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Functional analysis of OmpR and FixJ response regulators from *Burkholderia multivorans*: role in exopolysaccharide biosynthesis

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À minha família pois sem eles nada seria possível.

Aos meus colegas (e amigos) que acompanharam o meu percurso, apoiando todas as minhas decisões e consequências.

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ABSTRACT

Cystic fibrosis (CF) is a clinical syndrome characterized by chronic pulmonary infection, which is characterized by abnormally thick mucus secretions, creating favorable niches for bacterial colonization. Among these colonizers are the *Burkholderia cepacia* complex (Bcc) bacteria, a group of opportunistic pathogens particularly feared due to the unpredictable disease outcome in infected patients. Bcc bacteria produce several virulence factors, among them the exopolysaccharide (EPS) cepacian, produced by the majority of clinical isolates. Analysis of the mucoid morphotype of CF clinical isolates revealed an inverse correlation between EPS production and disease progression, being therefore important to understand the molecular mechanisms behind this ON/OFF switch. With that in mind, two *B. multivorans* early and a late isolates recovered from a single CF patient were grown under stress conditions and colonies showing lower mucoid recovered. After whole genome sequence of six variants we identified mutations in the *fixL* and *fixJ* genes encoding a two-component signal transduction system. Phenotypic characterization established that mutations in *fixJ*, but not *fixL*, cause loss of EPS biosynthesis. To investigate whether EPS biosynthesis regulation could be controlled by direct binding of FixJ protein to the promoter region upstream of the putative bceBCDEFGHIJ operon, EMSA assays were performed. Despite several attempts we were unable to demonstrate this direct binding. Nevertheless, we showed that these mutations seem to be involved in regulating biofilm formation and in catabolite control, suggesting an important role of these proteins in the adaptation of *Burkholderia* to the CF lung environment.

Keywords: *Burkholderia multivorans*, cepacian, FixL/FixJ, mucoidy, cystic fibrosis

RESUMO

A fibrose quística (FQ) é uma síndrome clínica caracterizada por infeção crónica pulmonar, identificada por secreções anormais de muco que criam nichos favoráveis para a colonização bacteriana. De entre os colonizadores, o complexo *Burkholderia cepacia* (Bcc) é um grupo de patogénicos oportunistas particularmente temido devido ao desfecho imprevisível que os doentes infetados podem ter. As bactérias do complexo Bcc apresentam diversos fatores de virulência, de entre os quais o exopolissacárido (EPS) cepaciano, produzido pela maioria dos isolados clínicos. Análises à morfologia mucosa dos isolados clínicos de FQ revelaram uma correlação inversa entre a produção de EPS e a progressão da doença, tornando-se importante a compreensão dos mecanismos moleculares por trás deste ON/OFF. Com isto em mente, dois isolados clínicos de *B. multivorans* recuperados, com diferença de 13 anos entre eles, de um único paciente com FQ foram crescidos em condições de stress e as colonias que apresentavam menos mucosidade foram recuperadas. Após sequenciação do genoma, seis variantes apresentavam mutações nos genes *fixL* e *fixJ* que codificam um sistema de transdução de sinal de dois componentes. A caracterização fenotípica levou à conclusão de que as mutações encontradas no *fixJ*, mas não no *fixL*, causavam a perda da biossíntese de EPS. De modo a investigar se a biossíntese do EPS podia ser controlada diretamente pela ligação da proteína FixJ à região promotora acima do operão bceBCDEFGHIJ, ensaios EMSA foram realizados. Após diversas tentativas não conseguimos demonstrar esta ligação direta. Mesmo assim, conseguimos demonstrar que estas mutações parecem estar envolvidas na regulação da formação de biofilmes e no controlo catabólico, sugerindo um papel importante destas proteínas na adaptação da *Burkholderia* ao microambiente do pulmão dos pacientes com FQ.

Palavras-chave: *Burkholderia multivorans*, cepaciano, FixL/FixJ, mucosidade, fibrose quística

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LIST OF ACRONYMS

Amp – Ampicillin

Bcc – *Burkholderia cepacia* complex

BM – *Burkholderia multivorans*

bp – Base pair

CDS – Coding DNA sequence

CF – Cystic fibrosis

Cm – Chloramphenicol

dNTPs – Deoxyribonucleoside triphosphate

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

1. Introduction

1.1 Cystic fibrosis, an overview

Cystic fibrosis (CF), the most common autosomal recessive disorder in Caucasians, establishes as a clinical syndrome characterized by chronic sinopulmonary infection, which presents an abnormally thick mucus secretion in the airways (**Figure 1**). Also, chronic infections and gastrointestinal abnormalities can be pointed as two of the typical symptoms (Lyczak et al. 2002).

In normal health conditions, the airways are covered by a thin layer of mucus composed of a complex mixture of mucins, proteins, lipids, ions and water secreted by epithelial cells lining airway surfaces, as well as submucosal glands that contribute to mucus secretion in the large airways. An elaborate mucociliary clearance (MCC) system provides important protective functions and contributes to the innate defense system of the lung (Wanner et al. 1996; Fahy & Dickey 2010). In CF patients the MCC system is defective and patients become more prone to infections.

Significant progress has been made in unraveling the disease mechanisms underlying impaired MCC and airway mucus obstruction in chronic lung diseases. The cause of the disorder is a genetic mutation targeting the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Rommens et al. 1989). The defect in the regulation of ion transport homeostasis in epithelial cells leads to a malfunction of many organs including pancreas, liver, intestine, and frequently the lungs. In the airways, the genetic defect impairs mucociliary clearance as well as antimicrobial defense creating a perfect niche for microbial colonization. Chronic infections by bacterial pathogens trigger airway inflammation and structural lung damage (Mall & Hartl 2014).

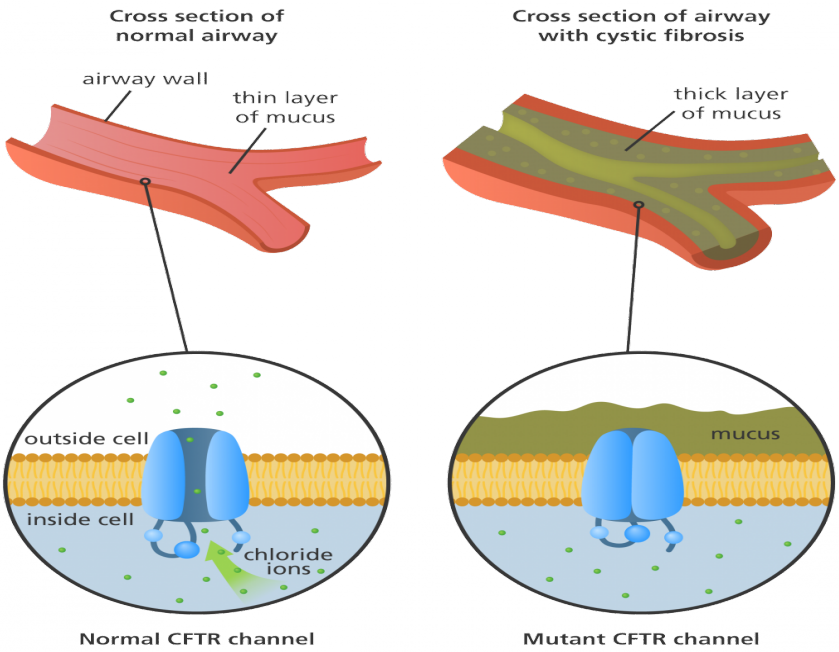

Figure 1 – Normal and mutant CFTR channels, with cross-sections of the effect on the airway (Adapted from yourgenome.org). In normal conditions a thin layer of mucus covers the lung whereas in patients with cystic fibrosis abnormally thick mucus characterize it.

Although the relationship between the host and the resident microorganisms is frequently commensal or symbiotic, countless microbial species have evolved to have a detrimental or even lethal effect on their mammalian hosts (Deitsch et al. 2009). Due to the fact that pathogenic microorganisms colonize airways of CF patients in infancy, chronic infections are established in the vast majority of cases. Most patients experience recurrent critical respiratory episodes and eventually die of respiratory failure resulting from infection (Lyczak et al. 2002).

Most studies of CF pathogens have focused on four major bacterial species: *Haemophilus influenza*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and, to a lesser extent, *Burkholderia cepacia complex*. Prevalence of these species in the airways changes over time and despite most pathogens are acquired from environmental reservoirs, patient-to-patient transmission also occurs (Harrison 2007).

1.2 Focusing on *B. cepacia complex*

Walter Burkholder, in 1940s, isolated the first bacteria from the *Burkholderia* genus (Burkholder 1950), which nowadays comprises more than 90 species. Among this genus we are particularly interested in the *Burkholderia cepacia complex* (Bcc). Bcc species have versatile lifestyles and exhibit conflicting biological features: some are pathogens for plants and immunocompromised individuals (Mahenthiralingam et al. 2008), whereas other show biotechnological potential for biocontrol, bioremediation and plant growth promotion (Parke & Gurian-Sherman 2001).

B. cepacia complex is a group, at the time of writing, comprising 22 phenotypically similar but genetically distinct bacterial species (Martina et al. 2017) that includes opportunistic pathogens causing in a smaller extent severe chronic infections in CF (Silva et al. 2013). However, the infections caused by Bcc bacteria are extremely difficult to eradicate because these bacteria have numerous metabolic enzymes, regulatory genes, transporters, and putative virulence determinants providing them with a great competitive capacity to move between different niches (Holden et al. 2009). Additionally, Bcc bacteria have an intrinsic antibiotic resistance (Nzula et al. 2002), making it very problematic to eliminate from infected hosts.

Burkholderia multivorans and *Burkholderia cenocepacia* are the most often isolated Bcc species from the respiratory tract of CF patients (Zlosnik et al. 2015). Although strains from all the Bcc species are capable of causing infections to CF patients, their prevalence varies geographically and regionally. While *B. multivorans* predominates in Europe, *B. cenocepacia* is predominant in North America (Govan et al. 2007). Infection with *B. multivorans* can also be brief due to acquisition of unique strains rather than patient-to-patient spread. Evidence suggests that in the CF lung *B. multivorans* can be replaced by *B. cenocepacia* (Zlosnik et al. 2015). This last species is one of the key Bcc species responsible for the risk of 'cepacia syndrome', a rapid fulminating pneumonia sometimes accompanied by septicemia, and its acquisition is often considered a contraindication to successful lung

transplantation (Abbott et al. 2016).

A number of remarkable features in CF airway infections distinguish it from the usual acquired forms of bronchitis. To successfully establish an infection, bacteria have to adhere to host mucosal or epithelial surfaces after entering into the respiratory tract of the CF patient. The condensed mucus layer, in case of the CF lung, provides an ideal environment for microbial colonization due to reduced efficacy of antimicrobial peptides, faulty mucus clearance, and improved inflammatory response (Boucher 2007). The ability to cross the epithelial barrier and gain access to the blood stream appears to be limited to Bcc strains, as other CF pathogens generally do not cause bacteremia (Leitão et al. 2010).

To understand Bcc pathogenicity, several studies have been undertaken. The airway epithelium plays a central role in the progression of CF lung disease via the production of numerous cytokines, chemokines, inflammatory enzymes, and adhesion molecules. During the interaction with the CF host, several virulence factors are known to play critical roles for the success of the pathogen (Leitão et al. 2010). One of these factors is the exopolysaccharide (EPS) cepacian produced by a majority of Bcc strains (Cunha et al. 2004; Zlosnik et al. 2008), and it will be here described.

1.3 Exopolysaccharide production: a not so rare phenomenon

The most abundant EPS produced by *Bcc* bacteria, is cepacian. This exopolysaccharide has been identified in both clinical and environmental isolates of *Bcc* bacteria (Ferreira et al. 2010) and the characterization of its chemical structure and composition showed that it 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, respectively and linked by (1→3) glycosidic bonds (reviewed in Ferreira et al. 2011).

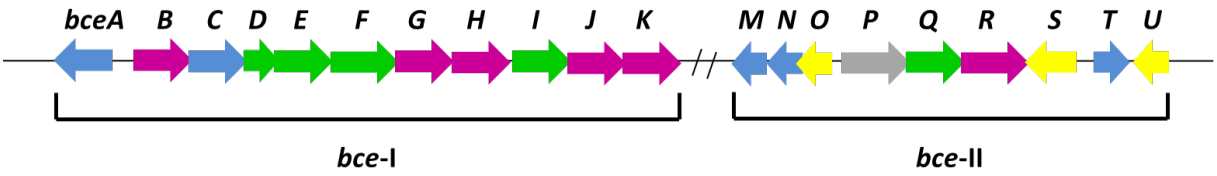

Figure 2 – Genetic organization of *bce*-I and *bce*-II clusters of genes encoding proteins involved in cepacian biosynthesis (Adapted from (Ferreira et al. 2011)). ■ Nucleotide sugar biosynthesis; ■ Glycosyltransferase; ■ Polymerization/Export; ■ Acyltransferase; ■ Unknown function. The two clusters are separated approximately by 155-314 kb in the genome of most *Burkholderia* species.

Genes involved in cepacian biosynthesis are located within *bce-I* and *bce-II* clusters (**Figure 2**), which are located in different loci of chromosome 2. The biosynthesis process is represented in the **Figure 3** and starts with the synthesis of the nucleotide sugar precursors, necessary for the repeat unit formation, and is catalyzed by isomerases, mutases, and epimerases, among other enzymes (Ferreira et al. 2010). The assembly of the repeat-units follows this step by the sequential addition of sugars to an isoprenoid lipid by dedicated glycosyltransferases. In the last step occurs the polymerization and export of the polysaccharide to the extracellular environment by a multienzyme complex composed by a repeat-unit translocase, a polysaccharide polymerase, an outer membrane protein and a protein tyrosine kinase, among others are involved in this process (Ferreira et al. 2010; Moreira et al. 2003).

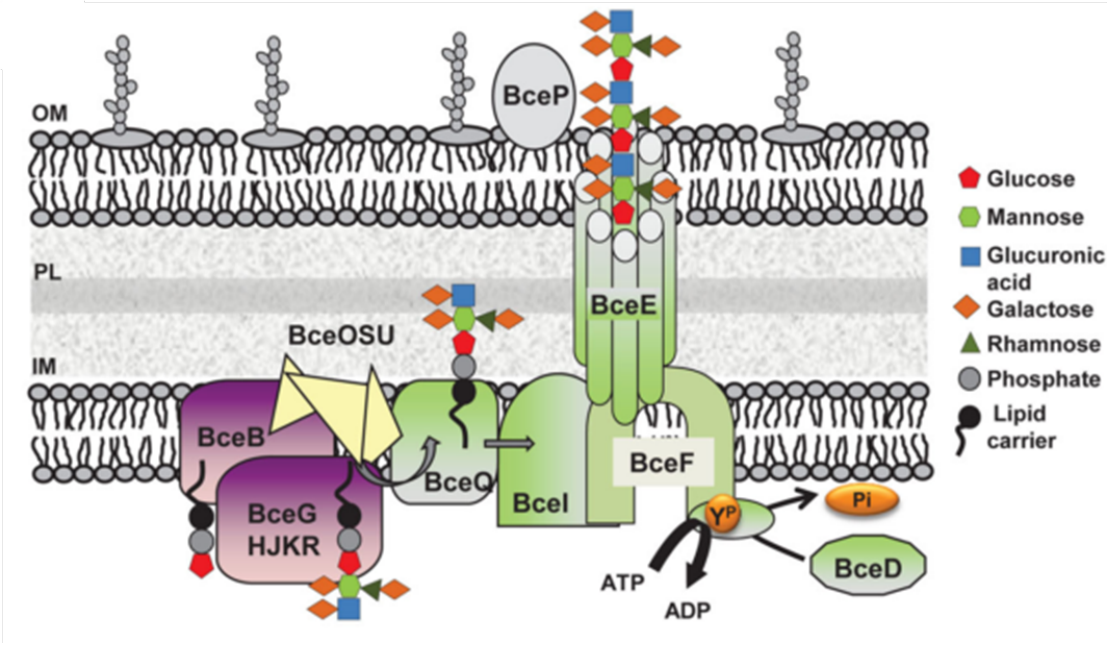

Figure 3 – Biosynthesis of the exopolysaccharide cepacian by *Burkholderia* (Adapted from (Ferreira et al. 2011)). Repeat-units of the polymer are assembled on a lipid carrier, in the cytoplasmic side of inner membrane in a reaction initiated by the BceB enzyme and continued by the other glycosyltransferases BceG, BceH, BceJ, BceK, and BceR and putative acyltransferases BceO, BceS and BceU. The lipid-linked repeat-units are translocated across the inner membrane by the putative BceQ membrane protein. Polymerization occurs at the periplasmic face of the inner membrane and is dependent on BceI. Wzy-dependent polymerization/export requires the activity of the BceF tyrosine kinase. BceD is a protein tyrosine phosphatase enzyme responsible for dephosphorylating BceF. BceE forms a channel structure for export of EPS chains to the outside. BceP, putatively involved in polysaccharide degradation, is depicted as associated to the outer membrane, as one of its possible locations. ATP - adenosine-5'- triphosphate; ADP - adenosine-5'- diphosphate; Y^P - phosphorylated tyrosine residue; Pi - inorganic phosphate; IM - inner membrane; OM - outer membrane; PL - peptidoglycan layer.

Several biological functions are attributed to exopolysaccharides but not all are fully known. Definitely, they contribute to create an appropriate hydrophilic niche where bacteria could more comfortably survive, by providing accumulation of micronutrients. Although EPS are considered one of the main components of bacterial biofilms, biofilm composition is rather variable and depends on several parameters including culture media and the nature of the support to which cell adhesion occurs (Flemming & Wingender 2010). Effectively, an important hallmark of Bcc is their aptitude to form biofilms, communities where bacteria live in a sessile lifestyle, protected from environmental insults and aggression from the immune system defenses of the host. In addition, Bcc bacteria in biofilms have been demonstrated to be more resistant to antibiotics, contributing to their persistence in the CF lung (Caraher et al. 2007).

Initially, EPS production by Bcc was considered a rare phenomenon. However, about 80% of the Bcc clinical isolates from Portuguese CF patients were found to produce variable amounts of the exopolysaccharide, cepacian. Cepacian was demonstrated to play a role in the establishment of thick biofilms, although not mandatory for the initiation of biofilm formation (Cunha et al. 2004).

In vitro studies have shown the ability of EPS to inhibit neutrophil chemotaxis and scavenge reactive oxygen species (Bylund et al. 2006). Moreover, using $gp91^{phox-/-}$ mice as an infection model, cepacian has been demonstrated to be a virulence factor (Sousa et al. 2007). A survey of more than 500 Bcc isolates from 100 chronically infected CF patients showed that strains of *B. cenocepacia*, the most virulent species of the complex, are most frequently nonmucoid but all Bcc species were able to express the mucoid phenotype due to EPS production. The high occurrence of nonmucoid isolates among strains of *B. cenocepacia* and the phenotypic switching typically from mucoid-to-nonmucoid raised the possibility of nonmucoid isolates being associated with augmented disease severity while the mucoid phenotype would be associated with persistence in the lungs (Zlosnik et al. 2008). That is the case for some *B. cenocepacia* and *B. multivorans* isolates, which, despite their absence of EPS biosynthesis ability, are highly infectious (Zlosnik et al. 2011).

Although the genes/proteins required for cepacian biosynthesis are well known, the regulatory elements that switch ON and OFF the expression of the *bce* genes is not yet determined. With the aim of identifying these regulators, several *Burkholderia* strains, able to produce exopolysaccharide, were exposed to stress conditions and nonmucoid variants were collected (Silva et al. 2013). Among these nonmucoid variants, we sequenced the genome of 10 colonies derived for *Burkholderia multivorans* VC13401 (BM11). Comparative genomic analysis mapped most of the mutations in two response regulators, one belonging to the OmpR-family (**Figure 4 a**) and the other to the FixJ family (**Figure 4 c**) (unpublished data). In both cases, the genes adjacent to the ones encoding the regulators, encode two histidine kinases: EnvZ and FixL, **Figure 4 b** and (**d**), respectively. These two component regulatory systems will be the focus of this work.

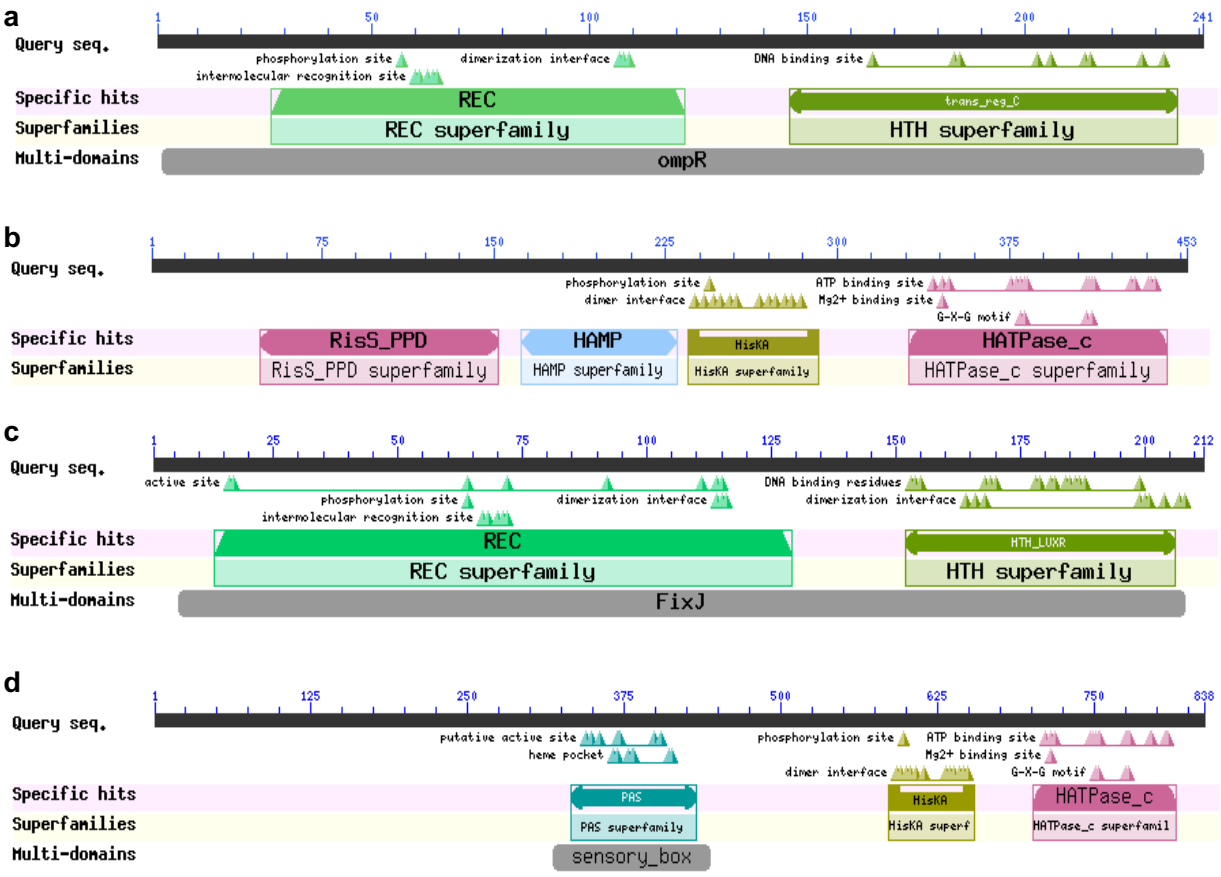

Figure 4 – Conserved domains in (a) response regulator OmpR with the cognate HK EnvZ (b) and (c) response regulator FixJ with the cognate HK FixL (d), obtained from NCBI database. (a) Two-component system response regulator similar to PhoP binds DNA upon phosphorylation and functions as transcriptional regulator: REC represents the receiver domain, HTH superfamily (Helix-turn-helix domains) a large family of mostly alpha-helical protein domains with a characteristic folds, and together form the OmpR, an osmolarity response regulator. (b) Histidine kinase EnvZ with RisS_PPD (periplasmic domain), HAMP (Histidine kinase, Adenylyl cyclase, Methyl-accepting protein, and Phosphatase), HisKA (phosphoacceptor domain) and HATPase_c (Histidine kinase-like ATPases). In other hand, (c) two-component response regulator, FixJ family, consists of REC and HTH domains. (d) Histidine kinase with a PAS sensor (found to bind ligands, and to act as sensors for light and oxygen in signal transduction) and/or ligand binding domain, similar to *E. coli* nitrogen regulator II, *Azorhizobium caulinodans* FixL and *Streptococcus mutans* CovS, having a variety of functions.

1.4 Two-component signaling systems: general structure and functions

Living organisms need to quickly detect environmental stimuli and respond accordingly in order to survive and proliferate. Signal transduction pathways control these detection and response mechanisms. In eukaryotes, signal transduction is frequently mediated by complex and branched pathways composed of multiple proteins, but prokaryotes have simpler systems, including 1-component systems and 2-component systems (TCSs) (Schaller et al. 2011).

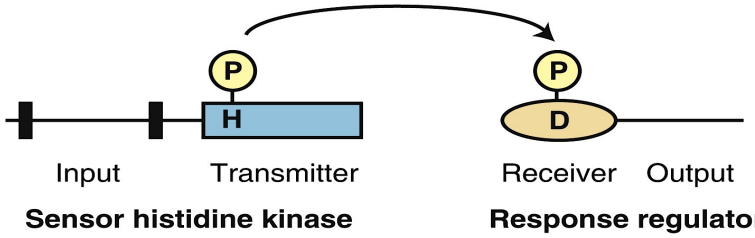

Figure 5 – General scheme of TCS in bacteria. The histidine kinase, through phosphorylation of a histidine (H) residue, detects the appropriate environmental signal and autophosphorylates from intracellular ATP. The sensor phosphokinase then transfers the phosphoryl group (P) to an aspartate residue (D) of the response regulator. Phosphorylation of the response regulator generally leads to its activation with an output. (Adapted from (Schaller et al. 2011))

For *B. multivorans* and most other opportunistic pathogens, the ability to sense external signals is critical for the transition from their environmental niche into the eukaryotic host, as well as for survival within specific niches in the host. TCS constitute a critical set of regulators, which act to sense environmental signals and respond by altering gene expression (Hoch 2000). Knowing to control virulence gene expression and host-pathogen interactions, these systems include a receptor histidine kinase (HK) that senses a specific signal and converts that input into a desired output over the phosphorylation of its cognate response regulator (RR) (**Figure 5**) (Capra & Laub 2012).

HK, the first component of TCS, detect the stimuli by a sensor domain regulating the HK through the transmitter domain (West & Stock 2001). The sensory domain mostly receives external environmental stimuli, which affect the balance between autophosphorylation and dephosphorylation of the HK (Gao & Stock 2013). The transmitter domain contains 2 subdomains: a dimerization domain with a conserved histidine residue and an ATPase domain that catalyzes phosphorylation of the conserved histidine. In most cases, histidine kinases are bifunctional such that, when not stimulated to autophosphorylate, they act as phosphatases for their cognate response regulators (Jin & Inouye 1993). The second component of TCS, the RR, usually has a phosphoryl group acceptor (receiver) domain followed by a diverse output (effector) domain. Effector domains in RR are characterized by their functional characteristics such as DNA-, RNA-, ligand-, or protein-binding abilities or enzymatic activity (Galperin 2010). Separation of the membrane-bound sensor and the soluble cytosolic regulator, which are linked via a phosphotransmission pathway, represents a tremendous evolutionary advance in prokaryotic signal transduction. The separation has allowed bacteria to dramatically expand and diversify their signaling capabilities (Capra & Laub 2012).

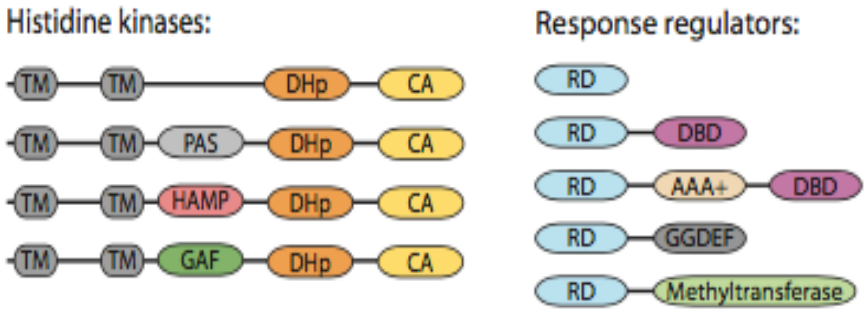

Figure 6 – Common domain organizations of HK and RR (Adapted from (Capra & Laub 2012)). For histidine kinases, the DHp (dimerization and histidine phosphotransferase) and CA (catalytic and ATPase) domains are shown with common intracellular domains PAS (Per Arnt Sim), HAMP (histidine kinase, adenyl cyclase, methyl-accepting proteins, and phosphatase), and GAF (cGMp-specific phosphodiesterase, adenyly cyclase and FhlA). Some kinases have multiple copies of such domains. Two TM (transmembrane) domains are shown on the kinases, but kinases can harbor from 1 to 13 TM domains. For response regulators, the conserved receiver domain is shown alone or with the common output domains, a DBD (DNA-binding domain), an AAA+ and DBD, a GGDEF domain involved in cyclic-di-GMP synthesis, or a CheB-like methyltransferase domain.

RRs are easier to group since they share a common well-conserved domain (RD, receiver domain), that catalyzes phosphotranfer from its cognate HK, and only differ in their effector domains (**Figure 6**) (Galperin 2006). HKs, in other hand, are hard to classify since family members do not necessarily share the same input domain. All histidine kinases contain two highly conserved domains, the dimerization and histidine phosphotransfer (DHp) domain, which harbors the conserved histidine that is the site of both the autophosphorylation and phosphotransfer reactions, and the catalytic and ATP binding (CA) domain. However, signal recognition domains tend to be more variable with, at least, one domain between the transmembrane and the DHp domains (Galperin 2005) (Figure 6).

1.4.1 Biological roles of EnvZ/OmpR TCS

Until now, a high number of TCSs have been identified in bacterial genomes, highlighting the impact of these systems on environmental adaptation of bacteria. The number of TCSs is related with bacteria genome size and metabolic versatility, with larger genomes and organisms that colonize different environments tending to encode more TCSs than bacteria with small genomes and living in a uniform habitat (Beier & Gross 2006).

Bacterial transcription factor OmpR is an extensively studied member of a subfamily of more than 50 response regulators characterized by sequence similarity in their C-terminal DNA-binding domain

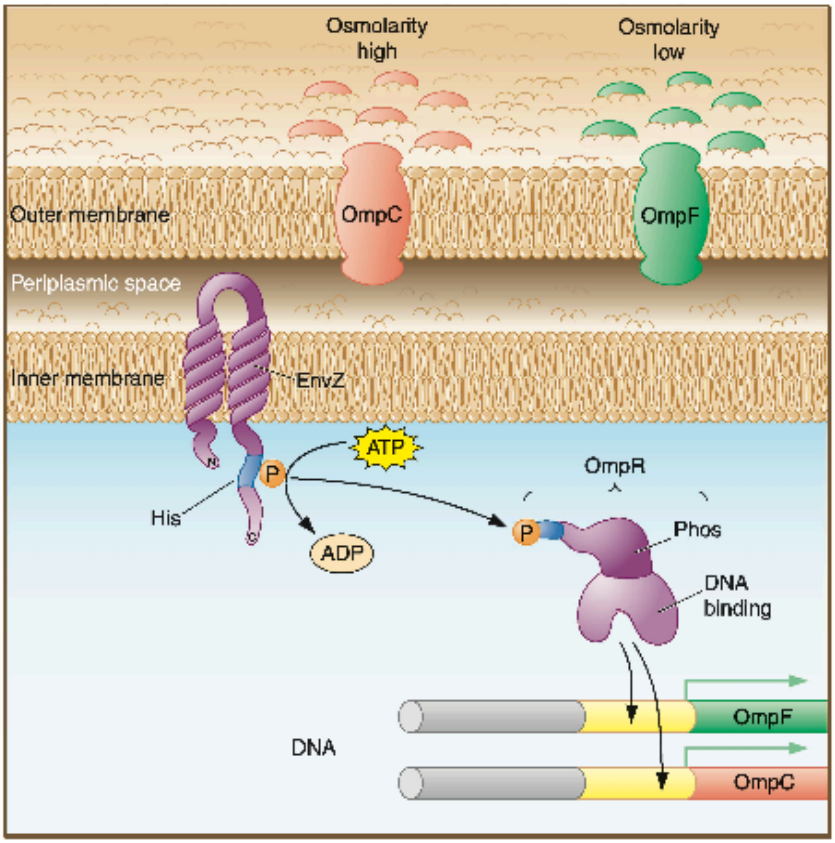

Figure 7 – EnvZ/OmpR system regulating the porin genes (Adapted from (Feng et al. 2003)). In low osmolarity medium, OmpF is present in higher amounts than OmpC in bacterial cell outer membrane. Otherwise, in high osmolarity media, OmpC is more abundant than OmpF. EnvZ/OmpR proteins, members of a TCS, regulate this process. EnvZ is an inner membrane sensor kinase phosphorylated by cytoplasmic ATP. It transfers the phosphoryl group to the response regulator OmpR. Phospho-OmpR binds to the regulatory regions of the porin genes and alters their expression.

(Mizuno 1997). *Escherichia coli* OmpR and the osmosensor HK EnvZ function as a TCS controlling the production of the outer membrane porins, OmpF and OmpC that enable bacterial cells to survive fluctuations in the osmolarity of the growth medium (Martínez-Hackert & Stock 1997).

Activation of EnvZ by an unknown signal leads to phosphorylation of OmpR at aspartate (Igo & Silhavy 1988). It is known that signals such as increasing osmolarity, temperature or acid pH (between others) induce the activity of the EnvZ-OmpR TCS, however, the specific signal detected by EnvZ is still unclear (Pratt et al. 1996).

OmpF and OmpC are porin proteins located in the outer membrane that allow polar molecules to cross this barrier, changing their amounts in response to medium osmolarity (Foster & Spector 2002). In low osmolarity medium, OmpF is present in higher amounts than OmpC in bacterial cell membrane. Otherwise, in high osmolarity, OmpC is more abundant than OmpF (Feng et al. 2003) (**Figure 7**).

Regulation of genes codifying for these proteins is mediated by *envZ* and *ompR* genes, which codify EnvZ/OmpR proteins. EnvZ is a transmembrane protein that acts as a sensor, suffering autophosphorylation in a histidine residue in response to increase of medium osmolarity. Subsequently, the phosphoryl group is transferred to the response regulator (OmpR), which increases the level of phosphorylated OmpR (OmpR-P) in the cell. At low osmolarity, OmpR-P binds to *ompF* promoter sites (with high affinity), activating the transcription of *ompF,* and subsequently increasing OmpF levels. When an increase in media osmolarity occurs, OmpR-P binds to *ompC* promoter sites (with low affinity), which result in OmpC activation. Besides osmolarity, the EnvZ-OmpR system is responsible for other functions in cellular physiology, including flagellar expression, cell division, fatty acid transport, microcin synthesis, curly fibers and acid tolerance, showing the versatility of TCSs systems (Foster & Spector 2002).

1.4.2 Biological roles of FixL/FixJ (LuxR) TCS

Bacterial responses to changes in pO_2 are crucial for balancing central metabolism as well as inducing cellular machinery needed for specialized metabolic pathways. FixL/FixJ, a bacterial O_2-sensing two-component signal transduction, was first identified in *Rhizobium meliloti* (de Philip et al. 1990). These two-component regulatory systems are found widely, even in *Mycobacterium tuberculosis,* a pathogen responsible for causing tuberculosis (Cho et al. 2009).

FixL proteins contain a HK domain and at least one PAS (Per-ARNT-Sim) domain, which contain the heme that bind O_2 (Monson et al. 1992). FixL has a relatively low O_2 affinity (*K*D ~ 100 μM) (Tanaka et al. 2006), allowing for physiological changes in pO_2 to alter the transcriptional activity of FixJ. Under hypoxic conditions, the unligated five coordinate high spin Fe(II) of FixL induces gene expression (Gilles-González et al. 1995) as this state of FixL autophosphorylates a conserved His residue within its HK domain. Phosphoryl transfer to a conserved Asp residue in the response regulator FixJ (Gilles-Gonzalez & Gonzalez 1993) induces a conformational change that leads to dimerization, thereby increasing affinity for the *fixK* promoter and inducing gene expression (Gouet et al. 1999). Upon binding of O_2, the Fe(II) converts to low spin six coordinate geometry, flattening the heme ring and disrupting hydrogen bonding interactions to the heme propionate groups. Most notable is the distal arginine residue breaking a salt bridge with a heme propionate group to help stabilize O_2, initiating the conformational change of FixL's FG loop needed to inhibit phosphorylation (Hiruma et al. 2007).

Oxygen controls the expression of the nitrogen-fixation genes of *Rhizobium meliloti*. When free oxygen concentration is reduced to microaerobic levels FixL and FixJ initiate the oxygen-response cascade. The *fixJ* product, a cytoplasmic regulator, seems to be modulating by *fixL* product, a transmembrane sensor. FixL and FixJ are homologous to a family of bacterial two-component regulators, for which the mode of signal transduction is phosphorylation (Gilles-Gonzalez et al. 1991).

On the basis of sequence similarities, many of the response regulators and cognate sensors can be classified into either the NtrC-, OmpR- or FixJ-type subfamily (Stock et al. 1990).

In rhizobial species, FixL histidine kinases bind a single molecule of iron protoporphyrin IX (heme b) through their amino-terminal PAS domain (Gilles-Gonzalez et al. 1991), which, in the presence of molecular oxygen, forms a ferrous (Fe^{2+}) oxyheme complex that inhibits kinase activity (Gilles-Gonzalez et al. 1994). Under low oxygen concentrations, oxygen dissociates from the iron center of heme leading to up-regulation of kinase activity, transphosphorylation of the FixJ response regulator, and increased transcription of genes required for nitrogen fixation (Fischer 1994).

A high number of nonsynonymous mutations in the *B. multivorans fixL* homolog (BMD20_10585 shown in **Figure 3** (d)) was recently reported in another whole genome sequencing study of isolates recovered from chronically infected CF-patients (Silva et al. 2016), suggesting that the FixLJ system has a similar role in all 3 Bcc species usually seen in CF patients (*B. cenocepacia*, *B. multivorans*, and *B. dolosa)*. The FixLJ system in *Rhizobium* and *Caulobacter* induce expression of genes required for fixing of atmospheric nitrogen in the absence of oxygen (Saito et al. 2003). Although *B. vietnamiensis* and *Burkholderia* species outside of the Bcc can fix nitrogen, most members of the Bcc, including *B. cenocepacia*, *B. multivorans*, and *B. dolosa*, lack the genes necessary for nitrogen fixation (Suárez-Moreno et al. 2012).

A recent study in *B. dolosa* report the functional significance of the *fixLJ* genes, finding not only that the pathway is induced by low oxygen, but also that it regulates a large number of genes and is critical for pathogenicity *in vivo* and intracellular invasion *in vitro* (Schaefers et al. 2017). Bcc ability to invade and/or survive within epithelial cells and macrophages is thought to represent an important aspect of Bcc pathogenesis (Cieri et al. 2002). Studies in *B. dolosa* found that *B. dolosa fixLJ* deletion mutant was fewer invasive towards A549 epithelial cells and THP-1 derived macrophages. It is possible that the *fixLJ* deletion mutant is either less able to invade the cell or less able to survive within the cell, or both. *fixLJ* deletion mutant is unable to regulate appropriate gene expression to survive within the intracellular environment once it is unable to detect the lower oxygen concentration found there (Schaefers et al. 2017).

FixL homologs in other pathogenic Gram-negative bacteria include one in *Brucella*, which was found to be necessary for intracellular survival (Roset & Almirón 2013). *Pseudomonas aeruginosa* has a FixL homolog called BfiS (biofilm initiation sensor) along with a FixJ homolog (BfiR), which, as their names suggest, have been shown to be critical for the irreversible attachment phase of biofilm development (Petrova & Sauer 2011). However, the clinical relevance of Bcc biofilm production is unclear. Indeed, one study found no correlation between the ability of Bcc isolates to form biofilm and clinical outcomes (Cunha et al. 2004). In another study that examined *P. aeruginosa* and/or Bcc infected CF lung tissue removed after transplant using species-specific antibodies to stain the lung tissue, Bcc bacteria were rarely found in biofilm-like structures while *P. aeruginosa* were often found in such structures (Saldias et al. 2008). These reports suggest that biofilm production may not be beneficial for Bcc infection.

B. dolosa FixLJ system has been suggested as an oxygen-sensing mechanism that regulates biofilm formation, motility, intracellular invasion, and virulence. It seems that *fixLJ* is required for *in vivo* persistence by limiting biofilm formation and allowing for survival within macrophages, which is known to be a low-oxygen environment (Schaefers et al. 2017).

AIMS OF THIS WORK

Burkholderia strains are able to produce different exopolysaccharides, but the most common is cepacian. The *bce* genes involved in this process are well known and encode several proteins whose function is the synthesis of the sugar-nucleotides, synthesis of the heptasaccharide repeat-unit, polymerization and secretion to the extracellular milieux. Despite this knowledge, the genes involved in the regulation of the *bce* genes expression is poorly uncovered. With that in mind, silva and collaborators (Silva et al. 2013) exposed the CF late isolate *B. multivorans* BM11 to different stress conditions and obtained several nonmucoid variants. To identify the mutations responsible for lack of cepacian biosynthesis, the genome of ten variants was sequenced. Data analysis identified a mutation in *bceF* gene encoding a tyrosine kinase involved in cepacian biosynthesis in one variant; eight variants had mutations in *ompR* gene; and the last variant had a mutation in *fixJ* gene. To evaluate whether this phenotypic trait switch also occurred in an early CF isolate from the same patient, we envisaged the following experiments:

1. Expose *B. multivorans* BM1 isolate recovered 13 years before BM11 to stress condition to see whether mucoid-to-nonmucoid switch is also occurring.
2. Colonies of interest (nonmucoid/less mucoid) will be randomly chosen and whole genome will be sequenced to identify possible mutations
3. Genetic complementation of the mutated genes will be done, to evaluate their relevance in the mucoid switch
4. Phenotypic characterization of the possible mutants, namely by evaluating growth, antibiotic resistance and biofilm formation
5. Biochemical studies to demonstrate whether the previously identified FixJ and OmpR response regulators bind to the bce promoter.

2. MATERIAL AND METHODS

2.1 Biological material

Several bacterial strains and plasmids used in this study are described in **Table 1.** *Burkholderia* strains were used to study EPS production or other phenotypic characteristics. *E. coli* strains were host cells for the cloning experiments or used for His_6-tag protein overexpression and purification.

Table 1 – Bacterial strains and plasmids used in this work.

Strain or Plasmid	Relevant characteristic(s)	Source/reference
Bacterial Strains		
B. multivorans		
C5568 (BM1)	Cystic fibrosis clinical isolate, mucoid. Canada (date of isolation: 30.11.1993)	Silva et al. 2016
BM1-1	Nonmucoid variants derived from BM1 after 21 days at 37ºC under nutrient starvation	This work
BM1-2		This work
BM1-3		This work
BM1-4		This work
BM1-5		This work
BM1-6		This work
BM1-7		This work
BM1-8		This work
BM1-9		This work
BM1-10		This work
D2095 (BM11)	Cystic fibrosis clinical isolate, mucoid. Canada (date of isolation: 1.6.2006)	Silva et al. 2016
BMV10	Nonmucoid variant obtained from BM11 after 21 days at 37ºC under nutrient starvation	Silva et al. (unpublished)
BMV10R	Mucoid revertant obtained from BMV10 after 14 days at 37ºC	Silva et al. (unpublished)
E. coli DH5α	*recA1* Δ*lacU*169, φ80 *lacZ*ΔM15	Invitrogen
Plasmids		
pWH844	Bacterial expression vector, 4978 bp, Amp^r	Schirmer et al. 1997
pUK21	*E. coli* cloning high copy vector 3089bp, $lacZ^+$, Kan^r	Vieira et al. 1991
pBBR1MCS	Broad-host-range cloning vector, 4.7 kb, $lacZ^+$, mob^+, Cm^r	Kovach et al. 1994
pLM127-13	pBBR1MCS_derivative expressing gene *bceF* of *B. contaminans* IST408 under control of the bce promoter, Cm^r	Ferreira et al. 2015
pLM014-3	pBBR1MCS_derivative expressing gene *fixJ* (*Bmul_1131*) of *B. multivorans* ATCC17616 under control of its own promoter, Cm^r	Moreira et al. (unpublished)
pRK2013	Mobilizing vector, ColE1 tra $(RK2)^+$, Kan^r	Figurski et al. 1979
pFB17-1	pWH844 containing the 832 bp BamHI/HindIII fragment containing the *ompR* gene from BM1, Amp^r	This study
pFB17-2	pWH844 containing the 800 bp BamHI/HindIII fragment containing the *fixJ* gene from BM1, Amp^r	This study
pFB17-3	pUK21 containing the 269 bp KpnI/NdeI fragment containing the *ompR* promoter from BM1, Cm^r	This study

Abbreviations: Kan^r: kanamycin resistance; Amp^r: ampicillin resistance; Cm^r: chloramphenicol resistance

2.2 Culture conditions

Burkholderia strains were grown in Lennox Broth (LB: 5 $g.L^{-1}$ NaCl; 5 $g.L^{-1}$ yeast extract; 10 $g.L^{-1}$ tryptone) or in EPS producing SM medium (12.5 $g.L^{-1}$ $Na_2HPO_4.2H_2O$; 3.0 $g.L^{-1}$ KH_2PO_4; 1.0 $g.L^{-1}$ K_2SO_4; 1.0 $g.L^{-1}$ NaCl; 20 $g.L^{-1}$ mannitol; 1.0 $g.L^{-1}$ casamino acids; 1.0 $g.L^{-1}$ yeast extract; 0.2 $g.L^{-1}$ $MgSO_4.7H_2O$; 0.001 $g.L^{-1}$ $CaCl_2.2H_2O$; 0.001 $g.L^{-1}$ $FeSO_4.7H_2O$) at 37ºC (Silva et al. 2013).

E. coli strains were grown in LB or Super Broth (SB) medium (32 $g.L^{-1}$ tryptone; 20 $g.L^{-1}$ yeast extract; 5 $g.L^{-1}$ NaCl).

The strains, when in use, were maintained on LB plates. Otherwise, the strains were conserved at -80 ºC with 30% glycerol.

2.3 Variation of colony morphology under nutrient starvation

To identify nonmucoid variants derived from mucoid *B. multivorans* BM1, this isolate was inoculated in 3 mL of SM medium (OD_{640nm} of 0.1) and maintained statically at 37 ºC for 21 days. At the end of this period, an aliquot was serially diluted and spread onto the surface of YEM agar plates (0.5 $g.L^{-1}$ yeast extract; 4 $g.L^{-1}$ mannitol; 15 $g.L^{-1}$ agar) and incubated at 37 ºC for 2 days. Colonies showing nonmucoid appearance were kept.

2.4 Growth rate and doubling time determination

Strains were grown at 37 ºC, 250 rpm, for 24 h in 100 mL of LB liquid medium, LB supplemented with NaCl (2.5 %) and LB supplemented with sucrose (20 %). Growth rates and doubling times were calculated from the exponential phase of growth. Two independent experiments, each with three replicates, were performed.

2.5 Phenotypic assays

2.5.1 Exopolysaccharide production

The amount of EPS produced was assessed based on the dry-weight of the ethanol-precipitated polysaccharide recovered from 50 mL cultures of the different strains grown in liquid SM medium over 5 days at 37 ºC with agitation (250 rpm), as described before (Ferreira et al. 2007). Bacterial cells present in the cultures were separated by centrifugation at 9000 rpm (Sigma-Aldrich 2K15) for 15 min. EPS was then precipitated from cell-free supernatants by the addition of 3 volumes of ethanol 96 %. After collection and evaporation, EPS was dried overnight and weighted. Two independent experiments, each with two samples per isolate, were performed.

2.5.2 Antimicrobial susceptibility

Antimicrobial susceptibility tests were based on the agar disc diffusion method (Bauer et al. 1966) using paper discs containing ciprofloxacin (5 µg), piperacillin (75 µg) plus tazobactam (10 µg), aztreonam (30 µg) and kanamycin (30 µg). The discs were applied onto the surface of Mueller-Hinton (Difco Laboratories) agar plates previously inoculated with 100 µL of a suspension at OD_{640nm} of 0.1 prepared from exponential-phase cells growing on LB medium for 5 h, at 37 ºC under 250 rpm, after overnight inoculation. Growth inhibition diameter was measured after 24 h (variants of BM1) or 48 h (variants of BM11) of incubation at 37 ºC. Two independent experiments, each with three replicates, were performed.

2.5.3 Biofilm formation

Biofilm formation assays were performed based on the method previously described (Ferreira et al. 2007). Overnight liquid cultures, grown at 37 ºC with agitation, of the different strains were diluted to a standardized OD640nm of 0.05. Subsequently, 200 µL of these cell suspensions were used to inoculate the wells of a 96-well polystyrene microtiter plate. Plates were incubated at 37 ºC statically for 48 h. Culture media and unattached bacterial cells were removed and the wells were cleaned with saline solution (three times, 200 µL for each rinse). Adherent bacteria were stained with 200 µL of crystal violet solution (1 % wt/vol) for 20 min at room temperature, and after three washes with 200 µL of saline solution each time, the dye associated with the attached cells was solubilized in 200 µL of 96% ethanol and the biofilm was quantified by measuring the absorbance of the solution at 590 nm using a microplate reader (Spectrostar nano, BMG LabTech). Three independent experiments, each with two 96-well plates, were performed.

2.6 DNA manipulation techniques

2.6.1 Genomic DNA extraction

Cells were harvested from LB plates, were ressuspended in 180 µL Buffer ATL (DNeasy® Blood & Tissue, QIAGEN). Proteinase K was added (20 µL) and the mixture incubated at 56ºC until cell lysis occurred (usually in 1-3 h). Then Buffer AL/ ethanol (200 µL of each premixed) was added and the mixture was pipet into the DNeasy Mini spin column, placed in a 2 ml collection tube and centrifuged at 8000 rpm for 2 min. Column was placed in a new collection tube and 500 µL of Buffer AW1 were added. After 1 min centrifugation at 8000 rpm, the flow-through was discarded and 500 µL of Buffer AW2 were added. After 3 min centrifugation at 14000 rpm the column was placed in a clean 1.5 ml microcentrifuge tube and the elution was done adding 50 µL of sterile water and centrifuged at 8000 rpm during 1 min.

DNA concentration was estimated and quality was checked using UV spectrophotometer (ND-1000 UV-Vis, NanoDrop Technologies, USA) and by agarose gel electrophoresis using standard procedures (Sambrook et al. 2001).

2.6.2 Plasmid DNA extraction

Plasmid DNA was extracted from cells grown in LB plates using the ZR Plasmid Miniprep™ kit (Zymo Research). Briefly, 200 µL of Buffer P1, 200 µL of P2 and 400 µL of Buffer P3 were added in this order and lysates mixed thoroughly. After 3 min centrifugation at 13200 rpm the supernatant was added to the Zymo-spin™ IIN column. After centrifugation (1 min at 13200 rpm), 200 µL of Endo-Wash Buffer followed by 400 µL of Plasmid Wash Buffer were added. After 1 min centrifugation at 13200 rpm, 60 µL of sterile water were used for the elution step. DNA concentration was estimated and quality was checked as referred above.

Screening of the correct plasmids during cloning experiments was carried out by a rapid and easy process. Cells of overnight cultures in LB solid were ressuspended in 200 µL of P1 solution (50 mM tris pH 8; 10 mM EDTA). Then, 200 µL of P2 solution (200 mM NaOH; 1% SDS) and 200 µL of P3 solution (3 M sodium acetate pH 5.5) were added and mixed by inversion. Centrifugation was performed and DNA present in the supernatant was precipitated with 500 µL isopropanol solution. After centrifugation 30 min supernatant was discarded and pellet was washed with ethanol 70%. The pellet was ressuspended in 70 µL of sterile water after dried in vacuum for 15 min at 45 ºC.

2.6.3 DNA amplification, restriction and ligation

Amplification of different genes was carried out by polymerase chain reaction (PCR). Each amplification contained 5 µL of genomic DNA (40 ng.µL^{-1}), 2.5 µL of Buffer 10X (Nzytech), 0.5 µL of 2.5 mM dNTPs (TaKaRa), 0.5 µL of each 25 nM primer solution (Stab Vida), 0.5 µL of $MgCl_2$ (50 mM) solution (Nzytech), 1 µL of DMSO (100 %), 12.5 µL of sterile water and 0.5 µL of taqmed polymerase (Nzytech). PCR amplification cycling parameters were optimized as follows: preincubation at 95 ºC for 5 min, 34 cycles of denaturation at 95 ºC for 30 seconds, annealing step at convenient temperature (**Table 2**) for 45 seconds and an extension step at 72 ºC for 2 minutes. A final extension step was carried out at 72 ºC for 10 minutes. Amplification procedure was performed in a Thermal Cycler Block (BioRad). The oligonucleotide specific primers sequences used for the amplification of the different genes were designed based on the genome sequence of *B. multivorans* BM1 using OligoPerfect™ Designer as shown in **Table 2**. The DNA amplification product was confirmed using a 0.8 % (w/v) agarose gel electrophoresis, carried out at 90 or 100 volts during 1h or 1h30, respectively, using NZYDNA ladder III (Nzytech) as ladder. After staining with GelRed (Biotium Inc.) solution, gel was visualized under short wave UV light in a transilluminator (BioRad).

Reaction mixtures with the amplification product were concentrated and purified using DNA Clean and Concentrator™ kit (Zymo Research) and eluted in 50 µL of sterile water.

Table 2 – Oligonucleotides used for PCR amplification. The recognition sites for restriction endonucleases are in bold

Gene	Primer Name	Primer Sequence (5' → 3')	Restriction site	Vector	Product size	Annealing T (ºC)
OmpR	HisOmpR_F	CGC**GGATCC**GAAACGAAAAA CCCCTCCA	BamHI	pWH844	832 bp	59
	HisOmpR_R	CCC**AAGCTT**GATCAGCAGGA AGGTGCG	HindIII			
FixJ	HisFixJ_F	CGC**GGATCC**AATAGTCCTGT CACCACCACCCA	BamHI		800 bp	59
	HisFixJ_R	CCC**AAGCTT**TACCGTTCGCC TGTCGCG	HindIII			
OmpR promoter	OmpRprom_F	CGG**GGTACC**GACCGTCCTCG CTTCTTTG	KpnI	pUK21	269 bp	61
	OmpRprom_R	CGC**CATATG**CATGAGCGGCA TCTTATCAC	NdeI			
EnvZ	EnvZ_F	CGC**CATATG**CGTATCGACCG GCGCCTT	NdeI		1545 bp	61
	EnvZ_R	GC**TCTAGA**GGATACTCCTGT ATGAGTTGGGAAGAAG	XbaI			
FixL	FixL_F	CCC**AAGCTT**AGATGTTCGCC GTGCATGC	HindIII	pBBR1MCS	3101 bp	-
	FixL_R	GC**TCTAGA**AGAAACTGCTCC GCGCTCG	XbaI			
	FixLA_F	CGG**GGTACC**GCCATTCAACG GTTTCCTG	KpnI		1951 bp	65
	FixLB_R	CGACGTACACCTCCTGCG	-			
	FixLC_F	ACGAAGCCGAGCTGCTGTT	-		1221 bp	-
	FixLD_R	GC**TCTAGA**ACGCGATAGCCG TTCGCCT	XbaI			

In the specific case of *envZ* gene the amplification product was recovered from the agarose gel using the Zymoclean™ Gel DNA Recovery (Zymo Research) according to the manufacturer's protocol. The purified products were used as inserts in further ligations and cloning procedures.

For the cloning procedure the inserts obtained from PCR were digested using the appropriate enzymes and cloned into appropriate vectors (**Table 2**). Digestion products were precipitated and the fragments/vectors were quantified using Nanodrop. After digestion the inserts and vectors were ligated using T4 ligase (TaKaRa). For this process 4 µL of vector plus 13 µL of insert were added to 2 µL of T4 buffer (TaKaRa) plus 1 µL of T4 ligase and the mixture were incubated overnight at 16 ºC.

The 832 bp (*ompR*) and 800 bp (*fixJ*) amplicons were digested with BamHI and HindIII and were

subsequently cloned into the BamHI/HindIII cloning sites of pWH844, yielding plasmids pFB17-1 and pFB17-2, respectively. The protein expression was controlled by the T5 promoter expressing the proteins with a 6×His-tag at N-terminus.

2.7 Bacterial cells transformation

2.7.1 Electrotransformation

To prepare electrocompetent cells *E. coli* DH5α cells was growth overnight at 37 ºC with orbital agitation (250 rpm). The starter culture was used to inoculate 750 ml of LB in order to start growth with 0.05 OD_{640nm}, at 37 ºC at 250 rpm. When the inoculum reached 1.2 ± 0.1 OD_{640nm}, cell suspension was centrifuged three times at 5000 rpm at 4 ºC during 15 min, with decreasing resupensions in cold de-ionized water, followed by two centrifugations at 5000 rpm at 4 ºC during 10 min with resuspension in decreasing volumes of glycerol 10 % (v/v).

For electrotransformation 100 µL of electrocompetent cells were transformed with 10 µL of foreign DNA using a Gene Pulser™ apparatus (BioRad) set at 2.5 kV, 400Ω resistance and 25 µF capacitance. After the electric pulse cells were incubated in LB medium for 1 h. After incubation the bacterial cultures were plated on appropriate selective medium.

2.7.2 Triparental conjugation

Triparental conjugation to *B. multivorans* strains was performed using the helper plasmid pRK2013. Cells of overnight cultures of the *Burkholderia* recipient, *E. coli* donor and *E. coli* helper strains (2:1:1) were harvested, washed with sterile 0.9 % (wt/v) NaCl solution and mixed. Supernatant was discarded and the pellet ressuspended in the rest of the solution remaining. Cells were scattered in LB agar plates and incubated for 24h at 30 ºC. Bacteria were then scraped and suspended in 1 mL of 0.9 % (wt/v) NaCl. Serial dilutions were performed and 100 µL were plated on selective medium with ampicillin (150 mg/L) and chloramphenicol (200 mg/L), enabling the positive selection of transconjugants. The samples were incubated 4 days at 37 ºC.

2.8 Overexpression and purification of recombinant proteins

2.8.1 Overexpression of OmpR and FixJ recombinant proteins

The overexpression of His_6-OmpR and His_6-FixJ proteins was carried out using *E. coli* DH5α as host cells. Overexpression of the 6×His-tagged proteins was performed by growing the cells in 250 mL of LB liquid medium supplemented with 150 mg/L ampicillin at 37 ºC and with orbital agitation (250 rpm). When the culture reached 0.5 OD_{640nm}, IsoPropyl-ß-D-ThioGalactoside (IPTG) was added (final

concentration 1 mM) and incubation was prolonged for 4 h. Bacteria were harvested by centrifugation at 7000 rpm for 10 min at 4 ºC, and ressuspended in 10 mL of start buffer (0.2 mM sodium phosphate, 0.5M NaCl, pH 7.4) containing 10 mM imidazole. Cell suspensions were kept at -80 ºC until purification. Aliquots of these cell suspensions were processed and protein overproduction in total extracts was assessed by 12.5 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) following standard procedures described in 2.8.3.

2.8.2 Extraction and purification

In order to purify OmpR and FixJ recombinant proteins, cells were disrupted (mechanical lysis of cells walls and membranes) by sonication (Branson Sonifier Sound Enclosure 250) during 9 cycles of 30 seconds each and placed 10 min in ice between each cycle. These were centrifuged at 17600×g for 5 min at 4 ºC. The pellet was discarded and the supernatant was centrifuged again in the same conditions for 1 hour.

A histidine affinity column (HisTrap™ FF, GE Helthcare) was used to purify the proteins as the genes were cloned into a plasmid with nucleotide sequence corresponding to a histidine tag (6×His). The elution of proteins was performed in ÄKTA system (GE Healthcare) with automatic increases in the concentrations of imidazole (20-500 mM). The buffer (rich in imidazole) was then exchanged to Phosphate Buffered Saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM of $Na_2HPO_4.2H_2O$ and 1.47 mM KH_2PO_4) in the same system but with a desalting column (HiPrep™ 26/10 Desalting, GE Healthcare). Proteins were stored at 4 ºC until further use.

2.8.3 Bradford and SDS-PAGE

Purified fractions were analyzed by SDS-PAGE and protein concentration was estimated by the method of Bradford. Particularly, 20 µL aliquots of protein samples were added to 10 µL of application buffer (2-mercaptoethanol; Sodium Dodecyl Sulphate (SDS); bromophenol blue; and glycerol). Then, samples were denatured at 95 ºC during 5 min and separated by electrophoresis in SDS-PAGE (**Table 3**) comparing with protein standard PageRuler™ Plus (ThermoScientific). The bands were visualized by the addition of BlueSafe (Nzytech).

For the quantification, samples were transferred to a 96-well plates and Quantification Protein Kit (Bradford, BioRad) was used. The determination of the total protein concentration, per sample, was achieved through the use of a calibration curve, on which were used the absorbance values of standard samples of bovine serum albumin, whose concentrations are known (provided by the kit).

Table 3 – Solutions and their quantity to prepare SDS-PAGE gels

	12.5 % Gel Resolving (mL)	4 % Gel Stacking (mL)
Solution I *	1.25	-
Solution II **	-	0.5
H_2O	1.64	1.22
30 % Acrylamide	2.08	0.27
TEMED	0.0025	0.002
10 % Ammonium Persulfate	0.05	0.02

*Solution I: Tris 1.5 M, 10 % SDS, HCl, H_2O, pH 8.9
**Solution II: Tris 0.5M, 10 % SDS, HCl, H_2O, pH 6.7

2.9 DNA binding Assays

To assess the ability of purified His_6-tagged OmpR and FixJ proteins to bind DNA, mobility shift assays in agarose and polyacrylamide gels were performed. For agarose gels different amounts of biotinylated probes (**Table 4**) were incubated for 30 min at 25 ºC in a total volume of 20 µL of DNA binding buffer (20 mM Tris-HCl pH 7.5, 50 mM KCl, 12 % (v/v) glycerol and 4 mM $MgCl_2$). After incubation, samples were mixed with one volume of 10x Gel Loading Dye (TaKaRa) and were loaded in a 1% (wt/v) agarose gel in 1x Running Buffer (90 mM Tris base, 90 mM Boric acid, pH 8.5). The gel was then electrophoresed at a constant voltage of 100V at 4 ºC, for an adequate time to visualize the DNA fragments. After electrophoresis gel was stained with a 3x staining solution of Gel Red™ (Biotium) and fragments were compared to O'Gene Ruler 50bp DNA ladder (Fermentas).

For the detection of complex formation between DNA probes bceB_P1, bceA_P2 and bceB_CDS (2 nM of each per ligation) and various amounts (between 0 and 2.4 µM) of 6×His-tagged purified proteins, incubation in 3x DNA Binding Buffer (120 µL Tris 1M pH 8, 300 µL KCl, 300 µL $MgCl_2$ 0.1M, 150 µL glycerol and 130 µL H_2O) for 30 min at 25 ºC was carried out. After addition of 0.2 volumes of DNA Gel Loading Dye, the reaction mixtures were directly subjected to electrophoresis on 6 % acrylamide gels (2 mL acrylamide 30 %, 5 mL TBE 1x, 0.5 mL glycerol, 2.5 mL H_2O, 100 µL of 10 % ammonium persulfate and 10 µL TEMED) in 1x TBE buffer (44.5 mM Tris base, 44.5 mM boric, 1 mM EDTA) at a constant current (30 mA) at 4 ºC. The DNA was electroblotted to Amersham Hybond™-N^+ positively charged nylon membranes (GE Healthcare) using the Trans-Blot® SD (BioRad) device apparatus at 15V for 50 min. Membranes were UV-crosslinked and the DNA probes were detected by chemiluminescence using the Chemiluminescent Nucleic Acid Detection Module kit (Thermo Scientific) according to the manufacturer's instructions. The visualization was carried out using FUSION software.

Table 4 – Sequence of oligonucleotides used in EMSA assays

Oligonucleotides	Primer Sequence (5' → 3') / Length	Modifications
bceB_P1_fw	CATGCCGATTTAATTCATCGACGACGCAGCAAGAATTGCCGATTATTTTTCGAAATACTTGTGCGCTTGCGGGAGGAATTTTCTTGTCGGTTCAATAGCGACATGCAACG (110 nucleotides)	5': Biotin-TEG
bceB_P1_rev	CGTTGCATGTCGCTATTGAACCGACAAGAAAATTCCTCCCGCAAGCGCACAAGTATTTCGAAAAATAATCGGCAATTCTTGCTGCGTCGTCGATGAATTAAATCGGCATG (110 nucleotides)	
bceA_P2_fw	GGCGTTGGCGGCCGAAGCGCGGAATTATTTTTTATTTTTTTATCGCGCTGCCGGGGATGCGCGGAAAATTTTCCCGGCGCAATGAAACGGTAATGATCGACAAAGGCGCCCGATTTTATTGT (122 nucleotides)	5': Biotin-TEG
bceA_P2_rev	ACAATAAAATCGGGCGCCTTTGTCGATCATTACCGTTTCATTGCGCCGGGAAAATTTTCCGCGCATCCCGCCCAGCGCGATAAAAAAATAAAAAATAATTCCGCGCTTTGGCCGCCAACGCC (122 nucleotides)	
bceB_CDS_fw	TGCAGCGCACGATGGTGCTGTTCGACTGCCTGCTCGTCGTCGTGTGCTTTCCGGCATTCGGCATCTATCAGTCCTGGCGCGGCAAGCGGCTCGTCGGGCT (100 nucleotides)	5': Biotin-TEG
bceB_CDS_rev	AGCCCGACGAGCCGCTTGCCGCGCCAGGACTGATAGATGCCGAATGCCGGAAAGCACACGACGACGAGCAGGCAGTCGAACAGCACCATCGTGCGCTGCA (100 nucleotides)	

3. RESULTS and DISCUSSION

3.1 Identification of genes involved in the mucoid-to-nonmucoid morphotype variation in *B. multivorans*

Since it was possible to obtain nonmucoid variants derived from later isolate of *B. multivorans* recovered from a cystic fibrosis patient (Silva et al., unpublished), we tested whether the first isolate, BM1, also had that property. For that, three independent cultures of *B. multivorans* BM1 were incubated in SM medium for 21 days statically. After that time of prolonged stationary phase, an aliquot was serially diluted and plated onto EPS producing YEM medium. Assessment of the mucoid morphotype of grown colonies led to observation that most of them were highly mucoid while a few of them were much smaller and apparently less or nonmucoid (**Figure 8**). Ten of these colonies were kept for further studies and were named BM1-1 to BM1-10.

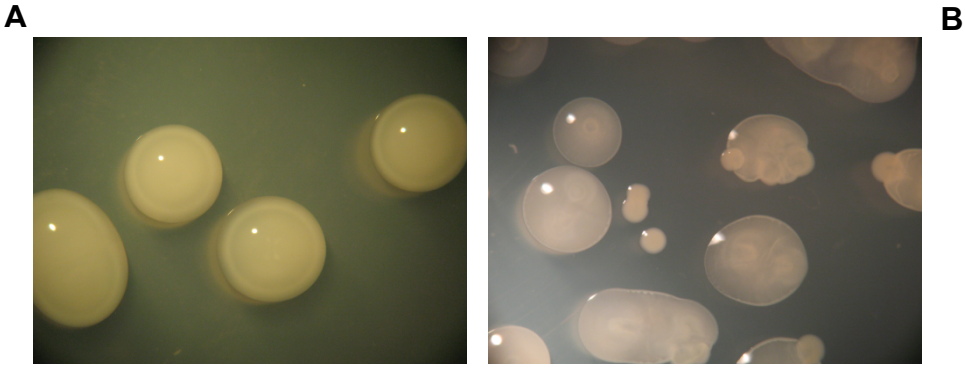

Figure 8 – Arising of *B. multivorans* BM1 variants with different mucoid morphology by exposure to prolonged stationary phase. Before incubation all colonies displayed the mucoid phenotype in YEM medium (A) while after 21 days it also observed several small colonies nonmucoid or slightly mucoid (B)

Four of these nonmucoid variants (BM1-1, BM1-2, BM1-3 and BM1-4) were chosen for whole genome sequence. Mapping reads against the genome of the reference strain *B. multivorans* BM1 identified the mutations present in these variants (analysis performed by Inês Silva). **Table 5** shows the results of this analysis. The nonmucoid variant BM1-1 has a single mutation (36bp insertion) in *bceF* gene encoding a tyrosine kinase involved in cepacian biosynthesis. BM1-2 shows a nonsynonymous R66W mutation in response regulator FixJ and a synonymous mutation in SecG protein. BM1-3 has a deletion of 21 amino acids in FixL sensor histidine kinase, the same gene found as mutated in BM1-4 variant, although here is a nonsynonymous substitution (V463G) (**Table 5**). In addition to these mutants, **Table 5** also includes a nonmucoid variant derived from *B. multivorans* BM11 (named BMV10) with the R66W substitution in FixJ protein, and a mucoid revertant BMV10R which besides the R66W mutation accumulates a mutation in FixL protein caused by the introduction of a STOP codon creating a truncated protein.

Table 5 – List of mutations present in the different nonmucoid variants comparing with the parental isolates

	Variant	Mutation	Annotation	Gene Locus	Description
BM1	BM1-1	+ 36bp	Coding (1025/2226 nt)	BMD20_03555 →	Exopolysaccharide biosynthesis protein (*bceF*)
	BM1-2	G → A	R66W (CGG → TGG)	BMD20_10580 ←	LuxR family transcriptional regulator (FixJ)
		A → G	A16A (CGG → GCG)	BMD20_25100 →	Preprotein translocase subunit SecG
	BM1-3	+ 8bp	Intergenic (+204/-162)	BMD20_02640 → / BMD20_02645	Cytochrome O ubiquinol oxidase / acetyltransferase
		Δ63 bp	Coding (1326-1388/2517 nt)	BMD20_10585 ←	Histidine kinase FixL
	BM1-4	A → C	V463G (GTG → GGG)	BMD20_10585 ←	Histidine Kinase FixL
BM11	BMV10	G → A	R66W (CGG → TGG)	BMD20_10580 ←	LuxR family transcriptional regulator (FixJ)
		A → G	E100G (GAG → GGG)	fusA →	Elongation factor G
	BMV10R	G → A	R66W (CGG → TGG)	BMD20_10580 ←	LuxR family transcriptional regulator (FixJ)
		A → G	E100G (GAG → GGG)	fusA →	Elongation factor G
		G → T	Y412* (TAC → TAA)	BMD20_10585 ←	Histidine kinase FixL
		+ 8bp	Intergenic (+399/+28)	sdhB → / ← BMD20_26080	Succinate dehydrogenase / cytochrome C

Mapping mutations to the conserved domains present in both proteins is shown in **Figure 9**. The mutation found in FixJ (R66W) located in the receiver domain in the vicinity from the phosphorylation site located in D64 (**Figure 9A**). The nonsynonymous substitution of R66W might interfere with phosphorylation of this domain by the sensor kinase FixL and perhaps with the binding of this regulator to target promoters. Two mutations in FixL protein are mapped in the second PAS domain (V463G and Δ442-463) (**Figure 9B**) and most likely will affect protein activity since no functional PAS domain is detected by in silico analysis (**Figure 9C**). The mutation introduced by a STOP codon at position 412 is within the two PAS domains of the sensory domain and leads to a truncated non functional protein.

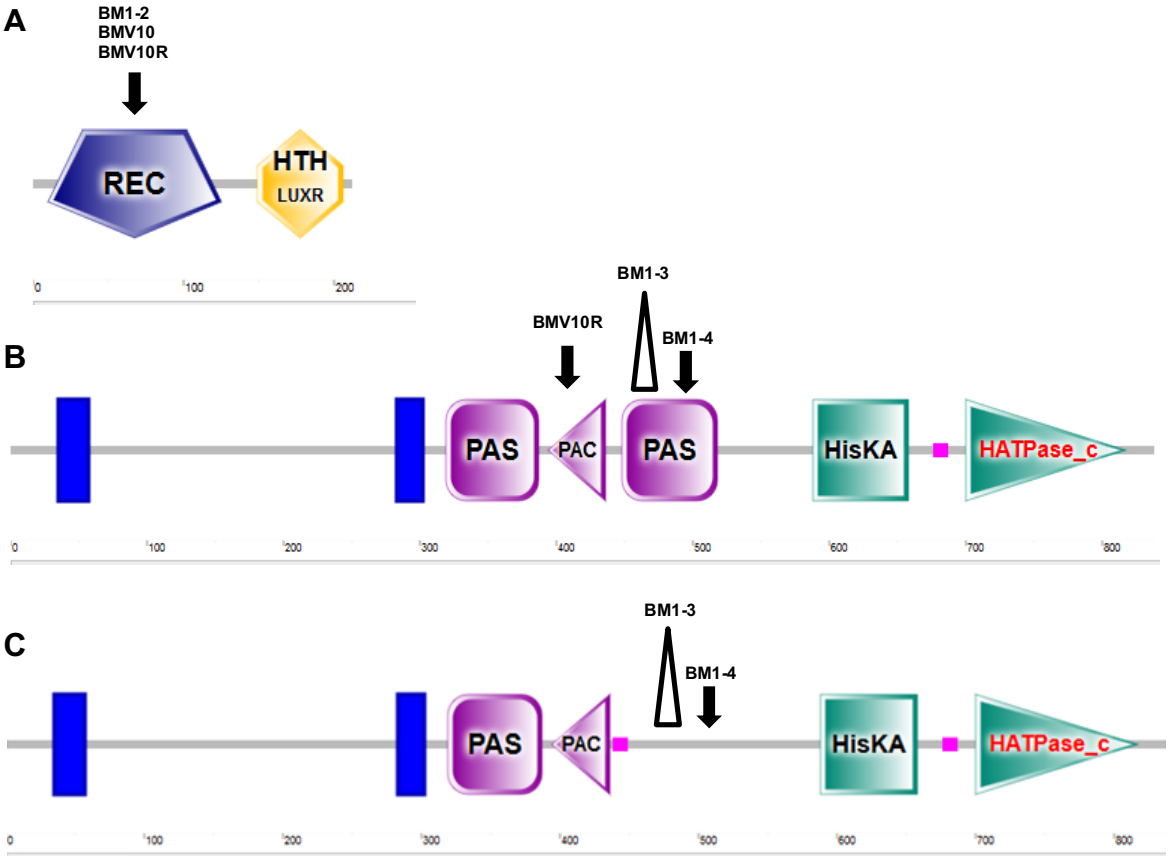

Figure 9 – Protein domains predicted by the SMART tool and mapping of mutations found in the *B. multivorans* variants. (A) Prediction for FixJ shows the receiver domain where phosphorylation takes place and the helix-turn-helix DNA-binding domain. (B) Prediction for FixL identifies the two transmembrane domains (blue), the sensor domain (PAS/PAC) and the histidine kinase domain where phosphorylation oh histidine takes place. (C) Mutations in the second PAS domain alter the *in silico* prediction for this domain.

3.2 Assessment of exopolysaccharide production in solid and liquid media

In order to evaluate the mucoid phenotype of the 10 variants they were grown in YEM agar. Variants BM1-1, BM1-2 and BM1-7 were nonmucoid, although we observed a switch to mucoidy in a few colonies of BM1-2 (**Figure 10**) and named them BM1-2R. Since BM1-1 has a mutation in *bceF* gene, we performed complementation analysis with plasmid pLM127-3 expressing gene *bceF* of *B. contaminans* IST408 from the bce promoter. Results shown in **Figure 10** confirm the recovery of the mucoid phenotype. Similar result was obtained when the fixJ mutant BM1-2 was complemented with the *B. multivorans* BM1 *fixJ* gene expressed from the promoter region upstream *fixL* gene (pLM014-3). Regarding BM1-7 variant, both pLM127-13 and pLM014-3 were mobilized to this strain, and the one complementing the mucoid phenotype was pLM127-13 (**Figure 10**), suggesting that this nonmucoid variant has a mutation in *bceF* gene.

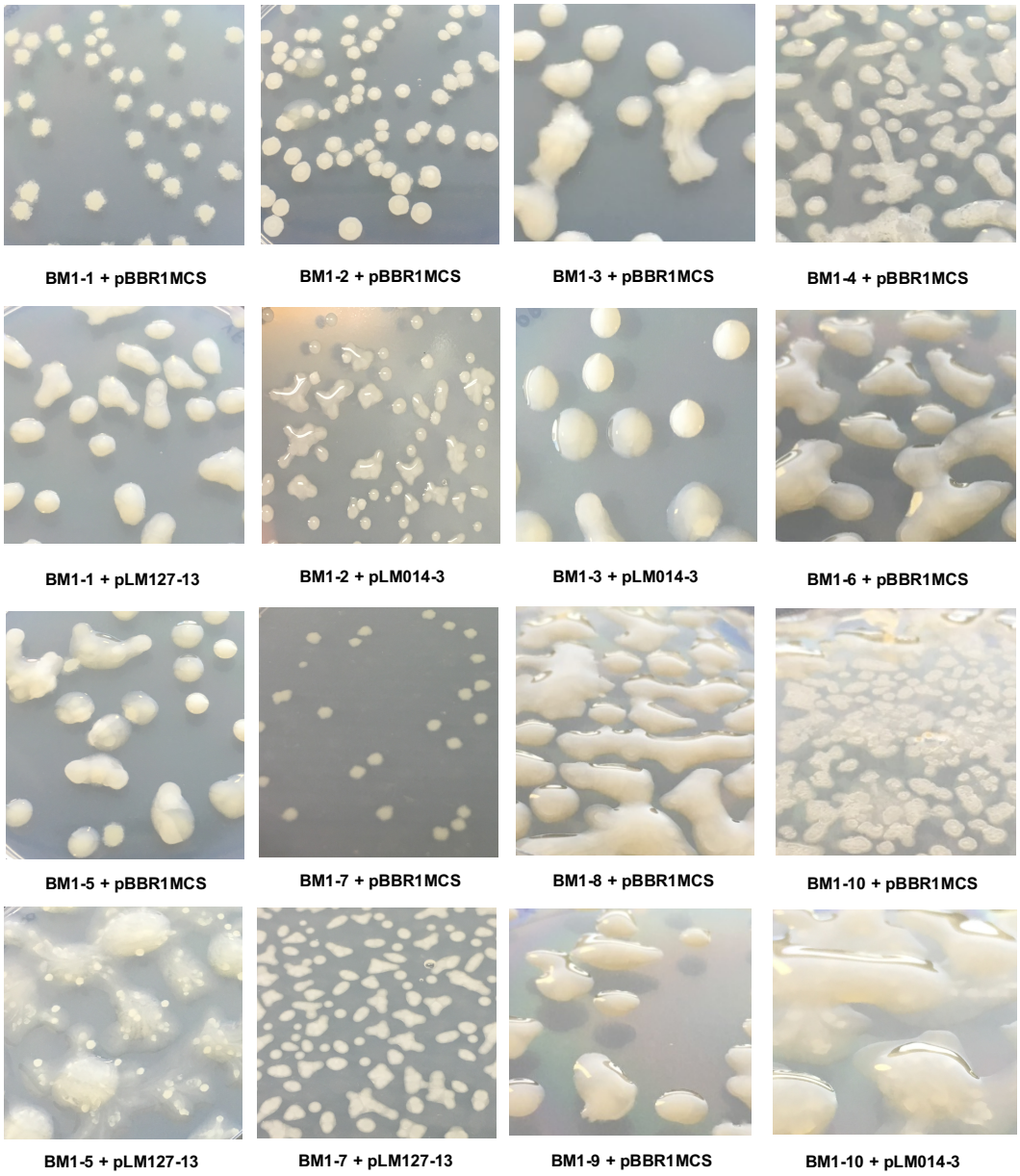

Figure 10 – Colony morphotypes in YEM agar of *B. multivorans* variants incubated for 4 days at 37 ºC after triparental conjugation.

Regarding the other variants, BM1-5 was highly mucoid (**Figure 10**) while BM1-3, BM1-4, BM1-6, BM1-8, BM1-9 and BM1-10 showed mucoidy, although less than BM1-5. This observation shows that in solid YEM medium BM1-3 (FixL Δ442-463) and BM1-4 (FixL V463G) still produce exopolysaccharide. Since we had pLM014-3 expressing *fixJ* but no construction for *fixL* was obtained (as will be described in **section 3.5**), we overexpressed *fixJ* gene in the seven mucoid variants. While from BM1-3 (FixL Δ442-463), BM1-5 and BM1-10 we were able to obtain the expected mucoid colonies, for variants BM1-4 (FixL V463G), BM1-6, BM1-8, and BM1-9, no colonies were obtained. Conjugation of pLM014-3 was attempted another time, and again no colonies were obtained, while when the vector pBBR1MCS was used, colonies were obtained. These results seem to indicate that

when *fixJ* gene is overexpressed in a wild-type FixL (or FixL Δ442-463) background cells are viable. In FixL the sensor domain is functional while in FixL(Δ442-463) is most like nonfunctional and phosphorylation of FixJ is regulated or non existing. Contrastingly, the overexpression of *fixJ* gene in the FixL (V463G) background might keep the sensory domain always active, leading to permanent phosphorylation of FixJ and having an adverse effect on the expression of genes related to cell viability. In line with these results variants BM1-6, BM1-8 and BM1-9 might have a similar FixL mutation as BM1-4. Further experiments are needed to confirm or reject the hypothesis of the deleterious effect of FixJ overexpression in certain FixL-mutant backgrounds.

Exopolysaccharide production was also evaluated in liquid SM medium by growing cultures for 5 days at 37 ºC under orbital agitation. Under these conditions BM1 parental isolate and BM1-5 (probably also parental) produce high-molecular weight EPS (**Figure 11**). As expected, variants BM1-2 and BM1-7 do not produce EPS, while a mucoid revertant colony of BM1-2 (BM1-2R) produces EPS. The remaining isolates (BM1-3, BM1-4, BM1-6, BM1-8, BM1-9 and BM1-10) although mucoid in solid medium, are unable to produce EPS (**Figure 11**). Regarding BM11 derivatives, nonmucoid BMV10 does not produce EPS but its revertant is able to produce this polymer. We have no explanation for this different behavior between solid and liquid medium, although we suspect it might be controlled by second messengers such as c-di-GMP or quorum sensing molecules secretion/import.

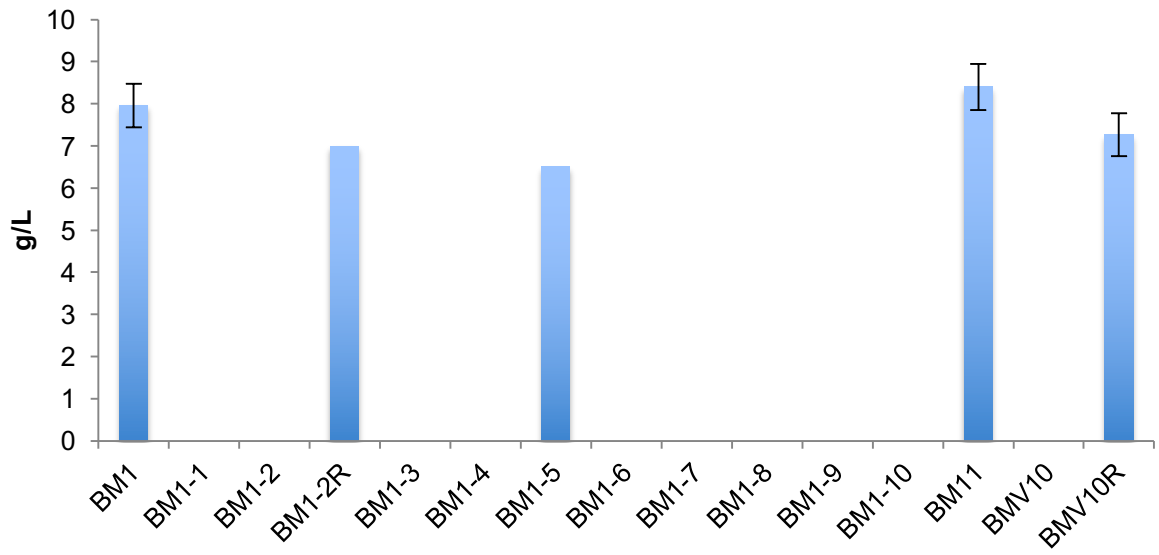

Figure 11 – EPS production in different strains. The amount of EPS produced was assessed based on the dry-weight of the ethanol-precipitated polysaccharide recovered from all strains grown in liquid SM over 5 days at 37 ºC with agitation. Error bars represent SD. The data are based on the mean values from the results of two independent cell cultivations, each with duplicates.

3.3 Growth of *B. multivorans* variants under different environmental conditions

To evaluate whether the different mutations introduced in *fixJ* and *fixL* genes had an effect on cell physiology, these were grown in LB broth or LB supplemented with 2.5 % NaCl or 20 % sucrose. Results obtained are shown in **Figure 12**. Two different patterns were observed, with the parental BM1 isolate and the BM1-1 (*bceF*) mutant having an exponential growth of 3-4 hours and then decelerating growth (**Figure 12a**). Contrastingly, BM1-2 (*fixJ*), BM1-3 (*fixL*) and BM1-4 (*fixL*) mutants have a much longer exponential phase, with a short deceleration period before entering stationary phase. Similar results were obtained for the *fixJ* and *fixJ*/*fixL* mutants derived from BM11 parental isolate. This result suggests that FixL/FixJ might be involved in metabolite control. This two component regulatory system might regulate the expression of genes involved in the metabolism of some carbon sources that are repressed in the presence of the preferential ones. In the case of this regulatory system being inactive, those alternative carbon sources can be used simultaneously with the preferred ones, prolonging exponential phase to a maximum period of time and then enter stationary phase due to nutrient depletion. Similar findings were observed for the two component regulatory system BphP/BphQ from *Acidovorax* sp. KKS102 (highly homologous to FixLJ) which controls the expression of genes for PCB/biphenyl degradation. In the presence of repressive carbon sources such as tricarboxylic acids the genes for PCB/biphenyl degradation are repressed and only when TCA metabolits decrease are they expressed (Ohtsubo, Y. et al., 2006).

Results from **Figures 12c** to **12f** where strains were grown under high osmotic conditions shows the expected growth inhibition with lower final biomass formation but, besides the already identified in charts **12a** and **12b**, there are no major differences. This suggests that FixLJ is likely not involved in osmotic stress adaptation.

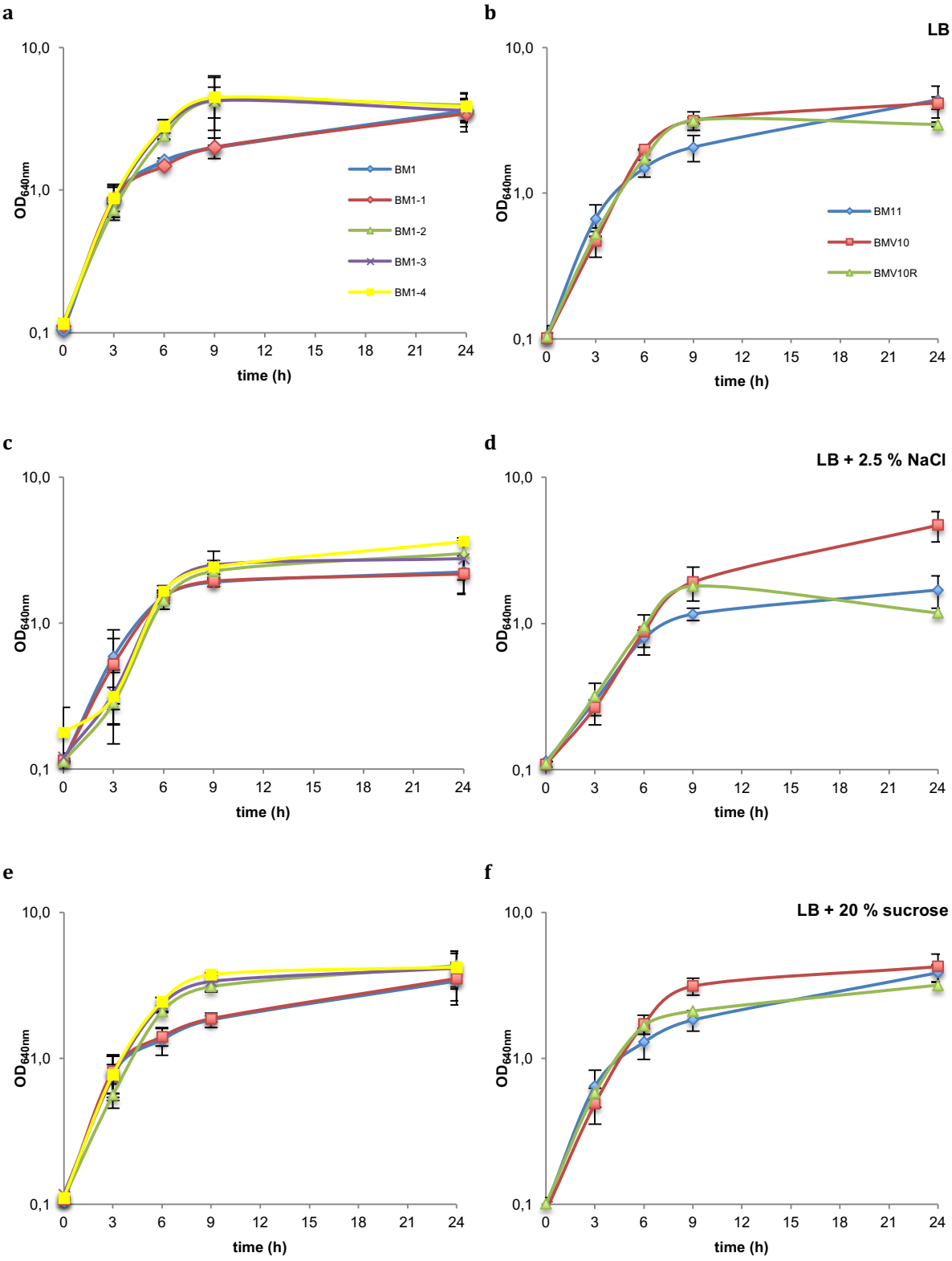

Figure 12 – Growth curves of isolates BM1 and BM11 with their respective nonmucoid variants. Strains were cultured in (a), (b) LB or (c), (d) LB supplemented with NaCl or (e), (f) LB supplemented with sucrose during 24 h at 37 ºC with orbital agitation. Left part of the figure represents BM1 (**blue**) and four variants (BM1-1 represented in **red**, BM1-2 in **green**, BM1-3 in **purple** and BM1-4 in **yellow**). Right part represents BM11 (**blue**) and two variants (BMV10 represented in **red** and BMV10R in **green**). Error bars show SD. The data are based on the mean values from the results of two independent cell cultivations.

3.4 Resistance against antimicrobials and biofilm formation

Since FixL of *B. dolosa* has been shown as regulating some virulence traits, we tested antibiotic resistance and biofilm formation ability, of our variants.

While analyzing **Figure 13 A**, three patterns can be distinguished. First, resistance to all the antibiotics tested is similar for BM1 and nonmucoid variant BM1-1. Second, nonmucoid BM1-2, harboring mutation in *fixJ*, showed slightly decreased resistance to piperacilin with tazobactam and to ciprofloxacin, but similar resistance to kanamycin and aztreonam compared to the mucoid isolate BM1. The third group, including variants harboring mutations in the HK FixL, BM1-3, -4 is shown to be less resistant to all the antibiotics tested, compared to the original mucoid isolate BM1.

In turn, from the analyses of **Figure 13 B**, resistance to ciprofloxacin is similar for all strains. Nonmucoid variant BMV10, harboring mutation in FixJ, showed deeply decreased resistance to piperacilin with tazobactam and to aztreonam but slightly increased to kanamycin compared to the mucoid isolate BM11. Whereas, BMV10R, harboring the same mutation of the nonmucoid variant BMV10 and an additional mutation in the HK FixL, showed an intermediated pattern between BM11 and BMV10R with exception of kanamycin where is completely resistant.

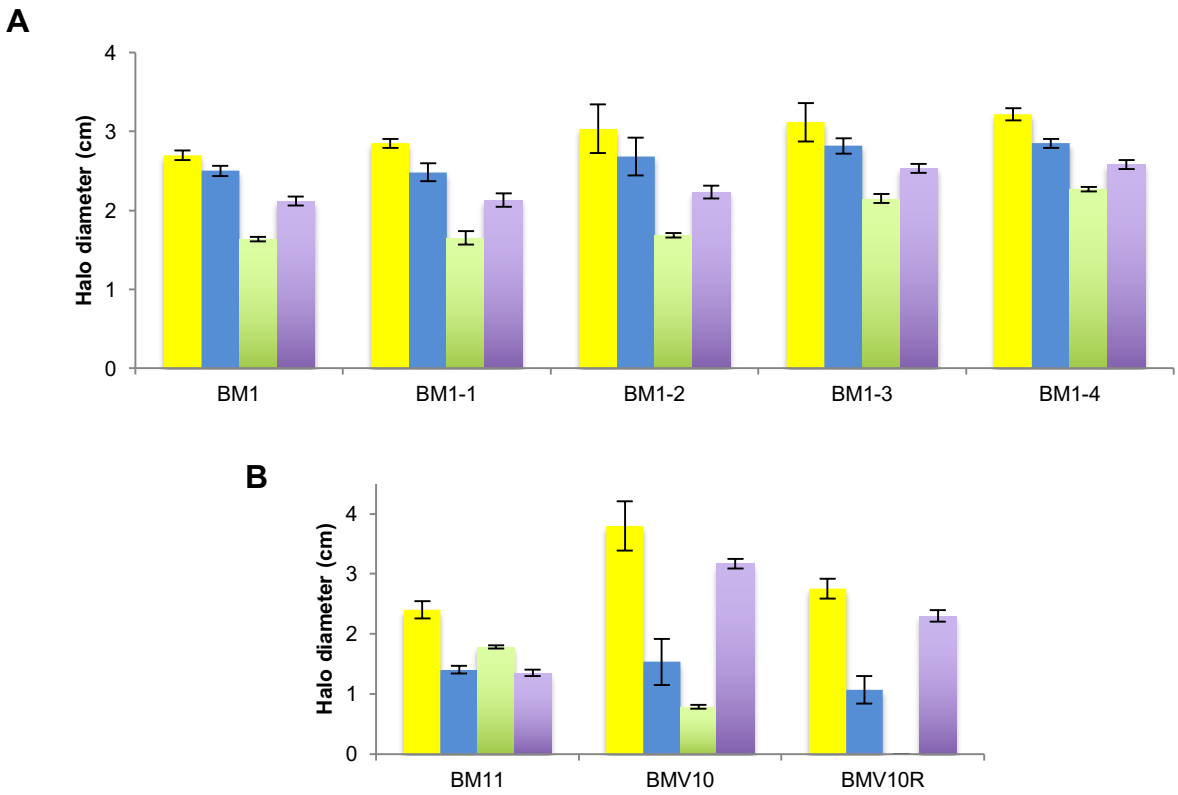

Figure 13 – Antibiotic resistance. Susceptibility tests were based on the agar disc diffusion method using paper discs containing piperacillin (75 µg) plus tazobactam (10 µg)(**yellow**), ciprofloxacin (5 µg)(**blue**), kanamycin (30 µg)(**green**) or aztreonam (30 µg)(**purple**). Halo diameter was measured after 24 h for BM1 and BM1 nonmucoid variants (a) or 48 h in case of BM11 and BM11 nonmucoid variants (b). Error bars show SD. Data are based on the mean values from the results of two independent cell cultivations, each with triplicates.

To determine biofilm production in polystyrene microtiter plate, mucoid BM1 and BM11 isolates and variants were grown for 48 h at 37 ºC statically (**Figure 14**).

Variants BM1-1, -2, -3 and -4 produced slightly higher amounts of biofilm compared to the mucoid isolate BM1. However, in (**B**) the nonmucoid variant produce less biofilm than the initial strain, while BMV10R shows no difference to the parental strain.

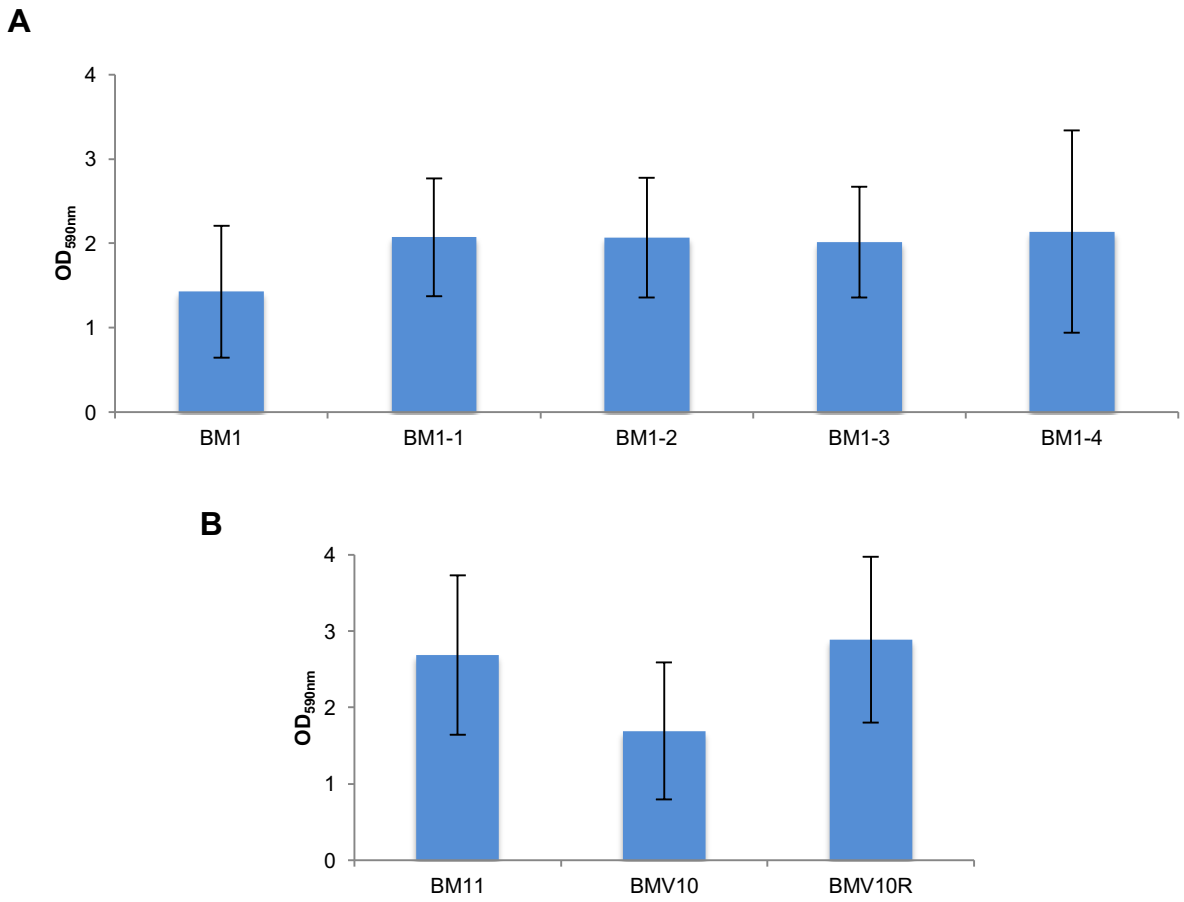

Figure 14 – Biofilm production. Bacterial cultures were used to inoculate 96-well plates incubated at 37 ºC statically for 48 h. Adherent bacteria were stained with crystal violet solution and solubilized in 96 % ethanol. Biofilm was quantified, by measuring the absorbance at 590nm, for (a) BM1 and BM1-1, BM1-2, BM1-3 and BM1-4 variants, and (b) BM11 and BMV10 and BMV10R variants. Error bars represent SD. The data are based on the mean values from the results of three independent cell cultivations, each with duplicates.

B. multivorans variants derived from BM1 produced more biofilm than the parental strain, which is the opposite of what was seen when *P. aeruginosa bfiSR* pathway (homolog to FixLJ) was inactivated (Petrova & Sauer 2009) and when we look for the nonmucoid variant of BM11. The clinical relevance of Bcc biofilm production is unclear. Indeed, one study found no correlation between the ability of Bcc isolates to form biofilm and clinical outcomes (Cunha et al. 2004). In another study that analyzed the presence of *P. aeruginosa* and/or Bcc in infected CF lung, Bcc bacteria were rarely found in biofilm-like structures while *P. aeruginosa* were often found in such structures (Schwab et al. 2014). In spite of this, due to the error associated on this method, more experiments need to be performed.

3.5 Cloning of *envZ* and *fixL* genes for complementation analysis

Since overexpression of mutated genes can help to understand their function, it it important to clone the two sensor kinases that directly or indirectly affect EPS production. These are FixL from this study and EnvZ from a previous study (Silva et al., unpublished). The genomic location of *fixLJ* and *envZ ompR* genes of *B. multivorans* BM1 is shown in **Figure 15**. Computational analysis predicts a single operon for *fixJ* and *fixL* genes with the promoter being located upstream of *fixL* gene. Similarly, the putative operon with *ompR* and *envZ* is expressed from a promoter upstream of *ompR* gene.

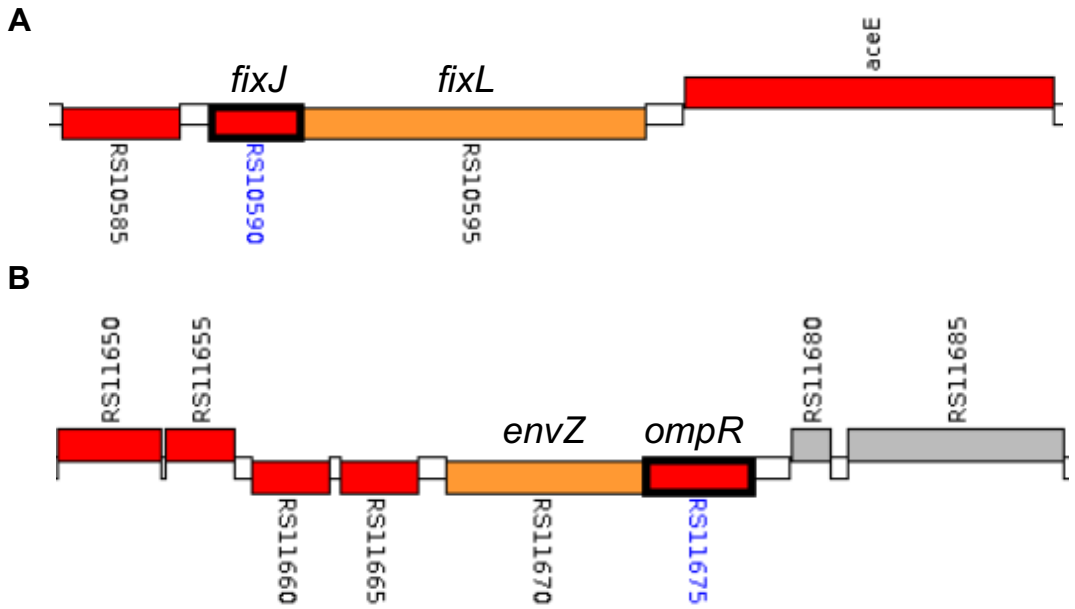

Figure 15 – Genomic location in *B. multivorans* BM1 of genes fixL/fixJ (A) and envZ/ompR (B) encoding two different signal transduction systems involved in mucoid-to-nonmucoid morphotype variation. Both set of genes are depicted in the complementary strand of chromosome 1.

The strategy for *fixL* region included the amplification of a single fragment of approximately 3100 bp comprising gene *fixL* and the upstream intergenic region, followed by cloning into pBBR1MCS vector. Despite several attempts we were unable to amplify the desired fragment. Therefore, primers were designed to amplify independently the first half (KpnI/XhoI, 1951 bp) and the second half (XhoI/XbaI, 1221 bp). PCR amplification of the 1951 bp fragment was achieved, but its cloning into pBBR1MCS was never obtained.

The cloning of *envZ* gene and the promoter region into pBBR1MCS would be achieved in two steps: 1) the 269 bp fragment with the promoter (KpnI/NdeI) and the 1545 bp fragment with the *envZ* gene (NdeI/XbaI) would be cloned into pUK21 vector and then 2) the KpnI/XbaI fragment would be cloned into pBBR1MCS. The amplification of the 269 bp fragment gave a single product (**Figure 16 A**) while the amplification of the 1545 bp product gave two bands (**Figure 16 B**). Due to that the band

with correct size was excised and purified from the gel. Cloning of the promoter region into pUK21 vector was achieved (**Figure 16 C**) and the obtained plasmid was named pFB17-3. The cloning of the *envZ* gene into pFB17-3 did not succeed.

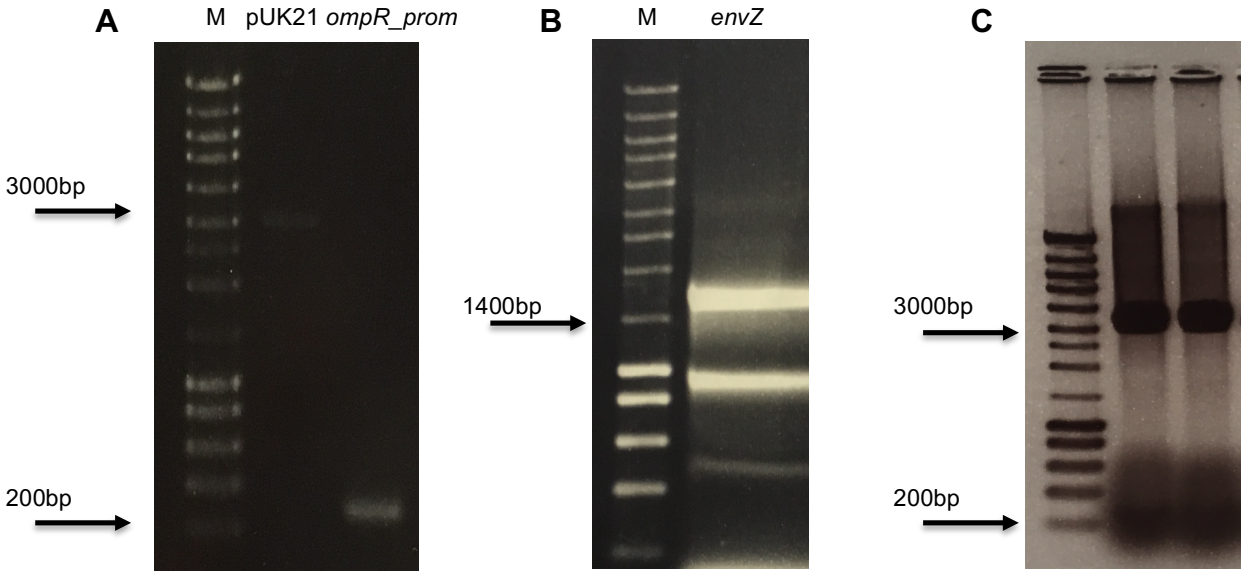

Figure 16 – Amplification of *ompR* promoter (A) and *envZ* (B) genes from *B. multivorans* BM1 genome. Electrophoretic separation in agarose gel of the mixture obtained after PCR amplification. M represent NZYDNA ladder III. Amplified DNA fragments of approximately 269 bp (*ompR* promoter) (A) and 1545 bp (*envZ*) (B) were obtained. Plasmid pFB17-3 was obtained (C).

3.6 Cloning procedures to express OmpR and FixJ in *E. coli*

Two recombinant plasmids, pFB17-1 and pFB17-2, were constructed to allow the overproduction of OmpR and FixJ proteins in *E. coli* cells. Plasmid pWH844 was used as expression vector. Standard genetic engineering techniques were used to generate recombinant plasmids containing both genes. For that, PCR amplification of the coding sequences from *B. multivorans* BM1 genomic DNA was performed. Since the main goal was the overexpression of the *ompR* and *fixJ* genes, the designed primers had to allow the amplification of a fragment with restriction enzymes recognition sites. Amplified DNA fragments of approximately 800 bp and 832 bp, corresponding to the expected size of *fixJ* and *ompR*, were obtained after electrophoretic separation of the PCR mixture (**Figure 17**).

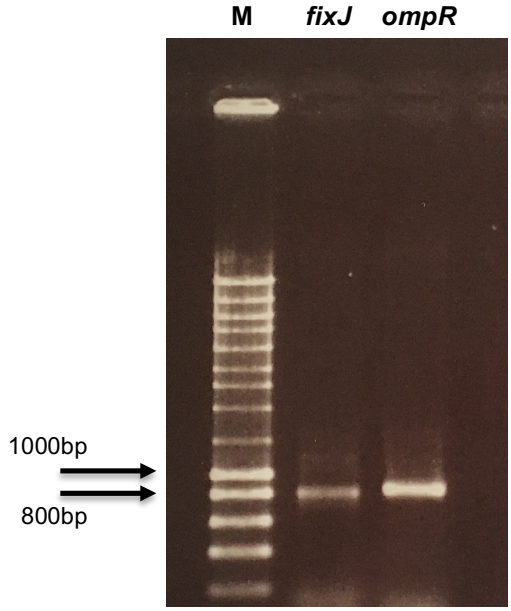

Figure 17 – Amplification of *ompR* and *fixJ* genes from *B. multivorans* BM1 genome. Electrophoretic separation in agarose gel of the mixture obtained after PCR amplification. M represent NZYDNA ladder III. Amplified DNA fragments of approximately 800 bp (*fixJ*) and 832 bp (*ompR*) were obtained.

After proceeding with digestion and ligation of the genes *ompR* and *fixJ* and the plasmid pWH844 (as described in Material and Methods section **2.5.3**), transformation of *E. coli* DH5α was carried out and candidates were selected in the presence of ampicillin, selective marker for the plasmid. The presence of the coding sequences was confirmed after plasmid DNA extraction and enzymatic digestion using HindIII and BamHI. Results show that 8 candidates (4 for each gene) had the expected restriction profile after digestion (**Figure 18**).

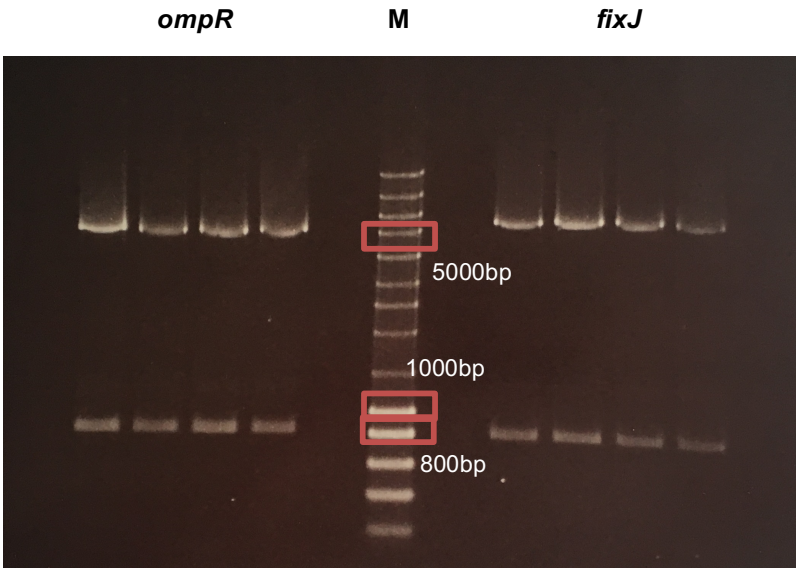

Figure 18 – pFB17-1 and pFB17-2 confirmation by restriction enzyme analysis. Electrophoretic separation in agarose gel of the mixture obtained after digestion with BamHI and HindIII of the putative candidates. M represent NZYDNA ladder III. Amplified DNA fragments of approximately 800 bp (*fixJ*), 832 bp (*ompR*) and 5000bp (pWH844) were obtained.

Since the presence of the correct size fragments was confirmed, one transformant of each gene was selected for proteins overexpression (as described in Material and Methods section **2.6.1**).

3.7 Overexpression and purification of recombinant proteins

For the overexpression of recombinant proteins *E. coli* was used as host cell. The proteins to be expressed from the recombinant plasmids have a fused 6×His-tag N-terminal allowing the desired purification by affinity chromatography. For this strategy, cells are grown in LB liquid medium supplemented with ampicillin. The desired proteins were overexpressed by addition of IsoPropyl-ß-D-ThioGalactoside (IPTG) to the bacterial cell culture. Aliquots before induction, at 4h and at 24h post-induction were visualized in SDS-PAGE as it will be shown later.

For protein purification, cells were disrupted by ultrasounds and purification was carried out in a histidine affinity column (as described in Material and Methods section **2.6.2**). Elution of proteins was performed with automatic increases of imidazole in the ÄKTA system (**Figure 19**).

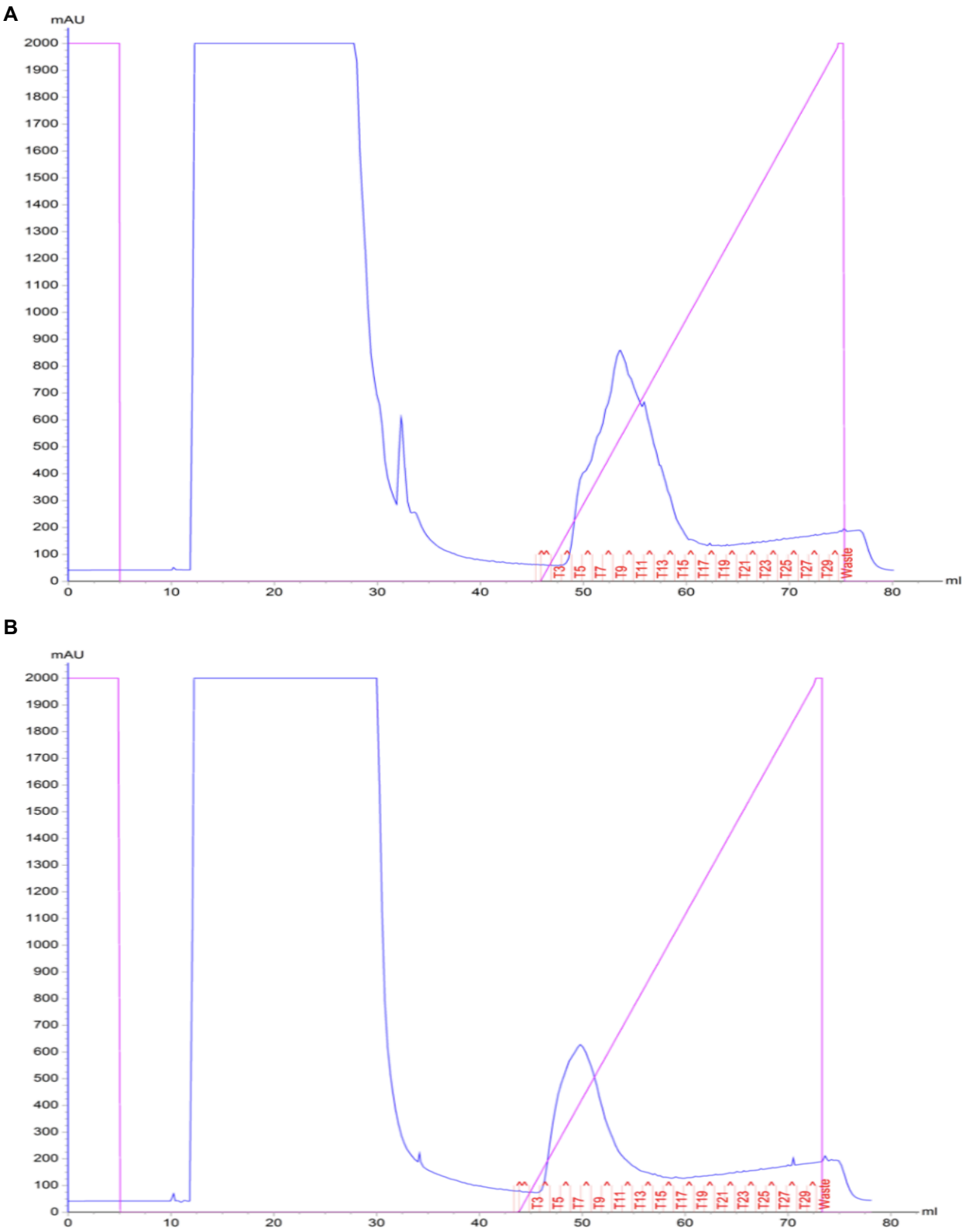

Figure 19 – Purification of His-tagged OmpR and FixJ proteins. Spectrum obtained by the ÄKTA system with automatic increase of imidazole. (A) shows the elution of OmpR protein and (B) shows FixJ.

Aliquots from the fractions corresponding to the elution were collected and analyzed by SDS-PAGE polyacrylamide gels followed by protein staining with BlueSafe. Results are shown in **Figure 20**.

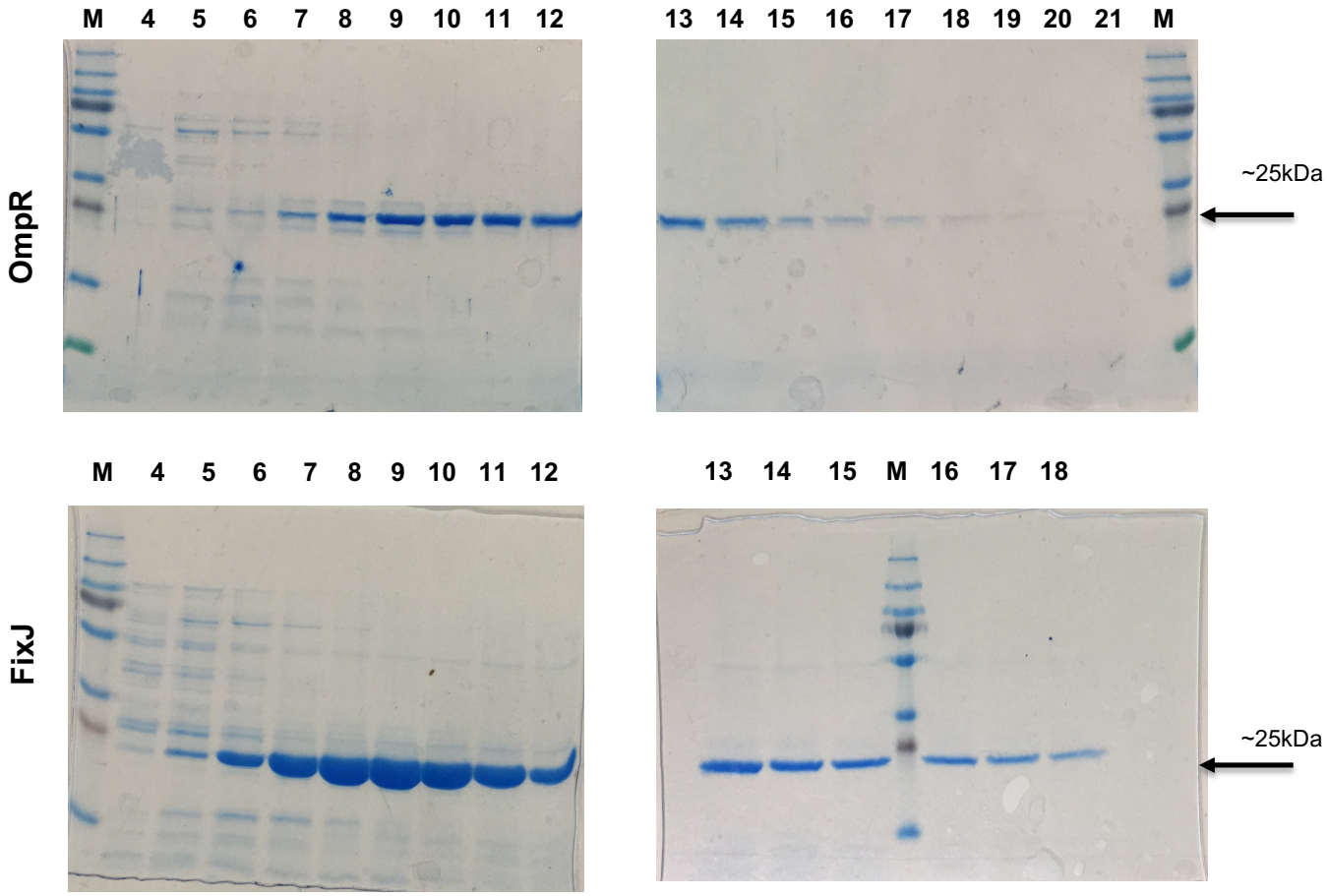

Figure 20 – Analysis of the elution profile of the His-OmpR and His-FixJ, purified by affinity chromatography. SDS-PAGE polyacrylamide gel obtained after affinity chromatography in ÄKTA system with an automatic increased gradient of imidazole. Gel was stained with BlueSafe (Nzytech). M represents PageRuler® plus prestained protein ladder (Thermo Scientific).

Figure 20 shows that, in an initial stage, fractions were contaminated with other proteins possessing higher and lower molecular weights than the expected. From each purification we choose the fraction that demonstrated less contamination (fraction 12 for OmpR and fraction 13 for FixJ) and compared them with the aliquots taken before induction and after the overexpression as shown in **Figure 21**.

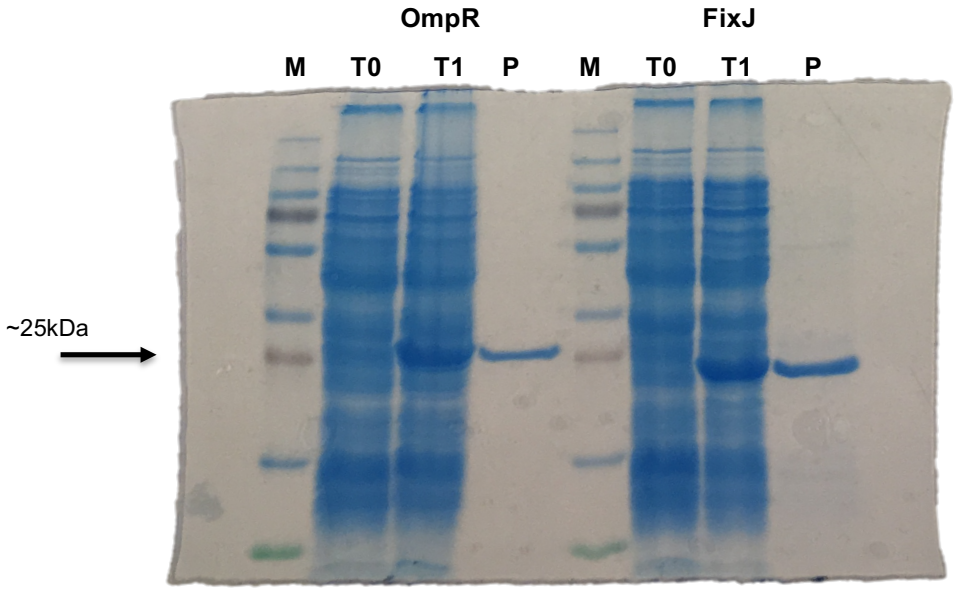

Figure 21 – Overexpression and purification of OmpR and FixJ proteins. SDS-PAGE polyacrylamide gel of the mixtures obtained before induction (T0), after 4h of IPTG supplementation (T1) and after ÄKTA system purification (P). M represents PageRuler plus.

In general, the elution profile of both proteins was similar. After collecting the purified fractions, the buffer was then exchanged to PBS with a desalting column. To asses the protein concentration we used the method of Bradford. Purified proteins were then used for EMSA tests. Probes were designed based on the regions before the start codon of the genes *bceA* and *bceB* and have a 5' end modification with Biotin-TEG allowing the detection by chemiluminescence (**Figure 22**).

bceA start codon

CGCGCGTGCCGACGGCAGGGGCGGCAGCGGCGACGTCGTGCGTGTCGGCCGTCACGGTCGGGGCATTCATGATGGCACTCTCCTCGATTGAAGTCAGTGC

CTTTCGTTGTAGCACGAAGTAAATCG**ACAATAAAATCGGGCGCCTTTGTCGATCATTACCGTTTCATTGCGCCGGGAAAATTTTCCGCGCATCCCCGGCA**

GCGCGATAAAAAAATAAAAAATAATTCCGCGCTTCGGCCGCCAACGCCCGCCCATCAACGGGTTCGGCCCGACGAAAAGTGTCCGAAATGTGCGTGCCGA

CGGAAATATCCGGGAAAA**CATGCCGATTTAATTCATCGACGACGCAGCAAGAATTGCCGATTATTTTTCGAAATACTTGTGCGCTTGCGGGAGGAATTTT**

CTTGTCGGTTCAATAGCGACATGCAACGTTTGAATCGAAGGTGCGGCACGGGCTGCACGAGGAGCTGTTCGGCAAACGACTGTTCAAAGAGGAAGCGGAC

bceB start codon

ATGTTGAGCGTGCTGGCGAGAGTCATCGATATCGCGATGGTCGTGGCGGGAGCGCTGATCGCCGCCGCGCTGCATCGCGGCAACGTGTGGTTCAGCGACC

TGCAGCGCACGATGGTGCTGTTCGACTGCCTGCTCGTCGTCGTGTGCTTTCCGGCATTCGGCATCTATCAGTCCTGGCGCGGCAAGCGGCTCGTCGGGCT

GATGGGCCGCGTCGCGTTCGCATGGCTCGTCGTCGAGCTCGCGGGCATCCTGATGAGTTTCAGCTTTCATCAGTCGGGCGAACTGTCGCGGCTGTGGCTC

Figure 22 – Intergenic region between *bceA* and *bceB* genes. Regions labelled as probes are in bold and underlined. Probe in black includes the putative promoter of *bceA* gene; probe in blue is the putative promoter of *bceB* gene; and probe in red is a negative control since it is located with the *bceB* coding region.

It was expected, if ligation between protein and probe occur, another band with higher molecular weight corresponding to the ligation. However, unfortunately, several tests were performed with different conditions and no significant binding to DNA was showed. Results from **Figure 23** and **24** show bands equals to the control (**Figure 23 A**). Considering these results it was hypothesized that possibly the non phosphorylation of the proteins make them in an inactive state. Another reason can be the probes chosen didn't represent the region where the ligation occurs. Finally, if everything represents the *in vivo* conditions, no ligation occurs between these proteins and the promoter regions of these genes. Additional work need to be accomplish in order to exclude the hypotheses.

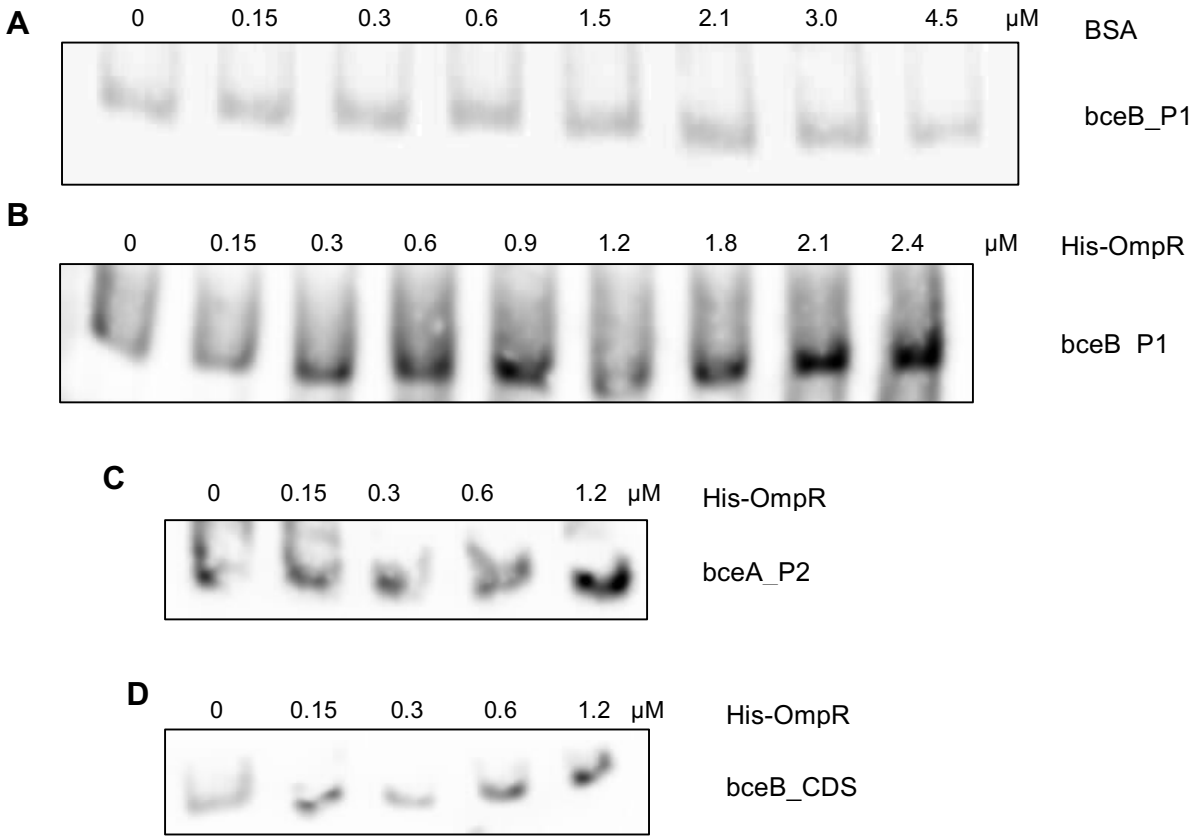

Figure 23 – Electrophoretic mobility shift assays using the DNA probes bceB_P1 (A and B), bceA_P2 (C) and bceB_CDS (D). Gel shift assays using increasing concentrations of protein. (A) Shows assays performed with BSA allowing control of the specificity of the ligation. (B) Assays with His-OmpR and the probe bceB_P1. (C) and (D) were the assays with His-OmpR and the probes bceA_P2 and bceB_CDS, respectively. For each assay with DNA double strand probes, 2.0 nM of the Biotin-TEG labeled probes were used.

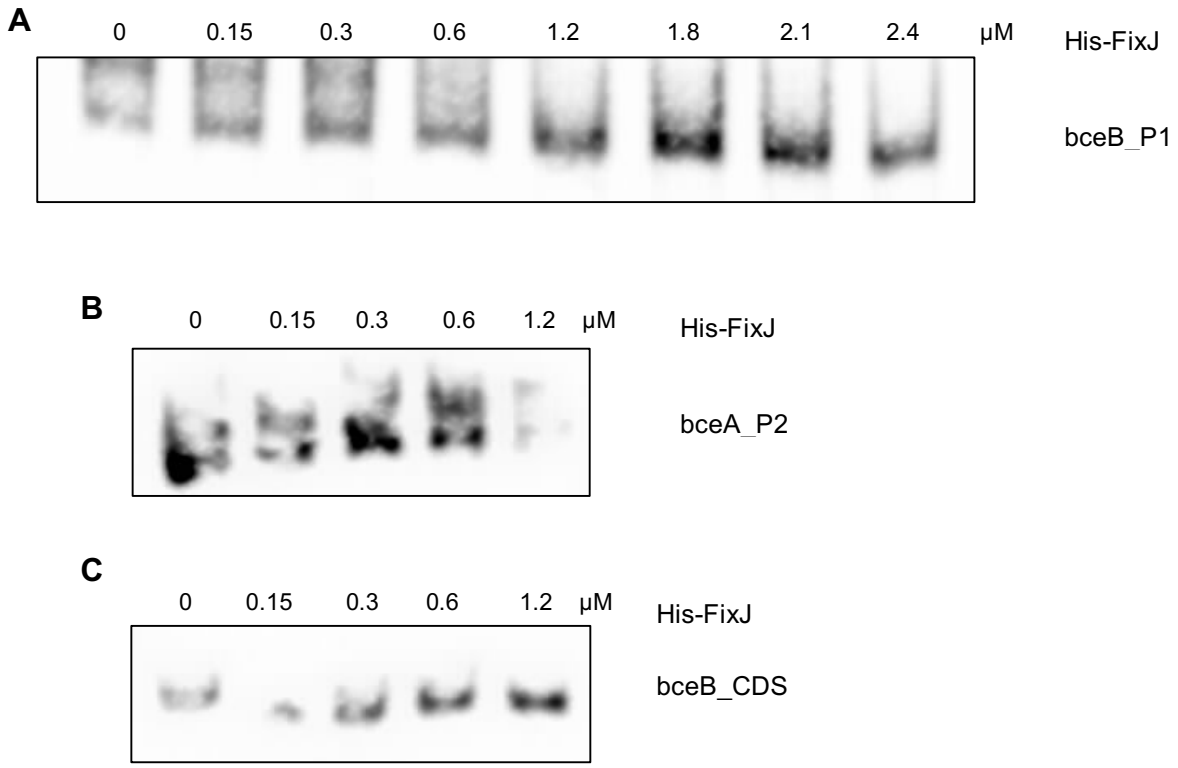

Figure 24 – Electrophoretic mobility shift assays using the DNA probes bceB_P1 (A and B), bceA_P2 (C) and bceB_CDS (D). Gel shift assays using increasing concentrations of protein. (A) Shows assays with His-FixJ and the probe bceB_P1. (C) and (D) were the assays with His-FixJ and the probes bceA_P2 and bceB_CDS, respectively. For each assay with DNA double strand probes, 2.0 nM of the Biotin-TEG labeled probes were used.

CONCLUSIONS

Although the genes/proteins required for cepacian biosynthesis are well known, the regulatory elements that switch ON and OFF the expression of the *bce* genes is not well characterized. In this work we focused on the phenotypical characterization of *B. multivorans* nonmucoid/less mucoid variants obtained *in vitro* from the early isolate BM1 and the late isolate BM11 harboring mutations in FixJ and FixL proteins. These two proteins form a two-component regulatory system that has been recently studied in *Burkholderia dolosa*. In that study, the authors reported the functional relevance of *fixLJ* genes in adaptation to low oxygen environments, but also in the regulation of a large number of genes and their critical role in pathogenicity *in vivo* and in intracellular invasion *in vitro* (Schaefers et al. 2017).

Living organisms need to quickly detect environmental stimuli and respond to survive and proliferate (Schaller et al. 2011). TCS constitute a critical set of regulators, which act to sense environmental signals and respond by altering gene expression (Hoch 2000). FixL/FixJ, a bacterial O_2-sensing two-component signal transduction, was first identified in *Rhizobium meliloti* (de Philip et al. 1990). FixL proteins contain a HK domain and at least one PAS (Per-ARNT-Sim) domain, which contain the heme that bind O_2 (Monson et al. 1992). The *fixJ* product, a cytoplasmic regulator, seems to be modulating by *fixL* product, the transmembrane sensor (Gilles-Gonzalez et al. 1991). *In silico* analysis of FixL and FixJ protein sequence from *B. multivorans* confirmed the presence of all domains typical from these proteins.

Despite the involvement of at least the FixJ protein in exopolysaccharide biosynthesis, we have shown a different growth behavior for the *fixJ* and *fixL* mutants grown in LB medium. The fact that these mutants have a much longer exponential phase and fast entrance into the stationary phase are indications that they might be involved in repressing the use of some carbon sources. While the wild-type strain uses the preferential carbon sources first and only later starts to use alternative nutrient sources, the *fixJL* mutants have these metabolic systems derepressed and enter stationary phase much earlier than the wild-type. The observation that growth in the presence of sucrose or NaCl does not change the already observed behavior is an indication that this regulator is not involved in stress response.

Interestingly it seems that *B. multivorans* nonmucoid variants derived from BM1 made more biofilm than the parental strain, while in the nonmucoid variant derived from BM11 we have the opposite effect. These might just be limitation of the technique, but we cannot exclude that it may be strain related. Although these isolates are recovered from the same patient, more than 50 different mutations are separating those (Silva et al., 2016). The clinical relevance of Bcc biofilm production is unclear. Indeed, one study found no correlation between the ability of Bcc isolates to form biofilm and clinical outcomes (Cunha et al. 2004). In alternative, a study that observed *P. aeruginosa* and/or Bcc infected

CF lung, Bcc bacteria were rarely found in biofilm-like structures while *P. aeruginosa* were often found in such structures (Schwab et al. 2014). In spite this, due to the error associated on this method, more experiments need to be performed.

Sensor kinases autophosphorylate an internal histidine residue by responding to an external signal, and then transfer the phosphoryl group to an aspartate residue of the response regulator. This phosphorylated regulator is then activating or repressing the expression of other genes. Our hypothesis is that FixL/FixJ could sense external signals and regulate the expression of the bce genes directing the biosynthesis of cepacian. This regulation could be direct (by the binding of FixJ to the bce promoter) or indirect, by regulating the expression of another regulator that then would bind to the bce promoter. For this study, EMSA assays were attempted, but our results did not confirm the direct ligation hypothesis. Still, we can also not exclude it. It could be that the recombinant protein produced in *E. coli* was not phosphorylated, or the promoter regions chosen for interaction do not include the full promoter. Similar results were obtained with the OmpR response regulator, also known to be involved in cepacian biosynthesis (Silva and Moreira, unpublished). In the future, additional work needs to be accomplished in order to investigate the different hypotheses.

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