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Phenotypic Evolution of *Burkholderia multivorans* during chronic lung infection of cystic fibrosis patients

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RESUMO

A fibrose quística (FQ) é uma doença genética autossómica recessiva que afeta maioritariamente indivíduos caucasianos. Esta doença afeta especialmente a função pulmonar dos pacientes, conduzindo a infeções pulmonares causadas por agentes bacterianos que em muitos casos levam à morte. Existem diversos agentes patogénicos responsáveis por esta patologia, entre os quais se destacam as bactérias pertencentes ao complexo *Burkholderia cepacia*. As vias respiratórias dos pacientes com fibrose quística constituem um ambiente complexo, contribuindo para que as bactérias, de forma a se adaptarem, tenham de sofrer mutações que levam a adaptações fenotípicas. Estas alterações fenotípicas contribuem para a persistência bacteriana nos pulmões do hospedeiro, aumento da resistência a antibióticos e diminuição da virulência aguda. Neste trabalho foi estudada a evolução fenotípica de de 40 isolados clínicas de *Burkholderia multivorans*, recolhidos de quatro doentes com fibrose quística, durante o período de 1995 a 2012. A estratégia adotada compreendeu a análise fenotípica dos mecanismos inerentes à adaptação bacteriana das vias respiratórios dos doentes com fibrose quística, tais como, suscetibilidade a antibióticos, motilidade, formação de biofilmes, taxas de crescimento, virulência em *Galleria mellonella* e adesão a células epiteliais. De forma geral, os últimos isolados recolhidos dos doentes com FQ, demonstraram aumento da resistência a antibióticos, menor motilidade, menor taxa de crescimento, maior formação de biofilmes e menor virulência em *Galleria mellonella.* Além disso, a presença de LPS O-antigénio, parece afetar a virulência em *Galleria mellonella* e adesão a células epiteliais.

PALAVRAS-CHAVE

Fibrose quística; *Burkholderia multivorans*; infeção respiratória crónica; evolução fenotípica; evolução bacteriana; adaptação no hospedeiro.

ABSTRACT

Cystic fibrosis (CF) is an autosomal recessive genetic disease that affects mainly caucasian individuals. This disorder affects especially the lung function of patients, leading to lung infections caused by bacterial agents, that are frequently lethal for patient. There are several pathogenic agents responsible for this pathology, although, bacteria belonging to *Burkholderia cenocepacia* complex (Bcc) have a prominent role. The respiratory tract of CF patients constitutes a complex environment for bacteria. In order to adapt, bacteria acquire mutations that lead to phenotypic adaptations. The phenotypic modifications promote bacterial persistence within the host lung, increased resistance to antibiotics but decrease acute virulence. In this work the phenotypic evolution of 40 *Burkholderia multivorans* retrieved from four CF patients during the period 1995 to 2012 was studied. The strategy adopted included a phenotypic analysis of the mechanisms inherent of bacterial adaptation to the respiratory tract of CF patients, such as antibiotic susceptibility, motility, biofilm formation, growth rates, *Galleria mellonella* virulence and adhesion to epithelial cells. Overall, the latter isolates retrieved from the CF patients investigated showed increased antibiotic resistance, decreased motility, decreased growth rates, increased biofilm formation and decreased virulence in *Galleria mellonella.* Moreover, the presence of the LPS O-antigen apparently, affect virulence in *Galleria mellonella* and adhesion to epithelial cells.

KEYWORDS

Cystic fibrosis, *Burkholderia multivorans*; chronic lung infection; phenotypic evolution; bacterial evolution; within-host evolution.

LIST OF ABBREVIATIONS

ABC – ATP-binding cassette

ATP – Adenosine triphosphate

Bcc – *Burkholderia cepacia* complex

cAMP – Cyclic adenosine monophosphate

CF – Cystic fibrosis

CFTR – Cystic fibrosis transmembrane conductance regulator

CFU – Colony forming unit

DNA – Deoxyribonucleic acid

EDTA – Ethylenediaminetetraacetic acid

EPS – Exopolysaccharide

FBS – Fetal Bovine Serum

LB – Luria-Bertani Broth

LPS – Lipopolysaccharide

MOI – Multiplicity of infection

mRNA – Messenger RNA

MSD – Membrane spanning domain

NBD – Nucleotide binding domain

OD – Optical density

PBS – Phosphate buffered-saline

QS – Quorum sensing

RNA – Ribonucleic acid

ROS – Reactive oxygen species

SCFM – Synthetic cystic fibrosis medium

SCVs – Small-colony variants

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

1.1 Cystic fibrosis

1.1.1. Cystic fibrosis overview

Cystic fibrosis (CF) is an autosomal recessive hereditary disease [1], affecting around 32 000 individuals in Europe and about 85 000 worldwide [2]. CF is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene, which encodes a membrane chloride channel that mediates anion transport across epithelia [3]. Although being multi-organ disease, CF affects mainly the lungs. The dominant cause of morbidity and mortality is lung disease, but other symptoms are manifested as pancreatic insufficiency, intestinal obstruction, elevate electrolyte levels in sweat and male infertility [4, 5]. This condition predisposes to respiratory infections due the production of thick mucus which results in defective clearance of bacterial pathogens [6]. Common pathogens causing infection in the respiratory tract of CF patients are *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Burkholderia cepacia* complex, among others [7]. These bacterial infections can lead to chronic infection in the lungs and consequently cause bronchiectasis, respiratory failure and ultimately death [8].

In 2014 the mean age at death for a patient with CF in Europe was below 29 years [9]. However, for the patients born in the past 15 years the mean expected lifetime in UK is now greater than 50 years [2]. Nowadays, early diagnosis, comprehensive multidisciplinary CF care, nutritional support, new inhaled therapies, new anti-infective treatment and lung transplantation have improved the survival of patients with CF [9]. Despite the scientific progress, CF patients still have a low quality of life and many limitations.

1.1.2. Cystic fibrosis transmembrane conductance regulator (CFTR)

CF results from mutations within the CFTR gene which is located on the long arm of chromosome 7. Protein CFTR is a 1490 amino acid integral membrane protein that regulates an ion channel for chloride in the epithelial cells of many organs [10]. CFTR belongs to the superfamily of ATP-binding cassette (ABC) transporters,[11] that functions as an ATP-gated low conductance chloride selective channel [12]. Despite the mainly function as chloride channel, CFTR has other functions as inhibition of sodium transport through the epithelial sodium channel, regulation of ATP channels, regulation of intracellular vesicle transport and acidification of intracellular organelles [13]. Like other ABC transporters, CFTR contains two membrane spanning domains (MSD1 and MSD2 which form the channel through the membrane and two nucleotide-binding domains (NBD1 and NBD2) that bind and hydrolyze ATP (Figure 1)[12,14]. However, CFTR has a unique large regulatory (R) domain, that connects the two halves of the protein and regulates the channel activity [14].

The absence of functional CFTR channels at the luminal surface of several epithelia tissues causes CF [12].

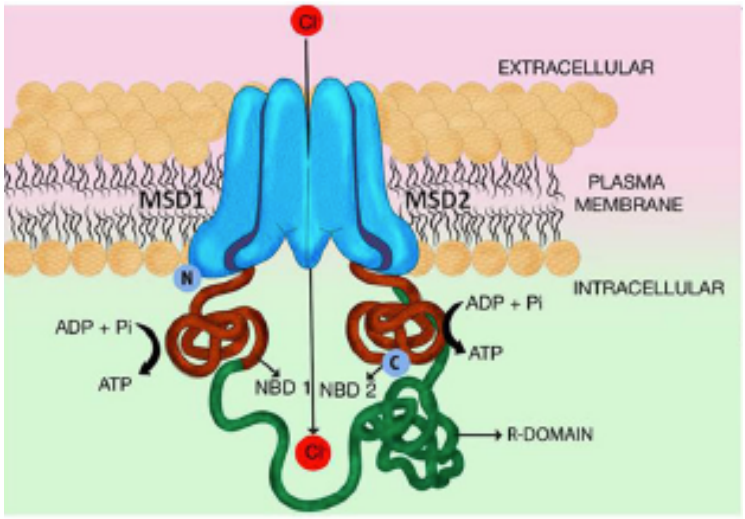

Figure 1 - Representation of CFTR channel at cell membrane, emphasizing the two membrane spanning domains (MSD1 and MSD2) and the two nucleotides binding domains (NBD1 and NBD2). Unique to CFTR, NBD1 is connected to the MSD2 by a regulatory domain (R) (adapted from Bhagirath *et al.* 2016) [15].

The spectrum mutation of this disease is made up of more than 1500 different mutations [13]. The most common mutation is the absence of phenylalanine at position 508, also known as F508del, which is present in at least one allele of 80-90% of CF patients [12,13]. CFTR mutations can interfere with the biogenesis, transport and activity of the channel and with its stability at the cell surface [12].

CFTR mutations have been classified in six classes according to their functional defect (Figure 2): Class I – mutations that impair protein production (mainly nonsense mutations); Class II – mutations that affect CFTR processing, causing the protein to misfold which leads to protein degradation (this class includes F508del mutations); Class III – mutations that disrupt channel regulation impairing gating; Class IV- mutations that decrease chloride conductance upon cyclic AMP (cAMP) stimulation, preventing a correct flow of ions through the channel pore; Class V – mutations that reduce CFTR protein levels, often by affecting splicing and generating a low amount of mRNA transcripts; and finally Class VI – mutations that decrease the retention and stability of CFTR at cell surface [12]. Therefore, it is important to identifying which are the mutations in the CFTR gene, once different ethnic or regional populations can have a different spectrum of CFTR variants, each one leading to a different degree of disease severity [16].

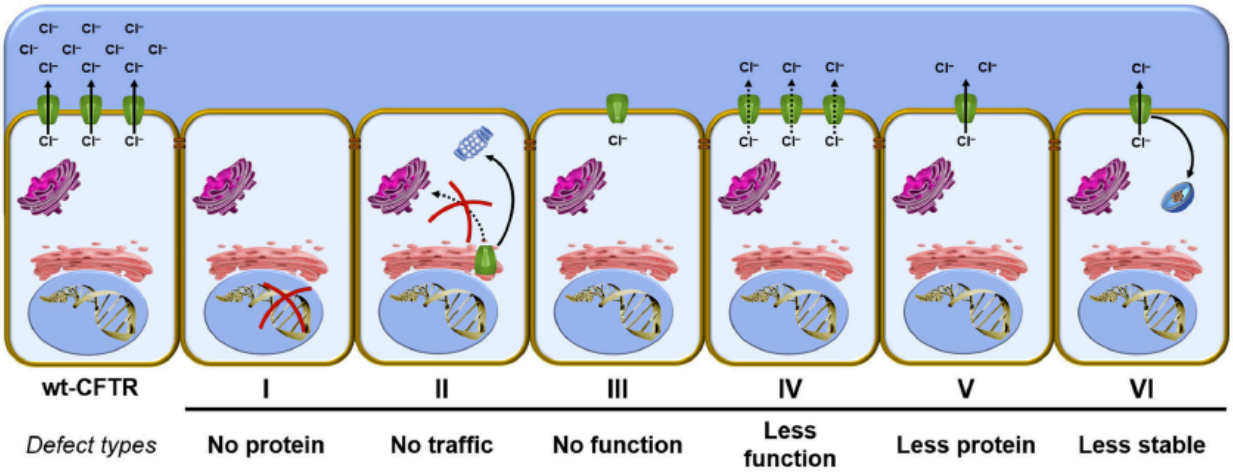

Figure 2 - Distribution of CFTR mutations into six functional classes according to their primary defect (adapted from Lopes-Pacheco 2016) [16]. Class I: no protein synthesis; Class II: impaired trafficking protein; Class III: defective channel gating; Class IV: reduced flow of ions through the channel; class V: decreased protein maturation; Class VI: less stable protein.

1.1.3. Pathogenesis in the cystic fibrosis lung

CF patients are born with apparently healthy lungs, but these are very conducive to acquisition of chronic, unrelenting bacterial infections of the airways in the first years of life [17]. The environment of the airways of patients with CF offers warm, humid and nutrient rich conditions for bacterial growth [18].

Lung infections in CF reflect the failure of the innate defense mechanisms of the lung against inhaled bacterial organisms [17]. There are some mechanisms that the lung develops against bacterial colonization such as mucociliary clearance, polymorphonuclear neutrophil phagocytosis, and local production of antibacterial cationic peptides. However, these systems are not enough under conditions of increased viscosity and osmolarity, resulting in chronic lung infection [19].

Human airways represent a highly compartmentalized environment, each one with distinctive characteristics (Figure 3): the paranasal sinuses, located in the upper airways, is where the mucus is produced, thus providing a site for bacteria reproduction; the sinus cavities (also in the upper airways) have less airflow and less exposure to antibiotics and host immune cells than the lower airways; finally, in the lower airways, the conductive zone refers mainly to bronchi, and the respiratory zone includes the respiratory bronchioles and the alveoli. The presence of bacteria in the respiratory zone is rare and frequently connected with lung destruction [18].

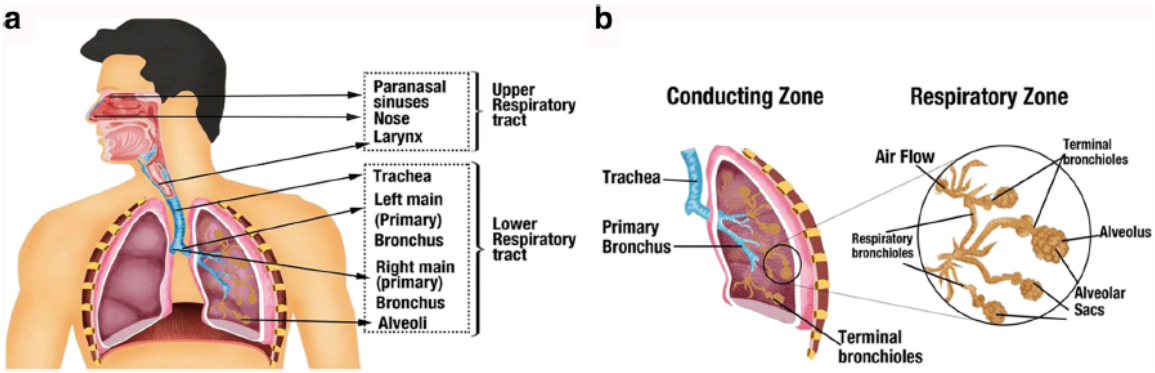

Figure 3 - Divisions of human respiratory system relevant to CF lung disease. **a -** the airway can be divided in upper and lower respiratory tract; **b** – Lower respiratory tract which is divided into conductive zone and respiratory zone. The conducting zones correspond to trachea, primary and terminal bronchioles. The respiratory zones allow the air exchange and consist of respiratory bronchioles and alveolar sacs. (adapted from Bhagirath *et al.* 2016).[15]

The airways of patients with CF are not entirely aerobic, especially because the deficient mucociliary clearance and development of a thick mucus layer. The combination of these two factors leads to the formation of mucus plugs that form a niche for microbes [17]. Within the plugs, a steep oxygen gradient forms with hypoxic or anoxic regions [20]. Microbes prepared to survive under diverse host conditions may have increased potential to chronically colonize the airways and impact patient outcome. Species assumed as aerobic microbes are multiple times prepared to survive in hypoxic or anoxic environments. For example, *Pseudomonas aeruginosa* can grow under all these conditions [21].

CF airways constitute a complex heterogeneous environment, allowing the presence of multiple niches, which means that infecting populations may genetically diversify to stablish sublineages, each with the ability to adapt under the conditions of its niche [22]. Multiple bacterial species exist in the respiratory tract of CF patients and a modest number is related with CF lung disease. In early childhood CF patients are often infected by *Staphylococcus aureus* and *Haemophilus influenzae*, while *P. aeruginosa* and the *Burkholderia cepacia* complex are more predominant in CF adults [23]. Meanwhile, many other organisms that co-inhabit the lungs of patients with CF, such as diverse fungi (including *Candida* ssp., *Malassezia* spp., and *Aspergillus* spp.) and some viruses (including influenza and syncytial viruses) have been found [24, 25].

Reverse to what would be expected, the primary sources of infection it is not the patient microbiota but patient-to-patient transmission and the environment [24]. Lungs of CF patients are abundant in organic compounds which is favorable for bacterial growth. Despite of that, bacteria must constantly evolve to adapt to limitations in specific growth factors, dehydration, leukocyte influx, the physical heterogeneity of the deteriorating lung tissue, and antibiotic therapy [19]. It is frequently observed that in order to adapt bacteria progressively loose the function of many genes typically associated to bacterial pathogenicity; however, in a chronic lung infection, these new conditions do not imply a decrease in virulence, but more likely a change in virulence [26]. Understand the complexity of these microbial infections, is very important for deducing the function and impact of infecting microbes as a community and not as

individuals. Moreover, it is critical to know which microbes do not contribute to the progression of the disease and those who have a pathogenic role in patient health condition [21].

Throughout their lives, CF patients receive many times antibiotic treatment for chronic infection management or during hospitalization for pulmonary exacerbation [27]. It is predictable that bacteria acquire antibiotic resistance, and microbial biology and CF lung physiology may contribute to this tolerance. As known, low oxygen environment or biofilm growth enhance drug resistance [28]. Despite the long antibiotic therapy, several microbial communities remain in the airways of CF patients [21]. Hypermutable strains are selected and predominate in chronic infection and are connected to the acquisition of mutations related to antibiotic resistance, biofilm growth, metabolic adaptation and virulence attenuation [29].

Nowadays, it is possible to use the application of genome-sequencing, transcriptomics, metabolomics and metagenomics technologies to improve the knowledge about evolutionary paths of bacteria infecting CF patients. Despite of that, it is important to continue to make efforts to better understand how bacterial pathogens evolve during infection in CF patients host since this will help to treat infections [18]. In the next sections will be reviewed the genomic and phenotypic evolution mechanisms that *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex bacteria develop in order to chronically infect CF patients.

1.2. *Pseudomonas aeruginosa* as a cystic fibrosis pathogen

1.2.1. *P. aeruginosa* and the cystic fibrosis lung environment

Pseudomonas aeruginosa is a Gram-negative bacterium capable of grow in many different environments both outside or within the host. It is an opportunistic human pathogen causing severe infections [30]. *P. aeruginosa* genome, is one of the largest in bacterial world, with 6000 genes and more than 500 regulatory genes suggesting that this bacterium has a great aptitude to move between different types of environments [26]. In CF patients, *P. aeruginosa* may persist in the respiratory tract for several decades [31].

P. aeruginosa has the capacity to obtain a virulence phenotype, for selection across the whole genome, that will increase the ability to cause chronic inflammation [32]. The genome of *P. aeruginosa* includes a vast number of virulence genes, for example: *lasR*, which encodes a transcriptional regulator that responds to one of the quorum sensing signals and regulate acute virulence factor expression and *exsA*, a gene encoding the transcriptional regulator for type III secretion [26, 31].

P. aeruginosa colonization of the airways and infection is the major cause to CF morbidity and mortality. More than 80% of the patients with CF die due to respiratory failure caused by chronic bacterial infection and associated airway inflammation. During the colonization process, *P. aeruginosa* has the capacity to adapt the to CF lung environment, therefore chronic lung infection is associated to phenotypic and genomic changes in the bacterium [15]. Preventing the chronic colonization of *P. aeruginosa* is essential because once establish, the infection becomes very difficult to eliminate and it is common that after an aggressive therapy, the patient is re-infected with *P. aeruginosa*. In many circumstances the colonization occurs by the same strain, or otherwise by the similar genotype [33, 34]. Even though it is not clear the way that patients acquire this bacterium, studies pointed to clinical exposures and social interactions zones. However, other risk factor in the acquisition of *P. aeruginosa* is the gender, since studies have shown that females are more susceptible than males [15].

Previously to the chronic airway infection is established, there is a period of intermittent colonization of the airway, in this phase CF patients are exposed to antibiotic therapy, therefore delaying the succeeding chronic infection. Chronic lung infection is characterized by the constant growth of *P. aeruginosa* in airway secretions and are connected with a higher degree of inflammation than found in intermittent colonization, and these conditions together lead to an increased lung function deterioration[18].

Many variants of the same infecting strain may exist, and it is very common to find within the CF lung heterogeneity within one population. Due to the different areas and conditions present in the respiratory tract, this heterogeneous *P. aeruginosa* resistant niches and the mutations that were acquired contribute to complicate therapeutic treatments and posteriorly cause pulmonary exacerbation in CF patients [15].

It is essential to continue studying microbial diversity in CF patients, once it will allow to develop treatments adapted to the individual needs of the patients over the course of their lives [35].

1.2.2. Genomic and phenotypic evolution of *P. aeruginosa* in cystic fibrosis

During the establishment of the infection, *P. aeruginosa* variants experience alterations in their phenotypes in order to adapt to the hostile environment of the lung, allowing its persistence [36]. The genotype modifications cause traits that diverge from environmental isolates, indicating a conserved evolutionary pattern in the adaptive process of *P. aeruginosa* to the CF airways [37].

In order to survive in the CF lungs, *P. aeruginosa* must overcome certain stressful conditions such as osmotic stress, competition from other colonizers, nutritional inadequacy, antibiotics and oxidative stress. *P. aeruginosa* overcomes these challenges by switching its gene expression, therefore chronic colonization is related with genotypic and phenotypic changes in the bacterium [38]. However, one of the most common mutations is in the *algU* gene encoding the regulator which is associated with the

responses to many different antibiotics and to environmental stresses such as osmotic shock and magnesium starvation [18, 39]. Under stress conditions, the AlgU (factor sigma σ^{22}) regulator is activated due to mutations in the *mucA* gene and these actions lead to a coordinated downregulation of central metabolism, motility and virulence, and upregulation of genes affecting membrane permeability and efflux (Figure 4). Investigations of *P. aeruginosa* isolates show that, in high frequency, the mucoid phenotype is caused by excessive production of the extracellular polysaccharide alginate, which forms a glycocalyx that covers the surface of bacterium. Production of alginate is part of the general envelope stress response of several bacterial species, protecting them from environmental stresses. Mucoid phenotype is caused by mutations in regulatory genes, including at various positions of the *mucA* gene. This gene encodes the anti-sigma factor MucA that binds to the sigma factor AlgU and avoids the transcription of the *alg* operon, which encodes the enzymes required for alginate synthesis. However, mutations deactivating the activity of MucA lead to activation of AlgU, resulting in elevated transcription of the *alg* operon, leading to alginate overproduction [18, 26, 40]. Alginate stimulates encapsulation and biofilm formation by *P. aeruginosa*, which results in a protection against the action of reactive oxygen species (ROS), antibiotics and host immune defenses [40].

Moreover, anti-sigma factor Muc-A is also connected with the activation of transcription of other genes that are related with stress response and virulence factor expression. Likewise, it can suppress the expression of type III secretion system (T3SS) genes, activating AlgU that actives several regulatory genes, such as AlgR, a global regulator, that affects the expression of various genes including T3SS. This mechanism suggests that there is a synchronization of two high-cost energy systems in order to bacteria persistence in CF airways [40].

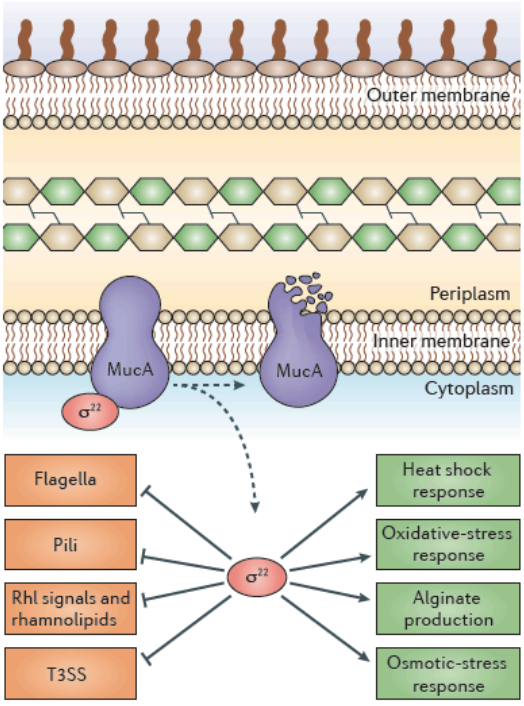

Figure 4 - Regulation network of MucA–AlgU. The function of the sigma factor AlgU is impeded through binding by the anti-sigma factor MucA. Mutations in *mucA* gene lead to release of AlgU, which activates the transcription of genes involved in alginate production and in the responses to heat shock, oxidative stress and osmotic stress, among others. AlgU also negatively regulates several *P. aeruginosa* virulence factors, such as flagella, pili, Rhl quorum sensing signals, Rhl-controlled rhamnolipids and the type III secretion system (T3SS) (adapted from Folkesson et al. 2012) [18].

Although alginate overproduction contributes to the success of chronic infection stage, non- and mucoid phenotypes can coexist. Non-mucoid isolates result from the persistence of *P. aeruginosa* wild type or the capacity to reverse mucoid phenotypes [26, 41]. Non-mucoid bacteria can still carry *mucA* mutation, indicating that this is a selective pressure mutation and it is vanished when a secondary mutation takes place. Subsequently, it is possible to conclude that the production of alginate implies a high energy consumption to bacteria, and also an unstable characteristic [42].

In effort to understand the mechanisms that command adaptation of *P. aeruginosa* to the CF airways, a study was conducted with two *P. aeruginosa* isolates collected from a CF patient over a period of 96 months. Comparison of these two genomes identified 68 mutated genes involved in virulence, specifically genes involved in antibiotic resistance, iron acquisition, quorum sensing and fatty-acid metabolism [32]. Also in this study, 91 new isolates were collected from 29 CF patients and posteriorly sequenced 24 genes and regulatory regions that were mutated in the first experiment and 10 genes and regulatory regions that are candidates for mutation, as determined from other studies. The most mutated gene in this sample is *mexZ*, encoding a negative regulator of *mexX* and *mexY* that encode the components of the MexXY-OprM multidrug-efflux pump. Loss-of-function mutations in *mexZ,* resulted in increased expression of *mexX* and *mexY,* which is associated to antibiotic resistance [32].

In a more recent work, Marvig and collaborators sequenced the genomes of 474 *P. aeruginosa* isolates collected longitudinally from the airways of young patients. This had the purpose to compare within-host evolution of 36 *P. aeruginosa* lineages to determine their evolutionary history. The authors found convergent molecular evolution in 52 genes, which are associated with acquisition of antibiotic resistance, remodeling of regulatory networks and loss of virulence factors and motility, suggesting that *P. aeruginosa* host adaptation is mediated by these mechanisms. Amongst these 52 pathoadaptive genes identified, ten of the genes (*gyrA*, *gyrB*, *mexA*, *mexB*, *mexR*, *mexS*, *mexZ*, *nalD*, *nfxB* and *oprD*) demonstrated to be involved in resistance against a series of antibiotics and eight genes (*bifA*, *lasR*, *morA*, *phaF*, *rbdA*, *retS*, *wspA* and *wspE*) are involved in regulation of biofilm growth. Moreover, a large number of the identified genes have functions related to phenotypes that are important during the process of infection, consistent with results from the previous study of Smith and co-workers [32]. Additionally, another 7 genes of the 52 (*algU*, *gyrA*, *gyrB*, *mexB*, *oprD*, *pelA* and *rbdA*) are homologous to genes previously found to be associated with cystic fibrosis infections in another study investigating the evolution of the *P. aeruginosa* DK02 lineage [22].

With the aim to study the role of bacteria on a path towards a “chronic infection phenotype”, Rau and colleagues collected *P. aeruginosa* isolates from three CF patients in the beginning of their colonization [26]. The experimental method combined transcriptional profiling and phenotypic characterization. Results allowed to conclude that for two patients occurred genetic changes that have led to the development of new bacterial phenotypes in the process of colonization. In one of the patients, the development of this new phenotype was caused by antibiotic resistance of bacteria associated to a mutation in *nfxB* gene, leading to upregulation of expression of the MexCD-OprJ efflux pump. In the

case of other patient, the phenotype acquired was bacterial mucoidy, caused by upregulation of the enzymes responsible for the alginate synthesis. For the third patient, there were no evidences of perceptible phenotypic changes during the persistence of *P. aeruginosa* [26].

Most of the chronic *P. aeruginosa* isolates are *lasR* mutants. This gene encodes the quorum sensing (QS) transcriptional regulator LasR. QS is used by bacteria do communicate, by using the concentration of a secreted molecule to quantify the prevailing cell density. When cells reach high density, they radically modify their pattern of gene expression. In this stage, wild-type *P. aeruginosa* produces virulence factors, forms biofilms and acquire antibiotic resistance. All these phenotypes are present in *lasR* mutants [40, 32]. Additionally, *lasR* mutants have the advantage of being able to use nitrate and nitrite as the terminal acceptor of electrons allowing *P. aeruginosa* to grow in anaerobic conditions. QS appears to contribute to *P. aeruginosa* pathogenesis during colonization and at acute infection stages [42].

Another phenotypic variation in chronic *P. aeruginosa* isolates is the rising of small-colony variants (SCVs). SCVs are typically hyperpiliated, hyperadherent and have the ability to form biofilm and reveal autoaggregative behavior and increased motility [43, 44]. Despite of that, SCVs contribute to increase resistance to multiple antibiotics, especially to aminoglycosides, supporting their persistence in CF airways and consequently damaging lung function. Mutations that result from SCV are very diverse and it is a challenge to understand the underlying molecular mechanisms. It is known, that this phenotype can appear from the increased expression of the *pel* and *psl* polysaccharide genes and contributes to elevate c-di-GMP levels that are associated to the ability to form biofilms, motility, and the expression of the type III secretion system, consequently persisting in the CF airways [44, 45].

During chronic infection, CF isolates demonstrated reduced motility, namely swimming and twitching, due the nonexistence of flagella and pili, respectively. Due to the absence of flagella, *P. aeruginosa* isolates are hard to phagocyte by macrophages and neutrophils, consequently weakening the host immune defenses, allowing its permanence in lung airways [42, 46]. Furthermore, *P. aeruginosa* tends to evolve to a chronic stage of biofilm-growth mode in which cells downregulate flagellum and pili once they are not necessary to move across sputum and epithelial surfaces. [47] The absence of pili is caused by mutations of *pilB*, which encodes an ATPase requested for the extension and retraction of pili, or defects in *pilQ* gene, needed to extrude the pilus through the bacterial external membrane.[48] However, the most predominant mutations are in *rpoN* gene (encoding sigma factor 54), which are present in the majority of CF isolates, and induce the loss of pili and flagella [49].

Studies also suggest that the need to adapt to heterogeneous and changing environments promote the emergence of hypermutable bacterial strains [19]. *P. aeruginosa* isolates were collected from chronic infected CF patients and was expected that they have a high frequency of hypermutable strains as the acquisition of mutations could be advantageous to speed up the adaptation to the CF airways environment. *P. aeruginosa* hypermutable strains occur to due mutations in *mutS* and *mutL* genes

encoding proteins of the repair system [50]. The constant changes in the environment of CF airways, contribute to a longevity of the hypermutable genotype, once bacteria always have the need to adapt to new CF lung conditions. These hypermutable strains confer a huge clinical challenge, especially due their resistance to a large spectrum of antibiotics and despite of that, it is likely that, they will become resistant to new compounds that may arise [19].

Feliziani and colleagues also studied the genetic adaptation process of *P. aeruginosa* in CF lung infection, confirming that it consists of mutations in specific genes, that consequently produce phenotypic advantages contributing for their longevity in the lung. In this work the authors characterized the mutations of 38 *P. aeruginosa* isolates from Argentinean CF patients and concluded that the most frequented mutated gene was *mexZ* (79%), followed by *mucA* (63%) and *lasR* (39%) as well as high prevalence of hypermutators (42%). Overall, the results indicate that mutations responsible for inactivation of *mucA*, *lasR* and *mexZ* arise by several different mechanisms [51].

Markussen and co-authors performed a study that combine phenotypic and genomic analyses with the aim of understanding diversification processes of an original *P. aeruginosa* strain into three distinct sublineages, during 32 year-period of chronic infection [52]. Phenotype profile showed that the accumulation of mutations displayed differences between the tree sublienages such as mucoidy, doubling time, metabolic performance and gene expression profiles. The patient was first colonized in the paranasal sinuses and later other two distinct sublineages colonized the lower airway. However, the three sublineages showed phenotypic differences, such as mucoidy and number of pathoadaptive genes, suggesting that they occupied different niches with different selective pressures in the CF patient airways. Overall, this experimental work provides evidence that the different environments of CF airways promote diversification and long-term coexistence of *P. aeruginosa* sublineages during chronic infections in CF patients [52].

In future investigations, it is imperative continue studying the mechanisms underlying *P. aeruginosa* adaptation to CF airways and especially understand *P. aeruginosa* pathogenesis at early stages, because it is the better way to avoid chronic infections, or at least preventing their progression.

1.3. *Burkholderia cepacia* complex as cystic fibrosis pathogen

1.3.1. *B. cepacia* complex and the cystic fibrosis lung environment

Burkholderia cepacia complex (Bcc) is a group of at least 20 closely related species, which are included in the β-proteobacteria subphylum, and emerged in the 1980s as important human pathogens, namely in patients suffering from CF. Bcc are Gram-negative bacteria and their genome consists in three chromosomes and genome size differs between 6.2 Mbp and 8.7 Mbp [53, 54].

Bcc bacteria can be found in the environment, such as water, soil and rhizosphere of crop plants[55]. This versatility has been mostly attributed to their metabolic complexity and their large genomes which have a wide array of insertion sequences which promoting high genome plasticity and consequently high adaptability to colonizing niches [56]. Bcc isolates have achieved several purposes, including biological control of plant pathogens, bioremediation and plant growth promotion. Despite of that, Bcc is vastly known for their pathogenic properties capable to cause infections in plants and in humans [57].

As reported before, the main pathogen responsible for respiratory infections in CF patients is *P. aeruginosa*, while Bcc bacteria infect a small percentage of patients [6, 58]. However, in the last years, Bcc bacteria have arisen as opportunistic pathogen, capable of causing severe infections in immunocompromised patients and CF patients [57]. Bcc infections are especially threatening for CF patients because they give rise to a variable clinical outcome, generating stages ranging from asymptomatic carriage until septicemia with acute respiratory failure known as "cepacia syndrome" [57, 59]. Once Bcc colonize the airways of CF patients, they were excluded from lung transplantation, since this prognosis increased dramatically the risk of death [6]. Although strains from every Bcc species are capable to cause infection in CF patients, the two most frequently isolated species are *B. multivorans* and *B. cenocepacia*[7].

The first source of transmission of these bacteria is patient-to-patient transmission, however, other sources of acquisition are possible. Bcc can survive in the surface of several materials, and patients can acquire these pathogens by touching the contaminated sources. Despite of that, "environmental" sources, such as, soil, water and plants have not been proved as contamination sources. Healthy individuals, including health care providers and family members of CF patients infected with these strains, are rarely infected [56].

The reasons for the rise of Bcc as an opportunistic pathogen in humans are unclear [60]. Bcc bacteria are difficult to exterminate, since they are recurrently resistant to the most used antibiotics[61]. Furthermore, during colonization of CF airways Bcc bacteria are exposed to selective pressures, as a result of the host immune defenses, antibiotic therapy and oxygen limitation [62]. These adaptations leading to their longevity, contribute to the emergence of new lineages, while maintaining their capacity of survival within the host. For these reasons, Bcc respiratory infections are very difficult to treat, and it is very common that treatment include triple antibiotic combinations to reach bactericidal activity [60].

In the future, it is important understand the mechanisms that permit the surveillance of these challenging bacteria and thus, develop new effective treatments against Bcc bacteria [63].

1.3.2. Genomic and phenotypic evolution of *B. cepacia* complex in cystic fibrosis

Most of the times the acquisition of Bcc by CF patients results in chronic lung infection. Bcc chronic infections normally involve one strain, although in particular cases, can occur prolonged infection involving two or more distinct strains or species and also, replacement of an initial infected strain with another [7, 62]. During the course of chronic infection, genomic and phenotypic variations occur due bacteria exposure to selective pressures [64].

In order to understand the pathogenicity of these bacteria, a particular phenotype studied was the capacity to produce exopolysaccharide (EPS), which is common in the majority of Bcc strains [65]. *In vitro* studies have shown the capacity of EPS to inhibit neutrophil chemotaxis and to neutralize reactive oxygen species [66], interfered with phagocytosis of bacteria by human neutrophils and facilitated persistent bacterial infection in mice[67]. Cunha and co-authors developed a study with 108 Bcc isolates obtained from 21 CF patients during a period of 7 years and concluded that approximately 80% to 90% of the Bcc isolates involved in CF respiratory infections are able to produce EPS [68]. The production of EPS has been linked to biofilm formation. However, it was demonstrated that, despite of EPS not being required for biofilm formation, it is involved in the formation of thick and mature biofilms produced *in vitro* by Bcc bacteria. Moreover, bacteria within the biofilms are more resistant to host immune defenses and antibiotic action, thus the production of EPS promote the persistence of Bcc bacteria in the airways of CF patients [68].

Zlosnik and colleagues analyzed 560 Bcc isolates from 100 CF patients, and conclude that all the Bcc species were able to express the mucoid phenotype. They also assessed the capacity of these bacterial isolates to switch their phenotype from mucoid and non-mucoid and vice-versa. Phenotypic changes were observed in 15 patients, with nine isolates of *B. multivorans*, three *B. cenocepacia*, and one *B. vietnamiensis* modifying their phenotype from mucoid to non-mucoid and two variations from non-mucoid were observed in *B. cenocepacia* and *B. vietnamiensis* [65]. Later on, Zlosnik and co-authors assessed Bcc isolates from the same 100 CF patients, and evidenced that infections with the non-mucoid phenotype are linked with more rapid deterioration in pulmonary function than patients infected with mucoid Bcc bacteria. Results obtained shown an inverse correlation between the extent of mucoid EPS production and the rate of decline in pulmonary function [69].

With the purpose to understand the role of Bcc mucoid and non-mucoid phenotype during lung infection, Silva and colleagues studied two *B. multivorans* clonal isolates sampled from chronically infected CF patients, where a mucoid to non-mucoid morphotypic transition had occurred [70]. In this work the authors combined analysis of transcriptional profiling and phenotypic assays and the results revealed that in the non-mucoid phenotype there is a reduction of the expression of several virulence factors in comparison with the mucoid isolate. Furthermore, the non-mucoid phenotype exhibits lower swimming and swarming motility, decrease in EPS production, increased biofilm formation and higher survival rate in minimal medium. The non-mucoid isolate demonstrated to be less virulent in *Galleria*

mellonella acute model of infection, presumably due to the decreased expression of transcripts encoding proteins related with virulence. Moreover, the non-mucoid variant had lower resistance to β-lactam antibiotics, likewise explained by the pleiotropic effects of the mutations that occurred in the isolate [70]. Afterwards, the same mucoid isolate was studied after being exposed to stress conditions, and the results allowed to conclude that mucoid to non-mucoid transition can be triggered by exposure of unfavorable conditions, such as oxidative and osmotic stress and susceptibility to antibiotics [71].

Another phenotypic trait of Bcc is the expression of the O-antigen repeats of lipopolysaccharide (LPS), a putative virulence factor [72, 73]. Studies revealed that expression of O-antigen was associated with reduced phagocytosis and reduce bacterial elimination [74]. Nevertheless, the O-antigen can interfere with adhesion to bronchial epithelial cells and stimulate the host immune defense, and its loss may contribute to a biofilm lifestyle, like it occurs most CF airways chronic infections [74, 63].

With the aim of understanding the adaptation process of *B. cenocepacia* during lung colonization of CF patients, Coutinho and co-authors studied 11 clonal isolates of this bacterium obtained from the same CF patient in a period of 3.5 years [75]. The findings support the concept that clonal expansion during CF airways colonization, are the result of mutations and selective pressures that occur in the lung environment. Several phenotypic assays were performed, thus, in general, the features of the isolate supposed to have initiated the infection were different from the subsequent isolates collected during the infection process. *B. cenocepacia* collected in the course of the infection, by comparison with the first isolate, showed higher resistance to several antibiotics, lower swarming motility and higher suitability to grow under iron limiting conditions. The authors, also showed the ability of *B. cenocepacia* to synthetize membranes with different fatty acid composition, which might be important for long-term lung colonization [75].

Later on, an additional experiment was carried out, using transcriptomic analysis, based on DNA microarrays. In this study genomic expression levels of the first *B. cenocepacia* isolate and another isolate obtained later – both from the same CF patient – were compared. Approximately 1000 genes were found to be differently expressed in the two isolates, indicating a noticeable alteration in gene expression. The upregulated genes of the recent clonal variant collected, include genes involved in translation, iron uptake, efflux of drugs and in adhesion to epithelial lung tissue [76].

Also regarding two clinical variants of *B. cenocepacia*, Madeira and co-authors used quantitative proteomics (2-D DIGE) to compare the expression profile of the first *B. cenocepacia* retrieved and a clonal variant collected after 3 years, both from the same CF patient. Proteomic analyses of the two isolates suggested that in the second isolate there are higher levels of protein synthesis and DNA repair processes, likewise contributing to promote long-term survival in the airways of the CF patient. This work allowed the identification of several proteins that contribute to the persistence of Bcc bacteria in CF airways, such as proteins involved in cell metabolism, translation, nucleotide synthesis and also proteins mediating iron uptake [77].

Lieberman and colleagues performed a retrospective study of 112 *Burkholderia dolosa* isolates recovered from 14 CF patients over a 16-year period, they sequenced the whole genome of the 112 *B. dolosa* isolates. Overall, the authors found 17 genes that acquired mutations that are attributed to the adaptation process of the pathogen to the host immune defense, such as antibiotic resistance, membrane synthesis, including LPS biosynthesis. Moreover, it was, also identified a mutation in the most-mutated gene, a homolog of *fixL*, and homologs of two other genes (*fnr* and *fixJ*). Homologs of these three genes have been implicated in several regulatory processes [78]. Afterwards, Lieberman and co-workers[79] studied the genotypic diversity of *B. dolosa* within patients with CF by re-sequencing individual colonies and whole populations from single sputum samples. This experiment allowed to conclude that the adaptation of *B. dolosa* within the CF airways requires mutations mostly as a diversification process and mutations rarely become fixed in a population. These diversifying lineages may coexist for many years, through selective pressures. Multiple mutations arise in genes related with antibiotic resistance, iron acquisition mechanisms and outer-membrane composition shown to be under strong selective pressure. In a more recent work, Roux and collaborators [80] studied the isolate AU0158 of *B. dolosa*, which was one of the responsible for an outbreak in the CF clinic at Boston's Children Hospital. The genome of AU0158 strain revealed a homolog of the *lafA* gene encoding a putative lateral flagellin, that in non-Bcc species, is used for movement on surfaces, attachment to host cells, or movement inside the host cells. The *lafA* gene is nonexistent in most of the Bcc isolates, suggesting that perhaps, *B. dolosa* is more ancestral than other members of the complex which possibly have lost these genes over time. Furthermore, a *lafA* deletion mutant in *B. dolosa* demonstrated increased swarming motility than wild-type due to an augmented number of polar flagella. Despite of that, did not seem to contribute to biofilm formation, host cell invasion or persistence over time.

In a recent work, Silva and co-workers performed a genomic and phenotypic study of 22 *B. multivorans* isolates recovered over 20 years of chronic infection from a single CF patient [63]. Genome comparison of 22 clinical isolates showed accumulation of several mutations over time of chronic lung infection and phylogenetic analysis showed that the initial strain diversified into several subpopulations, each one with their own mutations and able to coexist. Diversification of *B. multivorans* isolates seemed to be result of mutations affecting genes responsible for encoding regulatory proteins, proteins associated with envelope biogenesis and enzymes involved in lipid and amino acid and metabolism. Genomic analysis, also contributed to the identification of multiple mutations in a *fixL* homolog, which had been previously found in the study of *B. dolosa* clinical isolates carried out by Lieberman and collaborators.[78] In fact, among genes responsible for positive selection were *rpfR* (encoding a diguanylate cyclase/phosphodiesterase with regulatory functions), *plsX* (involved in the biosynthesis of phospholipids) and an *ompR*-like gene (OmpR is an osmolarity response regulator). Concerning phenotypic analysis, it was observed increased antibiotic resistance and biofilm formation and decreased motility and growth rate, these factors contributing to shift the bacteria toward a "chronic infection phenotype", which was connected to an augmented lung function deterioration.

Recently, an experiment that combined genomic and phenotypic analysis of 215 *B. cenocepacia* isolates from 16 CF patients over a period of 2 to 20 years was conducted [81]. Concerning phenotypes traits, the authors performed several assays such as growth in liquid media, swimming motility, biofilm formation, acute virulence in *Galleria mellonella*, with results showing considerable variation within patient bacterial series. Overall, 6 of the 16 series exhibited progressive decreases in motility, two decreased in acute virulence, seven showed changes in growth parameters, regarding biofilm formation there were two patient series that increasing this trait and three decreasing. The genomic analysis allowed identify four genes (*dnaK*, *papC*, *gcvA*, and *qseC*) that are associated with motility and biofilm formation. Moreover, authors observed gene losses in multiple longitudinal series, including loss of chromosome III and deletions in other chromosomes.

In order to adapt to a new and challenging environment of the CF airways, Bcc bacteria experience alterations in their genotypic and phenotypic profiles, contributing to their fitness, capacity of survival within the host and frequently, appearance of new lineages. Among the most common adaptive phenotypic traits of Bcc bacteria are increased antibiotic resistance and biofilm formation and decreased motility and growth rate. In future, it is important to use this knowledge to develop new diagnostics and effective treatments against Bcc bacteria.

1.4. Objectives

The purpose of this work is to study phenotypic evolution of 40 *B. multivorans* isolates collected from four distinct CF patients (P339, P342, P431 and P686) during the period 1995 to 2012. The strategy includes *in vitro* and *in vivo* phenotypic assays associated to bacteria adaptation to the CF lung environment, such as antibiotic resistance, swimming and swarming motility, biofilm formation, adhesion to epithelial cells, growth rate in SCFM medium and acute virulence in *Galleria mellonella.* Understand the adaptation mechanisms of this bacterium to the lung environment of CF patients is essential to fight and avoid the infection, and also to improve medical treatments and develop new therapies.

2. MATERIAL AND METHODS

2.1. Bacterial Strains and Growth Conditions

The bacterial isolates were sampled from four cystic fibrosis patients (patients P339, P342, P431 and P686) attending a clinic in Vancouver, Canada, and were provided by Prof. David P. Speert from the University of British Columbia. The bacterial isolates from each patient consists of a single clone of *Burkholderia multivorans*, except patient P686 which isolates P686-2 and P686-3 seem to be *Burkholderia cenocepacia*. Patient P339 isolates are 6 and were collected between 1995 and 2003 (Table 1); Patient P342 has 10 isolates sampled in the period of 2004 and 2011 (Table 2); Patient 431 isolates consist of 13 bacterial isolates collected between 1998 and 2006 (Table 3) and the last 11 isolates belong to patient P686 and were collected in the period of 2004 to 2011 (Table 4). The isolates were grown in lysogenity broth (LB medium) or in extracellular polymeric substance (EPS)-producing salt-mannitol (SM) medium at 37ºC with agitation at 250 rpm. SM medium is a medium with high carbon to nitrogen ratio to stimulate the production of EPS and its composition is: 12.5 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 1 g/L K_2SO_4, 1 g/L NaCl, 0.2 g/L $MgSO_4.7H_2O$, 0.001 g/L $CaCl_2.2H_2O$, 0.001 g/L $FeSO_4.7H_2O$, 1 g/L casaminoacids, 1 g/L yeast extract, 20 g/L D-mannitol.

Table 1 – Date of collection, identification (ID) and RAPD (randomly amplified polymorphic DNA) of *B. multivorans* isolates from patient P339.

Isolate	ID	Date of Collection	RAPD
P339-1	VC6880	21/02/1995	RAPD049
P339-2	VC9604	04/05/1999	RAPD049
P339-3	VC10336	24/08/2000	RAPD049
P339-4	VC11038	06/11/2001	RAPD049
P339-5	VC11508	05/09/2002	RAPD049
P339-6	VC12155	20/11/2003	RAPD049

Table 2 - Date of collection, ID and RAPD of *B. multivorans* isolates from patient P342.

Isolate	ID	Date of Collection	RAPD
P342-1	VC12539	23/09/2004	RAPD044
P342-2	VC12721	31/01/2005	RAPD044
P342-3	VC13492	15/09/2006	RAPD044b
P342-4	VC13732	28/03/2007	RAPD044b
P342-5	VC14645	02/02/2009	RAPD044b
P342-6	VC15079	13/01/2010	RAPD044b
P342-7	VC15604	18/05/2011	NA*
P342-8	VC15605	18/05/2011	NA*
P342-9	VC15918	17/12/2011	NA*
P342-10	VC16176	20/06/2012	NA*

*NA – non-available.

Table 3 - Date of collection, ID and RAPD of *B. multivorans* isolates from patient P431.

Isolate	ID	Date of Collection	RAPD
P431-1	VC9159	22/09/1998	RAPD029
P431-2	VC9410	12/01/1999	**RAPD025**
P431-3	VC9663	01/06/1999	**RAPD025**
P431-4	VC9937	09/11/1999	RAPD029
P431-5	VC10206	06/06/2000	RAPD029
P431-6	VC10207	06/06/2000	RAPD029
P431-7	VC10812	26/06/2001	RAPD029a
P431-8	VC11330	14/05/2002	RAPD029
P431-9	VC12100	21/10/2003	RAPD029
P431-10	VC12273	25/02/2004	RAPD029
P431-11	VC12274	25/02/2004	RAPD029
P431-12	VC12720	30/01/2005	RAPD029
P431-13	VC13473	01/08/2006	RAPD029

Table 4 – Date of collection, ID and RAPD of *B. multivorans* and putative *B. cenocepacia* isolates (P686-2 and P686-3) from patient P686.

Isolate	ID	Date of Collection	RAPD
P686-1	VC12675	15/12/2004	RAPD042
P686-2	VC12676	15/12/2004	**RAPD043**
P686-3	VC12677	15/12/2004	**RAPD043**
P686-4	VC12678	15/12/2004	RAPD042
P686-5	VC12735	10/02/2005	RAPD042
P686-6	VC13239	02/02/2006	RAPD042
P686-7	VC13240	02/02/2006	RAPD042
P686-8	VC13637	24/01/2007	RAPD042
P686-9	VC14586	05/12/2008	RAPD042
P686-10	VC15535	31/03/2011	RAPD042
P686-11	VC15917	15/12/2011	RAPD042

2.2. Antimicrobial susceptibility

In order to evaluate the susceptibility of the bacterial isolates against paper discs with antibiotics, the agar disc diffusion method was used [82]. The antibiotics discs used contained: piperacillin (100 μg) plus tazobactam (10 μg), kanamycin (30 μg) and ciprofloxacin (5 μg). First of all, the Mueller-Hinton agar (Sigma – Aldrich) plates were inoculated with 100 μL of bacterial cultures at an OD_{640} of 0.1 prepared from exponential-phase cells growing on LB liquid medium at 37ºC. Antibiotics discs were applied onto the surface of Mueller-Hinton agar plates and the growth inhibition zone diameter was measured after 24 hours of incubation at 37ºC. Results are the mean values from at least six replicates of two independent experiments, each with three discs per isolate.

2.3. Motility

2.3.1. Swimming assay

The swimming motility was assessed on swimming agar plates: 1% (wt/vol) tryptone, 0.5% (wt/vol) NaCl and 0.3% (wt/vol) noble agar (Difco) [63]. Plates were inoculated with 5 μL of bacterial culture at an OD_{640} of 1.0 and were incubated for 24 hours at 37ºC. The diameter of the swimming zone was then measured. Results are the mean values from at least eight replicates of two independent experiments.

2.3.2. Swarming assay

The swarming motility was assessed on swimming agar plates: 0.04% (wt/vol) tryptone, 0.01% (wt/vol) yeast extract, 0.0067% (wt/vol) $CaCl_2$ and 0.6% (wt/vol) bacto agar (Difco) [63]. Plates were inoculated with 5 μL of bacterial culture at an OD_{640} of 1.0 and were incubated for 48 hours at 37ºC. The diameter of the swarming zone was then measured. Results are the mean values from at least eight replicates of two independent experiments.

2.4. Biofilm formation

For biofilm formation assays, bacteria were grown in LB medium at 37ºC to mid-exponential phase and diluted to an OD_{640} of 0.05, and 200 μL samples of the cell suspensions were used to inoculate 96-well polystyrene microtiter plates. Plates were incubated at 37ºC statically for 48h. After this period, the wells were washed three times with 0.9% (wt/vol) NaCl. Adherent bacteria were stained with 200 μL of a 1% (wt/vol) crystal violet solution for 20 minutes at room temperature [83]. The following step was washing three times with 200 μL of 0.9% NaCl. The dye was solubilized with 200 μL of 96% ethanol and the biofilm formation is quantified by measuring the absorbance of the solution at 590 nm in a microplate reader (Spectrostar nano, BMG Lab Tech). Results are the means of data from at least eight replicates of three independent experiments.

2.5. Growth rate and doubling time determination

Isolates were grown overnight in LB medium at 37ºC. A volume was then centrifuged (2 minutes, 8000 rpm), and the pellet was washed with saline solution (0.9% NaCl) and used to inoculate a flask with 50 mL of synthetic cystic fibrosis medium (SCFM) [84], generating an initial optical density at 640 nm (OD_{640}) of 0.1. Flasks were incubated at 37ºC with agitation at 250 rpm, and growth rates were quantified by monitoring the OD_{640} for 24h. Growth rates were calculated from the exponential phase of growth from two independent experiments.

2.6. Virulence determination in *Galleria mellonella*

Killing assays were performed as described previously for Seed and Dennis.[85] *Galleria mellonella* were injected with cell suspensions containing a total colony forming units (CFU) of 1×10^6 in 10 mM $MgSO_4$ with 1.2 mg/mL ampicillin and incubated at 37ºC. Survival rates were evaluated during the following 72 hours post-infection. As a negative control, 10 mM $MgSO_4$ with 1.2 mg/mL ampicillin was used. Triplicates of 10 larvae were used in each experiment.

2.7. Adhesion to epithelial cells

The *B. multivorans* isolates studied were analyzed for adhesion to the bronchial epithelial cell line CFBE41o$^-$, derived from a patient homozygous for the cystic fibrosis transmembrane conductance regulator F508del mutation [86]. In this procedure host cell attachment was performed as described by Ferreira and colleagues [87]. Bacterial cells grown in SM medium during the period of 4-5 hours and were used to infect epithelial cells at a multiplicity of infection (MOI) of 10 (10 bacterial cells for 1 epithelial cell). Bacteria were applied to a 24-well plate previously seeded with CFBE41o$^-$ cells in minimum essential medium (MEM) supplemented with 10% (vol/vol) FBS and 1% (vol/vol) L-glutamine, and the plates were centrifuged at 700 x g for 5 minutes. Next, the plates were incubated for 30 minutes at 37 ºC in an atmosphere of 5% CO_2. Each well was washed three times with phosphate-buffered saline (PBS) to remove unbound bacteria and cells were lysed with lysis buffer (0.01 M PBS, 10 mM EDTA, 0.25% [vol/vol] Triton X-100; pH 7.4) for 20 minutes at 4 ºC. Serial dilutions were plated onto LB agar and adhesion was quantified by CFU counts after 48h of incubation at 37 ºC. Duplicates of each strain were performed per assay and the results presented were obtained from three independent experiments. Results are shown as the percentage of adhesion, which was calculated as the number of CFU recovered divided by the number of CFU applied to the epithelial cells multiplied by 100.

3. RESULTS

3.1. Analysis of *B. multivorans* collected from CF patient P339

3.1.1. *B. multivorans* from CF patient P339 overview

From CF patient P339 were sampled six bacterial isolates belonging to a single clone of *B. multivorans*, collected in the period between 1995 and 2003 (Figure 5a). To understand how the initial infecting strain evolved in this chronic infection, several phenotypic traits were investigated. Lung function for this patient was measured and represented in Figure 5b as forced expiratory volume in 1 second (FEV_1). In this patient lung function decline was decreasing 4.2% a year.

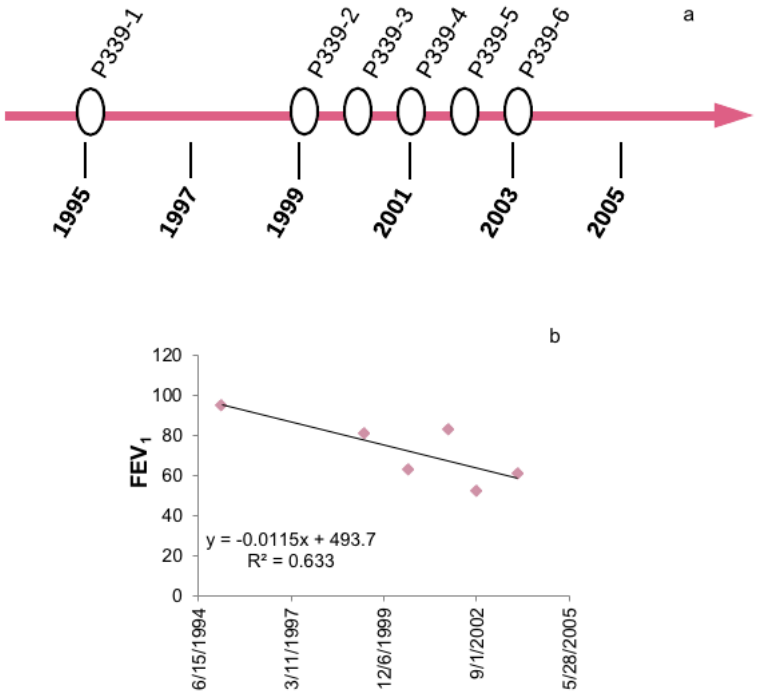

Figure 5 - *B. multivorans* isolates collected from CF patient P339 (a) and patient lung function decline as indicated by forced expiratory volume in 1 second (FEV_1) as percentage (b).

3.1.2. Antibiotic Resistance

Antimicrobial susceptibility was tested against the antibiotics piperacillin/tazobactam, ciprofloxacin, aztreonam and kanamycin. Piperacillin is a broad-spectrum β-lactam antibiotic that belongs to the penicillin class and is most commonly used with the β-lactamase inhibitor tazobactam; Ciprofloxacin is a quinolone antibiotic with one fluoro substitution, used to inhibit the bacterial enzyme DNA gyrase and

prevents replication of DNA during bacterial growth; Aztreonam is a β-lactam antibiotic and prevents bacterial cell wall synthesis; Kanamycin is an aminoglycoside antibiotic that inhibits protein synthesis.

The agar disc diffusion method [82] was used to test the antibiotic resistance of the isolates, and the shown result is the mean of the growth inhibition zone diameter (Figure 6).

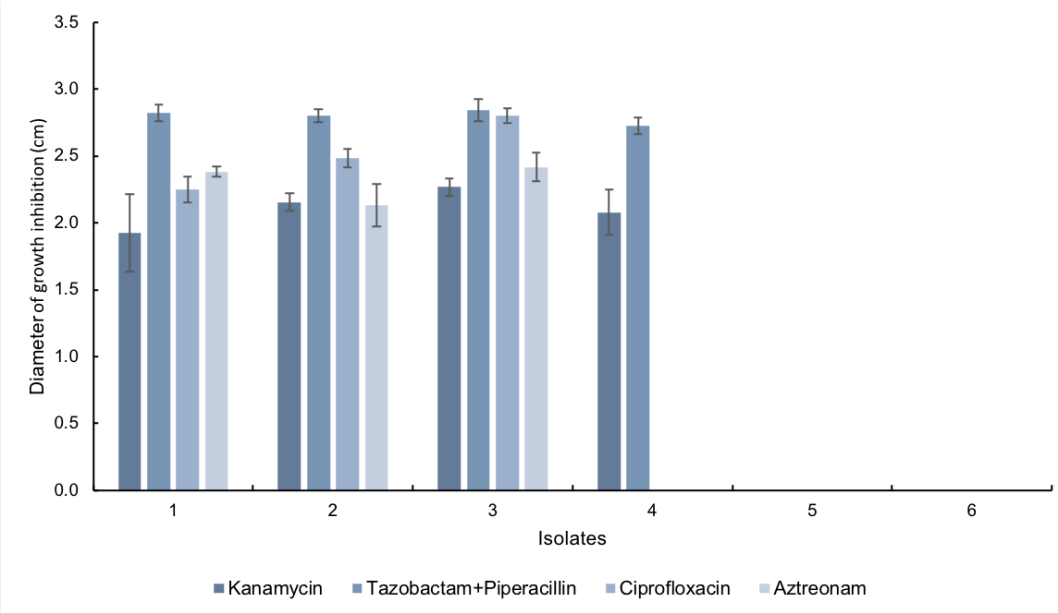

Figure 6 - Susceptibility to antibiotics (kanamycin, tazobactam/piperacillin, ciprofloxacin and aztreonam) for *B. multivorans* isolates recovered from patient P339 grown at 37 ºC after 24 hours as determined by measuring the diameter of cell growth inhibition (error bars represent the standard deviations of the mean values for three independent experiments).

Results show that the first three *B. multivorans* isolates were more susceptible to all the antibiotics assessed, while isolates 5 and 6 were completely resistant to all antibiotics. Moreover, isolate 4 was resistant to ciprofloxacin and aztreonam but reveals susceptibility to kanamycin and tazobactam/piperacillin. Overall, during the course of infection the isolates appear to acquire increased antibiotic resistance. Statistical significance of differences between the first isolates and the subsequent one was determined for aztreonam and kanamycin using ANOVA followed by Dunnett's multiple comparisons test. For susceptibility to aztreonam, differences were extremely significant for all isolates (*P*-value < 0.0001; supplementary figure 29), except isolate 3 that showed no statistically significance. For kanamycin, all isolates presented significant differences in susceptibility to this antimicrobial (*P*-value < 0.01, at least; supplementary figure 30).

3.1.3. Swimming and Swarming Motilities

The subsequent phenotypic test that was performed was motility, which is frequently associated to acute chronic infections in CF patients, since multiple studies show that bacteria tend to decrease their motility during the course of lung infection. Swimming assays were carried out in all 6 *B. multivorans*

isolates of patient P339 and swimming agar plates were incubated for 24 hours and the diameter of swimming zone was measured (Figure 7). Results indicate higher motility of the first isolate, which was null in isolate 2 or strongly reduced in the remaining isolates.

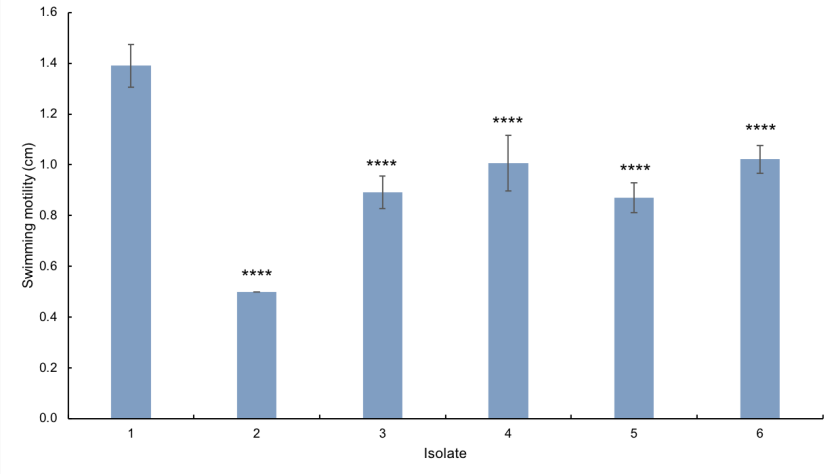

Figure 7 - Swimming motility of the *B. multivorans* isolates retrieved from CF patient P339, after growth in swimming agar plates for 24 h at 37ºC. Error bars represent the standard deviations of the mean values for three independent experiments (statistical significance of differences between the first isolate and the subsequent ones was determined: ****, P-value < 0.0001 by ANOVA followed by Dunnett's multiple comparisons test).

Swarming of 6 *B. multivorans* isolates was determined in swarming agar plates incubated for 48 hours and the diameter of swarming zone was measured (Figure 8). Results show reduced swarming motility except P339-3 which seem to have slightly higher motility.

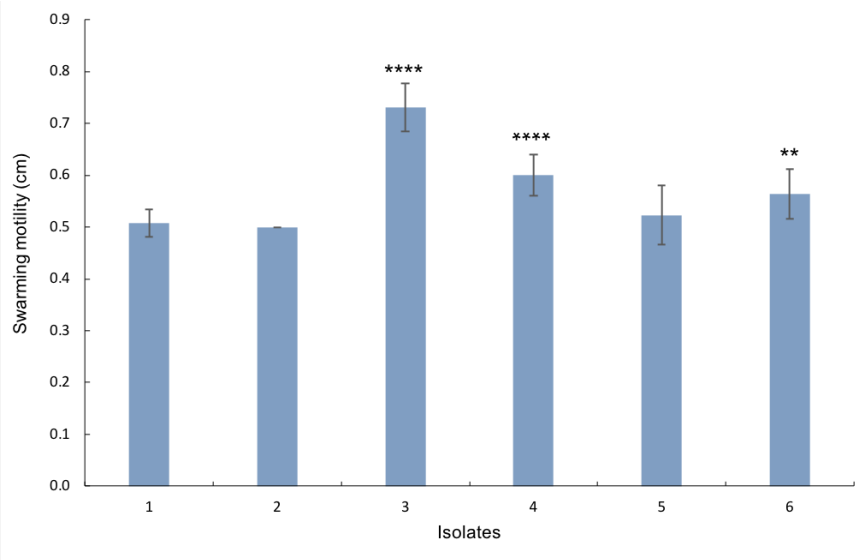

Figure 8 - Swarming motility of the *B. multivorans* isolates retrieved from CF patient P339, after growth in swarming agar plates for 48h at 37ºC. Error bars represent the standard deviations of the mean values for three independent experiments (statistical significance of differences between the first isolate and the subsequent ones was determined: **, P-value < 0.01; ****, P-value < 0.0001 by ANOVA followed by Dunnett's multiple comparisons test).

3.1.4. Biofilm formation

Another phenotypic trait that bacteria develop during chronic infections is the ability to form biofilms, that play an import role in the persistence of bacterial infection. Biofilms are communities within which bacteria can live, protected from host's immune defense. To evaluate biofilm formation of the bacterial isolates, sampled from CF patient P339, cell suspensions were grown in microtiter plates during 48h. After this period attached cell were stained with crystal violet and measured the absorbance at 590 nm (A_{590}). Results show that *B. multivorans* isolates 1 and 6 produced more biofilm than the remaining isolates which are drastically reduced in their ability to form biofilm (Figure 9).

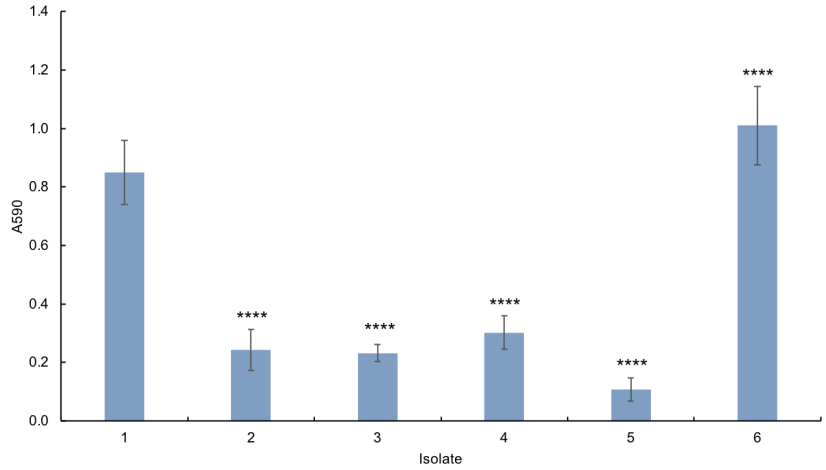

Figure 9 - Biofilm formation of the *B. multivorans* isolates retrieved from CF patient P339, after growth in polystyrene microplates for 48h at 37ºC. Error bars represent the standard deviations of the mean values for at least three experiments (statistical significance of differences between the first isolate and the subsequent ones was determined: ****, P-value < 0.0001 by ANOVA followed by Dunnett's multiple comparisons test).

3.1.5. Growth rate and doubling time determination

In order to evaluate differences in growth among the *B. multivorans* isolates retrieved from patient P339, growth rates were measured by monitoring the OD_{640nm} of isolates grown in synthetic cystic fibrosis medium (SCFM) [84]. Table 5 presents the results of doubling time and show that the first three have shorter doubling time than other three isolates.

Table 5 - Doubling times (in hours) calculated for the *B. multivorans* isolates sampled from CF patient P339, based on growth rates measured from cultures grown in synthetic cystic fibrosis medium (SCFM). Results are the means of data from two independent experiments.

Isolate	1	2	3	4	5	6
Doubling time (h)	1.4	1.3	1.4	2.5	3.2	3.5

3.1.6. Virulence determination in Galleria *mellonella*

To study the virulence of *B. multivorans* isolates we used *Galleria mellonella* larvae as infection model. *Galleria mellonella* are insects that in natural conditions inhabit beehives. These insects are frequently used as infection model for having similarities on innate immune system with mammals [88]. Ten larvae, for one experiment, were injected for each isolate and survival was tracked for 72 hours post-infection (results in figure 10). Results showed that the first two isolates were clearly more the virulent, followed by P339-3 and P339-5 isolates, and almost avirulent are P339-4 and P339-6.

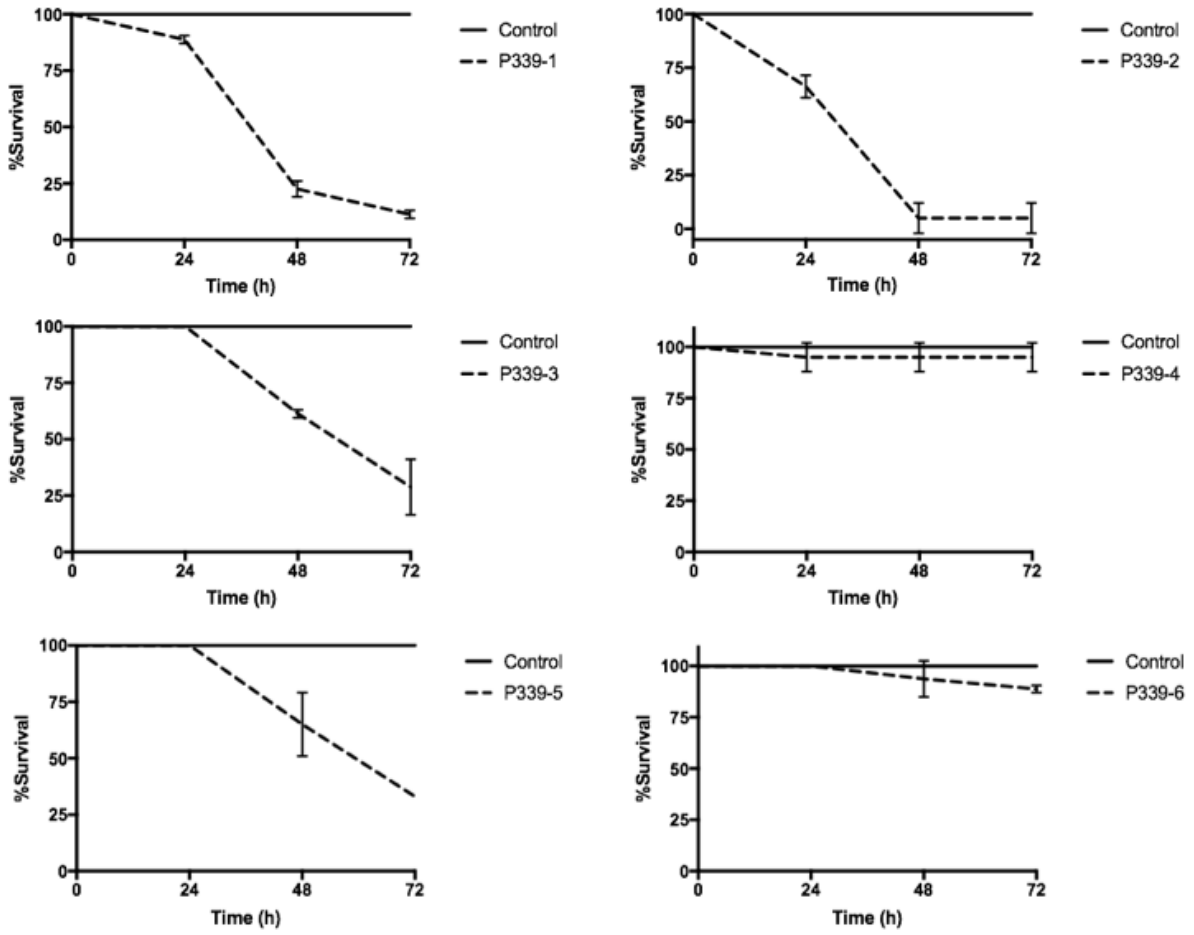

Figure 10 – Survival of *Galleria mellonella* larvae inoculated with *B. multivorans* isolates retrieved from patient P339. Triplicate groups of the 10 larvae were injected for each isolate and survival was tracked for 72 hours post-infection. Error bars represent the standard deviations of the mean values for at least two experiments.

3.1.7. Adhesion to epithelial cells

B. multivorans isolates recovered from patient P339 were analyzed for adhesion to the bronchial epithelial cell line CFBE41o$^-$, derived from a patient homozygous for the cystic fibrosis transmembrane conductance regulator F508del mutation. Epithelial cells were infected at a multiplicity of infection (MOI) of 10 (10 bacterial cells to 1 epithelial cell) and results are shown in figure 11, as percentage of adhesion.

Results show that the latter three isolates have increased adhesion to bronchial epithelial in comparison to the first ones.

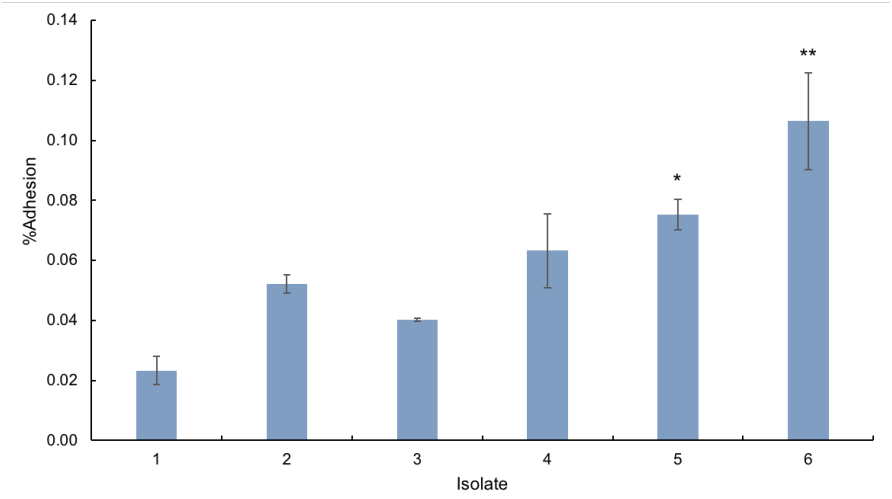

Figure 11 – Adhesion of 6 *B. multivorans* isolates recovered from patient P339 to the bronchial epithelial cell line CFBE41o$^-$. Results are shown as percentage of adhesion. Error bars represent the standard deviations of the mean values for at least two experiments (statistical significance of differences between the first isolate and the subsequent ones was determined: *, P-value < 0.05; **, P-value < 0.01 by ANOVA followed by Dunnett's multiple comparisons test).

3.2. Analysis of *B. multivorans* collected from CF patient P342

3.2.1. *B. multivorans* from CF patient P342 overview

CF patient P342 isolates are 10 and belong to a single clone of *B. multivorans*, sampled in the period between 2004 and 2012 (Figure 12a). Isolates 7 and were retrieved a time points of year 2011, while the other are single time-point isolates. Lung function decline in this patient (Figure 12b) is only available for the first years but it shows an estimated decreased of 2.5% a year.

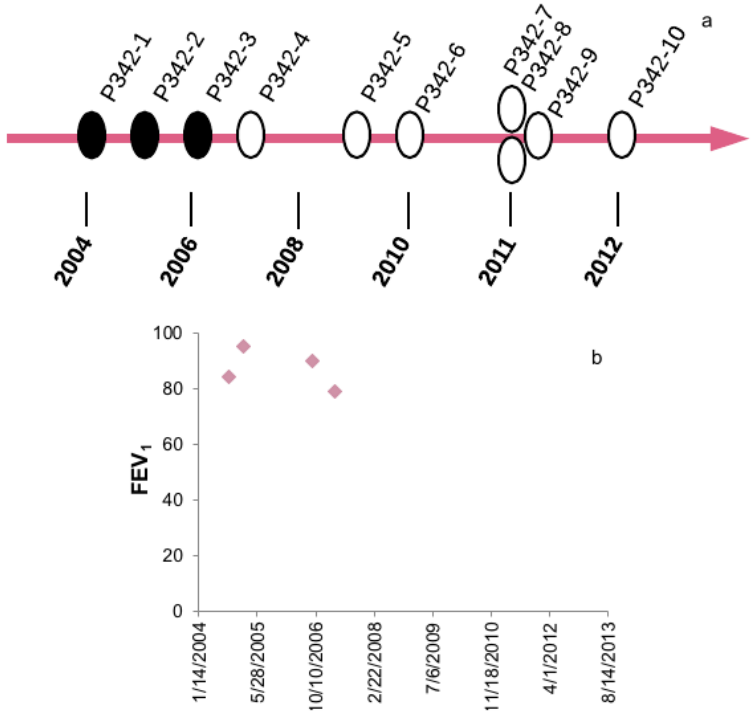

Figure 12 - *B. multivorans* isolates collected from CF patient P342 (a) and patient lung function decline as indicated by forced expiratory volume in 1 second (FEV_1) as percentage (b). Isolates in black produce the LPS O-antigen.

3.2.2. Antibiotic resistance

The susceptibility of each *B. multivorans* isolate sampled from patient P342 against antibiotics piperacillin/tazobactam, ciprofloxacin, aztreonam and kanamycin was tested. The results of the susceptibility against these antimicrobials are shown as the mean of growth inhibition zone diameter (Figure 13).

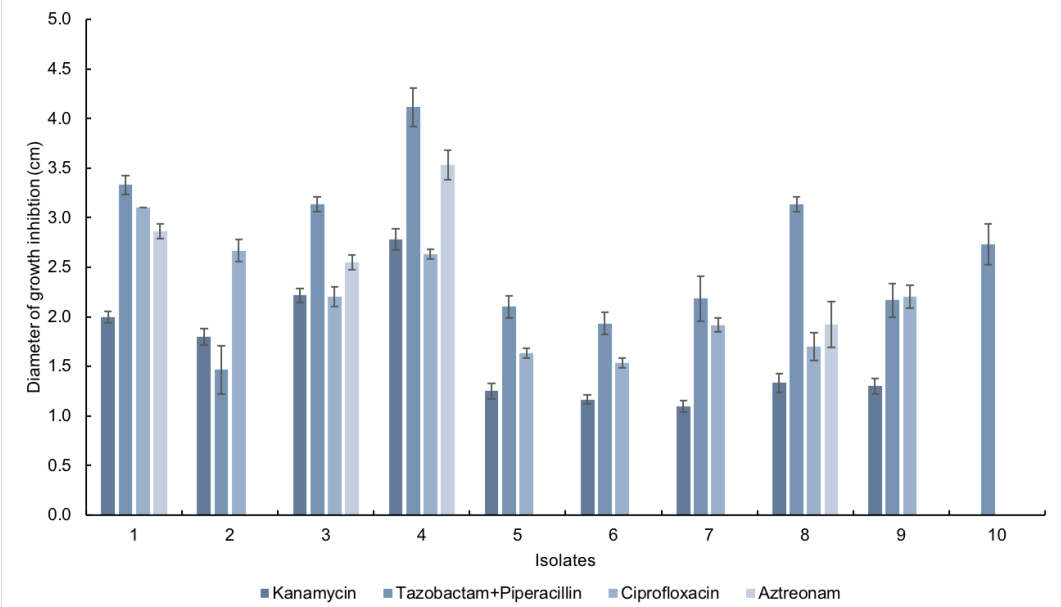

Figure 13 - Susceptibility to antibiotics (kanamycin, tazobactam/piperacillin, ciprofloxacin and aztreonam) for *B. multivorans* isolates recovered from patient P342 at 37 ºC after 24 hours incubation determined by measuring the diameter of cell growth inhibition. Error bars represent the standard deviations of the mean values for at least three experiments).

Results show that isolates 2, 5, 6, 7 and 9 were resistant to aztreonam and were less susceptible to the remaining antibiotics than other isolates. Furthermore, isolate 10 was resistant to three of the four antibiotics, only showing susceptibility to tazobactam/piperacillin. Isolates 1, 3, 4 and 8 were susceptible to the four tested antimicrobials, although isolate 4 seems to be more susceptible than others. For general, the latter isolates (P342-5 to P342-10) have increased antibiotic resistance. Statistical significance of differences between the first isolate and the subsequent ones was determined for susceptibility to aztreonam and kanamycin tested using ANOVA followed by Dunnett's multiple comparisons test, with significant differences for all isolates for the two antibiotics (*P*-value < 0.001, at least; supplementary figures 31 and 32).

3.2.3. Swimming and Swarming Motilities

Swimming assays were assessed for all 10 *B. multivorans* isolates from patient P342 (Figure 14). Results showed that isolate 1 displayed the highest swimming motility when compared to other isolates. Furthermore, isolates 4 to 10 decreased swimming motility in comparison with isolates 1 and 3.

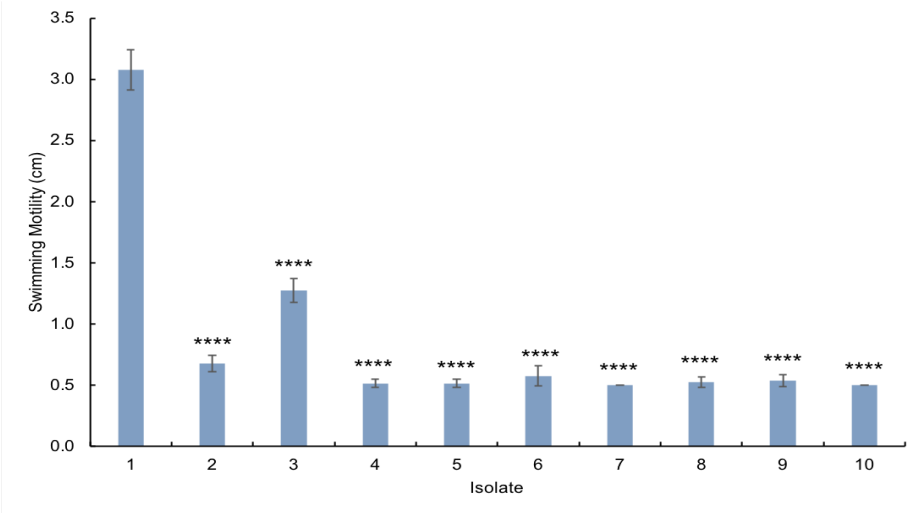

Figure 14 - Swimming motility of the *B. multivorans* isolates retrieved from CF patient P342, after growth in swimming agar plates for 24 h at 37ºC. Error bars represent the standard deviations of the mean values for three independent experiments. Error bars represent the standard deviations of the mean values for at least three experiments (statistical significance of differences between the first isolate and the subsequent ones was determined: ****, P-value < 0.0001 by ANOVA followed by Dunnett's multiple comparisons test).

Swarming motility was evaluated in the 10 *B. multivorans* isolates of patient P342 (Figure 15). Again, the first isolate displayed swarming motility in comparison with subsequent isolates tested, which were mostly non-motile.

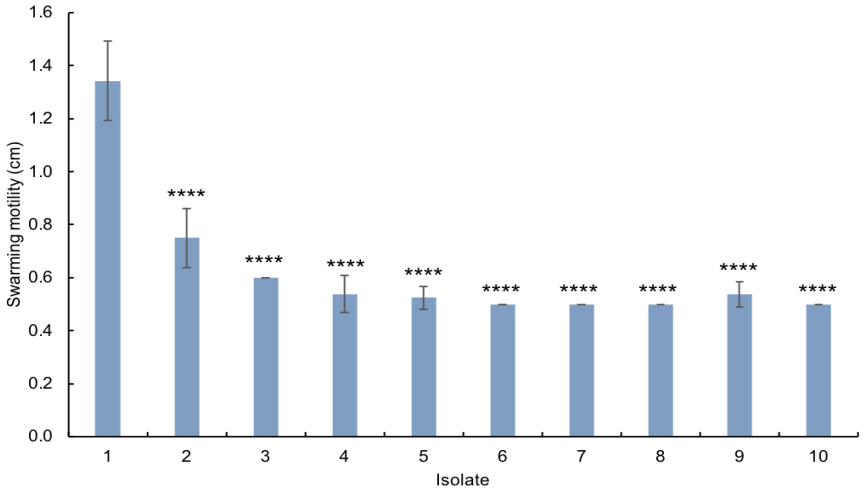

Figure 15 - Swarming motility of the *B. multivorans* isolates retrieved from CF patient P342, after growth in swarming agar plates for 48h at 37ºC. Error bars represent the standard deviations of the mean values for three independent experiments. Error bars represent the standard deviations of the mean values for at least three experiments (statistical significance of differences between the first isolate and the subsequent ones was determined: ****, P-value < 0.0001 by ANOVA followed by Dunnett's multiple comparisons test).

3.2.4. Biofilm formation

To evaluate biofilm formation of the bacterial isolates, sampled from CF patient P342, cell suspensions grown in microtiter plates during 48h. After this period, attached cells were stained with crystal violet and measured the absorbance at 590 nm (A_{590}). Results are represented in figure 16 and showed that isolates 1,2 and 10 produce more significantly biofilm biomass than other isolates.

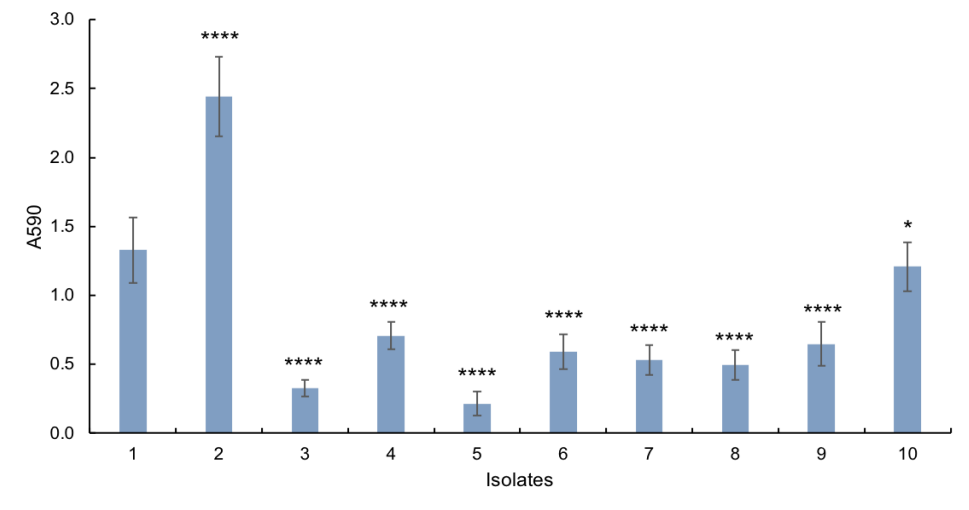

Figure 16 - Biofilm formation of the *B. multivorans* isolates retrieved from CF patient P342, after growth in polystyrene microplates for 48h at 37ºC. Error bars represent the standard deviations of the mean values for at least three experiments (statistical significance of differences between the first isolate and the subsequent ones was determined: *, P-value < 0.05; ****, P-value < 0.0001 by ANOVA followed by Dunnett's multiple comparisons test).

3.2.5. Growth rate and doubling time determination

Growth rates of the 10 *B. multivorans* isolates collected from patient P342 were measured by monitoring the OD_{640nm} of cell cultures in synthetic cystic fibrosis medium (SCFM).[84] Table 6 presents the results of doubling time and show that the first three isolates have shorter doubling time than the remaining isolates.

Table 6 - Doubling times (in hours) calculated for the *B. multivorans* isolates sampled from CF patient P342, based on growth rates estimated from cultures grown in synthetic cystic fibrosis medium (SCFM). Results are the means of data from two independent experiments.

Isolate	1	2	3	4	5	6	7	8	9	10
Doubling time (h)	1.5	1.5	1.7	3.3	3.1	2.5	3.3	3.5	2.7	3.0

3.2.6. Virulence determination in *Galleria mellonella*

Galleria mellonella was used as an infection model to assess virulence of the *B. multivorans* isolates retrieved from patient P342. Ten larvae, for one experiment, were injected for each isolate and survival was tracked for 72 hours post-infection (results in figure 17).

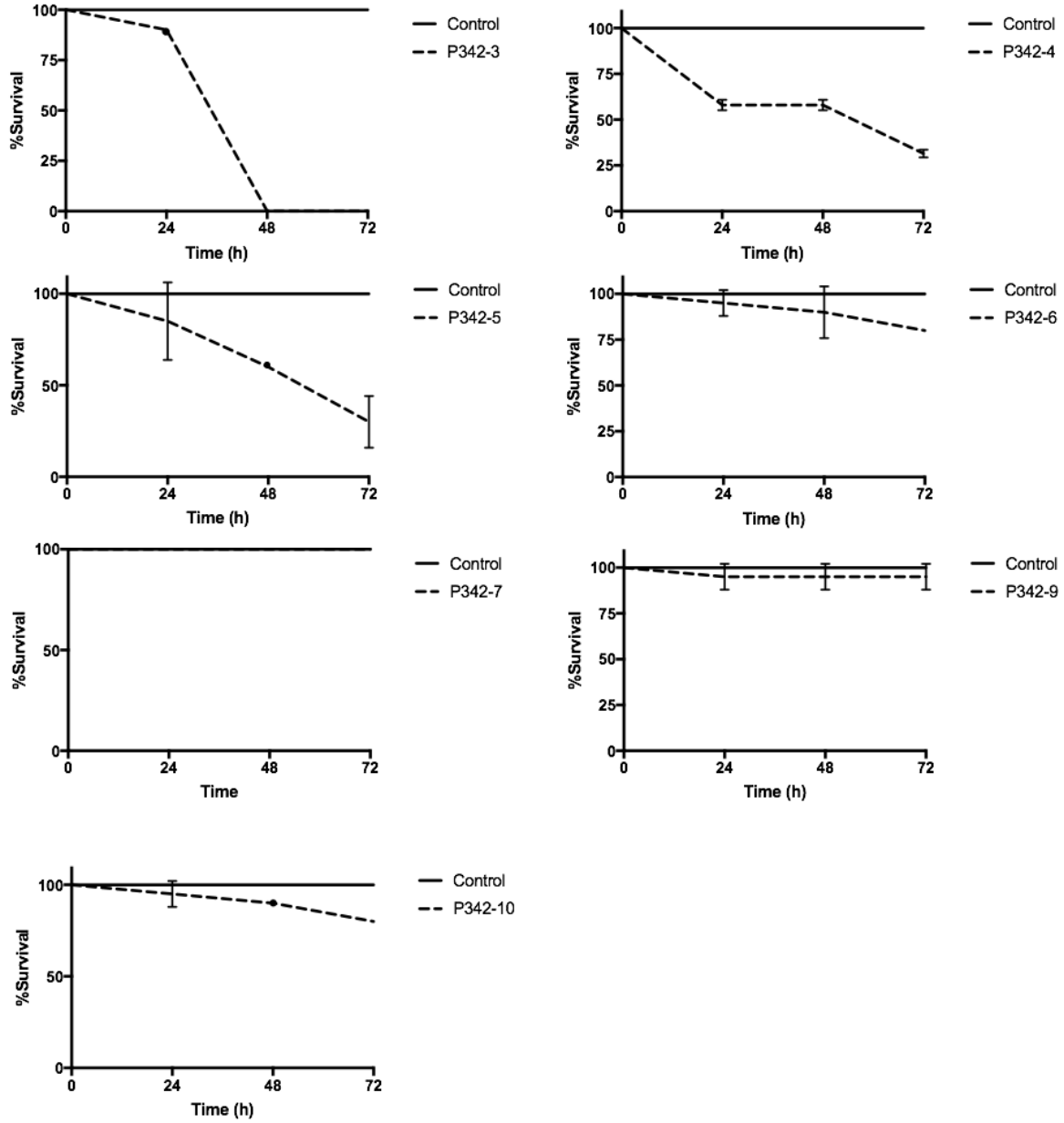

Figure 17 - Survival of *Galleria mellonella* larvae inoculated with *B. multivorans* isolates retrieved from patient P342. Triplicate groups of the 10 larvae were injected for each isolate and survival was tracked for 72 hours post-infection. Error bars represent the standard deviations of the mean values for at least two experiments.

Results show that isolate 3, which exhibit the LPS O-antigen was more virulent in comparison with the remaining isolates, being responsible for 0% survival 48 hours post-infection. Despite of that, it was visible among isolates without O-antigen, the later ones are much more attenuated than, for example, P342-4 and P342-5. Isolates P342-1, P342-2 and P342-8 were not tested due to lack of larvae.

3.2.7. Adhesion to epithelial cells

B. multivorans isolates recovered from patient P342 were analyzed for adhesion to the bronchial epithelial cell line CFBE41o$^-$, which were infected at a multiplicity of infection (MOI) of 10 (10 bacterial cells to 1 epithelial cell) and results are shown in figure 18, as percentage of adhesion. Results show that isolate 10 exhibit increased adhesion to bronchial epithelial cells in comparison with the previous isolates. Isolate P342-1, P342-2 and P342-8 were not tested.

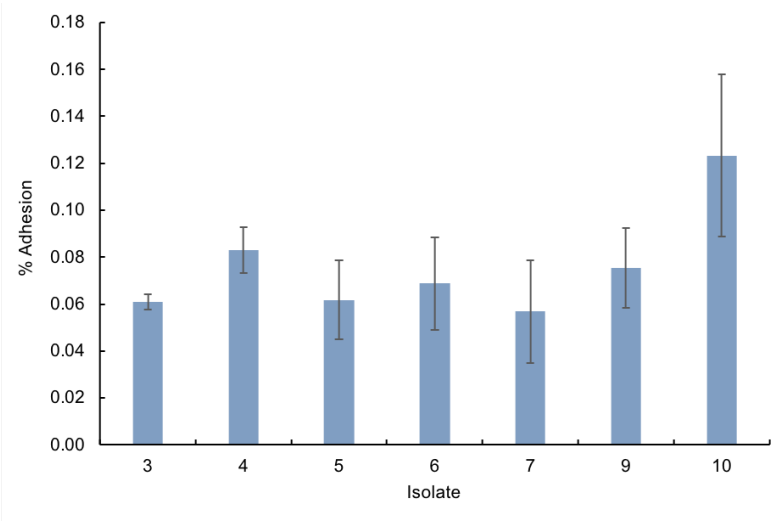

Figure 18 - Adhesion of *B. multivorans* isolates recovered from patient P342 to the bronchial epithelial cell line CFBE41o$^-$. Results are shown as percentage of adhesion. Error bars represent the standard deviations of the mean values for at least two experiments.

3.3. Analysis of *B. multivorans* collected from CF patient P431

3.3.1. *B. multivorans* from CF patient P431 overview

CF patient P431 is composed of thirteen bacterial isolates of *B. multivorans*, sampled in the period between 1998 and 2006 (Figure 19a). Isolates 2, 3 and 4 were sampled in different time points of year 1991; isolate pairs 5/6 and 10/11 were sampled at the same time of years 1999 and 2004, respectively. Genome sequencing results indicate that isolates P431-2 and P431-3 are from a different strain (RAPD025) while the remaining isolates are from RAPD029. Lung function decline in this patient (Figure 19b) decreased 10.3% a year.

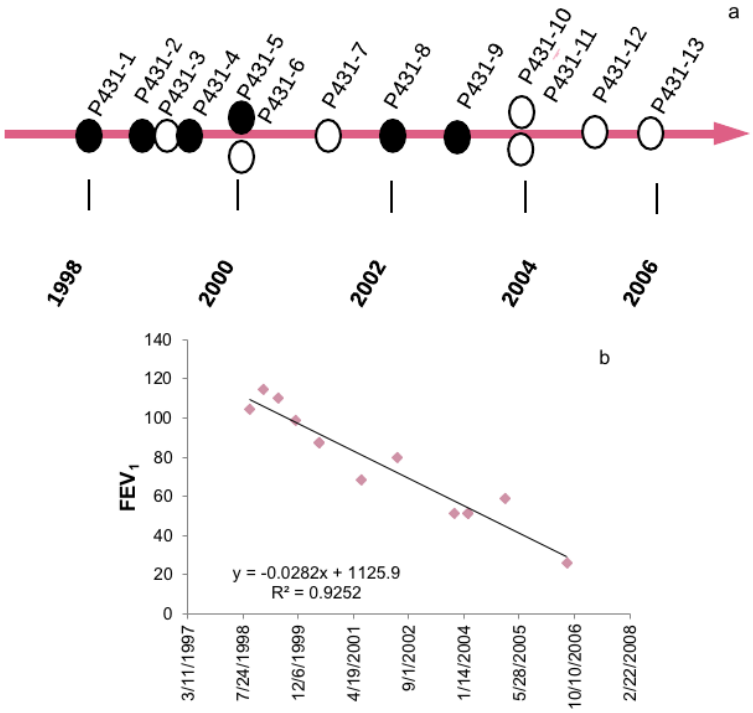

Figure 19 - *B. multivorans* isolates collected from CF patient P431 (a) and patient lung function decline as indicated by forced expiratory volume in 1 second (FEV_1) as percentage (b). Isolates in black produce the LPS O-antigen.

3.3.2. Antibiotic Resistance

The susceptibility of each *B. multivorans* isolate sampled from patient P431 against antibiotics piperacillin/tazobactam, ciprofloxacin, aztreonam and kanamycin was tested. The results of the susceptibility against these antimicrobials are shown as the mean of growth inhibition zone diameter (Figure 20).

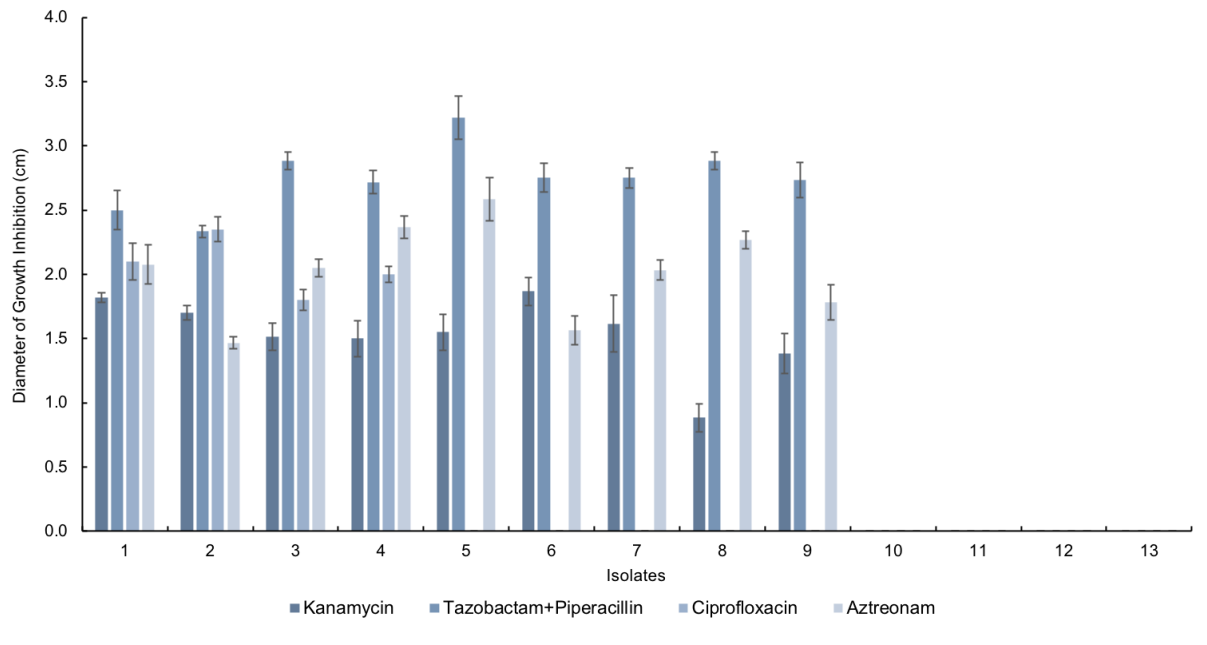

Figure 20 - Susceptibility to antibiotics (kanamycin, tazobactam/piperacillin, ciprofloxacin and aztreonam) for *B. multivorans* isolates recovered from patient P431 at 37 ºC after 24 hours incubation determined by measuring the diameter of cell growth inhibition (error bars represent the standard deviations of the mean values for three independent experiments).

Results show that isolates 1, 2, 3 and 4 were susceptible to all antibiotics tested. Isolates 5 to 9 revealed to be resistant to ciprofloxacin, but demonstrated susceptibility to kanamycin, tazobactam/piperacillin and aztreonam. Moreover, isolates 10, 11, 12 and 13 were fully resistant to the four antibiotics tested. Again, during the course of infection isolates seems to acquire antimicrobial resistance. Statistical significance of differences between the first isolate and the subsequent ones was determined for susceptibility to aztreonam and kanamycin using ANOVA followed by Dunnett's multiple comparisons test. For aztreonam, differences were significant for the majority of isolates (*P*-value <0.0001; supplementary figure 33), isolates 3 and 7 showed no statistically significant alterations. For kanamycin, isolates 7 to 13 differences were extremely significant (*P*-value < 0.0001) and isolates 2 and 6 showed no statistically significant alterations (supplementary figure 34).

3.3.3. Swimming and Swarming Motilities

Swimming assays were assessed for all 13 *B. multivorans* isolates of patient P431 as shown in figure 21. Results showed higher swimming motility to all *B. multivorans* isolates 7 RAPD029 up to P431-9, while the later four isolates have decreased motility.

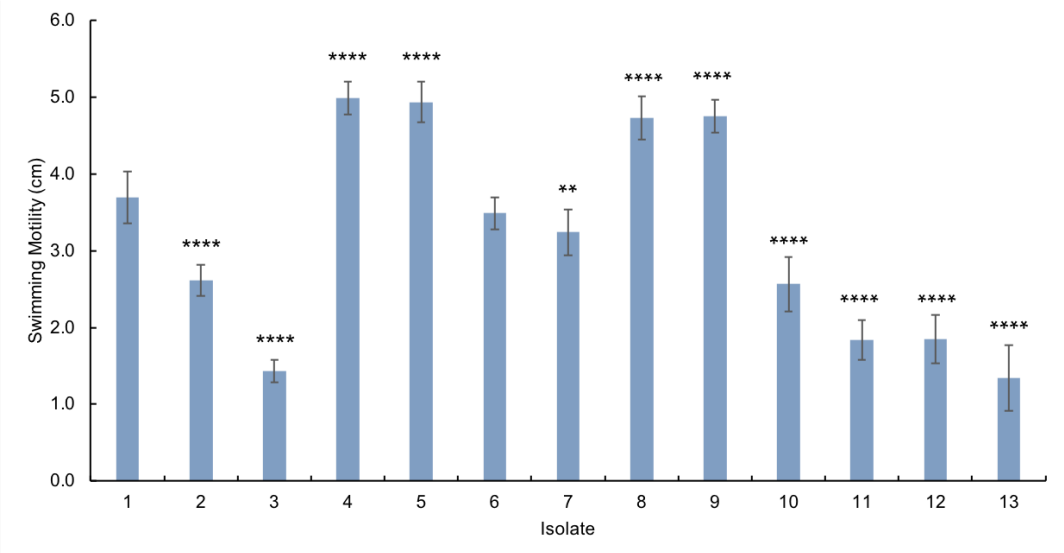

Figure 21 - Swimming motility of the *B. multivorans* isolates retrieved from CF patient P431, after growth in swimming agar plates for 24 h at 37ºC. Error bars represent the standard deviations of the mean values for three independent experiments (statistical significance of differences between the first isolate and the subsequent ones was determined: **, P-value < 0.01; ****, P-value < 0.0001 by ANOVA followed by Dunnett's multiple comparisons test).

Similarly, swarming motility shows the two already mentioned groups (Figure 22). The two *B. multivorans* RAPD025 isolates display reduced swimming and swarming motilities.

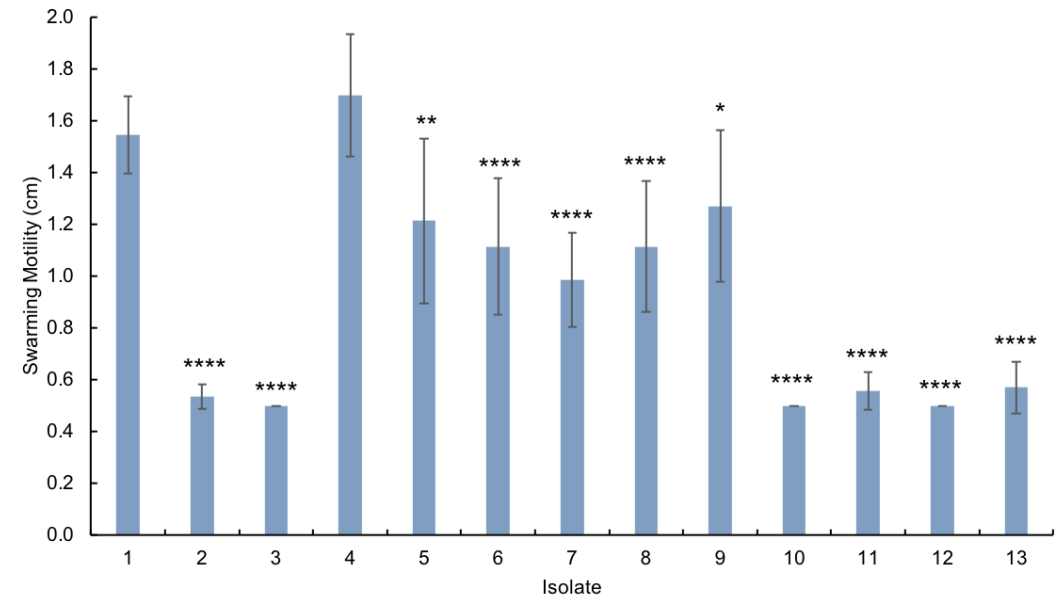

Figure 22 - Swarming motility of the *B. multivorans* isolates retrieved from CF patient P431, after growth in swarming agar plates for 48h at 37ºC. Error bars represent the standard deviations of the mean values for three independent experiments (statistical significance of differences between the first isolate and the subsequent ones was determined: *, P-value < 0.05; **, P-value < 0.01; ****, P-value < 0.0001 by ANOVA followed by Dunnett's multiple comparisons test).

3.3.4. Biofilm formation

To evaluate biofilm formation of the bacterial isolates, sampled from CF patient P431, cell suspensions grown in microtiter plates during 48h. After this period, attached cells were stained with crystal violet and measured the absorbance at 590 nm (A_{590}). Results showed that isolate 2 produced more biofilm than other isolates. Moreover, isolates 6, 8, 11 and 13 showed the lower biofilm formation (Figure 23). Isolates from RAPD029 seem to form two distinct cluster with P431-1, P431-4, P431-5, P431-7, P431-9, P431-10 and P431-12 producing higher biofilm than P431-6, P431-8, P431-11 and P431-13.

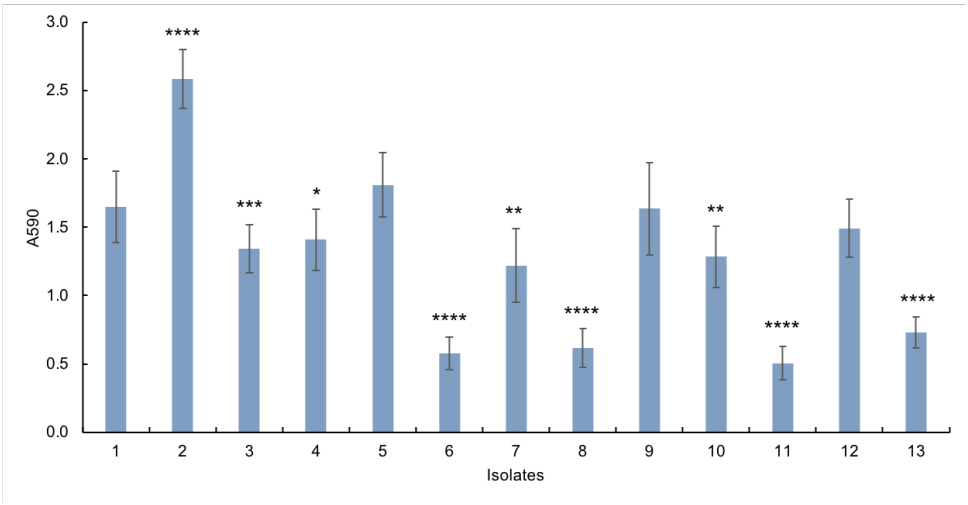

Figure 23 - Biofilm formation of the *B. multivorans* isolates retrieved from CF patient P431, after growth in polystyrene microplates for 48h at 37ºC. Error bars represent the standard deviations of the mean values for at least three experiments (statistical significance of differences between the first isolate and the subsequent ones was determined: *, P-value < 0.05; **, P-value < 0.01; ***, P-value < 0.001; ****, P-value < 0.0001 by ANOVA followed by Dunnett's multiple comparisons test).

3.4. Analysis of *B. multivorans* collected from CF patient P686

3.4.1. *B. multivorans* from CF patient P686 overview

CF patient P686 is composed of eleven bacterial isolates, with isolates 2 and 3 belonging to a single clone of *B. cenocepacia* sampled at the same time of 2004 while the remaining isolates consist of a single clone of B. *multivorans* collected in the period between 2004 and 2011 (Figure 24a). The pairs of isolates P686-1 and P686-4 and P686-6 and P686-7 were retrieved from patient P686 in a single time point of 2004 and 2006, respectively. Lung function decline in this patient (Figure 24b) is only available for the initial years, but it shows an estimated increase of 7.6%.

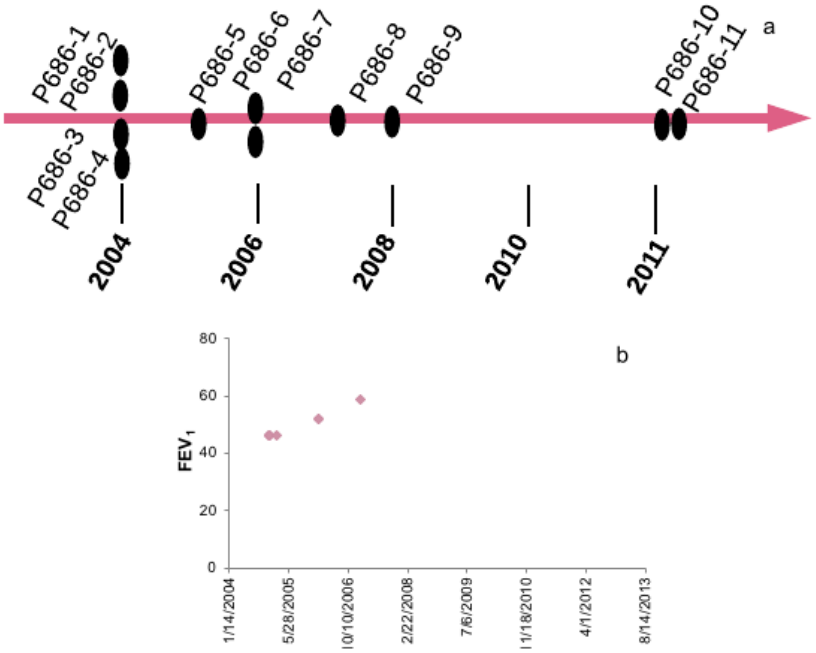

Figure 24 - *B. multivorans* isolates collected from CF patient P686, showing that all have O-antigen (a) and patient lung function decline as indicated by forced expiratory volume in 1 second (FEV_1) as percentage (b).

3.4.2. Antibiotic Resistance

The susceptibility of each *B. multivorans* and *B. cenocepacia* isolate sampled from patient P686 against antibiotics piperacillin/tazobactam, ciprofloxacin, aztreonam and kanamycin was tested. The results of the susceptibility against these antimicrobials are shown as the mean of growth inhibition zone diameter (Figure 25).

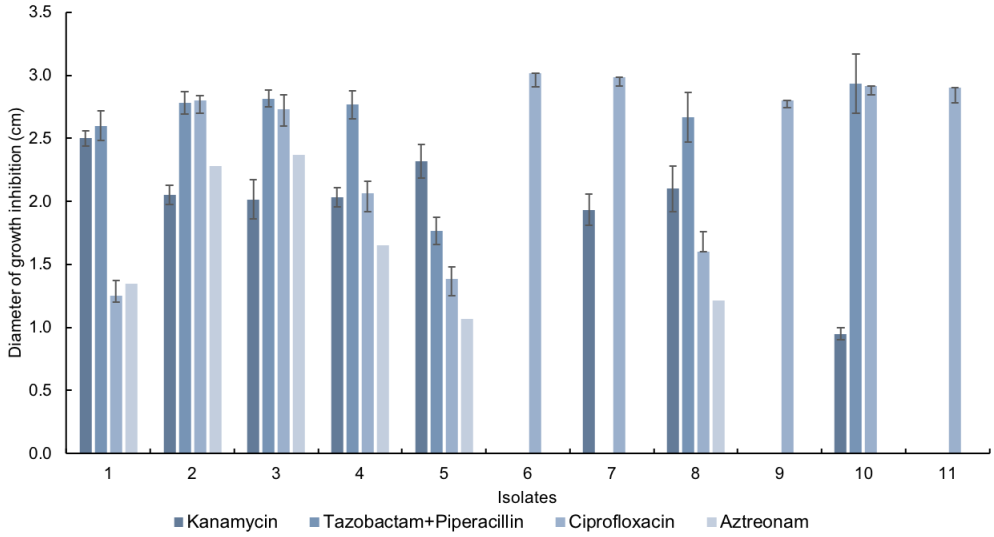

Figure 25 - Susceptibility to antibiotics (kanamycin, tazobactam/piperacillin, ciprofloxacin and aztreonam) for *B. multivorans* isolates recovered from patient P686 at 37 ºC after 24 hours incubation determined by measuring the diameter of cell growth inhibition (error bars represent the standard deviations of the mean values for three independent experiments).

Results show that bacterial isolates 1, 2, 3, 4, 5 and 8 were susceptible to all four antimicrobials tested. Furthermore, isolates 6, 9 and 11 were resistant to three of the four antibiotics but revealed susceptibility to ciprofloxacin. Isolate 10 was susceptible to kanamycin, tazobactam/piperacillin and ciprofloxacin, although demonstrated resistance to aztreonam. Finally, isolate 7 demonstrated susceptibility to kanamycin and ciprofloxacin. Statistical significance of differences between the first isolate and the subsequent ones was determined for susceptibility to aztreonam and kanamycin using ANOVA followed by Dunnett's multiple comparisons test. For aztreonam, all isolates showed significant differences (*P*-value < 0.0001; supplementary figure 36), except isolate 8 that showed no statistically significant alterations. Concerning kanamycin, also, all isolates showed significant differences (*P*-value < 0.0001; supplementary figure 37), except isolate 5 which significant differences were decreased (*P*-value < 0.05).

3.4.3. Swimming and Swarming Motilities

Swimming assays were assessed for all 11 bacterial isolates of patient P686, swimming agar plates being incubated for 24 hours and the diameter of swimming zone was measured (Figure 26). Results showed that with exception of isolate 11 which has the highest motility, after P686-4 all *B. multivorans* isolates show decreased motility when compared to the first isolate.

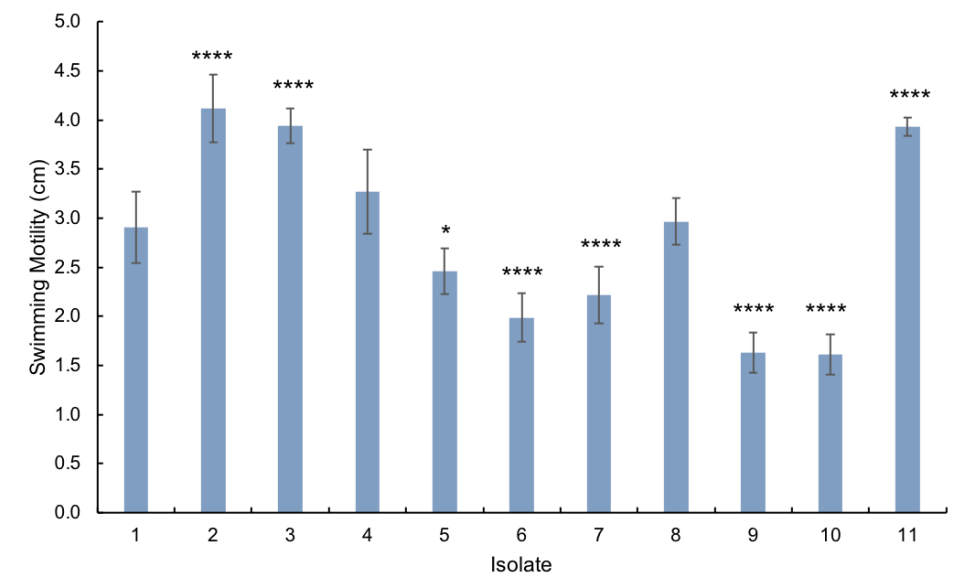

Figure 26 - Swimming motility of the bacterial isolates retrieved from CF patient P686, after growth in swimming agar plates for 24h at 37ºC. Error bars represent the standard deviations of the mean values for three independent experiments. Error bars represent the standard deviations of the mean values for at least three experiments (statistical significance of differences between the first isolate and the subsequent ones was determined: *, P-value < 0.05; ****, P-value < 0.0001 by ANOVA followed by Dunnett's multiple comparisons test).

Swarming motility was evaluated for 11 bacterial isolates of patient P686. Swarming agar plates were incubated for 48 hours and the diameter of swarming zone was measured (Figure 27). The majority of isolates from *B. multivorans* do not swarm, except for isolate 10. The two *B. cenocepacia* isolates are motile, both swimming and swarming (Figure 26 and 27).

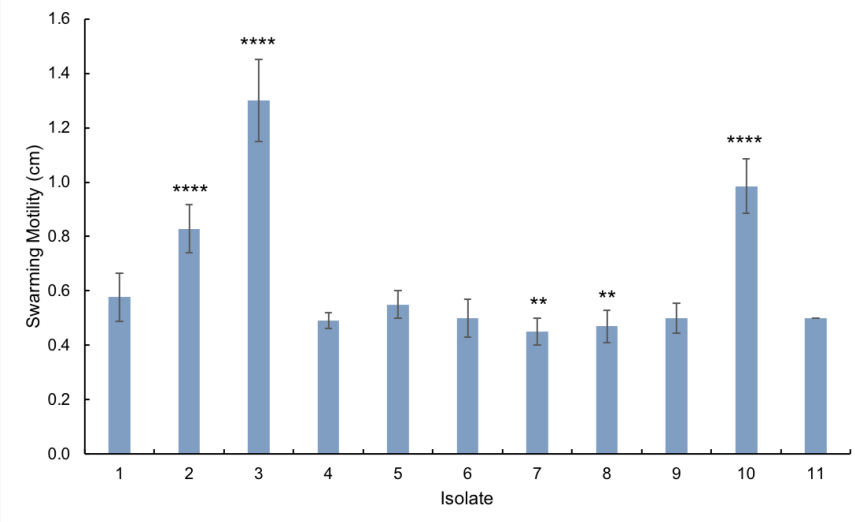

Figure 27 - Swarming motility of the bacterial isolates retrieved from CF patient P686, after growth in swarming agar plates for 48h at 37ºC. Error bars represent the standard deviations of the mean values for three independent experiments. Error bars represent the standard deviations of the mean values for at least three experiments (statistical significance of differences between the first isolate and the subsequent ones was determined: **, P-value < 0.01; ****, P-value < 0.0001 by ANOVA followed by Dunnett's multiple comparisons test).

3.4.4. Biofilm formation

To evaluate biofilm formation of the bacterial isolates, sampled from CF patient P686, cell suspensions grown in microtiter plates during 48h. After this period, attached cells were stained with crystal violet and the absorbance at 590 nm was measured (A_{590}). Results showed that isolates 1, 5, 10 and 11 displayed highest the production of biofilm while isolates 6 to 9 produced the lowest amount of biofilm than other isolates (Figure 28).

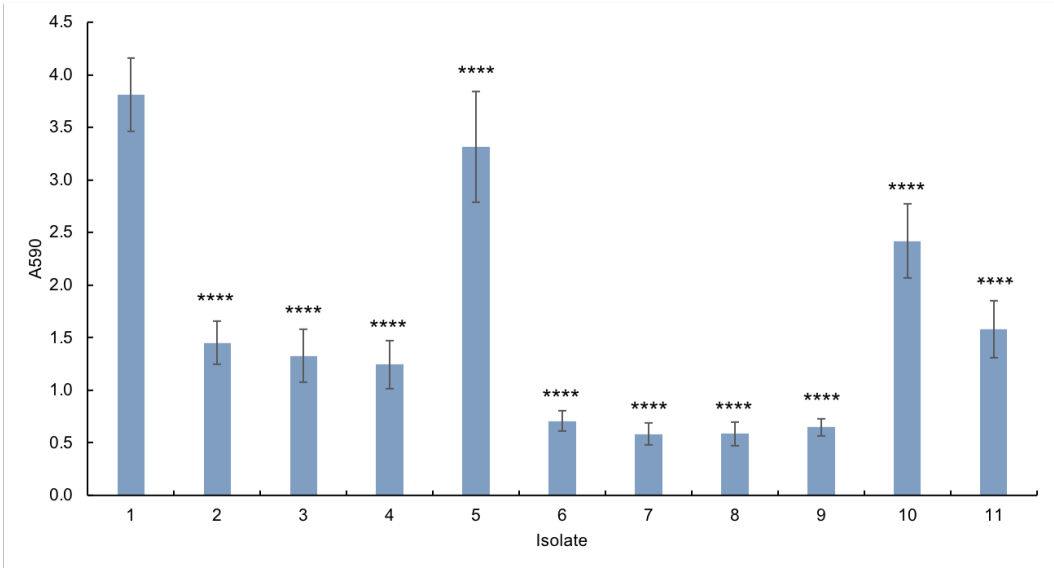

Figure 28 - Biofilm formation of the bacterial isolates retrieved from CF patient P686, after growth in polystyrene microplates for 48h at 37ºC. Error bars represent the standard deviations of the mean values for at least three experiments (statistical significance of differences between the first isolate and the subsequent ones was determined: ****, P-value < 0.0001 by ANOVA followed by Dunnett's multiple comparisons test).

4. DISCUSSION

The purpose of this work was to assess phenotypic evolution of 40 *Burkholderia multivorans* isolates collected from four distinct CF patients (P339, P342, P431 and P686) during the period 1995 to 2012 of chronic infection. The strategy includes phenotypic assays associated with *B. cepacia* complex bacteria adaptation to CF lung environment.

Concerning antibiotic resistance, the first three isolates of patient P339 were susceptible to all antibiotics tested and the last two isolates were resistant to all these antimicrobials. To contribute for this phenotype must be a frameshift mutation in a transcriptional regulator of the TetR family that controls an efflux pump [92]. Several studies [63, 75] demonstrated that during the course of chronic CF infections, bacteria tend to improve their resistance to antibiotics, derived from frequent and prolongated exposure to high doses of antibiotics, which promotes the emergence of strains more adapted to survive within the CF airways, promoting long-term infections. For the *B. multivorans* isolates retrieved from patient P342, isolates 1, 3, 4 and 8 were susceptible to all antibiotics assessed, though isolate 10 only showed susceptibility to tazobactam/piperacillin. Moreover, five isolates were completely resistant to aztreonam. *B. multivorans* isolates sampled from patient 431 revealed that the first four isolates were susceptible to all antibiotics, while the last four isolates were resistant to the four antibiotics tested. Additionally, isolates 5 to 9 were entirely resistant to ciprofloxacin but showed susceptibility to kanamycin, tazobactam/piperacillin and aztreonam. Finally, the 11 bacterial isolates sampled from patient P686 demonstrated susceptibility to, at least, one of the antimicrobials tested. All of the 11 bacterial isolates showed susceptibility to ciprofloxacin and the first five isolates and isolate 8 were susceptible to all antibiotics of this study. Isolates 6, 9, 11 only showed resistance to ciprofloxacin. Overall, it is possible to observe a general increase in antibiotic resistance over time in the four patients. For additional conclusions about antibiotic susceptibility, it would be necessary more clinic data, such as antibiotic administration regimes.

Motility of the bacterial isolates was assessed by measuring swimming and swarming in agar plates. Regarding motility of the *B. multivorans* isolates retrieved from patient P339, swimming motility is higher in the first isolate in comparison with subsequent isolates, although swarming motility increased in isolate 3. Despite of that, the swimming and swarming motility were substantially low in isolates of patient P339. Mutation analysis shows in the last five isolates a nonsynonymous mutation in gene *flil* encoding a flagellum-specific ATP-synthase [92]. This mutation could be the cause for the lower motility of P339-2 to P339-6. Concerning bacterial isolates of patient P342, it was visible that the first isolate displayed swimming and swarming by comparison with the remaining isolates. During the course of infection, bacteria decrease their motility as mechanism to adapt to the environment of lung [63]. This adaptation mechanism is recurrent in Bcc bacteria members. The last four *B. multivorans* isolates retrieved from patient P431 showed reduced motility when compared to the first isolate, as well as isolates 2 and 3. Surprisingly, the pairs 4/5 and 8/9 displayed swimming and swarming motility. Results of patient P686 are in agreement with the other patients, with the latter isolates showing decreased motility than the first

ones (except isolate 11 in swimming motility and isolate 10 in swarming motility). In order to evaluate more precisely motility of the bacterial isolates, it would be important understand the mutations that underlie the different bacterial isolates.

Another phenotypic trait assessed in this work was biofilm formation once Bcc bacteria that infect CF patients tend to change this trait during the course of infection. For patient P339, isolates 1 and 6 displayed higher biofilm formation in comparison with other isolates. Bacteria that live within biofilm are protected from several stresses, consequently studies demonstrated that one of the consequences of biofilm formation include resistance to antibiotics [89, 90]. Results showed that isolate 6 of patient P339 produced more biofilm and was, also, resistant to all antibiotics tested. Concerning biofilm formation of the isolates retrieved from patient P342, isolates 1, 2 and 10 produced higher amount of biofilm, than the subsequent isolates. According to this, isolates 2 and 10 produced more biofilm and showed increased resistance to one and three antibiotics tested, respectively. *B. multivorans* isolates 6, 8 e 11 from patient P431 showed low biofilm formation than the subsequent collected isolates. Moreover, isolate 10 agrees with the observation that biofilm lifestyle increase resistance to antimicrobials. For conclusion, bacterial isolates 6 to 9 retrieved from P686, demonstrated low biofilm formation by comparison with the remaining ones. Despite of that, isolates 10 and 11 revealed increased production of biofilm and were resistant to one and three classes of antibiotics assessed, respectively. Therefore, these results are in agreement with the findings previously reported that biofilm production increases antibiotic resistance.

In order to assess differences in growth among each bacterial strain, growth rates were measured in synthetic cystic fibrosis medium (SCFM) [84]. For *B. multivorans* isolates collected from patient P339, results show that the first three isolates decreased doubling times when compared with the subsequent isolates. Several studies have shown that during the course of chronic CF patients lung infections, *P. aeruginosa* and Bcc bacteria tend to decline their growth rates [26, 63, 81]. Therefore, it might be a response to new nutritional environment of CF airways or a consequence of vaster commitment in adhesion and biofilm production. A mutation that can be related with the decreased growth rate in P339-4 to P339-6 is a frameshift mutation in gene fixJ [92]. This gene encodes the response regulator of the two-component regulatory system FixL/FixJ which controls several phenotypes including regulation of metabolism. For patient P342, results showed that the first three isolates showed decreased doubling time by comparison with the remaining ones. These results support the findings that growth rate decreases during long-term lung infection of CF patients.

Galleria mellonella was used as an infection model to study the virulence of *B. multivorans* isolates, as the innate immune system of mammalians showed similarities to the immune system of these larvae. [88] For both patients (P339 and P342), the first isolates have increased virulence in comparison to the subsequent *B. multivorans* isolates. These results were in agreement with a study that reports decreased acute virulence caused by Bcc during the course of CF [81]. One trait relevant for virulence is the presence of O-antigen of the LPS. Since none of the P339 isolates presents O-antigen, differences

in virulence between the first two isolates and the remaining ones cannot be attributed to this. Nevertheless, analysis of mutations shows that isolates P339-3 to P339-6 display a nonsynonymous mutation in *cepR* gene encoding the major quorum sensing regulator CepR. This mutation might affect this protein function with influence in several phenotypes including virulence [92]. Isolate 3 of patient P342 showed increase virulence, responsible for 0% of survival, 48 hours post-infection. Considering that isolate 3 display the LPS O-antigen, it might be the cause for acute virulence when compared to the subsequent isolates. In a study with *B. cenocepacia* LPS has been shown to inhibit phagocytosis due it interaction with macrophages, consequently reducing bacteria elimination [74].

B. multivorans isolates were analyzed for adhesion to the bronchial epithelial cell line CFBE41o$^-$, derived from a patient homozygous for the cystic fibrosis transmembrane conductance regulator F508del mutation. Concerning *B. multivorans* isolates recovered from patient P339, which do not produce the LPS O-antigen, the latter isolates have increased adhesion than the first ones. Similar result was obtained for another set of longitudinal isolates collected from a single CF patient where adhesion to CF bronchial epithelial cells increased during infection [63]. Contrastingly, to this observation, in *P. aeruginosa,* adhesion to alveolar epithelial decreases during the establishment of long-term infection [91]. For patient P342, results evidenced that isolate 3, which produce the LPS O-antigen, showed lowest level of adhesion to epithelial cells. Moreover, in *B. cenocepacia* and *B. multivorans* was reported that, the O-antigen inhibits adhesion to epithelial cells [63, 74].

A last observation concerns lung function decline in the four patients. For three of the patients, lung function decreased during chronic infection, while in patient P686 it increases. Although this is pure speculation, P686 isolates still presents O-antigen while in the other patients isolates, this trait was lost in the initial stages of the infection. More data of this type need to be analyzed to evaluate if it exists a correlation between lung function decline and loss of O-antigen.

Overall, the later isolates retrieved from the CF patients investigated showed increased antibiotic resistance, decreased motility and growth rates and increased biofilm formation. The presence of the LPS O-antigen might have affected virulence in *Galleria mellonella* and adhesion to epithelial cells, showing higher virulence and decreased adhesion in the isolates that exhibit this trait. These phenotypic alterations in bacteria led to the establishment of chronic infection, that Bcc bacteria are frequently responsible. Moreover, for future investigations it is imperative study the genomic evolution of these patients, especially the mutations that underlie the phenotypes presented.

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6. APPENDIX

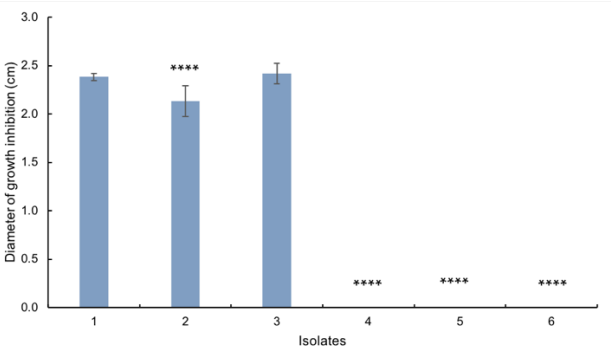

Figure 29 - Susceptibility to aztreonam for *B. multivorans* isolates recovered from patient P339 at 37 ºC after 24 hours incubation determined by measuring the diameter of cell growth inhibition. Error bars represent the standard deviations of the mean values for three independent experiments (statistical significance of differences between the first isolate and the subsequent ones was determined: ****, P-value < 0.0001 by ANOVA followed by Dunnett's multiple comparisons test).

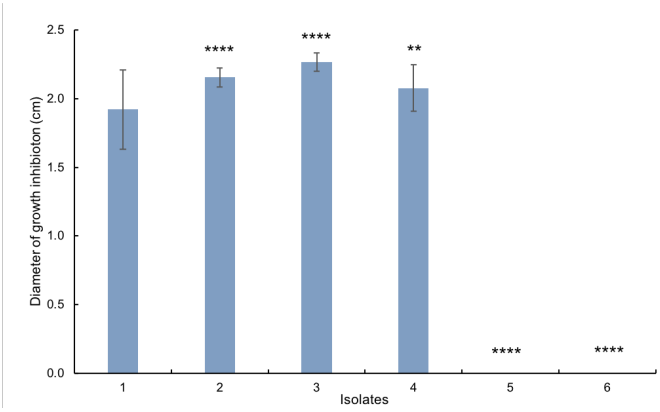

Figure 30 - Susceptibility to kanamycin for *B. multivorans* isolates recovered from patient P339 at 37 ºC after 24 hours incubation determined by measuring the diameter of cell growth inhibition. Error bars represent the standard deviations of the mean values for three independent experiments. (statistical significance of differences between the first isolate and the subsequent ones was determined: **, P-value < 0.01; ****, P-value < 0.0001 by ANOVA followed by Dunnett's multiple comparisons test).

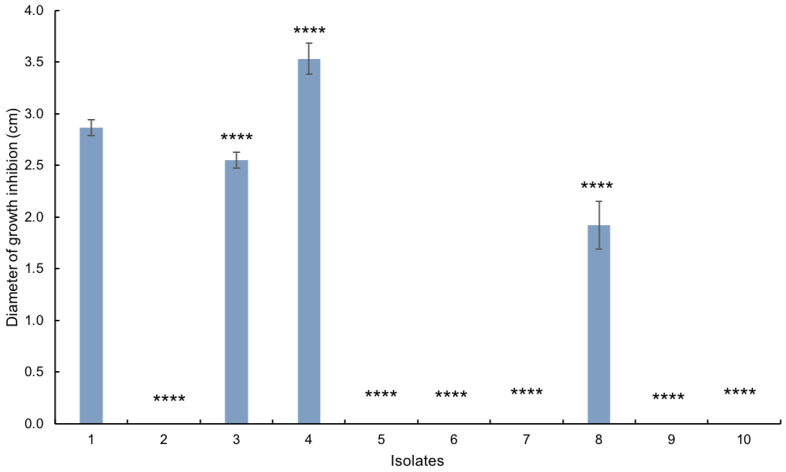

Figure 31 - Susceptibility to aztreonam for *B. multivorans* isolates recovered from patient P342 at 37 ºC after 24 hours incubation determined by measuring the diameter of cell growth inhibition. Error bars represent the standard deviations of the mean values for three independent experiments (statistical significance of differences between the first isolate and the subsequent ones was determined: ****, P-value < 0.0001 by ANOVA followed by Dunnett's multiple comparisons test).

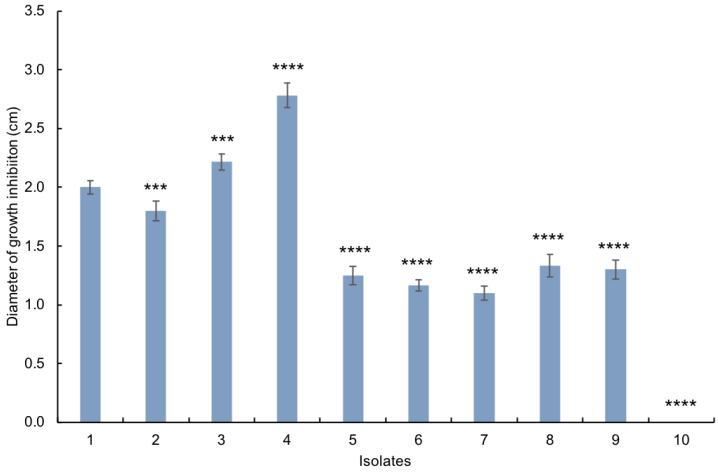

Figure 32 - Susceptibility to kanamycin for *B. multivorans* isolates recovered from patient P342 at 37 ºC after 24 hours incubation determined by measuring the diameter of cell growth inhibition. Error bars represent the standard deviations of the mean values for three independent experiments (statistical significance of differences between the first isolate and the subsequent ones was determined: ***, P-value < 0.001; ****, P-value < 0.0001 by ANOVA followed by Dunnett's multiple comparisons test).

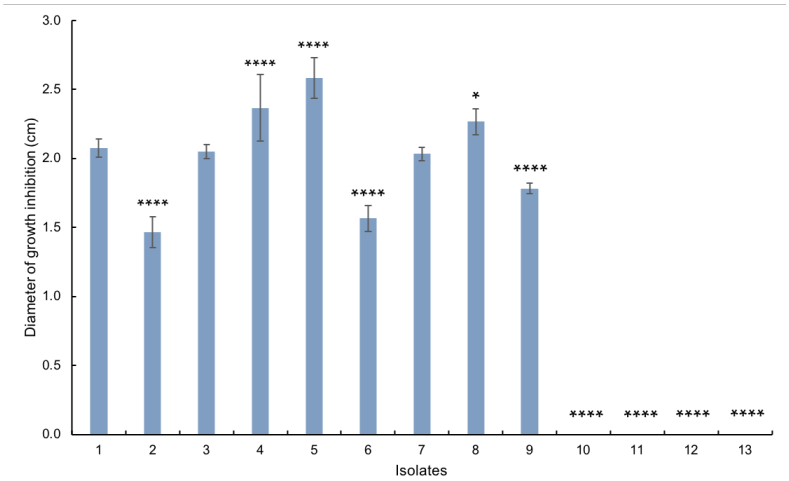

Figure 33 - Susceptibility to aztreonam for *B. multivorans* isolates recovered from patient P431 at 37 ºC after 24 hours incubation determined by measuring the diameter of cell growth inhibition. Error bars represent the standard deviations of the mean values for three independent experiments (statistical significance of differences between the first isolate and the subsequent ones was determined: *, P-value < 0.05; ****, P-value < 0.0001 by ANOVA followed by Dunnett's multiple comparisons test).

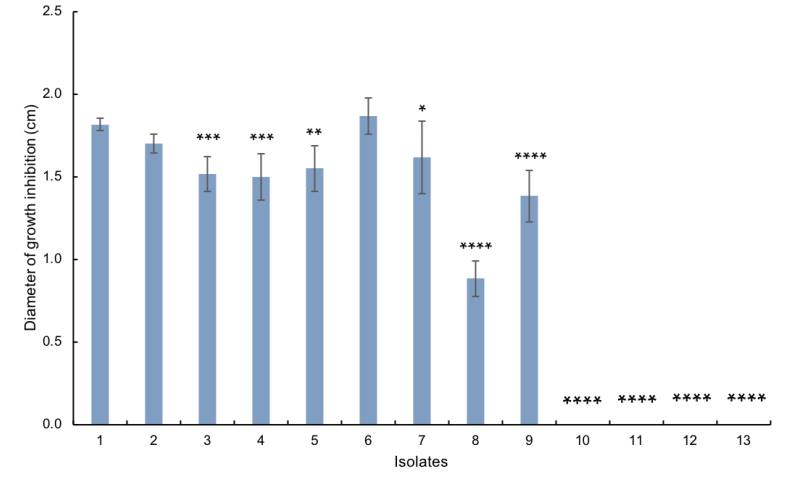

Figure 34 - Susceptibility to kanamycin for *B. multivorans* isolates recovered from patient P431 at 37 ºC after 24 hours incubation determined by measuring the diameter of cell growth inhibition. Error bars represent the standard deviations of the mean values for three independent experiments (statistical significance of differences between the first isolate and the subsequent ones was determined: *, P-value < 0.05; **, P-value < 0.01; ***, P-value < 0.001; ****, P-value < 0.0001 by ANOVA followed by Dunnett's multiple comparisons test).

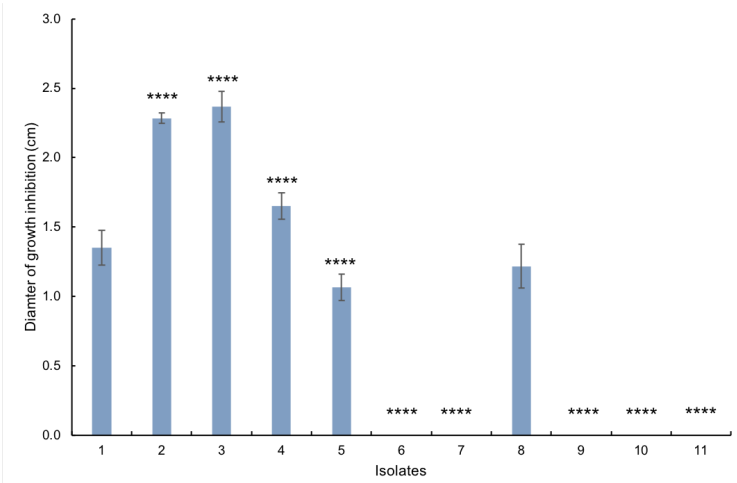

Figure 35 - Susceptibility to aztreonam for bacterial isolates recovered from patient P686 at 37 ºC after 24 hours incubation determined by measuring the diameter of cell growth inhibition. Error bars represent the standard deviations of the mean values for three independent experiments (statistical significance of differences between the first isolate and the subsequent ones was determined: ****, P-value < 0.0001 by ANOVA followed by Dunnett's multiple comparisons test).

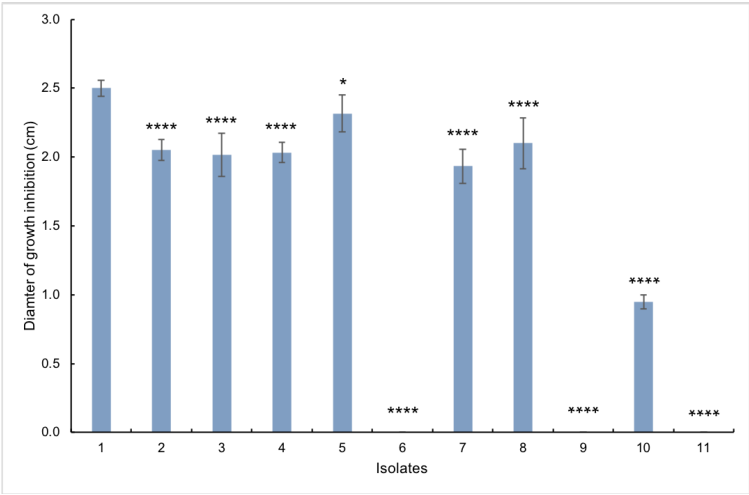

Figure 36 - Susceptibility to kanamycin for bacterial isolates recovered from patient P431 at 37 ºC after 24 hours incubation determined by measuring the diameter of cell growth inhibition. Error bars represent the standard deviations of the mean values for three independent experiments (statistical significance of differences between the first isolate and the subsequent ones was determined: *, P-value < 0.05; ****, P-value < 0.0001 by ANOVA followed by Dunnett's multiple comparisons test).