determination

Motility, including swimming, swarming and twitching, was assessed using the following procedures as described previously [19]. Swimming motility: 10^8 CFU/mL of each biofilm cell suspension was inoculated in TSA plates with 0,3 % (w/v) of agar and incubated for 24 h at 37 ºC in air conditions. Swimming was assessed by measuring the circular turbid zone around the inoculation point. Swarming motility: 10^8 CFU/mL of each biofilm cell suspension was inoculated in TSA plates with 0,5 % (w/v) of agar and incubated for 24 h at 37 ºC in air conditions. Swarming motility was indicated by irregular branching that appeared at the periphery of the colonies and this turbid zone was measured. Twitching assay: 10^8 CFU/mL of each biofilm cell suspension was inoculated in TSA plates with 1,5 % (w/v) of agar and incubated for 24 h at 37 ºC in air conditions. Twitching motility was indicated by a hazy zone of growth at the interface between the agar and the polystyrene surface and this hazy zone of growth was measured.

- Hemolysin production

Hemolytic activity of biofilm cells was assessed using Columbia Agar (CA; agar, 15 g/L, Liofilchem; Columbia broth, 43 g/L, Liofilchem) with 40 % (v/v) of sheep blood (Probiologica) plates. CAB plates were incubated 24 h at 37 ºC in air conditions. Hemolysin negative production was defined when no clearing zones were observed around the isolated colonies [19].

- Pyocyanin production

Pyocyanin production was determined for *P. aeruginosa* planktonic and biofilm populations. These assays were carried out on culture supernatants during growth in TSB as previously described [31]. Bacteria were pelleted at 15 000 *g* for 15 min and the free-cell supernatants containing pyocyanin were carefully collected and mixed with the same volume of chloroform. Mixtures were vigorously vortexed and centrifuged at 17 000 *g* for 1 min. Afterwards the inorganic phase was discarded and added to the organic phase, containing the pyocyanin (blue-green coloured), the same volume of 0.2 M HCl. Mixtures were vortexed and centrifuged at 17 000 *g* for 1 min. The pyocyanin-containing phase (pink layer) was collected and OD was measured at 520 nm using microtiter plate reader (Bio-Rek Synergy HT, Izasa). Concentration of pyocyanin, expressed as micrograms *per* millilitre of TSB supernatant, was determined by multiplying the OD by 17,072 [31].

Statistical Analysis

Results are reported in terms of population mean ± standard error of the mean (SEM). Values of biofilm formation (OD_{590} values), motility (zone diameters) and pyocyanin production (concentration values) represent means of 3 biological replicates and the value for each replicate is the average of 8 to 12 technical replicates for biofilm formation and 3 for motility and pyocyanin production. The significance of the difference between virulence factors expression among biofilm populations was analysed by using the one-way ANOVA and post-hoc by Tukey Multiple Comparison Test. Association of population diversity and the presence SCV, with virulence factors expression and antibiotic resistance was analysed using bivariate correlation and Pearson correlation, respectively. The statistical analysis was performed using SPSS version 20.0 (Chicago, Illinois, USA), and p values < 0.05 were considered significant.

3.3. Results

Colony morphology diversity

Planktonic and biofilm population diversity of the seven strains of *P. aeruginosa* (PA01, ATCC 10145, CECT 111, PA14 and 3 clinical isolates, PAI1, PAI2 and PAI3) was examined through colony morphology characterization. *P. aeruginosa* was grown overnight in planktonic state and in biofilms for 24 h and bacteria plated on TSA to evaluate population diversity. Prior to colony examination, knowing that reduce viable and culturable cell number could mask population diversity and lead to misinterpretations, the number of viable and culturable cells of each biofilm formed was determined. Figure 3.1A showed that all biofilms have identical number of cells (p > 0,05) indicating that further examinations could be done without an additional normalization step.

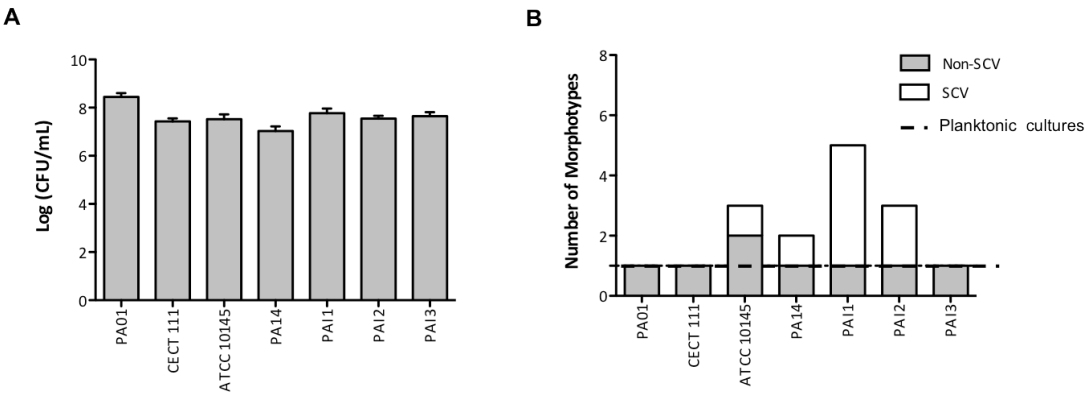

Figure 3.1. (A) Number of viable and culturable cells recovery from the distinct *P. aeruginosa* biofilms. The bars and whisker represent mean values ± SEM; (B) Diversity of biofilm populations expressed in number of colony morphology variants. Population diversity was determined after at least 5 independent assays.

Planktonic and biofilm population diversity was determined based on the number of distinct colony morphologies identified. Each *P. aeruginosa* colony formed by planktonic and biofilm bacteria on the solid media was inspected in terms of form, margin, surface, texture, size, presence of sheath, elevation, opacity, consistency and colour as described in Chapter 2, section 2.3. All planktonic populations did not demonstrate diversity being each *P. aeruginosa* population composed by only one morphotype (Figure 3.1B), called as wild-morphotype (WM). The WM of the majority of *P. aeruginosa* strains were large (~7,5 mm of diameter after 48 h onto TSA), dry, flat and opaque colonies with homogenous surface and rough texture (Table 3.2 and Figure 3.2). In contrast, 24 h-biofilms exhibited variable number of colony morphologies. *P. aeruginosa* biofilms could include in their populations exhibiting only one colony variant such as PA01, CECT and PAI3 biofilms or to be composed by several colony variants such as ATCC, PA14, PAI1 and PAI2 biofilms (Figure 3.1B). Colony morphology of biofilm cells demonstrated alterations in size and sheath and, less frequent, in texture in comparison with WM (Table 3.2 and Figure 3.2).

Among the colony biofilm variants, special focus was put in the inspection of mucoid variants and SCV due to their attributed importance related to biofilm resistance mechanisms. All morphotypes whose diameters were less than 3 mm were considered as SCV [18]. Results showed that all biofilms formed did not include in their populations mucoid variants, but SCV were frequently detected (never more than 20 % of frequency), with the exception of the biofilms formed by PA01, CECT111 and PAI3 (Figure 3.1B). Moreover, some biofilms were composed by more than one type of SCV, such as PAI1 and PAI2 (Figure 3.2). Comparing these results with those of section 2.2 of Chapter 2 some incongruities were noticed. In section 2.2, it was reported that ATCC biofilm populations included 3 distinct large colonies and no SCV (Figure 2.3 in Chapter 2) and PAI1 included 8 SCV and 2 large colonies (Figure 2.4 in Chapter 2). The differences could be explained by the different preparation of TSA plates. To perform the present study, it was followed all the guidelines described in section 2.2, in particular, it was standardized the height of solid medium among TSA plates to 0,5 cm. The study of the section 2.2 was performed without that concerns of height of solid medium. This experimental parameter was only detected as a factor that influences colony morphology but was not study and no standardised in section 2.2. The different height of solid medium may have produced variations in detection of colony morphologies. From now on, the analysis of the results was performed considering the information of biofilm population diversity obtained in the present study.

Despite the variable number and distinct colony morphologies, biofilm populations shared some characteristics. WM was present in all biofilm populations (Figure 3.2), with the highest prevalence, and rough SCV was frequently detected (Table 3.2), in 4 of 7 biofilms. Although these similarities, it was not found a colony morphology biofilm characteristic of *P. aeruginosa* sessile lifestyle, i.e., there was not a colony variant that has emerged in all *P. aeruginosa* biofilms.

Table 3.2. Detailed morphological description of the colonies identified in *P. aeruginosa* biofilm populations.

	Form	Margin	Sheath	Type of Surface	Texture	Consistency	Elevation	Opacity	Size	Colour
PA01										
planktonic	circular	undulate	present	homogeneous	rough	dry	flat	opaque	large	yellow
biofilm	circular	undulate	present	homogeneous	rough	dry	flat	opaque	large	yellow
CECT111										
planktonic	circular	undulate	present	homogeneous	rough	dry	flat	opaque	large	green
biofilm	circular	undulate	present	homogeneous	rough	dry	flat	opaque	large	green
ATCC 10145										
planktonic	circular	undulate	absent	homogeneous	rough	dry	flat	opaque	large	yellow
biofilm	circular	undulate	absent	homogeneous	rough	dry	flat	opaque	large	yellow
biofilm	circular	undulate	present	homogeneous	rough	dry	flat	opaque	large	yellow
biofilm	circular	undulate	absent	homogeneous	rough	dry	flat	opaque	small	yellow
PA14										
planktonic	circular	erose	present	homogeneous	rough	dry	flat	opaque	large	brown
biofilm	circular	erose	present	homogeneous	rough	dry	flat	opaque	large	brown
biofilm	circular	undulate	absent	homogeneous	rough	dry	flat	opaque	small	brown
PAI1										
planktonic	irregular	undulate	present	homogeneous	smooth[a]	dry	flat	opaque	large	yellow
biofilm	irregular	undulate	present	homogeneous	smooth[a]	dry	flat	opaque	large	yellow
biofilm	circular	entire	absent	homogeneous	smooth	dry	flat	opaque	small	yellow
biofilm	circular	entire	absent	heterogeneous	rough and smooth	dry	flat	opaque	small	yellow
biofilm	circular	entire	absent	homogeneous	rough	dry	flat	opaque	small	yellow
biofilm	circular	entire	present	homogeneous	rough	dry	flat	opaque	small	yellow
PAI2										
planktonic	circular	entire	absent	homogeneous	rough	dry	flat	iridescent	large	blue-green
biofilm	circular	entire	absent	homogeneous	rough	dry	flat	iridescent	large	blue-green
biofilm	circular	entire	absent	homogeneous	rough	dry	flat	iridescent	small	blue-green
biofilm	irregular	undulate	absent	homogeneous	wrinkled	dry	flat	iridescent	small	blue-green
PAI3										
planktonic	circular	undulate	present	homogeneous	rough	dry	flat	opaque	large	yellow
biofilm	circular	undulate	present	homogeneous	rough	dry	flat	opaque	large	yellow

[a] smooth texture with several surface element as “craters”

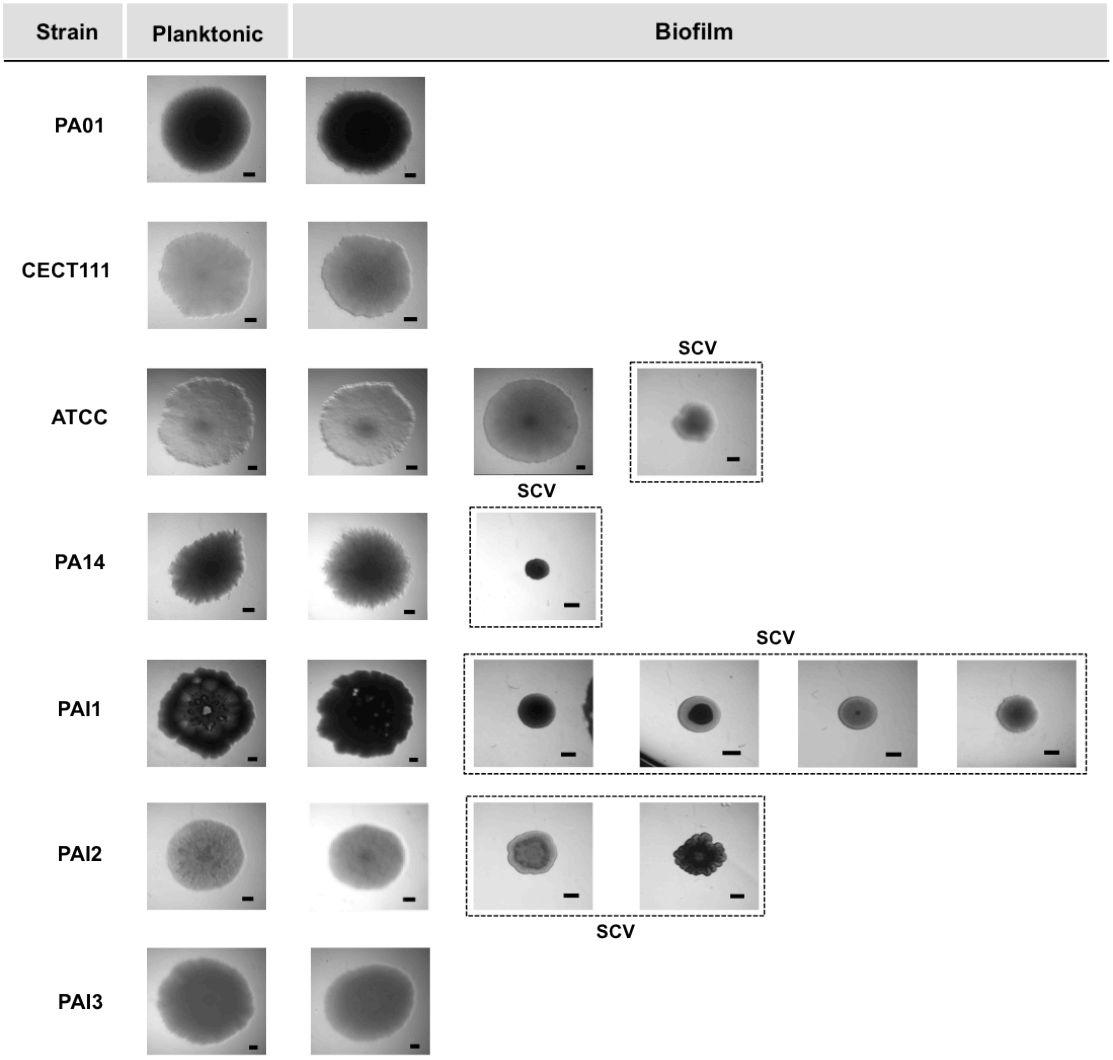

Figure 3.2. Appearance of colony morphology variants of *P. aeruginosa* planktonic and biofilm populations. Dashed line indicated SCV. Black bars = 1 mm.

Pathogenic profiling of *P. aeruginosa* biofilms

Because of the extensive colony variability within and among biofilms, the role of population diversity and, in particular, the SCV in biofilm pathogenesis was investigated. It was aimed to determine whether: 1) the population diversity was an indicator of augmented pathogenic potential of *P. aeruginosa* biofilms, i.e., whether biofilms with higher number of colony morphotypes exhibited more virulence potential and/or antibiotic resistance; and if so, 2) the presence of SCV was the responsible for the augmented biofilm virulence potential and/or resistance to antibiotics. To answer these questions, firstly the virulence factors expression and antibiotic resistance profiles of biofilm cells were determined to following perform the correlation among virulence and resistance profiles with population diversity and the presence of SCV.

It is undeniable the impressive arsenal of virulence factors that *P. aeruginosa* possesses with increase ability to cause damage to host. In this study, it was only analysed some virulence factors that have

particular impact in the context of CF–associated infections. The ability to form biofilm is one of the most important virulence factors of *P. aeruginosa* and it can be an indicator of the adaptive capacity of a strain to survive and grow in new environments as CF lungs [32, 33]. All *P. aeruginosa* biofilms demonstrated to be composed by identical number of viable and culturable cells (Figure 3.1A), but they can differ in the amount of matrix produced (Figure 3.3). Biofilm matrix has a crucial role in biofilm recalcitrance because act as protective barrier against antibiotics, host immune defences molecules, dehydration and ROS [34, 35]. Possible differences in the amount of matrix produced would be reflected in the total amount of biofilm biomass. Therefore, the CV staining method was used to determine the total amount of biofilm biomass (cells and matrix) and, thus, infer about the relative capacity of strains to form biofilms during 24 h. The results showed that *P. aeruginosa* strains formed biofilms with distinct total amount of biomass ($p < 0,05$, Figure 3.3), leading to conclude that they had produce different amounts of matrix.

PA01, ATCC and PAI1 displayed significantly increased biofilm amounts in comparison to the other strains, suggesting that these biofilms had a ”better” physiological barrier, the exopolysaccharide matrix. This matrix represents an ecological advantage in persistence in challenging environments as CF lungs. Despite the differences among *P. aeruginosa* strains, it should be highlight the impressive ability that all *P. aeruginosa* strains showed to form biofilms.

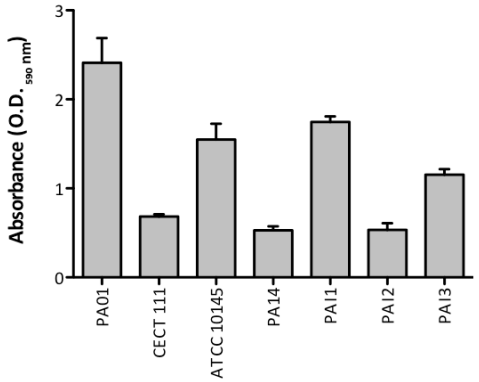

Figure 3.3. Total amount of biomass of 24 h *P. aeruginosa* biofilms measured by CV staining methods (biomass was quantified in terms of absorbance at 590 nm). The bars and whisker represent mean values ± SEM.

In addition to the different amount of exopolysaccharide matrix produced by biofilm cells, *P. aeruginosa* biofilms seemed to be composed by bacteria with different abilities to swim, swarm and twitch. Although the wide range of swimming, swarming and twitching motilities observed, it was verified that all *P. aeruginosa* biofilms seemed encompass niches where it was allowed the three types of motility (Figure 3.4A, B and C). The amount of pyocyanin produced by biofilm cells was also quite variable among strains (Figure 3.4D), but it was noticed a trend of *P. aeruginosa* to reduce pyocyanin production after switching to biofilm mode of growth.

Regarding the biofilm resistance to antibiotics, *P. aeruginosa* biofilms exhibited slightly differences in MIC values of amikacin and tobramycin, but according to CLSI susceptibility interpretation criteria [30] they

were equally categorised as sensitive to these antibiotics (Table 3.3). Ciprofloxacin and colistin MIC values were identical to all *P. aeruginosa* biofilms and categorised as resistant and sensitive, respectively.

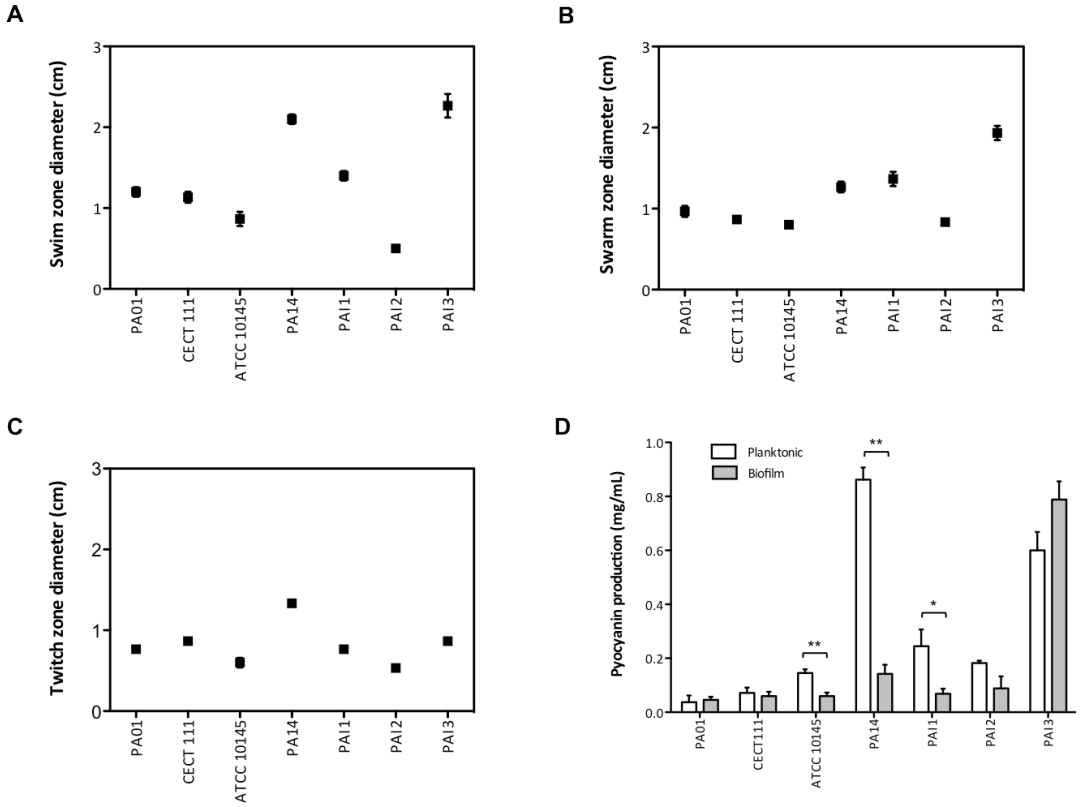

Figure 3.4. (A) Swimming, (B) swarming and (C) twitching motilities of biofilm-cells. Each point is the mean of the results from three biological replicates. (D) Pyocyanin production of planktonic and biofilm-cells of each *P. aeruginosa* strain. * $p < 0,05$; ** $p < 0,01$

Table 3.3. Characterization of the susceptibility profiles to amikacin (AK), ciprofloxacin (CIP), colistin (COL) and tobramycin (TOB) of the *P. aeruginosa* biofilm-cells.

Strain	MIC (mg/mL)			
	AK	**CIP**	**COL**	**TOB**
PA01	0,5 to 1	4	2	0,125 to 0,25
CECT 111	0,5	4	2	0,125
ATCC 10145	0,5 to 1	4	2	0,125
PA14	0,5	4	2	0,125
PAI1	0,5 to 1	4	2	0,125 to 0,25
PAI2	0,5 to 1	4	2	0,125 to 0,25
PAI3	1	4	2	0,25

The role of diversity and SCV within biofilms

To determine the role of population diversity and, in particular, SCV in the biofilm virulence potential, a statistical analysis of correlation (Pearson Correlation and Mann U Whitney test) was performed to ensure results reliability.

From the statistical results obtained, the distinct abilities to form biofilms were not related to the number of colony variants constituent of biofilms (Table 3.4), but, interestingly, they were associated with SCV (Table 3.5). That means populations without SCV tend to produce biofilms with increased biomass (Figure S3.1). Still regarding biofilm virulence potential, motility and pyocyanin production were not correlated with diversity of biofilm population and the SCV presence within biofilm populations (Table 3.4 and 3.5). In summary, the absence of SCV seemed to be associated with increased biofilm biomass.

As antibiotic resistance profiles of *P. aeruginosa* biofilms were identical, the statistical analysis was not performed because the assumption of variability for these factors was not accomplished.

Table 3.4. The association of diversity population with biofilm formation, swimming, swarming and twitching motilities, according Pearson correlation. Statistical significance was determined by two-tailed *p* value.

	Biofilm formation		Swimming		Swarming		Twitching		Pyocyanin production	
	χ^2	*p*-value	χ^2	*p*-value	χ^2	*p*-value	χ^2	*p*-value	χ^2	*p*-value
Diversity	-0,047	0,839	-0,039	0,868	0,153	0,509	-0,160	0,490	-0,148	0,522

Table 3.5. The association of SCV presence within populations with biofilm formation, swimming, swarming and twitching motilities, according Mann U Whitney. Statistical significance was determined by two-tailed *p* value.

	Biofilm formation		Swimming		Swarming		Twitching		Pyocyanin production	
	U	*p*-value	U	*p*-value	U	*p*-value	U	*p*-value	U	*p*-value
SCV	26,000	0,047	38,500	0,269	41,500	0,365	34,000	0,148	46,000	0,568

3.4. Discussion

P. aeruginosa infections in CF lungs generally persist despite the use of aggressive and long-term antibiotic treatments mainly because bacteria form antibiotic–resistant biofilms [36, 37, 38, 39]. To form biofilms bacteria need to undergo several changes to adapt to so spatially structure environment producing several colony variants with altered morphology. This study aimed to determine which were the

typical colony variants found in *P. aeruginosa* biofilms to better detect biofilm formation in further studies. To investigate the colony biofilm variants, isogenic populations of WM were allowed to form biofilms during 24 h in TSB. The results demonstrated that planktonic populations were homogenous composed by only one morphotype - the WM - that shared several colony traits among the seven *P. aeruginosa* strains, including circular form, homogenous type of surface, dry consistency, opaque opacity and large size.

Biofilm populations were much more complex indicating that biofilm mode of growth selected specific phenotypes. *P. aeruginosa* biofilm populations were not identical among the seven strains varying in number and colony morphological traits. The 24 h-biofilms were composed since only one morphotype until 5 distinct morphotypes. Although they shared the same genome that encodes the functional response of bacteria to external stimuli and the genes involved in regulation are highly conserved, the differences of biofilm population diversity among *P. aeruginosa* strains may be the result of the instability of the core elements that can function differentially [40]. Consequently, *P. aeruginosa* strains produced different biofilms with singular niches. The diversity of niches encouraged adaptive radiation and the emergence of biofilm niche-specialist observed in this study by colony morphology variation. Therefore, the differential diversity of biofilm population observed among strains could be supported by biofilms were formed by niches with different environmental conditions. Despite the variability of *P. aeruginosa* biofilm populations, they shared some characteristics. The WM of each strain was observed in all biofilms studied as previously reported in Chapter 2, section 2.2. Furthermore, the different biofilms could shared some colony variants, such as SCV and rough SCV, indicating that distinct biofilms could include similar niches in their structures. Similar environmental conditions and selective pressures within niches generate similar colony variants.

SCV have been intensively studied because of their particular characteristics, such as increased antibiotic resistance, twitching motility, increased fitness under stationary growth, biofilm formation ability, autoaggregative traits, auxotrophy and reduced respiration [18, 41, 42, 43]. Such variants are often isolated from laboratory biofilms [5, 7, 44], and more importantly from CF lungs [13, 44, 45]. The isolation of SCV from biofilms in this study reinforced the hypothesis that biofilms are present in CF lungs. Some authors have pointed out that SCV could be one of the specific-biofilm phenotypes that bacteria switch on in order to better adapt to biofilm lifestyle [16, 17, 18]. In this study, SCV was not detected in all biofilm populations, which could led to question whether SCV is indeed a specific-biofilm phenotype. But, it must be stressed that the observed diversification within biofilms is not necessarily indicative of sustained diversity and differences among populations could be transient. Population diversity could be dynamic changing over time, influenced by the surrounding environmental conditions and external factors exposure. More studies are needed to fully understand the biofilm population, its evolution and functionality over time and the influence of external factors over the population.

In this study, it was observed that WM dominated and SCV never made up more than 20 % of the biofilms, which led to conclude that SCV was only able to grow in some niches of biofilms and thus it was considered a colony variant niche specialist. The variability of morphological traits exhibited by SCV found

in some biofilms could be an indicator of distinct SCV niche specialists. Isolation of distinct SCV from biofilms it was reported previously [5, 13, 18, 41, 46].

Although the frequent detection of SCV in *P. aeruginosa* biofilms, they were not observed in all *P. aeruginosa* biofilms and thus SCV was not considered a colony variant characteristic of biofilm growth, but a strong hint. WM was present in biofilm populations but was also not considered a candidate because it was present in planktonic cultures making discrimination between the two modes of growth not possible. Therefore, it was conclude based on the gathered results that there was not a colony morphology marker for 24 h-biofilms of *P. aeruginosa*.

Although the inexistence of a colony morphology marker and given the distinct colony heterogeneity found among *P. aeruginosa* biofilms, it was sought to determine whether biofilms with increased population diversity and with SCV had augmented pathogenesis potential. Understanding the biofilm functionality, i.e., the role of population diversity and SCV within biofilms is crucial to CF-associated infections diagnosis, to improve the actual treatments of *P. aeruginosa* biofilm-associated infections and, consequently, to prevent chronic infections development. To test this hypothesis, it was characterized the virulence factors expression and antibiotic susceptibility profiles of the biofilm cells.

The virulence factors provide to bacteria tools to persist and cause damages in the host usually necessary for infection or disease development [32, 47, 48]. The ability to form biofilms, the production of extracellular (proteases and pyocyanin) and cell-associated (flagella and type IV pili) virulence factors are among the most important *P. aeruginosa* virulence determinants in CF context [19, 49, 50]. The biofilm cells of the seven *P. aeruginosa* strains exhibited quite diverse abilities to form biofilms, to swim, swarm and twitch and to produce pyocyanin. Among so much variability, it was noticed that all 24 h-*P. aeruginosa* biofilms were composed by niches where flagella expression was conserved as swimming motility of biofilm-cells was verified. It is known that bacteria inside a biofilm do not require extensive motility until the time they detach to colonize another available surface [19, 51, 52, 53]. However, biofilms are composed by stratified niches and conservation of flagella may be indicative of the niche localization inside the biofilms. These niches where flagella expression was conserved may be located at the top of biofilms where bacteria still conserved several planktonic-associated characteristics such as swimming motility. The results obtained from colony morphology characterisation supported this conclusion as the WM was detected in all biofilms. Therefore, WM could be the colony variant representative of this top biofilm niche. To determine the location of other biofilm niches, it would be interesting to individually characterize the remaining colony morphologies and predict the respective niche location. This issue should be addressed in near future since it could provide valuable insights about biofilms ecology, recalcitrance and exposure new target sites for antibiotics act on.

All *P. aeruginosa* biofilm cells exhibited in this study similar susceptibility profiles to the in-use CF antibiotics. Given these findings population diversity and SCV seemed not play a role in antibiotic recalcitrance of biofilms. But, regarding the biofilm virulence potential, the absence of SCV in populations was related to biofilms with increased biomass. This result contradicts some of the traditional concepts held in literature. SCV are typically considered excellent biofilm formers and thus it would be expected that

biofilms with SCV would have more biofilm biomass. Bofilms with SCV may have other advantages that biofilm without SCV do not. For instance, SCV may play a crucial role when communities face stressful conditions, such as pH alterations, osmotic stress, starvation, host immune molecules action. SCV could be responsible to ensure community survival and regrowth when a stress vanish [7, 54, 55, 56]. Under certain circumstances, subpopulations inside of biofilms might have a survival advantage over others resisting to the stressful conditions imposed. This question should be addressed in future studies to clarify the role of SCV in biofilms.

Current management of CF airway infections includes the use of antibiotics that reduce patient morbidity and mortality, but could also led to collateral effects as the emergence of antibiotic resistant bacteria [34, 57, 58]. Although at early CF infection stage it is expected bacteria growing in planktonic stage, increased concentrations of antibiotics are typically needed due to the adverse environmental surrounding conditions that affect the antibiotic action, such as extracellular DNA and hypoxia. The antibiotic treatments are being more aggressive in attempt to early eradicate bacteria, in particular *P. aeruginosa*, and to avoid persistence of infection. This trend had raised the need to attain higher organism-specific MIC. Determination of MIC is the first line technique of discovery for indicators of antibiotic activity against bacteria and indicates the lowest concentration of an antimicrobial agent to inhibit the growth of a particular organism. In this study it was determine the MIC of most commonly used antibiotics in early CF infections treatment, including ciprofloxacin, colistin, tobramycin and amikacin [57, 59, 60]. Based on the MIC values obtained in this study, it was hypothesized that early treatments could use concentrations of 4, 2, 1 and 0,25 mg/L of ciprofloxacin, colistin, amikacin and tobramycin, respectively. According pharmacodynamics and pharmacokinetics studies these concentrations could be use without adverse side effects in patients [61, 62, 63, 64, 65, 66, 67, 68]. The efficacy of antibiotic treatments using these concentrations was evaluated in Chapter 4.

3.5. Conclusions

Bacteria within biofilms are phenotypically different from their freely swimming counterparts. In this study, it was aimed to determine the characteristic colony morphologies of *P. aeruginosa* biofilm populations to enhance the detection of biofilm formation in further studies. The results revealed that biofilm populations varied in number of colony variants and in morphological traits. The increased diversity of colony variants inside biofilms resulted possibly due to bacteria be confronted with different selective pressures imposed in each biofilm-niche that favour optimized growth activities and thus the emergence of subpopulations with distinct colony morphologies. The variable colony variants among *P. aeruginosa* biofilms may be explained by the different ability of strains to form biofilms and with different regulatory responses despite belonging to the same species.

SCV was studied with particular attention due their supposed contribution to biofilm resistance against antibiotics. It was detected SCV within biofilm populations in opposite to planktonic cultures. This lead to conclude that these variants may result from adaptations undergone by bacteria to the specific sessile

lifestyle, making SCV a potential candidate to colony-biofilm marker. However, SCV was not observed in all *P. aeruginosa* biofilms, being no possible to consider SCV as candidate colony marker of biofilm growth but only a strong hint. In fact, none of colony variants identified in biofilm populations seemed to be a marker of *P. aeruginosa* biofilm growth.

The inconsistent diversity and presence of SCV among *P. aeruginosa* biofilms led to question the role of diversity and SCV in biofilm pathogenesis. Biofilm cells were characterized in order to understand the association between diversity and SCV with biofilm virulence potential and antibiotic resistance. Surprisingly, the increased diversity and the presence of SCV within biofilm populations was not found as an adaptive advantage in terms of antibiotic resistance. But, the absence of SCV was associated with increased biofilm formation ability. Anyway, population diversity and SCV could be an advantage in stressful conditions not tested in this study and be responsible for community survival and re-establishment. In these conditions SCV and higher number of distinct sub-populations may play a crucial role.

3.6. References

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3.7. Supplemental Material

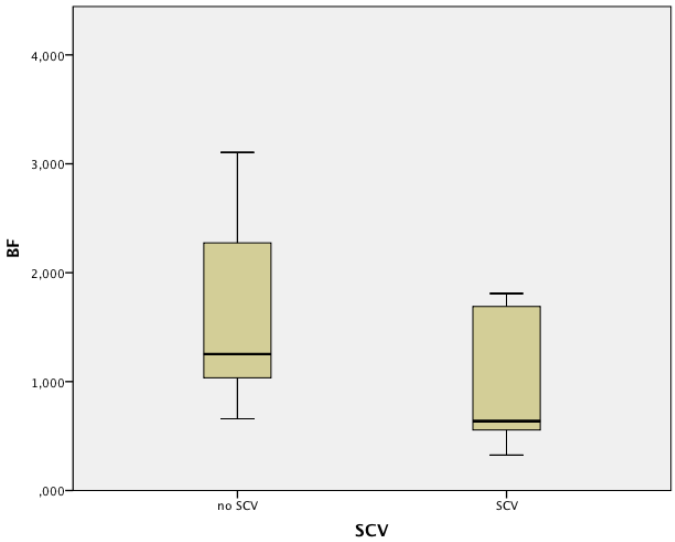

Figure S3.1. Boxplot of the total biofilm mass (cell and matrix) of biofilms with and without SCV. The black line within the box indicates the median of each dataset. The edges of rectangles correspond to the first and third quartile of the data set.

Chapter 4

Pseudomonas aeruginosa diversification at early infection stages in cystic fibrosis environment

4

Pseudomonas aeruginosa diversification at early infection stages in cystic fibrosis environment

Abstract

P. aeruginosa diversification has been pointed out as the major cause of chronic infections of CF patients, but little is known about the early stages of infection. The main goal of this study was to investigate the evolutionary processes associated with *P. aeruginosa* adaptation to the CF lungs and with early infection development. This study aimed to go further and intended to study *P. aeruginosa* evolutionary processes in relevant clinical scenarios for CF antimicrobial stewardship, such as different initiation timings of antibiotic treatments and antibiotic concentrations. Treatments could be initiated soon as first positive detection, called early antibiotic treatments, or could be initiated more lately, called delayed antibiotic treatments. The concentrations of the antibiotics administered to CF patients are variable which may impact on *P. aeruginosa* adaptation to the CF lungs and on the eradication success. To perform this study, bacteria were grown in artificial sputum medium (ASM) with and without early and delayed ciprofloxacin treatments with distinct concentrations. Population diversity was analysed through colony morphology sampled during ten days and mapped the temporal diversity variation within populations. The virulence factors expression and the antimicrobial resistance profiles of the distinct colony morphotypes were determined in order to identify the biological traits altered to *P. aeruginosa* achieve adaptation and/or to develop infection.

The results revealed clearly that the timing of antibiotic treatment initiation and the antibiotic concentration play a crucial role in early *P. aeruginosa* eradication. Early antibiotic treatments using adequate antibiotic concentrations, correspondent to biofilm-cells inhibitory concentrations, had strong possibilities to achieve successful eradication. In contrast, delayed antibiotic treatments exhibited quite variable successful eradication rates that demonstrated to be highly dependent of the adaptive ability of the *P. aeruginosa* strain. The constructed temporal diversity maps disclosed: 1) a trend of *P. aeruginosa* for diversification after initial CF environment colonization promoted by the CF environmental conditions; and 2) a propensity of inadequate ciprofloxacin treatments (early and delayed) in promoting diversification into new “fitter” variants impacting on eradication. Interestingly, all *P. aeruginosa* evolution/adaptation pathways included a common alteration, the impaired swimming motility. As so, this alteration was considered a potential disease marker for early *P. aeruginosa* adaptation and infection development. Furthermore, it was found that colony morphology variation was a sign of evolution/adaptation of *P. aeruginosa* and, more importantly, colony traits could be used as useful indicators of antimicrobial resistance profiles to some antibiotics, as well as to indicate the expression of some virulence factors proven by several statistical tests.

All these results will have soon impact on clinical diagnosis and will introduce great improvements in CF disease management. Such as, actual antibiotic treatments could be re-design adjusting the dosage of antibiotics take in account new standards, for instance the inhibitory concentration of biofilm-cells, early infections could me more easily detected using a impaired swimming motility as disease marker and resistance profiles and virulence factors expression could be anticipated by colony morphology observation.

4.1. Introduction

P. aeruginosa is the most prevalent pathogen in chronic infection stages contributing to lung function decline and ultimate mortality in CF patients [1, 2]. Although *P. aeruginosa* is the most common pathogen in adulthood, it is also frequently isolated from CF lungs of younger patients [3, 4]. Patients infected with *P. aeruginosa* at younger age generally have numerous respiratory complications and slower growth resulting in decreased quality of life and decreased chances of survival [5, 6, 7].

It is known that during infection development in CF lungs, *P. aeruginosa* switch from an acute environmental virulent pathogen, characteristic from early infection stages, to a CF-adapted pathogen typical from chronic infection stages [8, 9, 10]. Similar evolution and adaptation profiles were observed in distinct clonal lineages of CF-adapted strains suggesting parallel evolution of *P. aeruginosa* in CF airways driven by similar selective pressures [11, 12]. Genome sequence analyses have substantiated this conclusion because of the recurrent mutational patterns detected in independent *P. aeruginosa* lineages [13, 14, 15]. In addition to this parallel evolution, other studies have reported the evidences of intraclonal *P. aeruginosa* diversification [16, 17]. A significant level of phenotypic diversity is commonly observed from *P. aeruginosa* clones that display distinct colony morphologies, differences in QS regulators, antibiotic resistance and expression of virulence factors [8, 18, 19, 20, 21]. The specific causes and underlying mechanisms of intraclonal diversity are difficult to unveil and thus they are incompletely understood. Both pathways, parallel evolution and intraclonal diversification, seemed to be relevant in CF airway disease because ensure phenotypic diversity considered fundamental to the development of chronic infections.

Although all this knowledge about *P. aeruginosa* adaptation to CF lungs, it is not clear: i) which are the *P. aeruginosa* early adaptive changes after CF lungs colonisation; ii) which those changes are associated with the early infection development and transition to chronic stages; iii) what time bacteria switch and which are the environmental conditions that trigger this switching. This situation undermines the development of rationales for the selection of accurate antibiotic treatment regimes and achieving successful rates of *P. aeruginosa* early eradication. Early eradication is a central part of CF management as it avoids infection establishment and its progression to chronic stages. Inappropriate initial antibiotic treatments in addition to causing initial adverse effects in patient outcome can compromise the success of future antibiotic treatments. Therefore, CF disease management will greatly benefit from the understanding of *P. aeruginosa* early adaptive changes and the underlying mechanisms.

The ability to anticipate the bacterial pathogenic potential and the response to antibiotic treatments is also of interest. Predict antibiotic susceptibilities and the expression of virulence factors of the bacteria isolated from CF patients by, for instance, bacterial characteristics or colony features could made antimicrobial stewardship more assertive because it takes in account the particular characteristics of that specific infecting population. Therefore, to gain insights about the processes and mechanisms undergone by *P. aeruginosa* after initial colonization of CF lungs, this study investigated the early adaptation changes of three *P. aeruginosa* strains focusing on their phenotypic evolution, the environmental conditions that

trigger adaptation and the underlying mechanisms. Furthermore, it was attempted to correlate *P. aeruginosa* phenotypic characteristics (antibiotic susceptibilities and virulence factors expression), colony morphology traits, infection development and population diversity in order to determine putative disease markers and indicators of antibiotic failure.

4.2. Materials and Methods

Bacterial strains and culture conditions

P. aeruginosa PA01, ATCC 39324 and a clinical isolated non-CF related, PAI2, were used throughout this study. *P. aeruginosa* ATCC 39324, from now on referred as ATCC, is a mucoid strain and PA01 and PAI2 are dry strains. Bacteria were routinely cultured on TSB or TSA at 37 ºC. All strains were preserved in criovials (Nalgene) at – 80 ± 2 ºC to minimise putative adaptation to the laboratory environment. Prior to each experiment, bacterial cells were grown on TSA plates for 24 h at 37 ºC.

Artificial sputum medium preparation and bacterial growth conditions

Artificial sputum medium (ASM) was used to mimic the sputum of CF patients [22]. The ASM was prepared following the protocol of Sriramulu, D. (2010) [23]. ASM contained 5 g/L of mucin from pig stomach (Sigma-Aldrich), 4 g/L of DNA from salmon sperm (Sigma-Aldrich), 5,9 mg/L of diethylene triamine pentaacetic acid (DTPA, Sigma-Aldrich), 5 g/L of NaCl, 2,2 g/L of KCl and 5 g/L of casoamino acids (AMESRO). pH was adjusted to 7,0 with Tris base. ASM was sterilized in an autoclave at 110 ºC for 15 min and, after cooled, 5 mL of egg yolk emulsion (Fluka) was added. Two mL of ASM were transferred to each well of a 24 well-plate and inoculated on the top with 5 μL of bacterial cell suspensions obtaining a final cellular concentration in each well of 10^7 CFU/mL. *P. aeruginosa* inocula were obtained by growing each bacterial strain overnight in TSB at 37 ºC and 120 rpm in air conditions. The resulting cell suspensions were washed twice in sterile water by centrifugation (9000 *g*, 5 min) and further serial diluted to a final concentration of 4 x 10^9 CFU/mL. 5 μL of these bacterial suspensions were used to inoculate ASM. ASM cultures were incubated at 37 ºC for 10 days in static and air conditions. Static conditions were used in this study because the cilliary movements in CF lungs are typically reduced or absent [24, 25].

As referred in Chapter 1, antibiotic treatments should be administered to CF patients as soon as *P. aeruginosa* is detected in order to early eradicate bacteria and avoid infection development or progression for chronic stages. However, in some cases, *P. aeruginosa* could be detected later and, consequently, antibiotic treatments are also initiated later. In this study these two antibiotic regimes were investigated and to distinguish them, the formers were named as early antibiotic treatments and the others named as delayed treatments.

To mimic early antibiotic treatments, sub- and inhibitory concentrations of ciprofloxacin were added to the top of ASM cultures after 30 min of the bacterial inoculation. New antibiotic dosages were added to ASM every 24 h over 10 days. Delayed antibiotic treatments were mimicked adding sub- and inhibitory

concentrations of ciprofloxacin to ASM cultures after 5 days of bacterial growth. In this regime new antibiotic dosages were added to ASM every 24 h over the next 5 days. The concentrations of ciprofloxacin used in this study were based on the MIC of biofilm-cells of the three strains determined in Chapter 3. The inhibitory concentration of ciprofloxacin towards biofilm-derived cells was 4 mg/L and 0,5 and 1 mg/L were chosen as sub-inhibitory concentrations.

After 1, 3, 5, 7 and 10 days of bacterial growth in ASM, the content of wells was collected aseptically and was vigorously shaken to detach cells from the small aggregates and those adhered to mucin. From these cell suspensions that contained the whole population resident in ASM, called from now on as whole population, it was determined the number of viable cells, the population diversity through colony morphology diversity, the pyocyanin production, the mutation frequency and the antibiotic susceptibility. After numbering the viable cells, the whole resident population in ASM was divided in sub-populations according the colony morphologies identified. One colony morphotype represented one sub-population. The identified sub-populations were characterized in terms of virulence factors expression and antibiotic susceptibility. All experiments were performed at least 5 times.

Counting the number of viable cells

The number of viable cells obtained from ASM suspensions was determined through colony-forming unit (CFU). Cell suspensions were serial diluted, plated on TSA and incubated overnight at 37 °C. After that, the number of colonies were counted and verified after more 24 h of growth. Through the number of viable cells, it was constructed the growth kinetics of *P. aeruginosa* strains in ASM and the time killing curves of the *P. aeruginosa* strains grown in ASM and exposure to the different antibiotics treatments.

Colony morphology diversity

According the results obtained in Chapter 2, *P. aeruginosa* colonies should be grown at least 45 h to obtain complete and unchangeable morphotypes and to avoid that small colonies (slow growing bacteria) were overlooked. As so, to assess colony morphology diversity, bacteria were allowed to grow on TSA plates during 48 h at 37 °C in air conditions. Colony morphologies were observed by directly placing the petri plates on a magnifying glass (Olympus SZ-CTV) and photographed in a CCD camera (AVC, D5CE; Sony, Tokio, Japan). The morphological features of the colonies were annotated using the morphological criteria previously stipulated (Chapter 2). In brief, colonies were evaluated according their form, margin, type of surface, texture, size, sheath, elevation, opacity, consistency and colour.

After morphological characterisation of colonies, it was evaluated the stability of the morphological traits spreading repeatedly colonies in TSA plates, at least 10 times, and after 48 h of growth re-evaluated their morphology. This procedure allowed to distinguish the non- from the revertant phenotypes.

The distinct colony morphotypes were referenced with an identity number, for instance MT01. Identity numbers had not any correlation with the order of emergence or relationships with other colony

morphotypes within populations. It was used only to refer colonies with common features and distinguished them one from another.

Through colony morphology characterisation over ten days, it was constructed maps of the evolutionary profiles of the three *P. aeruginosa* strains in the several ASM growth conditions.

Characterisation of virulence factors expression

The bacteria derived from the different morphotypes were characterized according the virulence determinants more relevant in the CF context, namely biofilm formation, motility, auxotrophy, pigments and hemolysin production. All the tests were performed at least 5 times and each test used colonies of the identified morphotypes from the different biological assay. Moreover, in all tests 3 technical replicates were performed to ensure precision.

Pyocyanin production was the only parameter studied for both colony morphotypes and the whole populations collected after 1, 3, 5, 7 and 10 days of bacterial growth in ASM.

- Biofilm formation

The ability of colony morphotype-associated bacteria to form biofilms was assessed by the crystal violet (CV) method [26]. Colony morphotypes were suspended and diluted in TSB to obtain a final concentration of 10^7 CFU/mL. The cell suspensions were transferred to 96-well microtitre plates (200 µL *per* well) being then incubated at 37 ºC for 24 h under agitation (120 rpm) in air conditions. After 24 h of biofilm formation, the content of the plates was discarded and the adhered biomass to the plates was washed twice with sterile water in order to remove weakly attached cells. Afterwards, 200 µL *per* well of methanol were added and plates were allowed to stand for 15 min in order to fix the biofilm-mass. Methanol was discarded and plates were left to dry at room temperature. Biofilms were stained with 200 µL of pure CV for approximately 5 min and then rinsed thoroughly and repeatedly with tap water. Finally, the amount of biofilm formed was quantified by solubilization of the CV in 200 µL of 33 % (v/v) acetic acid. The optical density (OD) was measured at 590 nm using a microtiter plate reader (Bio-Rek Synergy HT, Izasa).

- Motility determination

Swimming, swarming and twitching were assessed using the following procedures: i) swimming motility - 10^8 CFU/mL of each colony morphotype-associated bacteria were inoculated in TSA plates with 0,3 % (w/v) of agar and incubated for 24 h at 37 ºC in air conditions. Swimming was assessed by measuring the circular turbid zone around the inoculation point; ii) swarming motility - 10^8 CFU/mL of each colony morphotype-associated bacteria were inoculated in TSA plates with 0,5 % (w/v) of agar and incubated for 24 h at 37 ºC in air conditions. Swarming motility was indicated by irregular branching that appeared at the periphery of the colonies being this turbid zone measured; iii) twitching assay - 10^8 CFU/mL of each colony morphotype-associated bacteria were inoculated in TSA plates with 1,5 % (w/v) of agar and

incubated for 24 h at 37 ºC in air conditions. Twitching motility was indicated by a hazy zone of growth at the interface between the agar and the polystyrene surface and this hazy zone of growth was measured.

- Auxotrophy testing

This metabolic trait was investigated by determining the ability of colony morphotypes-associated bacteria to grow on glucose M9 media (AMESRO) with and without 0,2 % (w/v) of casoamino acids [21]. Colony morphotypes were transferred to M9 medium plates with and without casoamino acids and incubated at 37 ºC. Evaluations were performed at 24 and 48 h of growth. Absence of growth on M9 media without casoamino acids was indicative of auxotrophy.

- Pigments production

The pyocyanin production was performed for each cell suspension obtained from ASM cultures and for each colony morphotype identified to assess the ability of the whole resident population and specific sub-populations to produce this pigment, respectively.

To determine pyocyanin production of the whole population it was performed a quantitative approach. These assays were carried out on culture supernatants during growth in ASM as previously described [27]. Bacteria from cell suspensions collected after 1, 3, 5, 7 and 10 days of growth in ASM were pelleted at 15000 *g* for 15 min and the free-cell supernatants containing pyocyanin were carefully collected and mixed with the same volume of chloroform. Mixtures were vigorously vortexed and centrifuged at 17000 *g* for 1 min. Afterwards, the inorganic phase was discarded and to the organic phase, containing the pyocyanin (blue-green coloured), was added with the same volume of 0.2 M HCl. Mixtures were vortexed and centrifuged at 17000 *g* for 1 min. The pyocyanin-containing phase (pink layer) was collected and OD was measured at 520 nm using microtiter plate reader (Bio-Rek Synergy HT, Izasa). Concentration of pyocyanin, expressed as micrograms *per* millilitre of ASM supernatant, was determined by multiplying the OD by 17,072 [27].

Due to the high number of morphotypes and biological and technical replicates, the production of pyocyanin for each colony morphotype-associated bacteria was assessed using a qualitative approach. So, pyocyanin production was determined visually observing the colour of colonies and the surrounding agar medium. Colonies exhibiting blue-green colour on TSA plates were considered pyocyanin producers [28].

The production of other pigments was also determined using a qualitative approach because of the abovementioned reasons. Pyoverdine and pyomelanin production were determined visually from colour of colonies and the surrounding agar medium. Colonies exhibiting green and brown colours on TSA plates were considered pyoverdine and pyomelanin producers, respectively [28].

- Hemolytic activity

The hemolytic activity of colony morphotypes-associated bacteria was assessed using Columbia Agar (CA; agar, 15 g/L, Liofilchem; Columbia broth, 43 g/L, Liofilchem) with 40 % (v/v) of sheep blood (Probiologica) plates (CAB). CAB plates were incubated 24 h at 37 ºC in air conditions. Hemolysin production was considered negative when no clearing zones were observed around the colonies.

Antimicrobial susceptibility test

Due to the large number of antimicrobial susceptibility analysis, including whole populations, colony morphotypes and the number of independent replicates (at least 5 replicates) for each whole population and colony morphotypes, it was selected the disk diffusion method for its simplicity and reproducibility in a high-throughput format. The antimicrobial susceptibility profile of each morphotype was determined using ten antibiotics commonly used to treat *P. aeruginosa* infections in CF context: amikacin (AK) 30 μg, aztreonam (ATM) 30 μg, ciprofloxacin (CIP) 5 μg, tobramycin (TOB) 10 μg, gentamicin (CN) 10 μg, ceftazidime (CAZ) 30 μg, cefepime (FEP) 30 μg, imipenem (IMI) 10 μg, piperacillin (PRL) 100 μg and ticarcillin /clavulanic acid (TTC) 85 (75+10) μg, all obtained from Liofilchem. Disk diffusion susceptibility testing was performed in accordance with the recommendations of NCCLS document M100-522 [29]. The cartridges of antibiotic disks were stored at -20 ºC and allowed to come to room temperature prior to use. The inhibition zones were measured (mm) after 18-21 h at 37 ºC in air conditions. Whole population and colony morphotypes-associated bacteria were assigned as resistant (R), intermediate resistant (I) or sensitive (S) as defined by NCCLS guidelines.

Determination of mutation frequencies

Hypermutability was assessed through determination of spontaneous mutation frequencies on TSA agar containing 300 mg/L rifampicin (Sigma-Aldrich) following ASM collection as described by Oliver *et al.* (2000) [30]. ASM cultures were plated on TSA plates with and without 300 mg/L of rifampicin and incubated at 37 ºC. The number of colonies growing after 24 h of incubation was counted and confirmed after more 24 h of incubation (48 h in total). The mutation frequency was calculated as the fraction of resistant mutant cells (median number of mutant cells/median total cell number). Colonies growing on TSA plates with 300 mg/L of rifampicin were streaked onto another plate with 300 mg/L of rifampicin in order to assess the stability of mutants and to avoid mutation jackpot (the recovery by chance of a vast number of mutants). These experiments were performed at least 3 times.

Statistical analysis

Four continuous variables (biofilm formation ability, swimming, swarming and twitching motilities) and twenty categorical phenotypic characteristics (10 antibiotic resistance profiles and 10 colony morphology traits) were analysed.

CFU data were analysed by Mann–Whitney test, a non-parametric procedure to assess a null hypothesis that two populations are the same. *In vitro* biofilm formation data (OD values) were analysed using Kruskal Wallis (assumption of normality was not verified). Differences in proportions of colony morphology traits *per* antibiotic susceptibility status were estimated by the Chi-square test or Fisher's exact test when appropriated. Measures of association between colony morphology traits and bacterial phenotypic features were determined by Kolmogorov-Smirnov Z test. This test does much the same as the Mann-Whitney U test, however it tends to have better power that the Mann-Whitney U in smaller samples as of this study [31]. The independent factors were colony form, margin, sheath, consistency, opacity and size. As colony colour had more than two categories, a Kruskal-Wallis test was performed. All statistical analyses were performed in SPSS version 20.0 (Chicago, Illinois, USA) considering as level of statistical significance $p < 0,05$.

4.3. Results

The initial adaptation and the intraclonal diversity of *P. aeruginosa* populations were monitored after *in vitro* initial CF environment colonisation and growth in ASM during 10 days. The intraclonal diversity of *P. aeruginosa* populations was studied with respect to colony morphology variation. Thus, the presence of distinct colony morphologies allowed supposing the existence of sub-populations within the whole populations grown in ASM. To characterize the ecology of the whole *P. aeruginosa* populations in early-infected CF environment and to infer about the adaptive alterations undergone by bacteria, it was performed a phenotypic characterization of all sub-populations detected along the 10 days.

This study used three *P. aeruginosa* strains, two dry (non-mucoid) strains (PA01 and PAI2) and one mucoid (ATCC 39324) strain. The mucoid strain was included in this study to assess whether initial colonisation by a mucoid strain represents indeed a risk factor for the development of faster and worse infection. Early colonisation of the CF airways with these strains is associated with an accelerated rate of decline in pulmonary function [32].

4.3.1. Intraclonal diversification of *P. aeruginosa* after initial colonization

The three *P. aeruginosa* strains used in this study demonstrated identical growth profile in ASM without the presence of ciprofloxacin (Figure 4.1). After one day of exponential growth, the three populations achieved the stationary phase with no difference in the number of viable cells between all the populations. However their microevolution profiles were dependent on the strain (Figure 4.2). *P. aeruginosa* PAI2 and ATCC populations exhibited colony morphology diversity after 3 days of growth in ASM, contrary to PA01 strain that demonstrated no population diversity. *P. aeruginosa* PAI2 population exhibited the highest diversity since it generated 4 new distinct morphotypes all catalogued as SCV, while ATCC strain generated only one new morphotype, a dry variant. (Table 4.1) In terms of colony morphology alterations, ATCC wild-morphotype (WM), MT01, generated colonies with dry consistency (MT12). Regarding PAI2

strain, WM generated colonies with small size and/or with irregular form and/or with mucoid consistency (Table 4.1). Interestingly, the coexistence of several morphotypes within planktonic/inoculum populations was not observed previously (Chapter 3), which may mean that bacteria switch to new phenotypes possibly to adapt to CF environmental conditions, for instance new nutritional conditions, pH, DNA and salts concentration.

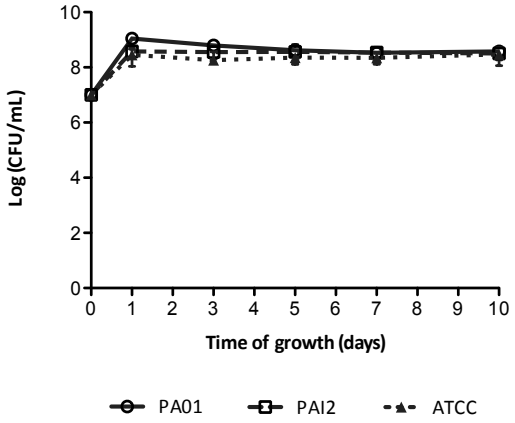

Figure 4.1. Growth kinetics of *P. aeruginosa* populations in ASM.

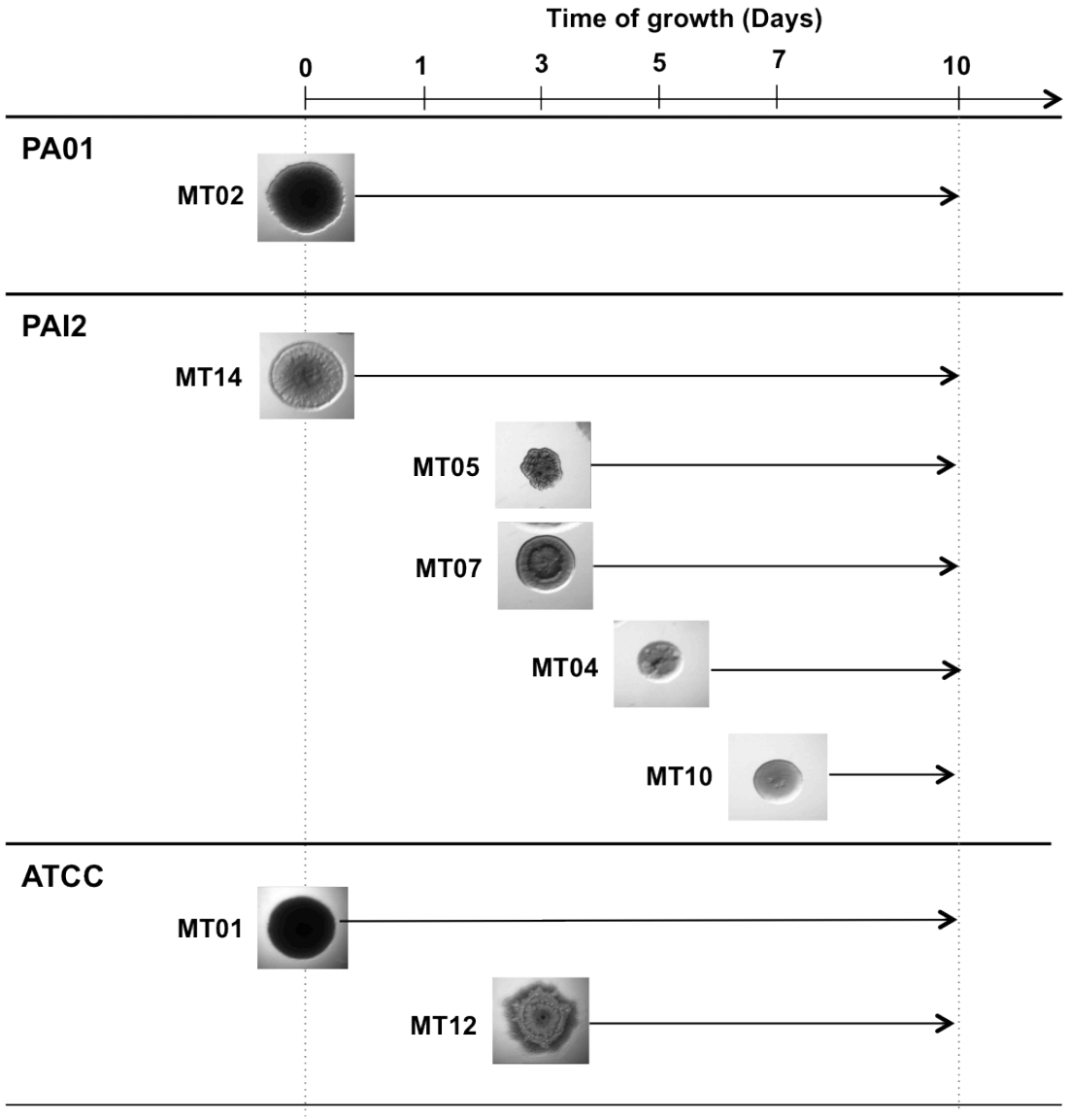

Figure 4.2. Evolutionary profiles of the three *P. aeruginosa* populations during 10 days of growth in ASM. Arrows represent the continuous presence of colony morphotypes within populations.

Table 4.1. Detailed morphological description of the colonies identified in *P. aeruginosa* populations grown from ASM with and without ciprofloxacin.

	Form	Margin	Sheath	Type of Surface	Texture	Consistency	Elevation	Opacity	Size	Colour
PA01										
MF02	circular	undulate	present	homogeneous	rough	dry	flat	opaque	large	yellow
MF17	circular	undulate	present	homogeneous	rough	moist	flat	opaque	large	yellow
PAI2										
MF14	circular	entire	absent	homogeneous	rough	dry	flat	iridescent	large	Blue-green
MF04	circular	undulate	absent	heterogeneous	smooth/rough	dry	flat	iridescent	small	Blue-green
MF05	irregular	undulate	absent	homogeneous	wrinkled	dry	flat	iridescent	small	Blue-green
MF07	circular	entire	absent	homogeneous	rough	dry	flat	iridescent	small	Blue-green
ATCC										
MF01	circular	undulate	present	homogeneous	smooth	mucoid	flat	opaque	large	beige
MF03	circular	undulate	present	homogeneous	rough	dry	flat	opaque	large	beige
MF12	circular	undulate	present	homogeneous	rough	dry	flat	transparent	large	beige
MF15	circular	undulate	present	homogeneous	rough	dry	flat	opaque	pinpoint	beige
MF19	circular	undulate	present	homogeneous	rough	dry	flat	opaque	small	beige
PA01 and PAi2										
MF10[a]	circular	entire	absent	homogeneous	smooth	mucoid	flat	opaque	small	yellow

[a] MF10 was observed in both PA01 and PAI2 populations.

Given the population diversity found in these CF populations, further research questions have emerged: was the population diversification indeed an indicator of *P. aeruginosa* adaptation to CF conditions and infection development? If so, which bacterial phenotypic traits could be considered indicators of initial adaptation and infection development? To answer these questions, the antibiotic resistance profiles and the expression of virulence factors of all colony morphotype-associated bacteria, representative of sub-populations, were investigated.

The *P. aeruginosa* PAI2 population demonstrated diversity either in antibiotic resistance and virulence factors expression. Two PAI2 morphotypes (MT04 and MT10) had an antibiotic resistance profile different of the WM, the MT14 (Table 4.2). MT04 and MT10 did not exhibit intermediate resistance against aztreonam and ticarcillin/clavulanic acid as MT14. Furthermore, MT10 exhibited intermediate resistance against ciprofloxacin in opposite to MT14 that was resistant. The distinct phenotypic features of MT04 and MT10 seemed influence the whole population response to ciprofloxacin and aztreonam (Figure S4.1 in Supplemental Material). The emergence of the sensitive morphotypes MT04 and MT10 within PAI2 populations at day 5 and 7, respectively, made populations more sensitive to antibiotics. For instance, the sensitivity of PAI2 population to ciprofloxacin and aztreonam increased from resistant to intermediate and intermediate to susceptible, respectively.

In terms of virulence potential, all the new PAI2 morphotypes exhibited impaired swimming motility (Figure 4.3A) and notable ability to form biofilms with exception of MT10 (Figure 4.3B). The expression of the other studied virulence determinants seemed to be unaltered (Table 4.3). Defective swimming motility and biofilm formation are characteristics typically associated with chronic isolates [24, 33], which may mean that PAI2 population had initiated its adaptation to CF conditions and started expressing some bacterial features fundamental to develop an infection.

Table 4.2. Antibiotic susceptibility profiles of PAI2 colony morphotypes-associated bacteria.

	CIP	**CN**	**TOB**	**AK**	**IMI**	**ATM**	**CAZ**	**FEP**	**TTC**	**PRL**
MT14 (WM)	R	S	S	S	S	I	S	S	I	S
MT04	R	S	S	S	S	S	S	S	S	S
MT05	R	S	S	S	S	I	S	S	I	S
MT07	R	S	S	S	S	I	S	S	I	S
MT10	I	S	S	S	S	S	S	S	S	S

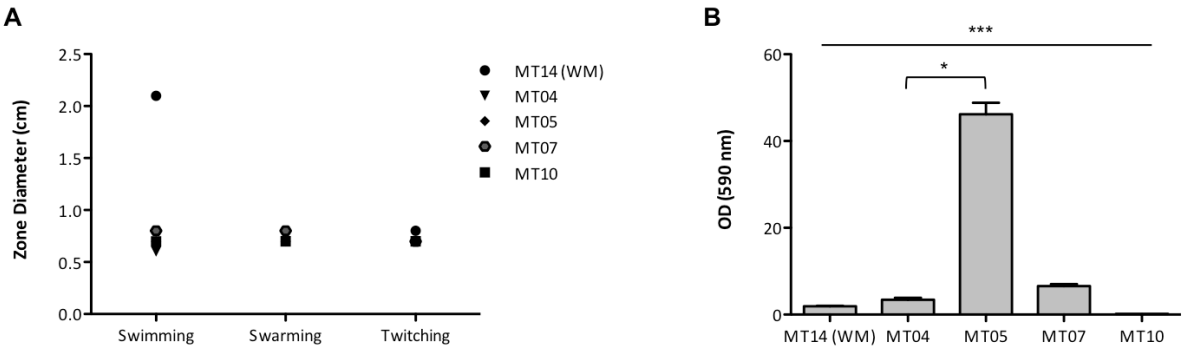

Figure 4.3. Phenotypic characterisation of the PAI2 colony morphotypes-associated bacteria: (A) swimming, swarming and twitching motilities and (B) ability to form biofilms. WM – wild-morphotype.

Table 4.3. Phenotypic profile of PAI2 colony morphology-associated bacteria related to hemolysin, pyoverdine, pyocyanin and pyomelanin production and auxotrophy.

	Hemolysin	Auxotrophy	Pigments production		
			Pyoverdine	Pyocyanin	Pyomelanin
MT14 (WM)	-	-	-	++	-
MT04	-	-	-	+[a]	+[b]
MT05	-	-	-	+[a]	+[b]
MT07	-	-	-	+[a]	+[b]
MT10	-	-	-	-	+[b]

[a] colonies exhibited blue-green colour when were grown surrounding each other.
[b] colonies exhibited brown colour when were grown in plates with lower colony concentration.

Concerning ATCC population, very little diversity was observed since the only one new morphotype, the MT12 (Figure 4.2), exhibited an antibiotic susceptibility profile identical to the WM, MT01 (Table 4.4). Moreover, no differences in overall population resistance against antibiotics were noticed (Figure S4.1 in Supplemental Material). Regarding virulence factors expression, some phenotypic heterogeneity was detected. MT12 exhibited impaired swimming motility (Figure 4.4A), better ability to form biofilms (Figure 4.4B) and pyocyanin production (Table 4.5) in comparison with ATCC WM, the MT01. Despite MT12 was a pyocyanin producer, the amount of pyocyanin found in the surrounding environment of the whole ATCC population was still very reduced in comparison with the whole PAI2 and PA01 populations (Figure 4.5). The reduced amounts of pyocyanin might be caused by the presence of only one sub-population pyocyanin producer and its reduced prevalence within population. Similarly to PAI2 population, it seemed that ATCC population had initiated its adaptation and started to develop infection.

Table 4.4. Antibiotic susceptibility profiles of ATCC colony morphotypes-associated bacteria.

	CIP	CN	TOB	AK	IMI	ATM	CAZ	FEP	TTC	PRL
MT01 (WM)	S	S	S	S	S	S	S	S	S	S
MT12	S	S	S	S	S	S	S	S	S	S

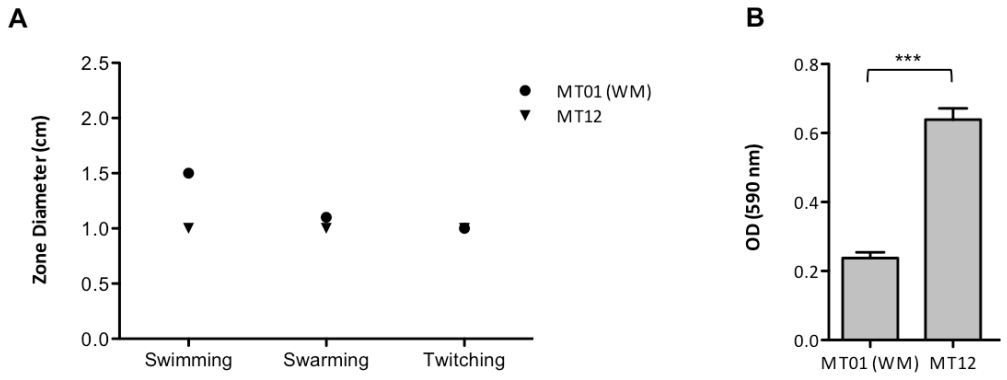

Figure 4.4. Phenotypic characterisation of the ATCC colony morphotypes-associated bacteria: (A) swimming, swarming and twitching motilities and (B) ability to form biofilms. WM – wild-morphotype.

Table 4.5. Phenotypic profile of ATCC colony morphology-associated bacteria related to hemolysin, pyoverdine, pyocyanin and pyomelanin production and auxotrophy.

	Hemolysin	Auxotrophy	Pigments production		
			Pyoverdine	Pyocyanin	Pyomelanin
MT01 (WM)	-	-	-	-	+
MT12	-	-	-	++	-

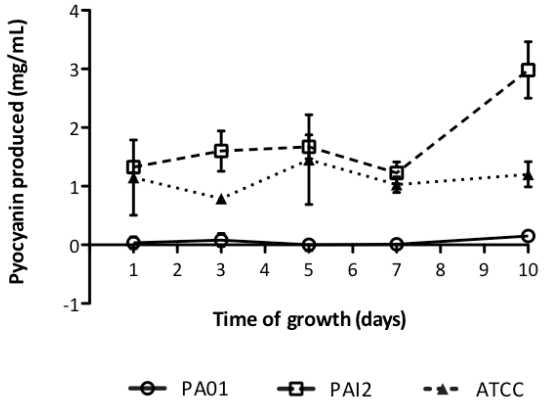

Figure 4.5. Pyocyanin production of PA01, PAI2 and ATCC populations grown in ASM along 10 days.

Concerning PA01 strain, its population did not exhibit diversification during all the 10 days, as only the WM, MT02, was observed (Figure 4.2). This event may mean that population did not start its adaptation and no infection was developed.

Taking together the results obtained for the three strains, it was verified that diversification can occur at early stages of disease and diversified populations was associated with *P. aeruginosa* adaptation and in a real scenario could be associated with infection development. This clonal diversification, however, was not caused by increased spontaneous mutations (Table S4.1 in Supplemental Material).

4.3.2. Effects of early ciprofloxacin treatments on *P. aeruginosa* populations

Typically, CF patients are subjected since first *P. aeruginosa* detection to extended antibiotic treatments in order to early eradicate infection and avoid progression to chronic stage. However, antibiotics generally do not reach the maximum (inhibitory) concentration in CF lungs due to, for instance the presence of mucin and extracellular DNA that interfere in antibiotic action. As so, bacteria are just exposed to sub-inhibitory concentrations. In this study, the impact of sub- and inhibitory concentrations of ciprofloxacin in *P. aeruginosa* growth, population diversity and evolution and response to ciprofloxacin treatment was evaluated. The inhibitory concentration of ciprofloxacin corresponded to the MIC of biofilm cells, 4 mg/L, previously determined (Chapter 3). Biofilm formation is characteristic of CF chronic stages and typically biofilm cells exhibited increased levels of antibiotic resistance [37, 74]. Therefore, the MIC of biofilm cells seemed a suitable therapeutic reference for early ciprofloxacin treatments. The sub-inhibitory concentrations tested were 0,5 and 1 mg/L.

The results obtained demonstrated that sub-inhibitory concentrations of ciprofloxacin were clearly inefficient to eradicate *P. aeruginosa* (Figure 4.6A and B). Some bacterial load reduction was indeed observed but only significant for the ATCC strain, where a 3 log reduction was attained using 1 mg/L of ciprofloxacin. Regarding the early ciprofloxacin treatment using 4 mg/L, it was observed PA01 and ATCC eradication, after three days of growth, and no interference in PAI2 growth (Figure 4.6 C).

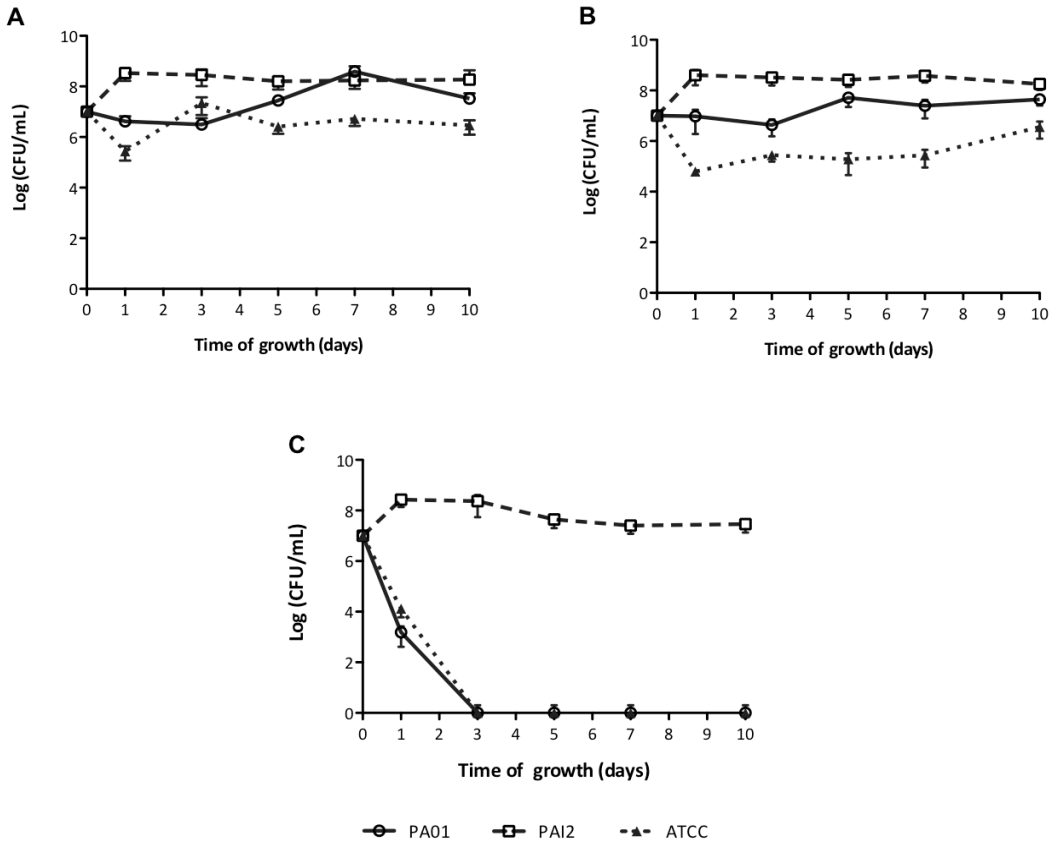

Figure 4.6. Time-killing curves of *P. aeruginosa* populations grown in ASM exposed to: (A) 0,5 mg/L; (B) 1 mg/L; and (C) 4 mg/L of ciprofloxacin.

The greatest impact of ciprofloxacin treatments seemed to be on population diversity. The exposure to sub-inhibitory concentrations, 0,5 and 1 mg/L, has changed the colony morphology diversity as new morphotypes have emerged (Figure 4.7 and 4.8). The WM of PA01 and ATCC switched to other variants with distinct colony traits giving rise to populations with distinct dynamics and structures in comparison with homologous populations grown only in ASM. In contrast, population diversity of PAI2 did not differ with ciprofloxacin treatment, as the same morphotypes were detected. However, these morphotypes emerged later, at day 7 and 10. It should be noted the negative selective pressure of 1 mg/L of ciprofloxacin that seemed to exert on PA01 and PAI2 populations. This selection was noticed because the no emergence of MT10 in PAI2 and PA01 populations (Figure 4.7 and 4.8). Among all the sub-populations within PAI2, this sub-population was the one who had exhibited intermediate resistance to ciprofloxacin.

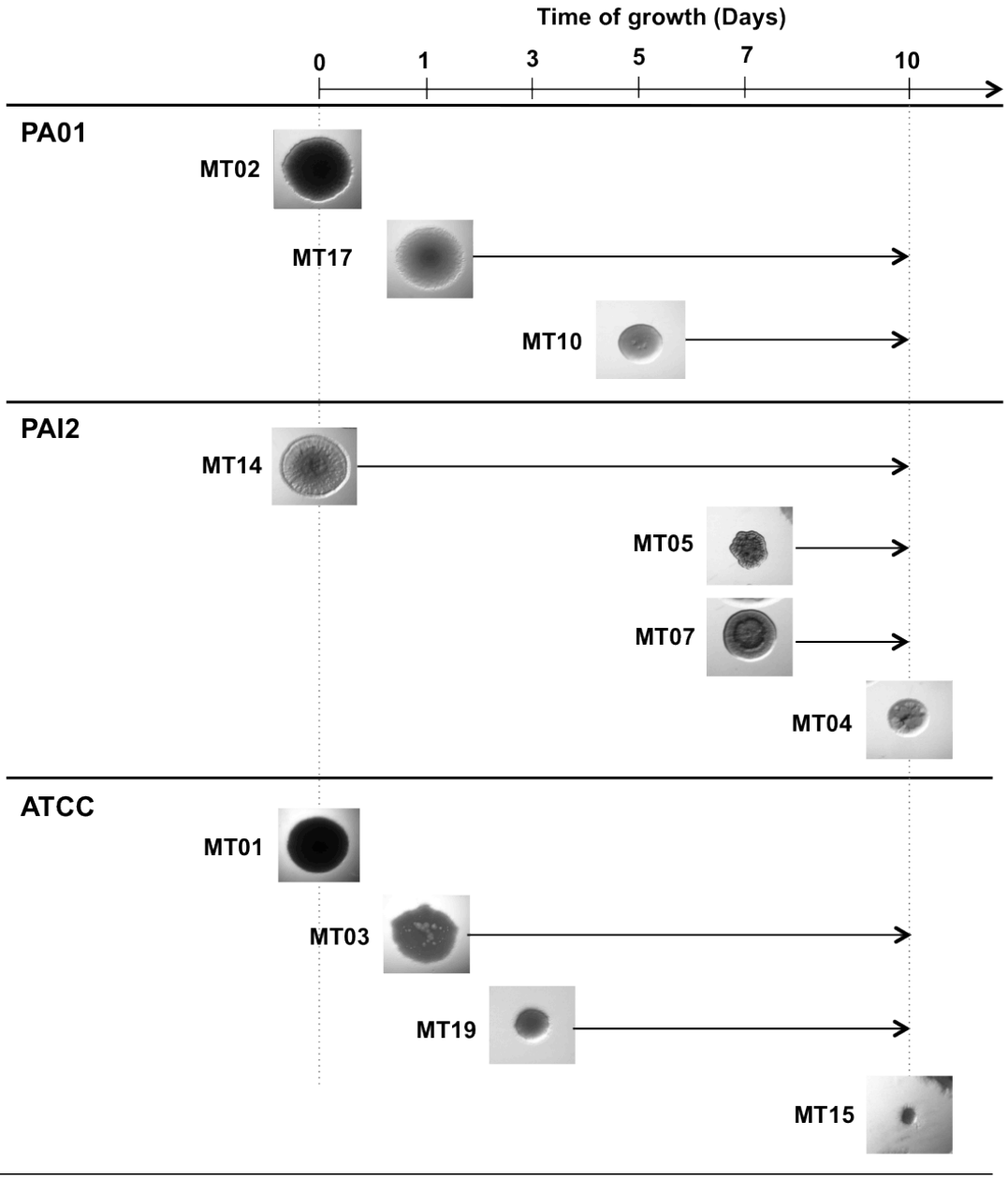

Figure 4.7. Evolutionary profiles of the three *P. aeruginosa* populations during 10 days of growth in ASM exposed to 0,5 mg/L of ciprofloxacin. Arrows represent the continuous presence of colony morphotypes within populations.

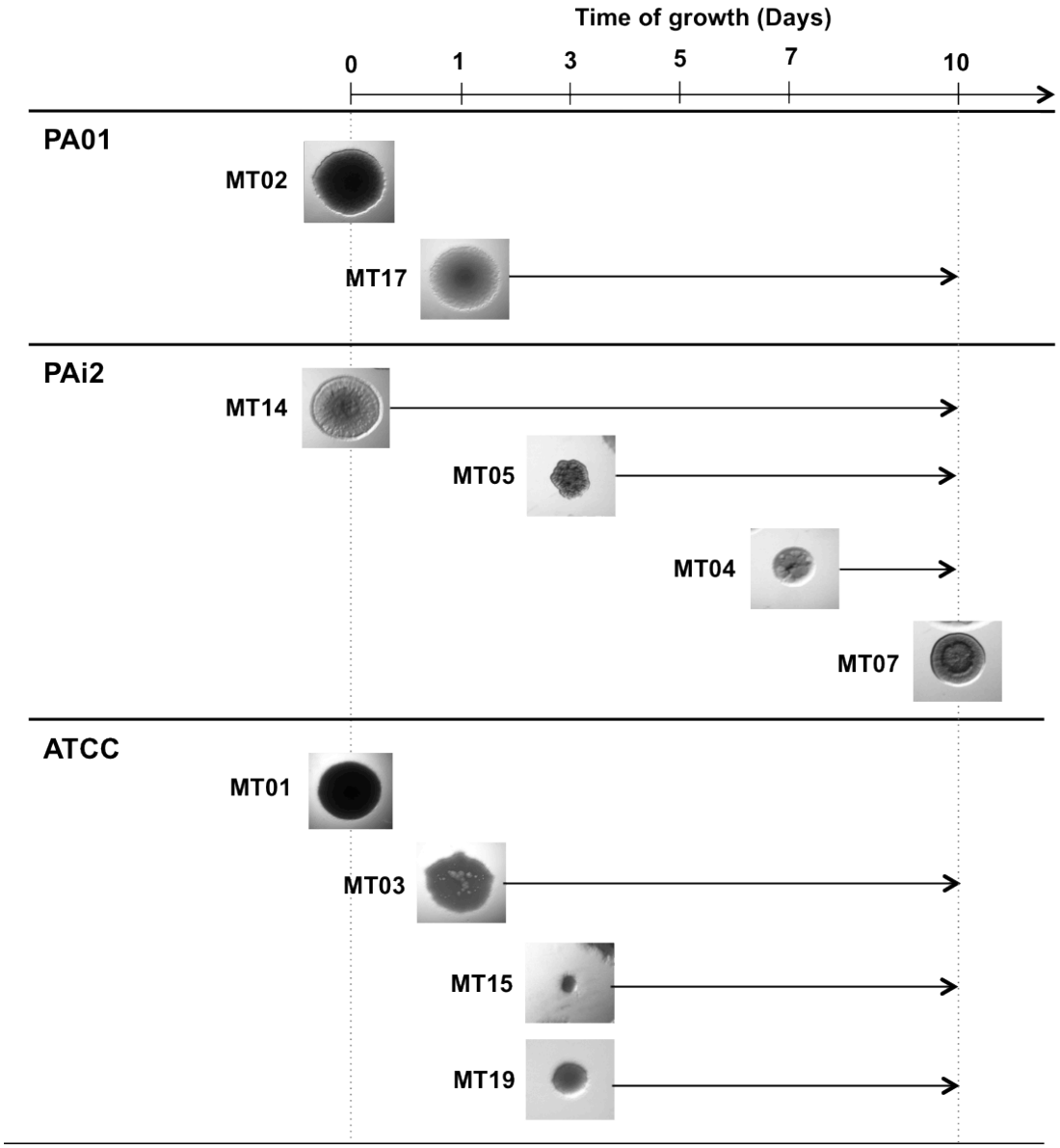

Figure 4.8. Evolutionary profiles of the three *P. aeruginosa* populations during 10 days of growth in ASM exposed to 1 mg/L of ciprofloxacin. Arrows represent the continuous presence of colony morphotypes within populations.

Among the new morphotypes emerged in PA01 and ATCC populations, it was observed moist variants (MT17), dry- and mucoid-SCV (MT19 and MT10, respectively) and pinpoint variants (MT15) (Figure 4.7 and 4.8 and Table 4.1). Through the emergence of these morphotypes, the respective populations became more diverse in terms of antibiotic resistance and virulence factors expression. In effect, all new morphotypes, with exception of MT03, exhibited distinct antibiotic resistance profiles in comparison with the respective WM (Table 4.6). Furthermore, the emergence of these new morphotypes altered the susceptibility of the whole population against the antibiotics (Figure S4.2, S4.3 and S4.4 in Supplemental Material).

Concerning the virulence factors expression, all new morphotypes, with exception of MT17, exhibited defective swimming motility (Figure 4.9A) as observed previously. Although the excellent biofilm formation ability of the new morphotypes, they did not revealed enhanced capacity than the respective WM (Figure 4.10). Interestingly, the treatment with sub-inhibitory concentrations of ciprofloxacin almost abolished the pyocyanin production of PA01 and ATCC populations (Figure 4.11).

Table 4.6. Antibiotic susceptibility profiles of colony morphotypes-associated bacteria. Grey shading marks the new morphotypes identified.

	CIP	CN	TOB	AK	IMI	ATM	CAZ	FEP	TTC	PRL
PA01										
MT02 (WM)	S	S	S	S	S	S	S	S	S	S
MT17	S	S	S	S	S	S	S	S	I	S
MT10	I	S	S	S	S	S	S	S	S	S
ATCC										
MT01 (WM)	S	S	S	S	S	S	S	S	S	S
MT03	S	S	S	S	S	S	S	S	S	S
MT19	S	S	S	S	S	S	S	S	I	S
MT15	R	S	S	S	S	S	S	S	I	S

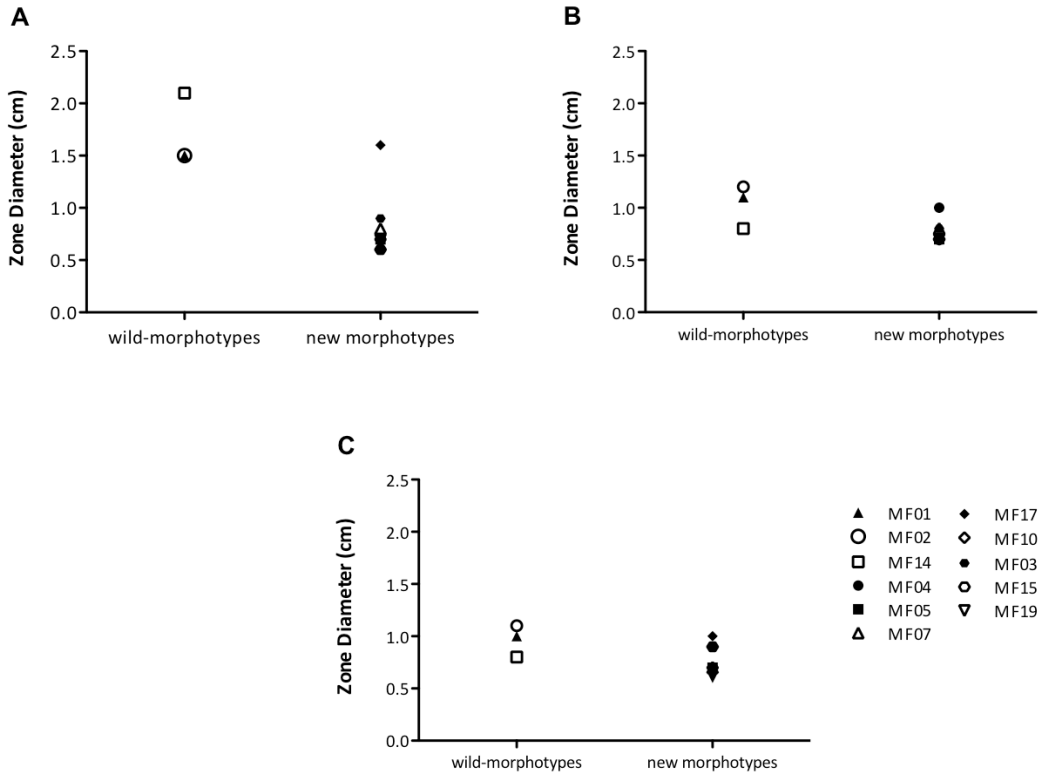

Figure 4.9. (A) Swimming, (B) swarming and (C) twitching motilities of colony morphotypes-associated bacteria.

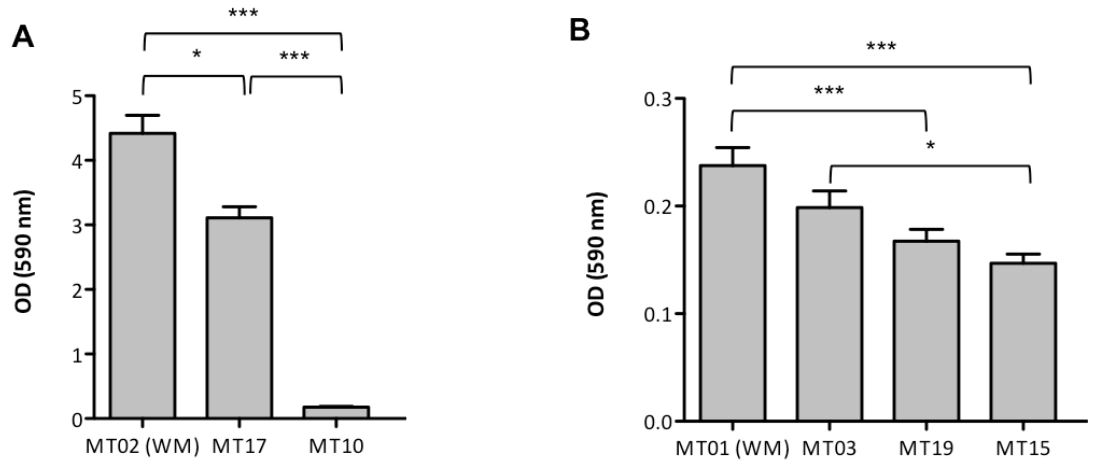

Figure 4.10. Biofilm formation abilities of colony morphotypes-associated bacteria of (A) PA01, (B) ATCC strains.

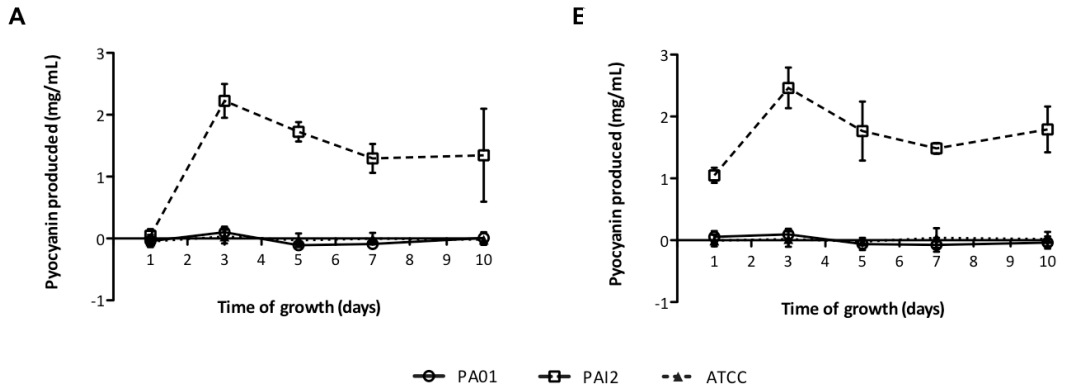

Figure 4.11. Pyocyanin production of PA01, PAI2 and ATCC populations grown in: (A) ASM with 0,5 mg/L of ciprofloxacin; (B) ASM with 1 mg/L of ciprofloxacin.

Negative selective pressure seemed to be exerted with the use of 4 mg/L ciprofloxacin (inhibitory concentration) because MT05 and MT07 were not observed within PAI2 population and MT10 within PA01 population (Figure 4.12). This negative selection seemed to be directed to sub-populations with intermediate resistance to aztreonam and/or to ticarcillin/clavulanic acid within PAI2 populations and directed to sub-populations with intermediate resistance to ciprofloxacin within PA01 populations (Table 4.2).

To investigate the mechanisms underlying the appearance of the new morphotypes within population under stressful conditions (exposure to sub- and inhibitory of CIP), the presence of mutators was assessed. The results obtained demonstrated that phenotypic heterogeneity was not caused by increased spontaneous mutations (Table S4.1 in Supplemental Material).

Taken together the results obtained, it was possible to conclude that treatments using sub-inhibitory concentrations of ciprofloxacin based on MIC of biofilm cells were ineffective to early eradicate *P. aeruginosa* and may trigger phenotypic switching to better “fitter” variants. Furthermore the emergence of the new variants, negative selection of less “fitter” variants within populations may enhance the ability of the whole population to survive in CF lungs. These events highlight that inadequate concentrations of antibiotics could be the driven force of *P. aeruginosa* diversification and adaptation. From the new emergent morphotypes it was verified that all of them exhibited impaired swimming motility.

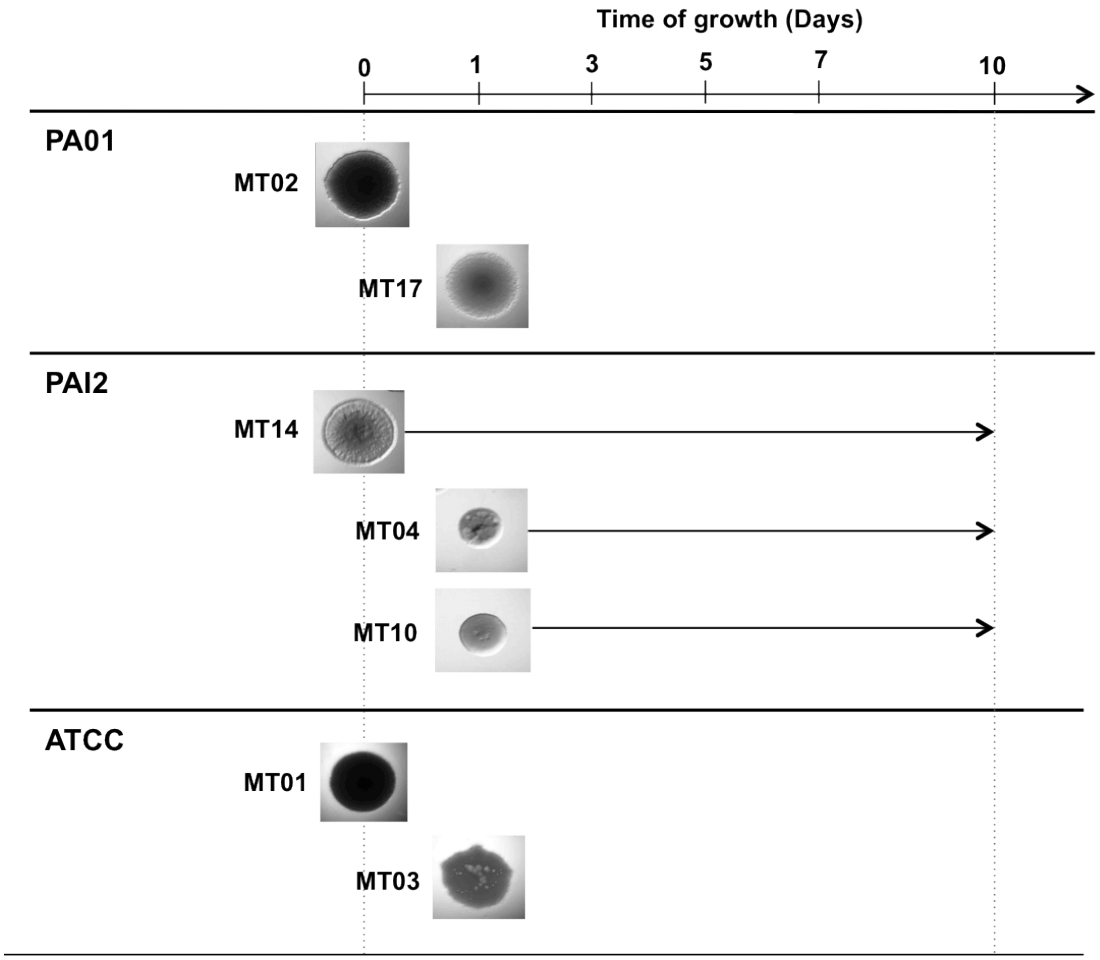

Figure 4.12. Evolutionary profiles of the three *P. aeruginosa* populations during 10 days of growth in ASM exposed to 4 mg/L of ciprofloxacin. Arrows represent the continuous presence of colony morphotypes within populations.

4.3.3. Effects of delayed ciprofloxacin treatment on *P. aeruginosa* populations

Delayed detection of *P. aeruginosa* is associated with the worsening of patient prognosis because bacteria may have initiated its adaptation to CF lungs and developed infection. Whether adaptation has been initiated, the changes undergone by *P. aeruginosa* might perturb the efficacy of antibiotic treatments. So, it is important to determine the window of opportunity to eradicate *P. aeruginosa* from CF lungs after a delayed detection that often leads to a delayed onset of an antibiotic treatment. Based on the previous results (described on section 4.3.1), it can be pointed out that *P. aeruginosa* populations started their adaptation at day 3. To mimic delayed antibiotic treatments, 5-day early-adapted populations were exposed to different ciprofloxacin treatments (using sub- and inhibitory concentrations of, respectively, 1 and 4 mg/L) to evaluate their efficacy and, in case of failure, the response of populations. The ciprofloxacin treatment using 0,5 mg/L was not tested at this stage because of the evident unsuccessful results in eradication of the three *P. aeruginosa* populations (described in section 4.3.2).

The results demonstrated that delayed antibiotic treatments using sub-inhibitory concentrations could have unpredictable results: complete inefficacy, bacterial load reduction or eradication as observed for

PAI2, PA01 and ATCC populations, respectively (Figure 4.13A). Similar time-killing curves were obtained when using the inhibitory concentration, even though slight differences were observed (Figure 4.13B). In effect, PA01 strain was eradicated after 48 h of antibiotic treatment and PAI2 underwent a little load reduction in the number of viable cells, approximately 1 log, but with no significant microbiological effect.

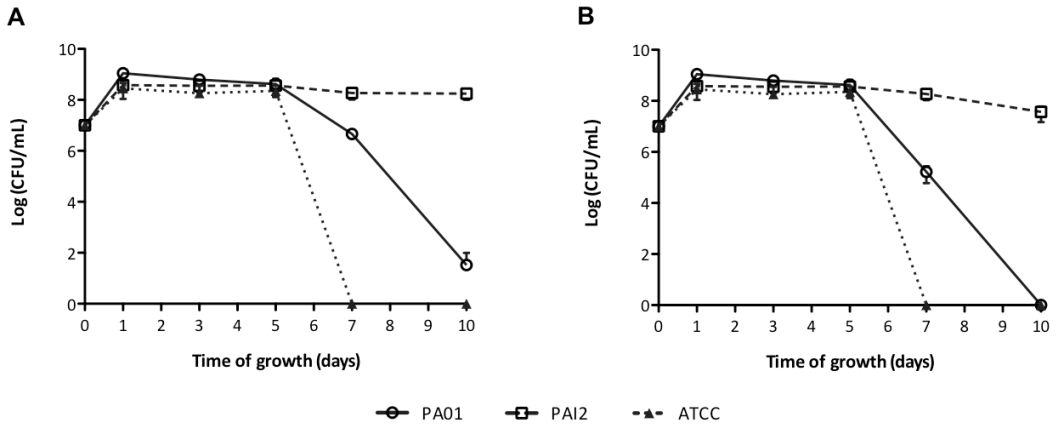

Figure 4.13. Time-killing curves of *P. aeruginosa* populations grown in ASM and exposed at day 5 to ciprofloxacin treatments of (A) 1 mg/L and (B) 4 mg/L and along the remaining 5 days.

Concerning the population diversity, sub- and inhibitory concentrations of delayed ciprofloxacin treatments have changed the dynamics and structure of the whole populations that survive (PA01 and PAI2 strains when using 1 mg/L and PAI2 when using 4 mg/L). PA01 population responded to 1 mg/L of delayed ciprofloxacin treatment identically to the early treatments because the same new morphotypes MT10 and MT17 had emerged again (Figure 4.14). MT17 variants still appeared after the application of 4 mg/L of ciprofloxacin (Figure 4.15), but they were eradicated in the course of the treatment. PAI2 population underwent also alterations in population diversity. Both ciprofloxacin treatments reduced greatly population diversity as happen in early treatments, but only for a few days (Figure 4.14 and 4.15). At day 10 populations were able to restore part or the whole diversity. ATCC population composed by the WM, the MT01, and MT12 did not survive to both delayed treatments.

No mutators were detected within populations under delayed ciprofloxacin treatments (Table S4.1 in Supplemental Material).

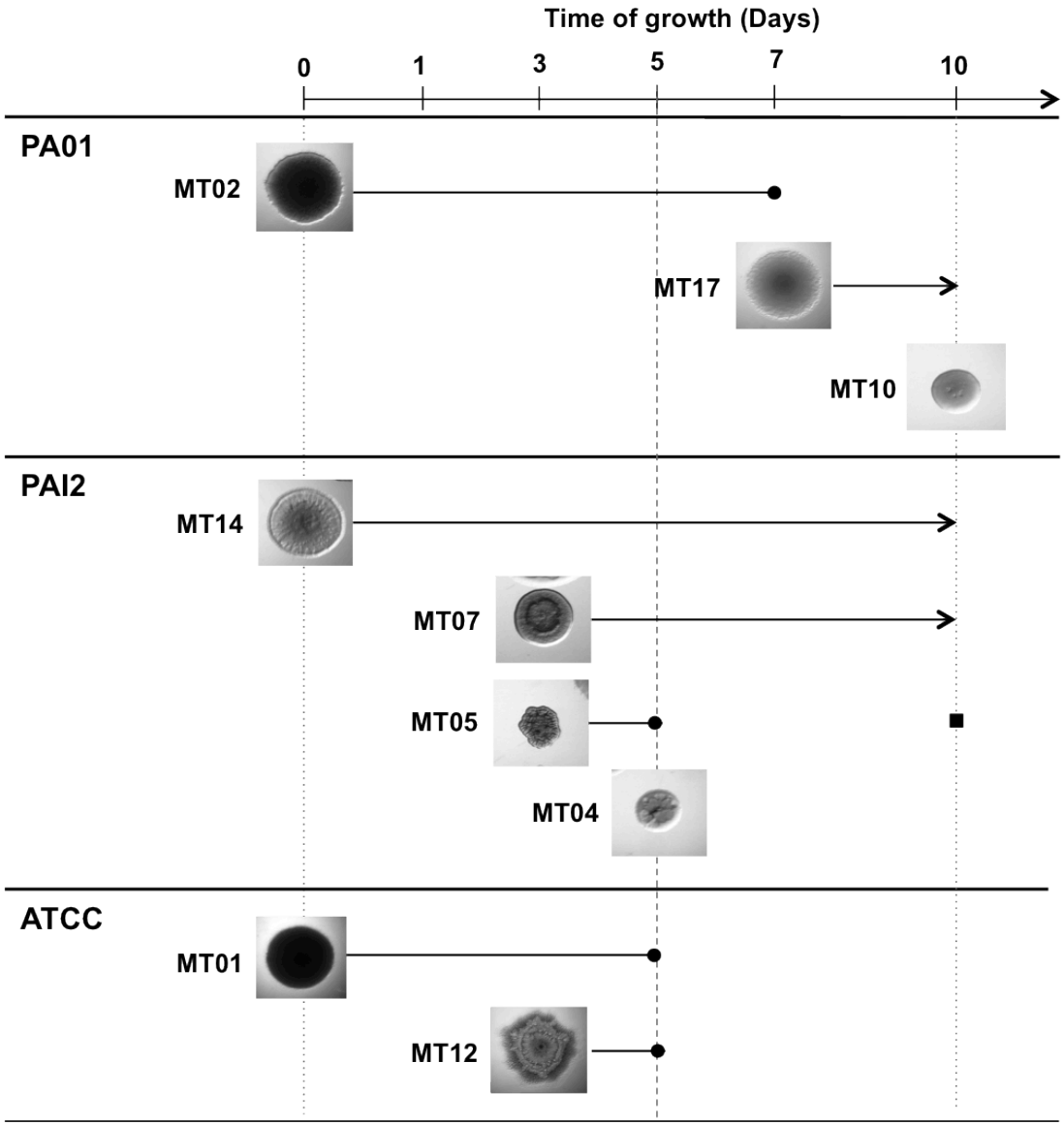

Figure 4.14. Evolutionary profiles of the three *P. aeruginosa* populations during 10 days of growth in ASM and exposed at day 5 to 1 mg/L. Arrows represent the continuous presence of colony morphotypes within populations. Black points represent the end of the morphotypes presence within populations and black squares re-emergence of morphotypes.

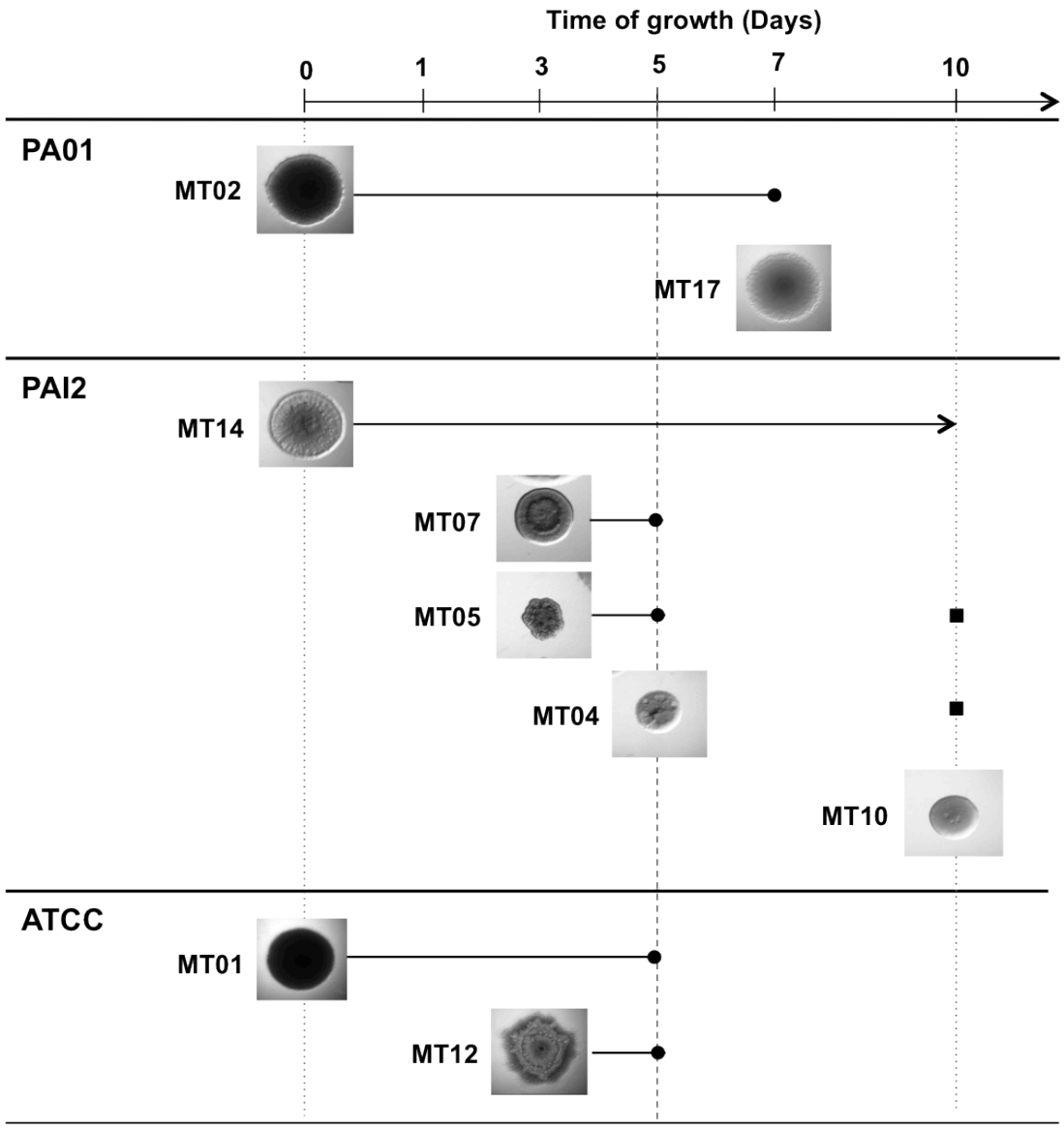

Figure 4.15. Evolutionary profiles of the three *P. aeruginosa* populations during 10 days of growth in ASM and exposed at day 5 to 4 mg/L. Arrows represent the continuous presence of colony morphotypes within populations. Black points represent the end of the morphotypes presence within populations and black squares re-emergence of morphotypes.

4.3.4. Association between colony morphology traits and antibiotic susceptibility

The significant association between mucoidy and antibiotic resistance described in literature [34] prompted to verify whether colony morphology traits were related to resistance against the antibiotic used in this study. As so, the results related to antibiotic susceptibility of all colony morphotypes identified and the colony morphotyping information were analysed following a statistical approach, in particular using correlation tests (Fisher's Exact test and Pearson Chi-square, when appropriate). The results obtained showed that the absence of sheath, iridescent opacity, small size and green-blue colour were significantly associated with resistance to ciprofloxacin (Table 4.7). The same colony traits with exception of small size were significantly associated with intermediate resistance to aztreonam. No colony traits were significantly associated with intermediate resistance or sensitivity to ticarcillin/clavulanic acid (Table S4.2 in

Supplemental Material). Therefore, based on this statistical approach sheath, opacity, size and colour were considered potential candidates to indicators of antibiotic susceptibility to aztreonam and ciprofloxacin.

Table 4.7. Categorical data analysis between ciprofloxacin resistance and colony morphology traits.

		Statistical test	χ^2	df	*p*-value
Colony morphology traits	**Ciprofloxacin resistance**				
	Form	Fisher's Exact Test			0,455
	Margin	Fisher's Exact Test			0,182
	Sheath	Fisher's Exact Test			0,015*
	Type of surface	Fisher's Exact Test			0,455
	Texture	Person Chi-Square	3,437 [a]	3	0,329
	Consistency	Person Chi-Square	2,037 [b]	2	0,361
	Opacity	Person Chi-Square	7,639 [b]	2	0,022*
	Size	Person Chi-Square	7,543 [b]	2	0,023*
	Colour	Person Chi-Square	7,773 [c]	2	0,021*
	Aztreonam resistance				
	Form	Fisher's Exact Test			0,250
	Margin	Fisher's Exact Test			0,250
	Sheath	Fisher's Exact Test			0,045*
	Type of surface	Fisher's Exact Test			1,000
	Texture	Person Chi-Square	4,000[d]	3	0,261
	Consistency	Person Chi-Square	1.333[e]	2	0,513
	Opacity	Person Chi-Square	8,000[e]	2	0,018*
	Size	Person Chi-Square	2,095[e]	2	0,351
	Colour	Person Chi-Square	8,000[f]	2	0,018*

* significant at level 0.05
[a] 8 cells have expected count less than 5. The minimum expected count is 0,45.
[b] 6 cells have expected count less than 5. The minimum expected count is 0,45.
[c] 6 cells have expected count less than 5. The minimum expected count is 0,91.
[d] 7 cells have expected count less than 5. The minimum expected count is 0,25.
[e] 5 cells have expected count less than 5. The minimum expected count is 0,25.
[f] 6 cells have expected count less than 5. The minimum expected count is 0,75.

4.3.5. Association between colony morphology traits and bacterial phenotypic features

It was hypothesized that colony morphologies could serve also as indicators of some bacterial virulence characteristics, as biofilm formation ability, swimming, swarming and twitching motilities. As so, a statistical approach was followed to verify the existence of correlation between these virulence factors expression and colony traits. From the statistical results obtained, using Kolmogorov-Smirnov Z test and Chi-square tests when appropriate, it was verified that swimming motility was significantly augmented in large colonies (Table 4.8 and Figure S4.5 in Supplemental Material) and twitching motility was significantly increased in colonies with sheath and in large colonies (Table 4.8 and Figure S4.6 in Supplemental Material). Regarding biofilm formation, significant differences in blue-green colour colonies in contrast with

yellow and beige colonies were detected (Table 4.6 and Figure S4.7 in Supplemental Material). In fact, blue-green colonies exhibited increased biofilm formation ability.

It seemed that beyond indicators of antibiotic susceptibility, sheath, size and colour revealed to be as well putative candidates for indicators of *P. aeruginosa* phenotypic characteristics.

Table 4.8. Differences of colony morphology traits in biofilm formation ability, swimming, swarming and twitching motilities using the Kolmogorov-Smirnov Z test (K-S Z).

		Biofilm formation		**Swimming**		**Swarming**		**Twitching**	
		K-S Z	***p*-value**	**K-S Z**	***p*-value**	**K-S Z**	***p*-value**	**K-S Z**	***p*-value**
Colony morphology traits	Margin	0,667	0,766	0,500	0,964	0,667	0,766	1,000	0,270
	Sheath	0,976	0,297	0,878	0,423	0,976	0,297	1,464	0,028*
	Consistency	0,667	0,766	0,833	0,491	0,667	0,766	0,667	0,766
	Opacity	1,140	0,149	0,513	0,955	0,684	0,738	1,140	0,149
	Size	0,969	0,305	1,368	0,047*	0,969	0,305	1,368	0,047*

* significant at level 0,05

Table 4.9. Differences of colony colour in biofilm formation ability, swimming, swarming and twitching motilities using Kruskal-Wallis.

	Biofilm formation		**Swimming**		**Swarming**		**Twitching**		**df[a]**
	χ^2	***p*-value**	**χ^2**	***p*-value**	**χ^2**	***p*-value**	**χ^2**	***p*-value**	
Colour	0,425	0,021*	1,478	0,809	3,005	0,478	7,753	0,223	2

* significant at level 0,05
[a] degrees of freedom

4.3.6. Association among phenotypic traits

In this study, the association among phenotypic traits was evaluated in order to verify whether the expression of a phenotypic trait could be an indicator of another. To verify this association, it was used the Pearson Correlation test. The results demonstrated that there was a strong positive association between swarming and twitching motilities and also a positive association between swimming and swarming motilities (Table 4.10). No phenotypic trait was significantly associated with biofilm formation ability.

The results obtained seemed thus pointed out that augmented swarming motility could predict augment swimming and twitching motilities.

Table 4.10. Pearson correlation between biofilm formation and swimming, swarming and twitching motilities.

	Biofilm formation	Swimming	Swarming	Twitching
Swimming	-0,156	-	0,632*	0,534
Swarming	0,473	0,632*	-	0,864**
Twitching	-0,277	0,534	0,864**	-

* significant at level 0,05
** significant at level 0,0001

4.4. Discussion

The understanding of *P. aeruginosa* evolution and diversification during the course of infections in CF lungs is of upmost importance regarding the antibiotic treatments to administer to patients, nevertheless these processes are poorly understood. Remain thus unknown what happens immediately after *P. aeruginosa* lungs colonization. The less amount of information regarding early infection stages is mainly due to the needed of continuous sampling the sputum, which is very uncomfortable for patients. The impossibility of serial sputum sampling limits the ability to determine the exact timing of the initiation of *P. aeruginosa* adaptation and the emergence of new phenotypes. Therefore, *in vitro* studies have a crucial role to start investigating what occurs to *P. aeruginosa* after enter in CF lungs.

Diversification of the infecting populations has been intensively studied, however limited to species identification and richness and anaerobic pathogen quantification mainly focused on chronic infections. Intraclonal diversification has been reported as a source of phenotypic heterogeneity with a role in bacteria adaptation to new environments [16, 35, 36, 37]. Despite these evidences, it remains unclear the causes of intraclonal diversity, how stable are the variants generated, their function within the population, which niche variants grow and their response to antimicrobial action of antibiotics. To address these issues, this study deeply analysed the diversity of three distinct populations of *P. aeruginosa* after simulated initial colonization of CF lungs.

The data obtained provided evidences of early *P. aeruginosa* diversity after ASM colonization. *P. aeruginosa* evolved generating diverse populations determined by the distinct colony morphotypes identified along 10 days of the experiment. The diversity of the *P. aeruginosa* populations of the three strains was distinct which may be explained by the different function of the central elements in genome, despite the genes involved in regulation were highly conserved. For instance, concentrations of sigma factors can vary according environmental conditions and across strains. This instability of the core elements of regulation is sufficient to generate distinct clonal diversification and adaptive pathways among strains [38]. Moreover, it was observed that these populations were also dynamic since colony morphotypes-associated bacteria exhibited divergent phenotypic characteristics that coexisted for 10 days. The results emphasized the role of intraclonal diversification in *P. aeruginosa* adaptation, in

particular at early stage, leading to conclude that a single clone in sputum may not represent the whole infecting population resident in CF lungs.

Phenotypic diversity is traditionally associated to adaptation to chronic infections [20, 21, 34], but some of colony morphotypes typically associated with chronic infections were observed at early stage, for instance mucoid- and rough-SCV, pinpoint and moist variants were detected in early populations. There is no single colony morphotype dominating at early stages of infections, but several morphotypes can evolve with distinct characteristics and not restricted to mucoid morphotypes.

In addition to the early diversity noticed in *P. aeruginosa* populations, results also demonstrated that such diversity could have long-term stability as pointed out during the 10 days of growth. The existence and co-existence of distinct variants suggested that they all play a role in infection development and progression to chronic stages. Furthermore, those earlier morphotypes maintained their colony features as well as their biological characteristics in subsequent cultures, at least 10 cultures, highlighting the stability of these variants, as previously reported by Markussen *et al.* (2014) [37].

The determination of the driven forces and the underlying mechanisms of intraclonal diversification of *P. aeruginosa* in ASM was considered of upmost importance and addressed in this study. The absence of mutators in populations grown in ASM seemed indicate that hypermutability was not the underlying mechanism of intraclonal diversification and suggested that spatial organisation in distinct niches in CF lungs might play a major role in the generation and maintenance of intra-population diversity. The rapid diversification of *P. aeruginosa* after initial ASM colonization was indicative of the existence of several niches within ASM that bacteria fill in leading to several sub-populations. This theory was previously proposed and named as adaptive radiation [18, 39]. The results of this study supported the adaptive radiation theory and reinforced the assumption that CF sputum is intrinsically a heterogeneous, complex and dynamic environment [40] highlighting the importance of spatial organisation and niche partitioning since early stages of infection. Other *in vitro* evolution studies have established this correlation between heterogeneous environments, evolution and maintenance of population diversity [41, 42]. However, conclusions about the characteristics of CF niches related to intraclonal variation are very difficult to reach because most of the studies analysed bacterial diversity from sputum or bronchoalveolar lavage fluid samples with lack of spatial resolution or intraclonal diversity concerns. Among the scarce studies that address these niches partitioning issues, Markussen *et al.* (2014) [37] found quite similar results as those reported in the present study. The authors showed that the initial infecting strain diverged into distinct sublineages immediately after initial lungs colonization possibly caused by niche partitioning. Both studies, Markussen *et al.* (2014) [37] and the present work, highlight the relevance of better understanding the interactions that take place between *P. aeruginosa* and the CF environment. The knowledge about those initial *P. aeruginosa* interactions could provide important insights into colonization, infection development and progression to chronic stages.

Biofilms are also heterogeneous environments and they are typically found in chronic infections [25, 33, 43]. After discarded the hypothesis of hypermutability to be the underlying mechanism of intraclonal diversification in ASM, it was considered interesting to verify whether population diversity was linked to the

presence of small aggregates or immature biofilms at this *in vitro* early stage of infection. Comparing the colony morphotypes derived from ASM cultures with those obtained in biofilm assays in Chapter 3, it was verified that some of new ASM morphotypes were identical to the colony morphotypes-biofilm associated. This evidence suggested that at early infection stages the *P. aeruginosa* mode of growth in CF lungs could be the aggregate mode (without or with very reduced amount of matrix) or immature biofilms that may further evolve to mature biofilms. Therefore, beyond niche partitioning the formation of small aggregates or immature biofilms in ASM seemed promote *P. aeruginosa* diversification.

Much attention has been directed towards the development of new drugs targeting biofilm formation, motility and QS-signals [44, 45, 46, 47, 48]. However, the answer for early eradication failure could pass in changing the design of the actual antibiotic treatment regimes, in particular, the dosage administered to patients. There is a continuous debate about antibiotics, combinations of antibiotics, concentrations and modes of administration that should be applied in the treatment of CF-associated infections [49, 50]. It is urgent to establish some guidelines about antibiotic therapy and this study aimed to gain insights about the most adequate concentrations of early treatments. In this study, the therapeutic dosage reference was established based on the MIC of biofilm cells previously determined in Chapter 3. Antibiotics commonly do not reach the expected maximum inhibitory concentration in CF lungs due to interferences in their action, such as the presence of mucin and extracellular DNA. To reach biofilm cells, antibiotics face similar obstacles and therefore the MIC of biofilm cells seemed an adequate therapeutic reference. From the results obtained, the MIC of biofilm cells demonstrated to be an accurate and useful reference for the antibiotic dosage for early treatments using ciprofloxacin monotherapy. Concentrations around MIC of biofilm cells have produced better bacterial eradication results than sub-MIC doses. Nonetheless, PAI2 strain survival to inhibitory concentrations of ciprofloxacin weakened the generalization of that assumption. The survival of PAI2 could be due to its culture history before the CF adaptation process implemented. Some authors advocated that the understanding of bacteria adaptations and their regulatory responses requires the contextualization of the culture history of bacteria, in particular of clinical isolates, such as the stress exposure, growth rate and mode of growth before the adaptive transition that is under studied [38]. PAI2 strain is a clinical isolate obtained from a non-CF environment that could have faced several unknown stresses and/or acquired some genetic material and/or mutations that provided to bacteria better fitness and greatest ciprofloxacin resistance and thus its success against ciprofloxacin MIC of biofilm cells. Transposing to real scenario, inefficacy of early ciprofloxacin treatments using MIC of biofilm cells could be verified whether CF patients acquire pathogens with pre-developed resistance to antibiotics, for instance from other patients.

The results related to early ciprofloxacin treatments using sub-MIC doses demonstrated that beyond contributing to the failure of bacteria eradication, inaccurate concentrations were the driven forces for *P. aeruginosa* diversification into better “fitter” variants. Furthermore, sub-MIC ciprofloxacin pressure has exerted selection within populations persisting the antibiotic resistant clones. Similar findings were also reported by Macia *et al.* (2006) [51] that found selection of populations with distinct antibiotic resistance levels in response to different ciprofloxacin concentrations.

Moreover, sub-inhibitory concentrations of antibiotics can function as signalling molecules regulating gene transcription, physiology and virulence potential [52, 53, 54, 55] and that alterations could be reflected on colony morphology variation. This regulation impacts on bacterial fitness increasing the survival in stressful environments. PA01 and ATCC populations under ciprofloxacin treatments greatly decreased their pyocyanin production. Pyocyanin production is under QS regulation and the sub-inhibitory concentrations of ciprofloxacin seemed to modulate QS networks in populations. This result challenges the traditional view of antibiotics action limited to reduce bacterial fitness and virulence potential and interestingly several authors previously described it [52, 56, 57, 58]. However, this result could be not verified in all *P. aeruginosa* populations. PAI2 population even under ciprofloxacin treatments maintained its pyocyanin overproduction. Similar feature was reported to the hypervirulent Liverpool epidemic CF strain of *P. aeruginosa* and correlated with periods of pulmonary exacerbations in some CF patients [20, 59]. The distinct pyocyanin production among *P. aeruginosa* populations in this study reflects that CF environments can comprise the coexistence of QS-positive and – defective bacteria [20, 60, 61]. QS-defective bacteria could be fitter facing other stressful conditions due to the biological cost of QS.

The importance of the antibiotic dosage boosts whether *P. aeruginosa* is detected later and antibiotic treatments are as well initiated later. The results obtained in this study evidenced that sub-MIC ciprofloxacin dosages of delayed treatments could have unpredictable results, since successful eradication to bacteria persistence. This range of antibiotic treatment results was highly dependent of *P. aeruginosa* strain ability to adapt to ASM. Some bacterial resistance to the treatments using reduced concentrations of ciprofloxacin was expected because *P. aeruginosa* is intrinsically resistant to the most fluoroquinoles [62].

The development of resistance to other unrelated antibiotics (antibiotics belonging to other classes) is a risk of antibiotic treatments and it has been particularly reported for ciprofloxacin [63]. The results obtained in this study demonstrated that ciprofloxacin could be used as an early antibacterial agent but some intermediate co-resistance emerged for aztreonam, monobactams antibiotic, and ticarcillin/clavulanic acid, a combination of β-lactam antibiotic with β–lactamase inhibitor. It should be addressed more studies to confirm if in fact ciprofloxacin treatments could induce co-resistance.

Nevertheless, the whole *P. aeruginosa* populations analysed in this study demonstrated to be susceptible to almost antibiotics. From the clinical point of view, this result is very welcome meaning that *P. aeruginosa* could be easily eradicate at this stage of infection. Although these satisfactory results of susceptibility, the antibiotic susceptibilities of colony morphotypes-associated bacteria were determined to monitored possible changes undergone by bacteria to achieve adaptation. From this analysis it was observed that antibiotic susceptibilities of the some colony morphotypes-associated bacteria were not identical to the antibiotic susceptibilities of the whole populations. For instance, PA01 populations grown in ASM under 0,5 mg/L of ciprofloxacin treatment exhibited sensitivity to ticarcillin/clavulanic acid, but MT17 and MT10 were intermediate resistant to this antibiotic. These results demonstrated that in fact some sub-populations have altered their susceptibility profile, which is of serious concern because it is unknown what happen after day 10. It is known through this study that whole populations are sensitive until day 10

but there is a possibility that the intermediate resistant or resistant sub-populations gain expression in number within populations after day 10 and the overall sensitive could be lost. These situations highlight the importance of testing the antibiotic susceptibility of colony morphotypes and the whole population separately. A sensitive population does not mean that resistant or intermediate resistant variants are not present and that soon, with or without antimicrobial treatment stimuli, population became resistant.

From the microbiological perspective, the distinct susceptibility profiles exhibited by whole populations and their derived morphotypes highlight the relevance of sub-populations interactions. However, so far, there is no relevant information on this matter, namely on the role that the distinct sub-populations may play on the different aspects of CF disease, as infection development, transition to chronic stages, bacteria persistence and exacerbations occurrence. In attempt to collect some information about the role of sub-populations in CF disease and to identify the alterations undergone by *P. aeruginosa* at this early stage of infection, all colony morphotypes were phenotypically characterised in terms of virulence factors expression. In this study, it was found that early clonal isolates varied in motility (swimming, swarming, twitching), biofilm formation ability and pigments production. Impaired swimming motility was a common feature among the all new clonal variants, seeming thus that bacteria lack flagella in order to adapt and survive to CF conditions and to antibiotic treatments. Motility in CF sputum is mechanically inhibited due to the presence of mucin as the FliD flagellar cap protein at the tip of the flagella binds to hitch cells and inhibits rotation [64]. Therefore, at one adaptation step swimming motility is inhibited and adherence promoted. Furthermore, defective swimming motility facilitates further adaptations, such as aggregation (biofilm formation), which confer protection against to antibiotics and host immune molecules, as described previously [65, 66] and reinforced by this study as some colony morphotypes-biofilm associated were detected. Data led to the hypothesis that impaired swimming motility could be associated with initial *P. aeruginosa* adaptation and early infection development. This premise is reinforced by Mayer-Hamblett *et al.* (2014) [28] that found an association between defective swimming ability and pulmonary exacerbations, worsening symptoms and declining lung function, the common signs of infection development [20, 67, 68] and by several other studies that described defective swimming ability as a characteristic of CF chronic isolates [28, 65, 66, 69].

The hypothesis that impaired swimming motility could be associated with initial *P. aeruginosa* adaptation and infection development contradicted some traditionally held concepts that mucoidy and/or emergence of SCV are the characteristic associated with infection development and transition to chronic stages and thus contributors to disease [70, 71]. Conversion to mucoid phenotype in this study was limited to one colony morphotype (MT10), susceptible to negative selection by exposure to ciprofloxacin and thus not considered a relevant feature at this infection stage. The detection of SCV was limited to one *P. aeruginosa* strain that did not allow considering as an infection development feature. As motility measures can be performed in a reproducible and quantitative way and it was a common trait among all new emergent colony morphotypes identified, defective swimming was considered a potential disease marker, as also proposed by Mayer-Hamblett *et al.* (2014) [28].

Acquisition of mucoid phenotypes even at early stages of infection development is typically associated with worsening of prognosis. Data obtained in this study did not support that assumption since the mucoid clones switched to dry variants after ASM colonization and were easily eradicated by early and delayed treatments using biofilm cells inhibitory concentrations of ciprofloxacin. Initial clearance of mucoid phenotypes was also reported by Troxler *et al.* [72].

Diversification of *P. aeruginosa* grown in ASM without ciprofloxacin seemed to be caused by phenotypic plasticity of *P. aeruginosa* and supported by the absence of mutators. It is well known the impressive ability of *P. aeruginosa* to adapt to so different environments diversifying into several phenotypes [8, 73, 74]. Changes in diversification during and after exposure to sub-inhibitory concentrations of antibiotics could result of mutagenesis induction, which causes variation and/or promotes hypermutability. Antibiotics can induce mutagenesis because trigger SOS response and thus increasing the expression of error-prone DNA polymerases leading to diversity within populations [75, 76]. Ciprofloxacin is a fluoroquinolone antibiotic that acts in DNA replication by interacting with DNA gyrase and with DNA topoisomerase IV [77, 78] that sub-inhibitory concentrations can induce mutations and recombinations and supporting the emergence of phenotypic variants and mutators [60, 79, 80]. Although the distinct phenotypic diversity of *P. aeruginosa* populations observed during the treatments with sub-MIC dosages of cirpofloxacin, no hypermutable phenotypes were detected. Therefore, there was no evidence that hypermutability was the underlying mechanism for diversification under antibiotic treatments. The absence of hypermutable phenotypes was similarly reported by other previous studies [42, 60, 81, 82].

As previously described, *P. aeruginosa* exhibited several different colony morphologies and it was hypothesized whether they could serve as indicator of antibiotic resistance and virulence factors expression. Due to the importance of these indicators in clinical diagnosis, it was used a statistical analysis to ensure results reliability. It was found that the absence of sheath, iridescent opacity and blue-green colour could be potential candidates to indicate antibiotic resistance. The small size revealed to be a good indicator but only for ciprofloxacin resistance. Concerning the virulence factors expression, the colony size revealed to be an indicator of swimming and twitching motilities, colony sheath demonstrated to be an indicator of twitching motility and colony colour an indicator of biofilm formation. These results emphasize the importance of colony morphology characterisation in the context of clinical diagnosis and in monitoring of *P. aeruginosa* adaptation and evolution. Despite the promising results, these colony traits need to be extensively studied to validate them.

4.5. Conclusions

The adaptive changes undergone by *P. aeruginosa* after initial colonisation of CF lungs are poorly studied. *P. aeruginosa* diversity is greatly associated with chronic stage infections and mucoidy remains the risk factor for the development of chronic infections and exacerbations. This study demonstrated the occurrence of *P. aeruginosa* diversification after *in vitro* initial colonisation and provided evidences that such diversification played a role in bacterial adaptation to the CF environment and early infection

development. These findings have great impact on CF disease management as they highlighted that "real" diversity can be underestimated when it is assumed that single or few isolates represent the entire population. Diversity underestimation can contribute to erroneous diagnosis with serious implications in antibiotic therapy efficacy. Moreover, it can also have implications in the information about *P. aeruginosa* adaptation to CF lungs provided by genomic, transcriptomic, metabolomic, proteomic and phenotypic studies. In effect, these studies are based on population diversity that, if poorly estimated, may provide defective information that not represents the true scenario about the infecting populations.

It is consensual that the delay of *P. aeruginosa* infection in CF patients is fundamental to avoid chronic infection establishment that is greatly associated with rapid lung function deterioration and increased antibiotic resistance. This study demonstrated that the antibiotic doses used in the early treatments have impact on *P. aeruginosa* populations. In fact, dosage of ciprofloxacin equivalent to the MIC of biofilm cells was able to eradicate bacteria in ASM. Conversely, sub-inhibitory concentrations were ineffective in bacterial eradication and constituted the driven forces for diversification towards better "fitter" variants and infection progression. Inadequate concentrations of antibiotics could even exert selection over population leading to only remain the resistant variants and boosting antibiotic resistance and persistence of the infecting population. This outcome is of upmost importance in clinical decision making, in particular, to re-design tailored antibiotic treatment regimes using accurate concentrations. Results also pointed out that MIC of biofilm cells seems to be an adequate therapeutic reference for early antibiotic treatments regimes.

Early intraclonal *P. aeruginosa* diversification seemed not be caused by hypermutability but by bacteria plasticity and niche specialization. In effect, the diversification of *P. aeruginosa* grown simply in ASM could result from niche partitioning reinforcing the assumption that CF lungs is one of the most complex environment within human host, highly structured with temporal and spatial variations, different environmental conditions (the niches), multiple connections among compartments and microorganism (intra- and interspecies interactions). Bacterial survival in ASM with ciprofloxacin seemed also not be caused by hypermutability but by the impressive versatility of *P. aeruginosa* to face stressful conditions triggering stress responses.

Bacteria resident in CF lungs undergone alterations that may have profound effects on disease status and clinical outcome specially whether these alterations have impact on bacterial fitness and resistance to antibiotics. Those alterations may represent useful tools for monitoring infection development and bacterial response to treatments. Therefore, in the present study, a substantial effort was directed towards finding early disease markers to enable prompt diagnosis and tailored antibiotic treatment before chronic infection development. Results showed that *P. aeruginosa* undergone multiple *in vitro* phenotypic changes after ASM colonization, but impaired swimming motility was the phenotypic trait most associated with CF adaptation instead of mucoidy as traditionally referred. Therefore, impaired swimming motility was considered a potential disease marker of early *P. aeruginosa* adaptation and infection development. It must be stressed that these results were obtained from *in vitro* experiments that could not exactly represent the *in vivo* scenario, so impaired motility should be assessed through *ex vivo* experiments and thus validated as disease marker.

In the present study, several colony morphologies usually associated to chronic stages were observed in early *P. aeruginosa* populations, such as SCV, rough, iridescent and moist variants. The presence of these colony variants in this *in vitro* study seems to indicate that CF lungs could be potential reservoirs of antibiotic resistant and increased virulent variants since the early stages of disease able to persist and to cause chronic infection.

Given the increased colony morphology diversification resulting from *P. aeruginosa* bacterial adaptation it was hypothesized that colony traits could be useful bacterial indicators of virulence factors expression and antibiotic susceptibility. It was found that some colony traits such as absence of sheath, iridescent opacity, small size and blue-green colour were indicative of ciprofloxacin resistance and aztreonam intermediate resistance. Likewise, the presence of sheath and large size were indicative of twitching motility and blue-green colour of increased ability to form biofilms. These findings highlight the relevance of colony morphology observation as an auxiliary and complementary diagnosis method.

The overall results achieved with the present work provide valuable insights about initial adaptation of *P. aeruginosa* in CF lungs, intraclonal diversity and disease markers. However, the results obtained could be strengthened if more strains of *P. aeruginosa* from non- and –CF environment were included. In effect, as observed, the genetic background of the strains plays a crucial role in bacteria adaptation. Despite strains belong to the same species and the genes involved in regulation are highly conserved, central elements in genome can function in different ways. Therefore, more *P. aeruginosa* strains should further be studied in order to broaden and generalise the main findings obtained. It must also be stressed that, in contrast with the described in literature, some virulence factors seemed not to be expressed in this experiment. This could be attributed to the lack of stimulus similar to those played by host cells and host immune molecules. The practicality of assessing several colony morphotypes limited the number of phenotypic traits evaluated. The lack of expression of some described virulence factors could be also caused by the reduced period of time of this study in the context of “real” CF infection development. Despite to be a considerable period of time for an *in vitro* study, ten days could not be the sufficient period of time to observe other *P. aeruginosa* alterations in virulence factors expression, as well as in antibiotic resistance. An early stage of infection in CF disease could last weeks to months.

4.6. References

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4.7. Supplemental Material

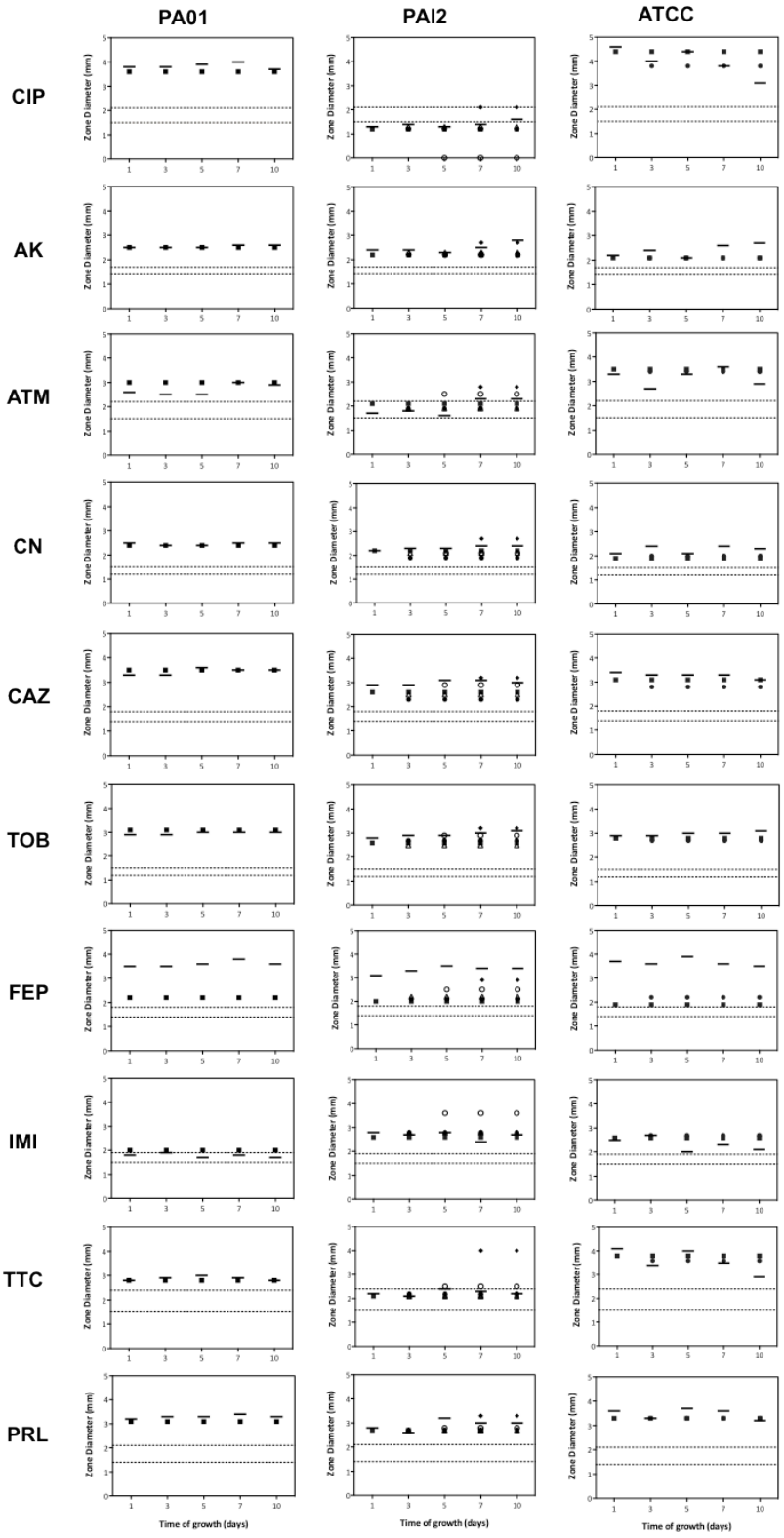

Figure S4.1 – Antibiotic susceptibility profiles of colony morphotypes that composed PA01, PAI2 and ATCC populations and antibiotic susceptibility profiles the respective whole populations (represented by (-) grown in ASM without ciprofloxacin.

Table S4.1. Rifampicin mutation frequencies of *P. aeruginosa* populations of PA01, U14 and ATCC grown in ASM without ciprofloxacin treatment; grown in ASM with 0,5 mg/L of ciprofloxacin; grown in ASM with 1 mg/L of ciprofloxacin; grown in ASM with 4 mg/L of ciprofloxacin.

	Mutation frequency (mutants per cell)		
	PA01	**U147016**	**ATCC 39324**
Without treatment			
Day 1	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$
Day 3	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$
Day 5	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$
Day 7	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$
Day 10	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$
0,5 mg/L			
Day 1	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$
Day 3	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$
Day 5	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$
Day 7	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$
Day 10	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$
1 mg/L			
Day 1	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$
Day 3	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$
Day 5	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$
Day 7	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$
Day 10	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$
4 mg/L			
Day 1	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$	$< 5 \times 10^{-11}$
Day 3	-	$< 5 \times 10^{-11}$	-
Day 5	-	$< 5 \times 10^{-11}$	-
Day 7	-	$< 5 \times 10^{-11}$	-
Day 10	-	$< 5 \times 10^{-11}$	-

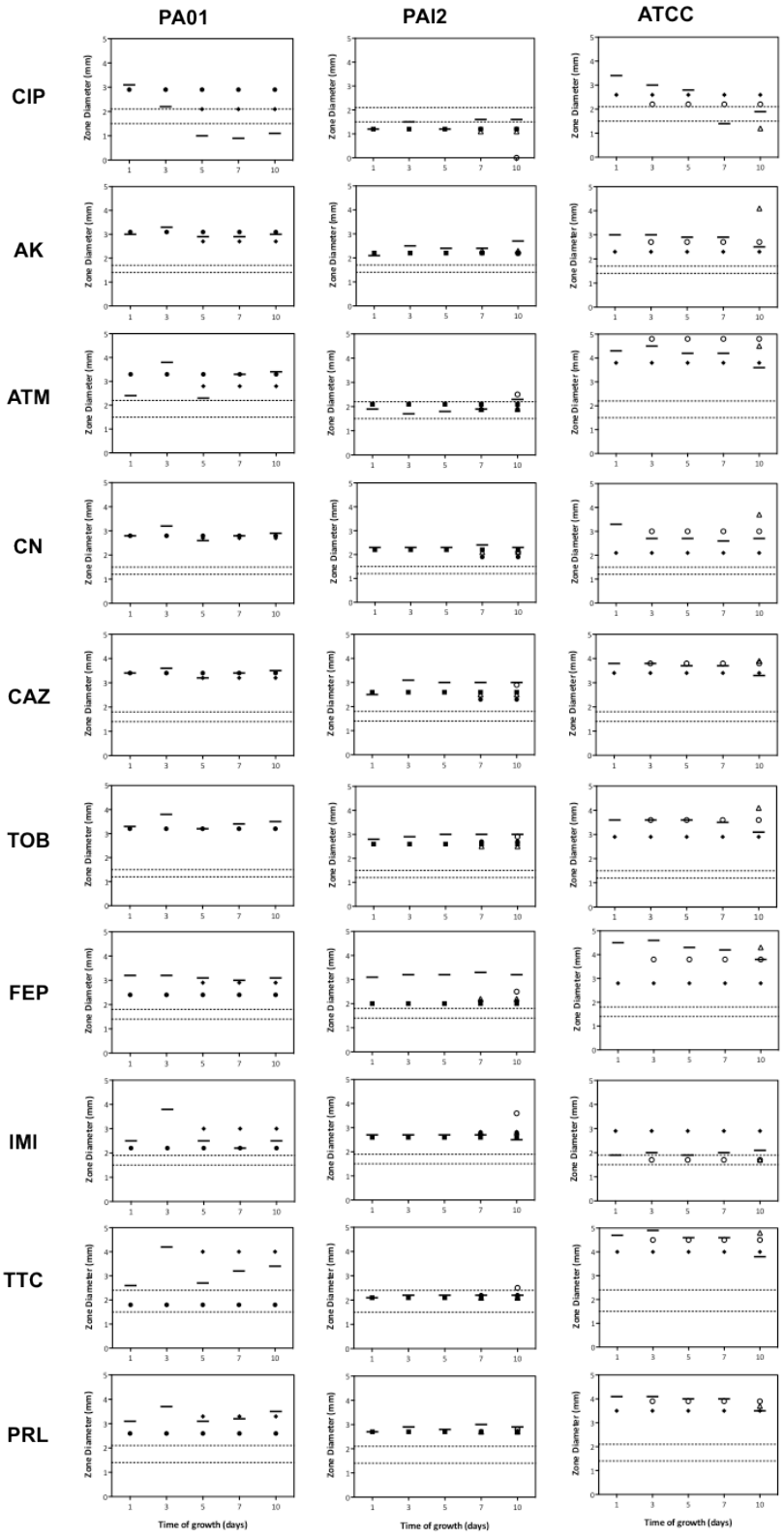

Figure S4.2 – Antibiotic susceptibility profiles of colony morphotypes that composed PA01, PAI2 and ATCC populations and the overall population (-) grown in ASM with 0,5 mg/L of ciprofloxacin.

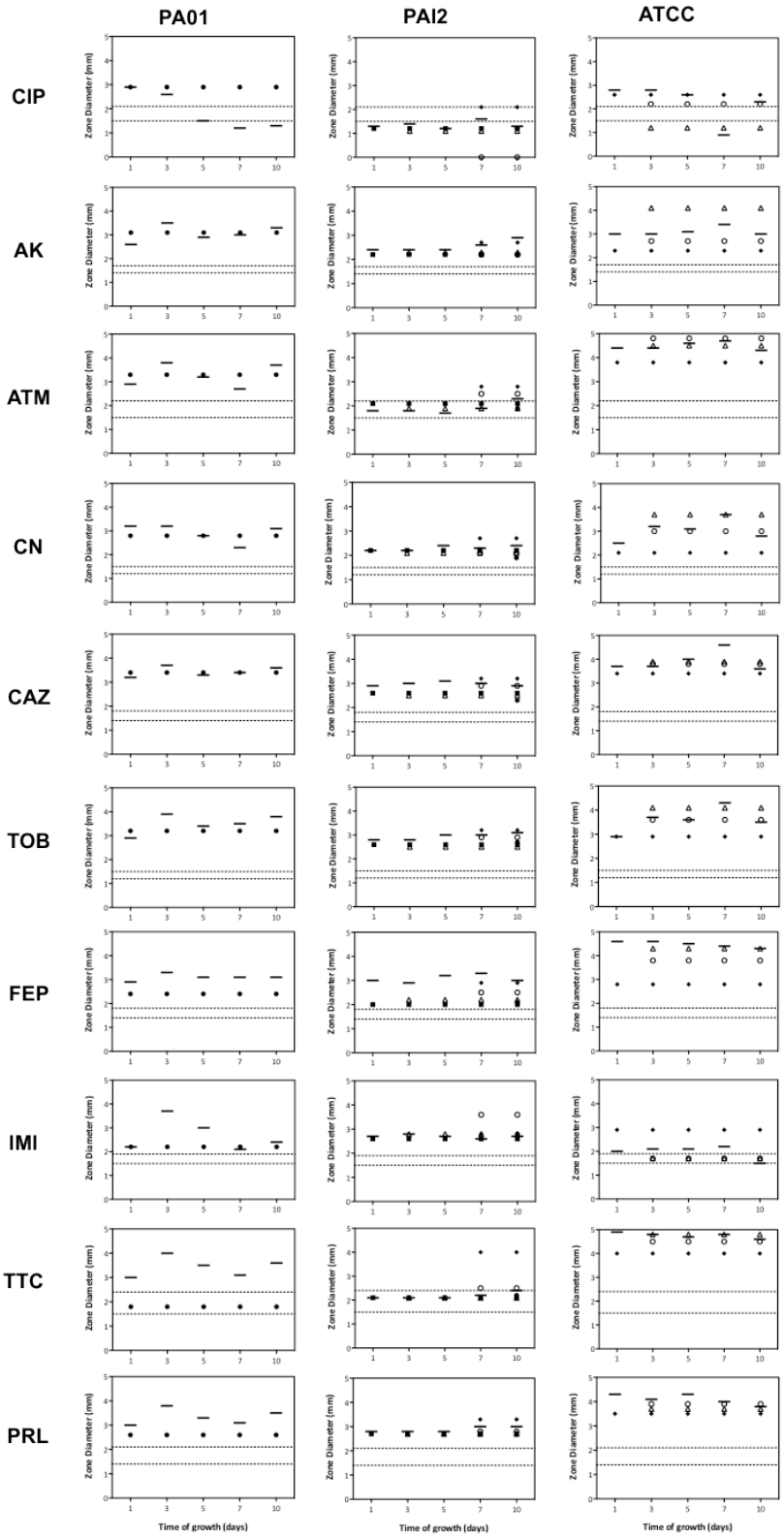

Figure S4.3 – Antibiotic susceptibility profiles of colony morphotypes that composed PA01, PAI2 and ATCC populations and the overall population (-) grown in ASM with 1 mg/L of ciprofloxacin.

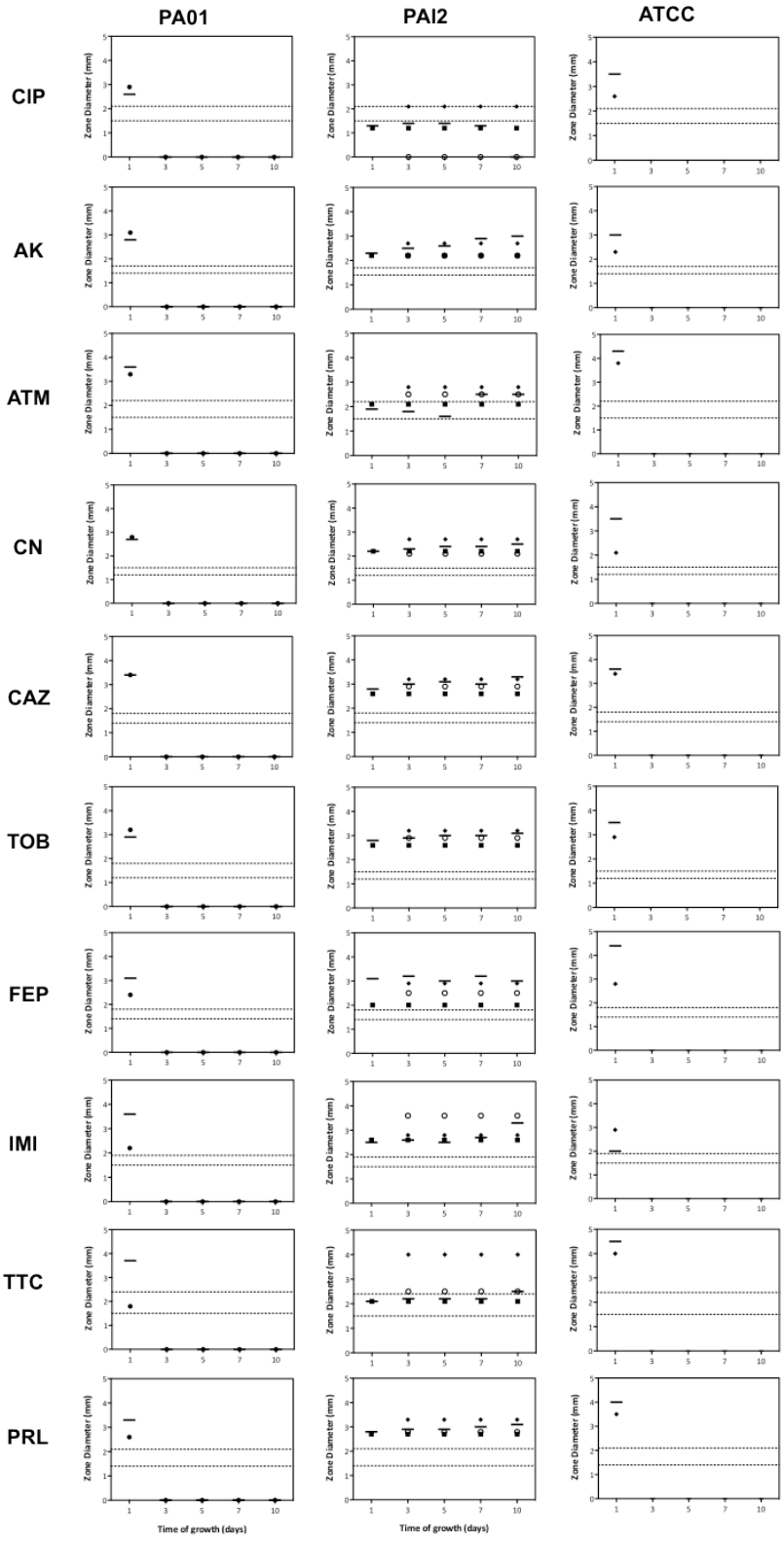

Figure S4.4 – Antibiotic susceptibility profiles of colony morphotypes that composed PA01, PAI2 and ATCC populations and the overall population (-) grown in ASM with 4 mg/L of ciprofloxacin.

Table S4.2. Categorical data analysis of between ticarcillin/clavulanic acid resistance and colony morphology traits.

		Statistical test	χ^2	df	p-value
Colony morphology traits	**Ticarcillin/clavulanic acid resistance**				
	Form	Fisher's Exact Test			1,000
	Margin	Fisher's Exact Test			1,000
	Sheath	Fisher's Exact Test			1,000
	Type of surface	Fisher's Exact Test			1,000
	Texture	Person Chi-Square	4,500 [a]	3	0,212
	Consistency	Person Chi-Square	3,111 [b]	2	0,211
	Opacity	Person Chi-Square	2,143 [b]	2	0,343
	Size	Person Chi-Square	1,143 [b]	2	0,565
	Colour	Person Chi-Square	1,533 [c]	2	0,465

[a] 8 cells have expected count less than 5. The minimum expected count is 0,5.
[b] 6 cells have expected count less than 5. The minimum expected count is 0,5.
[c] 6 cells have expected count less than 5. The minimum expected count is 1,5.

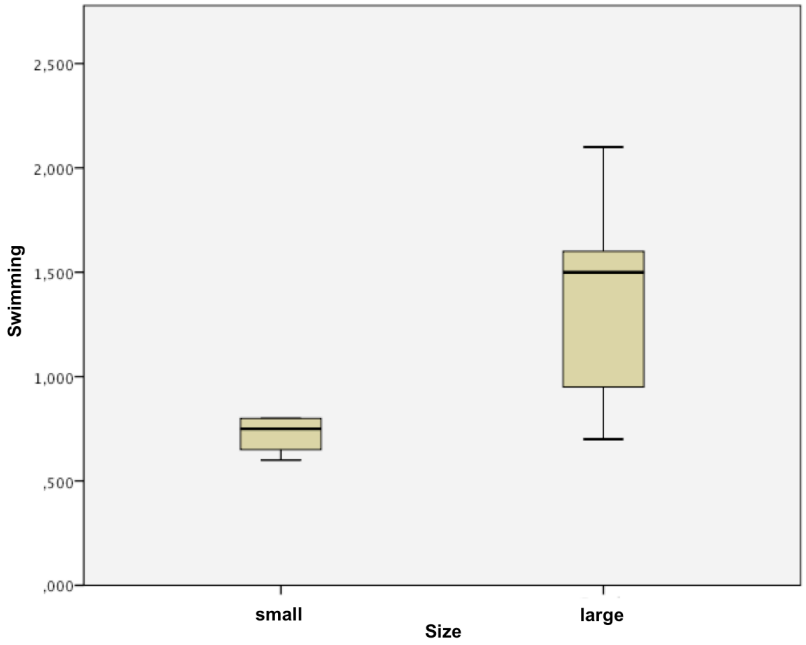

Figure S4.5 – Boxplots that display swimming motility data for colony size. The black bars in the boxes represent the median values.

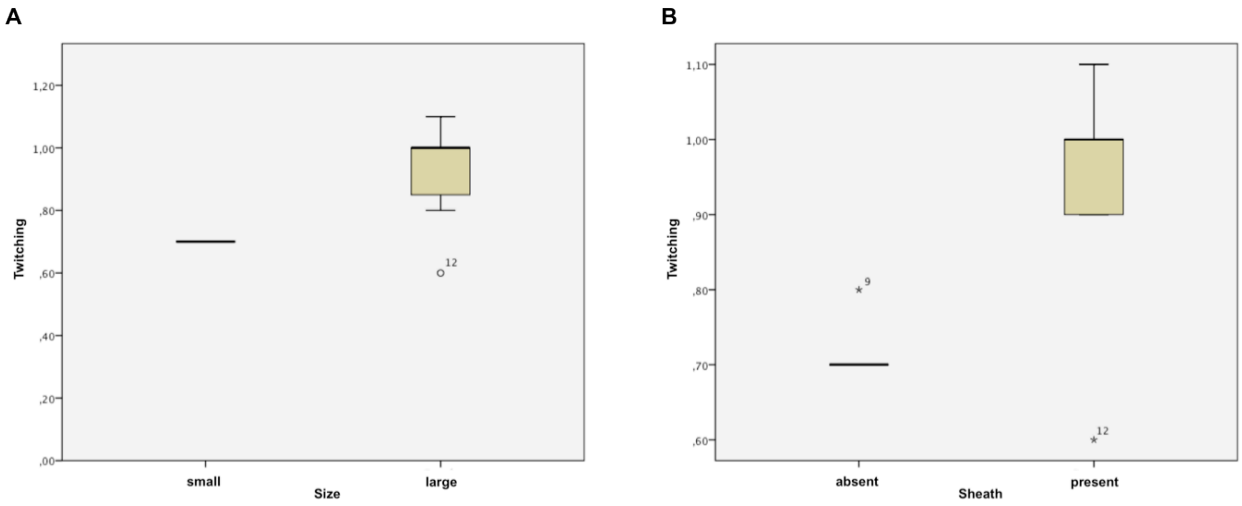

Figure S4.6 – Boxplots that display twitching motility data for (A) colony size and (B) sheath. The black bars in the boxes represent the median values.

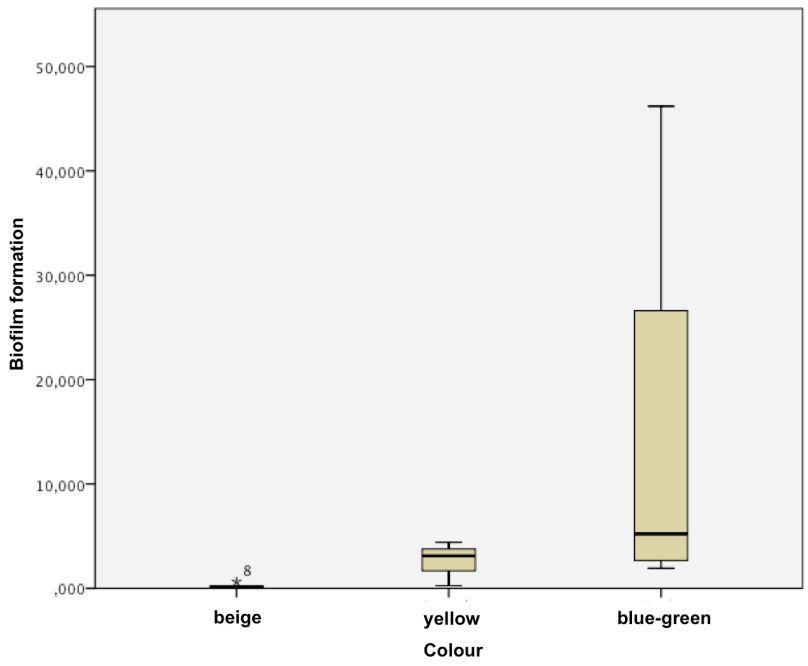

Figure S4.7 – Boxplots that display biofilm formation data for colony colour. The black bars in the boxes represent the median values.

Chapter 5

Morphocol: an ontology-based knowledgebase for the characterisation of clinically significant bacterial colony morphologies

5

MorphoCol: an ontology-based knowledge for the characterisation of clinically significant bacterial colony morphologies

Abstract

To transform the findings of this project and upcoming results into a valuable clinical decision making tool, colony morphology characterisation should be standardised. Notably, it is important to establish the minimum experimental information necessary to contextualise the environment that originated the colony morphology, and describe the main morphological features associated unambiguously. This chapter presents MorphoCol, a new ontology-based tool for the standardised, consistent and machine-interpretable description of the morphology of colonies formed by human pathogenic bacteria. MorphoCol, publicly Web-accessible knowledgebase, is an end-user means to search and compare annotated colony morphotypes using the Colony Morphology Ontology (CMO). CMO is the first controlled vocabulary addressing the specificities of the colony morphology of clinically significant bacteria. The ultimate aim of this work is to help correlate the morphological alterations manifested by colony-forming bacteria during different infections and disease stages with their response to the antimicrobial treatments administered, antibiotic resistance profiles and the expression of virulence factors. MorphoCol is the first tool to address bacterial colony morphotyping systematically and deliver a free of charge resource to the community. Hopefully, it may introduce interesting features of analysis on pathogenic behaviour and play a significant role in clinical decision making. Database URL: http://morphocol.org

5.1. Introduction

Colony morphology observation and characterisation is still today among the most used microbial identification methods by scientific and clinical laboratories because it can be performed through a simple procedure and it is fairly easy to integrate into the analytical pipeline of any laboratory (Figure 5.1). Although it disregards phenotypic variations that are not observable macroscopically as MALDI-TOF MS analysis demonstrated (Chapter 2), it points out those features that are observable macroscopically and keep emerging in association with antimicrobial susceptibility, virulence and persistence profiles. Clinical decision making applications can thus make use of these key morphological features as immediate indicators of microbial behaviour while reasoning about the most adequate antimicrobial therapies. However, colony morphologies vary widely, depending on the particular behaviour of the microbial species under different growth conditions, for instance different colonisation sites (lungs, bloodstream, urinary tract), or exposure to antibiotic agents with different modes of action. Also, the morphological traits exhibited by the colonies may be significantly affected by the procedures taken to isolate and grow the bacteria as demonstrated in Chapter 2, section 2.2. Colony morphology data management is thus a complex task because there is not available a structured and standardized formats, common terminology to describe colonies and minimum information standards for colony morphology origin (e.g. solid media, temperature, time of growth, agar concentration). Computer applications in assistance of phenotypic annotation and systematically analysis of morphotypes are therefore pivotal to manage and integrate data in an amenable, systematic and accurate way. It is very important to establish the minimum set of information to be part of the morphotype description and to employ harmonised vocabulary in both the biological contextualisation and the morphological characterisation of the observed colony.

Scientific literature is the main source of morphotypes, where they are often presented as exemplificative figures of what the researchers observed and are described informally. The fact that the description of colony morphologies does not yet follow predefined rules of annotation nor makes use of controlled vocabulary further hampers the automated classification, integration and interpretation of such data. In particular, biomedical ontologies are the key to standardise terminology and unambiguously describe relevant morphological features well as discretise the corresponding values (or establish some range of threshold, in the case of continuous features). Ontologies are a formalism to describe entities and their relationships and they attempt to replace the free-text descriptions of colony morphologies with equivalent computable descriptions [1]. Ontologies have become an important tool for structuring biological information since the introduction of Gene Ontology (GO) [2] and nowadays they are the standard to support knowledge representation of any scientific field. Through these computable descriptions it is able to draw inferences about the data and provide a practical way to capture the biologically relevant information about, for instance associations with antibiotic resistance, virulence factors expression and gene expression.

Because the collection and ability to compare existing data is imperative to move forward in colony morphology analysis, the present work had addressed the development of systematic computer-amenable approaches to colony morphology data organisation and standardisation. This chapter presents the rationale of a novel ontological framework in support of the characterisation of the colony morphology of clinically significant bacteria. The ontology created will be an integrative resource for the systematic, transparent and unambiguous characterisation of colony morphology traits in support of clinical diagnosis. The ontology will support a public Web repository of colony morphologies specially created to enable the macroscopic observation of morphotypes and the comparison of the morphological "output" of the species in different scenarios (e.g. antibiotic therapeutics and body localisation).

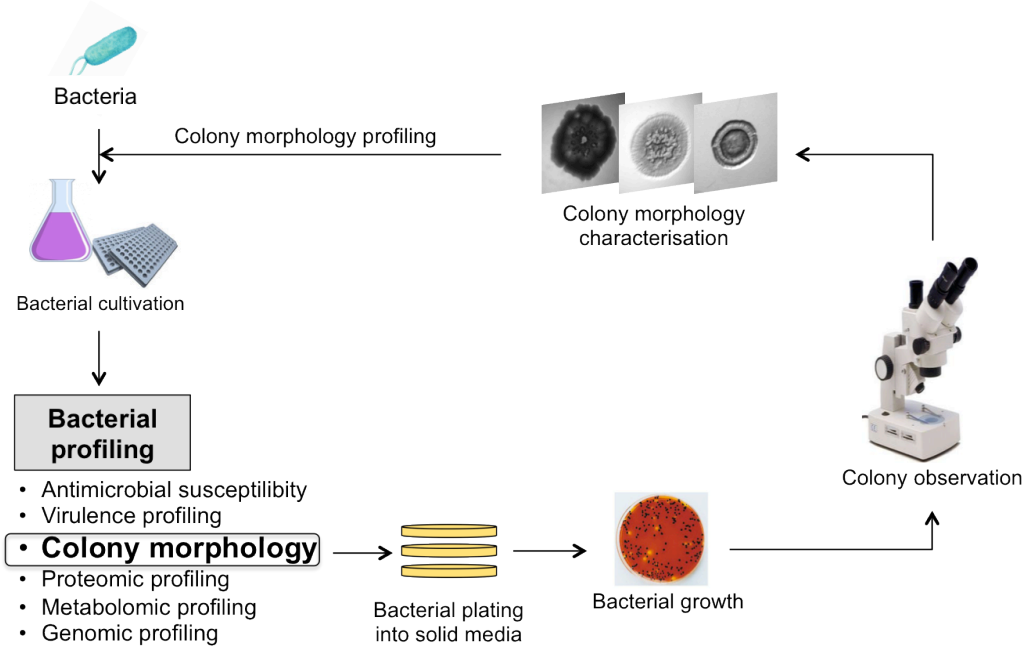

Figure 5.1. General workflow of bacterial profiling. Bacteria provided from samples are cultivated and further characterised using several methods in which colony morphology is included.

5.2. Design and Implementation

5.2.1. Considerations in ontology design

Harmonised and manual annotation guarantees the high quality of the colony morphotypes in MorphoCol repository. Likewise, it enables the search and comparison of morphotypes according to various aspects of morphological and biological characterisations. Ontologies are at the backbone of bioinformatics providing a significant contribution to the annotation of databases and high-throughput data analysis. Till date, no ontology has covered for the specifics of colony phenotypic traits. Therefore, CMO aims to characterise the main features of the morphology of bacterial colonies.

The design of CMO was based on the classification system of colony morphologies proposed in Chapter 2 section 2.3 (Figure 2.6) because this system was constructed following similar principles of an ontology design, including: 1) the revision of literature; 2) collection of the terms commonly used by authors of clinical, microbiological and medical studies in the characterisation of colony morphology; 3) analysis of these textual descriptors, evaluating the appropriateness of the associated semantics and identifying the common name and synonyms of each concept according to overall concordance and our expertise in the field; 4) manual validation of the descriptive ability of the set of concepts gathered against published descriptions of morphotypes. Furthermore, CMO was developed following other basic principles of the Open Biomedical Ontologies (OBO) Foundry [3], in particular:

- CMO is restricted to the morphological characterisation of bacterial colonies and, therefore, it contains just model concepts and relations that are relevant to the representation of colony data;

- CMO should be used for annotating data in databases and for textual documentation and as such, it should be understandable to people and unambiguously interpreted by software;

- CMO development follows a pragmatic approach that grants the ability to integrate new morphological descriptors as they arise without affecting the existing ontological structure;

- any bacterial colony morphology should be comprehensively described by a combination of CMO instances;

- whenever possible, CMO terms are cross-referenced to entries on other ontologies covering for phenotypic characterisation.

The ontology was constructed using the OBO-Edit editor, an open source platform that allows the editing of OBO-alike ontologies (http://oboedit.org/) [4]. Five pre-defined OBO tags were used to represent the CMO terms, including id, name, synonym, def and xref (Figure 5.2).

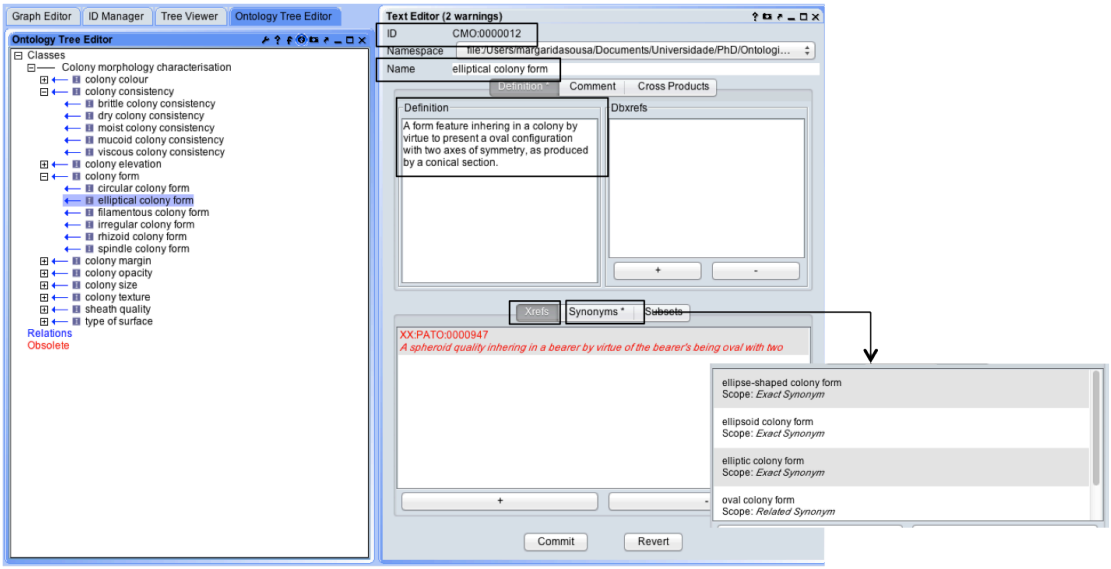

Figure 5.2. CMO viewed in OBO-Edit editor. OBO- Edit editor has multiple panels that can be used to display the overall graph structure of an ontology, id, name of the terms, definitions, external references (xrefs) and alternative names (synonyms).

5.2.2. MorphoCol knowledgebase

MorphoCol is a publicly Web-accessible knowledgebase that documents bacterial colony morphotypes, as comprehensively as possible, in order to enable the search and comparison of morphotypes across species (and strains) and conditions (namely, diseases and colonisation sites). Currently, the main source of information is scientific literature and manual curation grants the high quality of the data available. The curation pipeline (Figure 5.3) will be gradually incorporating automatic procedures, namely text mining processes, now that we have in hand an appropriate terminological resource and the minimum set of information necessary to comprehensively describe a morphotype. Likewise, the knowledgebase will enable the direct submission of morphotype data by authors, promoting a close interaction with the community.

MorphoCol server runs on a CentOS platform (version 5.6) with Apache HTTP server (version 2.2.22), MySQL Community Server (version 5.1.58) and PHP 5.5.3. Apache, MySQL and PHP technology are open-source and platform-independent software. Moreover, MySQL supports multi-threading and multi-user environments, and thus it is well-suited to support (increasing) real-world database usage. Currently, the Web server and all parts of the database are hosted at the Centre of Biological Engineering of the University of Minho, Portugal.

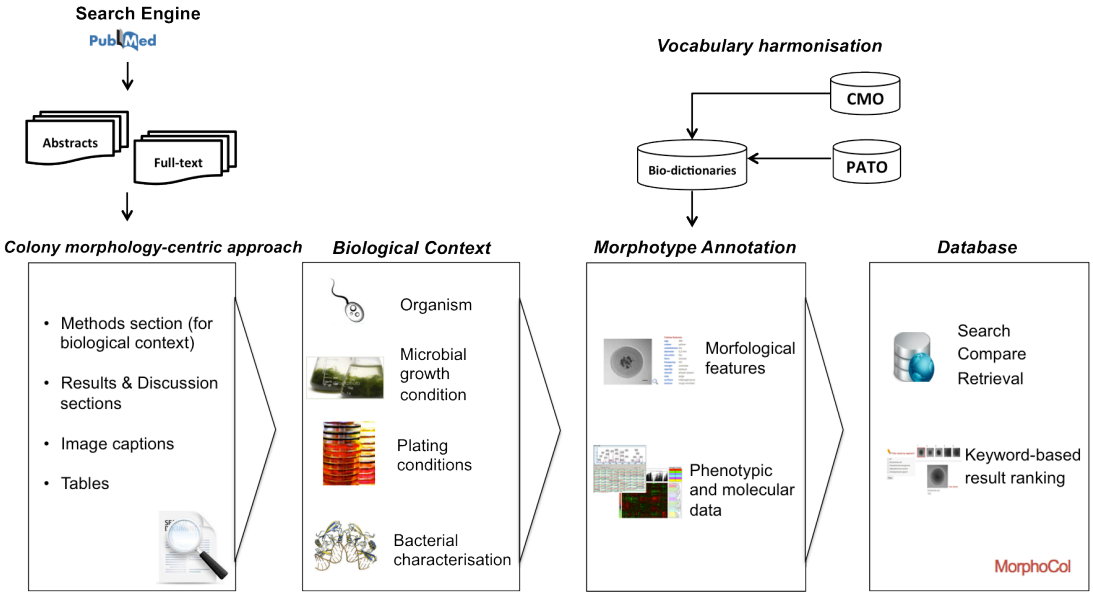

Figure 5.3. An overview of the information curation pipeline of MorphoCol. Colony morphotype curation starts with document retrieval via PubMed. Once all relevant information has been flagged, curators annotate the morphotypes using morphological features of colonies using CMO and PATO controlled vocabularies, and link morphotypes to the corresponding phenotypic and molecular data. MorphoCol search engine enables users to retrieve and compare morphologies, ranking results according to keywords of interest and grouped by bacterial species.

5.3. Results and Discussion

5.3.1. CMO structure and contents

Hierarchical ontologies, such as CMO, hold information about the structure of specific knowledge domain at varying degrees of detail allowing thus to have different levels of resolution. An important issue while defining the organisation of the CMO was the ability to perform updates without causing major changes in the structure. To this end, the high level nodes of the ontology represent the general concepts behind the morphological features more frequently discussed in literature, including ‘form’, ‘margin’, ‘type of surface’, ‘texture’, ‘sheath’, ‘opacity’, ‘elevation’, ‘consistency’, ‘size’ and ‘colour’ (Figure 5.4). CMO encompassed thus a total of 10 main categories and 45 sub-categories. Table 5.1 presents the definitions of the main concepts.

The CMO ontology files, including terms, definitions and relationships, are freely available at the MorphoCol Web knowledgebase for bacterial colony morphotypes (http://morphocol.org) and the portal of the international consortium Minimum Information About a Biofilm Experiment (MIABiE) (http://miabie.org).

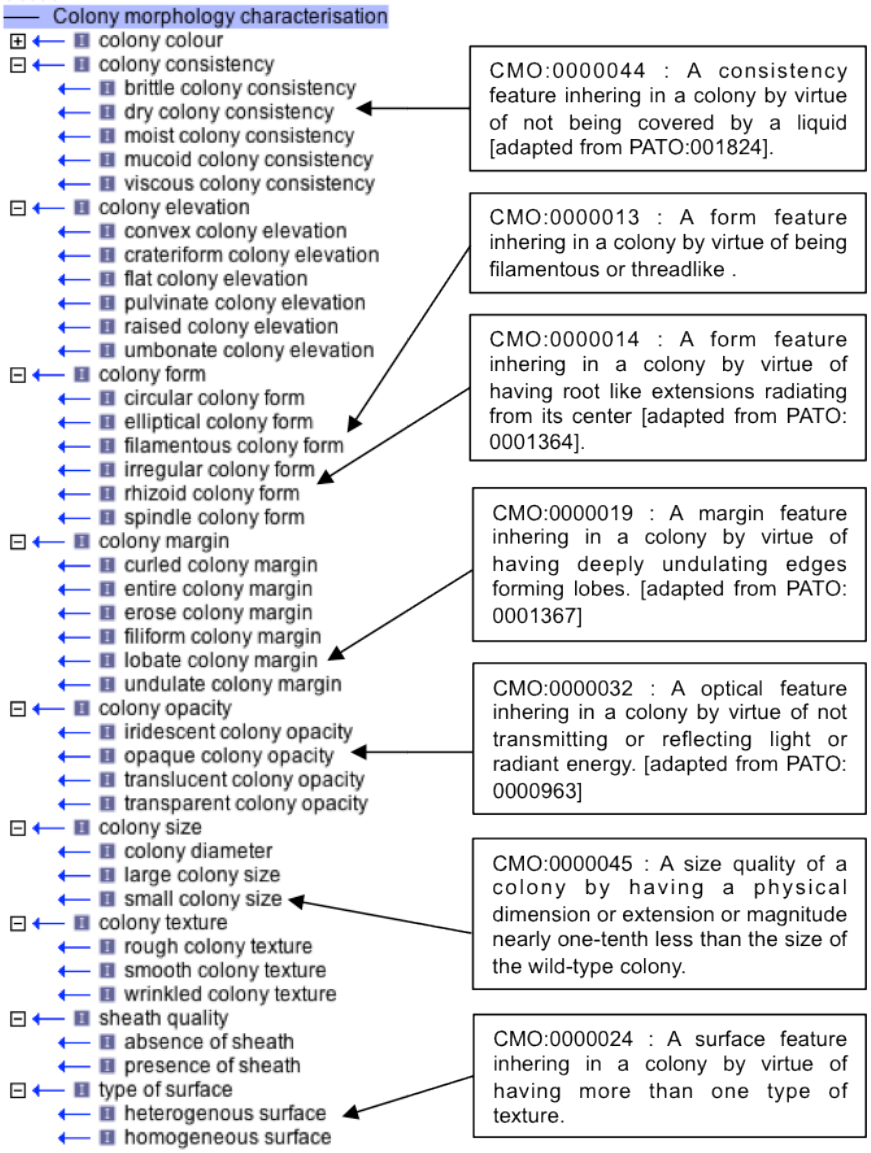

Figure 5.4. Structure of the CMO with definitions of some terms directly imported or adapted from the PATO.

Table 5.1 Conceptual definitions of the main categories of CMO. Each term is represent by an unique id (CMO:00000) and a name.

Entity	Description
colony form (CMO:0000001)	A morphological quality inhering in a colony by virtue of having a configuration.
colony margin (CMO:0000002)	A morphological quality inhering in a colony by virtue of having a limit zone.
Colony sheath (CMO:0000005)	A morphological quality inhering in a colony by virtue of having a closely enveloping part or structure after the margin and around the colony.
Type of surface (CMO:0000003)	A morphological quality inhering in a colony by virtue of having morphological features present on its outer layer.
colony texture (CMO:0000004)	A morphological quality inhering in a colony by virtue of having a characteristic physical structure or composition or representation of the appearance, organization and distribution of its surface elements.
Colony elevation (CMO:0000007)	A positional quality inhering in a colony by virtue of vertical distance of a point above or below a reference surface.
Colony consistency (CMO:0000008)	A physical quality inhering in a colony by virtue of having density, firmness, or viscosity.
Colony opacity (CMO:0000006)	An optical quality which obtains by virtue of the ability of the mass of the colony to absorb visible light.
Colony size (CMO:0000009)	A morphological quality inhering in a colony by virtue of having a physical magnitude.
Colony colour (CMO:0000010)	A composite chromatic quality composed of hue, saturation and intensity parts.

5.3.2. PATO as ontological reference

Multiple ontologies have been proposed in the domain of phenotypes. Some are specialised in the characterisation of species (typically, model organisms), such as the Mammalian Phenotype ontology (MP) [5], the Worm Phenotype ontology (WPO) [6], the Plant Trait ontology (PO) [7] and the Human Phenotype ontology (HPO) [8]. Others, like the Phenotype and Trait ontology (PATO) [9], are focused on integrating phenotypes across species, and reuse anatomy and process ontologies.

The analysis of existing phenotypic ontologies, such as PATO, MP, WPO, PO and HPO, revealed a number of terms in common. However, PATO is the only ontology that provides taxon-independent and general phenotypic descriptors, which is one of the main requirements of the CMO design.

No CMO term is defined identically to a PATO term. A total of 43 terms of PATO (Figure 5.4) were adapted to fit the CMO domain. For example, the term ‘circular’ (PATO:0000411) in PATO is defined as "a shape quality inhering in a bearer by virtue of the bearer's being such that every part of the surface or the circumference is equidistant from the center" and in CMO is defined as "a 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". So, the labels of the CMO terms were altered to prevent any confusion about the CMO and PATO terms and their definitions. For example,

'circular colony form' (CMO:0000011), 'smooth colony texture' (CMO:0000025), 'transparent colony opacity' (CMO:0000030), 'flat colony elevation' (CMO:0000034), 'viscous colony consistency' (CMO:0000041), and 'colony colour' (CMO:0000010). The adaptation of terms and definitions was also motivated by differences in the organisation of the PATO and the CMO, and affected 14 terms. For instance, the term 'lobate' (PATO:0001367) in the PATO is "a surface feature shape quality inhering in a bearer by virtue of the bearer's having deeply undulating edges forming lobes" whilst the term 'lobate colony margin' (CMO:0000019) in the CMO is not a surface feature shape quality, but rather a margin feature quality.

A total of 10 CMO terms did not find any correspondence with PATO due to semantics differences. For example, the term 'small' (PATO:0000587) is described in the PATO as "a size quality which is relatively low", but in the scope of bacterial colonies the definition of 'small colony size' (CMO:0000045) is more specific, i.e. "a physical magnitude 10 times smaller than the diameter of the wild-morphotype".

5.3.3. Web application – MorphoCol knowledgebase

The volume of phenotypic data on bacterial colonies is growing considerably due to the use of high-throughput experimental procedures and analytical methods, and the impressive ability of bacteria to diversify under several environmental conditions. The variety of morphotypes within and across species is great, and requires expert knowledge on the formation of colonies and on the pathogenic bacteria under study in order to describe the observed morphological features comprehensively. It is of upmost importance that experimental design and morphological annotation must be consistent in order to allow the systematic comparison of morphotypes across experiments (and laboratories), species, diseases and clinical samples. Currently, free-text description and searching is the basic form to report and retrieve information, which is extremely limited because of the inherent ambiguity and lack of accuracy and specificity. Researchers are in need of new resources and tools geared to systematically analyse morphotypes, across infections, body locations, antimicrobial treatments and a number of other conditions of clinical relevance. Web-knowledgebase and biomedical ontologies are the key to organise and standardise terminology and describe relevant morphological features unambiguously.

Concerning data organization in the MorphoCol repository, it should be made simple and intuitive so that the complexity of the studies would not affect the clear and unambiguous presentation of the colony morphology traits, the related results and biological contextualisation. In the scope of this bioinformatics initiative, a novel ontology on colony morphology was prepared to describe the overall concept of morphological traits of bacterial colonies. The immediate application of the CMO is the sharing of current understanding of the variation of colony morphology in microbial infections among domain experts, both clinicians and researchers. Furthermore, the CMO may be of help to

information retrieval applications, providing vocabulary and taxonomy that can be used for query expansion and semantic searching in this domain.

The MorphoCol knowledgebase is supported by the CMO and aims to help manage the fast proliferation of information about colony morphology. The description of colony morphotypes using CMO provides a practical way to capture the biologically relevant information about the morphotypes in a machine-readable form. Moreover, it allows to compare, combine and analyse different colony morphotypes obtained from different species, strains, patients and laboratories.

Currently, documentation efforts are focused on morphotypes exhibited by pathogenic bacteria causing respiratory infections, one of the most prevalent types of infection worldwide. Typically, respiratory human pathogens, such as *P. aeruginosa*, *S. aureus*, *Klebsiella pneumoniae* and *Dolosigranulum pigrum*. The identification of clinically significant colony morphotypes is of tremendous importance because colony observation is quite immediate and costless when compared to state-of-the-art identification methods. Even without pinpointing the strains involved, morphotypes comparison may provide insights on, for instance, the stage of infection (early, intermediate or chronic) and the resistance and virulence levels to be expected from the bacteria. To achieve those goals, colony morphology signatures should be self-contained, i.e. there should be enough information about the purpose and execution of the experiment as well as the analysis of results. As such, colony morphologies are to be documented with various descriptive metadata (Figure 5.5), that is data of data, including information about the experimental conditions. Experimental information is related to the “circumstances” in which colonies were formed since bacteria collection (in case of clinical samples, e.g. lungs, blood, urine, wounds, catheters), *in vitro* growth conditions (e.g. the type of culture - planktonic or biofilm -, type of solid media, temperature, pH, colony growth time, oxygen concentration), *a posteriori* bacterial characterisation (e.g. MIC and MBC determination, virulence factors expression and metabolomics, proteomic and transcriptomic profiles) and the statistical validation (i.e. the number of replicates and reproductions performed). The description of such information is of upmost importance because morphotypes are fully comparable only for similar methods under identical conditions. Also to effectively search, analyse and compare data performed in the same conditions it is need that metadata will be well organized and semantically integrated. Thus, the lexicon used should be universal, familiar and unequivocal.

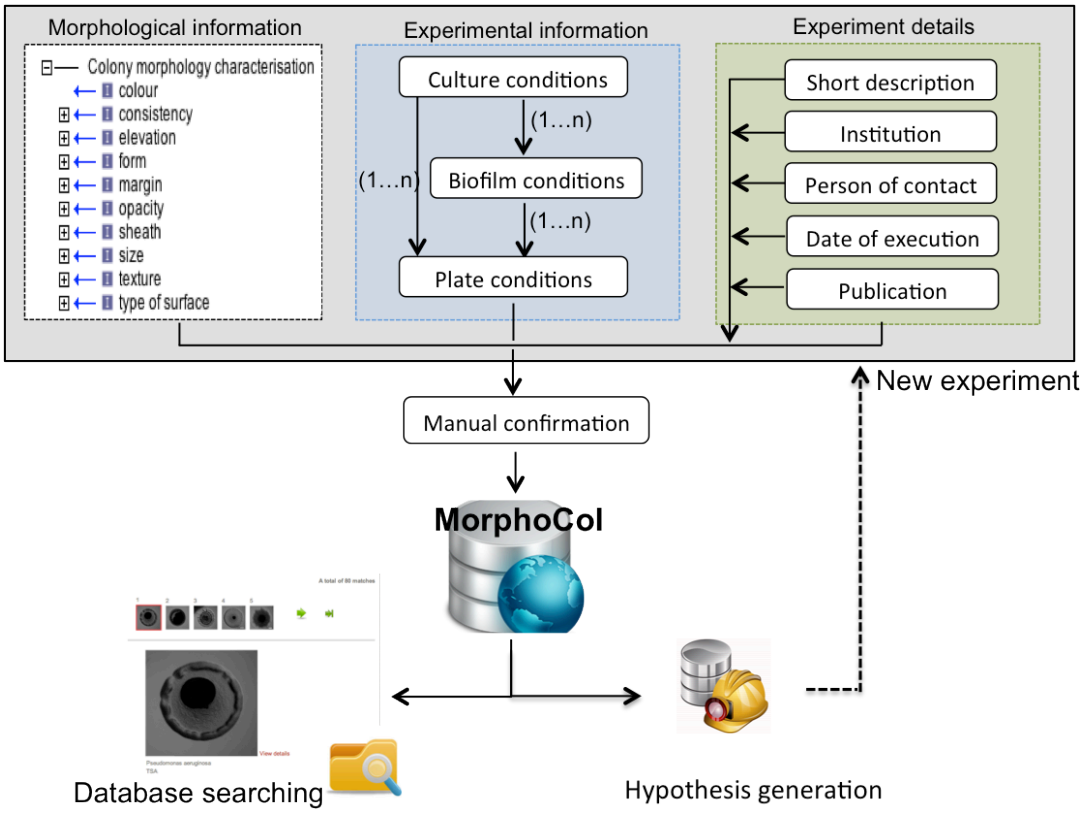

Figure 5.5 The flowchart of the MorphoCol annotation process. Colony data annotation consists of the curation of morphological, experimental metadata and details followed by manual confirmation. After validation, morphotype data record is available online for information retrieval and formulation of new hypotheses.

The major challenge to be faced in documenting colony morphology characterisation experiments and results is the complexity and variability of studies. The management of such heterogeneous metadata is complicated and raises several quality issues, such scarcity of standardised protocols, poor metadata quality and incomplete data sets, which affects significantly the quality of the results being stored in MorphoCol. This problem is not exclusive of colony morphology knowledge domain. Biofilm community has already detected difficulties of manage their heterogeneous data and metadata and has initiated efforts to overcome such barriers. The international Minimum Information About a Biofilms Experiment (MIABiE) initiative (http://miabie.org) is working on the harmonisation of biofilms procedures and associated data. To overcome some difficulties MIABiE initiative developed as well an ontology on biofilms and their study – the Biofilm Ontology (BO) [10] – and a web-repository – BiofOmics [11]. BO was designed to develop shared, structured and accurate vocabularies for the annotation of the general biofilm experimental workflow. For those colonies coming from biofilms MorphoCol is supported by BO. However, to accomplish the main goal of MorphoCol, to be a relevant auxiliary clinical decision making tool, information related to morphotypes must be indexed using whenever possible ontologies or controlled vocabulary accepted by scientific communities. Table 4.2 presents some of the intended ontologies and controlled vocabularies that will support MorphoCol in near future.

Table 5.2. Some predicted ontologies and controlled vocabularies that may support MorphoCol.

Ontology/ controlled vocabulary	Source	Reference
Chemical entities of biological Interest (CHEBI)	http://obo.cvs.sourceforge.net/obo/obo/ontology/chemical/chebi.obo	[12]
Infectious Disease Ontology (IDO)	http://www.bioontology.org/wiki/index.php/Infectious_Disease_Ontology	[13]
Gene ontology (GO)	http://obo.cvs.sourceforge.net/obo/obo/ontology/genomic-proteomic/gene_ontology.obo	[2]
Functional Genomics Investigation Ontology (FuGO)	http://sourceforge.net/projects/fugo/	[14]
MALDI imaging ontology (IMS)	http://www.maldi-msi.org/download/imzml/imagingMS.obo	[15]
PSI-Mass Spectrometry (MS)	http://psidev.cvs.sourceforge.net/viewvc/psidev/psi/psi-ms/mzML/controlledVocabulary/psi-ms.obo	[16]
PSI-Sample Processing and Separations (SEP)	https://psidev.svn.sourceforge.net/svnroot/psidev/psi/sepcv/trunk/sep.obo	[15]
PRIDE controlled vocabulary	http://code.google.com/p/ebi-pride/source/browse/trunk/pride-core/schema/pride_cv.obo	[17]
Protein ontology (PRO)	http://obo.cvs.sourceforge.net/obo/obo/ontology/genomic-proteomic/pro.obo	[18]
Phenotypic And Trait Ontology (PATO)	http://obo.cvs.sourceforge.net/obo/obo/ontology/phenotype/unit.obo	[9]

Some efforts had been initiated in indexing experimental information to colony morphotypes related to microorganism description, conditions of morphotype observation, *in vitro* growth conditions and phenotypic characterisation of morphotypes, in particular antimicrobial resistance profiles (Figure 4.5). Related to the conditions in which colonies are observed it is discriminate the solid media, temperature and oxygen availability used to originate the morphotype. At the colony source level, it is describe the origin of bacteria that form the morphotype. For instance, for *in vitro* studies it should be describe whether bacteria coming from a planktonic culture or biofilms. For clinical samples or others it should be describe all known characteristics of the sample, for instance, patient status (normal or infected), infection name, body location or fluid. Phenotypic data was also indexed to morphotypes and it was started integrating the antimicrobial susceptibility data due to the impact on clinical field. At this level authors should describe the agent, the method used to determine susceptibility (E-test, microdilution, disk diffusion method), the international standards used to categorize bacteria (e.g. EUCAST or CLSI) and the value of susceptibility (S – susceptible or sensitive, I – intermediate or R - resistant).

The morphotype data record is manually verified in order to ensure its quality and thus allow clinicians and researchers to consider such data in their analyses and the formulation of new hypotheses.

MorphoCol also “credits” information. The experiment is profiled in terms of authorship (institution and person of contact), the data scope, the date of execution, a summary of its main findings (produced by the authors), eventually other notes of interest and derived publications.

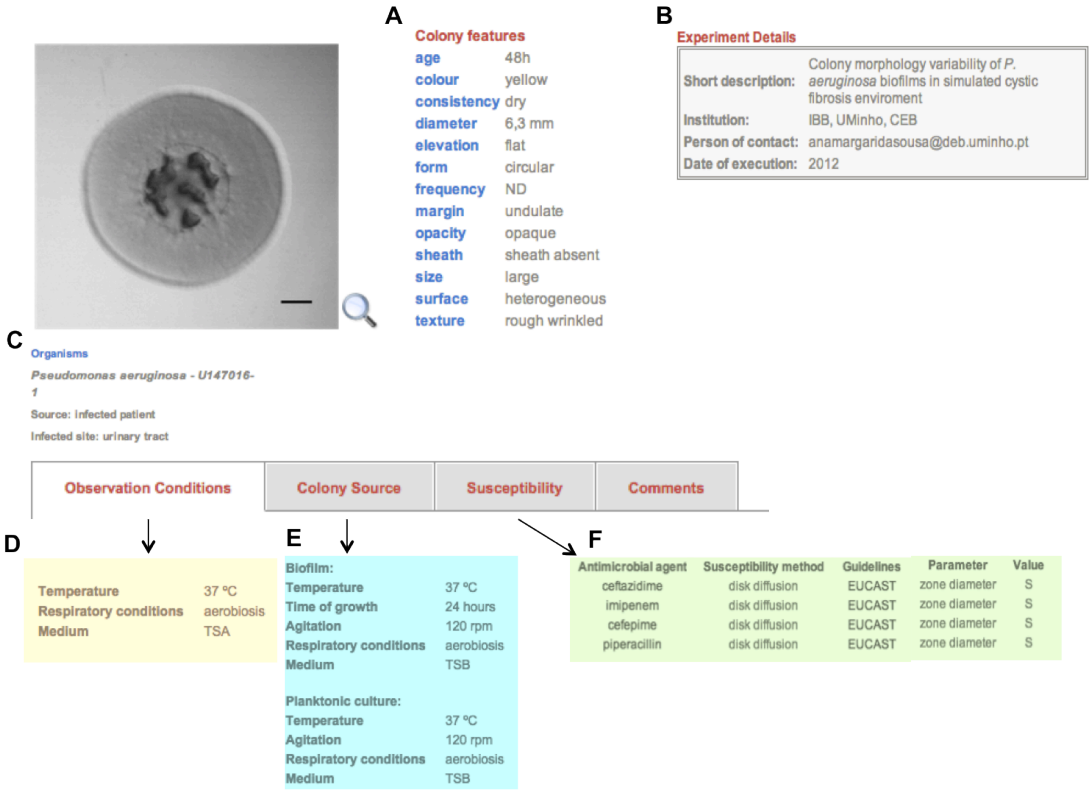

Figure 5.6. Different levels of information associated to a colony morphotype. (A) Morphological data: description of colour, consistency, diameter, elevation, form, margin, opacity, sheath, size, surface, texture; (B) Authorship; (C) Description of the microorganism; (D) Conditions in which morphotype was observed; (E) Colony source: description about the bacterial origin; (F) Antimicrobial susceptibility profile: discrimination of the bacterial response against antimicrobial agents.

5.4. Conclusions

Several authors have documented colony morphologies in clinical settings and have shown that morphological features may be indicative of underlying microbial cues, and most notably, of resistance and virulence responses [19, 20]. Nowadays, it is demand the clear association between morphological features and antibiotic resistance, virulence factors expression and the identification of biomarkers. The key to satisfying this need is the ability to describe different colony morphotypes in a consistent and structured way and also to sharing the clinical findings regardless source of the clinical samples. This led to the development of a specialised method of analysis that

aims to deliver useful inputs to more elaborated (and costly) studies, and assistance to clinical decision making.

The originality of this work lays on addressing colony morphotyping in a systematic, harmonised and computerised way. The development of the CMO, the first ever controlled vocabulary on colony morphology, and the MorphoCol knowledgebase are considered an important step forward for enabling the standardised and systematic annotation of morphotypes. For the first time, there is a knowledgebase dedicated to the management of data related to colony morphotypes, including morphological data and experimental metadata. This knowledgebase provides the basic means to enquire and compare the visual manifestations of bacterial evolution and adaptation processes across pathogenic microorganisms and infections. Although still in its infancy, MorphoCol aims to pave the way to the development of advanced clinical decision making applications, which may use morphological features as immediate indicators of microbial behaviour. These indicators can be used to guide more sophisticated (time-consuming and costly) analyses, such as proteome and transcriptome analyses. Also, they may be used to construct decision support models that help clinicians in determining or anticipating what may be expected in terms of a given microbial species resistance and resilience in a clinical incident. To the best of our knowledge this is the first public repository documenting bacterial colony morphology systematically.

In the short term, efforts will be focused on extending the description of the pathogenic potential of colony-forming bacteria, particularly regarding the expression of virulence factors, such as the ability to form biofilms, the production of toxins and quorum-sensing molecules. Moreover, the query tool will be complemented by a customisable comparison tool that looks for morphological similarities across species, infection sites and diseases. Therefore, the tool will provide insights on the most relevant traits of colony morphology under a given clinical scenario, which may be useful as predictive features of the virulence potential and resistance profile of bacteria causing the infection.

Currently, the system documents respiratory infection traits, but in the future it will cover other major infections regarding the urinary tract, bloodstream, chronic wounds, osteomyelitis and biomaterial-associated infections. MorphoCol will be thus of aid to the wider community of researchers and clinicians working in clinical microbiology.

MorphoCol welcomes contributions from any research group working on the characterisation of clinically relevant morphotypes, crediting data authorship and associated bibliographic references.

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Chapter 6

Final Remarks

6.1. General conclusions

While CF community awaits the development of effective therapies targeting CFTR mutations, the management of microbial infections remains the primary focus. CF lungs shelter an impressive number of microbial species, but *P. aeruginosa* has excelled due to the association with rapid poor lung function and impressive persistence in CF lungs despite the long and aggressive antibiotic courses. *P. aeruginosa* long persistence is mainly due to clonal diversification into well-adapted phenotypes to CF environmental conditions with diverse phenotypic traits altered. This phenotypic diversity is observed when isolates from CF lungs are plated on solid medium and exhibited diverse colony morphology variations, for instance mucoid consistency and small size. Furthermore, some colony morphological traits are also associated with altered virulence factors expression and antibiotic resistance. Therefore colony morphology variation is considered an indicator of clonal diversification.

The key factor to avoid clonal diversification, bacteria adaptation and chronic infections development is the early eradication of *P. aeruginosa*. To achieve a successful early eradication, it is need understanding the adaptation mechanisms used by *P. aeruginosa* so that effective antimicrobial strategies could be design and prevent antibiotic resistance, re-infection episodes and progression to chronic stages. As so, this study aimed to provide to CF community valuable insights about the early adaptation mechanisms used by *P. aeruginosa*, the respective driven forces and the early adaptation and infection development markers.

Colony morphology characterization is an old microbial technique that usually complements the microbial diagnosis. Despite its long use, it is not clear the impact of the experimental procedures on colony morphogenesis. Moreover, the vocabulary and concepts used to describe colonies is inconsistent among reports and it is not updated for years. Since *P. aeruginosa* clonal diversification and evolution would be evaluated based on colony variation, it was considered fundamental to verify first which parameters affect colony morphogenesis and the detection of colony diversity. All experimental factors analysed, including solid medium, time of colony growth, number of colonies *per* plate, bacteria mode of growth (planktonic and biofilm) and genetic background demonstrated to have impact on colony morphology definition. The perception of colony morphologies dependence of experimental conditions allowed fitting the actual performance of clinical diagnosis culture-based approaches and a set of guidelines was proposed for authors, clinicians and technicians to implement when performing colony morphology characterization. CF community will great benefit with similar experimental procedures of colony morphology characterisation when comparing results with other centres and studies, leading to a better comprehension of bacterial adaptation and evolution.

Some improvements at colony lexicon level were also performed. The vocabulary used to describe bacterial colony morphologies was updated and organized in a structured way. At the end, a classification system was proposed to unambiguously characterise colonies. This system was

deeply tested using several bacterial species and demonstrated to be adequate for colonies of any bacterial species. Modifications and updates to this classification system suggested by microbiological community are very welcomed. The system was constructed to be easily modified without structural constrains.

Colony morphology characterisation is a culture-dependent method and this significantly delays results delivery. Currently, microbial diagnosis demands high-throughput analysis and automated systems that can return rapid and reliable results. Therefore, culture-independent methods present an attractive approach to detect, identify and characterize pathogens. As colony morphology characterization has great potential in clinical diagnosis some efforts were performed to make colony morphology characterization an advanced method using MALDI-TOF MS. It was verified the capability of MALDI-TOF MS provide similar colony categorisation to the manual morphotyping. The results obtained revealed that MALDI-TOF MS did not return similar results than manual colony morphotyping. The distinct results obtained may due to the MALDI-TOF MS criteria were based on molecular profiles that could be not analysed by naked eyed as in colony morphology characterisation. As so, MALDI-TOF MS was not able to "replace" manual colony morphology characterisation, but it could be used as complementary method.

The extensive *P. aeruginosa* diversification in CF-adapted phenotypes could be also caused by the presence of biofilm growth within CF sputum. Biofilms are the underlying cause of chronic infections and thus their early detection is of upmost importance to CF disease management. Classically, there is a temporal window for successful eradication until bacteria switch to sessile lifestyle. Before biofilm formation, bacteria are more sensitive to antimicrobial agents and infections are thus more easily eradicated. After biofilm formation, *P. aeruginosa* is hardly eradicated mostly due to multifactorial resistance mechanism of biofilms. Therefore, the determination of the timing and the driven forces of bacteria switching mode of growth is upmost importance for early eradication treatments. As colony variants of biofilm cells were distinct from their planktonic counterparts as previously evidenced, the determination of the timing and the driven forces of bacteria switching to biofilms could be facilitated through a colony marker of biofilms. Therefore, it was verified whether *P. aeruginosa* biofilm cells had characteristic colony morphology. The results revealed that *P. aeruginosa* strains produced biofilms with distinct population diversity and a biofilm colony variant marker was not found. SCV was found as a strong indicator of biofilm growth.

Given the distinct *P. aeruginosa* biofilm populations with different number of colony variants with different morphological traits, it was investigated the role of population diversity in biofilm pathogenesis potential. Population diversity seemed to have no impact on biofilm pathogenesis, but the absence of SCV was associated with increased biofilm virulence potential, notably in the amount of biofilm mass produced.

To design effective early treatments for *P. aeruginosa* eradication, it is important the investigation of the adaptation mechanisms that *P. aeruginosa* uses when colonizes new environments and when challenges by antibiotic treatments. To closely mimic CF lungs environment, it was used ASM, an

experimentally determined composition of CF sputum that can support similar *P. aeruginosa* growth and adaptations as found in real CF lungs. Furthermore, different antibiotic regimes were simulated. Early and delayed ciprofloxacin treatments using different dosages, correspondent to sub- and MIC of biofilm cells, were tested to investigate the *P. aeruginosa* response. From the results obtained, it was concluded that substantial phenotypic diversity is likely to be present in *P. aeruginosa* populations shortly after CF lungs colonization. Ciprofloxacin concentrations had impact in success rates of the treatments. Early treatments using sub-inhibitory concentrations were ineffective in bacteria eradication and triggered population diversification into phenotypes better "fitter" to CF conditions. Delayed antibiotic therapies could have unpredictable results being the eradication results dependent of the adaptive ability of *P. aeruginosa* strains. Impaired swimming motility was the initial adaptation noticed in all *P. aeruginosa* strains exposure or not to ciprofloxacin treatments. This adaptation seemed to be driven by the environmental CF conditions, possibly by the presence of mucin. It was thus concluded that defective swimming motility is a potential disease marker of early stages. Colony morphology traits revealed to be promising indicators of antibiotic resistant and virulence expression.

Given that the ultimate aim is to rationalise morphological and biological associations within and across species, and across infections, molecular, proteomic, transcriptomic and genomic profiles, it was developed the first knowledgebase dedicate to bacterial colony morphologies, called MorphoCol. MorphoCol attempts to standardise annotation of morphotypes and, in addition, manage data related to colony-forming bacteria, such as phenotypic, transcriptomic and proteomic information. Although still "younger", MorphoCol in near future will provide the correlation of morphotypes to resistance and virulence cues, and enhance the understanding about the different responses of bacteria to antimicrobial therapies in acute and chronic infections. Altogether, these data will provide valuable inputs to clinical research and decision-making.

In summary, progresses were made in the identification of the initial *P. aeruginosa* adaptations and their driven forces, in identification of a promising disease marker and lastly in identification of new indicators of altered virulence potential and antibiotic resistance. These results will certainly impact on the diagnosis of CF-associated infections and in the antimicrobial stewardship of those infections.

6.2. Future research lines

The current challenge for clinicians and researchers is to translate the stages of *P. aeruginosa* adaptation steps and infection stages into clinical markers in order to perform adequate and effective clinical interventions that will significantly improve the therapeutic outcomes. In this project, some putative clinical markers of early stage of CF disease and promising indicators of altered virulence potential and antibiotic resistance were pointed out, but more work has to be done to validate these outcomes.

P. aeruginosa is remarkably plastic in its ability to colonize and growth in so different environments. This success needs an explanation and an overall picture of the adaptive and evolutionary pathway will be always valuable. CF lungs are extraordinary complex habitats where there are quite diverse environmental (pH, oxygen, DNA, amino acids, mucin concentrations, different antibiotic treatments) and biological conditions (other bacterial species, fungi and virus). To draw an adaptive and evolutionary profile of *P. aeruginosa*, it is essential to reflect the full complexity of this habitat in order to encompass all the adaptive possibilities. Nevertheless, it must be aware that evolution is somewhat unpredictable.

From an experimental point of view, it is a hard task to integrate all the environmental and biological ingredients into a single experiment. Clinical and microbiological studies are mainly based on cause-and-effect approach that helps to identify a likely cause of a problem. With such amount of possible clinical scenarios, it would be need to add a lot of variables at the same time resulting in a very hard task to find out the significant mechanisms used by *P. aeruginosa* to face certain clinical scenario and their driven forces. Therefore, *P. aeruginosa* evolutionary pathway and adaptation must be constructed “piece by piece” as a “puzzle”. The results obtained with this project are the first pieces of this quite complex puzzle. In fact, in this project, *P. aeruginosa* was studied alone, grown in common CF sputum conditions and in the presence and absence of ciprofloxacin, conditions that are a bit away from the real scenario. One of the most remarkable features of CF lungs is the impressive number of microbial species that can reside and co-exist. This feature is of upmost importance in the context of infections control and, in particular, *P. aeruginosa*-associated infections. It is thus paramount the detailed investigation of how *P. aeruginosa* diversity, pathogenesis and stress response are influenced by the presence of other pathogens and vice-versa. It is important determine 1) which are the organism or combinations of pathogens that augment the pathogenic potential of *P. aeruginosa*; 2) what is the impact of antibiotic therapies on mixed populations; 3) what are the interactions established among pathogens; and 4) what is their impact on antibiotic resistance. To answer these questions, it is important to previously know the adaptive mechanisms used by *P. aeruginosa* when grown alone in CF sputum in order to better identify the differential adaptive behaviour of *P. aeruginosa* when grown with other species.

After the development of this project and still regarding early CF infections, it would interesting to study the interactions between *P. aeruginosa* and *S. aureus*. The prevalence of *S. aureus* is mainly observed in younger patients with few years and frequently precedes *P. aeruginosa* colonization. Some evidences have pointed out that *S. aureus* somewhat prepare the CF environment for *P. aeruginosa* colonisation. During colonization, *S. aureus* secrete several exoproducts that could influence *P. aeruginosa* colonization and growth in CF lungs. These factors must be identified and their role in *P. aeruginosa* adaptation and microevolution understand. Furthermore, it would be important to determine the clinical implications of interspecies interactions concerning the efficacy of the antibiotic treatments. Through these experiments, more “pieces” could be added to the *P. aeruginosa* adaptation/evolution “puzzle”.

In chronic stages of CF disease, Bcc bacteria colonises CF lungs sharing the habitat with *P. aeruginosa* and sometimes also with *S. aureus* originating a polymicrobial community commonly found in CF patients. There is scarce information about the impact of the presence and interactions with Bcc on *P. aeruginosa* pathogenesis. The study of these mixed communities will bring out information helpful to construct the *P. aeruginosa* network with other bacteria and to understand how it is used to persist in CF lungs. Through the expected results of this project with those obtained with the previous experiments it could be possible to detect the (differential) mechanisms used by *P. aeruginosa* to persist in CF environment. All these experiments will help to assemble the quite complex "puzzle" of *P. aeruginosa* adaptation to CF lungs. Furthermore, these experiments with mixed communities (*P. aeruginosa* and *S. aureus* and *P. aeruginosa*, *S. aureus* and Bcc) will allowed gaining a temporal perspective of an infection course in CF patients.

Other pathogens could be successively added to the experiments in order to closely mimic the real microbiome including *H. influenza, S. maltophilia* and, most important, anaerobic species. Few published clinical reports have studied the impact of mixed communities containing anaerobic species and thus it is still unknown their clinical significance and the impact of the in-use antibiotic therapies on those species and communities.

In the study of *P. aeruginosa* adaptation of CF lungs, it could not be forgotten the influence of airway mammalian cells. To more closely mimic the real CF lung scenario, *P. aeruginosa* should be co-cultured with airway mammalian cells to simulate the signals that bacteria receive from airway cells immediately after CF lungs colonization. About this issue, it should be noted that still lacks an adequate animal model to study bacterial infections in CF context and by this reason the identification of the adaptation mechanisms is an hard task.

To construct the adaptive and evolutionary pathway of *P. aeruginosa* since initial CF colonization until chronic infection development based only on phenotypic data is important but it is not enough. The evolutionary pathway of *P. aeruginosa* is distributed to phenotypic, structural, metabolic, proteomic, regulatory and genomic rearrangements to culminate in optimized growth rate [#1325]. All the research lines afore described should follow a phenotypic approach, but integrating also metabolomic, proteomic and transcriptomic approaches. None of these approaches are powerful enough to provide a real picture of *P. aeruginosa* evolutionary pathway, but combined it would be possible to obtain a global picture about what happen to *P. aeruginosa* at several cellular levels. Therefore understanding interspecies and bacteria-host interactions, the "adaptomic" description of *P. aeruginosa* will be complete and effective and tailored therapies could be design to eradicate bacteria from CF lungs.