Genetic determinants and phenotypic changes of differential aggregation in *Burkholderia multivorans*

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Abstract: Some bacteria can accommodate to the surrounding environmental conditions by altering their growth and behavior. One such survival strategy is the formation of planktonic cellular aggregates, verified in cystic fibrosis (CF) patients. *Burkholderia multivorans*, one of the bacteria that afflicts these patients, was the focus of this work, which attempted to link this mode of growth with specific genes and mechanisms. As such, several mutants created by random mutagenesis of an aggregative clinical isolate, *Burkholderia multivorans* P0213-1, were screened for their distinct aggregation ability. Those selected were assessed for other aggregation-associated phenotypes like virulence, motility, antimicrobial resistance, surface-attached biofilm formation, adhesion, growth and exopolysaccharide production, and the disrupted genes identified in order to relate aggregation with specific mechanisms. Motility was confirmed to be key in planktonic cellular aggregation in this strain, apparently being required for approximation of free cells to initiate aggregate formation. Intracellular messengers might play a determinant role in this phenotype, although the specific mechanisms remain unknown. Other direct links were difficult to establish. However, some possible key players in cellular aggregation were identified. These involved transcriptional regulators, two-component systems, intracellular messengers, lipid metabolism and overall convergence on central metabolism, which has repercussions in numerous pathways. *Burkholderia contaminans* IST408 and the clinical isolate used in this study were also found to be a suitable model system for studying mixed planktonic cellular aggregates.

Keywords: Planktonic cellular aggregates, Cystic fibrosis, *Burkholderia multivorans*, Random mutagenesis, Mixed cellular aggregates.

Introduction

Cystic fibrosis (CF) is an autosomal recessive genetic disorder, affecting mostly Caucasians. It is caused by gene mutation on chromosome 7, encoding a chloride-conducting transmembrane channel called the cystic fibrosis transmembrane conductance regulator (CFTR), expressed in several organs. Malfunction of CFTR in the lungs leads to mucus retention, where highly dense and viscous secretions cripple ciliary activity and clearance from the airway, potentiating infection by bacteria and consequential harmful inflammation in the airway. Although CF infections are mostly dominated by *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex (Bcc) also plays a prominent role in these infections, among which the opportunistic pathogens *Burkholderia multivorans* was found to be one of the most prevalent species affecting CF patients[1]. Infections by Bcc bacteria can develop into "cepacia syndrome", associated with necrotizing pneumonia, bacteremia and sepsis, possibly resulting in death[2]. These are usually difficult to treat due to widespread multiple drug resistance. An established chronic infection can undergo genetic evolution, exerted by combinations of stresses. Once established, many species, such as *B. multivorans* can aggregate, without requiring a surface to attach. These are often called planktonic cellular aggregates. It appears that transition into this mode of growth requires the aforementioned genetic modulation, allowing the cells to survive environmental adverse conditions. Some adaptative mechanisms and cellular functions appear to be shared with those of biofilm formation, however this may not always be the case[3]. Transition into this mode of growth can provide resistance to or be induced by specific stresses such as antimicrobials, carbon availability or oxygen gradient. Pyruvate utilization, for example, under oxygen limiting conditions, has been suggested to have a role in redox balancing, a means to establish microcolonies[4]. Some elements subject to gene modulation are generally considered to be pivotal in controlling cellular aggregation, namely quorum sensing (QS), exopolysaccharide production, motility, chemotaxis and intracellular messengers, among others. In *B. cenocepacia*, for instance, CepIR QS system is crucial for biofilm development[5]. The aim of this work is to identify genes and possible mechanisms involved in planktonic cellular aggregation of the clinical isolate *B. multivorans* P0213-1. This is done by assessing random mutants originating from it for their differential ability to aggregate, as well as other aggregation related phenotypes. Though not definitive, motility and intracellular messengers were evidenced as being possible prominent players in this process. Other cell

functions such as lipid metabolism, transcriptional regulation and overall central metabolism also appear to be at play.

Materials and methods

Bacterial strains and growth conditions

E. coli-αDH5 used was grown at 37 °C in Lennox Broth (LB) with agar supplemented with Km_{50} (kanamycin 50 µg/mL), for plating under selective pressure. Clinical isolate *B. multivorans* P0213-1 was grown in LB plates at 37 °C. *B. multivorans* mutants derived from the clinical isolate were grown at the same temperature in plates of LB medium supplemented with Km_{500} (kanamycin 500 µg/mL) to maintain selective pressure of inserted transposon or in liquid SM medium[6] and 180 or 250 rpm orbital agitation, according to assay.

Transposon mutant library construction

A transposon mutant library of the clinical isolate P0213-1 was prepared by triparental mating with *E. coli* cells carrying either the helper plasmid, pRK600, or the donor plasmid, pTn*Mod*ΩKm. The procedure was performed as previously described, without enrichment[7].

Screening of planktonic cellular aggregation ability in transposon library mutants

A pre-inoculum was performed for each bacterial mutant, left growing overnight at 37 °C and 250 rpm orbital agitation in 3 mL of SM medium. Suspensions with OD_{640nm} = 0.1 were prepared in fresh medium and left to grow at 37 °C with 180 rpm orbital agitation for 48 hours. After this, each mutant was observed macroscopically and microscopically.

Observation of aggregation in mixed cultures

The same procedure as the screening assay was performed, but with two different fluorescent strains simultaneously. Before observation, aggregates were washed three times with NaCl 0.9 %. Fluorescent strains carried plasmid pIN25 or pIN29 expressing GFP or dsRed, respectively. Selection of these two strains resulted from several combinations of fluorescent aggregative strains until the best yielding pair was declared.

DNA manipulation and cell transformation techniques

Genomic DNA of *B. multivorans* strains was extracted using a previously described method[8]. Plasmid DNA extraction and isolation, DNA restriction, agarose gel electrophoresis, DNA amplification by PCR were performed using standard procedures[9]. Plasmid DNA extraction was performed both with and without ZR Plasmid Miniprep™. *E. coli* and *Burkholderia* electrocompetent cells were also obtained by standard procedures[9]. These were transformed by electroporation using a Bio-Rad Gene Pulser system (*E. coli* - 400Ω, 25 µF, 2.5 kV, *Burkholderia* - 200Ω, 25 µF, 2.5 kV) and grown for 1 hour before plating onto LB+Km_{50} or 4 hours before plating onto LB+Cm_{500} (chloramphenicol 500 µg/mL), respectively. Similarly, for co-culture assays, plasmid DNA with each fluorescent marker was extracted and electroporated into electrocompetent *Burkholderia* cells. Specifically, plasmid pIN25 and plasmid pIN 29, were independently introduced into three *Burkholderia* strains previously known to form considerable aggregates.

Microscopy analysis

Microscopical observation was performed using a Zeiss Axioplan microscope, equipped with an Axiocam 503 color Zeiss camera controlled with Zen software, and using a 10x0.3 NA and 100x0.3 NA objectives. Appropriate filters were used for fluorescence visualization.

Aggregate quantification and total biomass

Quantification of aggregates was performed as previously described[7].

Detection of plasposon in the genome of mutants

DNA amplification was performed by PCR, followed by agarose gel electrophoresis. Genomic DNA was used as template for 50 µL PCR reactions with 2 µL primers pTn*Mod*ΩKm-fw (GCAGAGCGAGGTATGTAGGC) and pTn*Mod*ΩKm-rev (TTATGCCTCTTCCGACCATC) each. PCR setup was as follows: denaturation at 94 °C for 1:30 minutes; 30 cycles of 30 seconds at 94 °C, 1 minute at 59 °C, and 1:30 minutes at 72 °C; final extension at 72 °C for 7 minutes; end of reaction cooling until 4 °C.

Identification of disrupted genes

Genes disrupted by plasposon insertion were identified by digestion of selected mutants' genomic DNA with EcoRI, followed by self-ligation of resulting fragment. Ligated product was electroporated into electrocompetent *E. coli* cells and plated onto LB+Km_{50}. From obtained colonies, plasmid DNA was extracted, run in agarose gel against its EcoRI digested counterparts for confirmation of plasmid presence. Once confirmed, plasmid DNA was sequenced by Sanger sequencing using primers kmR (CCTTTTTACGGTTCCTGGCCT) and oriR (GTGCAATGTAACATCAGAG). Some mutants were instead subject to whole-genome sequencing by Illumina short reads technology at Instituto Gulbenkian de Ciência (IGC).

In silico *analysis of nucleotide sequences*

For Illumina sequencing results, all reads, received in *fastq* files, were concatenated, joining the data. Statistics were run with FastQC software[10], before and after trimming. Reads were trimmed with Sickle[11], aligned using Burrows-Wheeler Aligner[12] and used to perform a reference assembly, with P0213-1 genome as the reference. Visual representation of both genomes is obtained with Geneious software[13], which also allows to see the reads, their location and BLAST sequences such as the plasposon region for confirmation of insertion or the corresponding reference gene disrupted in the mutant in search of homologs available at the National Center for Biotechnology Information (NCBI)[14]. BLAST algorithm was also used to compare flanking regions obtained by Sanger sequencing to database sequences. A BLASTN search was also conducted against the genome of *B. multivorans* ATCC 17616 using the *Burkholderia* Genome Database[15].

Phenotypic assessment

To assess growth of mutant strains, a pre-inoculum for each selected mutant and P0213-1 as control was performed and left growing at 37 °C and 250 rpm orbital agitation in 3 mL of SM medium. Suspensions were made for OD_{640nm} = 0.1 in fresh medium and left to grow at 37 °C with 180 rpm orbital agitation. OD_{640nm} readings were taken over time for a period of 24 hours. Growth rates and doubling times were calculated from the exponential phase of growth. Exopolysaccharide production in solid medium was tested by growing the bacteria for 48 hours at 37 °C in YEM (yeast extract mannitol medium) agar plates[16]. Results were assessed by visual observation of colonies' mucoid or nonmucoid morphotype. Resistance to piperacillin/tazobactam and aztreonam, as well as swimming and swarming abilities and biofilm formation were assessed following previously described procedures[7,17]. Virulence ability of mutants and wild-type strain was tested through killing assays previously described[18]. Larvae were injected with cell suspensions containing a total CFU of approximately $1x10^6$ in 10 mM $MgSO_4$ with 1.2 mg/mL ampicillin and incubated at 37 °C. Survival rates were assessed by counting dead larvae at 24, 48 and 72 hours. 10 mM $MgSO_4$ with 1.2 mg/mL ampicillin was used as a negative control. For each mutant isolate, ten larvae were used. *B. multivorans* isolates were also analyzed for adhesion to the bronchial epithelial cell line CFBE41o$^-$ derived from a patient homozygous for the CFTR F508del mutation. Host cell attachment was performed as previously described[19].

Statistical analyses

Error bars, and statistical significance of the data obtained was determined using one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. These analyses were performed using GraphPad Prism 8 software for Windows (GraphPad Software, Sand Diego California USA, www.graphpad.com). Kaplan-Meier survival curves also used the same software.

Results and discussion

In vitro *screening of* B. multivorans *mutants*

In order to identify genetic players involved in aggregate formation, several mutants from a previously and newly constructed plasposon mutant library were screened in SM medium to determine aggregation ability of each and differences when compared to the wild-type, P0213-1. Eleven mutants were selected due to their distinct phenotype (Fig. 1).

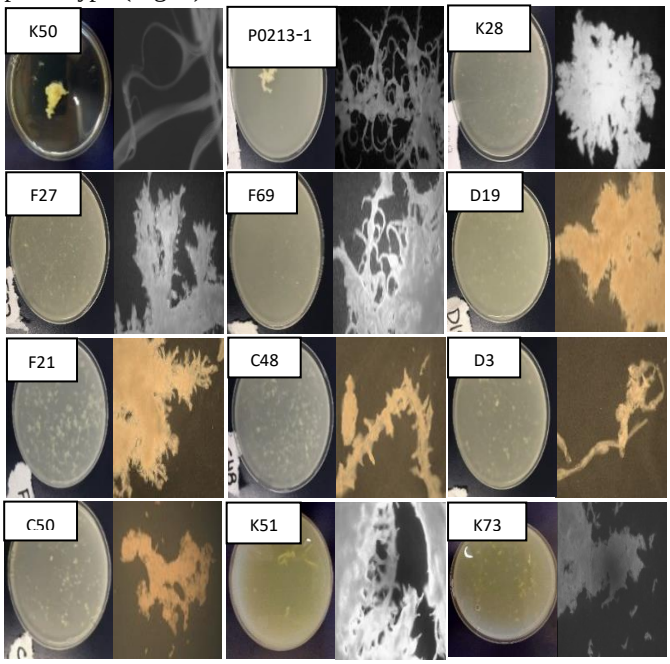

Figure 1 - Screening of the plasposon mutant library of B. multivorans P0213-1 for different abilities to form planktonic cellular aggregates*. For each strain a macroscopic image is shown (left) as well as a microscopic images (right).*

The group of mutants comprised by F27, D19, F69 and K28 appear to have notably less aggregates macroscopically visible than the wild-type strain and the remaining mutants. The following group composed by F21, C48, D3, C50, K51 and K73, appears to produce more noticeable aggregates, yet not as much as the wild-type, either due to bigger size or to a greater quantity of small aggregates. The last mutant, K50, displays an apparent increased aggregation compared to control.

Microscopically, the P0213-1 control produces very ramified and branched aggregates. Similar structures are

more evidently verified in F69 and K51. Possibly in D3, C48, F21, D19 and F27 as well, though not as prominent and without the curly like structure. C50, K28 and K73 appear to have a compact cloud like appearance with a few protrusions at the edges. K50 appears to present very long and ramified structures, however in a different manner than the wild-type, as they don't have the curly protrusions.

Identification of disrupted genes

Disrupted genes were identified by aligning the flanking regions sequence against GenBank database, as well as against *B. multivorans* ATCC 17616, in search of homologs, and the wild type genome.

Mutation in K28 pertains to a sequence apparently responsible for producing 23S rRNA, which takes part in peptidyl transferase activity of ribosomes, as well as protein synthesis and folding[20]. Mutant F27 has a disrupted dihydroorotase (*pyrC)* belonging to the *de novo* pyrimidine nucleotide biosynthesis, required to produce precursors of DNA and RNA synthesis. Mutant D19 has the plasposon inserted in the *ppsA* gene, encoding phosphoenolpyruvate (PEP) synthase, an enzyme of the gluconeogenesis pathway that converts pyruvate to PEP and has direct links to TCA cycle, lipid synthesis and the phosphotransferase system (PTS)[21,22]. The *tctD_2* gene was disrupted in mutant F69, despite being annotated as transcriptional regulatory protein TctD, it is more homologous to the response regulator PidR, involved in pigmentation regulation and possibly tolerance to ROS and UV light in *Burkholderia gluamae*[23]. Mutant F21 displayed the *paaC* gene disrupted, which belongs to a pathway where several carbon degrading pathways converge, specifically to the PaaABCDE monooxygenase which converts phenylacetyl-CoA into an epoxide[24]. In mutant C48, the transposon was found within an intergenic region upstream of the flagellar master transcriptional regulator encoding gene, *flhD*. It is responsible for controlling most genes related to motility and chemotaxis[25]. In mutant D3, the *hexR_2* gene was interrupted, encoding an HTH-type transcriptional regulator of the RpiR family with some homology with HexR. Most of these regulators are involved in sugar metabolism either as transcription repressors or activators[26]. C50 has the plasposon inserted into the *rsmB_2* gene, encoding a ribosomal RNA small subunit methyltransferase subunit B. These are involved in bacterial RNA modifications, required for fine-tuning of ribosome function and varying their expression according to environmental stresses[27]. Mutant K73, despite being annotated as a hypothetical protein, is homologous to an *iscX* gene in *B. multivorans* ATCC 17616. The protein it encodes does not have a defined role, but it is involved in FeS cluster assembly present in most enzymes[28]. Mutant K50 had a *xdhA_3* gene disrupted, which encodes a xanthine dehydrogenase molybdenum-binding subunit, required for purine salvage pathways, which provides bases required for DNA, RNA, cofactors, signaling molecules and carriers of energy. K51 mutation mapped into a gene encoding a hypothetical protein with unknown motifs or function.

In vitro *growth analysis and aggregate formation of selected mutants*

Growth behavior appeared to be relatively similar in all mutants (data not shown). Growth rates and doubling times (Fig. 2) were calculated for all strains during exponential phase of growth.

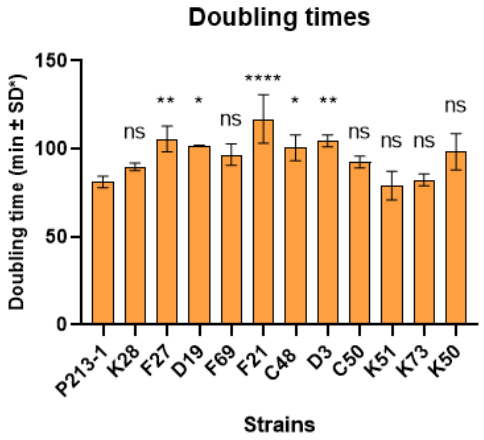

Figure 2 - Doubling time of the strains under study. *Significance: ns, not significant; *, P< 0.0332; **, P<0.0021; ****, P< 0.0001.*

Although growth behavior was relatively similar, doubling times reveal significantly decreased growth rates for mutants F21, F27, D19, D3 and C48, therefore it is possible that phenotypes observed were due to defective growth. For the remaining ones it appears that under these conditions, disrupted genes affect aggregation without affecting significantly growth kinetics.

Regarding mutant F27, reports state that in *P. aeruginosa* the independent deletion of either *pyrC* or *pyrC2*, did not affect growth as it is likely that expression of one of the genes compensates for the other[29]. In some other microorganisms, mutant strains unable to produce dihydroorotase of this pathway were shown to be unable to grow at different temperatures[30,31], possibly due to accumulation of toxic intermediaries. Opposite behavior was found in *Erwinia amylovora*, with similar growth to wild-type, suggesting that its plant hosts provide sufficient pyrimidines for growth[32].

Concerning mutant D19, it was found in *P. aeruginosa* that pyruvate fermentation alone does not sustain significant anerobic growth, expected in biofilms, but it provides bacteria with the metabolic capacity for long-term survival[33].

Aggregation quantification

Visually assessed aggregates were quantified to ensure initial phenotype screening. Percentage of free cells and aggregates of each bacterial mutant was calculated (Table 1). With the data from this assay, total biomass was also calculated.

***Table 1** - Selected mutants were grouped based on percentage of planktonic cellular aggregates formed.*

Percentage of planktonic cellular aggregates			
<25%	**> 25% and < 50 %**	**>50% and < 75%**	**>75%**
C50	D19	P0213-1	K50
C48	F27	F69	-
K51	D3	F21	-
-	K73	K28	-

According to the results, aggregation ability presents discrepancies with visual assessment, namely mutants F69 and K28, producing similar amounts of aggregates to the wild-type and mutants C50, C48 and K51 presenting much less. This may be due to unpredictable aggregate densities, rather than size or apparent quantity. In K28 it seems that the mutation in one 23S rRNA encoding gene alone is not enough to alter cell functioning and biofilm formation. However, it does appear to change either the size or structure. In mutant C50, perhaps translation of a specific subset of mRNA required for biofilm formation was affected by the impaired ability of translation of unmodified rRNA. Another possibility is that lack of methylation of 23S rRNA may impair the ability of the cell to sense specific stressors, thus not adapting as effectively to the environment. It was found that the lack of a gene responsible for the biosynthesis of an already modified nucleotide led to alteration of numerous bacterial properties, among them stress response, morphology, growth, antibiotic susceptibility and virulence[34], all of which associated with transition to a sessile mode of growth.

Regarding F21, F27, D19, D3 and C48, they also correspond to mutants that not only produce small or less aggregates and overall biomass but were also the ones found to have a significant decrease in growth, supporting the hypothesis that the aggregation phenotype is due to an influence of these mutations on growth. Regarding F27's mutation, in *P. aeruginosa*, inhibition of the uracil biosynthetic pathway, dependent on pyrimidine production, has been demonstrated to repress biofilms, as well as all three QS pathways also involved in biofilm development[35]. Such may be the case here, however a relationship between biofilm development and QS regulation is not yet established in this *B. multivorans* strain. Mutation of D19 is involved in central metabolism and its effect on aggregate formation is still not known due to the various associated pathways[7]. It is possible that despite likely accumulation of pyruvate and its role in promoting microcolony formation, the lack of conversion of pyruvate to PEP leads to an adapted use of pyruvate for pathways other than those that would promote biofilm formation. Also, considering the possible role in the PTS system, its functioning was shown to have a role on biofilm formation through regulation of extracellular DNA (eDNA) and capsular polysaccharide production in *Klesbiella pneuomoniae*[22]. This suggests a possible role of eDNA in aggregate formation of this mutant. Mutant C48 might have defected *flhD* gene expression, the motility master regulator. Transition to a sessile mode of growth and biofilm initiation is usually associated with loss of motility. In this case it is possible that the lack of motility posed an obstacle for biofilm formation, preventing swimming of the cells towards each other, making them unable to overcome repulsive forces on their own, resulting in reduced aggregation. Therefore, this master regulator appears to be necessary at least for initiation of biofilm formation, possibly losing motility during the maturation stage of biofilms. Mutant D3 produced significantly less cellular aggregates than the wild-type strain. SM medium emulates a stressful environment, triggering adaptative behavior. As such, some oxidative stress may be implied. HexR of *P. putida*, is a regulator of glucose catabolism through sensing of oxidative stress[36]. If this is the case here, this would be a mutation that would be important for NADPH production, and thus important to regulate redox status in addition to intracellular energy and tolerance to oxidative stress, which by not happening decreases the ability to form oxidative resistant aggregates[37].

Mutant K50 produces the highest amount of aggregates surpassing even P0213-1. Under different conditions this enzyme was found to trigger biofilm proliferation through oxidant production[38]. Destabilization of c-di-GMP concentration may happen due to this mutation. This key factor is known to control transition between motile and sessile lifestyles, with high levels of it indicating biofilm mode of growth. However, a decrease of c-di-GMP would be expected here due to blockage of a prominent c-di-GMP producing pathway. To support this, high concentrations of c-di-GMP resulting in

reduced biofilm formation[39] have been shown, indicative that low concentrations might induce it. Moreover, suppression of this pathway would also be expected to result in a decrease of (p)ppGpp formation, reducing positive regulation it has on RpoS, whose *rpoS* mutant in *P. aeruginosa* was shown to produce thicker biofilm structures[40]. Therefore, it may be that despite association of biofilm formation with high intracellular c-di-GMP, the opposite cannot be disregarded. It can only be concluded that c-di-GMP has a role on biofilm formation of this strain. Viable scenarios are that it may have a strain specific effect on this phenotype or that it is under such strict regulation that slight environmental changes might direct it to other genes than those initially expected in an attempt to increase survivability.

Surface-attached biofilms

Due to similarities with conventional biofilms, this ability in the selected mutants was also tested. Results are shown in Fig. 3.

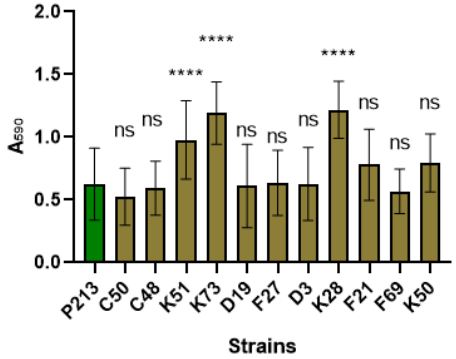

Figure 3 - Surface-attached biofilm formation of the P0213-1 and its derivative mutants. *Significance: ns, not significant; ****, P< 0.0001*

Of all tested mutants only K28, K51 and K73 displayed a significant increase when compared to P0213-1. When it comes to K51 and K73, despite these results, they correspond to two of the mutants that produce the least amount of aggregates. In the literature, similar opposite cases of phenotypical display were reported, supporting the fact that planktonic cellular aggregation and biofilm formation aren't necessarily correlated within the same strain. Indeed, cell aggregation has already been reported in bacteria with biofilm inhibiting mutations[3].

Swimming and swarming motilities

Motilities are a trait usually required for cellular aggregation, either for initiation or development of the process. As such, this phenotype was tested in selected mutants (Fig. 4).

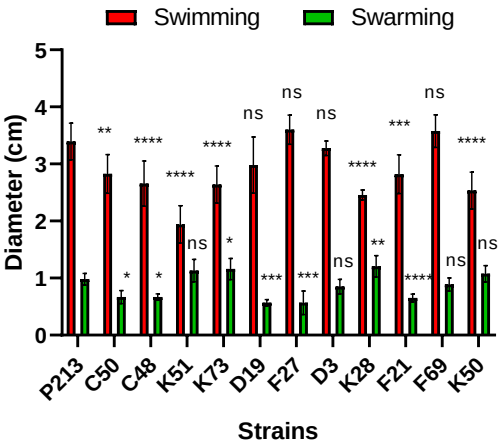

Figure 4 – Swimming and swarming ability of P0213-1 and its derivative plasposon mutants. *Significance: ns, not significant; *, P<0.0332; **, P<0.0021; ***, P< 0.0002; ****, P< 0.0001*

Mutant K28's significant decrease in swimming ability agrees with higher ability to produce surface-attached biofilms, which usually implicates the loss of motility. Swarming, which requires a surface also needed for biofilms, however, displayed a significant increase. It may be that the mutation may directly influence regulation of this type of motility or interfere with the production of a surfactant, usually required for swarming. In mutant D19, the PTS system is also responsible for chemotaxis towards their substrates by modulating CheA autophosphorylation[41]. It may be that altered concentrations of PEP prevents decrease of CheA autophosphorylation, associated with a decrease in PEP concentration, hampering the movement of bacteria up the carbohydrate gradient. And this may translate into reduced swarming motility found here. Regarding the *paaC* gene in mutant F21, a *B. cenocepacia* lacking the monooxygenase was found to have reduced swarming motility, likely due to inhibition of the *cepIR*, known to have an effect on motility capability[42]. Swimming motility in F21 also reduced significantly. A *P. aerugionosa* PAO1 was subject to extracellular PAA, which in F21 might accumulate intracellularly, leading to reduced swimming motility through inhibition of QS, suggesting that phenylacetate metabolism might impact motility through its effect on AHL-dependent factors[43]. C48 mutant presented reduced motility of both kinds. It seems that initial assumption that the plasposon meddled with expression of *flhD* gene is the most likely. Being the subunit of flagellar master transcriptional regulator that directly alters downstream genetic expression, it has control over most genes involved in flagellar synthesis, its movement and to some extent, chemotaxis, influencing both swimming and swarming motility.

There is presumably a direct consequence of this mutation. K50 shows a significant decrease in swimming motility, while differences in swarming motility were not significant. Aggregates formed by K50 were so great that this mode of growth could potentially abolish motility in order to favor sessile lifestyle under stressful conditions. It was found in *B. cenocepacia* H111 that high intracellular c-di-GMP, associated with this gene, caused deficiency in swimming motility[39].

EPS production

All mutants displayed ability to produce EPS, displaying a mucoid phenotype. Indeed, clinical isolates of *B. multivorans* frequently retain capacity for EPS production. These results (figures not shown) cannot delineate a clear relation between aggregate production and EPS production since the result is the same for the mutants producing large or small/few aggregates.

Antimicrobial susceptibility

One of the reasons CF afflicting bacteria are difficult to eradicate is due to diverse antibiotic resistance mechanisms, enhanced in this mode of growth. Thus, this phenotype was tested (data not shown). Regarding resistance to aztreonam, disrupted genes appear to not have altered significantly the resistance to this antibiotic under these conditions. As for piperacillin/tazobactam, significant differences were only verified for K51, with an increase in susceptibility, and for K73 and K28, with a decrease in susceptibility.

In mutant K28, an increase of resistance to antibiotics could be expected, since it is through binding of the V domain of 23S rRNA that antibiotics usually act[44]. Thus, a lack of this domain or alteration to its binding motif could decrease antimicrobial activity, as reported in several cases. This influence on antimicrobial resistance is usually associated to a mutation in more than one copy of the gene[45], which does not happen here. It is possible that in this strain, disruption of one copy is enough to destabilize effective binding of antimicrobials and their activity under the tested conditions. Another possibility is that destabilization of 23S rRNA results in a defect of rRNA subunit assembly, which might affect specific mRNA transcripts essential for antimicrobial action. V domain interaction with antibiotics usually regards macrolides, which were not used here.

Virulence in Galleria mellonella

According to the Kepler curves (data not shown), there was a very significant attenuation of virulence in mutant D19. C48 and C50 presented a nearly identical phenotype to the wild-type. Although D3 is also very similar, considerably more larvae were still alive at 48 hours. K50 was the most virulent strain tested, killing all larvae within 24 hours. F21, K28 and K73 all presented a slight increase of virulence than the wild-type. Although K28 and K73 end up with the same alive larvae at the end of the assay as P0213-1, much more larvae are already dead at 48 hours. Mutants F27, F69 and K51 were not tested.

Regarding mutant D19, absence of *ppsA* was found to have either no influence on virulence a reducing effect, in different strains and infection models of the bacteria[46,47]. An incomplete PTS in *Salmonella* also caused downregulation of multiple virulence determinants like flagella genes, the *lsr* operon, pathogenicity islands and the PhoQ regulon[48]. According to these reports, the role of this gene on virulence is species specific or possibly it depends on the infection model used. About mutant F21's *paaC,* in *B. cenocepacia, paaABCDE* mutants were found to result in accumulation of PAA-CoA, leading to an avirulent phenotype in *C. elegans*[49] by interacting with a putative transcriptional regulator that induces virulence in the absence of CepIR. This could be a relevant alternative pathway to accommodate to environmental conditions where QS signals do not accumulate but a virulent response is necessary. It may be that in F21 this alternative pathway is not at play or it depends on the infection model. Concerning mutant K73 reports show that mutants of *Edwardsiella ictalurid* lacking the *iscX* gene showed attenuation of virulence, even allowing the successful development of a vaccine of these bacteria, which granted protection to fish immune system against wild-type[50].

Although the specific role of IscX is undetermined in the ISC system[51], iron-metabolizing systems are known to be required for virulence in *Erwinia chrysanthemi* at least. Under iron-limited conditions it seems that the SUF system may take charge and thus ISC may become redundant on these occasions[52]. It may be that the ISC system, and possibly IscX, are required for invasion and initial establishment of the infection, upon which there is a transition to the SUF system. K50's mutation meddles with the purine catabolic pathway, and therefore with GMP, GTP and c-di-GMP concentration. Reports have shown that intracellular c-di-GMP determines the virulence of *B. cenocepacia* to *C. elegans* and *Galleria mellonella*[39]. Disruption of this pathway may reduce intracellular c-di-GMP, a statement supported by Schmid *et al.* 2017 who found that a high quantity of c-di-GMP attenuated virulence of *B. cenocepacia* in *G. mellonella* model. Therefore, it is possible that the opposite is true, which would support the idea that this pathway has a toll on pathogenicity. Inactivation of enzymes involved in

(p)ppGpp metabolism have also been shown to reduce the virulence of pathogenic species.

Adhesion to mammalian cells

Mutants C50, C48, F27, F21 and F69 and the wild-type strain were tested for their ability to adhere to mammalian cells. Since no significant alterations were found (data not shown), it appears that none of the mutations interferes with this ability, neither does it seem to be correlated with the planktonic cellular aggregation in this strain. Concurrently all mutants also displayed no significant alterations when it comes to the production of biofilms, which could potentially depend on the ability to adhere. Furthermore, all of them maintained ability to produce exopolysaccharides, known in some species to be important for adhesion to surfaces, possibly also implicated in adhesion to host cells. Contrary to these observations, all of them displayed either no significant reduction in motility, a phenotype shown in species to promote synthesis and functioning of motility appendages that enhance adherence of bacteria to surfaces and to each other, and possibly to host cells.

Co-culturing of Burkholderia strains

Co-localized bacteria may contribute to progressing changes of a bacterial community when it comes to its member composition, the evolution and severity of the infection, as well as mutational adaptations beyond those induced by the medium alone. *Burkholderia* has been shown to form co-cultures with different microorganisms[53,54]. Most of which inflict alterations to certain phenotypes, such as differential growth, enhanced biofilm formation, antimicrobial susceptibility and cross communication through AHL production. However, not all mixed cultures are beneficial for the intervening species. Secondary metabolites of one player may kill the other[53], or one species eventually dominates the other through more efficient substrate consumption[54].

B. multivorans P0213-1 expressing dsRed and *Burkholderia contaminans* IST 408 expressing GFP were co-cultured in solid medium, as described, to determine if either would have a killing action upon the other. No such thing was apparent. Afterwards, co-culture assays in liquid medium were performed. The results (data not shown) were not quite clear. It appears that microscopically P0213-1 dominated, possibly due to production of larger aggregates. Growth rates of both species can also be different, promoting the dominance of one over the other. Visualization of fluorescence was not certain at times, with the possibility of red regions being due to high background or the color produced by the matrix under the scope of the filter used, regardless of actually fluorescing or not. Despite this, in some images it appears that both bacteria can form mixed aggregates, with the GFP-expressing strain being predominantly enveloped by the dsRedexpressing P0213-1. The structure of the aggregates, does not seem to be maintained, lacking the very branched and curly like structures of the wild-type of P0213-1, as well as the large clumps usually visible in the medium. We determined that among the tested strains this pair appears to be the most promising for further studies on co-cultured aggregates. Presuming both species integrate the aggregates, it may be that this would have an effect on the capacity of the bacteria to adapt to stressful media as the size and structure of the wild-type was not maintained. There is also a need to optimize visualization of the fluorescence in aggregates and different strains might have to be used.

Conclusion and future perspectives

B. multivorans planktonic aggregation in a CF context is a theme with scarce information, therefore, there is a need to engage in studies dedicated to this bacterium, understanding cellular behavior and seeking weaknesses that might lead to therapeutic targets. The objective of this work was to identify genes that could possibly mediate *B. multivorans* P0213-1 aggregation, and consequently, with its ability to invade and establish infection within a host mediated by several phenotypes. As such, random mutants were obtained from the aggregative clinical isolate P0213-1, whose disrupted genes and different phenotypes were studied. For many of the selected mutants, a direct link was not found due to widespread repercussions in several pathways. Ribosomal activity appears to play a key part in aggregation formation. Minding mutant C50 it is possible that abnormal ribosome composition, which may distort ability to synthesize proteins or contact exogenous substances, leads to reduced aggregation of the bacteria, possibly mediated by an interference with motility, also found to be reduced in this mutant. Coincidentally, mutant C48 possibly affected in the motility master regulator, presented nearly identical phenotypes to C50. In this case the link with aggregation appears to be clear, with the formation of these structures requiring motility either initially for free cells to come together or/and after formation so as to provide a degree of mobility to the aggregates, that allows nutrient scavenging. Carbon catabolism is also implicated in aggregate formation. If not for the quantity, then for the structure of the aggregates. D19 PEP mutation is likely to be key in a pathway or specific component that may be a common factor between virulence and aggregation. Pyrimidine

synthesis identified in F27 and FeS cluster assembly identified in K73 also appear to be required for appropriate transition to planktonic cellular aggregation. However, this most likely pertains to general harmonized cell functioning rather than specific aggregation requirements since pyrimidines are essential for DNA and RNA integrity and proper cell signaling and iron-sulfur prosthetic group is required for correct functioning of the majority of enzymes. This is further supported by the fact that K73 displayed significant changes in all phenotypes, as it most likely influences a much wider range of functions. Intracellular messengers (p)ppGpp and c-di-GMP also seem to be key, possibly due to their widespread function in sensing, activating and repressing enzyme activity, bridging intracellular and intercellular communication and response in tandem with environmental changes. It confirms several reports that implicate these molecules with an aggregation type of growth. The implication of these molecules is so dispersed that to better understand what and how it influences aggregation, following their concentration over time through transcriptomic analysis may be a hypothesis. As it is, further studies are required to try to identify more genes and mechanisms that influence aggregation in *B. multivorans*. Targeted mutagenesis on genes thought to be associated with aggregation is a possible strategy. Furthermore, due to complexity of CF lung environment, these studies could be performed in the presence of other representative strains, more closely emulating stresses and interactions that might induce this mode of growth.

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