Role of two-component regulatory systems in mucoid morphotype variation in *Burkholderia multivorans*

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Abstract

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

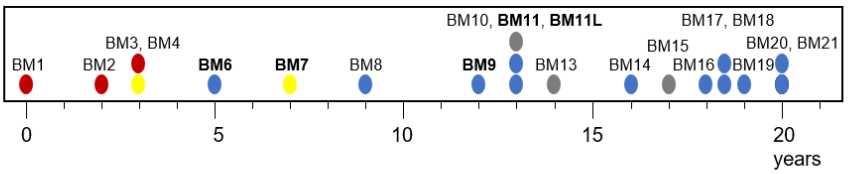
INTRODUCTION

It is known that bacteria from the *Burkholderia cepacia* complex (Bcc) and *Pseudomonas aeruginosa*, among other opportunistic pathogenic species have the capacity to infect the lungs of patients suffering from cystic fibrosis (CF) disorder. This disorder is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein (Staab, 2004). If this protein is defective or absent, there's an obstruction of the airways due to the accumulation of thick and viscous mucus. (Livraghi et al., 2007; Snouwaert et al., 1992). This provides a rich environment for opportunistic human pathogens, such as Bcc strains or *Pseudomonas aeruginosa* species (Mcdaniel et al., 2015). Both species possess many virulence factors, such as polysaccharides cepacian and alginate, respectively (May et al., 1991; Herasimenka et al., 2007). However, studies have shown that mucoid-producing EPS Bcc strains can switch to a nonmucoid variant (devoid of EPS production). This nonmucoid variants were related with a rapid decline function in animal infection models (Conway et al., 2004; Silva et al., 2011; Sousa et al., 2007). Consequently, understanding the mechanism behind this switch became important for the clinical outcome of CF patients. To address this, Silva et al., sequenced the genomes of 22 *Burkholderia multivorans* sequential isolates recovered over 20 years from a CF patient in which mucoid-to-nonmucoid transitions had already occurred. Through phylogenetic analysis based on the type of mutations accumulated it was possible to identify an early clade C1, from the first isolate (BM1), that quickly diversified into clades C2, C3 and C4. (Silva et al., 2016). In later studies, nonmucoid variants were successfully

obtained *in vitro* from clade C1 (Bica, 2018) and clades C2 and C3 (Silva et al., 2018) mucoid isolates by exposing those isolates to stress conditions known to trigger mucoid-to-nonmucoid switch. Mapping of mutations revealed that clade C1 BM1-derived nonmucoid variants harbored mutations in *fixL* and *fixJ* genes of a two-component regulatory

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

A



B

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

Figure 1 - Temporal distribution of the *B. multivorans* mucoid clinical isolates recovered from a single CF patient with indication of the clade inferred by phylogenetic analysis (adapted from Silva et al., 2016) (A). The four clades are **C1 (red)**, **C2 (yellow)**, **C3 (blue)**, and **C4 (green)**. Mutation common to clades C2, C3 and C4, but absent from isolates of clade C1. The locus_tags used are from *B. multivorans* ATCC 17616 **(B).**

system. Contrastingly, C2 and C3 clades-derived variants mainly accumulated mutations in the *ompR* gene also from a two-component regulatory system, with a single exception where *fixJ* was the target (Silva et al., 2018). Since isolates from clades C2 and C3 include later isolates when compared to C1 isolates, it could be that this phenotypic transition of mucoid-to-nonmucoid dependent on OmpR or FixJ was caused by a particular genetic background. Since nonmucoid variants are yet to be obtained *in vitro* for the isolates of clade C4 (BM10, BM13 and BM15), the primary aim of this work was to evaluate if this mucoid-to-nonmucoid switch was also occurring in isolates from clade C4, and if it was the case, to investigate if the mutations related with the emergence of nonmucoid variants in this clade was related to clade C1 or C2, C3 clades. With this in mind, we designed the following experiment: expose triplicates of *B. multivorans* BM10, BM13 and BM15 isolates to stress conditions to see whether mucoid-to-nonmucoid switch was also occurring; recover random colonies of interest (nonmucoid/less mucoid) and perform whole genome sequencing to identify possible mutations; perform phenotypic characterization of the mutants obtained.

RESULTS AND DISCUSSION

The *B. multivorans* isolates analyzed in this study were recovered from one patient chronically colonized by *B. multivorans* in which the mucoid-to-nonmucoid transition had already occurred (Zlosnik et al. 2008). *B. multivorans* isolates were first obtained in 1993 (BM1) and were recovered periodically until 2013, yielding a total of 22 isolates for analysis (Figure 1). 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 evaluate which molecular mechanisms could be underlying the emergence of nonmucoid variants, triplicates of mucoid BM11 was maintained statically for 21 days in SM medium. *In vitro*, the mucoid clinical isolate BM11 gives rise to stable nonmucoid variants in response to known stress inducing conditions, like prolonged stationary phase, presence of antibiotics, and osmotic and oxidative stresses (Silva et al. 2013). The same experiment was carried out for the mucoid isolates BM10, BM13 and BM15 and it was

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

Figure 2 – Mutations identified in the *B. multivorans* variants when compared to BM10 isolate as reference. Locus_tag used is from *B. multivorans* ATCC17616. **syn**, synonymous; **nonsyn**, nonsynonymous mutation.

possible to obtain colonies displaying a less mucoid phenotype on YEM agar solid medium only from isolate BM10. Fifteen of these colonies were initially kept for further studies and were named C1 to C15 (C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12, C13, C14, C15). To confirm which colonies were indeed *Burkholderia*, a PCR was carried out for the amplification of gene *ldhA*, which encodes the enzyme D-lactate dehydrogenase of *B. multivorans* ATCC

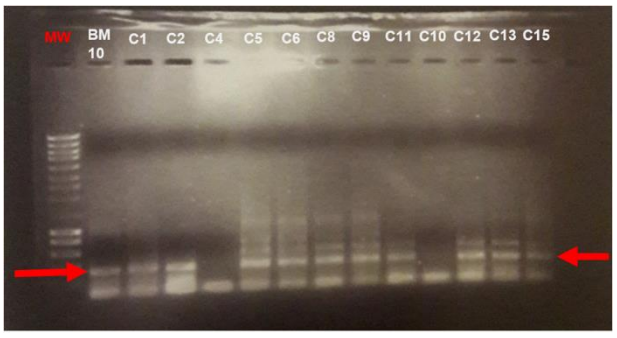


Figure 3 - Electrophoretic separation in 0.8% agarose gel of the PCR products corresponding to a fragment of the *ldhA* gene of *B. multivorans* with an estimated size of 555 bp (indicated by the red arrows)**.** The molecular weight (MW) marker used to estimate DNA size is the NZYDNA Leader.

16716 (Gomes, 2018) (Figure 3) confirming that ten colonies were *Burkholderia* (C1 , C2, C5, C6, C8, C9, C11 C12, C13, C15) (Figure 4). Of those, nine colonies (C1, C2, C5, C6, C8, C9, C12, C13, C15) were chosen for whole genome sequence and mutations in the *ompR* gene and *envZ* gene were found, as it can be observed in Figure 2.

Assessment of exopolysaccharide production in solid and liquid media

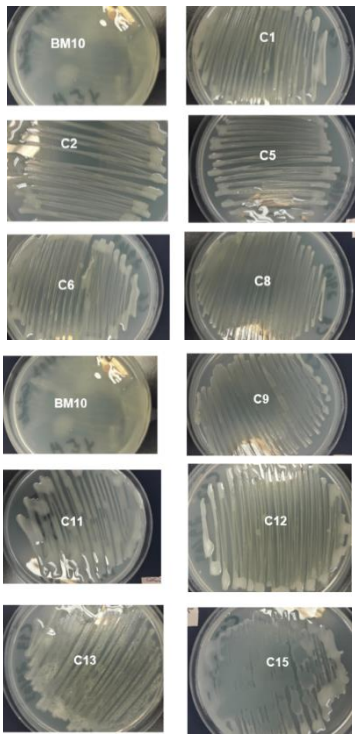


Figure 4 - Confirmation of the less mucoid phenotype of variant colonies derived from *B. multivorans* BM10. For each is shown a petri dish with YEM solid agar medium and it can be observed that the mutant colonies display a reduced mucoidy when compared with the parental strain (BM10).

In order to evaluate the mucoid phenotype of the 10 variants they were grown in YEM solid agar medium. Unlike the nonmucoid variants previously obtained, none of the variants were nonmucoid, displaying a much less mucoid phenotype (Figure 4). Based on the

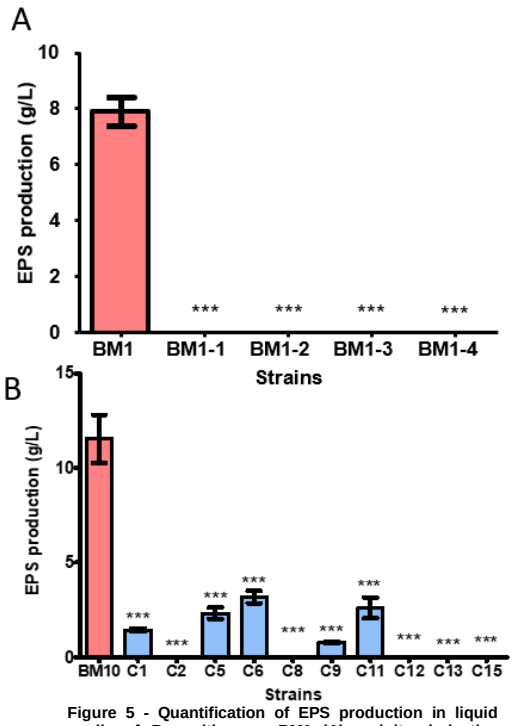


Figure 5 - Quantification of EPS production in liquid media of *B. multivorans* BM1 (A) and its derivative nonmucoid variants and of *B. multivorans* BM10 and its derivative less-mucoid variants (B) was measured by the dry-weight of the ethanol-precipitated polysaccharide recovered from all strains grown in liquid SM medium over 3 days at 37ºC under 250 rpm orbital agitation. Error bars correspond to the standard deviations of the mean values of at least two independent experiments. Significance level (one-way ANOVA followed by Dunnett's multiple comparison test) between the wild-type (BM1 or BM10) and the mutants was determined: ***, P<0.001.

results obtained from whole-genome sequencing, complementation assays were performed with two selected colonies) that harbored mutations in *ompR* and *envZ* locus (colony C2 and colony C8) but also in one selected colony that only harbored mutations in the *ompR* locus (colony C12). The referred complementation was made through triparental conjugation using the plasmid pLM014-5, which consisted of the vector pBBR1MCS including the amplified region of the promoter and coding sequence of gene *ompR*, to evaluate the role of *envZ* in the producing-exopolysaccharide mucoid phenotype. Results suggested that the producing-exopolysaccharide mucoid phenotype involves a fully functional OmpR and EnvZ proteins, since the mucoid phenotype was reverted in colony C12 (which only harbored mutations in *ompR* locus) but not in colonies C2 and C8 (which had accumulated mutations in both *ompR* and *envZ* genes). Exopolysaccharide production was also evaluated in liquid SM medium by growing cultures 4 days at 37 ºC under 250 rpm orbital agitation. Under these conditions BM10 parental isolate produced a

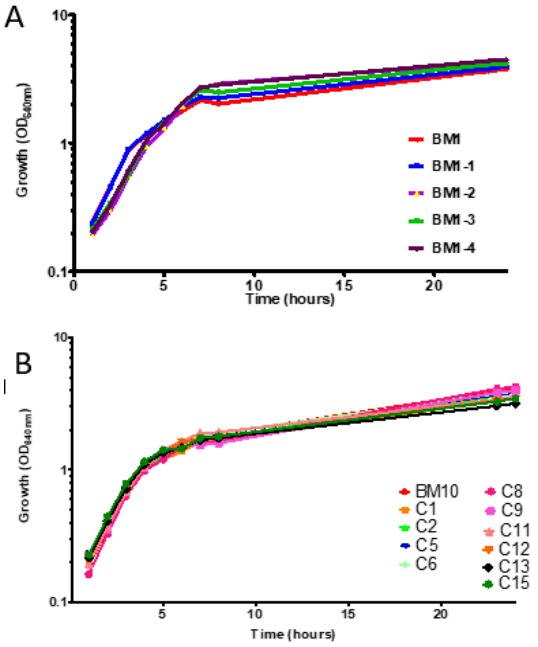


Figure 6 - Growth curves obtained for *B. multivorans* BM1 and its derivative nonmucoid variants (A) and for *B. multivorans* BM10 and its derivative less-mucoid variants (B). Cultures were grown in LB medium at 37ºC, 250 rpm of orbital agitation, and OD_{640nm} was measured for 24 hours. Results are the means of data from three independent experiments. The standard deviation is below 5%.

high molecular weight EPS. The mutants (C1, C2, C5, C6, C8, C9, C11, C12, C13, C15) however, despite revealing a reduced mucoidy associated with production of reduced EPS in YEM solid agar medium, were unable to produce EPS in liquid medium. Regarding BM1 derivatives, nonmucoid BM1-1, BM1-2, BM1-3 and BM1-4 were also unable to produce EPS in liquid medium (Figure 5). There is no explanation for this different behavior between solid and liquid medium.

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

To evaluate if the mutations accumulated by the mutants could cause any growth impairment, the growth kinetics of the mutants was estimated. To do this, cultures of *B. multivorans* BM1, BM10 and its variants were grown in liquid LB medium for 24 hours and under 250 rpm orbital agitation. Results obtained are shown in Figure 6. The majority of the less-mucoid variants did not have a statistically significant difference in the doubling time by comparison with the mucoid parental strain *B. multivorans BM*10, with the exception of variants C8 and C12, as it can be observed in table 2. These results suggest that mutations accumulated

in the *ompR* gene don't represent a major impairment in growth kinetics within the tested conditions.

Antimicrobial resistance

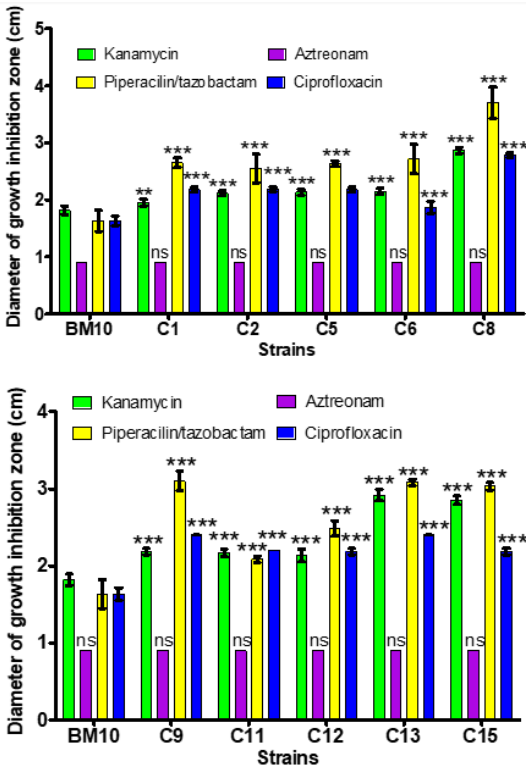


Figure 7 - Susceptibility of *B. multivorans* BM10 and its derivative less-mucoid variants to antibiotics (Ciprofloxacin, Aztreonam Piperacillin + Tazobactam and Kanamycin) was measured by the diameter of cell growth inhibition, after growth for 24h at 37ºC. Error bars correspond to the standard deviations of the mean values of at least two independent experiments. Significance level (one-way ANOVA followed by Dunnett's multiple comparison test) between the wild-type (BM1) and the mutants for each antibiotic tested was determined: ns, not statistically significant; *, P<0.05; ***, P<0.001.

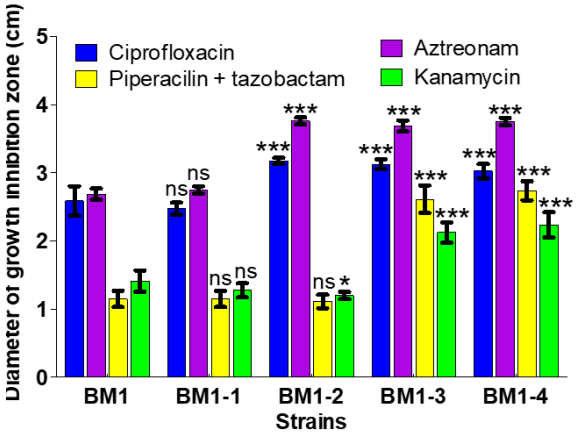


Figure 8 - Susceptibility of *B. multivorans* BM1 and its derivative nonmucoid variants to antibiotics (Ciprofloxacin, Aztreonam Piperacillin + Tazobactam and Kanamycin) was measured by the diameter of cell growth inhibition, after growth for 24h at 37ºC. Error bars correspond to the standard deviations of the mean values of at least two independent experiments. Significance level (one-way ANOVA followed by Dunnett's multiple comparison test) between the wild-type (BM1) and the mutants for each antibiotic tested was determined: ns, not statistically significant; *, P<0.05; ***, P<0.001

Since the *ompR and fixL/fixJ* genes have been implicated in the regulation of some virulence traits, such as resistance to antimicrobials, we tested the antibiotic resistance of the variants derived from *B. multivorans* BM10, by comparison with the parental strain (*B. multivorans* BM10) to Ciprofloxacin, Aztreonam, Piperacillin + Tazobactam and Kanamycin. The antimicrobial susceptibility was determined by measuring the diameter of the growth inhibition zone after 24 hours of incubation at 37°C. Results for the BM10-derived variants are shown on Figure 7 and it can be noted that the resistance to Aztreonam was not affected by the mutations in *ompR* since there was no statistically significant difference between the wild-type and the mutant strains. As for the other antimicrobials tested there was an overall increase in antibiotic susceptibility in all the tested variants, with variant C8 having a higher susceptibility to Piperacillin + Tazobactam and Ciprofloxacin and the variants C8, C13 and C15 having a higher susceptibility to Kanamycin, by comparison with the other tested variants. As for BM1-derived nonmucoid variants, results are shown on Figure 8, where it can be observed that the resistance to Ciprofloxacin, Aztreonam Piperacillin + Tazobactam and Kanamycin was similar between variant BM1-1 and the parental strain *B. multivorans* BM1; it can also be noted that there was an overall increase in Aztreonam and Ciprofloxacin susceptibility in nonmucoid variants BM1-2, BM1-3 and BM1-4 and that this increase was similar among the three variants; finally nonmucoid variants BM1-3 and BM1-4 registered an increase in Piperacillin + Tazobactam and Kanamycin susceptibility by comparison to the parental strain *B. multivorans* BM1 and this increase was also similar between these two variants.

Swimming and swarming motilities

Motility is also an important factor upon adaption of *Burkholderia multivorans* to the microenvironment of the CF lung. Some studies have associated fluctuations in the swimming and swarming motilities between mucoid and less mucoid/nonmucoid isolates (Silva et al., 2018; Schaefers et al., 2017). Consequently, swimming and swarming motilities were considered to be important features to test in both *B. multivorans* BM1 derived nonmucoid variants and *B. multivorans* BM10-derived less-mucoid variants. To do this, swimming plates containing 1% (wt/vol) tryptone, 0.5%

(wt/vol) NaCl, 0.3% (wt/vol) noble agar (Difco) (Kamjumphol et al., 2013) and swarming plates containing 0.04% (wt/vol) tryptone, 0.01% (wt/vol) yeast extract, 0.0067% (wt/vol) CaCl2, 0.6% (wt/vol) bacto agar (Difco) (Silva et al. 2018) were statically incubated for 24 hours (swimming) or 48 hours (swarming), at 37ºC after inoculation. Then, the motility zone diameter was measured (in cm) for each plate. Regarding swimming motility, results for the four *B. multivorans* BM1-derived nonmucoid variants BM1-1, BM1-2, BM1-3 and BM1-4 can be observed on Figure 9A, where it can be noted that there was an overall decrease in swimming motility while *B. multivorans* BM10-derived less-mucoid variants C8, C9, C11, C12, C13 and C15 presented a statistically significant increase in swimming motility and the variants C1, C2, C5 and C6

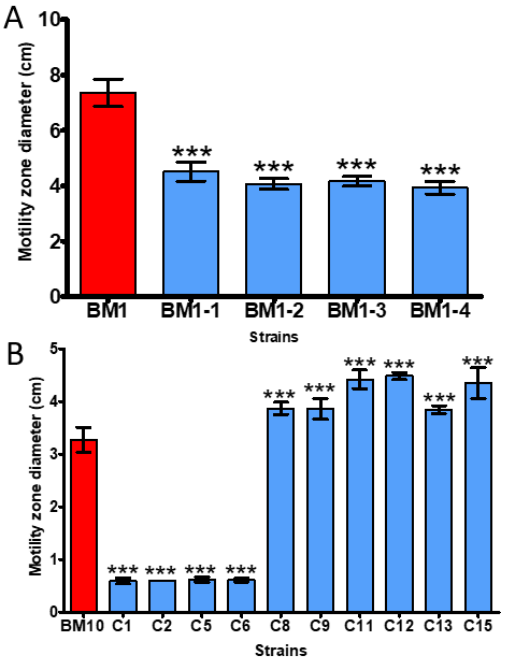


Figure 9 - Swimming motility of *B. multivorans* BM1 (A) and its derivative nonmucoid variants and swimming motility of *B. multivorans* BM10 and its derivative less-mucoid variants (B) was measured by the motility zone diameter after growth for 24h at 37°C. Error bars correspond to the standard deviations of the mean values of at least two independent experiments. Significance level (one-way ANOVA followed by Dunnett's multiple comparison test) between the wild-type (BM1 or BM10) and the mutants was determined: ns, not statistically significant; *, P<0.05; ***, P<0.001.

presented a statistically significant decrease in swimming motility, by comparison with the wild-type *B. multivorans* BM10 (Figure 9B). Regarding swarming motility, results for the *B. multivorans* BM1-derived nonmucoid variants can be observed on Figure 10C, where it can noted that variant BM1-1 did not have its swarming motility affected and the remaining three nonmucoid variants BM1-2,

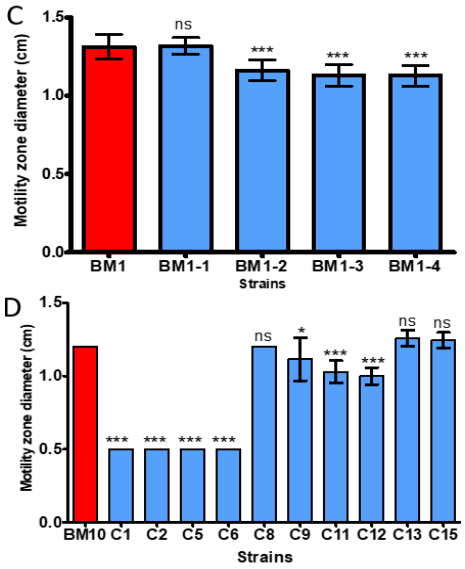


Figure 10 - Swarming motility of *B. multivorans* BM1 (C) and its derivative nonmucoid variants and swimming motility of *B. multivorans* BM10 and its derivative less-mucoid variants (D) was measured by the motility zone diameter after growth for 24h at 37°C. Error bars correspond to the standard deviations of the mean values of at least two independent experiments. Significance level (one-way ANOVA followed by Dunnett's multiple comparison test) between the wild-type (BM1 or BM10) and the mutants was determined: ns, not statistically significant; *, P<0.05; ***, P<0.001.

BM1-3 and BM1-4 registered a statistically significant decrease in swarming motility, by comparison with the parental strain *B. multivorans.* As for *B. multivorans* BM10-derived less-mucoid variants, results can be observed on Figure 10D, and it can be noted that variants C8, C13 and C15 did not have their swarming motility affected and that the less mucoid variants C1, C2, C5, C6, C9, C11 and C12 presented a statistically significant decrease in swarming motility, by comparison with the wild-type strain *B. multivorans* BM10. It can also be observed that the diameter measured in cultures C1, C2, C5 and C6 corresponds to the size of the inoculum, indicating that these variants lost the capacity for both swimming and swarming motilities.

Surface-attached biofilm formation

Another phenotype that some studies have shown to be affected by the mucoid-to-nonmucoid switch is the capacity that *B. multivorans* strains have to establish stable surface-attached biofilms in the lungs of CF patients (Ferreira et al., 2007; Silva et al., 2011; Silva et al., 2018; Schaefers et al., 2017). With this data in mind, it was important to evaluate and compare the ability of the mucoid strains and its less/nonmucoid variants in producing surface-attached biofilms. To test this, the *B. multivorans* strains under study were grown in microtiter plates for 48 hours and after this

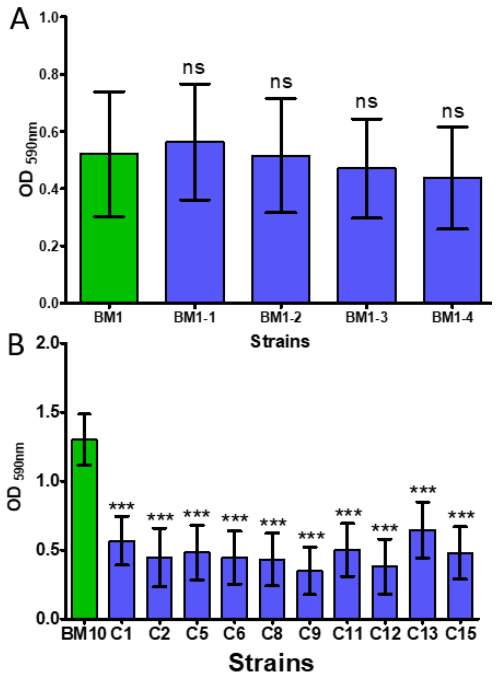


Figure 11 - Surface-attached biofilm formation of *B. multivorans* BM1 (A) and its derivative nonmucoid variants and of *B. multivorans* BM10 and its derivative less-mucoid variants (B) was determined by absorbance measurement at 590 nm after growth for 48h at 37ºC in polystyrene microplates. Error bars correspond to the standard deviations of the mean values of at least seven independent experiments. Significance level (one-way ANOVA followed by Dunnett's multiple comparison test) between the wild-type (BM1 or BM10) and the mutants was determined: ns, not statistically significant; ***, P<0.001.

period, they were stained with crystal violet and washed, and the absorbance of the retained dye in the microtiter plate wells, was measured at 590 nm (A_{590nm}). Regarding the mucoid *B. multivorans* BM1 and its nonmucoid variants, results can be observed on Figure 11A, where it can be seen that there was no statistically significant difference between the mucoid wild-type *B. multivorans* BM1 and the nonmucoid strains *B.* multivorans BM1-1, BM1-2, BM1-3 and BM1-4. Regarding the mucoid *B. multivorans* BM10 and its less mucoid variants, there was a statistically significant decrease in surface-attached biofilm formation, by comparison with the parental strain *B. multivorans* BM10 (Figure 11B). Through observation of the Figure 11B, it can also be noted that BM10-derived less mucoid variants display reduced biofilm formation when compared to the wild-type strain, but no significant differences were observed in the production of surface-attached biofilms among the mutants.

CONCLUSIONS

Chronical infections established in CF patients by Bcc strains remain the deadliest for CF patients, since studies have shown that this bacteria have an intrinsic resistance to many antimicrobial peptides, can cause a life threatening condition, "cepacia syndrome" and possess several different virulence factors that they can activate or inhibit when there is a shift on the environmental conditions (Zlosnik et al., 2011). One well characterized virulence factor is the exopolysaccharide cepacian, which is produced by mucoid clinical isolates retrieved from the lungs of CF patients (Cunha et al., 2004). Although the structure and genes involved in cepacian biosynthesis are already identified (Moreira et al., 2003; Ferreira et al., 2010), the set of genes/molecular mechanism responsible for regulating cepacian biosynthesis are unknown. Nevertheless, studies performed in sequential isolates obtained over the course of a chronical infection, have shown that bacteria in order to adapt to the environment of the CF lungs can alter their surface determinants, switching from a mucoid to a nonmucoid variant unable to produce the EPS cepacian. These nonmucoid variants were shown to be related with rapid decline in lung function (Zlosnik et al., 2011). Therefore, understanding the mechanisms associated with the emergence of nonmucoid variants became important in the clinical context. With this data in mind, Moreira and her team made efforts to characterize a genomic and functional evolution of a chronic infection of a CF patient, in which mucoid-to-nonmucoid transitions had already occurred (Silva et. al, 2016). In that work, sequential clonal isolates that had been sampled over 20 years from the lungs of a CF patient were sequenced and their mutations were mapped against the first isolate (BM1), revealing the mutations that each of the 22 isolates has accumulated over the course of the chronic infection. This allowed the construction of a phylogenetic tree that defined the existence of four different clades, based on the type of mutations of each isolate. Clade C1 includes the isolates BM1, BM5, BM2 and BM3; clade C2 includes the isolates BM4 and BM7; clade C3 includes the isolates BM6, BM11,BM22,BM12, BM8, BM18, BM9, BM14, BM20, BM21, BM16, BM17 and BM19 and finally clade C4 including isolates BM10, BM13 and BM15. . Clade C1 isolates had mostly given rise to nonmucoid isolates through mutations

accumulated in *fixL/fixJ* genes; as for clades C2 and C3 isolates, the emergence of nonmucoid isolates in those clades was mainly achieved through the accumulation of mutations in the *ompR* gene. Later on, nonmucoid variants were successfully obtained *in vitro* from clade C1 (Bica, 2018) and clades C2 and C3 (Silva et al., 2018) mucoid isolates by exposing those isolates to stress conditions known to trigger mucoid-to-nonmucoid switch and mutations mapped in the same genes (*fixL/fixJ* in clade C1 and *ompR* gene in clades C2 and C3), depending on the genetic background of the isolate. The main focus of this work was to evaluate if the mucoid-to-nonmucoid switch was also occurring in isolates from clade C4. Our main results showed that the switch was also occurring in isolates from clade C4 and less mucoid isolates were obtained by exposing the clade C4 BM10 mucoid isolate to stress conditions known to trigger the mucoid-to-nonmucoid switch. Random colonies of interest were kept and the whole genome sequencing of nine of those colonies showed that all the variants had accumulated mutations in the *locus* of the genes ompR, with some mutants also harboring mutations in the *envZ locus.* Both genes are involved in a two-component regulatory system and had already been identified has likely targets for mutations related with emergence of nonmucoid variants on previous studies. (Silva et al., 2016:Silva et al., 2018). To evaluate if those mutations could affect some phenotypical traits related with cell envelope composition, the less mucoid/nonmucoid variants obtained where characterized and compared with the parental strain BM10 regarding their growth, resistance to antimicrobials, ability to establish surface attached biofilms, swimming and swarming motility and production of exopolysaccharide in both liquid and solid EPS-producing media. Our main results appear to agree with the results obtained by previous studies that have linked mutations in the *ompR* gene with changes in some of those phenotypical traits, such as the formation of biofilms or an increase in antimicrobials susceptibility (Silva et al., 2018). To evaluate the importance of the Histidine Kinase EnvZ in the mucoid producing EPS phenotype, selected less mucoid mutants that harbored mutations in *ompR* or in *ompR* and *envZ* were complemented by triparental conjugation with the plasmid vector pLM014-5, which contained the promoter and the coding region of the gene *ompR*. As a result, the mucoid phenotype was reverted in the mutant harboring mutations only in the *ompR locus*, but the expression of the vector in the mutants for *envZ* and *ompR* did not restored the mucoid phenotype, suggesting that an intact HK EnvZ and RR OmpR are needed for the production of the EPS cepacian. Since there was no genetic construction available for the promoter and coding region of the *envZ* gene, no complementation was performed with this gene, and for future work it could be suggested the construction of a plasmid vector containing the promoter and coding sequence of the *envZ* gene, in order to see if the mucoid phenotype would be restored in the mutants harboring mutations for both *envZ* and *ompR* genes, through the expression of both plasmids (the one that would be constructed containing the promoter and sequence region of *envZ* and the plasmid vector pLM015-5), fully confirming the suggested importance of the HK EnvZ protein in production of the highly mucoid phenotype.

MATERIALS AND METHODS

Biological material

The various bacterial strains and plasmids used in this study are described in Table X. *Burkholderia* strains were used to study EPS production or other phenotypic characteristics. *E. coli* strains were used for the complementation assays through triparental conjugation).

Culture conditions

The *Burkholderia* strains were grown in Lennox Broth (LB: 5 g. L Nacl, 5 g. L yeast extract: 10 g. L and 10 g. L tryptone) or in SM medium (12.5 g. L $Na_2HPO_4.2H_2$ 3.0 g. L KH_2PO_4, 1.0 g. L K_2SO_4, 1 g. L NaCl, 20 g. L mannitol, 1.0 g. L casaminoacids, 1.0 g. L yeast extract, 0.2 g. L $MgSO_4.7H_2O$, 0.001 g. L $FeSO_4.7HO$), used to induce the production of EPS (Silva et al. 2013). *E. coli* strains were grown in LB medium. The strains, when used were maintained on LB plates. All the strains were conserved at -80ºC, with 30% glycerol (table 1).

Inducing the morphotype switch under prolonged incubation

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

2 days. The colonies that displayed a slightly less mucoid or nonmucoid morphotype, were kept.

Growth rate and doubling time determination

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

Exopolysaccharide production

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

Antimicrobial susceptibility

To assess the antimicrobial susceptibility of the strains, the agar disc diffusion method (Bauer et. al. 1996) was used. Paper discs containing ciprofloxacin (5 µg), piperacillin (75 µg) + tazobactam (10 µg), aztreonam (30 µg) and kanamycin (30 µg) were used. The discs were placed onto the surface of Mueller-Hinton (Sigma-Aldrich) agar plates that had been previously inoculated with 100 microliters of a suspension of bacterial cells at an OD_{640nm} of 0.1. The cells were grown at 37ºC, 250 rpm, after overnight inoculation. Growth inhibition diameter was measured after 24 h of incubation at 37ªC. Three independent experiments were performed.

Biofilm formation

Biofilm formation assays were performed based on the method previously described (Ferreira et al. 2007). Overnight liquid cultures, grown at 37ºC 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 37ºC 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.

Swimming and Swarming motilities

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

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

DNA manipulation techniques

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

Genetic complementation

Complementation assays were performed by triparental conjugation. The receptor strains were nonmucoid variants C8 and C12 and the wild-type strain BM10. The donor was *E. coli* containing pBBR1MCS on pLM14-5 harboring the *ompR* gene. The helper was *E. coli* with pRK2013. Selection was in YEM medium supplemented with 200 mg/mL of chloramphenicol and 40 mg/mL of gentamycin.

Genome sequencing and reference assembly

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

Statistical analyses

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

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