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Role of two-component regulatory systems in mucoid morphotype variation 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 September 2018 - October 2019, under the supervision of Prof. Doctor Leonilde de Fátima Morais 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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Abstract

Patients with cystic fibrosis (CF) accumulate thick and viscous mucus in their airways, providing a rich environment for thriving of pathogenic bacteria. Among them is the *Burkholderia cepacia* complex, a group of bacteria which poses serious threat to CF patients due to their resistance to antibiotics and ability to cause life-threatening necrotizing pneumonia. *Burkholderia* strains possess several virulence factors, among them, the exopolysaccharide cepacian, produced by most of the clinical isolates. Additionally, studies have shown that *Burkholderia* strains are able to switch from a mucoid cepacian producing morphotype to a nonmucoid one within the host, and these nonmucoid isolates have been associated with rapid decline in lung function. Recently, mutations in *B. multivorans* genes *envZ*/*ompR* and *fixL*/*fixJ*, which encode two signal transduction regulatory systems have been linked to the emergence of nonmucoid variants in three different lineages (C1, C2, and C3) within a CF chronic respiratory infection. Here, we tested whether the genetic background dictated whether loss of the mucoid phenotype was via OmpR/EnvZ or FixJ/FixL transduction systems. By cultivating isolate BM10 from lineage C4, belonging to the mentioned CF patient, under stress conditions, we were able to obtain variants with reduced mucoidy which showed indel mutations in the *ompR/envZ* locus. Additionally, we tested whether these mutations affected other cell wall-dependent phenotypes and show differences in motility, biofilm formation and antimicrobial resistance. Overall, our data confirmed the importance of OmpR regulator in mucoid switch, but depending on the genetic background of the isolates, other mechanisms for mucoid switch might exist.

Keywords: *Burkholderia multivorans*, EnvZ/OmpR, FixL/FixJ, mucoid switch, cystic fibrosis, two-component regulatory system

Resumo

Os doentes com Fibrose Quística (FQ) acumulam muco espesso nos pulmões, potenciando a proliferação de bactérias patogénicas oportunistas do complexo *Burkholderia cepacia.* Estas, devido à sua resistência intrínseca a antibióticos e capacidade de causar uma pneumonia necrotizante, que pode ser fatal, provocam uma redução significativa na saúde destes doentes. As estirpes de *Burkholderia* possuem vários factores de virulência, como o exopolissacárido cepaciano, produzido pela maioria dos isolados clínicos. Tem-se demonstrado que no microambiente do pulmão dos hospedeiros, estas bactérias conseguem alterar o seu fenótipo de mucoso e produtor de exopolissacárido para não-mucoso. Este último fenótipo foi correlacionado com um rápido declínio na função pulmonar. Recentemente, mutações nos genes *envZ/ompR* e *fixL/fixJ* de *Burkholderia multivorans*, que codificam dois sistemas reguladores de dois componentes, implicaram-nos no surgimento de variantes não-mucosas em três das quatro linhagens (C1, C2 e C3) provenientes da mesma infecção respiratória crónica. Aqui testámos se o património genético ditava se a perda do fenótipo mucoso ocorria via OmpR/EnvZ ou FixJ/FixL ou outro mecanismo. Ao cultivar o isolado BM10 da linhagem C4, obtido a partir da referida infecção crónica sob condições de stress, foi possível obter variantes que revelaram mutações de inserção/eliminação nos genes *ompR/envZ.* Adicionalmente, testámos se essas mutações poderiam afectar outros fenótipos dependentes da integridade da parede celular, tendo-se obtido diferenças na motilidade, formação de biofilmes e resistência a antibióticos. Os dados obtidos confirmam a importância do regulador OmpR na alteração do fenótipo mucoso nesta linhagem. No entanto, dependendo do património genético dos isolados, outros mecanismos estarão associados.

Palavras-chave: *Burkholderia multivorans*, EnvZ/OmpR, FixL/FixJ, alteração de fenótipo mucóide, fibrose quística, sistemas reguladores de dois componentes

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List of Acronyms

Amp – Ampicillin

Bcc – *Burkholderia cepacia* complex

BM – *Burkholderia multivorans*

bp – base pair

CF – Cystic Fibrosis

Cm – Chloramphenicol

EPS – Exopolysaccharide

HK – Histidine Kinase

Kan – Kanamycin

LB – Lennox broth

OD – Optical density

PCR – Polymerase chain reaction

RR – Response Regulator

SD – Standard deviation

SM – S medium with mannitol

TCS – Two-component system

YEM – Yeast extract mannitol medium

DNA – Deoxyribonucleic acid

RNA – Ribonucleic acid

1. Introduction

1.1. Cystic Fibrosis infections by *Burkholderia cepacia* complex bacteria

The genus *Burkholderia* comprises more than 100 species of bacteria that were isolated from very diversified ecological niches, such as soils, plants rhizosphere and phytosphere, invertebrate intestinal tracts and respiratory tracts of humans (Vandamme & Peeters, 2014) and within those species, there's a group of closely related species, termed the *Burkholderia cepacia* complex (Bcc). These species include strains that can have biotechnological applications and can be used in biocontrol or bioremediation, but also includes other strains that can infect plants and animals. At the moment, the Bcc group is composed of more than 20 species of *Burkholderia* and the majority of isolates were recovered from the lungs of patients with Cystic Fibrosis (CF). This genetic disease is considered the most potentially lethal and is related with a defective or absent CFTR protein, a cAMP-regulated chloride ion channel. This ion channel has been associated with the role of regulating the cyclic AMP-dependent endocytosis and exocytosis in epithelial cells (Snouwaert et al., 1992). In normal conditions, human airways are covered with a thin and mobile mucus layer that is composed of mucins, proteins, lipids, ions and water secreted by secretory cells and by submucosal glands, in the large airways (Figure 1). Contrastingly, in the conditions verified by CF patients, an abnormal functioning of the epithelial surfaces of the respiratory, digestive and reproductive tracts, originates a large and thick accumulation of mucus that obstructs the airways and provides a rich environment for bacteria, that can cause infection, inflammation and in chronic cases, death (Fahy & Dickey 2010; Maisonneuve et al., 2012; Livraghi & Randell, 2007).

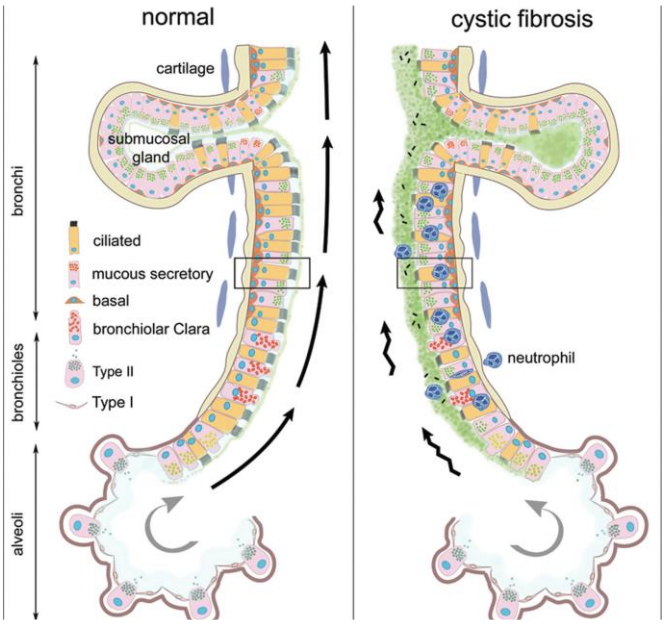


Figure 1 – Schematic representation comparing the respiratory tract in normal conditions and in cystic fibrosis. In normal conditions, the existence of a functional CFTR protein, regulates mucus clearance and hydration. In cystic fibrosis, the epithelial abnormalities verified by a non-functional or absent CFTR protein, leads to the accumulation of thick and viscous mucus that provides a rich environment for the development of infections by opportunistic bacteria (adapted from Livraghi & Randell, 2007).

The microbiology of the CF lung is complex, and it is characterized by infections with opportunistic Gram-positive and Gram-negative bacteria, frequently belonging to *Staphylococcus aureus, Pseudomonas aeruginosa* and *Burkholderia cepacia* complex (Bcc) strains (Zlosnik et al, 2011). These microorganisms possess several virulence factors, such as numerous metabolic enzymes, transporters, regulatory genes and putative virulence determinants that they can activate or suppress at different stages of infection. Those virulence factors provides them with the necessary tools to move and adapt to different ecological niches, like the conditions provided by the microenvironment of the CF lung, where they can cause inflammation and damage to the tissues (Drevinek & Mahenthiralingam, 2010; Holden et al., 2009). Although *Pseudomonas aeruginosa* remains the most prevalent species isolated from the lungs of CF patients, infections by bacteria of the Bcc are still the most dangerous for patients, since they can spread from patient to patient, have an intrinsic resistance to most classes of antibiotics and can cause various types of symptoms that range from mild asymptomatic carriage to a rapid decline in lung function of CF patients. In some rare cases, the infection can progress to a fatal bacteremic invasion manifested by a progressive necrotizing pneumonia, termed “cepacia syndrome” (Zlosnik et al., 2011; Govan et al., 2007).

Within the Bcc group, *Burkholderia cenocepacia* and *Burkholderia multivorans* appears to be the most prevalent species in the CF lungs, accounting for at least 85-97% of all Bcc infections (Mahenthiralingam et al., 2005; Pope et al., 2010; Kenna et al., 2017).

1.2. Exopolysaccharides of the *Burkholderia cepacia* complex

Exopolysaccharides are high molecular weight polymers with sugar bases that are synthetized and secreted by many organisms. Their importance has been studied in a wide variety of microorganisms, with several studies showing that these EPS play a crucial role in the adaptation of bacteria to different environments where microorganisms can experience stress conditions. The EPS can form a hydrated anionic matrix that surrounds and protects cells and also have hygroscopic properties that appear to reduce the loss of water by the cells, increasing survival rate during periods of drying and desiccation (Potts, 1994). The exopolysaccharides also protect the cells against metal ion stress, mostly high concentration of Fe^{2+} and Zn^{2+}, since they have metal-binding-properties that might be explained by the presence of the carbonyl, carboxyl and hydroxyl groups within the EPS structure that allows them to complex cations and scavenge metals (Potts, 1994). It is also known that the exopolysaccharides are vital in the establishment of symbiotic or pathogenic relationships of bacteria with plants and animals and that, among proteins and DNA, are a major component of the biofilm mature structures, contributing, for example, for the persistence of chronic *Pseudomonas aeruginosa* lung infections in CF patients (Hentzer et al., 2001).

The production of different types of exopolysaccharides in the *Burkholderia* genus is highly variable and appears to be strain dependent, with some strains having the ability to produce a wide variety of exopolysaccharides, while other strains only have the capacity to produce one type of exopolysaccharide (Silva et al., 2011). It is known that bacteria from the *Burkholderia cepacia* complex can produce at least seven different types of polysaccharides (Ferreira et al., 2011), which constitutes one of the many virulence factors that allow the bacteria from the Bcc to invade and thrive in the CF lungs. Some studies have associated its presence with resistance to antimicrobial peptides produced by epithelial and phagocytic cells, such as the human antimicrobial peptides cathelicidin LL-37 and beta-defensin hBD-3, as well as peptides produced by other mammals (Benincasa et al., 2009) and evasion of the host immune system, through inhibition of neutrophil chemotaxis and scavenging of reactive oxygen species (Bylund et al., 2006). The most studied exopolysaccharide produced by the *Burkholderia* genus is cepacian, which has been identified in different species of the genus, including environmental isolates associated with plants and in both environmental and clinical isolates, being present in 80-90% of Bcc strains recovered from the lungs of CF patients (Cunha et al., 2004). Consequently, its structure and the genes involved in cepacian biosynthesis have already been identified in previous studies.

Cepacian structure is composed of a branched acetylated heptasaccharide repeat-unit with D-glucose, D-rhamnose, D-mannose, D-galactose, and D-glucuronic acid in the ratio 1:1:1:3:1 and the genes encoding the proteins involved in the synthesis of cepacian are located in the *bce*-I and *bce*-II gene clusters (Figure 2) (Moreira et al., 2003; Ferreira et al., 2010). These clusters appear to be well conserved in all sequenced *Burkholderia* genomes, confirming the cepacian dominance among the different types of EPS's produced by *Burkholderia* species, with the exception of the intracellular endosymbiotic *Burkholderia rhizoxinica* HKI 454, which lacks both clusters and all strains of *Burkholderia mallei*, which only have the *bce*-II cluster. These species have genome sizes that range from about 3.6 Mbp and 5.2-5.9 Mbp, respectively, while the majority of *Burkholderia* species have genome sizes ranging from 6.3-9.7 Mbp, suggesting that these two species suffered a significant genome size reduction, accompanied with the loss of many genes (Ferreira et al., 2010). The *bce*-I and *bce*-II gene clusters can be located together in the same genomic region or separated by hundreds of kilobase pairs, as it can be seen in Figure 2. Based on this genomic characteristic, the bacteria can be grouped in three distinct groups, with group 1 including clinical and environmental isolates from the Bcc that have the two *bce* clusters in different genomic regions, group 2 containing pathogenic non-Bcc isolates that also have the two clusters in different genomic locations and finally group 3 containing non-pathogenic rhizosphere and plant-associated environmental strains, formerly belonging to the genus *Burkholderia* and currently assigned to the new genus *Paraburkholderia*. These strains have the *bce*-I and *bce*-II genes in the same genomic region (Ferreira et al., 2011; Sawana & Gupta, 2014).

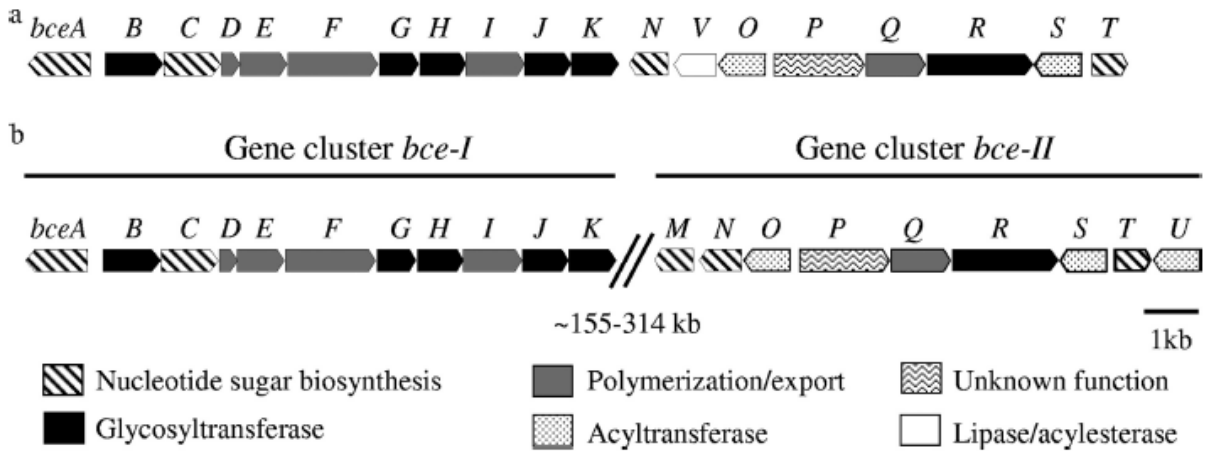


Figure 2 – Organization of the gene clusters *bce*-I and *bce*-II, which are involved in cepacian biosynthesis. In some species of the genus *Burkholderia*, the clusters are in the same genomic region while in other species, the two clusters are located in different genomic regions (adapted from Ferreira et al., 2010).

The production of cepacian by *Burkholderia* depends on external factors, since they are unable to produce the EPS on LB medium but are able to produce the exopolysaccharide in medium rich in glucose or mannitol and poor in nitrogen. The biosynthesis of cepacian starts with the formation of activated sugar-nucleotide precursors required for the synthesis of the repeat-unit building blocks (Richau et. al, 2000b). This process is catalyzed by several enzymes encoded by the *bce*-I and *bce*-II gene clusters, which have isomerase, pyrophosphorylase, dehydrogenase, among other activities. After this step, the heptasaccharide repeat-unit is assembled, by the addition of the sugars to an isoprenoid lipid carrier, a reaction catalyzed by glycosyltransferase enzymes encoded by genes of the two *bce* clusters. Following the repeat unit assembly, the next steps in cepacian synthesis are polymerization and export of the exopolysaccharide to the cell's environment, through polysaccharide polymerase, flippase, tyrosine kinase, and tyrosine phosphatase, all encoded within the genes of the two *bce* clusters, as it can be seen in Figure 3 (Ferreira et al., 2011). Studies performed in *Burkholderia cepacia* grown in exopolysaccharide-producing liquid medium suggested that cepacian productions starts when bacteria enter in the stationary phase and it appears to be a stable phenotype (Richau et al., 2000). Mutants defective for the *bce* genes have shown the importance of cepacian to the formation of mature biofilms, since mutants unable to produce cepacian exhibited a much thinner biofilm, when compared with the parental strain (Ferreira et al., 2007) and this observation lead to the hypothesis that by promoting the formation of mature biofilms, exopolysaccharides can enhance the ability of bacteria to thrive in CF lung, leading to the impossibility of an efficient eradication of Bcc infections (Ferreira et al., 2011).

Associated with the production of exopolysaccharide is the mucoid phenotype, which results in colonies with shiny morphology in solid agar media, as opposed to its isogenic nonmucoid phenotype variants unable to produce the exopolysaccharide, resulting in rough colonies in solid agar media (Bernier et al., 2007). Often, colonizer species of CF lungs, such as *P. aeruginosa* and bacteria belonging to the Bcc are mucoid but can suffer alterations on the expression of their surface determinants to facilitate adaptation to the conditions experienced in this microenvironment, switching between mucoid and nonmucoid phenotypes (Zlosnik et. al, 2008; Silva et al., 2018).

The importance of this phenotypic variation for the adaptation of bacteria to the CF lungs microenvironment was demonstrated in two pairs of sequential isolates, with the first pair of isolates belonging to *Burkholderia cenocepacia* and the second pair belonging to *Burkholderia multivorans*. In the first pair, the isolates were recovered within 10 months of each other and the authors found mutations in the quorum sensing regulator, which encoded the gene *cepR*, and also a deletion of a genomic region belonging to the pathogenicity island, that was present in the mucoid isolate (Zlosnik and Speert, 2010; McKeon et al., 2011). In the second pair of sequential isolates, which were recovered in a period of 6 months, the mucoid isolates were more susceptible to nutrient depletion medium, produced less biofilm, presented a higher expression of genes associated with virulence, higher growth rate under microaerophilic condition, increased motility and more virulence in the animal infection model *Galleria mellonella* (Silva et al., 2011).

In *Pseudomonas aeruginosa*, it is known that the initial colonization of the CF lungs is made by nonmucoid strains that during the course of the infection switch to a mucoid phenotype that starts producing alginate, which is associated with a higher risk of morbidity and mortality (Pedersen et al., 1992). In order to evaluate if the same phenotype variation occurred in the *Burkholderia* genus, a study was conducted with 560 Bcc sequential isolates, recovered from 100 CF patients that were screened for EPS production through analysis of the mucoid phenotype in EPS-producing medium (Zlosnik et al., 2008). The results not only confirmed the ability of the majority of isolates to produce EPS, but also resulted in the observation of fifteen mucoid phenotypic variations, with thirteen phenotypic variations from mucoid-to-nonmucoid in *Burkholderia multivorans, Burkholderia cenocepacia* and *Burkholderia vietnamiensis*, and two phenotypic variations from nonmucoid-to-mucoid *in Burkholderia cenocepacia* and *Burkholderia vietnamiensis*. Since it was already known from previous studies that the phenotypic variations in *P. aeruginosa* typically occurred from a nonmucoid to a mucoid phenotype (with production of an exopolysaccharide, alginate), the high number of mucoid-to-nonmucoid phenotypic variations registered in *Burkholderia cenocepacia*, the most virulent species of the Bcc, did not match with the established pattern for *P. aeruginosa*, suggesting that as opposed to *P. aeruginosa*, the nonmucoid isolates of *B. cenocepacia* were probably associated with an increase in disease severity and the mucoid isolates with long term persistence in the CF lungs (Zlosnik et al., 2008).

The relationship of the mucoid phenotype switch in the adaptation to the CF lungs and disease progression it is not known yet, since experimental data has suggested that bacteria producing the EPS are more favorable to persist in the lungs, since they can avoid the immune system (Bylund et al., 2006). On the other hand, studies have suggested that bacteria unable to produce the exopolysaccharide have a higher ability to produce more biofilm and also have higher survival rate under nutrient limitation (Silva et al., 2011) meaning that they could also be adapted to persist in the lungs, although when comparing the virulence of the mucoid and non-mucoid phenotype, studies performed in animal models of infection, such as *Galleria mellonella*, the BALB/c mouse pulmonary infection model or the gp91$^{phox-/-}$ mouse, all showed a higher mortality for the *Burkholderia* strains producing the exopolysaccharide, suggesting that the mucoid strains are more virulent than the nonmucoid strains (Conway et al., 2004; Silva et al., 2011; Sousa et al., 2007). These observations suggest that the production of exopolysaccharides by *Burkholderia* is tightly regulated as a response to external conditions, which can be crucial to bacterial colonization and adaptation to different environments and hosts.

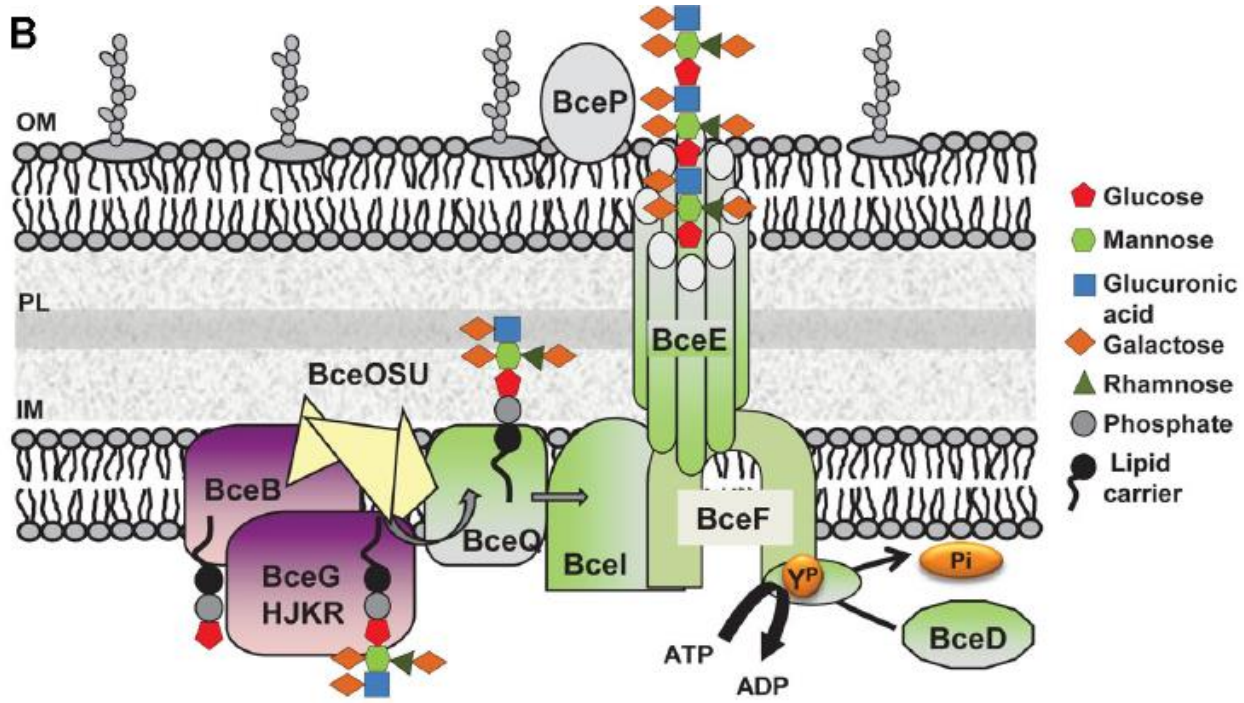


Figure 3 – Metabolic pathway for the biosynthesis of the exopolysaccharide cepacian by *Burkholderia*, with the different enzymes involved in cepacian assembly, polymerization and export to the extracellular environment (adapted from Ferreira et al., 2011).

1.3. Regulation of cepacian biosynthesis

Although the identified *bce*-I and *bce*-II gene clusters encode most of the structural proteins necessary for the synthesis of cepacian, little is known about the genes that are associated with the regulation of cepacian biosynthesis.

In *Pseudomonas aeruginosa*, studies have shown that the *algU* and *mucA* genes encode a transcription factor and anti-sigma factor, respectively that appear to be responsible for the control of the biosynthesis of alginate, through the regulation of its operon (Ramsey and Wozniak, 2005). Contrastingly in *Burkholderia cepacia*, studies involving a knockout mutant for RpoE protein, the *B. cepacia* AlgU homolog proved that this gene was not needed for the synthesis of cepacian by the knockout mutant (Devescovi & Venturi, 2006), suggesting that the regulation of its production was related with another molecular mechanism that was still unknown.

Some studies showed evidences of the involvement of quorum sensing regulation at the transcriptional level and protein tyrosine phosphorylation/dephosphorylation at post-translational level. Studies on the plant-associated *Paraburkholderia kururiensis* M130, *Paraburkholderia xenovorans* LB400 and *Paraburkholderia unamae* MTI-641 indicated that the N-acyl homoserine lactones (AHL) synthase BraI and the quorum sensing transcriptional regulator BraR might be involved in the regulation of the EPS biosynthesis, since knockout mutants for the *braI* and *braR* genes showed a decrease in mucoidy and in the total sugar content, when compared with the wild type and this phenotype was successfully reverted through the artificial supplementation of 3-oxo-C14-HSL or 3-oxo-C12-HSL to the culture medium, suggesting that quorum sensing positively controls the production of exopolysaccharide in this plant-associated species (Suarez-Moreno et al., 2010; Sawana & Gupta, 2014). Nevertheless, the BraI/BraR system is not conserved in the *Burkholderia* genus and 3-oxo-AHLs are not produced by several species, such as the ones belonging to the *Burkholderia cepacia* complex which have a quorum sensing system based on CepI and CepR, that produces and responds to C6-HSL and C8-HSL (Sokol et al., 2003).

Another mechanism that could be involved in the regulation of cepacian biosynthesis at the transcriptional level is mediated by the RNA chaperone Hfq, through the regulation of target mRNAs by small regulatory non-coding RNAs (Sousa et al., 2010). The deletion of the *hfq* gene on the strain *Burkholderia cepacia* IST408, resulted in a significant decrease in the production of cepacian, but it is not known if this result is due to the effect of a small RNA molecule or if it is related with pleiotropic effects caused by the loss of the *hfq* gene.

In the post-translational regulation of cepacian, it is known that cycles of phosphorylation and dephosphorylation, by BY-kinases and phosphotyrosine phosphatase proteins such as BceF and BceD, respectively, control the amount and the molecular weight of several bacterial exopolysaccharides (Vincent et al., 2000; Wugeditsch et al., 2001), but the molecular

mechanisms behind regulation of cepacian by these two proteins has not yet been identified (Ferreira et al., 2011).

Another molecular mechanism that could be related with the regulation of exopolysaccharide production is the binding of second messengers to the specific targets of the pathways involved in exopolysaccharide production, since it is now accepted that second messengers, like c-di-GMP, have an important role in the regulation of biofilm formation in many species of bacteria, because experimental data suggest that high intracellular c-di-GMP induces the production of extracellular biofilm matrix components (in which the exopolysaccharide is included), and low intracellular concentration of this second messenger promotes single cell motility and suppresses the production of the biofilm components, through binding of specific effectors that can be proteins or RNA, altering their structure or output function (Ryan et al., 2012).

Studies made by Fazli and collaborators (2017) showed that the second messenger c-di-GMP could bind to the bacterial enhancer-binding protein BerB (for *Burkholderia* exopolysaccharide regulator B), which in turn could also bind to the promoter region of the gene *berA*, encoding the transcription factor protein BerA. Upon binding of c-di-GMP to BerB, this protein activated the expression of the *berA* gene, that was mediated through the binding of an alternative sigma factor RpoN and resulted in an increase of the expression levels of the protein BerA, that stimulated the production of a biofilm-stabilizing exopolysaccharide termed *bepA-L* (for *Burkholderia* exopolysaccharide locus A to L) (Fazli et al., 2013; Fazli et al., 2011; Fazli et al., 2017). Whether c-di-GMP is also involved in regulating cepacian biosynthesis is unknown.

Two-component regulatory systems were also implicated in regulation of cepacian biosynthesis One of them is the NtrB Histidine Kinase and the NtrC Response regulator. The Ntr system monitors the intracellular ratio of glutamine to α-ketoglutarate. Under nitrogen limiting conditions, the sensor Histidine Kinase NtrB phosphorylates the Response Regulator NtrC, which together with the alternative sigma factor σ^{54} activates transcription (Reitzer, 2003). *Burkholderia cenocepacia* H111 mutants in sigma factor σ54 and NtrC displayed decreased expression of the bce-I and bce-II gene clusters, as well as reduced amount of cepacian production (Lardi et al., 2015; Liu et al., 2017). The second regulatory system required for cepacian biosynthesis is the OmpR/EnvZ, with OmpR being the response regulator and EnvZ the sensor histidine kinase (Silva et al., 2018). Further detail on these genes will be given in the next section.

1.4. Two-Component Regulatory Systems

Free living organisms, such as bacteria, have the capacity to modulate their gene expression in response to changes in their environment. They are equipped with tightly regulated molecular mechanisms that allows them to activate or repress the appropriate set of genes, in order to adapt and colonize new ecological niches, which sometimes are associated with inhospitable and harsh conditions for the organisms (Mitrophanov & Groisman, 2008).

A well-studied molecular mechanism is the two component regulatory system (TCS), which represents the predominant system in which bacteria transduce extracellular signals (Hoch 2000; Stock et al. 2000; Mascher et al., 2006), having been identified in eubacteria, archaea and also in a reduced number of eukaryotes. These TCS are highly sophisticated and have been incorporated in a variety of cellular signaling pathways (Stock et al., 2000), serving as a molecular response from the organism to physical and chemical changes in the environment, such as temperature, pH, oxygen pressure, osmolarity, autoinducer compounds, the redox state of electron carriers and the contact with host cells (Beier et al, 2006). This occurs through a basic stimulus-response coupling mechanism, in which an organism sense and respond to variations in those conditions. As the name suggests, the TCS consists of two mainly components: A Histidine Kinase (HK) protein, which contains a conserved kinase residue and a Response Regulator (RR) protein, which contains a conserved regulatory domain. When extracellular stimuli is sensed by the HK, it autophosphorylates and transfers the phosphoryl group to the RR protein, as it can be seen in Figure 4, activating the downstream effector domain, that will produce a specific response to the stimuli, such as DNA binding and transcriptional control, perform enzymatic activities, bind to RNA, or engage in protein–protein interactions (Gao et al. 2007).

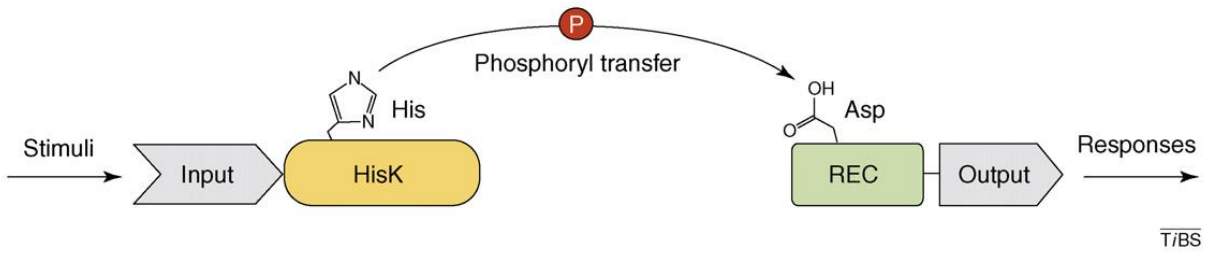


Figure 4 – Schematic representation of the functioning of a typical two-component response regulator. External stimuli is sensed by the sensor Histidine Kinase (in yellow), which autophosphorylates and transfers a phosphoryl group to the aspartate residue of the Response Regulator (in green), activating it (adapted from Gao et al. 2007).

1.4.1. EnvZ/OmpR transduction system

The OmpR is one of the most studied transcription factors, being an example of a typical response regulator involved in a widespread TCS. The family of OmpR proteins is present in several types of bacteria, suggesting that the OmpR-like proteins are one of the most common transcription factors found in bacteria (Mizuno et al., 1997).

This two-component regulatory system comprises the HK EnvZ and the RR OmpR which controls osmoregulation of bacteria, through porins OmpC and OmpF, at the transcriptional level. The HK EnvZ functions as a sensor, monitoring the external osmolarity and communicating this information to the RR OmpR, through phosphorylation and dephosphorylation. The phosphorylated OmpR (OmpR-P) serves as a transcription factor and regulates the expression of the *ompF* and *ompC* genes, which encode the porins OmpC and OmpF, proteins that assemble as trimers in the outer membrane of Gram-negative bacteria, forming aqueous channels that allow the passive diffusion of small hydrophilic molecules across this hydrophobic barrier. The expression level of the genes encoding porins OmpF and OmpC is highly influenced by a wide variety of environmental conditions, such as pH, osmolarity, temperature, the concentration of some toxins and the growth phase (Mattison et. al, 2002; Pratt et al., 1996).

It is known from previous studies that *Burkholderia* mucoid clinical isolates, able to produce the exopolysaccharide cepacian, if grown under stress conditions, such as prolonged incubation, subinhibitory concentration of antibiotics, extreme temperatures and osmotic, nitrosative and/or oxidative stresses, can undergo a phenotype switch, originating nonmucoid variants that are unable to produce the exopolysaccharide (Silva et al, 2013). In a recent study it was determined that one of the major causes of the emergence of the nonmucoid phenotype was associated with nonsynonymous mutations and small *indels* in the gene encoding the RR OmpR (Silva et al, 2018). The authors randomly chose fourteen nonmucoid variants derived from *Burkholderia multivorans* isolates, to evaluate the hypothetic molecular mechanisms underlying the phenotype switch from mucoid-to-nonmucoid and through whole-genome sequencing, they were able to compare the genome sequence of the nonmucoid with the previously sequenced genome of its respective mucoid ancestor, to identify possible genes that could be associated with the phenotype switch described. They found out that one of the nonmucoid variants had accumulated a frameshift mutation in the *bceF* gene encoding a tyrosine kinase, which is one of the enzymes involved in the biosynthesis of cepacian, and that the other thirteen nonmucoid variants had point mutations and small insertions and deletions in a gene within the locus tag BMD20-RS11675. This gene encoded a putative RR protein that has homology with the RR OmpR from the TCS EnvZ/OmpR and it was located in chromosome 1 of *B. multivorans* and downstream of its transcription was the gene BMD20-RS11670, which encoded a putative sensor HK that had homology with the EnvZ HK. This genetic organization appeared to be highly conserved within more than 800 sequenced strains of *Burkholderia*, with the amino acid identities of all OmpR proteins ranging from 95 to 100%. The closest homologs of *Burkholderia multivorans* OmpR in

well-studied bacteria include the OmpR proteins from *Escherichia coli*, *Salmonella enterica* and *Shigella flexneri*. The proteins AruR, ArcA, WalR, VirG and PetR share a protein identity of 30 to 45% within these species and have conserved domains such as the N-terminal receiver domain with the phosphorylation site (D57), the site of Mg^{2+} coordination (13DD14), T85 and K107 that interact with the phosphoryl group and Y104, which functions as a rotamer in the OmpR of *E. coli*. The C-terminal effector DNA-binding domain is also well conserved within the species, containing the hydrophobic core of OmpR from *E. coli.* The amino acids that are identified has being important for DNA binding in *E. coli*, also appear to be conserved in the homologs, including OmpR_BURMU. The mutations that resulted in the emergence of the nonmucoid variant were grouped in three different groups, depending on their location (Figure 5). The first group is related with the DNA-binding domain that was disrupted in some mutants; the second group is related with mutations in the receiver domain, with the mutant BM7-nmv1 having a 3-bp deletion, which removed valine at position 11, near the aspartate residues involved in Mg^{2+} ion coordination during phosphorylation, BM11L-nmv3 having a 15-bp deletion, which removed the amino acids 52DLLVL56, that formed a beta sheet with the aspartate residue at position 57, where phosphorylation takes place. The third group accumulated several indels of variable size that caused frameshift mutations and truncated/non-functional OmpR proteins.

The loss of a functional OmpR protein appeared to be related with an increase in growth, adhesion to lung epithelial cells and biofilm formation in a high osmolarity medium. An increase in the swimming and swarming motilities was also reported for the *Burkholderia multivorans* mutants lacking a functional OmpR. The phenotypes of antibiotic resistance, biofilm formation at low osmolarity and virulence in the infection model *Galleria mellonella* suffered a significant decrease in the *Burkholderia multivorans* mutants, when compared with the parental strain (Silva et al., 2018). A transcriptomic analysis showed that the loss of the *ompR* gene affected the expression of 701 genes, with many associated with outer membrane composition, motility, stress response, iron acquisition and uptake of nutrients. The authors also performed *in trans* complementation with the native *ompR* gene in the nonmucoid variants originated from different types of stress conditions (such as prolonged stationary phase for 14 days at 37ºC, 42ºC and 7 days under subinhibitory concentrations of ciprofloxacin, amikacin and 1% NaCl), to evaluate if the nonmucoid phenotype could be reverted. Production of the EPS cepacian was restored in all the variants, confirming the reversion to the mucoid phenotype. These results suggested that the accumulation of mutations in the *ompR* gene is independent of the stress condition that originated the nonmucoid variant and it also suggested that the *ompR* locus is one of the most common targets of mutations, during the mucoid-to-nonmucoid phenotype switch (Silva et al., 2018).

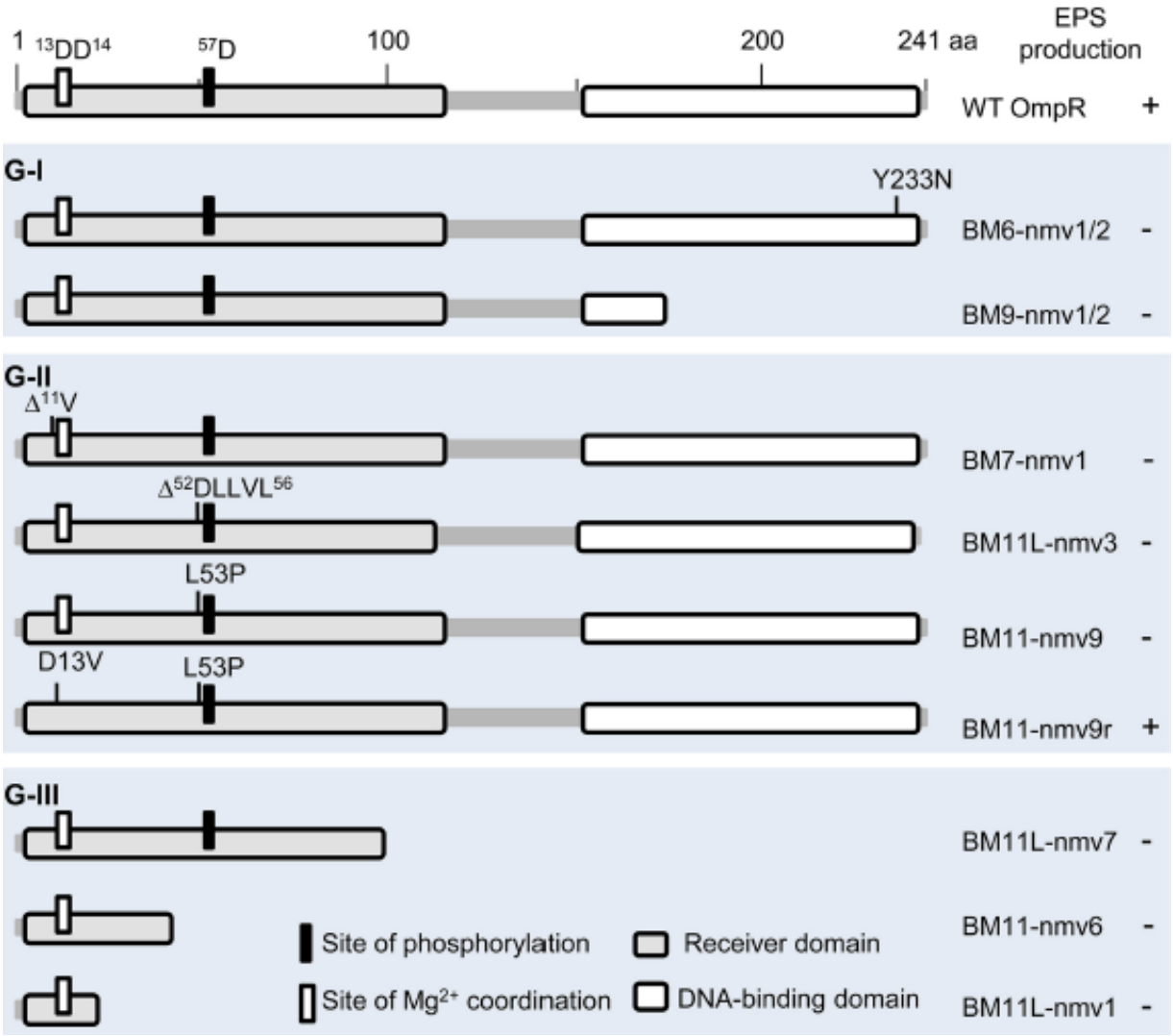


Figure 5 – Schematic representation of the three different groups of mutations, based on the location of the mutation in the *ompR* gene. Effect of mutations in EPS production is also indicated (adapted from Silva et al., 2018).

1.4.2. FixL/FixJ transduction system

The FixL/FixJ was described for the first time as an oxygen sensitive two-component regulatory system involved in the regulation of symbiotic nitrogen fixation by *Sinorhizobium meliloti*. The activation of the system is dependent on the concentration of environmental oxygen available in the gas phase. In microaerobic conditions, the Histidine Kinase FixL phosphorylates the Response Regulator FixJ, resulting in the transcription of the *nifA* and *fixK* genes. In the presence of oxygen, the FixJ protein remains in an unphosphorylated and inactive state, as it can be seen in Figure 6. The FixJ protein is organized in a typical modular arrangement, with a phosphorylatable N-terminal receiver domain and a C-terminal transcriptional activator domain. The FixJ receiver domain inhibits the latent activity of the C-terminal domain within the native protein, and this inhibition is relieved by phosphorylation of the receiver domain. Phosphorylation also induces dimerization of the response regulator, a process mediated by the receiver domain that significantly enhances the affinity of the response regulator to the *fixK* promoter (Birck, 1999; Tuckerman et al., 2001).

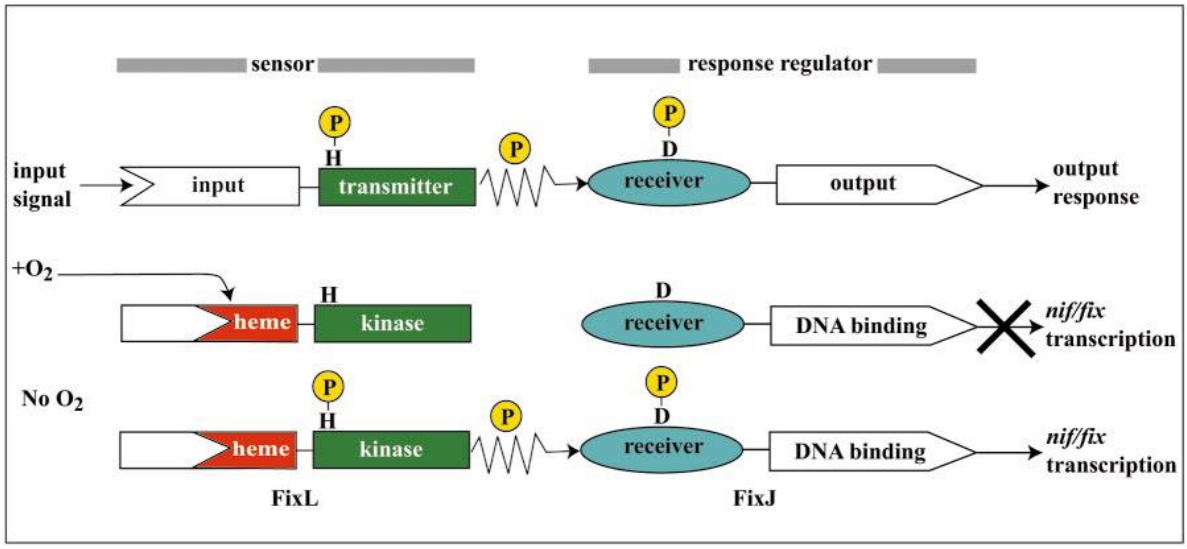


Figure 6 – Schematic representation of the functioning of the FixL/FixJ two-component regulatory system. The sensor Histidine Kinase FixL has a heme-binding domain (in red), that allows oxygen binding, inhibiting the autophosphorylation of the HK FixL. When oxygen is absent, the HK FixL can autophosphorylate the RR FixJ, which in turn promotes the transcription of the genes involved in the fixation of atmospheric nitrogen (adapted from Tuckerman et al., 2001).

Schaefers and his team sequenced the genomes of 112 *Burkholderia dolosa* isolates recovered from 14 CF patients, over 16 years, from the Boston Children Hospital's outbreak and discovered that a subset of genes having three or more point mutations contained almost 18 times more nonsynonymous mutations. These unexpected results lead to the hypothesis that those genes were under positive selection. One of the genes, BDAG_01161 (AK34_969), had a total of 17 nonsynonymous mutations and encoded a protein that was similar to FixL, the HK of the two-component regulatory system in *Sinorhizobium* and *Caulobacter* (Lieberman et al., 2011). The homolog proteins FixJ and FixL of *Burkholderia dolosa*, had domains that appeared to be consistent with their putative function as a two-component regulatory system, with FixL predicted to have a heme-binding site, where oxygen binding could occur. *Burkholderia dolosa fixK* (BDAG_04180, AK34_4936) had a homology with *Burkholderia cenocepacia* BCAM0049 that was found to be upregulated during growth in low concentrations of oxygen.

Since a high number of nonsynonymous mutations was also recently reported in the *Burkholderia multivorans* FixL homolog BMD20_10585, in a whole-genome sequencing study of isolates recovered from CF patients with chronic infections (Silva et al., 2016), it is suggested that the FixL/FixJ TCS may have a similar role in the three most common Bcc species isolated from CF lungs (*Burkholderia cenocepacia, Burkholderia multivorans*, and *Burkholderia dolosa*).

As previously reported, the FixL/FixJ TCS can induce the expression of genes required for atmospheric nitrogen fixation, under microaerobic environmental conditions, in *Rhizobium* and *Caulobacter*, but the majority of *Burkholderia* species in the Bcc group lack the genes necessary for nitrogen fixation, with only *Burkholderia vietnamiensis* and *Burkholderia* outside of the Bcc group having the ability to fixate nitrogen from the atmosphere.

Schaefers and his team tried to gain insights into the functions of *Burkholderia dolosa* FixJ/FixL and its association with pathogenesis and found out that the FixL/FixJ TCS was induced by conditions of low oxygen, regulated a wide variety of genes and was critical for pathogenicity *in vivo* and intracellular invasion *in vitro*. Indeed, *Burkholderia dolosa* mutants lacking the TCS FixL/FixJ and growing in conditions of low oxygen were unable to induce the transcription of a *fixK-lacZ* reporter gene, had an altered expression of ~11% of the genome and their virulence was attenuated in the murine lung infection model. The FixL/FixJ mutants also had decreased motility and were less invasive, when compared with the parental strain, but were able to make more biofilm (Schaefers et al., 2017).

These results were consistent with the previous reports that the *Burkholderia cenocepacia fixK* homolog (BCAM0049) was upregulated when grown in conditions of low oxygen (Pessi et al., 2013). Other FixL homologs in pathogenic Gram-negative bacteria include the one from *Brucella*, which was discovered to be needed for intracellular survival (Roset et al., 2013) and the one from *Pseudomonas aeruginosa*, which has a FixL homolog called BfiS (biofilm initiation sensor) and a FixJ homolog, BfiR, which were found to be critical for the development of a mature biofilm, because mutants lacking the TCS BfiS/BfiR registered a drastic decrease in virulence in the

murine infection model, when compared with the *P. aeruginosa* parental strain (Petrova & Sauer, 2009).

Interestingly, in *Burkholderia dolosa*, the deletion mutant for the *fixLJ* genes had an increased ability to make biofilm, when compared with the parental strain, which is the opposite result of the deletion of the *bfiS*/*bfiR* pathway in *P. aeruginosa*.

1.5. Aims of this work

It is known through several studies that bacteria belonging to the *Burkholderia cepacia* complex alter their genetic and phenotypic profiles upon adaption to the microenvironment that they face upon colonization of the lungs of CF patients. One genetic alteration that Bcc bacteria strains suffer is a morphotype switch, in which they change from a mucoid to a nonmucoid phenotype (Zlosnik et. al, 2008; Silva et al., 2018). This phenotypic switch is important in the clinical context, because it has been associated with rapid decline in lungs function (Zlosnik et al., 2011). However, the genes and molecular mechanisms that could trigger it within the host remain mostly unknown. Since chronic infections are easily established in the lungs of CF patients and this morphotype switch can occur over the course of infection by *B. multivorans,* Moreira and her team made efforts to characterize a genomic and functional evolution of a chronic infection of a CF patient, in which mucoid-to-nonmucoid transitions had already occurred (Silva et. al, 2016). In that work, sequential clonal isolates that had been sampled over 20 years from the lungs of a CF patient were sequenced and their mutations were mapped against the first isolate (BM1), revealing the mutations that each of the 22 isolates has accumulated over the course of the chronic infection. This allowed the construction of a phylogenetic tree that defined the existence of four different clades, based on the type of mutations of each isolate. Clade C1 includes the isolates BM1, BM2, BM3, BM5; clade C2 includes the isolates BM4 and BM7; clade C3 includes the isolates BM6, BM11,BM22,BM12, BM8, BM18, BM9, BM14, BM20, BM21, BM16, BM17 and BM19 and finally clade C4, including isolates BM10, BM13 and BM15 (Figure 7). In a follow-up study, Silva and co-authors, obtained *in vitro* nonmucoid variants from mucoid isolates of clades C2 and C3, by exposing these *B. multivorans* isolates to stress conditions known to trigger mucoid-to-nonmucoid morphotype switch. After sequencing the genome of selected nonmucoid variants and mapping it against their parental strains, the mutations were mapped onto the *ompR* gene (Silva et al., 2018). Later on, Bica (2018) obtained *in vitro* nonmucoid variants from clade C1 isolate BM1 and mutations mapped into *fixJ* and *fixL* genes, encoding another two-component regulatory system. Since isolates from clades C2 and C3 include later isolates when compared to C1 isolates, it could be that this phenotypic transition of mucoid-to-nonmucoid dependent on OmpR or FixJ was caused by a particular genetic background. Figure 7B shows several mutations present in all isolates from clades C2, C3 and C4, but not C1, and one could be involved in mediating mucoid switch via OmpR/EnvZ or FixJ/FixL.

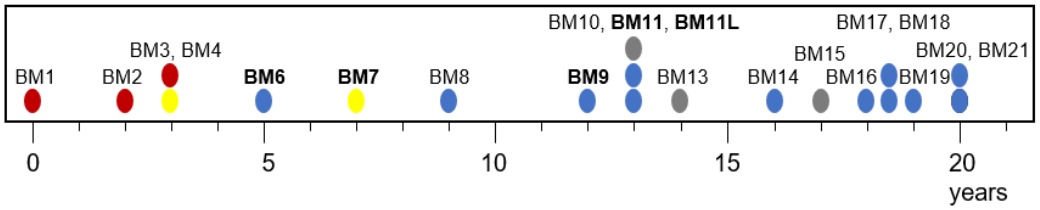
Since nonmucoid variants are yet to be obtained *in vitro* for the isolates of clade C4 (BM10, BM13 and BM15), the primary aim of this work was to evaluate if this mucoid-to-nonmucoid switch was also occurring in isolates from clade C4, and if it was the case, to investigate if the mutations related with the emergence of nonmucoid variants in this clade were related to mutations in clade C1 or clades C2 and C3. To do this, the following experiments were designed:

- Expose *B. multivorans* BM10 isolate recovered 13 years after BM1 to stress conditions, to see if the mucoid-to-nonmucoid switch was also occurring.

- Chose random colonies that might be of interest (appear to be dry or less mucoid in solid media) for whole genome sequencing, to reveal the mutations that they have accumulated.
- Phenotypic characterization of the possible mutants, through analyzing their growth, antibiotic resistance, biofilm formation, and swimming and swarming motilities.

Additionally, phenotypic characterization of four nonmucoid variants (BM1-1, BM1-2, BM1-3, BM1-4) previously obtained *in vitro* from the mucoid isolate BM1 (Bica, 2018) was also performed, through analyzing their growth, antibiotic resistance, biofilm formation and swimming and swarming motilities.

A



B

Locus_tag	Gene	Annotation	Mutation Effect
Bmul_5122	rpfR	diguanylate cyclase/ phosphodiesterase (BDSF receptor)	E185D
Bmul_4619	accD	acetyl COA carboxylase, carboxyl transferase subunit beta	T240M
Bmul_4436		transcriptional regulator/2-aminoadipate aminotransferase	A362A
Bmul_4315		two component heavy metal response transcriptional regulator	E229D
-		LysR transcriptional regulator	A24E
Bmul_3456		DNA-binding protein	I228M
Bmul_1333		OmpR-like response regulator	- (intergenic)
Bmul_1680		ABC transporter permease	P12T
Bmul_1751	emrA	multidrug resistance efflux pump membrane protein	Q97H
Bmul_2184	plsX	glycerol-3-phosphate acyltransferase	- (intergenic)

Figure 7- Temporal distribution of the *B. multivorans* mucoid clinical isolates recovered from a single CF patient with indication of the clade inferred by phylogenetic analysis (adapted from Silva et al., 2016) (A). The four clades are **C1 (red)**, **C2 (yellow)**, **C3 (blue)**, and **C4 (green)**. Mutation common to clades C2, C3 and C4, but absent from isolates of clade C1. The genes locus_tag used is from *B. multivorans* ATCC 17616 **(B).**

2. Materials and methods

2.1. Bacterial strains and growth conditions

The *Burkholderia* strains were grown in Lennox Broth (LB: 5 g/L NaCl, 5 g/L yeast extract: 10 g/L and 10 g/L tryptone) or in SM medium (12.5 g/L $Na_2HPO_4.2H_2O$, 3.0 g/L KH_2PO_4, 1.0 g/L K_2SO_4, 1 g/L NaCl, 20 g/L mannitol, 1.0 g/L casaminoacids, 1.0 g/L yeast extract, 0.2 g/L $MgSO_4.7H_2O$, 0.001 g/L $FeSO_4.7H_2O$), used to induce the production of EPS (Silva et al. 2013). *E. coli* strains were grown in LB medium. The strains, when used (table 1) were maintained on LB plates. All the strains were conserved at -80ºC, with 30% glycerol.

Table 1. Bacterial strains and plasmids used in this work

Bacterial strains – *Burkholderia multivorans*		
Bacterial strains or plasmids	**Description**	**Reference or source**
Burkholderia multivorans P205-1 (BM1)	Cystic fibrosis clinical isolate from Canada (mucoid) Date of isolation: 30-11-1993	Silva et al. 2016
Burkholderia multivorans P205-10 (BM10)	Cystic fibrosis clinical isolate from Canada (mucoid) Date of isolation: 01-06-2006	Silva et al. 2016
BM1-1	Nonmucoid variants derived from BM1 after 21 days at 37ºC under nutrient starvation	Bica (2018)
BM1-2		Bica (2018)
BM1-3		Bica (2018)
BM1-4		Bica (2018)
C1	Less mucoid variants derived from BM10 after 21 days at 37ºC, under nutrient starvation.	This study
C2		This study
C5		This study
C6		This study
C8		This study
C9		This study
C11		This study
C12		This study
C13		This study
C15		This study
Plasmids		
pBBR1MCS	Broad-host-range cloning vector, 4.7 kb, *lacZ*$^+$, *mob*$^+$, Cm^r	Kovach et al. 1994

pLM014-5	pBBR1MCS_derivative expressing gene *ompR* (Bmul_1131) of *B. multivorans* ATCC17616 under control of its own promoter, Cm^r	Silva et al. 2018
pRK2013	Mobilizing vector, ColE1 tra^+ (RK2), Kan^r	Figurski and Helsinki, 1979

Abbreviations: Cm^r, chloramphenicol; Kan^r, kanamycin.

2.2. Inducing the morphotype switch under prolonged incubation

In order to retrieve nonmucoid or slightly less mucoid variants derived from mucoid *B. multivorans* BM10, this strain was inoculated in 5 mL of liquid SM medium (OD_{640nm} of 0.1) and triplicates were maintained statically for 3 weeks (21 days) at 37ºC. After this period of time, an aliquot was taken, serially diluted and spread onto the surface of YEM agar plates (0.5 g/L yeast extract, 4 g/L mannitol and 15 g/L agar) and incubated at 37ºC for 2 days. The colonies that displayed a slightly less mucoid or nonmucoid morphotype, were kept.

2.3. Growth rate and doubling time determination

The strains were grown at 37ºC, 250 rpm, for 24h in 50 mL of LB or SM liquid medium. Growth rates and doubling times were obtained from the exponential phase of growth. Three independent experiments were performed.

2.4. Exopolysaccharide production

The amount of EPS produced was assessed through the dry-weight of ethanol-precipitated polysaccharide recovered from 50 mL cultures of the different strains grown in liquid SM medium over 4 days at 37ºC, 250 rpm, as described in Ferreira et. al 2007. Bacterial cells present in the cultures were separated through centrifugation at 9000 rpm (Eppendorf) for 15 minutes. The EPS was then precipitated from the cell-free supernatants by the addition of 3 volumes of ethanol 96%. After collection and evaporation, the EPS was weighted. Three independent experiments were performed.

2.5. Antimicrobial susceptibility

To assess the antimicrobial susceptibility of the strains, the agar disc diffusion method (Bauer et. al. 1996) was used. Paper discs containing ciprofloxacin (5 µg), piperacillin (75 µg) + tazobactam (10 µg), aztreonam (30 µg) and kanamycin (30 µg) were used. The discs were placed onto the surface of Mueller-Hinton (Sigma-Aldrich) agar plates that had been previously inoculated with 100 microliters of a suspension of bacterial cells at an OD_{640nm} of 0.1. The cells were grown at

37ºC, 250 rpm, after overnight inoculation. Growth inhibition diameter was measured after 24 h of incubation at 37ºC. Three independent experiments were performed, at least.

2.6. Biofilm formation

To assess biofilm formation, the method previously described by Ferreira et. al. 2007 was used. Cultures of the different strains were grown in LB liquid medium, overnight, at 37ºC, 250 rpm. 200 microliters of these cell suspensions, with a standardized OD 640nm of 0.05, were used to inoculate the wells of 96-well polystyrene microtiter plates. After, the plates were statically incubated at 37ºC for 48 h. The media containing the unattached bacterial cells was removed and the wells were subsequently cleaned three times with 200 microliters of saline solution (0.9%) per well. The remaining adherent bacteria were stained with 200 microliters of crystal violet solution 1% (wt/vol) for 20 minutes at room temperature. After this period, the wells were cleaned, three times, with 200 microliters of saline solution (0.9%) per well. The wells were then solubilized in 200 microliters of 96% ethanol, each, and the biofilm formation was quantified through measuring the absorbance of the ethanol solution at 590 nm using a microplate reader (Spectrostar nano, BMG LabTech). Three independent experiments were performed.

2.7. Swimming and Swarming motilities

To evaluate the swarming motility of the different strains under study, swarming agar plates containing 0.04% (wt/vol) tryptone, 0.01% (wt/vol) yeast extract, 0.0067% (wt/vol) CaCl2, 0.6% (wt/vol) bacto agar (Difco) (Silva et al. 2018) were spot inoculated with 5 microliters of culture (OD 640nm of 1.0). After, the plates were incubated for 48 h, at 37ºC and the diameter of the swarming motility was measured. Three independent experiments were performed, at least.

To evaluate the swimming motility of the different strains under study, swimming agar plates containing 1% (wt/vol) tryptone, 0.5% (wt/vol) NaCl, 0.3% (wt/vol) noble agar (Difco) (Kamjumphol et al., 2013) were spot inoculated with 5 microliters of culture (OD 640 nm of 1.0). After, the plates were incubated for 24 h, at 37ªC and the diameter of the swimming motility was measured. Tree independent experiments were performed, at least.

2.8. DNA manipulation techniques

Genomic DNA from *B. multivorans* was extracted by using the DNeasy Blood & Tissue kit of Qiagen using the recommendations of the manufacturer. DNA amplification by PCR and agarose gel electrophoresis were performed using standard procedures. Primers used to amplify an internal fragment of the *ldhA* gene of 555 bp were: Forward 5' - TTCAACCATGTCGACCTCGC – 3'; Reverse 5' – CTCTTCGTAGACGTCGAGGC – 3' (Gomes, 2018).

2.9. Genetic complementation

Complementation assays were performed by triparental conjugation. The receptor strains were nonmucoid variants C8 and C12 and the wild-type strain BM10. The donor was *E. coli* containing pBBR1MCS or pLM014-5 harboring the *ompR* gene. The helper was *E. coli* with pRK2013.

Selection was in YEM medium supplemented with 200 µg/mL of chloramphenicol and 40 µg/mL of gentamycin.

2.10. Genome sequencing and reference assembly

Genomic DNA of the nonmucoid variants was sequenced by the Illumina short reads technology at Instituto Gulbenkian de Ciência, Oeiras. Reads were concatenated and trimmed using software Sickle (Joshi & Fass, 2011) in order to remove primer adapters and low-quality sequences. Then, it was performed a reference assembly against the BM1 isolate genome using BWA-MEM (Burrows-Wheeler Aligner) (Li and Durbin, 2010). The *Geneious* software (Kearse et al. 2012) was used to map mutations against the reference genome.

2.11. Statistical analyses

The statistical analysis was performed by assessing the statistical significance of the difference in the data determined using the one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test or Turkey's multiple comparison test and using the Mantel-Cox test which were performed using GraphPad Prism software v.5.04 for Windows (GraphPad Software, San Diego California USA, www. Graphpad.com) (Swift, 1997). Differences were considered statistically significant, when the P-value was inferior to 0.05.

3. Results

3.1. Isolation of nonmucoid variants in *B. multivorans*

Since it has been shown through several studies that is possible, under laboratory stress conditions, to obtain nonmucoid variants from mucoid isolates retrieved from the lungs of cystic fibrosis patients, we tested whether clade C4 isolates, similarly to C1, C2 and C3 clades isolates could also originate nonmucoid variants, while being incubated under stress-inducing conditions. With that aim, three independent cultures of mucoid *B. multivorans* BM10, BM13 and BM15 (Figure 8-I) were statically incubated for 3 weeks (21 days). After that time of prolonged stationary phase, an aliquot was taken, serially diluted and inoculated onto EPS-producing Yeast Extract Mannitol (YEM) agar medium. Screening for nonmucoid variants revealed that among the colonies displaying a highly mucoid phenotype, there were smaller colonies that appeared to be slightly less mucoid or nonmucoid (Figure 8-II). Twelve of these colonies derived from isolate BM10 were kept for further studies – C1, C2, C4, C5, C6, C8, C9, C10, C11, C12, C13 and C15. From isolates BM13 and BM15, no nonmucoid variants were obtained, and that experiment was not repeated.

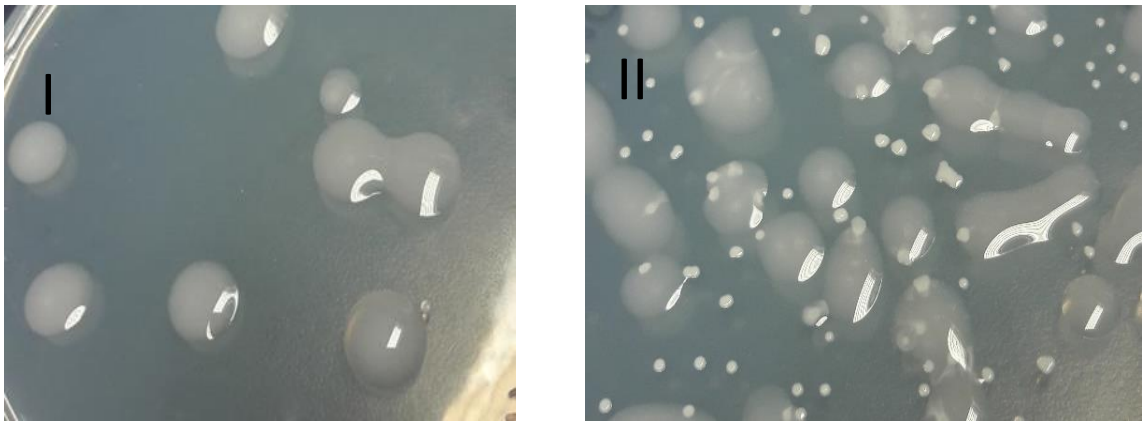


Figure 8 – Emergence of *B. multivorans* BM10 variants with different types of mucoid morphology after growth under stress induced conditions. Prior to the incubation, all colonies displayed the mucoid phenotype **(I),** but after 21 days in prolonged stationary phase, it was also possible to identify smaller colonies with reduced mucoidy, by comparison with the parental strain BM10 mucoid colonies **(II)**.

3.2. Confirmation of the identity of the nonmucoid variants by PCR

To assess if the twelve isolated variants belonged to the *Burkholderia* genus, a confirmation PCR was performed using a pair of primers that had been designed in a previous study to amplify a fragment of the *ldhA* gene, encoding D-lactate dehydrogenase of *B. multivorans* ATCC 17616 (Gomes, 2018). The results of the PCR determined that from the twelve initial isolates, ten (C1, C2, C5, C6, C8, C9, C11, C12, C13, C15) were most likely *Burkholderia* and the remaining two (C4 and C10) were considered contaminants, as it can be seen in Figure 9 by the lack of the 555 bp amplification product. Consequently, the ten nonmucoid variants were kept and had their DNA extracted for whole-genome sequencing.

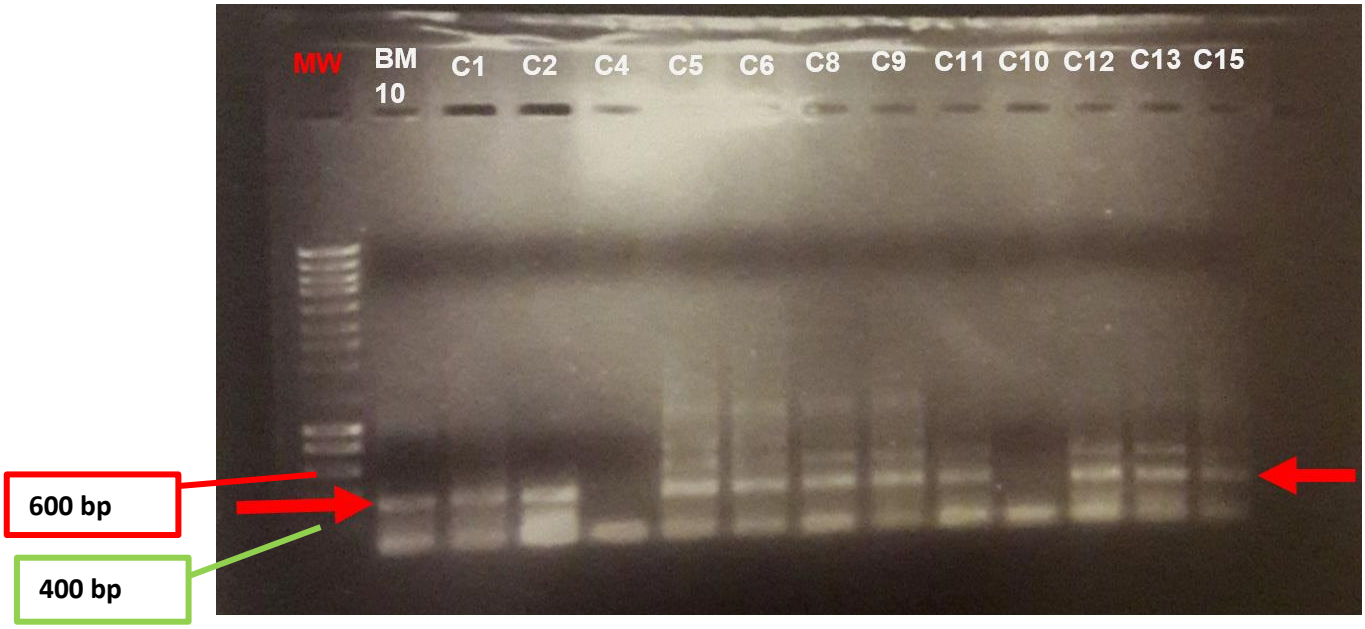


Figure 9 - Electrophoretic separation in 0.8% agarose gel of the PCR products corresponding to a fragment of the *ldhA* gene of *B. multivorans* with an estimated size of 555 bp (indicated by the red arrows)**.** The molecular weight (MW) marker used to estimate DNA size is the NZYDNA Leader III.

3.3. Assessment of exopolysaccharide production in solid and liquid media

With the aim of evaluating the mucoid phenotype, the ten *B. multivorans* BM10-derived variants, together with BM10 serving as positive control, were grown in EPS-producing YEM agar solid medium. All the ten variants seem to present a less mucoid phenotype, when compared with the parental strain *B. multivorans* BM10 (Figure 10), but they are not fully nonmucoid like the previous variants derived from C1, C2 and C3 clades isolates (Bica, 2018; Silva et al. 2018).

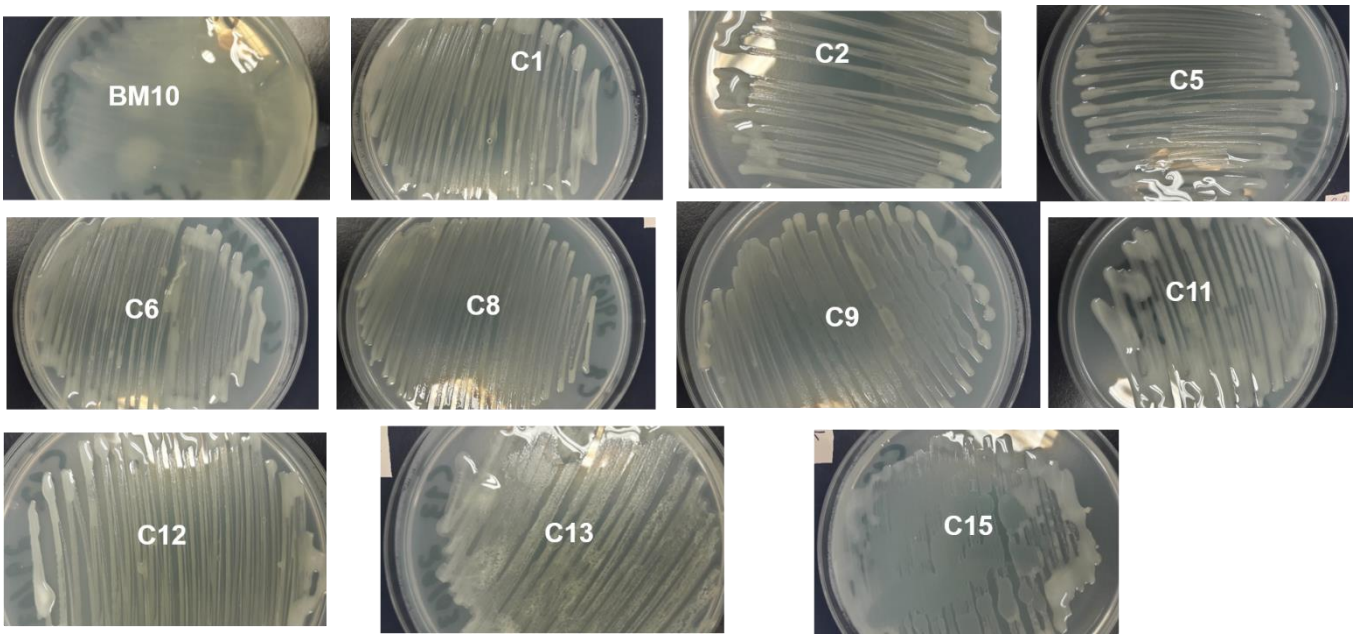


Figure 10 – Confirmation of the less mucoid phenotype of variant colonies derived from *B. multivorans* BM10. For each is shown a petri dish with YEM solid agar medium and it can be observed that the mutant colonies display a reduced mucoidy when compared with the parental strain (BM10).

As mentioned earlier, the mucoid morphotype of *B. multivorans* is associated with the production of exopolysaccharides, which might be cepacian alone or in combination with other polysaccharides. To test if the mutated variants under study and the ones derived from BM1 isolate (Bica, 2018), which were much less mucoid or nonmucoid when compared with their respective parental strain, were producing exopolysaccharides, cultures were grown in liquid SM medium for 4 days, at 37ºC and under 250 rpm orbital agitation. After this period, the cultures were centrifuged to remove cells, and the supernatant was precipitated with cold ethanol. Precipitates were left to dry under static incubation at 60ºC. The dry-weight of these precipitates was obtained and it was possible to observe that under these conditions, the *B. multivorans* BM10 wild-type strain produced approximately 12 g/L of a high-molecular weight EPS (Figure 11A). Regarding the BM10-derived variants none was producing high-molecular weight EPS, although C1, C5, C6, C9 and C11 showed a white powder after ethanol precipitation that might be low-molecular weight EPS. As control it was also assessed EPS production in BM1 and the four

nonmucoid variants (BM1-1, BM1-2, BM1-3 and BM1-4) with the results showing 8 g/L for BM1 mucoid parental isolate and no EPS production for the variants, confirming previous data (Figure 11B) (Bica, 2018). Together this result shows that variants derived from BM10 are much less mucoid in YEM agar plates, but not completely nonmucoid. In liquid media, they seem to be unable to produce high-molecular weight polysaccharide.

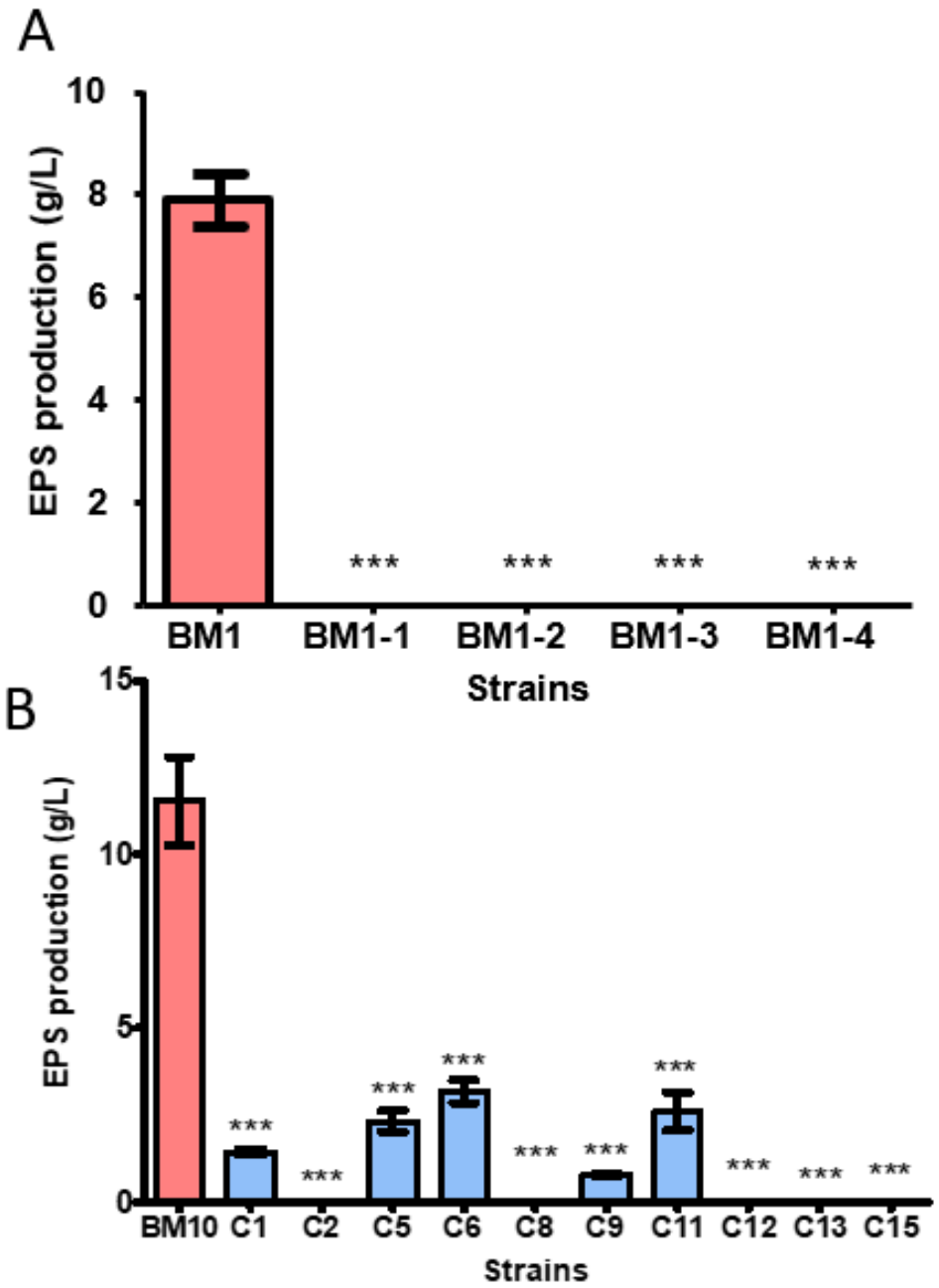


Figure 11 - Quantification of EPS production in liquid media of *B. multivorans* BM10 and its derivative less mucoid variants (A) and of *B. multivorans* BM1 and its derivative nonmucoid variants (B). The amount of EPS was measured by the dry-weight of the ethanol-precipitated polysaccharide recovered from all strains grown in liquid SM medium over 4 days at 37ºC under 250 rpm orbital agitation. 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 (BM1 or BM10) and the mutants was determined: ***, P<0.001.

3.4. Whole genome sequencing of the variants

To determine which mutations might have caused the decrease of the mucoid phenotype, nine of the variants had their whole genome sequence determined with a coverage of more than 30x. Alignment of the Illumina reads against the BM10 isolate genome led to the identification of several mutations as depicted in Figure 12.

A closer look at the mutations reveals that all variants have mutations in *ompR*/*envZ* genetic locus, but none in *fixJ/fixL* locus. Variants C1, C2, C5, C6, C8, C9 and C15 had a deletion of approximately 1 kb comprising half of the *ompR* gene and part of the *envZ* gene (Figure 13A). This mutation results in the loss of this two-component regulatory system in those variants. Contrastingly, variants C12 and C13 present an insertion of 17 nucleotides close to the beginning of the *ompR* gene, causing a frameshift and a truncated aberrant protein (Figure 13B). Alignment of reads in these two last variants indicate a heterogenous population since some of the reads did not seem to have this insertion. That might indicate instability of this mutation, with part of the population reverting to the wild-type genotype.

Regarding additional mutations present in the variants, it is observed that C12 and C13 show an additional mutation each, involving a nonsynonymous mutation in a cold shock-like protein CspA (for C12) and an HTH-type transcriptional regulator PuuR (for C13).

The remaining variants accumulated 12 additional mutations, most of them nonsynonymous, in genes encoding proteins such as putative multidrug resistance protein, a tyrosine recombinase, and F1 capsule anchoring protein. Also, a frameshift mutation in a nonribosomal peptide encoding gene was observed. C15 has an extra mutation in uridylate kinase while C8 and C9 shown a mutation in RNA polymerase-binding transcription factor DksA (Figure 12). According to the mutation profile, C1, C5 and C6 seem to be clonal. Similarly, C8 and C9 are also clonal.

This number and type of mutations is significantly different from the ones found in variants derived from BM1 in which BM1-1 displays a 36 bp insertion in the *bceF* gene coding for a tyrosine kinase involved in cepacian biosynthesis; BM1-2 has a nucleotide substitution in *fixJ* gene and in a gene encoding preprotein translocase subunit SecG; BM1-3 has a deletion of 63 nucleotides in *fixL* and a 8-bp insertion in the gene encoding cytochrome O ubiquinol oxidase; and BM1-4 has a nucleotide substitution in *fixL* (Bica, 2018).

Contig	Gene name	Annotation	ATCC_17616	Type of mutation	BM10	C1	C2	C5	C6	C8	C9	C12	C13	C15
contig000008	qorA_1	Quinone oxidoreductase 1	Bmul_0988	syn	G	A	A	A	A	A	A	G	G	A
contig000012	-	putative multidrug resistance protein EmrY	-	-	T	C	C	C	C	C	C	T	T	C
contig000013	adiA_2	Biodegradative arginine decarboxylase	Bmul_0862	nonsyn	C	T	T	T	T	T	T	C	C	T
contig000014	-	Dimodular nonribosomal peptide synthase	-	frameshift		-10acgccctcat	-10acgccctcat	-10acgccctcat	-10acgccctcat	-10acgccctcat	-10acgccctcat			-10acgccctcat
contig000015	-	tyrosine recombinase XerC	-	-	C	G	G	G	G	G	G	C	C	G
contig000019	pyrH	Uridylate kinase	Bmul_1259	nonsyn	C	C	C	C	C	C	C	C	C	T
contig000028	hsdR	Type I restriction enzyme EcoR124II R protein	-	nonsyn	A	G	G	G	G	G	G	A	A	G
contig000034	aaeA_1	P-hydroxybenzoic acid efflux pump subunit AaeA	Bmul_6150	nonsyn	C	T	T	T	T	T	T	C	C	T
contig000038	cspA	Cold shock-like protein CspA	Bmul_0775	nonsyn	T	T	T	T	T	T	T	A	T	T
contig000048	-	**Transcriptional regulatory protein OmpR**	Bmul_1333	frameshift								+17gacgaccgcgcctgcg	+17gacgaccgcgcctgcg	
contig000048	-	**Transcriptional regulatory protein OmpR and begining EnvZ**	Bmul_1333	frameshift		-997nt	-997nt	-997nt	-997nt	-997nt	-997nt			-997nt
contig000050	-	Maleylpyruvate isomerase	Bmul_0491	-		-6tcgtat	-6tcgtat	-6tcgtat	-6tcgtat	-6tcgtat	-6tcgtat			-6tcgtat
contig000061	caf1A	F1 capsule-anchoring protein	-	syn	A	G	G	G	G	G	G	A	A	G
contig000085	shdC	Phenolic acid decarboxylase subunit C	Bmul_3609	nonsyn	T	A	A	A	A	A	A	T	T	A
contig000094	-	hypothetical protein	Bmul_4109	nonsyn	G	A	A	A	A	A	A	G	G	A
contig000112	-	HTH-type transcriptional regulator PuuR	Bmul_3262	-	T	T	T	T	T	T	T	T	C	T
contig000132	-	between P0205-1_05295 and P0205-1_05296	between Bmul_0461 and_0462	-			+1t							
contig000175	dksA_2	RNA polymerase-binding transcription factor DksA	Bmul_3085	nonsyn	T	T	T	T	T	G	G	T	T	T
				SNPs/indels	0	9	9	9	9	10	10	1	1	10
					0	3	4	3	3	3	3	1	1	3

Figure 12 – Mutations identified in the *B. multivorans* variants when compared to BM10 isolate as reference. Locus_tag used is from *B. multivorans* ATCC17616. **syn**, synonymous; **nonsyn**, nonsynonymous mutation.

A

B

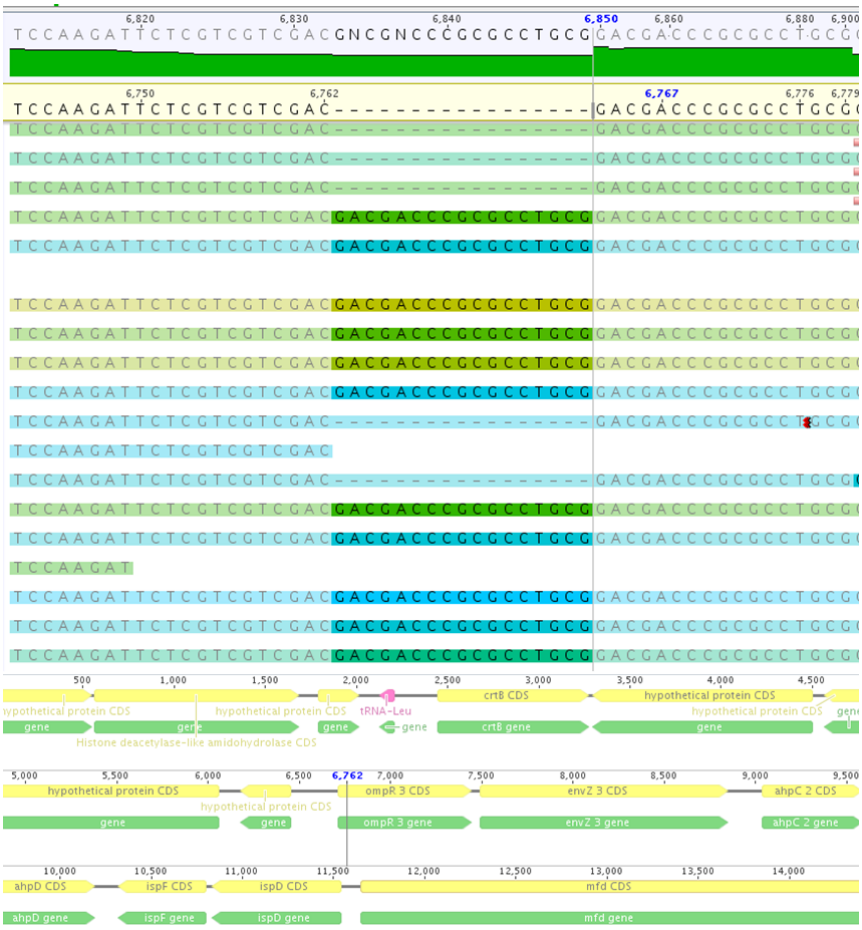


Figure 13 – Printscreen of *Geneious* software images of C1 (A) and C12 (B) showing alignment of reads to BM10 genome (upper panel of A and B) and genomic location of the mutations (lower panel of A and B). C1 variant has a 997 nucleotides deletion while C12 has as insertion of 17 nucleotides.

3.5. Genetic complementation of the mutants through triparental conjugation

To assess if the highly mucoid morphotype could be restored in the less-mucoid *B. multivorans* BM10-derived mutants, triparental conjugation was performed in selected mutants, namely *B. multivorans* C2, C8 and C12. To test this, the mutants were grown with the genetic construct plasmid pLM014-5, obtained from a previous study (Silva et al., 2018). This plasmid contains the promoter and the *ompR* gene of *B. multivorans* ATCC 17616. The expression of the plasmid in the mutants resulted in the restoration of the highly mucoid phenotype in the mutant *B. multivorans* C12 (Figure 14). In the plates where the mobilized plasmid was the empty pBBR1MCS vector, it was also possible to observe a few mucoid colonies (data not shown), confirming the reversion of the phenotype in some cells of the C12 variant population.

Complementation of C2 and C8 with both empty vector and vector with *ompR* gene led to very few colonies and no difference in mucoidy was observed (data not shown). However, this results could suggest that the presence of a functional *ompR* gene is not sufficient for the production of high-molecular weight EPS in *B. multivorans*, given the fact that the expression of the *ompR* in the mutant C12, which only had accumulated mutations in the *ompR* gene restored the highly mucoid phenotype observed in the parental strain *B. multivorans* BM10, but it did not restored the highly mucoid phenotype in mutants C8 or C2, which had accumulated mutations in the *ompR* gene but also in the beginning of the *envZ* gene, suggesting that the production of high-molecular EPS, involves the presence of both functional EnvZ and OmpR proteins and not just a functional OmpR protein.

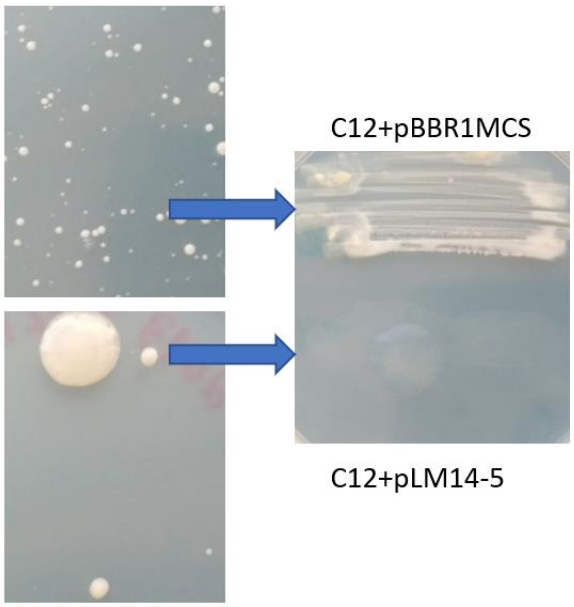


Figure 14 – Complementation of variant C12 by introducing the empty vector pBBR1MCS or the vector with the *ompR* gene and its promoter amplified from *B. multivorans* ATCC 17616 (left panels). One colony of each type was grown in YEM agar solid medium for further confirmation of the mucoid phenotype (right panel).

3.6. Assessment of growth in LB medium

It is important to estimate the growth kinetics of the mutant strains to study if the mutations accumulated by the mutants could cause any growth impairment by comparison with their respective wild-type strains. To determine the growth properties of each strain under study, liquid cultures of *B. multivorans* BM1, *B. multivorans* BM10 and their respective mutants were grown in liquid LB medium for 24 hours under 250 rpm orbital agitation, as it can be seen in Figure 15. In the case of mutants derived from *B. multivorans* BM1, there were statistically significant differences in the doubling time of the mutant strains when compared with the wild-type, as it can be observed in table 2. For the mutants obtained from *B. multivorans* BM10, the majority of less-mucoid mutant strains did not have a statistically significant difference in the doubling time by comparison with the mucoid parental strain, with the exception of variants C8 and C12, as it can be observed in table 2. These results suggest that the *bceF* and *fixL/fixJ* genes could affect the growth kinetics while mutations in the *ompR* gene did not represent a major impairment in growth kinetics within the tested conditions.

Table 2 - Doubling time of *B. multivorans* BM1 and its derivative nonmucoid variants and of *B. multivorans* BM10 and its derivative less-mucoid variants.

Bacterial strains under study (min ± SD*)		**Doubling time (min ± SD*)**	
BM1		64.68 ± 2.743	
BM1-1		75.85 ± 0.4874 (**)	
BM1-2		72.09 ± 2.509 (*)	
BM1-3		77.16 ± 2.445 (***)	
BM1-4		71.82 ± 3.803 (*)	
Bacterial strains under study (min ± SD)	**Doubling time (min ± SD*)**	**Bacterial strains under study (min ± SD)**	**Doubling time (min ± SD*)**
BM10	73.59 ± 1.801	C11	73.03 ± 0.2276 (ns)
C1	78.83 ± 2.459 (ns)	C12	80.79 ± 1.857 (*)
C2	75.34 ± 3.313 (ns)	C13	76.15 ± 3.389 (ns)
C5	79.09 ± 0.3963 (ns)	C15	76.26 ± 4.620 (ns)
C6	79.28 ± 1.368 (ns)		
C8	85.75 ± 1.376 (***)		
C9	78.44 ± 3.642 (ns)		

*** Statistical difference in the doubling time of the mutant strains was tested in comparison with the wild-type (BM1 or BM10).** Significance level (one-way ANOVA followed by Dunnett's multiple comparisons test): ns, not statistically significant; *, P<0.05; **, P<0.01; ***, P<0.001.

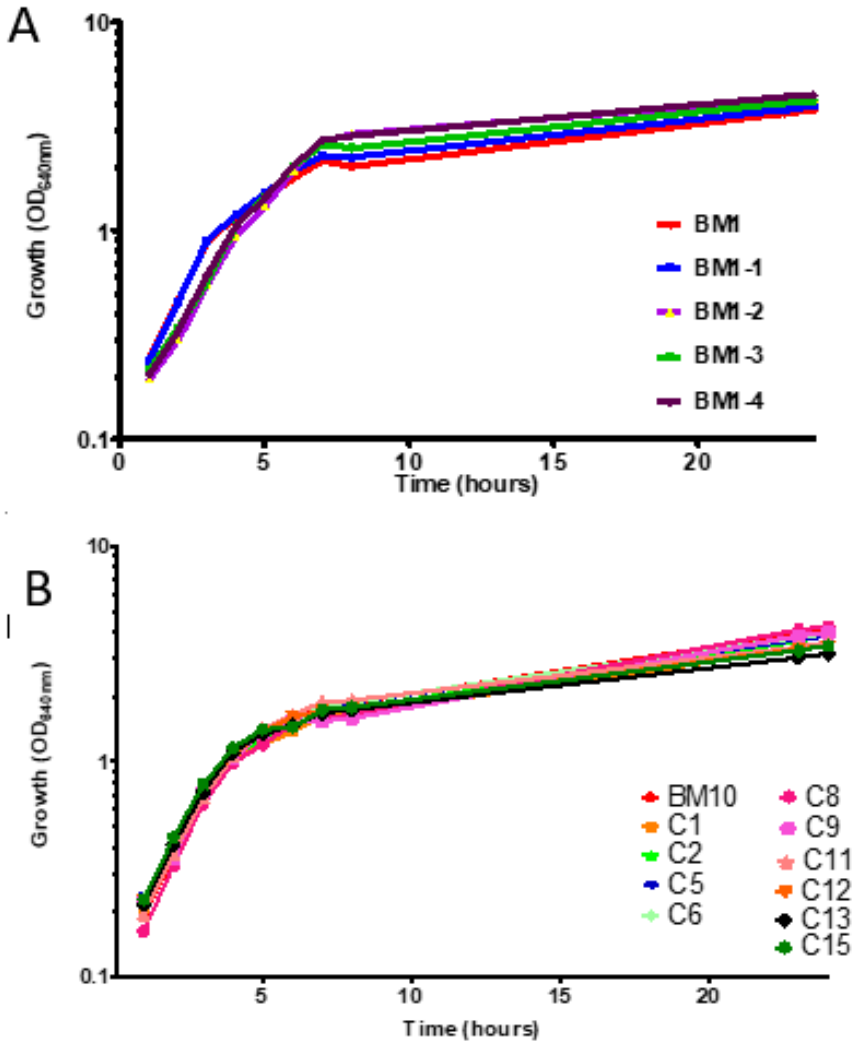


Figure 15 - Growth curves obtained for *B. multivorans* BM1 and its derivative nonmucoid variants (A) and for *B. multivorans* BM10 and its derivative less-mucoid variants (B). Cultures were grown in LB medium at 37ºC, 250 rpm of orbital agitation, and OD_{640nm} was measured for 24 hours. Results are the means of data from three independent experiments. The standard deviation is below 5%.

3.7. Resistance against antimicrobial agents

Since some previous studies have linked the presence of the *ompR* and *fixL* genes with certain phenotypes, such as antimicrobial susceptibility, virulence and biofilm formation (Silva et al., 2018; Schaefers et al., 2017), the antimicrobial susceptibility was tested for *B. multivorans* BM1 and its derived mutants and also for *B. multivorans* BM10 and its derived mutants. The antimicrobial susceptibility of the mutants and its respective wild-type strain was tested against Ciprofloxacin, Aztreonam, Piperacillin plus Tazobactam and Kanamycin. Ciprofloxacin is a fluoroquinolone that works by inhibiting DNA replication (Drlica, 1999); Aztreonam is a β-lactam that works by inhibiting cell wall biosynthesis (Sykes, 1985); Piperacillin is a β-lactam antibiotic

that belongs to the class of Penicillin and works by targeting the penicillin binding proteins (PBPs) and thus inhibiting cell wall biosynthesis and is widely used in combination with the β-lactamase inhibitor Tazobactam (Fortner et al,, 1982; Bryson et al., 1994); and Kanamycin, which is an aminoglycoside antibiotic that works by binding irreversibly to specific 30S-subunit proteins and 16S rRNA, inhibiting bacterial protein synthesis (Le Goffic et al., 1979).

The antimicrobial susceptibility was determined by measuring the diameter of the growth inhibition zone after 24 hours of incubation at 37°C (Figures 16 and 17).

Regarding isolate BM10 and its derived mutants, by looking at Figure 16, it can be seen that the resistance to Aztreonam was not affected by the mutations since there was no statistically significant difference between the wild-type and the mutant strains. Regarding the remaining tested antimicrobials, there was a statistically significant difference between the wild-type and the mutant strains, with an overall increase in antibiotic susceptibility displayed by all the tested mutant strains when compared with the wild-type *B. multivorans* BM10. Variant C8 appeared to be slightly more susceptible to Piperacillin + Tazobactam and Ciprofloxacin than the other tested mutant strains. The mutant strains C8, C13 and C15 appeared to be slightly more susceptible to Kanamycin than the other tested mutants.

Regarding BM1 and its mutants, by looking at Figure 17, it can be observed that the resistance to Ciprofloxacin, Aztreonam Piperacillin + Tazobactam and Kanamycin was similar between *B. multivorans* BM1-1 and the wild-type *B. multivorans* BM1. It can also be observed that there was an overall increase of Aztreonam and Ciprofloxacin susceptibility in mutant strains *B. multivorans* BM1-2, BM1-3 and BM1-4 and that their resistance was similar among the three strains, when compared with the wild-type. Finally, it can also be noted that there was an increase in Piperacillin + Tazobactam and Kanamycin susceptibility in *B. multivorans* BM1-3 and BM1-4 and that the resistance of the two strains to this antibiotics was similar, when compared with the wild-type *B. multivorans* BM1 (Figure 17).

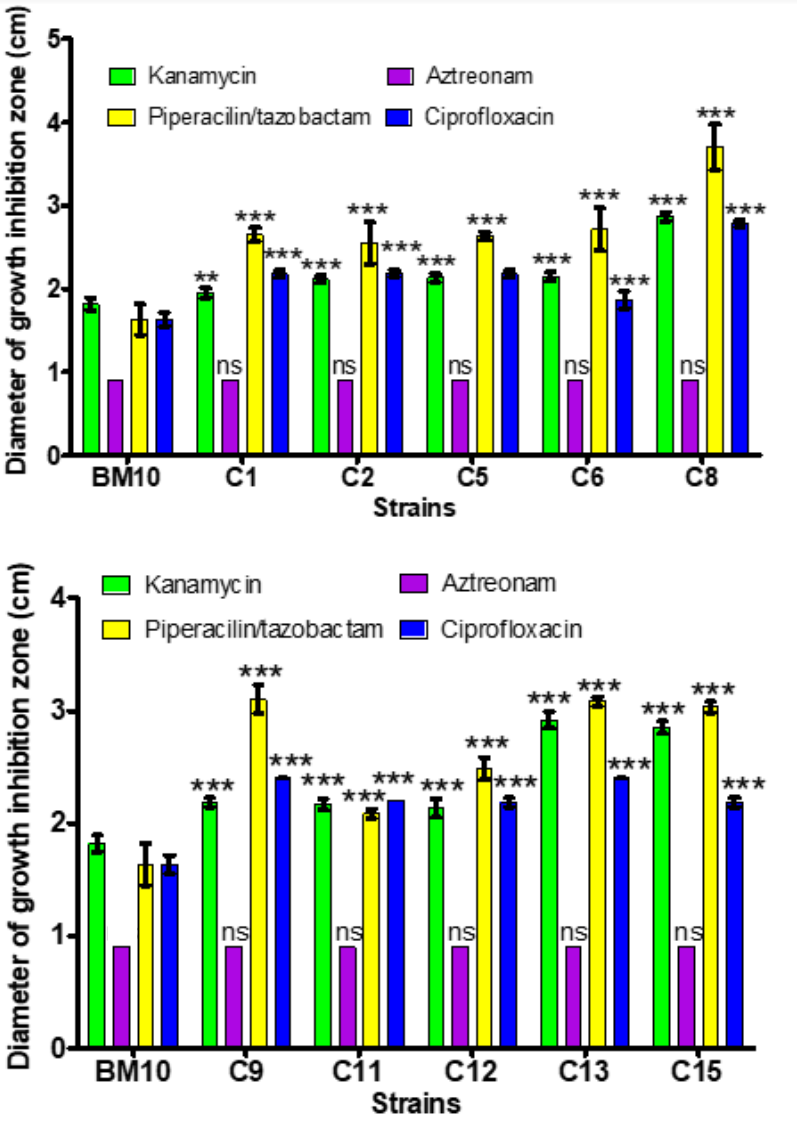


Figure 16 - Susceptibility of *B. multivorans* BM10 and its derivative less mucoid variants to antibiotics (Ciprofloxacin, Aztreonam, Piperacillin + Tazobactam and Kanamycin) was measured by the diameter of cell growth inhibition, after growth for 24h at 37ºC. Error bars correspond to the standard deviations 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 (BM10) and the mutants for each antibiotic tested was determined: ns, not statistically significant; **, P<0.01; ***,

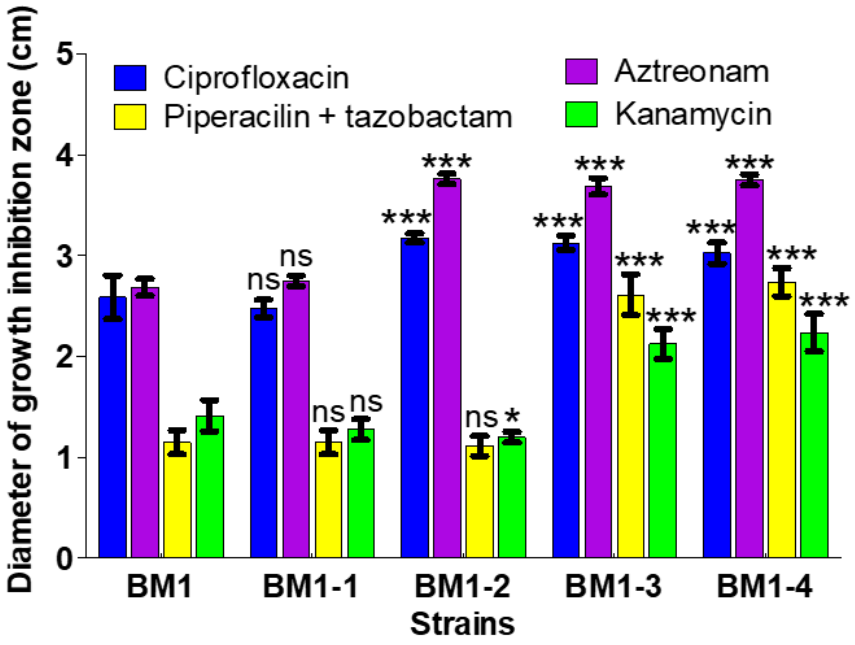


Figure 17 - Susceptibility of *B. multivorans* BM1 and its derivative nonmucoid variants to antibiotics (ciprofloxacin, aztreonam piperacillin/tazobactam and kanamycin) was measured by the diameter of cell growth inhibition, after growth for 24h at 37ºC. Error bars correspond to the standard deviations 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 (BM1) and the mutants for each antibiotic tested was determined: ns, not statistically significant; *, P<0.05; ***, P<0.001.

3.8 Swimming and swarming motilities

Motility is also an important factor upon adaptation of *Burkholderia multivorans* to the microenvironment of the CF lung. Some studies have associated fluctuations in the swimming and swarming motilities capacity between mucoid and less mucoid/nonmucoid isolates (Silva et al., 2018; Schaefers et al., 2017). Consequently, it was considered to be important to test the swimming and swarming motilities of the mucoid *B. multivorans* BM1 and its nonmucoid mutants and also of the mucoid *B. multivorans* BM10 and its less mucoid strains. To test the motility of the *B. multivorans* strains, swimming and swarming plates were incubated for 24 hours or 48 hours, respectively, at 37ºC after inoculation. After this period, the motility zone diameter was measured (in cm) for each plate and the results are shown in Figures 18 and 19.

Regarding swimming motility, we can observe in Figure 18A that the mutants obtained from *B. multivorans* BM1 presented a statistically significant decrease in swimming motility, when compared with the parental strain *B. multivorans* BM1. Regarding mutant colonies obtained from *B. multivorans* BM10, some presented a statistically significant increase in swimming motility, namely C8, C9, C11, C12, C13 and C15, while others presented a statistically significant decrease in swimming motility, namely C1, C2, C5 and C6, in comparison with the wild-type *B. multivorans* BM10 (Figure 18B). It can also be observed that the diameter measured in cultures

C1, C2, C5 and C6 corresponds to the size of the spotted inoculum, indicating that these mutants lost the capacity for swimming motility.

Regarding swarming motility, we can observe in Figure 19A that three of the nonmucoid mutants obtained from *B. multivorans* BM1, namely *B. multivorans* BM1-2, BM1-3 and BM1-4 presented a statistically significant decrease in swarming motility, when compared with the mucoid wild-type *B. multivorans* BM1 and that the swarming motility capacity of *B. multivorans* BM1-1 was not affected by the mutation that it harbors, since there was no statistically significant difference between this strain and the parental strain *B. multivorans* BM1. Referring to BM10 and its respective less mucoid variants, C8, C13 and C15 did not have their swarming motility capacity affected, since there was no statistically significant difference between them and the parental strain *B. multivorans* BM10. Variants C1, C2, C5, C6, C9, C11 and C12 presented a statistically significant decrease in swarming motility, in comparison with the wild-type strain *B. multivorans* BM10 (Figure 19B). It can also be noted that variants C1, C2, C5 and C6 displayed a similar pattern between swimming and swarming motilities and that in both cases, the diameter measured in the plates matched the size of the spotted inoculum, indicating that these mutants are nonmotile due to the loss of both swimming and swarming motilities.

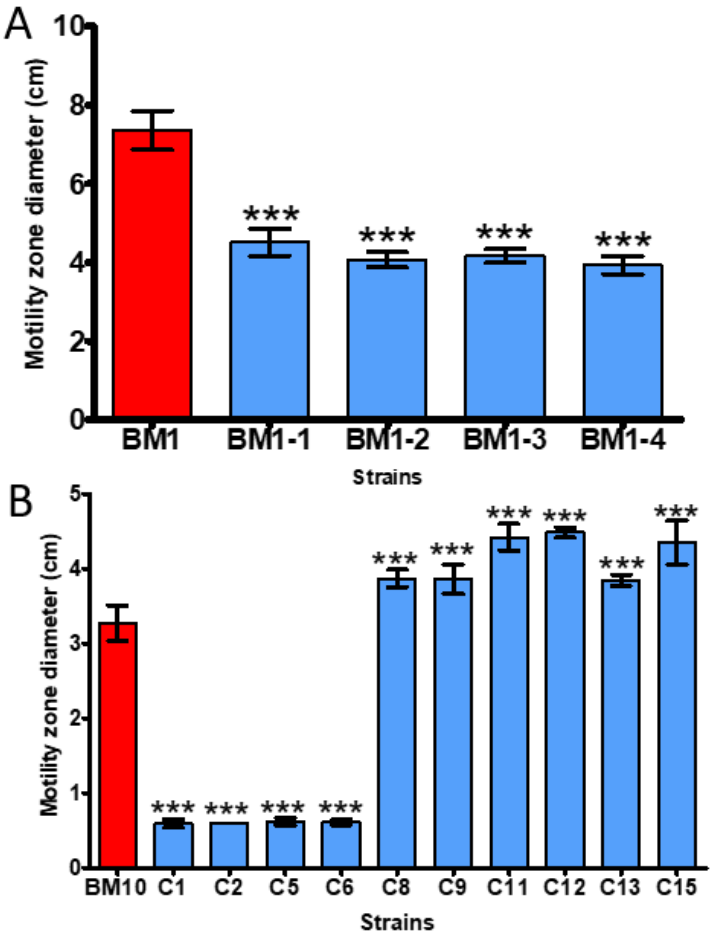


Figure 18 - Swimming motility of *B. multivorans* BM1 (A) and its derivative nonmucoid variants and swimming motility of *B. multivorans* BM10 and its derivative less-mucoid variants (B) was measured by the motility zone diameter after growth for 24h at 37ºC. Error bars correspond to the standard deviations 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 (BM1 or BM10) and the mutants was determined: ***, P<0.001.

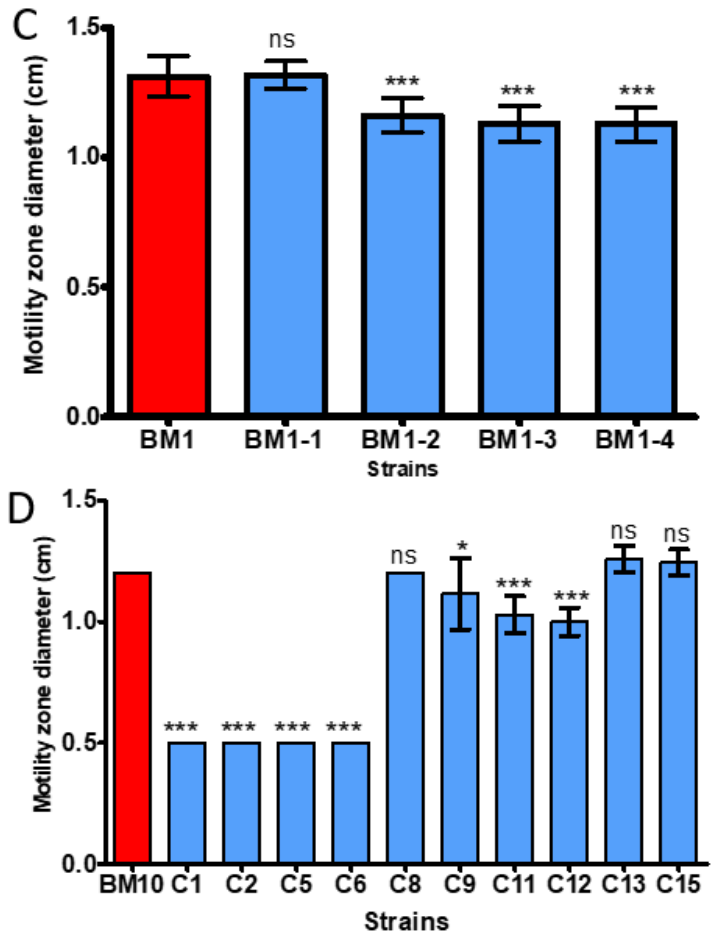


Figure 19 - Swarming motility of *B. multivorans* BM1 (C) and its derivative nonmucoid variants and swarming motility of *B. multivorans* BM10 and its derivative less-mucoid variants (D) was measured by the motility zone diameter after growth for 48h at 37ºC. Error bars correspond to the standard deviations 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 (BM1 or BM10) and the mutants was determined: ns, not statistically significant; *, P<0.05; ***, P<0.001.

3.9 Surface-attached biofilm formation

Another phenotype that some studies have shown to be affected by the mucoid-to-nonmucoid switch is the capacity that *B. multivorans* strains have to establish stable surface-attached biofilms (Ferreira et al., 2007; Silva et al., 2011; Silva et al., 2018; Schaefers et al., 2017). With this data in mind, it was important to evaluate and compare the ability of the mucoid strains and its less/nonmucoid variants in producing surface-attached biofilms. To test this, the *B. multivorans* strains under study were grown in microtiter plates for 48 hours and after this period, they were stained with crystal violet and washed, and the absorbance of the retained dye in the microtiter plate wells, was measured at 590 nm (A_{590nm}), as it can be observed in Figure 20.

Regarding the mucoid *B. multivorans* BM1 and its nonmucoid variants, there was no statistically significant difference between the mucoid wild-type *B. multivorans* BM1 and the nonmucoid

strains *B. multivorans* BM1-1, BM1-2, BM1-3 and BM1-4 (Figure 20A). As for the mucoid *B. multivorans* BM10 and its less mucoid variants, there was a statistically significant decrease in surface-attached biofilm formation, in comparison with the mucoid wild-type strain *B. multivorans* BM10. Through observation of the Figure 20B, it can also be noted that all variants display reduced biofilm formation when compared to the wild-type strain, but no significant differences were observed between them.

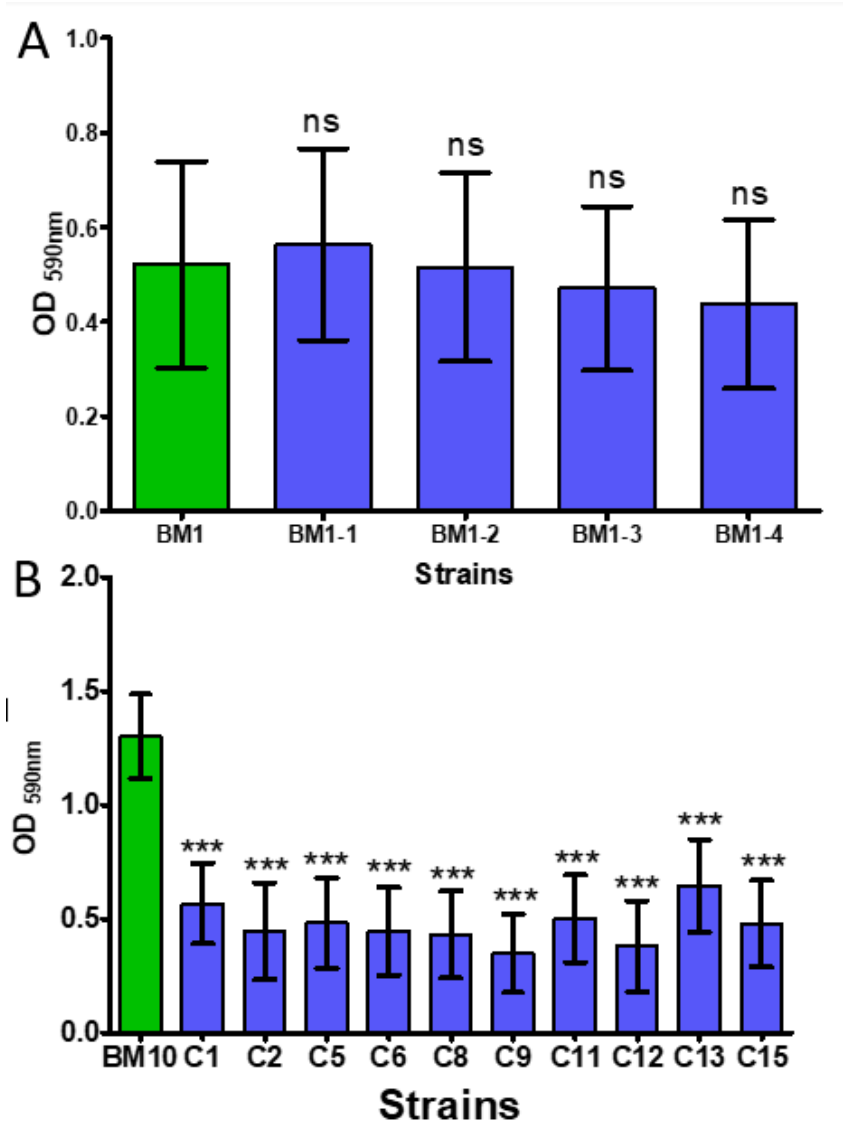


Figure 20 - Surface-attached biofilm formation of *B. multivorans* BM1 (A) and its derivative nonmucoid variants and of *B. multivorans* BM10 and its derivative less-mucoid variants (B) was determined by absorbance measurement at 590 nm after growth for 48h at 37ºC in polystyrene microplates. Error bars correspond to the standard deviations of the mean values of at least seven independent experiments. Significance level (one-way ANOVA followed by Dunnett's multiple comparison test) between the wild-type (BM1 or BM10) and the mutants was determined: ns, not statistically significant; ***,

4. Discussion

Bacteria often encounter harsh environments with poor nutrient availability and where different stress conditions may be experienced by them. In order to survive, they need to adapt either through genetic mutations or changes at the gene expression level. One of those examples is the conversion from a mucoid to a nonmucoid colony morphotype, which has been found to be experienced by *B. multivorans* clinical isolates grown under different stressful conditions (Silva et al., 2018). The stress conditions used on those *in vitro* studies mimic some of the microenvironmental challenging conditions experienced by bacteria within the lungs of CF patients, where the conversion of a mucoid to a nonmucoid morphotype over the course of *Burkholderia* chronic lung infections is well documented (Zlosnik et al., 2008). With the aim of determining how *Burkholderia* adapts to CF lungs and is able to establish chronic lung infections, Silva and collaborators sequenced the genome of 22 sequential isolates recovered from a CF patient during 20 years of chronical lungs infection. Based on the type of mutations identified, they were able to group the 22 isolates on different lineages/clades that evolved simultaneously (Silva et al., 2016). According to Figure 7, clades C1 and C2 appear in an early phase of infection; clade C3 is the dominant and clade C4 appears in a latter phase, when the infection is already well established. Since isolates from clades C2 and C3 were shown of being capable of switching from a mucoid to a nonmucoid phenotype through the accumulation of mutations within the *ompR* gene and isolates from clade C1 were shown to give rise to nonmucoid variants through accumulation in the *fixL/fixJ* genes (Silva et al., 2018; Bica, 2018), it was the aim of this work to determine if the mucoid-to-nonmucoid switch was also occurring in nonmucoid variants obtained from clade C4 isolates, through exposition of mucoid isolates from this clade to stress conditions. Our data confirmed that prolonged stationary phase induced by static incubation in SM liquid medium (21 days at 37ºC), also triggered the mucoid-to-nonmucoid switch in isolates from C4 clade. The next question was to evaluate if the mutations that triggered the loss of mucoidy mapped in the *ompR*/*envZ*, *fixL/fixJ locus* or in another unknown set of genes/molecular mechanisms. Successful genome sequence of nine less mucoid mutants derived from the parental strain *B. multivorans* BM10, revealed that the mutations most likely involved in this morphotype switch mapped into the *ompR/envZ* locus, similarly to the previous data obtained for clades C2 and C3. This poses the question on whether cells regulating mucoid conversion through two different two-component regulatory systems is dependent on their genetic background. With that in mind, Figure 7 shows a group of mutations present in all isolates from clades C2, C3 and C4, but not in C1 clade isolates. As C1 isolates give rise to nonmucoid variants whose mutations mapped to the *fixL/fixJ locus,* perhaps one of the presented mutations is responsible for the triggering of mutations in *ompR/envZ* in the other clades, instead of *fixL/fixJ* locus. Of notice, isolates from clades C2, C3 and C4 accumulated a mutation in the promoter region of the *ompR* gene, which increased the expression of this gene in two isolates tested from clades C3 and C4 (Silva et al., 2016). It is then possible that under stress conditions this genetic region is more exposed to mutations and to the activity of DNA repair mechanisms. Another

interesting early mutation occurred in the *rpfR* gene encoding the BDSF receptor. Changes at the quorum sensing level influence the way cells respond to stress experienced within the surrounding microenvironment, but whether this translates into an alteration of the mutation rate in unknown. Comparing the high number of accumulated mutations on some of our mutants and the ones obtained by Silva and collaborators, and Bica and collaborators this was an unexpected result. Effectively, most of the previously identified nonmucoid variants had 1-4 different mutations. Here, except for variants C12 and C13, all other less mucoid variants have more than 12 different mutations (Figure 12). This seems to be a rapid evolution, considering that cultures were evolved within the comprised period of 21 days. Under more extreme and sustained periods of stress, the inherent plasticity of bacterial populations may be insufficient to maintain fitness, which selects for mutants with superior growth or resistance, at expense of plasticity and may result in rapid evolution (Silva et al., 2018). Mutations in *ompR*/*envZ or fixL/fixJ* seem to affect differently some phenotypes. One of those phenotypes is biofilm formation, in which *fixL/fixJ* mutations do not show differences in comparison to the wild-type BM1 isolate, while BM10 derived variants show decreased biofilm formation. Another difference was observed in swimming motility with BM1-derived variants having decreased motility while six of the BM10-derived variants show increased motility. Unexpectedly, mutants C1, C2, C5 and C6 are nonmotile, which contrasts to previous *ompR* mutants derived from clade C2 and C3 which showed increased motility (Silva et al., 2018). The antibiotic resistance profile in BM10-derived variants is in accordance with the one obtained by Silva and collaborators, in which it was observed that mutations in the *ompR* gene increased the susceptibility to antimicrobial compounds such as piperacillin (Silva et al., 2018). Analyzing the mucoid/nonmucoid phenotype of the variants it was observed that while variants derived from clades C1, C2, C3 formed nonmucoid dry colonies, the ones derived from clade C4 isolates are still shiny, although both types are unable to produce polysaccharides of high-molecular weight in liquid medium. This unforeseen difference might be explained by mutations present in clade C3 but absent in clade C4 isolates. One such mutation is a nonsynonymous mutation in *fixL* gene that is present in all isolates of clade C3. Perhaps disruption of *ompR* gene in a wild-type FixL background abolishes high-molecular weight EPS, but not the shiny morphotype observed in YEM agar plates. In conclusion, this study provides evidence that mutations triggering mucoid-to-nonmucoid switch likely depend on the genetic background. Besides we confirmed that the OmpR RR influences several traits related to cell envelope composition, playing a key role in *Burkholderia.*

5. References

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