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

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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.

INTRODUCTION

Cystic fibrosis (CF) is the most common life-threatening inherited disease in the developed world. Abnormally thick mucus secretions in the airways, leading to chronic bacterial infection, persistent inflammation and ultimately respiratory failure, characterize it (Lyczak et al. 2002).

Burkholderia cepacia complex (Bcc) is a group of, at the time of writing, 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). *Burkholderia multivorans* and *Burkholderia cenocepacia* are the most often isolated Bcc species from the respiratory tract of CF patients (Zlosnik et al. 2015).

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 them is the exopolysaccharide (EPS) cepacian, produced by the majority of clinical isolates (Cunha et al. 2004). Analysis of the mucoid morphotype of more than 500 Bcc isolates from 100 chronically infected CF patients revealed thirteen cases of mucoid-to-nonmucoid transition (Zlosnik et al. 2008). An inverse correlation between EPS production and disease progression, with the nonmucoid ones being associated to increased disease severity while the mucoid phenotype would be associated with persistence in the lungs, has been shown (Zlosnik et al. 2011).

Living organisms need to quickly detect environmental stimuli and response accordingly in order to survive and proliferate. 2-component systems (TCS), knowing to control virulence gene expression and host-pathogen interactions, consist of a sensor histidine kinase (HK) and the cognate response regulator (RR), those together sense environmental changes and regulate gene expression in order to adapt to new conditions (Capra & Laub 2012). For example, EnvZ is the HK associated to the OmpR response regulator and, together, they control *E. coli* responses to osmolarity of the medium (Igo & Silhavy 1988).

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

RESULTS AND DISCUSSION

The CF patient from whom *B. multivorans* isolates analyzed in this study were recovered is one of the nine patients chronically colonized by *B. multivorans* in which a colony morphotype mucoid-to-nonmucoid transition was identified previously (Zlosnik et al. 2008). During the course of these patients regular semiannual examinations, *B. multivorans* was first isolated in 1993 (BM1) and recovered periodically until 2013, yielding a total of 22 isolates for analysis. Throughout these chronic infections, mucoid-to-nonmucoid morphotype variation occurs, with the two morphotypes exhibiting different phenotypic properties. This collection of 22 isolates was sequenced and analyzed phenotypically (Silva et al. 2016). To understand which molecular mechanisms trigger mucoid to nonmucoid variants, mucoid BM11 was

Table 1 - 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

maintained statically for 21 days in SM medium. *In vitro*, the mucoid clinical isolate BM11 gives rise to stable nonmucoid variants in response to prolonged stationary phase, presence of antibiotics, and osmotic and oxidative stresses (Silva et al. 2013). The same experiment was carried out for the first isolate and 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. Ten of these colonies were kept for further studies and were named BM1-1 to BM1-10.

Four of these nonmucoid variants (BM1-1, BM1-2, BM1-3 and BM1-4) were chosen for whole genome sequence. Mutations in FixJ gene (one variant) and FixL gene (two variants) were found as shown in **Table 1**. In addition to these mutants, **Table 1** also includes a nonmucoid variant derived from *B. multivorans* BM11 (named BMV10) with a mutation in FixJ protein, and a mucoid revertant BMV10R which besides this mutation accumulates a mutation in FixL protein.

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 and named them BM1-2R. Since BM1-1 has a mutation in *bceF* gene, we performed complementation analysis with plasmid pLM127-3. Results confirm the recovery of the mucoid phenotype. Similar result was obtained when the fixJ mutant BM1-2 was complemented with pLM014-3. Regarding BM1-7 variant, both pLM127-3 and pLM014-3 were mobilized to this strain, and the one complementing the mucoid phenotype was pLM127-3, indicating that this nonmucoid variant has a mutation in *bceF* gene.

Regarding the other variants, BM1-5 was highly mucoid 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*, 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. 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.

Exopolysaccharide production was also evaluated in liquid SM medium by growing cultures 5 days at 37 ºC under orbital agitation (**Figure 1**).

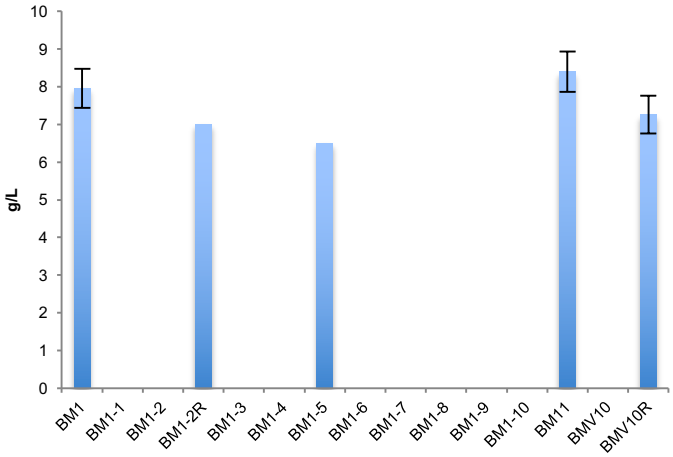

Figure 1 – EPS production in different strains. The amount of EPS produced was assessed based on the dry-weight of the ethanol-precipitated polysaccharide recovered from all strains grown in liquid SM over 5 days at 37 ºC with agitation. Error bars represent SD. The data are based on the mean values from the results of two independent cell cultivations, each with duplicates.

Under these conditions BM1 parental isolate and BM1-5 (probably also parental) produce high-molecular weight EPS. 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. 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.

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 2**. 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 2a**). 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 (**Figure 2b**). 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.

Results when strains were grown under high osmotic conditions shows the expected growth inhibition with lower final biomass formation but, besides that, there are no major differences. This suggests that FixLJ is likely not involved in osmotic stress adaptation.

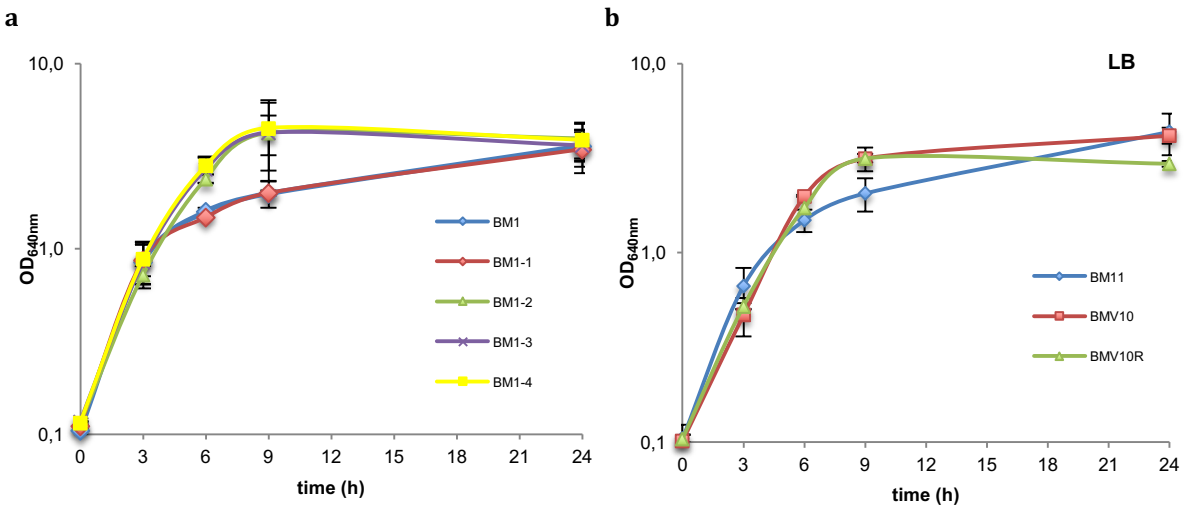

Figure 2 – Growth curves of isolates BM1 and BM11 with their respective nonmucoid variants Strains were cultured in (a), (b) LB or LB supplemented with NaCl or with sucrose (results not shown) during 24 h at 37 ºC with orbital agitation. (a) represents BM1 (**blue**) and four variants (BM1-1 represented in **red**, BM1-2 in **green**, BM1-3 in **purple** and BM1-4 in **yellow**). (b) 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.

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.

Resistance to all the antibiotics tested is similar for BM1 and nonmucoid variant BM1-1. 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. 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, 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.

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 3**).

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.

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 spite of this, due to the error associated on this method, more experiments need to be performed.

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

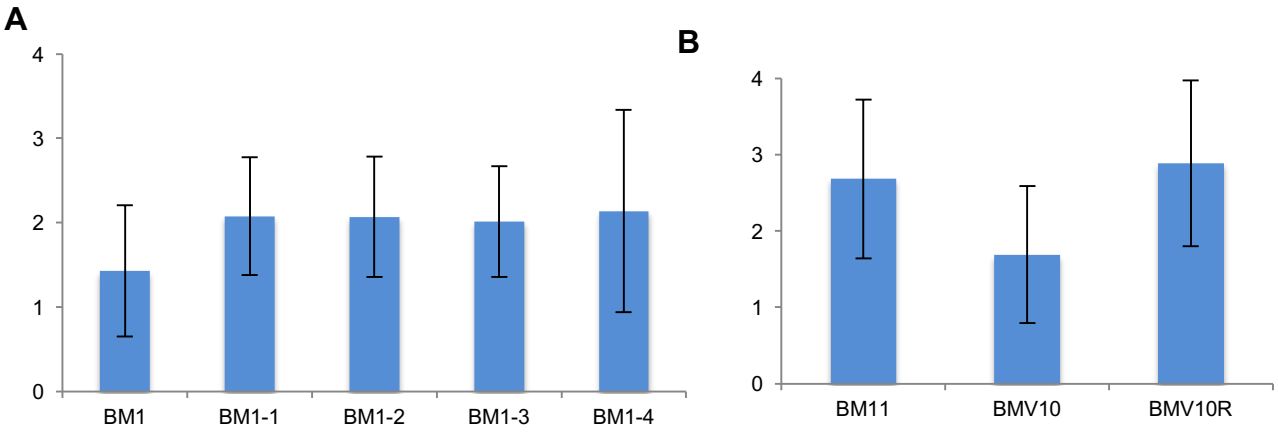

Figure 3 – Biofilm production. Bacterial cultures were used to inoculate 96-well plates incubated at 37 ºC statically for 48 h. Adherent bacteria were stained with crystal violet solution (x %) and solubilized in 96 % ethanol. Biofilm was quantified, by measuring the absorbance at 590nm, for (a) BM1 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.

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.

After proceeding with digestion and ligation of the genes *ompR* and *fixJ* and the plasmid pWH844 (as described in Material and Methods), 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.

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

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 and at 4h 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). Elution of proteins was performed in the ÄKTA system.

From each purification we choose the fraction that demonstrated less contamination and compared them with the aliquots taken before induction and after the overexpression as shown in **Figure 4**.

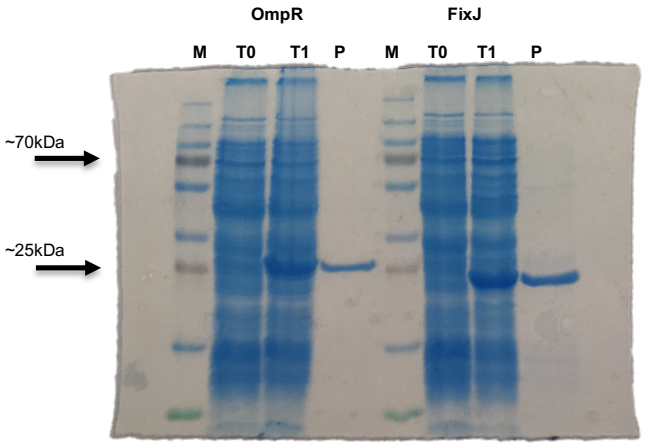

Figure 4 – Overexpression and purification of OmpR and FixJ proteins. SDS-PAGE polyacrylamide gel of the mixtures obtained before induction (T0), after 4h of IPTG supplementation (T1) and after ÄKTA system purification (P). M represents PageRuler plus.

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.

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 5** show bands equals to the control (**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.

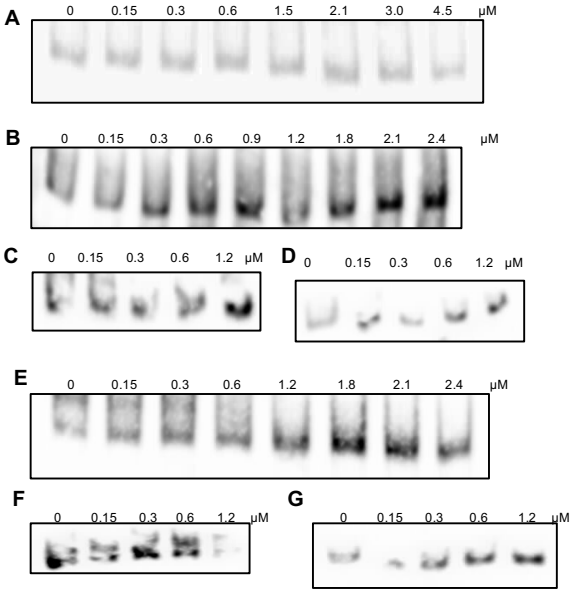

Figure 5 – Electrophoretic mobility shift assays using the DNA probes bceB_P1 (A, B and E), bceA_P2 (C and F) and bceB_CDS (D and G). Gel shift assays using increasing concentrations of protein. (A) Shows assays performed with BSA allowing control of the specificity of the ligation. (B and E) Shows assays with His-OmpR (B) or His-FixJ (E) and the probe bceB_P1. (C and F) and (D and G) were the assays with His-OmpR (C/D) or His-FixJ (F/G) 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.

MATERIALS AND METHODS

Biological material

Several bacterial strains and plasmids used in this study are described in **Table 2.** *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.

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 supplemented with the respective antibiotic when necessary. Otherwise, the strains were conserved at -80 ºC with 30% glycerol.

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.

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.

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.

Table 2 – 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, Canada (date of isolation: 30.11.1993)	Silva et al. 2016
BM1-1, -2, -3, -4, -5, -6, -7, -8, -9 and -10	Nonmucoid variants derived from BM1 after 21 days at 37ºC under nutrient starvation	This work
D2095 (BM11)	Cystic fibrosis clinical isolate, 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

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.

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.

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.

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.

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 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. 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.

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. 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.

Electrotransformation

To prepare electrocompetent cells *E. coli* DH5α cells was 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.

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.

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.

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.

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 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).

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 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 and various amounts 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.

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