Genetic determinants of cell aggregation in *Burkholderia multivorans*

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Preface

The work presented in this thesis was performed at the Institute for Bioengineering and Biosciences of Instituto superior Técnico (Lisbon, Portugal), during the period from September 2018 to March 2019, under the supervision of Professor Leonilde Moreira.

Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

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Resumo

A Fibrose quística é a doença genética mais comum e letal. A mortalidade elevada resulta tipicamente da insuficiência respiratória causada por infecções bacterianas nos pulmões. *Pseudomonas aeruginosa* e *Burkholderia* são os agentes patogénicos mais prevalentes nos pulmões de pacientes com fibrose quística. A formação de biofilmes é clinicamente relevante uma vez que ajuda as bactérias a resistir mais eficientemente à acção de antibióticos, conferindo-lhes ao mesmo tempo uma maior protecção contra a resposta imunitária do hospedeiro. Estudos recentes demonstram que, em infecções pulmonares as bactérias normalmente formam agregados com outras bactérias através de componentes da própria matriz e de muco. Assim, as infecções crónicas caracterizam-se pela presença de agregados suspensos nos tecidos ou lúmen. Os mecanismos envolvidos na formação de agregados por parte da espécie *Burkholderia* ainda não são claros, por esse motivo neste trabalho pretendia-se investigar os determinantes genéticos da agregação celular por parte da *Burkholderia multivorans.* Para este propósito, construiu-se uma biblioteca de mutantes de inserção a partir de um isolado clínico, e seleccionaram-se seis mutantes com capacidade de agregação reduzida. Caracterizou-se cada um dos mutantes em termos de fenótipos como mobilidade, produção de exopolissacáridos, susceptibilidade a antibióticos e taxa de crescimento. Após confirmação da inserção do plasposão, três regiões flanqueantes foram sequenciadas com sucesso. Dos genes interrompidos, um codifica a fosfoenolpiruvato sintase, outro faz parte de um sistema de secreção tipo VI, enquanto o último está envolvido num sistema regulador de dois componentes.

Palavras-Chave

Agregados Celulares, Biofilmes, Fibrose Quística,
Burkholderia multivorans, Biblioteca de mutantes

Abstract

With mortality typically resulting from respiratory failure due to chronic pulmonary bacterial infections, cystic fibrosis (CF) is the most common, and lethal, genetic disease in Caucasians. *Pseudomonas aeruginosa* and *Burkholderia* are the most prevalent pathogens in CF patient's lungs. Biofilm formation is an important clinical problem since it helps bacteria resist more efficiently to antibiotic's action while conferring a greater protection from the immune response of the host. Recent data has demonstrated that in pulmonary infections bacteria usually attach to their fellow bacteria likely by means of matrix components and mucus. Thus, it appears that chronic infections can be characterized by aggregates suspended within host tissue or lumen. The mechanisms involved in the formation of planktonic aggregates by *Burkholderia* species are not clear, so this work aimed to investigate the genetic determinants of cell aggregation in *Burkholderia multivorans*. For this purpose, a library of transposon mutants of a clinical isolate was created, and six mutants were selected for their reduced ability to aggregate. Each of these mutants was characterized concerning phenotypes such as motility, exopolysaccharide production, antibiotic susceptibility and growth rates. After confirmation of the plasposon insertion, three flanking regions were successfully sequenced. Of the genes disrupted one encodes a phosphoenolpyruvate synthase, other is a part of a type VI secretion system, and the last is involved in a two-component regulatory system.

Keywords

Planktonic aggregates, Biofilm, Cystic fibrosis,
Burkholderia multivorans, Transposon mutant library

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List of Abbreviations

ABC - ATP-binding cassette

AHL - N-acyl-homoserine lactones

Bcc - *Burkholderia cepacia* complex

BLAST – Basic Local Alignment Search Tool

c-di-GMP - Cyclic di-GMP

CF – Cystic Fibrosis

CFTR - Cystic Fibrosis Transmembrane Conductance Regulator

DGC - Diguanylate Cyclase

eDNA - Extracellular DNA

EPS - Exopolysaccharide

GMP - Guanosine Monophosphate

IL-8 - Interleukin-8

LB - Lennox Broth

LdhA - Lactate dehydrogenase

LPS - Lipopolysaccharide

MAP - Mitogen-Activated Protein

MBIC - Minimal Biofilm Inhibitory Concentration

MIC - Minimum Inhibitory Concentration

NBD - Nucleotide Binding Domain

NCBI - National Center for Biotechnology Information

OD_{640} - Optical Density 640 nm

PDE - Phosphodiesterase

PEP - Phosphoenolpyruvate

PTS - Phosphoenolpyruvate Phosphotransferase System

QS- Quorum-Sensing

ROS - Reactive Oxygen Species

sRNA - Small noncoding RNA

T6SS - Type VI Secretion System

TCS - Two Component Signal

TLR - Toll-like receptors

Wt – Wild-type

YEM - Yeast Extract Mannitol Medium

1. Introduction

1.1. Cystic fibrosis

Cystic fibrosis (CF) is the most common genetic disease, in Caucasians, of which about 1 in 2500 newborns is affected. It is an autosomal recessive monogenetic disease. Therefore, heterozygotes from two carriers have a one in four chance of inheriting the mutation and being affected with CF, whereas the ones who carry one normal CF allele and one mutant allele are entirely asymptomatic and are denoted carriers (Collins, 1992). The disease's main pathology is the accumulation of viscous mucus at the epithelial surfaces of organs including the lungs, pancreas gut and testes, which results in blockages, infection, inflammation and ultimately, organ failure (Cant et al., 2014).

CF arises from mutations in a 189 kb gene located on chromosome 7, encoding a 1480 amino acid polypeptide, named cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is a member of the ATP-binding cassette (ABC) transporter family of membrane proteins which forms a channel at epithelial apical cell membrane (Cant et al., 2014). The ABC family of proteins has highly conserved domains which include a membrane-spanning region, containing six membrane-spanning peptides, followed by a nucleotide binding domain (NBD), which supplies energy to drive the opening and closing of the ion channel through ATP binding and hydrolysis. The membrane-spanning domain of ABC family proteins typically consist of six membrane-spanning regions and the length of the hydrophilic loops that connect the membrane-spanning regions is highly conserved, suggesting that the spatial topology of the membrane-spanning regions is crucial for channel function (Lyczak et al., 2002). As it can be seen in Figure 1, the CFTR contains two transmembrane domains and two NBDs, differing from most ABC family proteins because it has a highly charged central domain, containing a number of serine residues which are targets of PKA mediated phosphorylation, therefore called R (regulatory), and also has long N- and C-terminal extensions about 80 and 30 residues in length, respectively (Cant et al., 2014; Collins, 1992). It is the only known member of the ABC family that acts as an ion channel, and has been shown to act as a HCO_3^- channel and to regulate Cl^-/ HCO_3^- exchangers (Cant et al., 2014).

There are currently 2065 mutations listed in the CFTR mutation database, however a 3-bp deletion in exon 10 which results in the loss of a single amino acid (phenylalanine at codon 508, hence its designation as ΔF508) accounts for approximately 70% of the mutant alleles (Collins, 1992; Lyczak et al., 2002). Considerable effort has been expended to correlate specific mutations with the phenotype of disease produced. The mutations have been classified into 5 groups: Class 1 mutations cause a defect in CFTR protein synthesis, such as a premature stop codon; Class 2 mutations, include the common mutation ΔF508, are translated into full-length nascent polypeptide chains but are defective in folding and are thus targeted for degradation rather than trafficked to the plasma membrane; Class 3 mutants of CFTR are able to reach the PM but have channel gating defects that decrease channel opening time and decrease chloride flux, e.g. the second most common mutation is G551D; Class 4 mutants reach the plasma membrane, but have decreased channel conductance even when the gate is open; and Class 5 represent a fully functional CFTR at the PM but with reduced abundance due to defective mRNA splicing. Class 1-3 cause severe disease phenotypes, whereas class 4 and 5 are generally mild-disease

causing mutations. It should be noted that some CFTR mutations can have more than one phenotype (Cant et al., 2014).

1.2. Cystic Fibrosis in the lungs

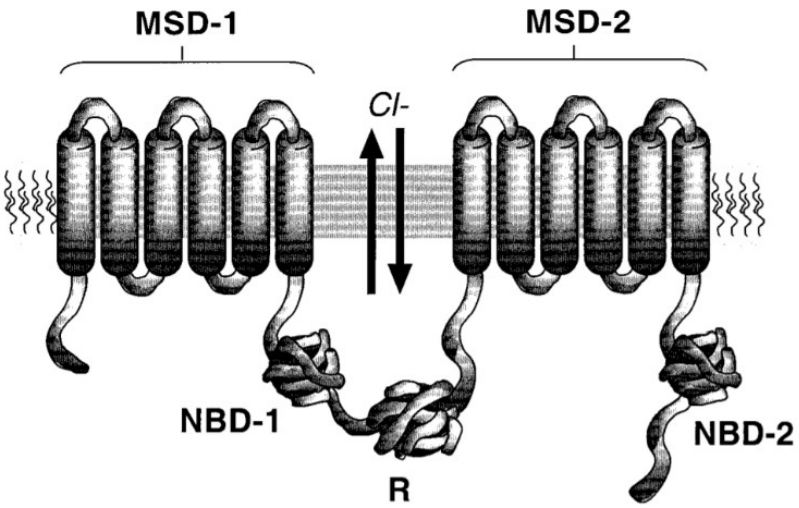


Figure 1. Schematic diagram of the proposed structure of CFTR. A member of the ABC family, CFTR consists of a tandem repeat of the ABC motif. This motif comprises a nucleotide ordinary domain (composed of six transmembrane stretches of amino acids) followed by an NBD. In CFTR, the two occurrences of this motif are separated by a regulatory (R) domain. Each NBD is able to bind and hydrolyze ATP to operate chloride channel function: hydrolysis of ATP by NBD-1 opens the chloride channel, while ATP hydrolysis by NBD-2 closes the channel. Channel function is further regulated by phosphorylation of serine residues in the R domain. Retrieved from (Lyczak et al., 2002)

With ultimately, 80 to 95% of patients with CF succumbing to respiratory failure, the most troublesome clinical features are the prominent neutrophilic inflammation, mucus in airways, progressive bronchiectasis and the chronic pulmonary bacterial infection (Lyczak et al., 2002; Stoltz et al., 2015). Since CFTR is expressed early during development, there is the possibility that the airway obstruction and air trapping observed in infants with cystic fibrosis, might, at least in part, be congenital in nature (Stoltz et al., 2015).

Inflammation is observed early in the course of the disease and several studies showed higher interleukin-8 (IL-8) levels and significant neutrophil recruitment prior to overt infection in both CF infants and CF human fetal xenografts. Nevertheless, the presence of significant inflammation before infection in CF airways remains a controversial hen-egg issue. While the surrounding pro-inflammatory CF microenvironment substantially affects neutrophil phenotype and function, the intrinsic CFTR defect also seems to modulate neutrophil homeostasis and granule release. Collectively, CFTR function in neutrophils has been associated with granule exocytosis and phagocytosis, as well as sustained neutrophilic lung inflammation in response to infection (Laval et al., 2016).

In the healthy respiratory system, the upper respiratory tract is colonized by a wide variety of microorganisms comprising the normal flora while the lower respiratory tract is maintained in a sterile state by the various innate defenses of the host. These defenses consist of physical barriers and

endocytic/phagocytic barriers. Failure of any of these innate defenses results in susceptibility to pulmonary infection (Lyczak et al., 2002).

One important defense to protect lungs against infection is the mucociliary action of the airway epithelium. A mucus layer that lines the airway lumen, this layer is biphasic, consisting of an upper, viscous layer that serves to trap particulates and microorganisms and a lower, more fluid layer in which the cilia beat synchronously, creating a steady current that continually moves the mucus layer upward toward the nasopharynx. When functioning normally, this clearance system traps foreign bodies in the mucus and subsequently carries them to the nasopharynx, where they are expectorated and swallowed (Lyczak et al., 2002). Cystic fibrosis alters the electrophysiological properties across airway epithelia through two processes: the reduction of the CFTR mediated anion (chloride and bicarbonate) transport, which results in lower water content in the periciliary fluid and thus in abnormally dense mucous; and the inhibition of epithelial sodium channel–mediated sodium absorption that conducts to amiloride-inhibitable sodium hyperabsorption, dehydrating the airways and reducing the height of the periciliary liquid layer (Castellani & Assael, 2017; Stoltz et al., 2015). This directly links impaired mucociliary transport to loss of CFTR anion transport, indicating that defective mucociliary transport is a primary abnormality that is not dependent on infection, inflammation, or remodeling. Nevertheless, advancing infection and bronchiectasis might further disrupt mucociliary transport and fuel disease progression. Data also suggest that the environment of the submucosal gland lumen into which mucus is initially secreted probably alters its properties, causing abnormal detachment (Stoltz et al., 2015). The effects of CF in mucociliary action are represented in Figure 2.

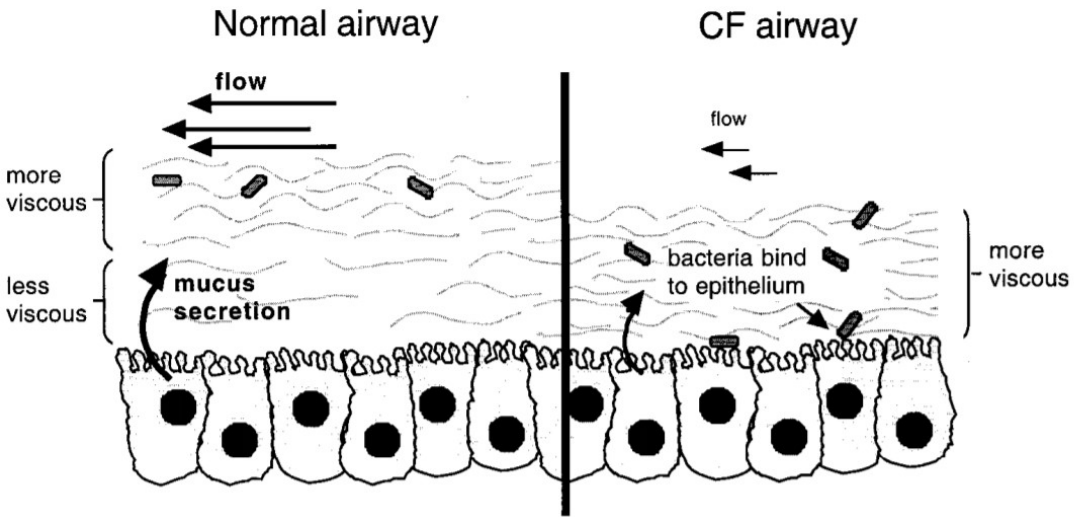


Figure 2. Comparison of mucociliary clearance in the normal and the CF airway. The normal airway epithelium is covered with a biphasic mucus layer consisting of a viscous upper layer and a more fluid lower (periciliary) layer. Concerted beating of epithelial cell cilia causes the mucus to flow unidirectionally toward the esophagus, carrying with it any microorganisms trapped in the mucus. In the CF airway, alterations in either mucus secretion, mucus reabsorption, or both cause the mucus layer to become uniformly viscous, such that beating of the epithelial cilia is no longer enough to propel the mucus toward the esophagus. Bacteria can therefore persist in the airway. Retrieved from (Lyczak et al., 2002)

Another prominent innate defense of the airway is the complex soup of antimicrobial peptides, proteins, and lipids in airway-surface liquid, many of which have individual as well as synergistic effects that rapidly kill bacteria. CF patient's airway-surface liquid has impaired antimicrobial activity which is not

due to a decreased abundance of antimicrobials in airway-surface liquid. Instead, loss of CFTR eliminates bicarbonate secretion by airway epithelia, and as a result the pH of the airway-surface liquid of these epithelia is low, and the secretions from their submucosal glands are also abnormally acidic, which inhibits the airway-surface liquid antimicrobial activity (Stoltz et al., 2015). Figure 3 represents these differences in pulmonary immune defenses of healthy versus cystic fibrosis individuals.

Lack of CFTR function in the bronchial epithelia is also involved in increased inflammatory response ultimately facilitating infection and chronic bacterial colonization of the lung. Microorganisms typically involved in pulmonary chronic infection are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Stenotrophomonas maltophilia*, and *Burkholderia cepacian* (Castellani & Assael, 2017).

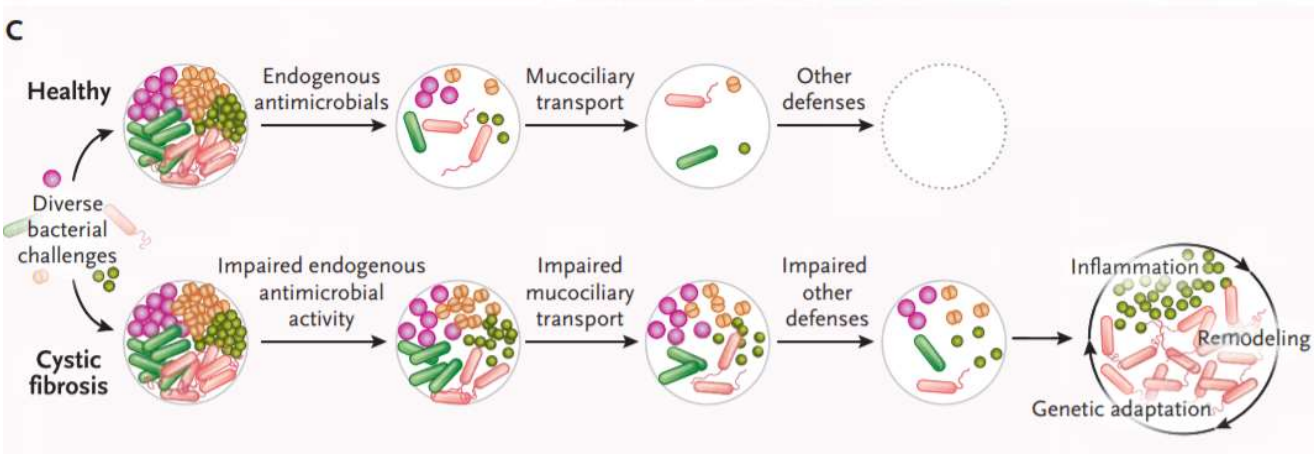


Figure 3. Pulmonary immune defenses of healthy versus cystic fibrosis individuals. When bacteria enter healthy airways (top), they are killed by the antimicrobial activity of airway-surface liquid, mucociliary transport sweeps them out of the lung, and other defenses, including phagocytic cells, eradicate them to maintain sterile lungs. In persons with cystic fibrosis (bottom), antimicrobial activity and mucociliary transport are less effective than in healthy persons, and other defenses may also be impaired. Eventually, the host defenses are overwhelmed, and bacteria proliferate, with inflammation, remodeling, immunity, and genetic adaptation in the bacteria influencing the species that will dominate. In addition, the resulting inflammation and airway remodeling may further enhance or impair host-defense mechanisms. Retrieved from (Stoltz et al., 2015)

1.3. Burkholderia cepacia complex (Bcc)

The *Burkholderia cepacia complex* is a group of closely related Gram-negative, non-spore-forming bacilli belonging to the β-Proteobacteria. These species share a high (>97.5%) level of 16S rRNA gene sequence similarity and moderate (30–60%) DNA–DNA hybridization values. Their unusually large genomes (7.5–8.5 Mb) with a DNA G+C base composition of approximately 67 % consist of multiple replicons and provide them not only with unsurpassed metabolic capacities, but also with genotypic and phenotypic characteristics that defy our need to classify bacteria in well delineated groups (Vandamme & Dawyndt, 2011) At present, the *B. cepacia* complex contains 25 defined species, table 1, which are notable for their ability to metabolize a wide range of organic compounds, to thrive in many different environments and for causing respiratory tract infections in susceptible individuals (Martina et al., 2017, Weber & King, 2016, Bach et al., 2017,.Coenye, 2010; Suppiger et al, 2013)

Pseudomonas cepacian (former designation for *Burkholderia*) was initially described as a pathogen of onions and for many years was identified only sporadically in association with human infections. The first reports of *Burkholderia cepacia* infection in CF patients appeared in the late 1970s and early 1980s and were associated with a fast deterioration in respiratory function characterized by necrotizing pneumonia, bacteremia, and sepsis (Isles et al., 1984; Laraya-Cuasay et al., 1977). This "cepacia syndrome" was observed for as many as 20% of infected patients (Isles et al., 1984; Tablan et al., 1985). During the mid-1990s, comprehensive taxonomic studies to demonstrate that bacteria being identified as *B. cepacia* strains comprised several distinct species were conducted. These experiments found them to be unusually closely related, forming a distinct phylogenetic group. These species were referred to as "genomovars" of the *B. cepacia* complex, until distinguishing phenotypic characteristics were identified, which then allowed each new species to receive a formal binomial designation (Lipuma, 2010).

Table 1. Twenty-five defined species within the Burkholderia cepacia complex and respective former genomovar designation ([1] Martina et al., 2017, [2] Weber & King, 2016, [3] Bach et al., 2017, [4] Coenye, 2010; [5] De Smet et al., 2015., [6] Ong et al., 2016 [7] Peeters et al., 2013 [8] Adams et al., 2017

Species	Former genomovar	Ref.
B. alpina	I	[2]
B. ambifaria	II	[4]
B. anthina	III	[4]
B. arboris	IV	[4]
B. catarinensis	V	[3]
B. cenocepacia	VI	[4]
B. cepacia	VII	[4]
B. contaminans	VIII	[4]
B. diffusa	IX	[4]
B. dolosa		[4]
B. lata		[4]
B. latens		[4]
B. metallica		[4]
B. multivorans		[4]
B. paludis		[6]
B. pseudomultivorans		[7]
B. puraquae		[1]
B. pyrrocinia		[4]
B. seminalis		[4]
B. stabilis		[4]
B. stagnalis		[5]
B. territorii		[5]
B. ubonensis		[4]
B. vietnamiensis		[4]
[Pseudomonas] mesoacidophila		[8]

1.4. Epidemiology

Prevalence of Bcc infection in CF patients, ranges from 0 to 40%, depending on the medical center, and increases with age (Zlosnik et al., 2015). Similar to other opportunistic pathogens such as *Pseudomonas aeruginosa*, Bcc strains do not usually infect healthy individuals but only those that are immunocompromised. Bcc differs from other opportunistic pathogens because it has to be acquired in

the hospital setting (nosocomially) or from other environments since they are not usually carried as commensal organisms (Mahenthiralingam et al., 2005).

Bcc infections usually occur later in the course of CF pulmonary disease, Bcc species initially cause transient infections in the CF airways before a single strain becomes established and causes chronic infection marked by episodes of exacerbations (Hauser et al., 2011). Overall, infection with Bcc in CF patients is generally chronic and correlates with poorer prognosis, longer hospital stays and an increased mortality. In fact, a significant proportion of patients with CF succumb to a rapidly progressive necrotizing pneumonia after acquisition of Bcc bacteria. The poor outcome in lung transplant recipients has led many centers to consider Bcc infection an absolute contraindication to transplantation. However, the clinical outcome observed in patients infected with the same clonal strain of Bcc species is quite variable, which makes it difficult to predict the risks and outcome of Bcc infections, while many CF patients remain colonized with Bcc for years without an apparent adverse impact on clinical course, others may be only transiently colonized. The basis for this differential clinical outcome has not been elucidated, and both host and strain factors probably contribute to the observed disease pathogenesis (Mahenthiralingam et al., 2005).

Most of the early studies on *Burkholderia* and CF simply refer to "*B. cepacia*" or Bcc, however, recently reports have begun to tease out the contributions to disease of individual species within the Bcc (Hauser et al., 2011). It seems that the ability of Bcc bacteria to cause infection in patients with CF is not species dependent, since several Bcc species have been recovered from infected CF patients. However the greatest percentage of Bcc infections in CF patients occur with *B. cenocepacia* and *B. multivorans* strains (Lipuma, 2010; Mahenthiralingam et al., 2005). *B. cenocepacia* is the species that has been most closely associated with high rates of morbidity and mortality as well as the development of the cepacia syndrome (Hauser et al., 2011). A major *B. cenocepacia* lineage, ET-12, was responsible for arguably the largest CF epidemic across Canada and the United Kingdom in the late 1980s and throughout the 1990s (Mahenthiralingam et al., 2005; Zlosnik et al., 2015). Early studies identified it as the most prevalent CF species in North America and Europe, however, after implementation of new infection control measures in 1995, *Burkholderia multivorans* became the most prevalent species (Zlosnik et al., 2015). As shown in Figure 4 representing the distribution of *Burkholderia* species among U.S. CF patients between 1997 and 2007.

In Portugal, from January 1998 to June 2002 the incidence and prevalence of infection with Bcc bacteria, ranged from 1.2 to 7.8% and from 7.1 to 13.3%, respectively. At the time of first isolation of Bcc bacteria, the age of the CF patients varied between 2 months and 19 years, with an estimated mean age of 7.7 years and were predominantly (70%) of the female gender, although the CF population examined included an identical percentage of males and females (Cunha et al., 2003). Between 1995 and 2002 a study confirmed the prevalence of *B. cenocepacia* (52%) but observed that a significant percentage (36%) of the patients at the CF center of Hospital de Santa Maria were colonized or infected with *B. cepacia*. During the 3.5-year surveillance period of a study (2002 to 2005), 85% of Bcc-positive CF

patients under surveillance at the Hospital Santa Maria CF center harbored strains of the species *B. cepacia* (Cunha et al., 2007).

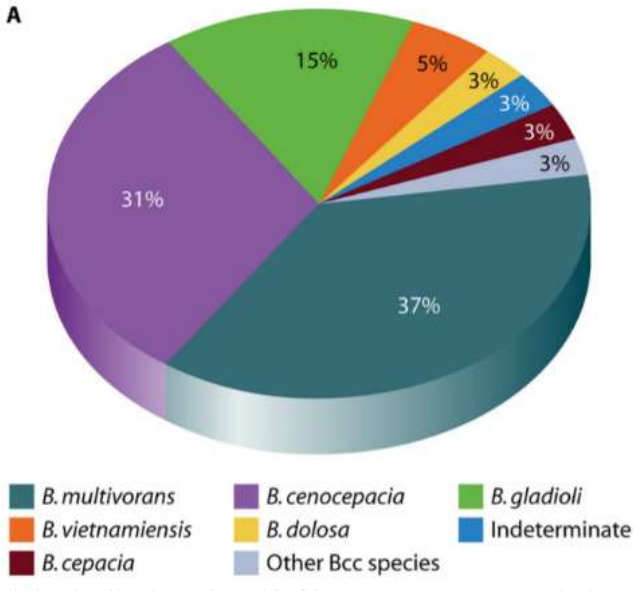


Figure 4. Distribution of Burkholderia species among U.S. CF patients. The proportions of CF patients infected with various Burkholderia species are shown. The data is based on 2,024 CF patients who were infected with Burkholderia species and whose isolates were referred to the Burkholderia cepacia Research Laboratory and Repository (University of Michigan) between 1997 and 2007. "Other Bcc species" indicates patients infected with B. cepacia complex species other than those specified in the chart. "Indeterminate" refers to patients infected with strains that phylogenetically are members of the B. cepacia complex species but that could not be definitively placed into one of the defined species in this group. Retrieved from (Lipuma, 2010)

In an 18-year observational study, cumulative mortality of *B. cenocepacia*-infected patients was 43%, compared to 16% of those infected with *B. multivorans*. The incidence of cepacia syndrome was 13% with *B. cenocepacia* versus 5% with *B. multivorans*. It is important to note that in the studies referenced above, *B. cenocepacia* infection at each center was usually due to a single epidemic strain. Thus, one cannot assume that all strains of *B. cenocepacia* are as virulent (Hauser et al., 2011).

Two of the major problems arising from infection of CF patients with Bcc species are their high intrinsic resistance against antibiotics and biocides, impeding effective medical treatment, and their high transmissibility between patients (Huber et al., 2002). The factors that establish Bcc infection are still undefined, but putative virulence determinants that have been described include cable pili, lipopolysaccharide (LPS), extracellular protease, lipase, hemolysin, a melanin-like pigment, and siderophores. The roles of these factors in lung infections in CF patients remain to be clarified, as their presence does not necessarily correlate with the severity of disease (Chung et al., 2003)

1.5. Phenotypic traits of Bcc strains

1.5.1. Biofilms

Biofilms can be defined simply and broadly as communities of microorganisms that are attached to a surface. They can comprise a single microbial species or multiple microbial species and can form on a range of biotic and abiotic surfaces. Although mixed-species biofilms predominate in most environments, single-species biofilms exist in a variety of infections and on the surface of medical implants (Toole et al., 2000) Biofilm growth helps to protect the resident bacteria from environmental stresses, such as desiccation, nutrient limitation, and predation. (Suppiger et al., 2013).

Biofilm communities produce an extracellular matrix that serves to hold them together, its formation is a coordinated process among community members and can differ from species to species (Suppiger et al., 2013). In most biofilms, the microorganisms account for less than 10% of the dry mass, whereas the matrix can account for over 90%. The matrix is the extracellular material, mostly produced by the organisms themselves, in which the biofilm cells are embedded. It consists of a conglomeration of different types of biopolymers — known as extracellular polymeric substances (EPS) — that forms the scaffold for the three-dimensional architecture of the biofilm and is responsible for adhesion to surfaces and for cohesion in the biofilm (Flemming & Wingender, 2010). Studies indicate that biofilms are a stable point in a biological cycle that includes initiation, maturation, maintenance, and dissolution. Bacteria seem to initiate biofilm development in response to specific environmental cues, such as nutrient availability. Evidence accumulated over the past few years has established that the formation of a mature biofilm on an abiotic surface comprises three distinguishable developmental steps, which are represented in Figure 5: (Huber et al., 2002; Kim & Lee, 2016; Toole et al., 2000)

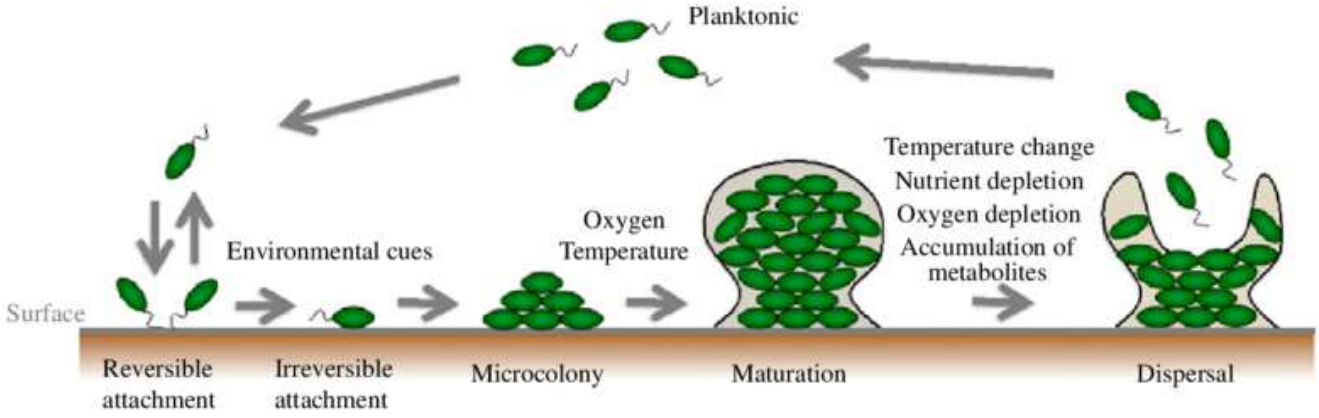


Figure 5. Biofilm formation and selected environmental factors that affect each stage of biofilm formation. The environmental cues control the concentration of second messengers such as cAMP and c-di-GMP. These second messengers control biofilm-related factors such as cell appendages, surface proteins, EPS, and cell motility. Quorum Sensing is also involved in shaping the biofilm by controlling the above biofilm-related factors. Retrieved from (Toyofuku et al., 2016)

(i) Initial attachment of surface-adherent individual cells to a surface. This happens through the action of flagella and pili and the formation of a single layer of planktonic cells on the surface.

(ii) Aggregation of these cells to microcolonies, making the attachment irreversible

(iii) Differentiation of the microcolonies into a mature biofilm by building a mushroom-shaped and highly porous structure in the matrix composed of self-producing EPSs.

(iv) Return to planktonic mode by detachment when they are nutrient deprived, Presumably, this starvation response allows the cells to search for a fresh source of nutrients and is driven by well-studied adaptations that bacteria undergo when nutrients become scarce

Biofilm formation on *P. aeruginosa* infections in CF patients is an important clinical problem since it helps bacteria to resist more efficiently to antibiotic's action while conferring them a greater protection from the immune response of the host (Cunha et al., 2004). The biofilm facilitates their evasion of the antimicrobial actions of neutrophils by impeding migration of leukocytes at their exofacial surface. Mucoid *P. aeruginosa* are also more resistant to phagocytosis by neutrophils compared to non-mucoid counterparts, by virtue of cell surface alteration which renders the bacterium unrecognizable (Murphy & Carahera, 2015). Shedded cellular components of necrosed neutrophils become part of the exopolymeric matrix of the biofilm, serving to further enhance its biomass while preserving the antibiotic resistance characteristic of the biofilm (Bjarnsholt et al., 2009; Parks et al., 2009). A similar situation seems to prevail for *Burkholderia*, a close relative of *Pseudomonas.* Indeed, biofilm formation has also been observed for the Bcc bacteria in abiotic surfaces and on well- differentiated human epithelial cells (Schwab et al., 2002) and *B. cepacia* is thought to coexist with *P. aeruginosa* are as mixed biofilms in the lungs of patients suffering from CF (Caceres et al., 2014; Cunha et al., 2004). Overall, the production of biofilms is associated to a significant increase of resistance against the host immune system and antibiotic treatment in *Burkholderia* (Caraher et al., 2007).

1.5.2. Planktonic Aggregates

Medically relevant bacterial biofilms were generally defined as "multi-cellular surface associated communities embedded in a self-made exopolymeric matrix". Therefore, most attention within medical research has been given to the study of biofilm bacteria that are clearly surface attached (Alhede et al., 2011). However, recent *ex vivo* data has yielded valuable insights in the spatial distribution of bacteria in chronic infections, demonstrating that bacteria do not inevitably attach to surfaces. Rather, they attach to their fellow bacteria likely by means of matrix components and mucus, and they seem to establish impenetrable barriers to the host. Thus, it appears that opposed to harboring bacteria firmly attached to a surface, chronic infections can be characterized by aggregates suspended within host tissue or lumens of organs (Bjarnsholt et al., 2009).

Two clearly identified phenotypes associated with biofilms are the aggregation of cells and a significant increase in the capacity to cope with antibiotic treatments. Studies have shown that non-surface attached aggregates, also called planktonic aggregates or tight microcolonies, exhibit the required survival capability in the hostile human environment and possess inherent antibiotic tolerance to

antibiotics and phagocytes (Alhede et al., 2011). *P. aeruginosa's* planktonic aggregates also share other biofilm features, like the dependency on cyclic di-GMP, eDNA, bacteriophage, and dispersal based on carbon, nitrogen, or oxygen limitations (Schleheck et al., 2009). Therefore, planktonic aggregates should be considered to fully represent the biofilm growth phenotype (Alhede et al., 2011). The major hallmarks of *in vivo* biofilms are thus aggregated bacteria, which tolerate the host defense and high concentrations of antimicrobial agents even over longer times (Bjarnsholt et al., 2013).

A study from Schleheck *et al.* found that, on liquid culture, the planktonic aggregates formed by *P. aeruginosa* PAO1 range from 10 to 400 μm in diameter, comprise up to 90% of the total biomass and disperse into single cells upon carbon, nitrogen, or oxygen limitation (Schleheck et al., 2009). It has been shown that 95 % of *P. aeruginosa* found in the CF lung are present at a distance greater than 5 mm from the epithelial cell surface in non-surface-attached tight microcolonies (Mccarthy et al., 2014). The same is true for Bcc bacteria in CF patients lungs, which are reported to grow as a single cells, but are more often found in small clusters (Schwab et al., 2014).

The genetic mechanisms involved in the formation of planktonic aggregates are not clear, however, at least two regulators have been shown to influence its formation *P. aeruginosa*. LTTR BvlR's inactivation prevented microcolony formation, possibly mediated through the repression of fimbria based surface attachment, but the mechanisms subjacent are still unknown (Mccarthy et al., 2014). Mutants on the two-component regulator MifR form thin and unstructured microcolonies (Petrova et al., 2012). This phenotype suggests that fermentation of pyruvate is required for microcolony formation, since deactivation of genes encoding lactate dehydrogenase and aconitate hydratase abrogated microcolony formation in similar manner. A possible justification for this phenomenon, are the oxygen-limiting but energy-rich conditions that cells experience within aggregates that forces *P. aeruginosa* to use pyruvate fermentation as a means of redox balancing (Petrova et al., 2012).

A study with Bcc bacteria, including *B. multivorans,* by Silva *et al.* showed that the LTTR LdhR and the D-lactate dehydrogenase LdhA are implicated in the formation of planktonic cellular aggregates and biofilms properties possibly relevant in natural environments and within hosts (Silva et al., 2017).

1.5.3. Exopolysaccharide (EPS)

Extracellular polysaccharides or exopolysaccharides (EPSs) are high-molecular weight sugar-based polymers that are synthesized and secreted by many microorganisms. The importance of their production has been studied in many bacteria, being implicated in biofilm formation (Ferreira et al., 2011). EPS immobilize biofilm cells and keep them in close proximity, thus allowing for intense interactions. Owing to the retention of extracellular enzymes, a versatile external digestive system is generated, sequestering dissolved and particulate nutrients from the water phase and allowing them to be utilized as nutrient and energy sources. The matrix also acts as a recycling center by keeping all of the components of lysed cells available. This includes DNA, which may represent a reservoir of genes for horizontal gene transfer. EPS can also serve as a nutrient source, although some components of EPS are only slowly biodegradable and, owing to the complexity of EPS, complete degradation of all

components requires a wide range of enzymes. The matrix protects organisms against desiccation, oxidizing or charged biocides, some antibiotics and metallic cations, ultraviolet radiation, many (but not all) protozoan grazers and host immune defenses (Flemming & Wingender, 2010).

The bacteria from the Bcc have the ability to produce EPS and at least seven different exopolysaccharides have been identified and their structure determined. However, cepacian is the most common EPS produced by *Burkholderia* since it has been identified in different species and on several different Bcc isolates obtained from various CF centers in different countries. Furthermore, genes encoding proteins involved in cepacian synthesis are well conserved among *Burkholderia*, suggesting that cepacian is a common feature among the genus (Ferreira et al., 2011; Sist et al., 2003). Cepacian, whose structure is represented in Figure 6, is composed of a branched acetylated heptasaccharide repeating unit made up of D-glucose, D-rhamnose, D-mannose, D-galactose, and D-glucuronic acid (1:1:1:3:1). This similarity suggests that cepacian, is specific to Bcc bacteria, and the pathway leading to the nucleotide sugar precursors that are necessary to its biosynthesis was proposed (Ferreira et al., 2011; Sist et al., 2003).

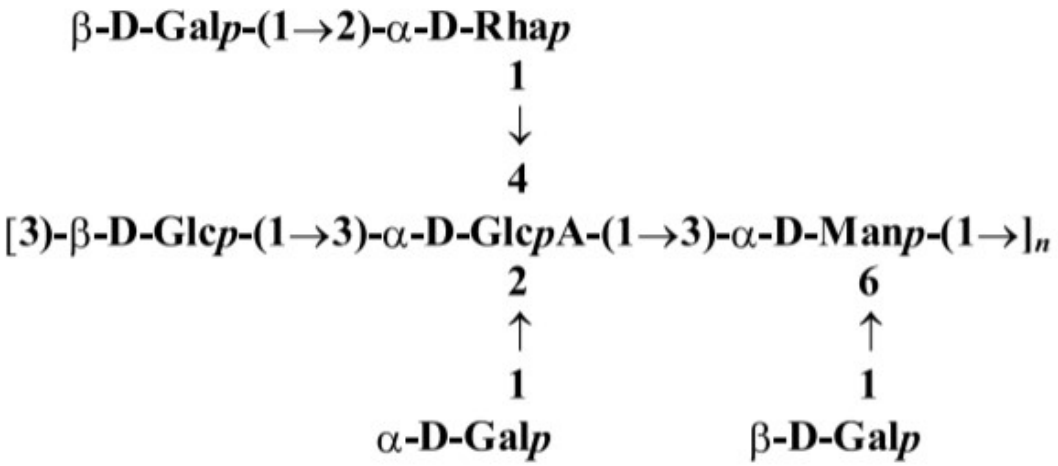


Figure 6. Repeating unit of Cepacian. The structure includes one or more acetyl group per repeating unit whose position is unknown. Retrieved from (Sist et al., 2003).

It was postulated that EPSs, particularly cepacian, could be determinant of Bcc species virulence on airway infections of CF patients and its importance in pathogenicity was assessed by several *in vitro* and *in vivo* studies. Although these studies seem to indicate that mucoid isolates persist longer in the lungs (Conway et al., 2004) and are associated with higher mortality (Sousa et al., 2007), non-mucoid strains were responsible for the major clinical outbreaks and are associated to a worst clinical outcome (Govan et al., 1993). A hypothesis derived from these observations is that mucoid strains would be favored in chronic lung infections while non-mucoid strains would be more prone to virulence in acute infection (Zlosnik et al., 2011).

Nevertheless, polysaccharides mediate bacterial resistance against antimicrobial peptides produced by epithelial and phagocytic cells, so production of EPS in the lungs of CF patients could contribute to a decreased efficacy of the host defense response and the concomitant establishment of a persistent

infection by these bacteria (Benincasa et al., 2009). Also, the overproduction of alginate by clinical isolates of *P. aeruginosa* is considered to contribute to biofilm formation and to the long-term survival of the bacterium in the lungs of CF patients (Cunha et al., 2004). Exopolysaccharides are one of the main constituents of mature biofilms, which are associated to a significant increase of resistance against the host immune system and antibiotic treatment in *Burkholderia*. In fact, mutants constructed on EPS genes have confirmed the importance of cepacian in the formation of mature biofilms (Ferreira et al., 2007). By promoting the formation of mature biofilms the EPS may enhance bacterial survival in CF lung, which leads to the impossibility to efficiently eradicate Bcc infections (Ferreira et al., 2011).

1.5.4. Quorum-Sensing (QS)

Quorum-sensing (QS) is a cell density-dependent regulatory mechanism used by bacteria to coordinate gene expression by the aid of diffusible self-produced signal molecules (Suppiger et al., 2013). The *Vibrio fischeri* autoinduction is the best-studied QS phenomena. The LuxI protein synthesizes an AHL signal (N-acyl homoserine lactone) while LuxR is an AHL receptor protein that activates or represses gene expression by binding to a consensus sequence (the so-called lux box) in the promoter regions of target genes (Suppiger et al., 2013). Cells are fully permeable to AHL, so during growth it accumulates in the medium until it reaches a sufficient concentration. At this critical point it triggers synthesis of specific luminescence enzymes, thus allowing cells to sense their own population density (Kolibachuk & Greenberg, 1993).

In Gram-negative bacteria, QS usually consists of a transcriptional regulator of the LuxR family that binds to AHLs synthesized by LuxI homologs and regulates the expression of target genes (Subramoni & Sokol, 2012). In the Bcc AHL production is strain-dependent with respect to both the quantity and the type of AHL molecules signal. However, within the Bcc the CepIR QS system is fully conserved. It consists of CepI which directs the synthesis of N-octanoyl-homoserine lactone (C8-HSL) and minor amounts of N-hexanoyl-homoserine lactone (C6-HSL), and the LuxR homolog CepR, which has the highest affinity for C8-HSL and can function as both a positive and negative regulator of target genes (Subramoni & Sokol, 2012; Suppiger et al., 2013).

Phenotypic assays as well as global transcript and protein analyses using *cepIR* mutant strains have shown that AHL-mediated QS controls various functions, including swarming motility, biofilm formation and the production of virulence factors, such as proteases, siderophores and toxins, which ensure that pathogenic traits are only expressed when the bacterial population density is high enough to overwhelm the host before it is able to mount an efficient response.

1.5.5.Motility and Chemotaxis

The main ways of movement in bacteria are through flagella (swimming or swarming), pili (twitching), or other mechanisms that occur in the absence of identified appendages, referred to as gliding (Alexandre, 2015).

Chemotaxis is the movement of an organism in response to a chemical stimulus allowing motile bacteria to rapidly escape conditions that limit growth by orienting their movement toward a more favorable niche. It controls flagellar motility by regulating the frequency at which the flagellar motor changes its direction of rotation or the speed at which the flagellar motor rotates. It also controls twitching, but the mechanisms involved are distinct from those controlling flagellum-dependent chemotaxis (Alexandre, 2015).

Bacteria swim by rotating of one or more rigid helical flagella. Flagella are extremely effective organelles of locomotion that permit bacteria to achieve speeds exceeding many cell body lengths per second. Cells are propelled forward when flagella turn counterclockwise, while changes in course are affected when one or more flagella turn clockwise. Swimming cells can perform chemotaxis by moving up or down chemical gradients, using an elaborate signaling system that modulates the counterclockwise/clockwise bias of the motors (Belas, 2014).

Swarming: is a bacterial flagella-dependent motile behavior that allows cells to move over surfaces in a coordinated manner and expand the population to new locations. The process of swarming is distinct from swimming in that swarming is a multicellular process that occurs on solid surfaces or in viscous liquids, and requires differentiation of a vegetative swimmer cell into a specialized cell type called a swarmer cell (Belas, 2014).

Besides the undisputable importance of flagella for motility, studies have linked flagella to the dissemination of bacteria from local infection sites to other organs, and to virulence traits as the adherence to and invasion of epithelial cells, the formation of biofilms and the induction of host inflammatory responses (Arora et al., 2000; Moens et al., 1996; Toole et al., 2000; Urban et al., 2004).

The flagellum can be subdivided into three substructures that are assembled in a temporal sequence. The first component to be assembled is the basal body, which anchors the flagellum to the cell membrane, provides the power for rotation, and secretes the more distal components. The next component is the hook, which is connected to the basal body and serves as a flexible universal joint changing the angle of flagellar rotation. The third structure is the helical filament, which is composed primarily of the protein flagellin, one of the most abundant proteins made by the cell (Belas, 2014).

Bcc bacteria are motile organisms that possess a single, long, polar flagellum responsible for swimming and swarming motility. Members of the Bcc express one of two types of flagellin that are distinguished by size (55 kDa for type I and 45 kDa for type II) and restriction fragment length polymorphism patterns of the *fliC* gene (Urban et al., 2004). Five types of pili were identified in Bcc strains: mesh (Msh), filamentous (Fil), spine (Spn), spike (Spk), and cable pili (Cbl). Although the cable pilus Cbl has an important role in the adhesion to epithelial cells (and is therefore thought to be of clinical relevance), only a few strains from the Bcc harbor the *cblA* gene coding for this pilus and not all *cblA*-positive strains produce the pilus (Suppiger et al., 2013).

Studies involving Bcc bacteria's CF lung infections revealed that isolates from the majority of chronic infections most commonly display the motile phenotype throughout the infection and do not normally

switch to nonmotile (Zlosnik et al., 2014). As mentioned, the production of functional flagella is associated with virulence in models of mucosal infection since it facilitates invasion into epithelial cells *invitro* and promotion of inflammatory markers (Urban et al., 2004; Zlosnik et al., 2014). Upon infection, the host flagellin is recognized by Toll-like receptors (TLR) and a signaling cascade is initiated, causing activation of nuclear factor B, as well as members of the mitogen-activated protein (MAP) kinase family. This pathway activation ultimately results in the transcription of a variety of host defense genes, including those for IL-8, IL-6, tumor necrosis factor and IL-1. In CF lung disease great amounts of Cytokines, specially IL-8, promote tissue damage and decreased pulmonary function, most profoundly through the recruitment of neutrophils (Lyczak et al., 2002; Mukaida, 2003). Neutrophil infiltration, in conjunction with other immune mediators, leads to high concentrations of neutrophil oxidants and enzymes in the lung environment, which stimulate mucus secretions, plugging airways and further damaging the lung. This cycle of damage results in a frustrated immune system that, despite a vigorous response, is unable to clear the infection (Urban et al., 2004)

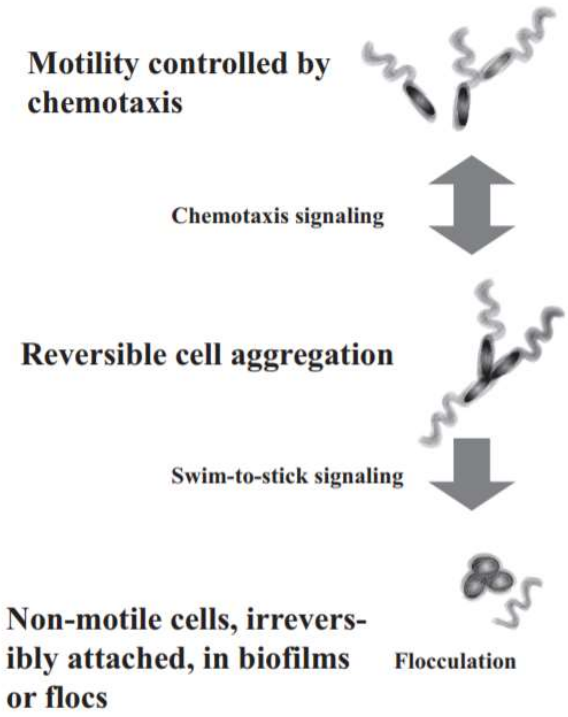


Figure 7. Chemotaxis signaling modulates motility patterns promoting transient cell-cell interactions. The double arrows indicate reversible events. The unidirectional arrows represent a committed transition to irreversible attached state. Retrieved from (Alexandre, 2015)

According to an increasing number of reports, motility is one of the factors contributing to the ability of bacteria to form biofilms or to flocculate. Biofilm formation starts with the transient adhesion of the cells to a surface, or other cells. This process is dependent of physicochemical interactions between the bacteria and the surface being strongly influenced by both hydrodynamic effects and Brownian motion (Conrad, 2012). If motile bacteria get sufficiently close to the surface, the hydrodynamic effect of the surface affects them resulting in the bacteria spending longer times swimming close to the surface (Vigeant et al., 2002). Weak adhesions though van der Waals, electrostatic, and hydrophobic interactions, are more likely to occur due to the prolonged residence near the surface (Conrad, 2012; Flemming & Wingender, 2010). Hydrodynamic trapping of swimming bacteria may thus promote transient cell-surface contacts.

A recent model predicts that for bacteria swimming in a very confined space, the activity of bacterial flagella alone can alter the hydrodynamic conditions to such an extent that it might trigger aggregation under these conditions (Cheng et al., 2014). The model, represented in Figure 7, proposes that the initial

weak adhesions might lead to irreversible attachment via mechanisms that likely involve sensing of surfaces, such as hindrance of flagellar rotation acting as a mechanosensing signal for the cell to deploy EPS and adhesins for permanent attachment. Increased cell density in cell aggregates might also trigger attachment via quorum sensing (Alexandre, 2015).

1.5.6.Extracellular DNA (eDNA)

It is widely accepted that microorganisms produce and release a great deal of DNA which is an important and abundant matrix component of many single- and multispecies cultured biofilms (Flemming & Wingender, 2010; Whitchurch et al., 2002). Recent studies further confirmed this hypothesis through the discovery of a filamentous net of DNA in the aggregate of an aquatic Gram-negative bacteria (Böckelmann et al., 2006; Böckelmann et al. 2007). The extracellular DNA (eDNA) involved in formation of bacterial aggregates is usually double-stranded and more or less similar to the whole-genome DNA of the bacteria within the aggregate, there is also evidence that DNA release occurs through cell lysis, or through lysis of released DNA-containing membrane vesicles (Allesen-holm et al., 2006). However, whether eDNA is circular or linear and what proteins, if any, are associated with the secondary structure and maintenance of the eDNA is still unknown (Steinberger & Holden, 2005).

eDNA present in biofilms seems to be important for several different function, such as surface attachment and biofilm strengthening, to help confer antibiotic resistance, to act as a nutrient source during starvation, to promote colony spreading and structuring and to serve as a gene pool for horizontal gene transfer (Chiang et al., 2013; Dominiak et al., 2011; Mann et al., 2009; Molin & Tolker-nielsen, 2003; Whitchurch et al., 2002).

Studies have shown that *P. aeruginosa* produces a large amount of (eDNA) during alginate biosynthesis (Whitchurch et al., 2002). The eDNA is generated via a mechanism which is dependent on acyl homoserine lactone and *Pseudomonas* quinolone signaling, as well as on flagella and type IV pili. In surface attached biofilms, eDNA is located primarily in the stalks of mushroom-shaped multicellular structures, with a high concentration especially in the outer part of the stalks forming a border between the stalk-forming bacteria and the cap forming bacteria (Allesen-holm et al., 2006; Böckelmann et al., 2007).

1.5.7.Cyclic di-GMP

First messengers receive signals at the cell surface relaying them to cytoplasmatic second messengers which in turn target molecules within the cell. This process results in biochemical changes in the cell through the amplification of the original signal into an intracellular signal. Cyclic di-GMP (c-di-GMP), is a cyclic diguanylate used by most bacteria to regulate a myriad of biological processes (Tamayo et al., 2007).

The synthesis of c-di-GMP from two molecules of GTP is catalyzed by diguanylate cyclases (DGCs), enzymes characterized by the presence of a GGDEF domain, whereas the breakdown of c-di-GMP is catalyzed by phosphodiesterases (PDEs), enzymes noted for possessing an EAL or an HD-GYP domain

(Kuchma et al., 2007). GGDEF, EAL, and HD-GYP domains are often fused with periplasmic or membrane-embedded ligand-binding sensory domains like PAS, REC, GAF and globin, that is involved in oxygen sensing. This indicates that these proteins could take part in sensing environmental cues, modulating its enzymatic activity to regulate the intracellular c-di-GMP level (Romling et al., 2013).

Through the binding to the effectors, c-di-GMP directly influences the phenotypes of cells. The production of adhesins and extracellular matrix components is induced by high internal levels of c-di-GMP, as well as the inhibition of flagellar motility, enabling the formation of biofilms and aggregates. On the other hand, if levels are low the planktonic growth mode is privileged (Fazli et al., 2014). This interaction occurs through the association with proteins with c-di-GMP binding motifs, with proteins that do not have known c-di-GMP binding motifs or through the association with RNA molecule. So far, several classes of c-di-GMP receptors have been identified; PilZ domain receptors, I-site receptors (modified GGDEFF domain), inactive EAL domain receptors, and likely HD-GYP domain receptors (Romling et al., 2013). This regulatory pathway is represented in Figure 8.

In *P. aeruginosa*, c-di-GMP-dependent signaling is part of a regulatory network controlling surface associated group behaviors, including swarming motility and biofilm formation (Bernier et al., 2011; Kuchma et al., 2007; Merritt et al., 2010). A simple increase in the intracellular c-di-GMP level facilitates biofilm formation or the sessile mode of life, whereas a decrease facilitates the dispersal or planktonic mode of life (Romling et al., 2013). However, *P. aeruginosa* encodes close to 40 DGCs and PDEs that are predicted to function in the synthesis and/or degradation of c-di-GMP and there seems to exist an apparent disconnection between the total levels of c-di-GMP and the resulting phenotypes, at least for the early biofilm formation events (Merritt et al., 2010). Although, under some circumstances, the total level of c-di-GMP may act as a control mechanism, Christen et al. obtained date that indicate the possible existence of subcellular pools of c-di-GMP (Christen et al., 2010). Such pools could be produced by the specific localization and/or localized activation of DGCs, through the action of proteins that efficiently bind and/or degrade c-di-GMP this way limiting diffusion of c-di-GMP, or by the different availability or activity of c-di-GMP receptors that are specific for a particular phenotype (Hengge, 2009).

There is also evidence that the c-di-GMP signaling system is involved in the regulation of biofilm formation in the Bcc bacteria and Fazli et al. have identified a c-di-GMP responsive transcriptional regulator. Binding of c-di-GMP to this regulator, Bcam1349, enhances the protein's affinity for the promoter DNA region of Bcam1330–Bcam1341 gene cluster which regulates expression of cellulose, fimbriae biosynthesis and exopolysaccharide production promoting biofilm formation (Fazli et al., 2011; Fazli et al., 2013). Fazlil et. al also demonstrated that the enhancer-binding protein BerB binds to c-di-GMP and activates the alternative sigma factor RpoN -dependent transcription of the *berA* gene encoding a c-di-GMP responsive transcriptional regulator. In response, BerA protein production increases and induces production of Bep exopolysaccharide that stabilizes biofilms (Fazli et al., 2017).

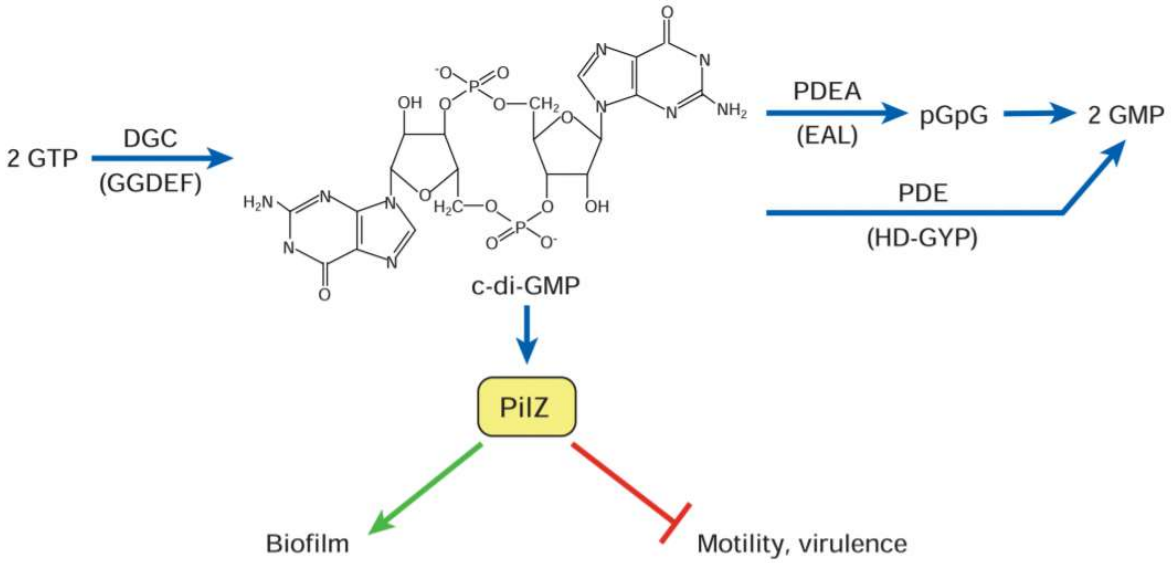


Figure 8. c-di-GMP regulatory pathway. c-di-GMP is synthesized from two GTPs by GGDEF domain DGCs. c-di-GMP is hydrolyzed by EAL domain PDEAs into the linear pGpG before being hydrolyzed by other PDEs into two GMPs. HD-GYP domain PDEs hydrolyze c-di-GMP completely into two GMPs. Numerous studies have shown that c-di-GMP activates EPS production and biofilm formation but inhibits motility and virulence. These processes are regulated by c-di-GMP through a variety of pathways, as discussed in the text. PilZ domain proteins are one class of c-di-GMP sensor, although others likely exist. The mechanism(s) by which PilZ domain proteins transduce changes in c-di-GMP into downstream physiological effects is unknown. Retrieved from (Tamayo et al., 2007)

In Bcc bacteria, the bceF gene encodes a BY-kinase primarily involved in cepacian exopolysaccharide biosynthesis. Ferreira et al. studied the phenotypic effects of the inactivation of *bceF* on a strain of *B. cepacia (ΔbceF),* and found it affected exopolysaccharide production, resistance to stress, motility, biofilm formation, and virulence. Despite the impaired biofilm formation abilities, *ΔbceF* had higher levels of cyclic-di-GMP. However, it must be considered that the level of cyclic-di-GMP and the transcriptional control dependent on this secondary messenger are not the only factors for biofilm formation. In fact, the inactivation of the *bceF* gene had a remarkable effect on the number of genes differentially expressed (Ferreira et al., 2013).

1.5.8.Amino Acids

Amino acids are abundant in the CF sputum (Palmer et al., 2005) and recent studies indicate they could represent a key factor for *P. aeruginosa* growth in this environment (Palmer et al., 2010; Palmeret al., 2007). Bernier et al. identified seven amino acids (arginine, ornithine, isoleucine, leucine, valine, phenylalanine, and tyrosine) which increased the extent of biofilm formed on an abiotic substratum compared to glucose (Bernier et al., 2011). This increase could result from the additional source of nitrogen, in addition to the ammonia provided in the medium, by the amino acids. However, the contribution of this nitrogen to the biofilm phenotype is not yet clear (Bernier et al., 2011). On the other hand, proline seems to promote planktonic growth, (Bernier et al., 2011) while glutamate is known to induce biofilm dispersion (Sauer et al., 2004).

Arginine and pyruvate are the only two known substrates to be fermented by *P. aeruginosa* (Eschbach et al., 2004). Therefore, considering that the CF lung might represent an anoxic or microaerophilic environment (Alvarez-Ortega & Harwood, 2007) and the oxygen-limiting conditions within bacteria aggregates, growth in the presence of this amino acid allows the generation of ATP via substrate level phosphorylation.

D-Amino acids are produced by several bacteria in stationary phase (Lam et al., 2009) and are generated by racemases (Yoshimura & Esaki, 2003). Kolodkin-Gal et al. found that *P. aeuriginosa's* mutants on gene *ylmE* or *racX*, whose predicted products exhibit sequence similarity to known racemases, alone showed a modest delay in biofilm disassembly. However, double mutant cells for the putative racemases were substantially delayed in disassembly. Cells engineered to overexpress *ylmE* were blocked in biofilm formation, further attesting the hypothesis (Kolodkin-Gal et al., 2010).

On the same study, medium containing D-amino acids was shown to have the ability to prevent biofilm formation, but also to disrupt already established biofilms. Indeed, D-tyrosine and a D-amino acid mixture, were active in inhibiting biofilm formation in a liquid medium, as well as on a solid medium (Kolodkin-Gal et al., 2010). The fact that this effect is prevented by D-alanine, seems to indicate it results from the incorporation of D-amino acids into the peptide side chains of peptidoglycan in place of the terminal D-alanin. TasA fibers, precursors for amyloid-like fibers, are anchored to the cell wall. The incorporation of D-amino acids into the cell wall might disengage the fibers from their anchor, resulting in the observed disassembly of biofilm structures (Kolodkin-Gal et al., 2010).

Given that many bacteria produce D-amino acids, these amino acids may provide a general strategy for biofilm disassembly. If so, then D-amino acids might prove widely useful in medical and industrial applications for the prevention or eradication of biofilms (Kolodkin-Gal et al., 2010).

1.5.9.sRNA

Small noncoding RNAs (sRNAs) bind with proteins, or other RNA molecules exerting regulatory functions. Since Romeo et al. discovered the RNA-binding proteins CsrA and sRNA CsrB and found their roles in *E. coli* biofilm formation and dispersal, sRNA have been found to have an important role in the bacterial regulatory networks controlling the switch between the planktonic and the biofilm mode (Romeo et al., 1993)

One well-studied example of such control is *P. aeruginosa* GAC signal-transduction network, represented in Figure 9. GacS is a transmembrane sensor kinase, which induces the phosphorylation of its cognate regulator, GacA, which can also be phosphorylated by sensor kinase LadS and blocked by RetS. Phosphorylated GacA stimulates the production of two sRNAs: RsmY and RsmZ, which have multiple binding sites for the regulatory protein RsmA. As a translational repressor, RsmA binds to the target mRNAs to promote bacterial motility and repress biofilm formation. Sequestration of RsmA by sRNAs RsmY and RsmZ switches planktonic mode to biofilm formation (Kay et al., 2006).

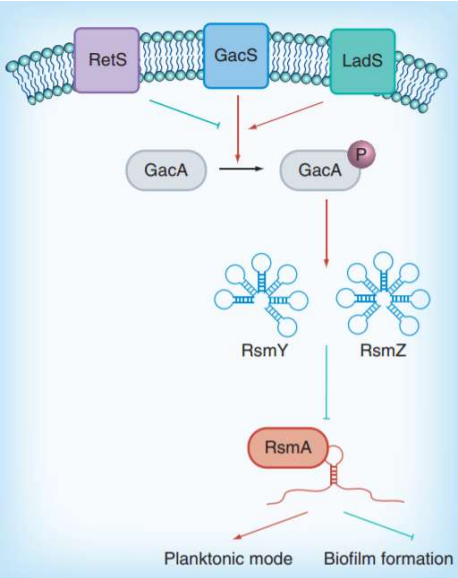


Figure 9. Pseudomonas aeruginosa GAC regulatory network. Phosphorylation of regulatory protein GacA by GacS and LadS but inhibited by RetS. Phosphorylated GacA promotes the production of RsmY and RsmZ. that sequester RsmA and block its mRNA-binding function. RsmA would prevent mRNA, translation and increased cell motility with decreased biofilm formation.

1.5.10. Stress Factors

The intrinsic properties of pathogens alone, or the existence of an innate immune defect on CF patients, does not account for the typical antimicrobial resistance and chronic nature of CF infections. In fact, it would seem that conditions produced by the basic CF defect somehow induce these infection phenotypes. Several studies suggest that biofilm formation could be responsible for the phenotypes associated with chronic infections (Staudinger et al., 2014).

Within the CF lung, colonizing bacteria face an environment with high osmolarity, heterogeneous distribution of oxygen and nutrients, high concentration of antimicrobials, and constant challenge by the host immune defenses. These factors exert a selective pressure in colonizing bacteria and are thought to be the driving force of microevolution in pathogens (Silva et al.,2013). As a result of inflammation, bacteria are exposed to high levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are generated primarily by neutrophils as part of the host's innate immune response. ROS and RNS contribute to mutations that confer an adaptive advantage to bacteria in CF patient's airway (Ciofu et al., 2005). Consistent with this notion, ROS and RNS react robustly with biomolecules including DNA, protein and lipids. DNA reactions result in pro-mutagenic and cytotoxic DNA lesions. Pro-mutagenic lesions are damaged DNA bases that can make non-Watson–Crick base pairs, resulting in nucleotide misinsertions during replication. If unrepaired, mutations result in the ensuing replication phase (Hassett et al., 2009)

Genetic analysis of *P. aeruginosa* isolates from independent CF patients revealed that mutations such as loss-of-function in the *lasR* gene are common targets of mutation. These include adaptations that make *P. aeruginosa* exhibit a less aggressive form of life. This happens due to attenuation in the expression of virulence factors, immunostimulatory products such as flagella and LPS and the initiation of a biofilm mode of growth. Chronically persisting *P. aeruginosa* undergoes metabolical adaptation to the aerobic microenvironment found within mucin plugs and consequently upregulate metabolic

enzymes and metabolite transporters for anaerobic survival. Increased levels of OprF expression may indicate improved NO_3^-/NO_2^- utilization. Although several adaptive changes are conserved, there remains extensive *P. aeruginosa* phenotypic diversity, as demonstrated by the comparison of multiple isogenic isolates recovered from single CF patients (Hogardt & Heesemann, 2010).

Studies have shown that Bcc nonmucoid isolates form more biofilm than the mucoid isolates and have virulence attenuation in animal models of infection. This colony morphotype variation in bacteria reflects their adaptation to the surrounding environment, whether it occurs by phase variation or by irreversible mutational events. Since phase variation is a spontaneous and reversible process, it is likely that the mucoid-to-nonmucoid switch occurring in *Burkholderia* exposed to stress conditions results from irreversible mutations (Silva et al., 2013).

1.5.10.1. Antimicrobials

One of the most significant aspects of the biofilm growth mode is that microorganisms escape the host immune response and are much less susceptible to antibiotics than are their planktonically grown counterparts.

Several hypotheses explain the antibiotic tolerance of bacterial biofilms including physical barriers to antibiotic penetrance from extracellular matrix that biofilms produce (Pugliese & Favero, 2002; Stewart, 1996), the production of periplasmic glucans (Mah et al., 2003), slow growth (Anwar et al., 1992) and/or the presence of metabolically inactive persister cells within a biofilm that are inherently tolerant to antibiotics (Lewis, 2005).

Efflux pumps allow bacteria to pump intracellular toxins out, including antibiotic drugs. Although they are also expressed in planktonic cells, some efflux pump genes are upregulated in biofilm, indicating that they contribute to its increased antibiotic resistance. Zhang et al. identified a novel *P. aeruginosa* efflux pump gene PA1874–1877 which confers resistance to tobramycin, gentamicin and ciprofloxacin and whose expression level is much higher in biofilms than in planktonic cells (Zhang & Mah, 2008).

Inside biofilms there is limited availability of oxygen and nutrients, therefore cells, especially those in the deep layers, have a slow metabolic rate, as well as growth rate and division rate. These features make biofilm bacteria more insensitive to antibiotic drugs, especially those that target dividing cells (Ashby et al., 1994). Within biofilms there is also a small subpopulation of cells called persister cells whose growth rate is zero or extremely close to zero. Most of the antibiotics that are currently used, which target processes which are relevant for cell growth or division, are not effective against persister cells. Therefore, these cells act as disease reservoirs that could reactivate into infectious particles once the antibiotic stress has been removed(Lewis, 2007).

For planktonic cells, suspension with an antimicrobial agent rapidly exposes all cells to its full dose. Conversely, the rate of transport of antimicrobial agents into biofilms is important. Considering that the rate of antibiotic penetration through a biofilm is decreased in comparison to the rate of transport through a liquid, then the bacteria may be exposed to a gradually increasing dose of the antibiotic and may have

time to mount a defensive response to the agent (Stewart, 1996 ; Jefferson et al., 2005). Experimental data suggests that this retardation is often compound and/or biofilm specific. This is clearly illustrated by the penetration of antimicrobial agents into the alginate-containing *P. aeruginosa* biofilm. Aminoglycoside antibiotics, such as gentamicin and tobramycin, bind to alginate making it's penetration considerably slower than that of β-lactam antibiotics (Gordon et al., 1988). In addition, the presence of inactivating compounds can greatly influence the effective penetration; for example, extracellular β-lactamase activity can have a dramatic effect on the penetration of penicillins in *P. aeruginosa* biofilms (Bagge et al., 2004).

Studies on *B. multivorans*, *B. cenocepacia* and *B. dolosa* strains have shown that for the β-lactams meropenem and piperacillin-tazobactam the minimal concentrations required to inhibit biofilms (minimal biofilm inhibitory concentration (MBIC)) were considerably higher than the minimum inhibitory concentrations (MICs) for planktonic cells (Caraher et al., 2006). The combination of multiple antibiotics to increase the *in vitro* effect against Bcc biofilm isolates is not straightforward. A study by Dales et al. showed that 59% of 47 Bcc isolates recovered from CF patients were resistant to all 27 double antibiotic combinations tested, while 18% of all isolates were resistant to all 67 triple antibiotic combinations. The most effective double antibiotic combination against Bcc biofilms was meropenem combined with high-dose (200 μg/ ml) tobramycin (35% of all isolates were inhibited), while the most effective triple antibiotic combination contained meropenem, piperacillin tazobactam and high-dose tobramycin (53% of all isolates were inhibited) (Dales et al., 2009).

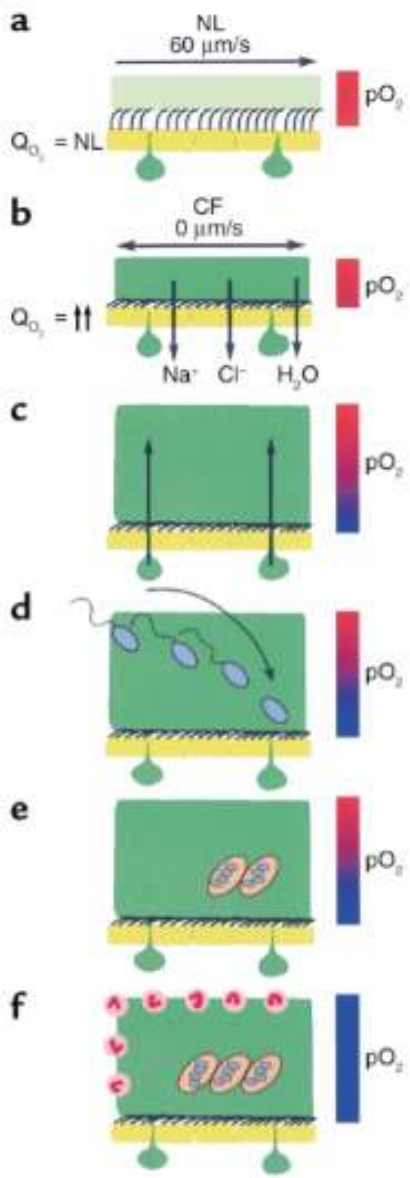
Treatments with acetic acid, chlorhexidine, hydrogen peroxide and NaOCl do not result in the eradication of Bcc biofilms. This reduced susceptibility to oxidizing disinfectants not only has implications for infection control but, as these oxidative agents are being produced by neutrophils as part of the endogenous defense against microorganisms (Saldías & Valvano, 2009), may also have implications for pathogenesis. Reduced activity of a cetylpyridinium chloride nanoemulsion against Bcc biofilms has also been reported and the median increase in MBIC and minimum biofilm eradicating concentration compared with the respective MICs and minimum bactericidal concentrations for the six strains tested was 12-fold and 11-fold, respectively (Lipuma et al., 2008).

As it was mentioned before, Bcc nonmucoid clinical isolates form more biofilm than the mucoid isolates and have virulence attenuation in animal models of infection. Since CF patients are often exposed to antibiotic therapy, the presence of antibiotics of clinical relevance could be a factor triggering the mucoid morphotype variation. A study by Zlosnik et al. found that for two *Burkholderia* clinical isolates, the presence of ciprofloxacin and ceftazidime could induce a mucoid to nonmucoid phenotypic switch, therefore privileging aggregate formation. In addition to this antibiotics (Zlosnik et al., 2011). Silva et al. have also found that amikacin and kanamycin trigger the nonmucoid morphotype variation of *B. multivorans* D2095, and ciprofloxacin induced mucoid-to-nonmucoid morphotype variation in three other Bcc isolates from different species. These observations raise the question on whether antimicrobial therapy, by possibly triggering morphotype variation within the CF lung, contributes to disease progression (Silva et al., 2013).

1.5.10.2. Oxygen and Nitrate

Given that CF patients suffer from pulmonary insufficiency, it is highly likely that oxygen tension plays a part in the stability of the genomic content of infecting strains, as well as the metabolic behavior of bacteria within the airway mucus.

Studies have shown that *P. aeruginosa* preferentially binds to mucus rather than epithelial cell surfaces Compared with a normal airway epithelial function CF airway epithelia excessively absorb Na^+, Cl^- and water from the lumen, depleting the periciliary liquid layer, having impaired mucus clearance (Jiang et al., 1993). Accelerated Na+ absorption is fueled by an increased turnover rate of ATP-consuming Na+-K^+-ATPase pumps leading to a two- to threefold increases in CF airway epithelial O_2 consumption (Peckham et al., 1997).The persistent mucin secretion into stationary mucus generates plaques/plugs. The combination of thickened mucus and raised O_2 consumption by CF epithelia generates steep O_2 gradients within adherent mucus. Bacteria deposited on thickened mucus have the ability to penetrate into hypoxic zones (Worlitzsch et al., 2002).



P. aeruginosa's response to hypoxia stress seems to involve increased alginate production which seems to be part of the process that forms planktonic aggregates, the predominant phenotype of *P. aeruginosa* in CF airways. Interestingly, *Staphylococcus aureus* also responds to the hypoxic environment of CF mucus with a switch from nonmucoid to a mucoid phenotype (Cramton et al., 2001). *P. aeruginosa*'s capacity to proliferate in hypoxic mucus further generates a fully anaerobic condition in patients with persistent CF airways infection, and alginate production is maintained by anaerobic conditions (Hassett et al., 2009). The reduced O_2 tension in the

Figure 10. Schematic model of the pathogenic events hypothesized to lead to chronic P. aeruginosa infection in airways of CF patients (a) On normal airway epithelia, a thin mucus layer (light green) resides atop the PCL (clear). A normal rate of epithelial O_2 consumption produces no O_2 gradients within (denoted by red bar). (b–f) CF airway epithelia. (b) mucus becomes adherent to epithelial surfaces, and mucus transport slows/stops. (c) Persistent mucus hypersecretion (denoted as mucus secretory gland cell units; dark green) with time increases the height of luminal mucus plugs, generating steep hypoxic gradients (blue color in bar) (d) P. aeruginosa bacteria deposited on mucus surfaces penetrate actively and/or passively into hypoxic zones within the mucus masses. (e) P. aeruginosa adapts to hypoxic niches within mucus masses with increased alginate production and biofilm formation (f) Aggregates resist secondary defenses, including neutrophils, rendering the now mucopurulent mass hypoxic (blue bar). Retrieved from (Worlitzsch et al., 2002)

mucopurulent intraluminal contents of CF airways may, therefore, be one variable contributing to the persistence of *P. aeruginosa* aggregates in CF airways, as represented in Figure 10.

A study by Yoon et al. found that the titers of outer membrane protein OprF antibodies were nearly identical to titers produced in *in vitro* anaerobic biofilms, while Son et al. measured *P. aeruginosa's* clinical isolates gene expression and found that a large number of upregulated genes were involved in anaerobic respiration (Son et al., 2007; Yoon et al., 2002). These studies further confirm that *P. aeruginosa* growing as biofilms during chronic infection in the CF lung are subjected to anaerobic conditions, thus biofilm formation in the CF airways is dynamically different to that of surface attached biofilms reminiscent of catheter, ventilator or stint biofilms from a genetic, physiological and morphological standpoint (Hassett et al., 2009).

Under conditions of oxygen limitation, growth of *P. aerguinosa* is predominantly driven by denitrification, in which nitrate or nitrite serve as the terminal electron acceptors through the action of four reductases (Petrova et al., 2012). The activation/up-regulation of the components of the denitrification pathway has been repeatedly observed within *in vitro* biofilms and during persistent *P. aeruginosa* infections, with sensing and processing of nitrate and other intermediate forms playing an essential role in the establishment, maintenance, resistance, and dispersal of biofilms *in vitro* and *in vivo* (Alvarez-Ortega & Harwood, 2007; Hassett et al., 2009).

1.5.10.3. Pyruvate

P. aeruginosa was shown to be capable of utilizing pyruvate fermentation for survival under conditions of oxygen limitation in the absence of nitrite and nitrate (Eschbach et al., 2004). The process involves the conversion of pyruvate to lactate, acetate, and/or succinate, with experimental evidence suggesting that the lactate and acetate producing branches of the pathway are predominant. Inactivation of lactate dehydrogenase (LdhA), which converts pyruvate to lactate and regenerates NAD^+, severely impairs pyruvate fermentation and compromises survival on pyruvate under conditions of electron abundance (Eschbach et al., 2004). Consistent with a role of pyruvate fermentation as a microcolony-specific adaptation mechanism, an *ldhA* mutant was impaired in biofilm microcolony formation with exogenous nitrate or pyruvate being unable to restore microcolony formation (Petrova et al., 2012).

Studies of *P. aeruginosa* growing under anaerobic, aerobic and oxygen-limiting conditions, strongly suggest that pyruvate is used as a means of redox balancing. Consistent with a role of LdhA in regenerating reducing equivalents under oxygen-limiting conditions, expression of LdhA in *P. aeruginosa* wild-type (Wt) correlated with a significant increase in biofilm biomass accumulation and microcolony formation and decreased NADH/NAD^+ ratios (Petrova et al., 2012).

A recent study by Goodwine et al. demonstrated that pyruvate depleting conditions coincided with loss of the *P. aeruginosa* biofilm biomass and that loss was due to dispersion. More specifically, the dispersion seems to be due to impaired maintenance of redox balance and cellular homeostasis, induced by pyruvate deprivation (Goodwine et al., 2019). It had been demonstrated that *P. aeruginosa*

produces and secretes pyruvate under favorable conditions but to utilize pyruvate under anoxic conditions to sustain survival (Petrova et al., 2012). It is likely that *Pseudomonas aeruginosa* biofIm cells localized at the periphery and interstitial anaerobic regions of biofilm microcolonies produce and secrete pyruvate that not only diffuses out into the surrounding environment but also toward the central anoxic core of microcolonies. Overall, targeting pyruvate availability potentiates the efficacy of antimicrobial agents in killing biofilm cells and thus, improve the ability to eradicate persistent biofilm infections (Goodwine et al., 2019).

A study by Silva et al. implicated the D-lactate dehydrogenase, LdhA, in the formation of planktonic cellular aggregates and biofilms by *B. multivorans,* indicating that pyruvate fermentation could also be a relevant adaptation for this strain within CF patient's lungs (Silva et al., 2017b).

1.5.10.4. Iron

Iron, which is the most important metal for living organisms, in catalytic form can trigger the Fenton chain reaction and amplify the generation of ROS with deleterious effects to cells, contributing heavily to subsequent clinical complications in the lungs of CF patients. Reid et al. have demonstrated the existence of a significant relationship between the increase of sputum iron content and the decrease of lung function (Reid et al., 2002). It is important to underline that in healthy humans the lower respiratory tract, as well as all mucosa, contains a very low free iron concentration (10^{-18} M) as this element is bound predominantly to iron-chelating proteins, as transferrin and lactoferrin (Weinberg, 1978). This iron concentration is very far from those inducing significant ROS formation, and to that which is required by bacteria to grow and survive (10^{-6} M). Conversely, the iron concentration in CF patient sputum is very high, showing a median value of $63x10^{-6}$ M and ranging between 17 to $134x10^{-6}$ M (Reid et al., 2002; Stites et al., 1998).

Lactoferrin, a cationic glycoprotein able to chelate two Fe^{3+} ions per molecule with high affinity, is synthesized by exocrine glands and neutrophils in infection and inflammation sites (Valenti & Antonini, 2005). In the CF airway secretions, the high concentrations of proinflammatory cytokines, including Interleukin-8, recruit neutrophils which synthesize and secrete lactoferrin, which as a consequence, is found at higher concentrations in airway secretion of CF patients than in healthy humans (up to 0.1 and 0.01 mg/ml, respectively) (Sagel et al., 2009). Lactoferrin exerts multiple antimicrobial functions both dependent on and independent of its iron-withholding ability. Although the mechanism of action is not fully elucidated, lactoferrin also demonstrates anti-inflammatory activity, which contributes to protection of the mucosa from inflammation-related damage (Valenti et al., 2011).

It is well known that high iron availability increases bacterial virulence and promotes bacterial growth (Schaible & Kaufmann, 2004). Moreover, *P. aeruginosa* and Bcc bacteria, similarly to other aerobic bacteria, are able to acquire Fe^{3+} through various mechanisms including: i) the synthesis of proteases to degrade host Fe-binding proteins leading the iron release; ii) reduction of insoluble Fe" to soluble Fe^{2+} by membrane reductases; iii) production of siderophores, low-molecular iron-binding compounds, which compete with transferrins for iron acquisition (Berlutti et al., 2005).

The presence of siderophores in CF patient sputa has been well documented by Haas et al., indicating that the iron overload in CF patient airways could enhance the colonization and persistence of *P. aeruginosa* and *Burkholderia* even if subjected to aggressive and long-term antibiotic therapy (Haas et al., 1991). Conversely, Singh et al. have demonstrated that the iron deficiency, induced *in vitro* by apo-lactoferrin as iron chelating molecule, decreased microbial virulence by inhibiting the *P. aeruginosa* PAO1 biofilm formation and development. In fact, under iron-stress condition, *P. aeruginosa* PAO1 is highly motile with a specialized form of motility, named switching motility, activated. Consequently, the bacterium disperse on the surface instead of adhering, so there is no biofilm formed. On the other hand, 100% iron-saturated lactoferrin did not inhibit the formation of biofilm (Singh et al., 2002). In fact, *P. aeruginosa* PAO1 and *B. cenocepacia* PV1 cultured under low-iron availability (1μM Fe^{3+}) were in free-living form while under iron-loaded (10 and 100 μM Fe^{3+}) abundant aggregates evolving in biofilm were observed (Berlutti et al., 2005). *P. aeruginosa* mutants inactivated in the high affinity pyoverdine iron acquisition system only form thin unstructured biofilms even when grown in iron-sufficient medium, thus further demonstrating that iron controls microcolony formation (Banin et al., 2005). Coenye et al. also showed that physiological concentrations of the iron-binding glycoprotein lactoferrin inhibited the growth of planktonic and sessile Bcc cells, and that the biofilms formed in the presence of lactoferrin contained significantly less biomass than untreated biofilms after 24 h. However, several Bcc strains were capable of overcoming this antibiofilm effect and at 48 h treated biofilms were very similar to untreated ones (Coenye, 2010)

1.5.10.5. Mannitol

A subset of strains of *P. aeruginosa* (PAO1 and FRD) grown as biofilms on an abiotic surface (plastic) were approximately 1000-fold more sensitive to tobramycin treatment with co-administration of mannitol and tobramycin (Barraud, et al., 2013). However, an additional clinical isolate remained resistant to tobramycin, even with co-administration of mannitol and tobramycin, suggesting that tobramycin sensitization by mannitol is strain specific and is not generalizable (Price et al., 2015).

Price et al. used an *in vitro* model system of bacterial biofilm formation on CF airway cells in order to investigate the relevance of mannitol and tobramycin synergy to the CF patient population. *P. aeruginosa* grown in this model system recapitulates several key aspects of chronic biofilm formation, including microcolonies formation, expression of genes associated with biofilm growth, induction of quorum sensing, requirement for genes necessary for biofilm formation on abiotic surfaces and, of clinical importance, high-level antibiotic tolerance consistent with biofilms in clinical settings (Anderson et al., 2008; Moreau-Marquis et al., 2008). All the strains tested (PA14, PAO1, mucoid clinical isolate FRD1, and five other clinical strains) did not present increased sensibility to the co-administration of mannitol and tobramycin. Mannitol is currently used as a therapeutic that hydrates mucus allowing for increased mucociliary and cough clearance of retained secretions in the airways of individuals with CF (Bilton et al., 2013). The model system does not produce mucus, therefore the effect that mucus viscosity/ hydration has on tobramycin efficacy was not tested. Thus, is formally possible that better hydration of the airways may allow better penetrance and consequently better effectiveness of

antibiotics. However, in the clinical trial, it was not observed any change in the *P. aeruginosa* load in patients given mannitol versus placebo control despite maintaining their current CF therapies including inhaled antibiotics (Bilton et al., 2013), supporting the idea that tobramycin and mannitol do not synergize in the treatment *of P. aeruginosa* biofilms formed on airway cells.

Mannitol is one of several sugars and sugar alcohols that can induce the overproduction of EPS by members of the Bcc, resulting in a extreme mucoid phenotype (Bartholdson et al., 2008). Since colonization with BCC was amongst the exclusion criteria for participation in the mannitol powder clinical trials, the impact of this osmolyte therapy on the course of infection in *Burkholderia*-infected patients has yet to be determined.

Irrespective of the role played by EPS during Bcc infection of the CF lung, it is now apparent that growth on mannitol rich medium can have EPS-independent effects on *B. multivorans* that impacts virulence-associated traits. A comparison of isogenic EPS-proficient and EPS-deficient strains revealed that the adherence of a clinical isolate of *B. multivorans* to mucin was enhanced following growth in mannitol rich medium. This enhanced adherence was independent of EPS production and was instead associated with the upregulation of newly identified fimbrial and afimbrial adhesins (Denman & Brown, 2013).

A study by Denman *et al*. found that growth of *B. multivorans* in the mannitol rich medium has a profound effect on the transcriptome and resulting phenotype of the Bcc bacteria, influencing biofilm formation and resistance, epithelial cell invasion, motility, and virulence in the *Galleria mellonella* infection model. This phenotypic response appears largely independent of EPS production (Denman et al., 2014).

1.5.11. *Burkholderia* multivorans planktonic aggregates

Not much is known regarding the formation of planktonic aggregates by Bcc bacteria. However, Silva *et al.* have shown that both environmental and clinical isolates of various Bcc species form planktonic aggregates in salts media with different carbon sources like fructose, glucose or mannitol (Silva et al., 2017).

The most recent work concerning *Burkholderia multivorans* planktonic aggregates was done by Ferreira (2018), where screening of a genetic library created through random insertional mutagenesis resulted on a selection of a number of mutants presenting different aggregation phenotypes. The identification of the disrupted genes resulted in several different results with no apparent connection between genetic mechanisms and phenotypes identified. However, off the mutants selected, some are noteworthy, namely mutant G38. G38 presented a reduced ability to produce macroscopic aggregates when compared to its ancestor, also displaying decreased swarming motility, exopolysasccharide production, and growth rate in SCFM. The *gltB* gene encodes a glutamate synthase whose malfunction might cause accumulation of glutamine and subsequent inactivation of the NtrBC signaling system, which is known to regulate swarming motility. The mutant E70 displayed a lower capacity to form aggregates and presented a disruption on the *ppsA* gene, which encodes a phosphoenolpyruvate synthase that converts

pyruvate into phosphoenolpyruvate and is involved in formation AHL in *B. cenocepacia*. B74 had *rpsI* gene interrupted, which encodes a ribosomal protein, S9, involved in 30S subunit assembly being therefore involved in translation. One mutant, which produced large planktonic aggregates, but nevertheless displayed decreased virulence and capacity to produce surface attached biofilms, presented a disruption of the gene that encodes a xanthine dehydrogenase molybdenum-binding subunit XdhA, which is an enzyme required for purine salvage pathways, which is important for energy and GMP/GTP maintenance, and subsequently, c-di-GMP. Purine is also essential for the synthesis of components which include it in its structure, such as DNA, RNA, cofactors, signaling molecules and carriers of energy which integrate purine in its structure. Taking this into account, Ferreira (2018) suggested that c-di-GMP could possibly be involved in a key in mechanism in the different formation of the two types of biofilms. Furthermore, ribosomal proteins, quorum-sensing control and cell membrane constitution may contribute for aggregate formation, as well as proteins involved in nitrogen and lipid metabolism (Ferreira, 2018).

Gomes (2018) tested the impact of different stresses on a clinical isolate ability to aggregate, while most stresses seem to induce the aggregate phenotype, osmotic stress and nitrogen excess impact seems to be strain specific. Ciprofloxacin was found to induce the formation of planktonic aggregates on isolates that previously did not display this phenotype and resulted on a great increase on aggregate biomass percentage (Gomes, 2018).

Taking into account previous works concerning the LdhA enzyme, its impact on the aggregation phenotype was tested. For that purpose an *ldhA* mutant of a clinical isolate presenting the aggregation phenotype was constructed and its ability to aggregate evaluated. Compared to the wild-type it showed a general decrease in aggregation, while the overexpression of LdhA resulted in a strong increase, further confirming its role on aggregation (Gomes, 2018).

An evolution assay of 30 days was performed using a clinical isolate which was previously shown not to present the ability to aggregate. During the assay the phenotype changed and the genome of the colony obtained from the last time point was sequenced, and the acquired mutations were analyzed. Mutations in the genes *amiC*, *nlpD*, and *flp* were considered relevant. The first encodes a peptidoglycan hydrolase involved in peptidoglycan degradation which has a role in septum cleaving during cell division, the second encodes for a lipoprotein, NlpD, which anchors to the membrane and interacts with the peptidoglycan, also influencing cell division by modulating AmiC, while the third one is related to the expression of adhesive Flp pili, possibly leading to alteration of pili production and higher cell adhesion. These results seem to indicate that cell division and adhesion play key roles in planktonic aggregation of *B. multivorans* (Gomes, 2018).

1.6. Aims of this Thesis

CF patient's infection with Bcc is generally chronic and correlates with poorer prognosis, longer hospital stays and an increased mortality. Recent studies suggest that opposed to harboring bacteria firmly attached to a surface, chronic infections can be characterized by aggregates suspended within host

tissue or lumen (Bjarnsholt et al., 2009). These aggregates share the same hallmarks as biofilm growth, with some classifying them as such, which consists of a more efficient resistance to antibiotic's action while conferring them a greater protection from the immune response of the host. Therefore, a promising way to enhance antibiotic therapy or to uncover novel treatment options for Bcc chronic infections is to understand the genetic adaptation involved in the formation of planktonic aggregates.

The aim of this work is to study mechanisms responsible for the formation of cellular aggregates in *Burkholderia multivorans*. The approach taken makes use of a cystic fibrosis isolate able to form large cellular aggregates to construct a transposon mutant library, followed by screening to identify mutants with reduced ability to form cellular aggregates. After identification of mutated genes, the effect of each mutation not only in cellular aggregation, but also in other phenotypes such as: motility, exopolysaccharide production, antibiotic susceptibility and growth rates, will be characterized. The objective is to understand not only the genes involved in cellular aggregates formation, but also, it's possible impact on other phenotypes. An aggregation kinetics assay will also be performed in order to allow a better understanding of the processes enabling aggregate formation.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in table 2. The *E. coli* strain were grown at 37ºC in Lennox Broth (LB) with or without agar, supplemented with kanamycin (50 µg/ml) or chloramphenicol (25 µg/ml) when required to maintain selective pressure. *Burkholderia multivorans* P0213-1, a clinical isolate from a cystic fibrosis patient (patient P0213), and its derivative strains were grown in LB or in SM medium (12.5 g/l $Na_2HPO_4.2H_2O$, 3 g/l KH_2PO_4, 1 g/l K_2SO_4, 1 g/l NaCl, 0.2 g/l $MgSO_4.7H_2O$, 0.01 g/l $CaCl_2.2H_2O$, 0.001 g/l $FeSO_4.7H_2O$, 1 g/l yeast extract, 1 g/l casamino acids, pH 7.2), supplemented with 20 g/l of D-mannitol with or without agar, supplemented with kanamycin (500 µg/ml) when required to maintain selective pressure, at 37ºC with 180 or 250 rpm of orbital agitation.

Table 2. Strains and plasmids used in this study.

Bacterial strains or plasmids	Description	Reference or source
Burkholderia multivorans		
***Burkholderia* multivorans P0213-1**	Cystic fibrosis isolate, Canada Date of isolation 13-02-1996 ID: VC7495	D.P Speert University of British Columbia
F11	P0213-1 derivative with the plasposon pTnMod-Kmr, inserted in *ppsA* gene	This work(M. R. Ferreira, 2018)
F82	P0213-1 derivative with the plasposon pTnMod-Kmr, inserted in the genome	This work
C93	P0213-1 derivative with the plasposon pTnMod-Kmr, inserted in *tctD* gene	This work
C84	P0213-1 derivative with the plasposon pTnMod-Kmr inserted in the genome	This work
C85	P0213-1 derivative with the plasposon pTnMod-Kmr, inserted in the genome	This work
D23	P0213-1 derivative with the plasposon pTnMod-Kmr, inserted in the genome	This work
Escherichia coli		
DH5-α	DH5α *recA1* Δ(*lacZ*YA-argF)U169 ϕ80d*lacZ*ΔM15	Gibco BRL
Plasmids		
pTnModΩkm	Carrying a Km^r Plasposon with pMB1oriR	(Dennis & Zylstra, 1998)
pRK600	ColE1 *oriV*; $RP4tra^+$ RP4oriT; Cm^r helper in triparental matings	(Kessler, 1992)

2.2. Transposon mutant library construction

A transposon mutant library of the clinical isolate *B. multivorans* P0213-1 (recipient) was constructed through triparental conjugation using an *E. coli* carrying the pRK600 plasmid as a helper and an *E. coli* carrying the pTnModΩkm plasmid as a donor (Figure S1). The donor strain was inoculated in 3 mL of LB with kanamycin, while the helper strain was inoculated in 3 mL of LB with chloramphenicol and the recipient strain was inoculated in 3 mL of LB. The three cultures were incubated at 37ºC with orbital agitation of 250 rpm for 5 hours. Following incubation, 1 mL of the recipient liquid culture was mixed with 1 mL of both helper and donor. The final mixture was transferred to a LB plate at 30ºC for 24 hours. This conjugation mixture was then resuspended in 1ml of LB, 100 µl were plated onto 10 LB plates supplemented with 500 µg/ml kanamycin and 40 µg/ml gentamicin and the plates were incubated at 37ºC for 24 hours. The mutant colonies suspension was mixed to the same volume of 60% of glycerol into a well of 96-well plates and frozen at - 80ºC with. The mutant colonies were numbered, from 1 to 96 and named as following: K5 (mutant of cystic fibrosis isolate P0213-1 from well 5 of plate K).

2.3. Screening of transposon insertion libraries

Each bacterial mutant was grown overnight in 3 mL of SM medium at 37ºC with 250 rpm of orbital agitation. Suspensions with Optical Density 640 nm (OD_{640}) 0,1, were prepared with fresh SM medium and incubated at 37ºC with 180 rpm of orbital agitation for 48 hours. After this time, each mutant was observed macroscopically and microscopically for aggregates and compared with wild-type (*B. multivorans* P0213-1) strain.

2.4. Microscopy analysis

B. multivorans strains grown in SM medium for 48 hours were visualized on Zeiss Axioplan microscope, equipped with an Axiocam 503 color Zeiss camera, using a 10x 0.3 NA objective, and controlled with the Zen software.

2.5. DNA manipulation techniques

Genomic DNA from *B. multivorans* strains was extracted using a protocol previously described (Meade et al., 1982). Plasmid DNA isolation and purification, DNA restriction, agarose gel electrophoresis, DNA amplification by PCR and *E. coli* transformation were performed using standard procedures.

2.6. Detection of the plasposon in the genome of mutants

To verify if the plasposon was inserted into the genome of selected mutants, a DNA amplification by PCR was performed, followed by agarose gel electrophoresis. Genomic DNA was used as template for 50 µl PCR reactions with 2 µM of primers pTnModΩKm-fw (5'-GCAGAGCGAGGTATGTAGGC-'3) and pTnModΩKm-rev (5'-TTATGCCTCTTCCGACCATC-'3) and a Taq DNA polymerase enzyme. The conditions for the amplification were the following: initial denaturation at 94ºC for 1:30 minutes; 30 cycles of 30 seconds at 94ºC, 1 minute at 59ºC, and 1:30 minutes at 72ºC; final extension at 72ºC for 7 minutes.

2.7. Identification of the genes disrupted by the plasposon

The insertion position of the plasposon was determined in selected mutants through the digestion of genomic DNA with EcoRI (an enzyme that does not have any cutting sequence the plasposon), followed by fragment self-ligation. The ligation mixture obtained was electroporated into electrocompetent *E. coli* cells using a Bio-Rad Gene Pulser II system (400 Ω, 25 µF, 2.5 kV) that after being grown on LB medium at 37ºC with orbital agitation of 250 rpm for 1 hour, were plated on the selective LB medium supplemented with kanamycin (50 µg/ml). Plasmids were then recovered using the ZR Plasmid Miniprep™-Classic kit (Zymoresearch®), following the manufacturer´s instructions. The recovered plasmids were sequenced using primer kmR (5'-CCTTTTTACGGTTCCTGGCCT-'3) and oriR (5'-GTGCAATGTAACATCAGAG-'3), through a Sanger sequencing system at the Instituto Gulbenkian de Ciência (Portugal). The BLAST (Altschul et al., 1997) algorithm was then used to compare sequences of the gene disrupted to database sequences available at the National Center for Biotechnology Information (NCBI). A BLASTN search was also conducted against the genome sequence of the clinical isolate *B. multivorans* P0213-1 available in our group, but not yet deposited in any repository

2.8. Growth curves and doubling time estimation

Cells from an overnight culture were inoculated with an OD_{640} of 0.1 in 30 mL fresh LB. The culture was incubated at 37ºC with 180 rpm of orbital agitation. OD_{640} readings were taken over an 8 hour period and then at 23 and 24 hours. Growth rates were calculated from the exponential phase of growth from at least two independent experiments. The doubling time was calculated from the growth rate of the exponential growth phase.

2.9. Quantification of cellular aggregates and free cells

The quantification of cellular aggregates and free cells was made based on a protocol previously described (Haaber et al., 2012), with some adjustments. Cells from an overnight culture were inoculated with an OD_{640} of 0.1 in 30 mL fresh LB. After the culture was incubated at 37ºC with 180 rpm of orbital agitation for 48h it was transferred to a 50 mL Falcon tube and centrifuged at 1400 rpm at 25ºC for 30 seconds. After centrifugation, cell suspensions were left to settle for 10 minutes. The supernatant was removed by pipetting and placed in a new 50 mL Falcon tube. After a few quick-spins, the suspensions containing the aggregates were transferred to 2 mL, previously weighted, Eppendorf tubes. The 50 mL Falcon tubes containing the free cells were centrifuged for 10 minutes at 4000 rpm and 25ºC, allowing the separation of free cells from the growth medium. The resulting pellet was resuspended in 5 mL of growth medium and after a 2 minute centrifugation at 13400 rpm all free cells of each isolate were collected. Two 2 mL Eppendorf tubes were obtained for each strain, one containing all the aggregates and another with all the free cells. These tubes were placed, open, at 60ºC during at least 72 hours until completely dry and presenting a brown color. The weight of all the Eppendorf tubes containing the samples was then measured.

2.10. Aggregation Kinetics

A kinetics assay was performed in order to evaluate aggregate formation throughout cell growth. For that, cells from an overnight culture were inoculated with an OD_{640} of 0.1 in 30 mL fresh SM medium. The 24 separate flasks were incubated at 37ºC with 180 rpm of orbital agitation in two different time frames, in order to obtain data from four intercalated 12h periods. Time points were measured every two hours and a quantification of cellular aggregates and free cells as previously described.

2.11. Exopolysaccharide production

YEM (yeast extract mannitol medium) plates containing 4 g/L mannitol, 0.5 g/L yeast extract and 15 g/L agar were used to evaluate the production of exopolysaccharide. After inoculation, YEM plates were incubated for 48 h at 37°C and the mucoid phenotype due to exopolysaccharide production was observed through visual inspection.

2.12. Antimicrobial susceptibility

Antimicrobial susceptibility of wild-type and its derivative strains was assessed based on the agar disc diffusion method (Bauer et al., 1966). Exponential-phase cells growing on LB medium at 37ºC were suspended at a 0.1 OD_{640}. Müller-Hinton agar (Sigma-Aldrich) plates were inoculated with 100 µl of the referred suspension. Paper discs (BD BBL Sensi-Disc) containing aztreonam (30 µg), piperacillin (75 µg) plus tazobactam (10 µg), were applied onto the surface of the inoculated plates. The diameter of the growth inhibition zone was measured after 24 hours of incubation at 37°C. Results are the mean values from 3 replicates.

2.13. Motility

2.13.1. Swarming motility

The swarming agar plates containing 0.04% (wt/vol) tryptone, 0.01% (wt/vol) yeast extract, 0.0067% (wt/vol) $CaCl_2$, 0.6% (wt/vol) bacto agar (Difco) were spot inoculated with a 5 µl drop of a culture with a 1.0 OD_{640}. After inoculation, swarming plates were incubated for 48 h at 37°C and the diameter of the swarming zone was measured. Results are the means of data from 3 replicates.

2.13.2. Swimming motility

The swimming agar plates containing 1% (wt/vol) tryptone, 0.5% (wt/vol) NaCl, 0.3% (wt/vol) noble agar (Difco) were spot inoculated with a 5 µl drop of a culture with an 1.0 OD_{640}. After inoculation, swimming plates were incubated for 24 h at 37°C and the diameter of the swimming zone was measured. Results are the means of data from at least six replicates of two independent experiments.

2.14. Statistical analyses

The statistical significance of differences in the data was determined using the one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test which were performed using GraphPad Prism software 8.0.1 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Differences were considered statistically significant for P-values lower than 0.05.

3. Results and Discussion

3.1. Aggregation Kinetics of Wild-type P0213-1

In order to study the evolution of aggregation formation throughout the growth, a kinetic analysis was performed for 48h, at 37℃ with an orbital agitation of 180 rpm. The results obtained are presented on Figure 11.

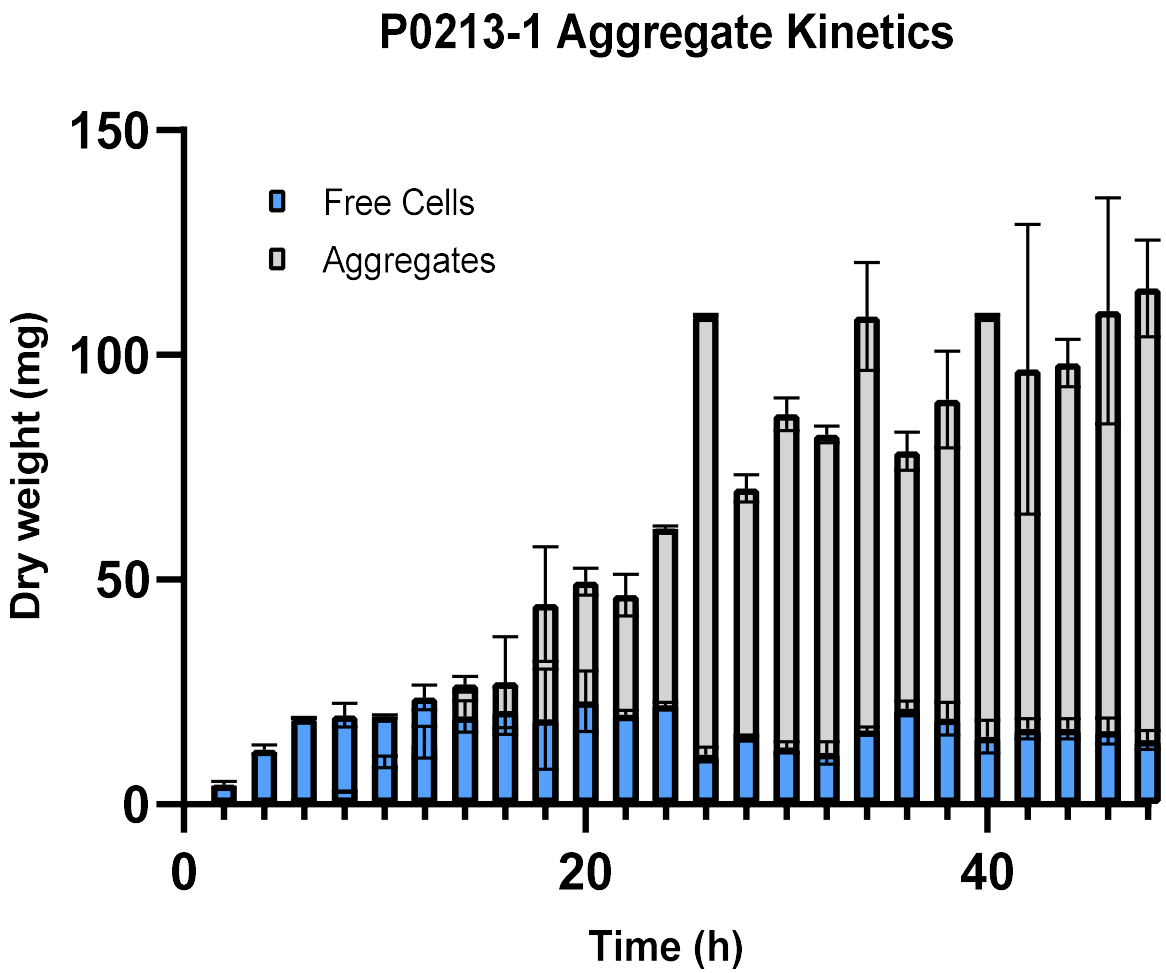


Figure 11. Dry weight of planktonic aggregates and free cells of wild-type (P0213-1) grown in LB medium, at 37℃ and 180 rpm of orbital agitation for 48h. Error bars correspond to the standard deviations of the mean values of three independent experiments

It can be noted that on the first eight hours of growth there is virtually no aggregate formation, which starts after about 14 hours of growth. The free cells dry weight seems to remain approximately constant throughout aggregate formation, while the aggregates dry weight seems to increase steadily.

3.2. Screening of *Burkholderia multivorans* plasposon mutants

When grown in SM medium, which possesses a high carbon to nitrogen ratio, P0213-1 can grow both as free cells and planktonic aggregates. These aggregates can range from macroscopic to microscopic structures. Previous work by Ferreira (2018) resulted in a construction of a plasposon mutant library comprising 900 mutants of isolate P0213-1 of *B. multivorans.* Of this library, 30 mutants were chosen based on the different aggregation phenotype they presented. In order to identify the genetic players and molecular mechanisms involved in the formation of bacterial aggregates, these mutants were screened to further confirm the aggregation phenotype compared to the wild-type, P0213-1. For this, mutants and wild-type strains were incubated at 37 °C with 180 rpm orbital agitation for 48 hours in SM medium and visually assessed at microscopic and macroscopic level. Of the original selection, only 6 mutants were considered relevant (Figure 12), since they seem have a reduced ability to form large aggregates.

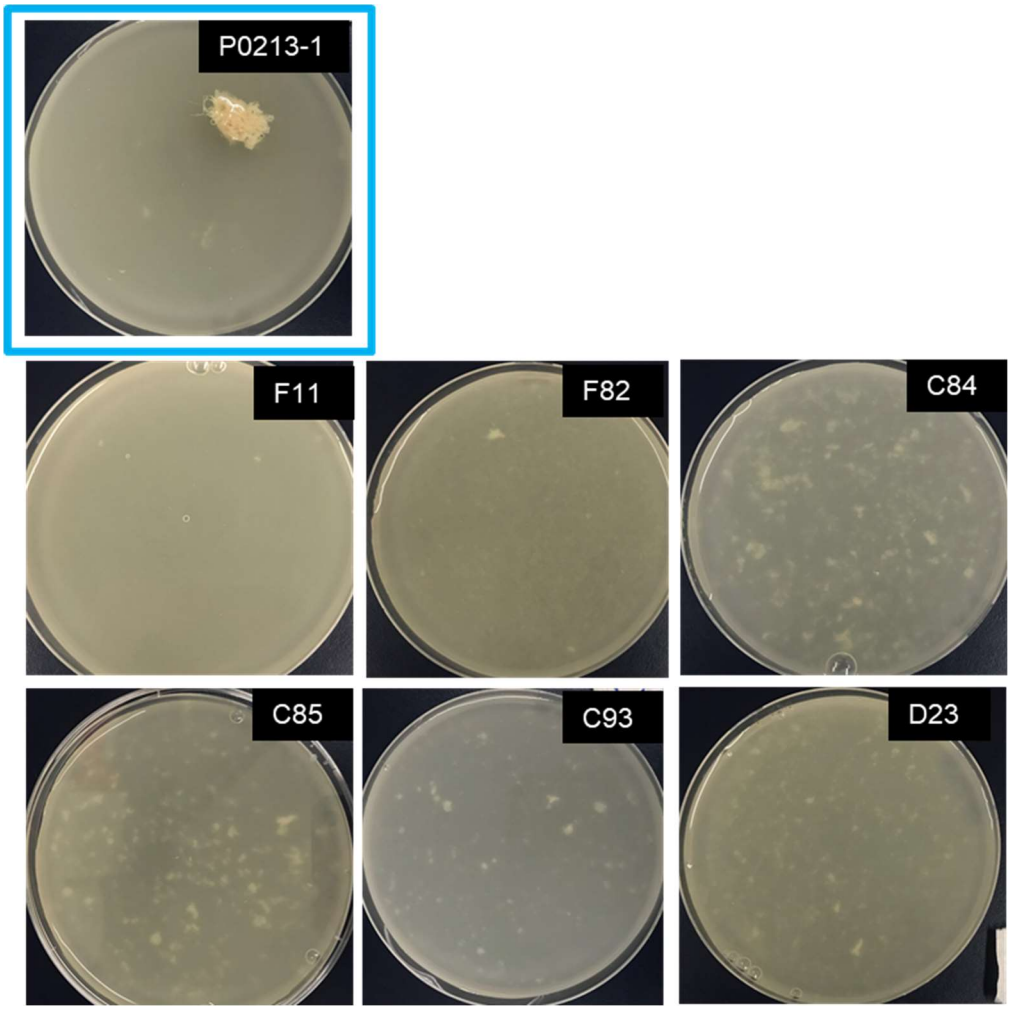


Figure 12. Macroscopic screening of the plasposon mutant library of B. multivorans P0213-1 for different abilities to form planktonic cellular aggregates. Each culture was grown in SM medium at 37ºC, 180 rpm of orbital agitation, for 48 hours

As it can be accessed by visual observation, mutants selected present fewer and smaller aggregates than the Wt. Microscopic evaluation seems to indicate that the smaller aggregates also have a different structure than the ones produced by the Wt. As it can be observed on Figure 13, while the fist seem to have a cloudy unstructured structure, the Wt presents highly structured aggregates presenting many ramifications.

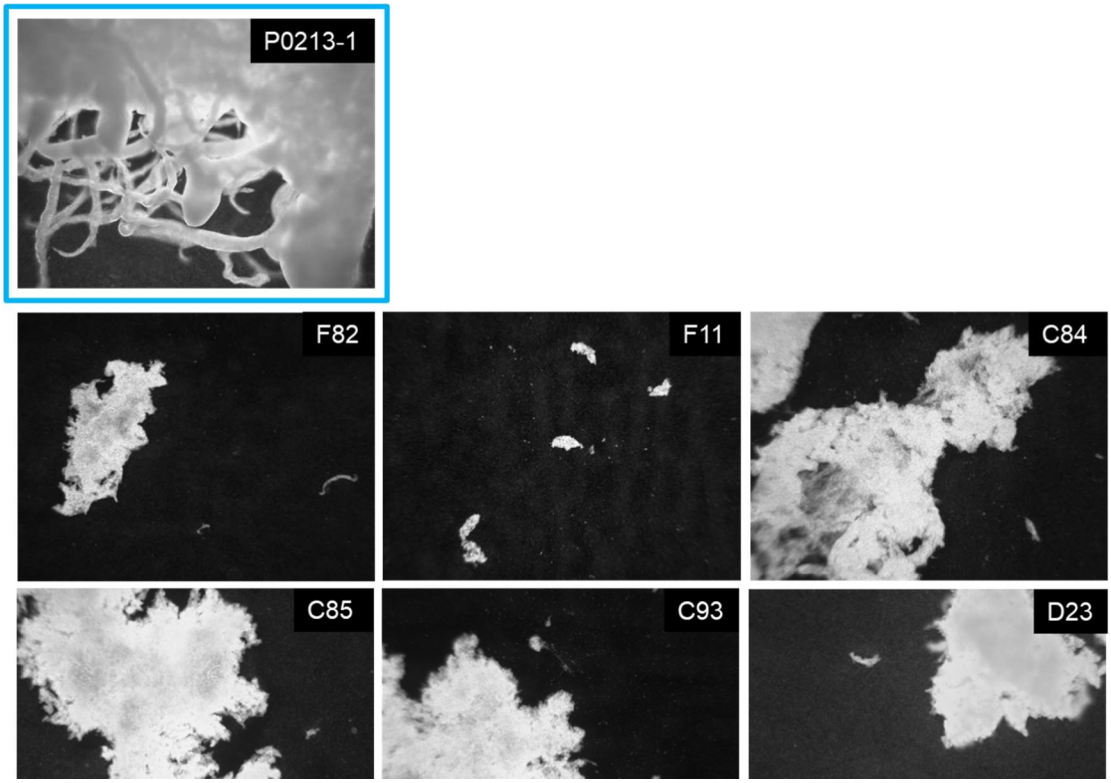


Figure 13. Microscopic screening of the plasposon mutant library of B. multivorans P0213-1 for different abilities to form planktonic cellular aggregates. Each culture was grown in SM medium at 37ºC, 250 rpm of orbital agitation, for 48 hours. The same scale is present in all pictures

3.3. Detection of the plasposon in the genome of mutants

In order to confirm that alteration of aggregation phenotype observed was the result of the plasposon insertion into the genome and consequent disruption of a gene. The mutant's genomic DNA was extracted, and a PCR amplification was performed using primers designed to amplify the plasposon's internal region, with a specified size of 1161 bp. An electrophoresis in an agarose gel was run on the amplification product. As it can be observed from Figure 14 all selected mutants display a band with size between 1000 and 1400 bp, corresponding to the expected size of the plasposon fragment, namely of 1161 bp. As it was expected, the wild-type P0213-1 did not present a band, confirming the plasposon absence and indicating that the insertion of the plasposon is the most likely the cause of the altered phenotype.

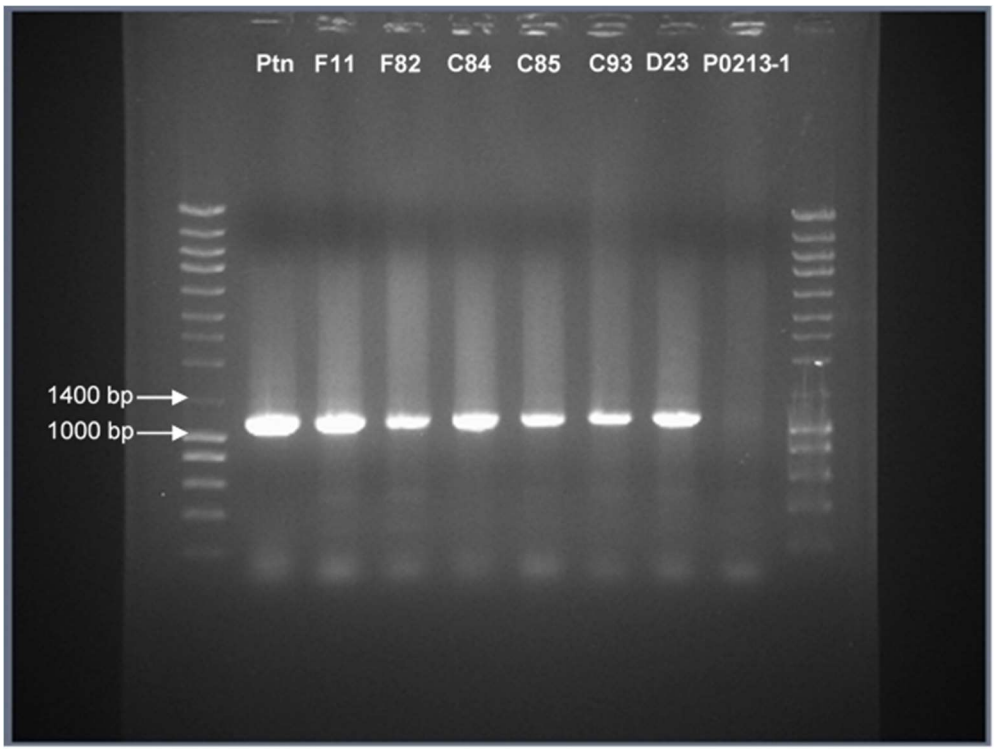


Figure 14. Electrophoretic separation in 0.8% agarose gel of the PCR products of selected mutants, corresponding to an internal region of the pTnModΩKm plasposon with an estimated size of 1161 bp. Amplification using the plasposon as target DNA was used as a positive control and the Wt as a negative control. DNA marker is the NZYDNA Leader III.

3.4. Identification of the genes disrupted by the plasposon

After confirming the plasposon insertion in the genome, the identification of the disrupted gene was made through the sequencing of the flanking regions of the site where the plasposon was inserted. The whole genome was restricted with a restriction endonuclease without recognition sequences within the plasposon, such as EcoRI, and the fragments obtained were subsequently self ligated and electroporated into electrocompetent *E. coli* cells. This procedure allowed for the obtention of colonies corresponding to each mutant. The plasmids were then extracted and after confirming its size the flanking regions were Sanger sequenced. By aligning the sequences obtained (Table S2) against *B. multivorans* ATCC 17616 genome, the disrupted genes were identified.

Table 3. Genes disrupted by the plasposon were identified in the B. multivorans selected mutants.

B. multivorans mutant	Chromossome	Start position	Locus Tag	Gene Name	Annotation	Homolog in B. multivorans ATCC 17616
F11	1	2 422 886	PROKKA_02266	*ppsA*	Phosphoenolpyruvate synthase	Bmul_1274
C93	1	447 574	PROKKA_00419	*tctD*	Two component transcriptional regulator	Bmul_0075
F82	1	772 615	PROKKA_00729	-	Type VI secretion protein	Bmul_2926

Mutant F11 is disrupted in the *ppsA* gene, figure 15, which encodes a phosphoenolpyruvate synthase, an enzyme that converts ATP and pyruvate into AMP, phosphoenolpyruvate and Pi. This conversion is essential for gluconeogenesis and as such PEPs is required for growth when pyruvate or lactate are the sole carbon sources. Upon binding of ATP to the N-terminal domain of PEPs, a catalytic residue within the central domain, His-421 hydrolyzes the β-phosphate. This generates AMP, Pi and a distinct phospho-enzyme intermediate. The C-terminal domain of the phosphorylated enzyme binds to pyruvate and transfers the β-phosphate group through a swiveling domain mechanism, to generate phosphoenolpyruvate (PEP) (McCormick & Jakeman, 2015). In another study, Ferreira (2018) found that a mutant disrupted in the same gene presented the same phenotype observed here. It was noted that the disruption could result in the accumulation of pyruvate and acetyl-CoA and perhaps an increase of lipid metabolic reactions (Ferreira, 2018).

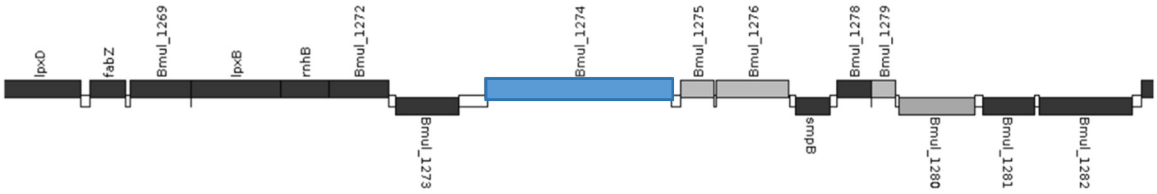


Figure 15. Gene disrupted in mutant F11 by insertion of the plasposon and its neighborhood in the B. multivorans genome. Gene ppsA is highlighted in blue. Both upstream and downstream genes encode hypothetical proteins

The gene encoding PEPs, *ppsA*, has been implicated in the glucose-phosphate stress response of *Escherichia coli*, (Richards et al., 2013) and is up-regulated in rifampin-resistant *Mycobacterium tuberculosis* (Bisson et al., 2012). Conversely, disruption of *ppsA* in *Xanthomonas campestris* resulted in a significant reduction in virulence (Tang et al., 2005) and Veselova et al. reported that the same disruption in *B. cenocepacia* increased its ability to produce N-acyl-homoserine lactones (AHL), the signal molecules of the quorum sensing system, which is known to influence biofilm formation (Veselova et al., 2012).

Phosphoenolpyruvate Phosphotransferase System (PTS), Figure 15, is used for the transport of sugars and requires phosphate transfer from PEP to enzyme I (EI) to the histidine protein (HPr) or the homologous protein FPr and then to an EII complex. A membrane-associated EIIB then transfers the phosphate from EIIA to the specific sugar that is transported across the membrane by the EIIC/D complex (Lazazzera, 2010). The mutation of the *ppsA* gene results in the blockage of the step of conversion of pyruvate into phosphoenolpyruvate which in turn might result in the decrease of the PEP levels in the cell, possibly leading to a disruption of the PTS. The PTS system has long been known to participate in regulation that impinges on metabolism, including chemotaxis, inducer exclusion, and catabolite repression (Deutscher et al., 2006; Saier, 1989). Recently, it has also been implicated in regulating processes not directly related to metabolism, such as virulence gene expression. Studies with *Vibrio cholerae* and *Bacillus cereus* have shown that this system is involved in the regulation of biofilm formation (Houot et al., 2010; Xu et al., 2014), while others have indicated a possible link between the PTS sugar import and c-di-AMP synthesis (Corrigan & Gründling, 2013).

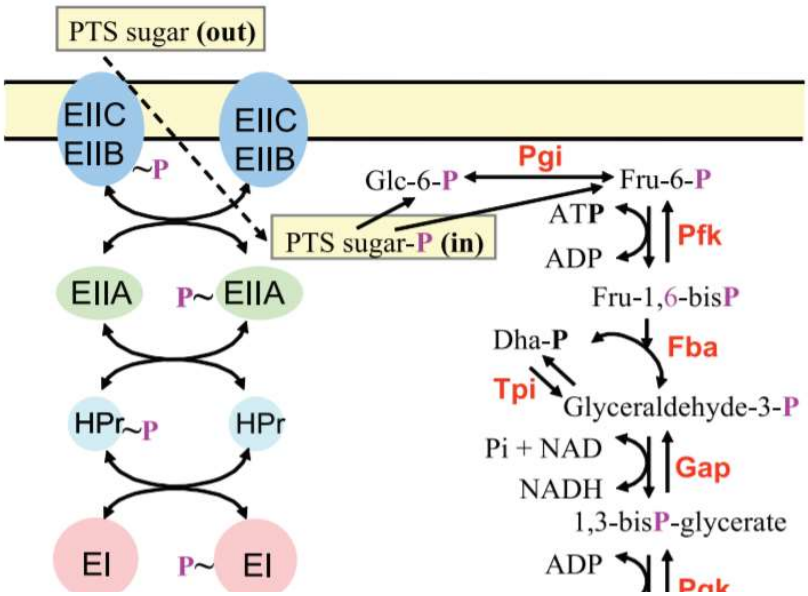


Figure 16. Carbohydrate transport and phosphorylation by the PTS and their coupling to glycolysis. Carbohydrates are transported and concomitantly phosphorylated by the PTS. The phosphorylated carbohydrate feeds into glycolysis, normally at the glucose-6-P or fructose-6-P level. Two phosphoenolpyruvate molecules are usually formed in glycolysis, one of which is used to drive the transport and initial phosphorylation of the carbohydrate. As a result, the phosphorylation state of the PTS proteins depends on both the concentration of extracellular carbohydrates and the ratio of internal phosphoenolpyruvate and pyruvate. Abbreviations for enzymes (in boldface type) are as follows: Pgi, phosphoglucose isomerase; Pfk, phosphofructokinase; Fba, fructose-1,6-bisphosphate aldolase; Tpi, triose-phosphate isomerase; Gap, glyceraldehyde-3-phosphate dehydrogenase; Pgk, phosphoglycerate kinase; Pgm, phosphoglycerate mutase; Eno, enolase; Pyk, pyruvate kinase. Retrieved from (Deutscher et al., 2006)

Sequencing of the flanking regions of the mutant F82 places the plasposon in a gene encoding a type VI secretion system protein (homologue to Bmul_2926), Figure 17.

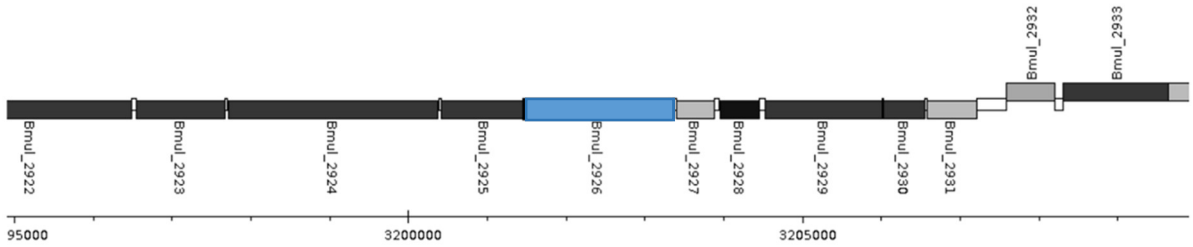


Figure 17. Gene disrupted in mutant F82 (homologue to Bmul_2926, highlighted in blue) by insertion of the plasposon and its neighborhood in the B. multivorans genome. This gene seems to be part of an operon constituted by Bmul_2928, Bmul_2927, Bmul_2926, Bmul_2925, Bmul_2924, Bmul_2923, Bmul_2922 and Bmul_2921

The T6S system is conserved in numerous gram-negative pathogens that interact closely with eukaryotic cells and it has been recognized as an important contributor to pathogenesis in many bacteria, having been studied in several pathogenic organisms, including *Vibrio cholerae*, *Francisella tularensis, Escherichia coli, and P. aeruginosa* (Bingle et al., 2008; Pukatzki et al., 2009). It has been implicated in several diverse processes, including biofilm formation, toxin delivery, virulence, and fitness in chronic infection (Bingle et al., 2008; Jani & Cotter, 2010; Schwarz et al., 2010)

A study of the virulence genes of *B. cenocepacia* identified three transposon insertion mutants that were attenuated in the rat agar bead model of chronic lung infection. Further analysis indicated that these mutants had insertions within T6SS genes, demonstrating that the T6SS is required for the virulence of *B. cenocepacia in vivo* (Aubert et al., 2008).

At present, it is unclear in what way the T6SS is involved in aggregate formation by *B. multivorans*. As it has been shown that a surface-localized protein that is part of a high-affinity manganese uptake ABC transporter system functions as an adhesin (Kolenbrander et al., 1998) it seems possible that some components of the secretion machinery itself could be important for aggregate formation. However, there is also the possibility that the T6SS is involved in the export of a yet unknown protein required for biofilm development.

Mutant C93 presents a disruption in gene *tctD* which is part of a two component regulatory system, Figure 19. These systems usually consist of a membrane-associated sensor (histidine kinase protein) that monitors environmental signals and a response regulator (receiver) whose function is modulated by phosphotransfer from its cognate histidine kinase. Two component systems are often in charge of regulating the adaptation of bacteria to new environments, as is the case when *B. multivorans* colonizes the lungs of CF patients. This happens through the modification of gene expression patterns required for bacterial survival. Sensor histidine kinases respond to a wide range of signals, including those encountered during infection (Aubert et al., 2008).

Together with other factors, two component signal (TCS) transduction systems are responsible for regulating biofilm formation in several bacteria (Chin et al., 2015).

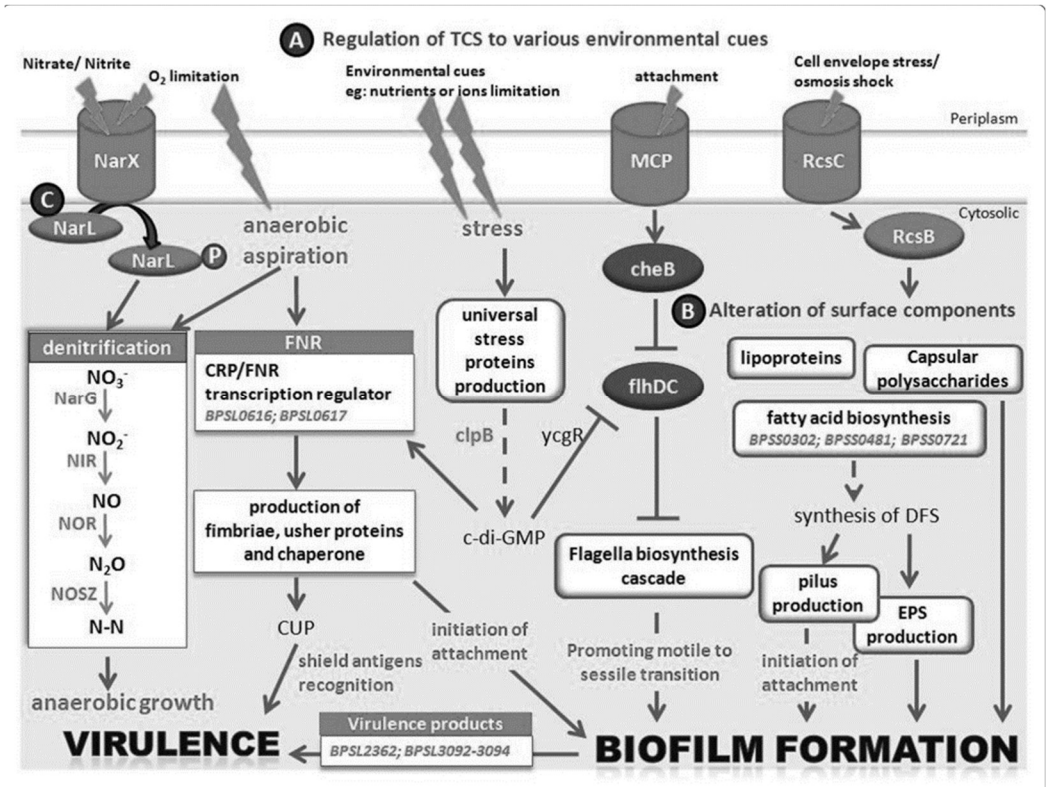


Figure 18. TCS response to various environmental cues regulating biofilm formation in Burkholderia. Retrieved from (Chin et al., 2015)

As is represented in Figure 18, a study with a *Burkholderia* strain revealed that several genes related to the TCS were up regulated during biofilm formation. The mechanism proposed suggests that in response to varied environmental signals, *Burkholderia* regulates the TCS to adapt to environmental changes by transcribing genes that encode proteins involved in the alteration of surface components and components crucial to initiate attachment of planktonic bacterial cells. As the biofilm grows, an oxygen-limited environment forms within the biofilm forcing bacteria to adjust metabolism for anaerobic growth (Chin et al., 2015). Therefore, it is possible that a disruption of Bmul_0075 might impact gene expression and consequently results in an impaired ability to form aggregates.

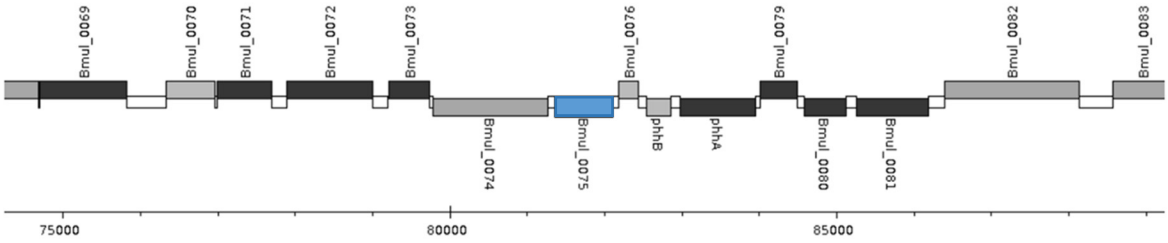


Figure 19. Gene disrupted in mutant C93 by insertion of the plasposon and its neighborhood in the B. multivorans genome. Gene tctD is highlighted in blue. Upstream is a gene encoding and integral membrane sensor signal transduction histidine kinase

3.5. *In vitro* growth analysis of selected mutants

Taking into account that mutations could impair growth and therefore result in altered aggregation properties, growth kinetics of selected mutants were assessed. Since cell growth is measured through following OD_{640} evolution over time, growth was evaluated in LB medium as results in less aggregation and therefore more reliable measures. P0213 and the selected mutants were grown at 37℃ with 180 rpm orbital agitation and measurements were taken hourly for 8 hours and then at 23 and 24h. The growth curves obtained are represented in figure 20.

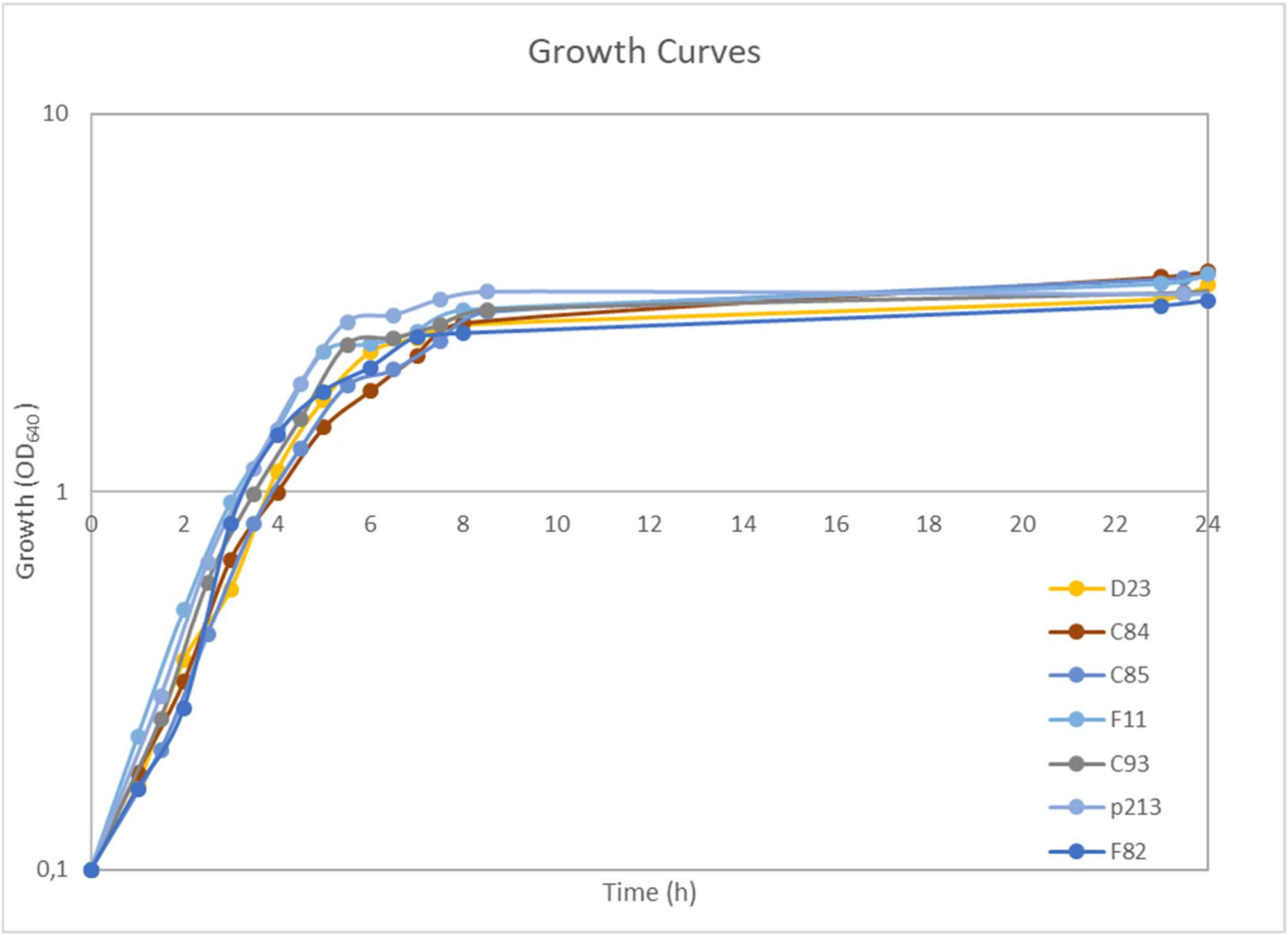


Figure 20. Growth curves of the strains under study. Cultures were grown in LB medium at 37ºC, 180 rpm of orbital agitation, and OD_{640} was measured for 24 hours. Results are the means of data from three replicates. The standard deviation is below 5%.

The slight differences observed between the growth of the wild-type and the mutants were found to be not significative. Using the points corresponding to the exponential phase, between the first and fifth hour, the doubling time for each mutant was calculated, and the results thus obtained are presented in table 4.

Table 4. Doubling time of the strains under study. Cultures were grown in LB medium at 37ºC, 180 rpm of orbital agitation, and OD_{640} was measured for 24 hours. The doubling time was calculated from the growth rate of the exponential growth phase. Statistical difference in the doubling time of the mutant strains was tested in comparison with the wild-type (P0213-1). Significance level (one-way ANOVA followed by Dunnett's multiple comparisons test): ns, not statistically significant

Bacterial strain	Doubling time (min ± SD*)
P0213-1	84.7 ± 2.665 (ns)
F11	80.9 ± 2.875 (ns)
F82	84.0 ± 1.572 (ns)
C84	88.0 ± 5.464 (ns)
C85	86.2 ± 1.484 (ns)
C93	83.3 ± 3.449 (ns)
D23	76.7 ± 2.454 (ns)

Analyzing Figure 20 and table 4, it can be observed that mutants D23 and F11 are the ones with the fastest growth, however, this difference was deemed not statistically significant. Despite the fact that the doubling time calculated for each mutant and the Wt, was similar, after 48h the amount of biomass formed by the Wt and the mutants seems to be considerably different. Taking into account the microscopic and macroscopic differences observed, this seems to be due to aggregate formation. Moreover, all mutants seem to end up with approximately the same biomass formed. This leads to the conclusion that the mutations observed are not interfering with growth, and, therefore, the phenotypic alterations observed result of impaired aggregate formation abilities.

The method used to assess growth, measuring the OD_{640}, does not account for the aggregates, so even after 23 and 24h, when there is already a significant amount of aggregates formed, the mass of free cells remains approximately constant (section 3.1 2.1.Aggregation Kinetics of Wild-type P0213-1), justifying the apparent stationary phase and similarity between biomass formed observed.

3.6. Quantification of cellular aggregates and free cells

Although macroscopic visual analysis allows the identification of different aggregation phenotypes between the mutants selected and the Wt, a more thorough quantification was performed. Following the procedure described on the materials and methods section, the dry weight of both planktonic aggregates and free cells produced by each mutant was quantified. The results in terms of percentage thus obtained are presented in Figure 21A.

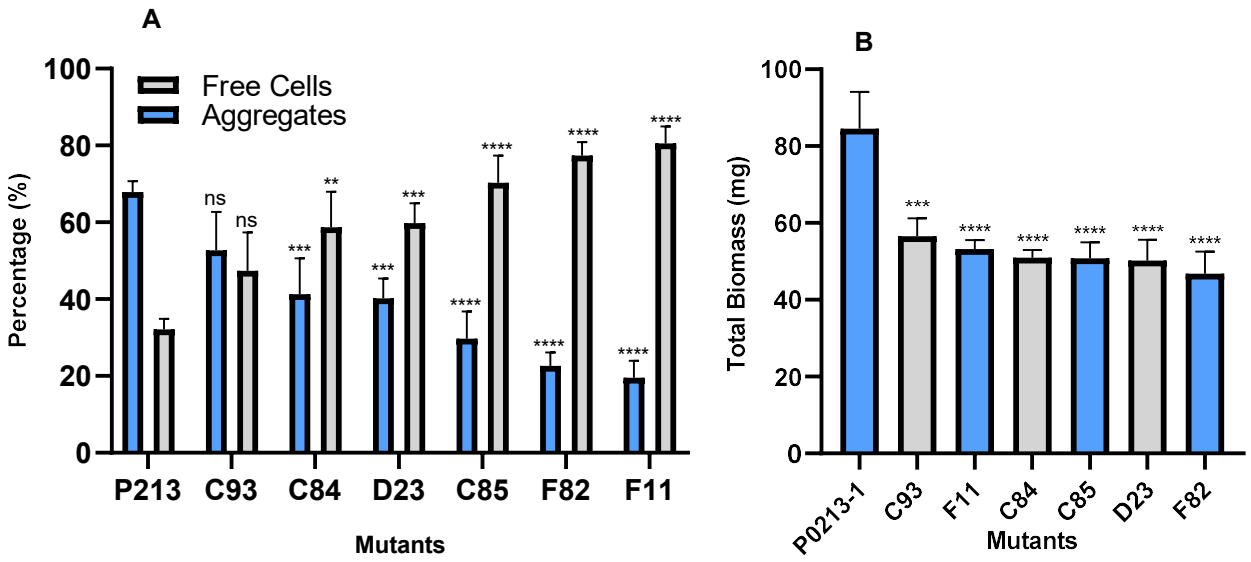


*Figure 21. **A** - Quantification of cellular aggregates and free cells of wild-type (P0213-1) and its derivative mutants. **B** – Total biomass (dry weight of aggregates + free cells). Measured after incubation at 37ºC with 180 rpm of orbital agitation for 48h. Error bars correspond to the standard deviations of the mean values of three independent experiments. Significance level (one-way ANOVA followed by Dunnett's multiple comparison test) between cellular aggregates and free cells of the wild-type (P0213-1) and cellular aggregates and free cells of the P0213-1 derivative mutants was determined: *, P<0.05; ***, P<0.001; ****, P<0.0001; ns, not statistically significant*

The results obtained seem to confirm the visual analysis performed with most mutants presenting a lower percentage of aggregates vs free cells than the Wt. In fact, only C93 was considered not statistically significant. However, total biomass (aggregates + free cells) of P0213-1 is considerably higher than the remaining mutants (results shown on Figure 21B) which justifies the macroscopic difference between mutant C93 and the Wt. The remaining results are according to the microscopic and macroscopic analysis, further confirming the decrease in planktonic aggregates formation.

In most biofilms, the microorganisms account for less than 10% of the dry mass, whereas the matrix can account for over 90%. The matrix constituted of extracellular material, in which the biofilm cells are embedded, consists mostly of conglomeration of EPS that forms the scaffold for the three-dimensional architecture of the biofilm and is responsible for cohesion in the biofilm (Flemming & Wingender, 2010). Aggregate formation seems to happen throughout stationary phase which indicates that as in biofilm formation, most of the dry weight of aggregates is not due to cell growth, but rather other extracellular material, as EPS.

3.7. Exopolysaccharide Production

Exopolysaccharides are one of the main constituents of mature biofilms, which are associated to a significant increase of resistance against the host immune system and antibiotic treatment in *Burkholderia,* therefore, the mutant's exopolysaccharide production was also accessed.

To determine the production of exopolysaccharide, each mutant was inoculated onto YEM agar plates at 37ºC for 48 hours. Then, through visual inspection, it was possible to evaluate their mucoid phenotype.

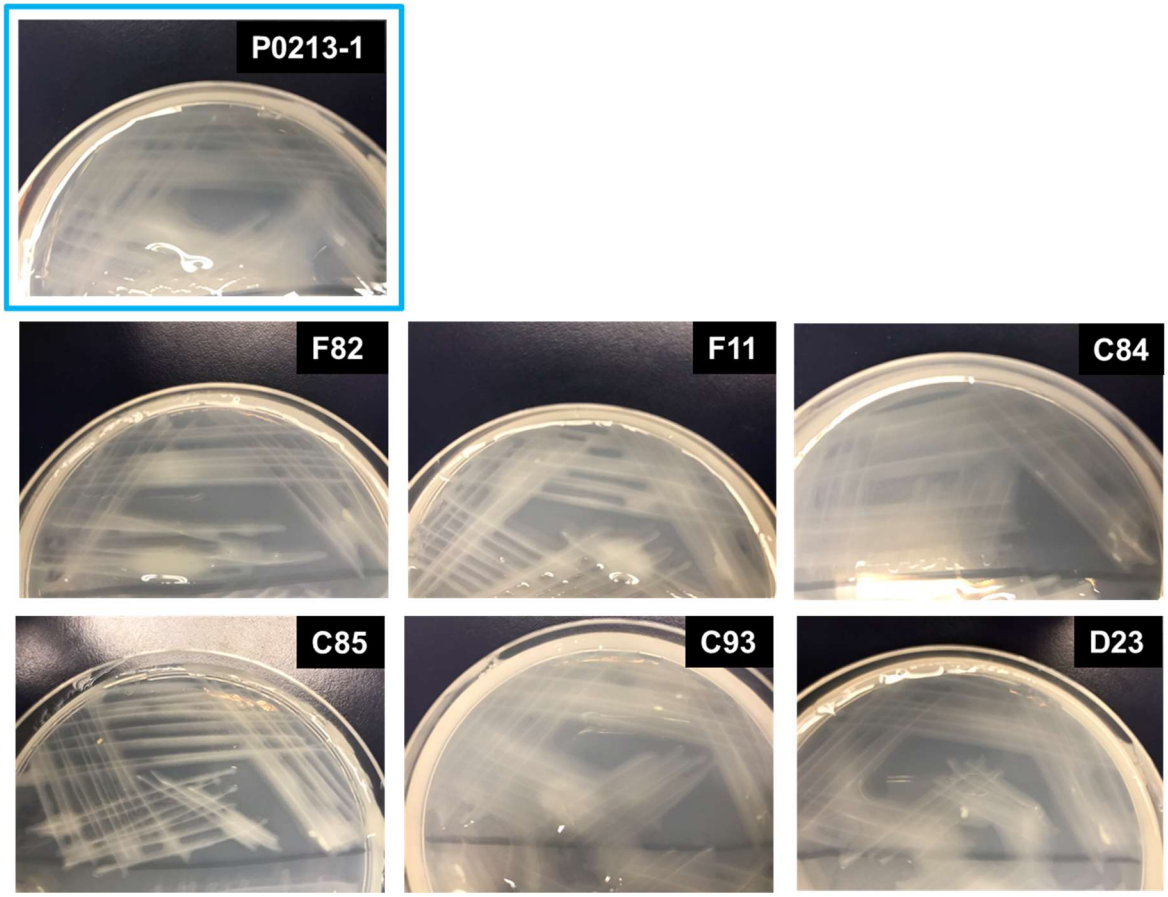


Figure 22. Evaluation of mucoid phenotype in yeast extract mannitol medium (YEM) after 48 h of incubation at 37°C for wild-type (P0213-1) and its derivate mutants

All mutants replicate the mucoid phenotype of the Wt. Studies have shown that Bcc nonmucoid isolates form more biofilm than the mucoid isolates and have virulence attenuation in animal models of infection (Silva et al., 2013). On the other hand results by Silva et al. and Ferreira (2018) have shown that under some conditions EPS does not seem to be relevant for cellular aggregate formation (Silva et al., 2017).

3.8. Swimming and swarming motilities

According to an increasing number of reports, motility is one of the factors contributing to the ability of bacteria to form biofilms or to flocculate. Biofilm formation starts with the transient adhesion of the cells a surface, or other cells (Conrad, 2012). Studies involving Bcc bacteria's CF lung infections revealed that isolates from the majority of chronic infections most commonly display the motile phenotype throughout the infection and do not normally switch to nonmotile

To evaluate the motility of mutants, swimming and swarming agar plates were incubated for 24 hours and 48 hours, respectively, at 37ºC after inoculation. Then, motility zone diameter (mm) was measured for each of them and the results are presented in figure 23.

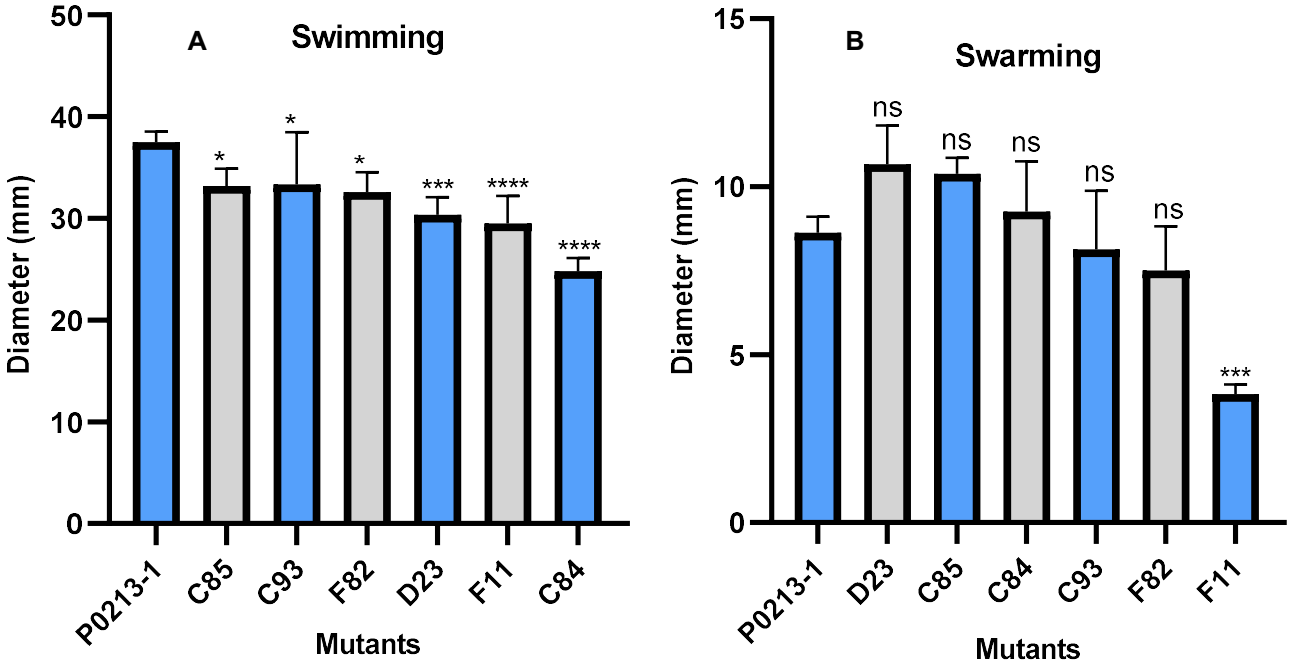


*Figure 23. **A** - Swimming motility of P0213-1 and its derivative mutants measured as the motility diameter after growth for 24h at 37ºC. **B** - Swarming motility of the P0213-1 and its derivative strains measured as the zone diameter after growth for 48h at 37ºC.Error bars correspond to the standard deviations of the mean values of at least three independent experiments. Significance level (one-way ANOVA followed by Dunnett's multiple comparison test) between the wild-type (P0213-1) and the mutants was determined: **, P<0.01; ***, P<0,001*

In order to successful aggregate cells must approach each other, either through swimming motility or Brownian motion. The process of swarming is distinct from swimming in that swarming is a multicellular process that occurs on solid surfaces or in viscous liquids. Therefore, swarming motility could be more relevant for the formation of surface-attached biofilm. Considering that this study focuses on planktonic aggregates instead of surface attached biofilms, it is not surprising that most mutants do not present statistically relevant alterations in swarming abilities. F11 is the only mutant presenting a statistically significant difference of swarming capacity. As previously mentioned, the PTS system is known to participate in the regulation of chemotaxis (Deutscher et al., 2006). Therefore, the disruption of the ppsA gene with its possible impact on the PTS system, could be resulting in impaired swarming ability.

On the other hand, bacteria swim by rotating of one or more rigid helical flagella, the activity of bacterial flagella alone can alter the hydrodynamic conditions to such an extent that it might trigger aggregation. Most mutants present impaired ability to swim, which might relate to their impaired ability to aggregate.

3.9. Antibiotic Resistance

One of the most significant aspects of the biofilm growth mode is that microorganisms escape the host immune response and are much less susceptible to antibiotics than are their planktonically grown counterparts. Antimicrobial susceptibility of all mutants and Wt was tested against aztreonam and piperacillin plus tazobactam. Both aztreonam and piperacillin are β-lactams that inhibit cell wall biosynthesis while tazobactam is a β-lactamase inhibitor.

The antimicrobial susceptibility was determined by measuring the diameter of the growth inhibition zone after 24 hours of incubation at 37°C.

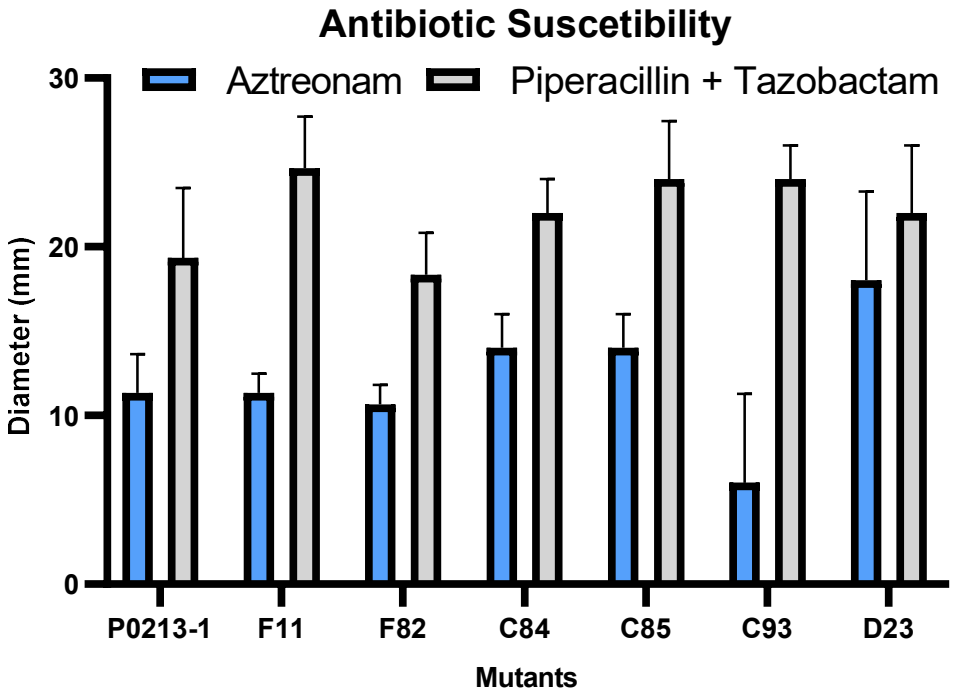


Figure 24. Antibiotics, aztreonam and piperacillin+ tazobactam, susceptibility of P0213-1 and its derivative mutants was measured as the diameter of cell growth inhibition. After growth for 24h at 37ºC. Error bars correspond to the standard deviations of the mean values of at least two independent experiments. Significance level (one-way ANOVA followed by Dunnett's multiple comparison test) between the wild-type (P0213-1) and the mutants for each antibiotic tested was determined.

Significance levels between the Wt and the mutant strains was determined, and it was found that the differences in susceptibility were not statistically significant for either one of the antibiotics tested. This results were expected since there seem to be several causes for antibiotic tolerance of bacterial biofilms which include physical barriers to antibiotic penetrance from extracellular matrix (Pugliese & Favero, 2002; Stewart, 1996), the production of periplasmic glucans (Mah et al., 2003), slow growth (Anwar et al., 1992) and/or the presence of metabolically inactive persister cells within a biofilm that are inherently tolerant to antibiotics (K Lewis, 2005). None of these factors are relevant on the mode of growth tested, therefore a different kind of analysis should be conducted to access whether mutant strains are more susceptible than Wt aggregates.

4. Conclusion Remarks and Future Perspectives

Cystic fibrosis is the most common genetic disease in Caucasians. CF mortality typically results of respiratory failure due to chronic pulmonary bacterial infection. *Burkholderia* infections are amongst the most common, with prevalence ranging from 0 to 40%, depending on the center, and increasing with age. Biofilm formation on *P. aeruginosa* infections is an important clinical problem since it helps bacteria resist more efficiently to antibiotic's action while conferring a greater protection from the immune response of the host. *Burkholderia* is a close relative of *Pseudomonas* and also has the capacity to form biofilm. However, recent data has demonstrated that in pulmonary infections bacteria do not inevitably attach to surfaces, rather, they attach to their fellow bacteria likely by means of matrix components and mucus. Thus, it appears that opposed to harboring bacteria firmly attached to a surface, chronic infections can be characterized by aggregates suspended within host tissue or lumen. The genetic mechanisms involved in the formation of planktonic aggregates are not clear.

Screening of a library of insertion mutants of a *Burkholderia multivorans* clinical resulted in the selection of six mutants with reduced ability to aggregate. Sequencing of flanking regions resulted in the identification of three genes thought to be relevant for aggregation ability. The first encodes a phosphoenolpyruvate synthase, other is a part of a type VI secretion system, and the last is involved in a two-component regulatory system.

The inactivation of the PEP synthase can result in the accumulation of pyruvate, acetyl-CoA or in a possible increase of lipid metabolic reactions. There is also the possibility that it can somehow affect the PTS which in turn has been shown to be involved in virulence, regulation of biofilm formation, sugar import and c-di-AMP synthesis.

The T6S system has been recognized as an important contributor to pathogenesis in many bacteria, being implicated in several diverse processes, including biofilm formation, toxin delivery, virulence, and fitness in chronic infection. Although it is unclear in what way the T6SS is involved in the aggregation capacity of *B. multivorans*, it is proposed that the system could be involved in the export of a yet unknown protein or even that some components of the secretion machinery itself could work as adhesins.

Two component systems are often in charge of regulating the adaptation of bacteria to new environments, as is the case when *B. multivorans* colonizes the lungs of CF patients. It seems that in response to varied environmental signals, *Burkholderia* regulates the TCS by transcribing genes that encode proteins involved in the alteration of surface components and components crucial to initiate attachment of planktonic bacterial cells.

The deeper understanding of the complex mechanisms regulating cellular aggregation could be a key factor to the development of potential therapeutics to improve treatments against Bcc bacteria. Little has been done so far regarding this problematic, so there is still a lot of work that needs to be done. Besides the three selected mutants whose interrupted genes still need to be identified, there are still more than three hundred mutants of the recently constructed library left to screen. Other approaches to the study

of planktonic aggregates are also being employed, namely an evolution assay, the exposure to specific stresses and the production of mixed aggregates through co-culture of different strains.

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6. Supplementary Material

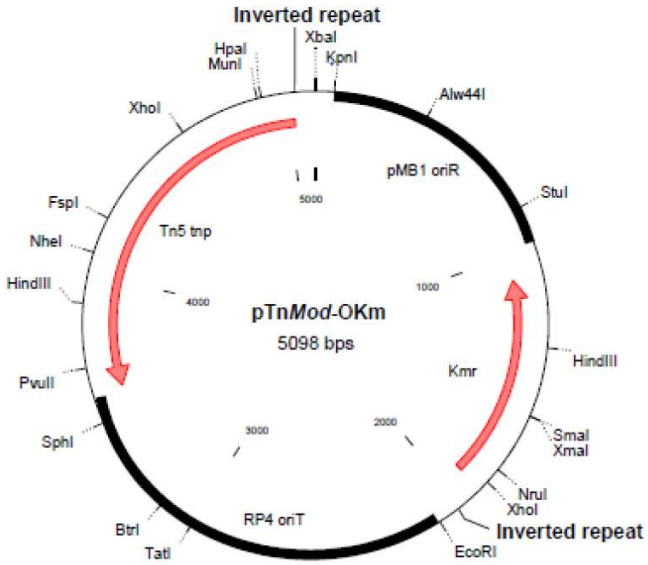

Figure S1 Physical map of transposon pTModΩKm. Transposon is composed of genes that encode a transponase, two inverted repeats and a selectable marker, kanamycin in this case. The sequence between inverted repeats is the unique part of transposon that remain inserted in genome. Size is roughly 2000 bp. Retrieved from (Dennis & Zylstra, 1998).

Table S1. Primers used in this work

Primers	Sequences
pTn*Mod*ΩKm – fw	5’ GCAGAGCGAGGTATGTAGGC ‘3
pTn*Mod*ΩKm – rev	5’ TTATGCCTCTTCCGACCATC ‘3
KmR	5’ CCTTTTTACGGTTCCTGGCCT ‘3
OriR	5’ GTGCAATGTAACATCAGAG ‘3

Table S2. Flanking sequences of the plasposon insertion obtained from the plasmids recovered from the mutants

F11	
Seq A	ATAAATGGGTTGGTTTTCTCCCCCCCCCCCGAGCTCTTAATTAATTTAAATCTAGACTAGTG CGGCCGCACTTGTGTATAAGAGTCAGGCGACGCATGACCGCGCGCGACGCTCTCGACGG CCTTCTTCAGATCCTGATCGACGTTCGGATACTCGAGGATCGCCTTCGGGTGCACGCCGA TGTTGTTGTTGATGATGAACTCGAGACGCGCGAGGCCGACGCCGGCGTTCGGCAGCTGC GAGAAGTCGAATGCGAGCTGCGGGTTGCCGACGTTCATCATGATCTTCACCGGAATTTCC GGCAGTTCGCCGCGCTGCACTTCGGTGACTTCCGTCTCGAGCAGCCCGTCGTAGATCTTG CCTTCGTCGCCTTCCGCGCACGACACGGTGACGAGCGCGCCGTCCTTCAGCACGTCGGT CGCGTCGCCGCAGCCGACCACGGCCGGCACGCCGAGCTCACGCGCGATGATCGCCGCG TGGCATGTGCGCCCGCCACGGTTCGTGACGATCGCCGACGCGCGCTTCATTACCGGCTC CCAGTTCGGGTCGGTCATGTCGGCGACGAGCACGTCGCCCGGCTGCACGCGTTCCATTT CGGACGGATCCTGGATCACGCGTACGGGGCCCGCACCGATCTTCTGGCCGATCGCACGG TCGGTCGCGGAGCACCTGCGACTGGCCCTTCAGCTTTGAATCGCTGCTCGGCCTTGGTC GCTCGCCTGGCTCTTCACCGTCTCCGGGGCGCGCCTGCAGAATGAAGATCTTGCCGTCA CGCCGTCCTTGGCCCACTCATGTCCATCGGACGCTGGGTAGTGCTTTCTCGATATGAACG GCGTACTTCTAGAATTCGATCTCGTCGGCATCAGTGAATCAAGTACCGGTTGCGCTGATC GATAAGAACGACAACCGTACTTACACGGCCTGACTCGGACGGATGCGTGAATCCGATCTT GGAC
Seq B	TTTTACTTTTTTTTGGTCATAGAGAACAGAGTGTTTTTACCGCCTGGAACTTTTTTTACGCG GTCGGCTGCTGTGGTCAGAAGCAGTTAACTTCTTGGTTCGCCGCGTGCTTTTTTTACAACA AGATGACCTGAGGCGACGCGTAAATGCCGGGCGGCTTTCTCTTTCTTTGATCATTGACTGT CCGAATCAACCTGAAAAGTTTCATCATCATTTGTTTTTCTTCCACAACTAGCCGGATCAATA TTACCCTAGCCTGGCTTTTCGTGGCGCGTCACACTACTTTTCCGACTTAATCTCGCAGTTA TTCGTCCTTTTTGTCGCGCCTTTCCGCGCGTGCGTGACCAAATGGGCCGGACCAACATCA AGATCATGGTGCCGTTCGTGCTCTCCGCGAAGCAGGCGAAACGCGTCACCGGCCTGCTC GAAAATTTCAGACTGAAGCGCGGCGAGAACGGGTTGTTTTTCGTGATGAGGTGCAAAGTG ACGACTGACTCAATTCTCGCGGAAGTGTCCCTGGAGTGCTTCGACGGTATCTCGATCGGA TCCAAAGACCTTTGCACCTCACGCTGGGCCGCGAGGCGATTTTGGTATGGAATTTGCGGG ACGACGAATTAAACCAGTACGAAACTTCAGTAAAGATCCTGCTGAAGCGCGAGATCGATG CGTGTCGCATGATGGGCAAGCACGTAGGTGTCTGCGTGCTAGGCCGGGACAATCATCCA GAGTTTGCTCAATGGCTGA
F82	
Seq A	AGTCGTCGTCGAGCTTCTTGTTGATCCGCGCGCCGAGCAGCGCGAACGACTCGATCATCC GCTCGACGTGCGGATCCTCGCAGTGCTCGCCGGACAGCGCGAGGCGCGCCGCGATCTT CGGATAGCGTTCGGCGAAATCCCGCGAATAGCGCCGCAAAAACGATAATTCGCGCTCGTA GTACGGCAGCAATTCTTCCATCGACGACCCCGAACCTCTTCCATTCCTTGCCGGACCCTG TTCCGCCGCCGTGCGGTCCGGCCCGCGCGGCGGC
Seq B	CATGTTCTTTCCTGGTACCGTCGACGTGCATGGCGCGCCGGCGATCGCGGCCGGCCTAG GCGGCCAGATCTGATCAAGAGACAGACCCGGAATTCTGATTACGCCGCGTTCTTCGCTGC GCAATCGGTGCAGAAACCGAAAAAATACAGCACCGACAGCGCGAACGCGAACGCCGTGC TCTCTGCCCAGCTTCAGTACATCTTCTCGGTATCGCGCGTTGCGCATTACCTGAAGGGGA TGATGCGGGACAAGATCGGCAGCTTCGCGTCGGCGCAGAACGTGGAGACCTTCCTCAAC CGGTGGATTTCGCAATACGTGCTGCTCGACGACAACGCGACGCAGGAGC

C93	
Seq A	ATTCCGTCGATGCCCGGCAGGCCGAGGTCGAGGATCACGAGTTCGTGGCGGTTTTGTGC CAGCGCCTGTTCGGCAAAGATGCCGTCGTGCACCATGTCGACGGTGAAGCCAGCCTGTT CGAGGCTGCTCTGGATACCGCGTGCGATGGGGCGGTCGTCTTCGATCAGAAGGAGTCGC ATGATGTTCGCTCAAGTACAATGGGTTGGCGGTTCAGGCAC
Seq B	GAATGACCGGTGCCGGCCCCGCCGCGCGGCGCATGCCGCGCGCGAGCGCCGTTACCGA ACGAGGTGCAACATGGTCCACAAGCTCACATCAGAAGAACGCAAGACGCGACTCGAACG CCTGCCGCAATGGTCGGCGGTACCGGGCCGCGATGCGATCCAGCGCAG