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Genetic determinants of cell aggregation in *Burkholderia multivorans*

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ABSTRACT

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

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive monogenetic disease (Collins, 1992). Which arises from mutations in a 189 kb gene on chromosome 7, encoding a 1480 amino acid polypeptide, named cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is a member of the ATP-binding cassette (ABC) transporter family of membrane proteins which forms a channel at epithelial apical cell membrane (Cant, Pollock, & Ford, 2014).

With ultimately, 80 to 95% of patients succumbing to respiratory failure, the most troublesome clinical features of CF are the prominent neutrophilic inflammation, mucus in airways, progressive bronchiectasis and the chronic pulmonary bacterial infection (Lyczak et al., 2002; Stoltz et al., 2015).

Prevalence of Bcc infection in CF patients, ranges from 0 to 40%, depending on the center, and increases with age (Zlosnik et al., 2015). Similar to other opportunistic pathogens such as Pseudomonas aeruginosa, Bcc strains do not infect healthy individuals but only those that are immunocompromised. Two of the major problems arising from infection of CF patients with Bcc species are their high intrinsic resistance against antibiotics and biocides, impeding effective medical treatment, and their high transmissibility between patients (Huber et al., 2002). The factors that establish Bcc infection are still undefined, putative virulence determinants that have been described include cable pili, lipopolysaccharide (LPS), extracellular protease, lipase, hemolysin, a melanin-like pigment, and siderophores. The roles of these factors in lung infections in CF patients remain to be clarified, as their presence does not necessarily correlate with the severity of disease (Chung et al., 2003).

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

Two clearly identified phenotypes associated with biofilms are the aggregation of cells and a significant increase in the capacity to cope with antibiotic treatments. Studies have shown that non-surface attached aggregates, also called planktonic aggregates or tight microcolonies, exhibit the required survival capability in the hostile human environment and possess inherent antibiotic tolerance to antibiotics and phagocytes (Alhede et al., 2011). *P. aeruginosa's* planktonic aggregates also share other biofilm features, like the dependency on cyclic di-GMP, eDNA, bacteriophage, and dispersal based on carbon, nitrogen, or oxygen limitations (Schleheck et al., 2009). Therefore, planktonic aggregates should be considered to fully

represent the biofilm growth phenotype (Alhede et al., 2011). The major hallmarks of in vivo biofilms are thus aggregated bacteria, which tolerate the host defense and high concentrations of antimicrobial agents even over longer times (Bjarnsholt et al., 2013).

The genetic mechanisms involved in the formation of planktonic aggregates are not clear, however A study with Bcc bacteria, including *B. multivorans*, by Silva et al. showed that the LTTR LdhR and the D-lactate dehydrogenase LdhA are implicated in the formation of planktonic cellular aggregates and biofilms properties possibly relevant in natural environments and within hosts (Silva et al., 2017).

In order to investigate the genetic determinants of cell aggregation, a. library of insertion mutants of a clinical isolate of *Burkholderia multivorans* was created, and six mutants were selected for their reduced ability to aggregate. The sequencing of the flanking regions of insertion of the plasposon allowed the identification of three disrupted genes: one encodes a phosphoenolpyruvate synthase, other is a part of a type VI secretion system, and the last is involved in a two-component transcriptional regulator. Each of these mutants was also characterized concerning phenotypes such as motility, exopolysaccharide production, antibiotic susceptibility and growth rates.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The bacterial strains used in this study are listed Burkholderia multivorans P0213-1 (Cystic fibrosis isolate, Canada from 13-02-1996 ID: VC7495 by Dr.P Speert University of British Columbia), Escherichia coli DH5-α (DH5α recA1 Δ(lacZYA-argF)U169 ϕ80dlacZΔM15 from Gibco BRL. The plasmids used were pTnModΩkm (carrying a Kmr Plasposon with pMB1oriR) (Dennis & Zylstra, 1998) and pRK600 (ColE1 oriV; RP4tra+ RP4oriT; Cmr helper in triparental matings) (Kessler, 1992).

The *E. coli* strain was grown at 37ºC in Lennox Broth (LB) with or without agar, supplemented with kanamycin (50 µg/ml) when required to maintain selective pressure. *Burkholderia multivorans* P0213-1, a clinical isolate from a cystic fibrosis patient (patient P0213), and its derivative strains were grown in LB or in SM medium (12.5 g/l Na2HPO4.2H2O, 3 g/l KH_2PO_4, 1 g/l K_2SO_4, 1 g/l NaCl, 0.2 g/l $MgSO_4.7H_2O$, 0.01 g/l $CaCl_2.2H_2O$, 0.001 g/l $FeSO_4.7H_2O$, 1 g/l yeast extract, 1 g/l casamino acids, pH 7.2), supplemented with 20 g/l of D-mannitol with or without agar, supplemented with kanamycin (500 µg/ml) when required to maintain selective pressure, at 37ºC with 250 or 180 rpm of orbital agitation.

Transposon mutant library construction

A transposon mutant library of the clinical isolate *B. multivorans* P0213-1 (recipient) was constructed through triparental conjugation using an *E. coli* carrying the pRK600 plasmid as a helper and an *E. coli* carrying the pTnModΩkm plasmid as a donor (Figure A1). The donor strain was inoculated in 3 mL of LB with kanamycin, while the helper strain was inoculated in 3 mL of LB with chloramphenicol and the recipient strain was inoculated in 3 mL of LB. The three cultures were incubated at 37ºC with orbital agitation of 180 rpm for 5 hours. Following incubation, 1 mL of the recipient liquid culture was mixed with 1 mL of both helper and donor. The final mixture was transferred to a LB plate at 30ºC for 24 hours. This conjugation mixture was, then resuspended in 1ml of LB and 100 µl were plated onto 10 LB plates supplemented with 500 µg/ml kanamycin and 40 µg/ml gentamicin, were incubated at 37ºC for 24 hours. The mutant colonies suspensions were each mixed with the same volume of 60% of glycerol into a well of 96-well plates and frozen at - 80ºC with. The mutant colonies were numbered, from 1 to 96 and named as following: K5 (mutant of cystic fibrosis isolate P0213-1 from well 5 of plate K).

Screening of transposon insertion libraries

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

Microscopy analysis

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

DNA manipulation techniques

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

Detection of the plasposon in the genome of mutants

To verify if the plasposon was inserted into the genome of selected mutants, a DNA amplification by PCR was

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

Identification of the genes disrupted by the plasposon

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

Growth curves and doubling time estimation

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

Quantification of cellular aggregates and free cells

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

Aggregation Kinetics

A kinetics assay was performed in order to evaluate aggregate formation throughout cell growth. For that, cells from an overnight culture were inoculated with an OD_{640} of 0,1 in 30 mL fresh SM medium. The 24 separate flasks were incubated at 37ºC with 180 rpm of orbital agitation in two different time frames, in order to obtain data from four intercalated 12h periods. Time points were measured every two hours and a quantification of cellular aggregates and free cells as previously described.

Exopolysaccharide production

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

Antimicrobial susceptibility

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

Motility

Swarming motility

The swarming agar plates containing 0.04% (wt/vol) tryptone, 0.01% (wt/vol) yeast extract, 0.0067% (wt/vol) $CaCl_2$, 0.6% (wt/vol) bacto agar (Difco) (Silva et al., 2016) were spot inoculated with a 5 µl drop of a culture with a 1.0 OD_{640}. After inoculation, swarming plates were incubated for 48 h at 37°C and the diameter of the swarming zone was measured. Results are the means of data from 3 replicates.

Swimming motility

The swimming agar plates containing 1% (wt/vol) tryptone, 0.5% (wt/vol) NaCl, 0.3% (wt/vol) noble agar (Difco) were spot inoculated with a 5 µl drop of a culture with an 0.1 OD_{640}. After inoculation, swimming plates were incubated for 24 h at 37°C and the diameter of the swimming zone was measured. Results are the means of data from at least six replicates of two independent experiments.

Statistical analyses

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

RESULTS AND DISCUSSION

Screening of *B. multivorans* plasposon mutants

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

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

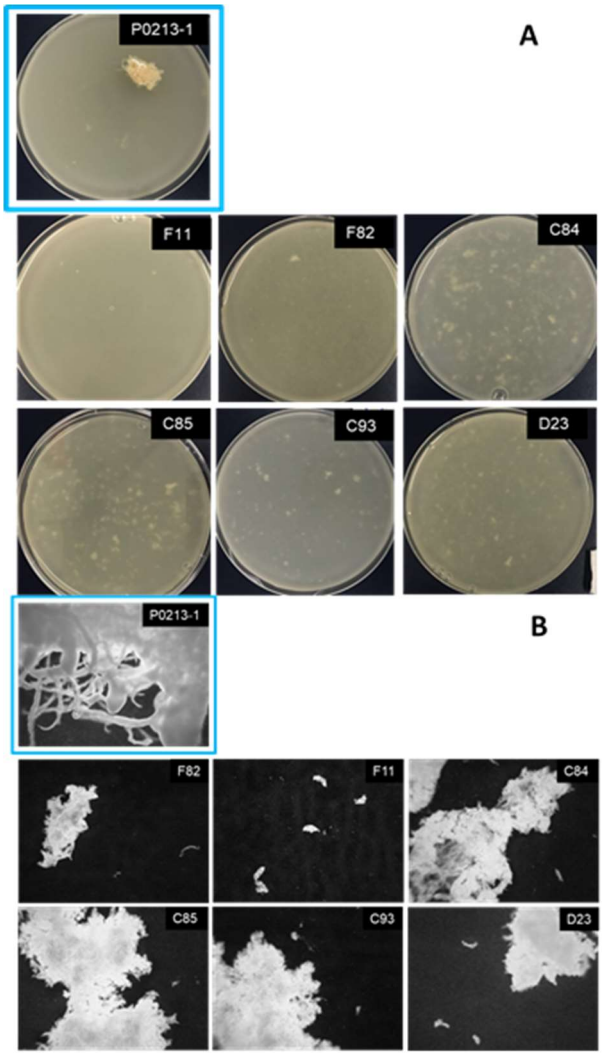

Figure 1. Macroscopic (A) and microscopic (B) screening of the plasposon mutant library of B. multivorans P0213-1 for different abilities to form planktonic cellular aggregates. Each culture was grown in SM medium at 37°C, 180 rpm of orbital agitation, for 48 hours. The same scale is present in all pictures

Detection of the plasposon in the genome of mutants

In order to confirm that alteration of aggregation phenotype observed was the result of the plasposon insertion into the genome and consequent disruption of a gene that induced. The mutant's genomic DNA was extracted, and a PCR amplification was performed using primers designed to amplify the plasposon's internal region, with a specified size of 1161 bp. An electrophoresis in an agarose gel was run on the amplification product

All selected mutants display a band with size between 1000 and 1400 bp (Figure A2), corresponding to the expected size

of the plasposon fragment, namely of 1161 bp. As it was expected, the wild-type P0213-1 did not present a band, confirming the plasposon absence and indicating that the insertion of the plasposon is the most likely the cause of the altered phenotype.

Identification of the genes disrupted by the plasposon

After confirming the plasposon insertion in the genome, the identification of the disrupted gene was made through the sequencing of the flanking regions of the site where the plasposon was inserted. The whole genome was restricted with a restriction endonuclease without recognition sequences within the plasposon, such as EcoRI, and the fragments obtained were subsequently self ligated and electroporated into electrocompetent *E. coli* cells. This procedure allowed for the obtention of colonies corresponding to each mutant. The plasmids were then extracted and after confirming its size the flanking regions were Sanger sequenced. By aligning the sequences obtained (Table A2) against *B. multivorans* ATCC 17616 genome, the disrupted genes were identified.

Table 1. Genes disrupted by the plasposon were identified in the B. multivorans selected mutants

Mutant	Gene Name	Annotation	Homolog in ATCC 17616
F11	*ppsA*	Phosphoenolpyruvate synthase	Bmul_1274
C93	*tctD*	Two component transcriptional regulator	Bmul_0075
F82	-	Type VI secretion protein	Bmul_2926

Mutant F11 is disrupted in the ppsA gene, Figure A3, which encodes a phosphoenolpyruvate synthase, an enzyme that converts ATP and pyruvate into AMP, phosphoenolpyruvate and Pi. This conversion is essential for gluconeogenesis and as such PEPs is required for growth when pyruvate or lactate are the sole carbon sources. Upon binding of ATP to the N-terminal domain of PEPs, a catalytic residue within the central domain, His-421 hydrolyzes the β-phosphate. This generates AMP, Pi and a distinct phospho-enzyme intermediate. The C-terminal domain of the phosphorylated enzyme binds to pyruvate and transfers the β-phosphate group through a swiveling domain mechanism, to generate phosphoenolpyruvate (PEP) (McCormick & Jakeman, 2015). In another study, Ferreira (2018) found that a mutant disrupted in the same gene presented the same phenotype observed here. She noted that the disruption observed could result in the accumulation of pyruvate and acetyl-CoA and perhaps an increase of lipid metabolic reactions (Ferreira, 2018).

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

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

Sequencing of the flanking regions of the mutant F82 places the plasposon in an homologue of Bmul_2926 encoding a type VI secretion system protein, Figure A4.

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

A study of the virulence genes of *B. cenocepacia* identified three transposon insertion mutants that were attenuated in the rat agar bead model of chronic lung infection. Further analysis indicated that these mutants had insertions within

T6SS genes, demonstrating that the T6SS is required for the virulence of *B. cenocepacia* in vivo (Aubert et al., 2008).

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

Mutant C93 presents a disruption in gene *tctD* which is part of a two component regulatory system, Figure A5. These systems usually consist of a membrane-associated sensor (histidine kinase protein) that monitors environmental signals and a response regulator (receiver) whose function is modulated by phosphotransfer from its cognate histidine kinase. Two component systems are often in charge of regulating the adaptation of bacteria to new environments, as is the case when *B. multivorans* colonizes the lungs of CF patients. This happens through the modification of gene expression patterns required for bacterial survival. Sensor histidine kinases respond to a wide range of signals, including those encountered during infection. (Aubert et al., 2008)

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

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

In vitro growth analysis of selected mutants

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

Table 2. Doubling time of the strains under study. Cultures were grown in LB medium at 37ºC, 180 rpm of orbital agitation, and OD_{640} was measured for 24 hours. The doubling time was calculated from the growth rate of the exponential growth phase. Statistical difference in the doubling time of the mutant strains was tested in comparison with the wild-type (P0213-1). Significance level (one-way ANOVA followed by Dunnett's multiple comparisons test): ns, not statistically significant

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

Analyzing the data obtained it can be observed that mutants D23 and F11 are the ones with the fastest growth, however, this difference was deemed not statistically significant. Moreover, all mutants seem to end up with approximately the same biomass formed. This leads to the conclusion that the mutations observed are not interfering with growth, and, therefore, the phenotypic alterations observed result of impaired aggregate formation abilities.

Quantification of cellular aggregates and free cells

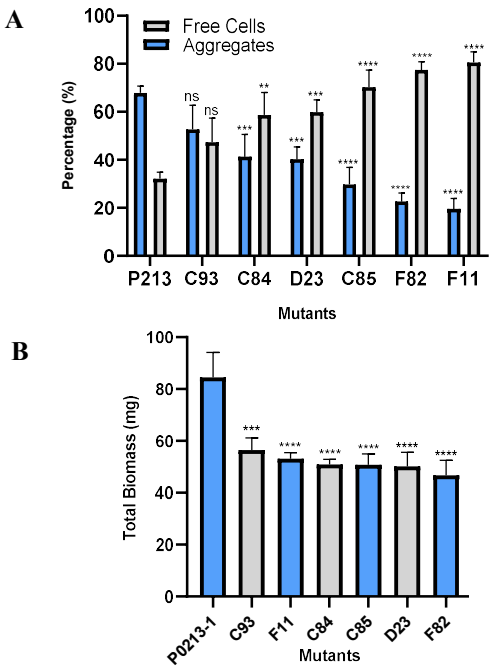

*Figure 2. – **A**- Quantification of cellular aggregates and free cells of wild-type (P0213-1) and its derivative mutants. **B** -Total biomass (dry weight of aggregates + free cells Measured after incubation at 37ºC with 180 rpm of orbital agitation for 48h. Error bars correspond to the standard deviations of the mean values of three independent experiments. Significance level (one-way ANOVA followed by Dunnett's multiple comparison test) between cellular aggregates and free cells of the wild-type (P0213-1) and cellular aggregates and free cells of the P0213-1 derivative mutants was determined: *, P<0.05; ***, P<0.001; ****, P<0.0001; ns, not statistically significant*

Although macroscopic visual analysis allows the identification of different aggregation phenotypes between the mutants selected and the Wt, a more thorough quantification was performed. Following the procedure described on the materials and methods section, the dry weight of both planktonic aggregates and free cells produced by each mutant was quantified. The results in terms of percentage thus obtained are presented in Figure 2.

The results obtained seem to confirm the visual analysis performed with most mutants presenting a lower percentage of aggregates vs free cells than the Wt. In fact, only C93 was considered not statistically significant. However, total biomass (aggregates + free cells) of P0213-1 is considerably higher than the remaining mutants (results shown on Figure A7), which justifies the macroscopic difference between mutant C93 and the Wt. The remaining results are according to the microscopic and macroscopic analysis, further confirming the decrease in planktonic aggregates formation.

Aggregation Kinetics

Despite the fact that the doubling time calculated for each mutant and the Wt, was similar, after 48h the amount of biomass formed by the Wt and the mutants seems to be considerably different. Taking into account the microscopic and macroscopic differences observed, this seems to be due to aggregate formation. In order to study the evolution of aggregation formation throughout the growth, a kinetic analysis was performed for 48h, at 37ºC with an orbital agitation of 180 rpm. The results obtained are presented on Figure 3.

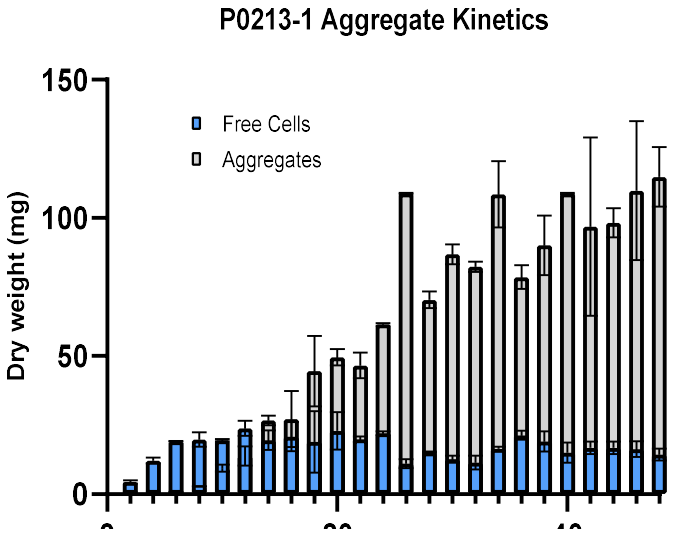

Figure 3. Dry weight of planktonic aggregates and free cells of wild-type (P0213-1) grown in LB medium, at 37ºC and 180 rpm of orbital agitation for 48h. Error bars correspond to the standard deviations of the mean values of three independent experiments

Considering aggregate formation, it can be noted that on the first eight hours of growth, when most of the time points for the growth curves were measured, there is virtually no aggregate formation. The method used to access growth, measuring the OD_{640}, does not account for the aggregates, so even after 23 and 24h, when there is already a significant amount of aggregates formed, the mass of free cells remains approximately constant, justifying the apparent stationary phase and similarity between biomass formed observed on section 3.4.

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

Exopolysaccharide Production

Exopolysaccharides are one of the main constituents of mature biofilms, therefore, the mutant's exopolysaccharide production was also accessed.

To determine the production of exopolysaccharide, each mutant was inoculated onto YEM agar plates at 37ºC for 48 hours. Through visual inspection, it was possible to access that all mutants replicate the mucoid phenotype of the Wt. Studies have shown that Bcc nonmucoid isolates form more biofilm than the mucoid isolates and have virulence attenuation in animal models of infection (Silva et al., 2013). On the other hand, results by Silva et al. and Ferreira (2018) have shown that under some conditions EPS does not seem to be relevant for cellular aggregate formation (Silva et al., 2017).

Swimming and swarming motilities

According to an increasing number of reports, motility is one of the factors contributing to the ability of bacteria to form biofilms or to flocculate (Conrad, 2012). Studies involving Bcc bacteria's CF lung infections revealed that isolates from most chronic infections usually display the motile phenotype throughout the infection and do not normally switch to nonmotile.

To evaluate the motility of mutants, swimming and swarming agar plates were inoculated, and after 24 hours and 48 hours, respectively, at 37ºC the motility zone diameter (mm) was measured

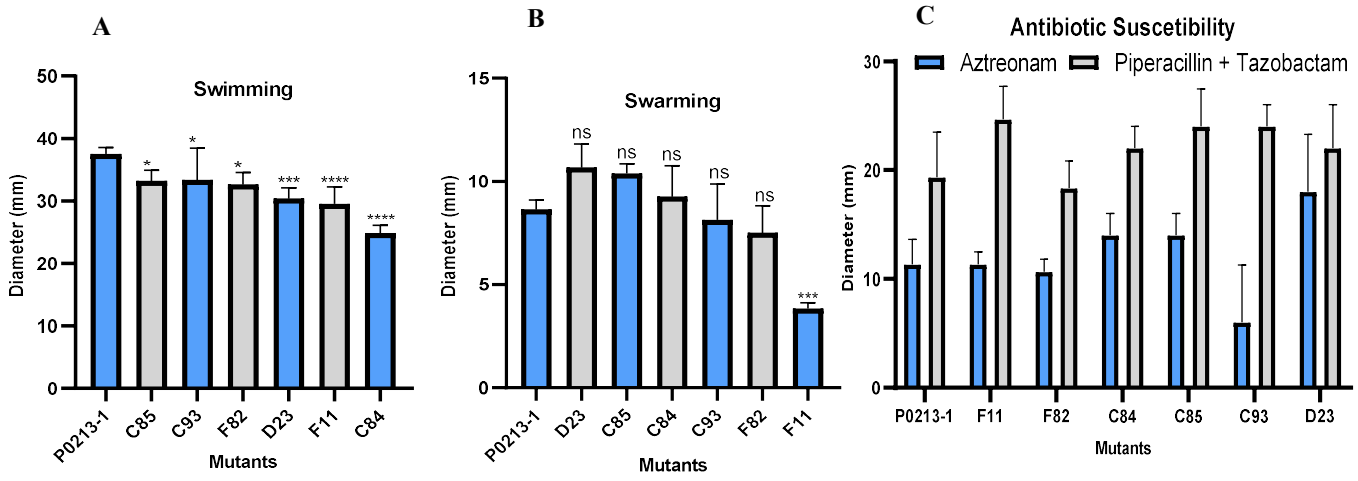

*Figure 4. A - Swimming motility of P0213-1 and its derivative mutants measured as the motility diameter after growth for 24h at 37ºC. B - Swarming motility of the P0213-1 and its derivative strains to antibiotics measured as the zone diameter after growth for 48h at 37ºC. C - Antibiotics, aztreonam and piperacillin+ tazobactam, susceptibility of P0213-1 and its derivative mutants was measured as the diameter of cell growth inhibition. After growth for 24h at 37ºC. Error bars correspond to the standard deviations of the mean values of at least three independent experiments. Significance level (one-way ANOVA followed by Dunnett's multiple comparison test) between the wild-type (P0213-1) and the mutants was determined: **, P<0.01; ***, P<0,001*

In order to successful aggregate cells must approach each other, either through swimming motility or Brownian motion. The process of swarming is distinct from swimming in that swarming is a multicellular process that occurs on solid surfaces or in viscous liquids. Therefore, swarming motility could be more relevant for the formation of surface-attached biofilm. Considering that this study focuses on planktonic aggregates instead of surface attached biofilms, it is not surprising that most mutants do not present statistically relevant alterations in swarming abilities.

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

Antibiotic Resistance

One of the most significant aspects of the biofilm growth mode is that microorganisms escape the host immune response and are much less susceptible to antibiotics than their planktonically grown counterparts. Antimicrobial susceptibility of all mutants and Wt was tested against aztreonam and piperacillin plus tazobactam.

The antimicrobial susceptibility was determined by measuring the diameter of the growth inhibition zone after 24 hours of incubation at 37°C. Through the significance levels it was found that the differences in susceptibility were not statistically significant for either one of the antibiotics tested. This results were expected since there seem to be several causes for antibiotic tolerance of bacterial biofilms which include physical barriers to antibiotic penetrance from extracellular matrix (Pugliese & Favero, 2002; Stewart, 1996), the production of periplasmic glucans (Mah et al., 2003), slow growth (Anwar et al., 1992) and/or the presence of metabolically inactive persister cells within a biofilm that are inherently tolerant to antibiotics (Lewis, 2005). None of these factors are relevant on the mode of growth tested, therefore a different kind of analysis should be conducted to access whether mutant strains are more susceptible than Wt aggregates.

CONCLUSION

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

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

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

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

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

The deeper understanding of the complex mechanisms regulating cellular aggregation could be a key factor to the development of potential therapeutics to improve treatments against Bcc bacteria. Little has been done so far regarding this problematic, so there is still a lot of work that needs to be done. Besides the three selected mutants whose interrupted genes still need to be identified, there are still more than three hundred mutants of the recently constructed library left to screen. Other approaches to the study of planktonic aggregates are also being employed, namely an evolution assay, the exposure to specific stresses and the production of mixed aggregates through co-culture of different strains.

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