# Long-term evolution of *Burkholderia multivorans* bacteria during chronic respiratory infections of cystic fibrosis patients

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Cystic fibrosis is an autosomal recessive genetic disorder that affects mainly the pulmonary functions of patients, creating a particular environment for airway infections with opportunistic pathogens that might have fatal consequences. Chronic infections with *Burkholderia cepacia* complex bacteria have been associated with a worse clinical outcome for patients. As a complex and challenging environment, the cystic fibrosis airways display several stress sources that lead to the emergence of bacterial variants with improved capacity to subsist in that environment. The aim of this work was to assess the genomic and phenotypic evolution of 37 *Burkholderia multivorans* isolates sequentially retrieved from two infected cystic fibrosis patients over 18 years. The approach included genome sequencing of all isolates and the identification of mutations among the isolates of each patient, and the study of phenotypes associated with adaptation to the cystic fibrosis airways. Alterations in exopolysaccharide production, susceptibility to antibiotics, biofilm formation, motility and growth rate are reported here, with the latter isolates collected from both patients displaying increased resistance to antibiotics, increased biofilm formation, decreased motility and decreased growth rates. Furthermore, the most relevant mutations distinguishing the latter isolates from the first isolates occurred in genes that may relate to antibiotic resistance, lipid metabolism and regulation of biofilm formation, motility and virulence, with the identification of the global regulator *fixL* as a possible candidate for regulating traits that impact *Burkholderia cepacia* complex bacteria adaptation to the host. Insights into the strategies underlying the adaptive evolution of bacteria during the course of cystic fibrosis chronic infections may provide novel targets for the development of therapies.

**Keywords:** Cystic fibrosis; *Burkholderia multivorans*; bacterial evolution; chronic respiratory infections; comparative genomics

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## Introduction

Cystic fibrosis (CF) is the most common inherited chronic disease among Caucasians[1]. The genetic defect underlying the CF disorder results from mutations in the gene that encodes a membrane chloride channel, known as the cystic fibrosis transmembrane conductance regulator (CFTR)[2]. This condition causes the impairment of mucociliary clearance of bacterial pathogens and consequently originates respiratory infections that can ultimately lead to death[3]. Although the respiratory tract of patients with CF is colonized with a complex polymicrobial community, infections with *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex (Bcc) bacteria are associated with a worse clinical outcome[4]. During chronic long-term infections, pathogens have to adapt to a new environment, facing the numerous stress sources presented by the CF airways, such as osmotic stress due to the viscous mucus produced, mediators of host immune response, antibiotics and the microaerophilic conditions[3]. Under these strong selection pressures, bacteria usually gain the ability to evade the host immune

response and become more resistant to antibiotics, giving rise to genetic variants with improved capacity to survive in the CF environment.

Several studies have been conducted to identify genes associated with adaptive evolution of *P. aeruginosa* in the airways of chronically infected CF patients. The most commonly described phenotypic traits of *P. aeruginosa* isolates on an evolutionary path towards adaptation to the CF airways are overproduction of the exopolysaccharide (EPS) alginate, resistance to antibiotics, decreased motility, increased biofilm formation and loss of virulence factors[5–7]. This phenotypic profile has been designated the 'chronic infection phenotype' and, given that it has been reported in isolates retrieved from different CF patients in distinct clinical settings, it seems to be a result of parallel evolution of different genotypes of *P. aeruginosa*[5].

Bcc bacteria are opportunistic pathogens and infection with these bacteria in CF patients may cause a fatal necrotizing pneumonia with respiratory failure known as '*cepacia* syndrome'[8]. Although strains of every Bcc species can cause infection in CF patients, the two most frequently isolated species are *B. cenocepacia* and *B. multivorans*[9]. One of the most frequently reported phenotypic alterations in Bcc bacteria chronically infecting CF patients is the variation in colony mucoidy associated with EPS production. Sequential *B. cenocepacia* and *B. multivorans* isolates from CF patients have been shown to undergo mucoid to non-mucoid transitions[10,11], but it still isn't clear whether EPS production associates with increased virulence or persistence in the lungs[12]. Nonetheless, it has been shown that infection with non-mucoid Bcc isolates caused accelerated lung function deterioration in comparison with infection with mucoid isolates[13]. EPS production by members of the Bcc has been associated with the formation of biofilms[14], which is another phenotype that experiences alterations during the adaptation of pathogens to the CF airways. Bacteria within biofilms are protected from environmental stresses and have been shown to be significantly more resistant to antibiotics[15]. In a phenotypic assessment of 11 *B. cenocepacia* sequential clonal isolates sampled from a chronically infected CF patient over 3.5 years, it was observed that the first *B. cenocepacia* isolate showed increased antimicrobial susceptibility against antibiotics, increased swarming motility and decreased ability to grow under iron limiting conditions, in comparison with the subsequent isolates[16]. Similar studies on *Burkholderia dolosa* have also been performed, showing that isolates retrieved later on in the course of infection display increased antibiotic resistance and decreased motility[17]. Moreover, in a retrospective study of 112 *B. dolosa* isolates recovered from 14 CF patients over a 16-year period, genomic analysis of the isolates identified 17 genes that acquired mutations in several patients, indicating a parallel adaptive evolution[18]. The mutated genes were associated with resistance to antibiotics and membrane composition, including lipopolysaccharide (LPS) biosynthesis[18]. Concerning LPS biosynthesis, several isolates recovered from chronically infected CF patients lack the O-antigen[19,20], suggesting that this might be an adaptive mechanism to improve fitness in the CF airways. In a work that combined genome sequencing and phenotypic profiling of 22 *B. multivorans* isolates recovered from a CF patient during a 20-year chronic infection, mutations that evolved independently in multiple isolates affected genes encoding regulatory proteins and enzymes involved in lipid and amino acid metabolism[20]. Furthermore, the phenotypic assessment showed increases in antibiotic resistance and biofilm

formation, and decreases in motility and growth rate during the adaptive trajectory of the isolates, directing the bacteria toward a 'chronic infection phenotype', which was associated with the period of accelerated lung function decline[20]. More recently, a study combining whole-genome sequencing and phenotypic assays on 215 *B. cenocepacia* isolates sampled from 16 CF patients over a span of 2 to 20 years of infection was conducted[21], with results showing progressive phenotypic changes in several patient bacterial series leading to decreased motility, growth rate and acute virulence. Alongside the phenotypic assessments, the genomic analysis provided insights into genetic variations that could underlie phenotypic changes, with the identification of four candidate genes that are associated with motility and biofilm formation (*dnaK*, *papC*, *gcvA* and *qseC*). Although genomic and phenotypic characterizations of longitudinally collected Bcc isolates from CF patients with chronic infections have provided insights about evolutionary strategies underlying the adaptive processes that take place during colonization of the CF airways, a molecular understanding of these processes is still lacking.

The aim of this work is to analyse the genomic and phenotypic evolution of 37 *B. multivorans* isolates that were sequentially retrieved from two infected CF patients (patients P213 and P426) over 15 to 18 years of chronic infection. The approach comprises the identification of important mutations among the isolates sampled from each patient and the assessment of phenotypes associated with bacterial adaptation to the CF airways, such as EPS production, resistance to antibiotics, biofilm formation, motility, and growth rate. Insights into the adaptive strategies that drive within-host bacterial evolution during colonization of CF airways are expected to lead to improved therapies against these infections.

## Materials and Methods

### Bacterial strains and growth conditions

Bacterial isolates were sampled from two cystic fibrosis patients (patients P213 and P426) attending a clinic in Vancouver, Canada, and were provided by Prof. David P. Speert from the University of British Columbia. 16 bacterial isolates consist of a single clone of *B. multivorans* sampled from patient P213 at certain time points in the period between 1996 and 2010 (table 1) and 21 isolates consist of a single clone of *B. multivorans* sampled from patient P426 at certain time points in the period between 1997 and 2014 (table 1). Isolates were grown in LB medium at 37ºC with agitation at 250 rpm.

### Exopolysaccharide production

Mucoidy was assessed on yeast extract mannitol medium (YEM) containing 4 g/L mannitol, 0.5 g/L yeast extract and 15 g/L agar[11]. After inoculation, YEM plates were incubated for 48 h at 37°C and mucoidy was then observed by visual inspection.

### Antimicrobial Susceptibility

To evaluate susceptibility of isolates against antibiotics, the agar disc diffusion method[22] was used. Müller-Hinton agar (Sigma-Aldrich) plates were inoculated with 100 µl of a suspension at an $OD_{640}$ of 0.1 prepared from exponential-phase cells growing on LB medium at 37ºC. Paper discs (BD BBL Sensi-Disc) containing aztreonam (30 µg) were applied onto the surfaces of the inoculated plates. The diameter of the growth inhibition zone was measured after 24 h of incubation at 37°C. Results are the means of data from at least six replicates of three independent experiments, each with three discs per isolate.

### Biofilm Formation

Bacteria were grown in LB medium at 37°C to mid-exponential phase and diluted to an $OD_{640}$ of 0.05. 200 µL samples of the cell suspensions were used to inoculate 96-well polystyrene microtiter plates. Plates were incubated at 37°C statically for 48 h, after which the wells were washed three times with

0.9% (wt/vol) NaCl. The biofilm was stained with 200 µL of a 1% (wt/vol) crystal violet solution for 20 minutes at room temperature[23], followed by washing three times with 200 µL of 0.9% NaCl. The dye was then solubilized with 200 µL of 96% ethanol and the solution's absorbance at 590 nm ($A_{590}$) was measured in a microplate reader (Spectrostar nano, BMG LabTech). Results are the means of data from at least eight replicates of three independent experiments.

**Swimming Motility**

Motility was assessed on swimming (1% [wt/vol] tryptone, 0.5% [wt/vol] NaCl, 0.3% [wt/vol] noble agar [Difco]) agar plates[20]. Plates were spot inoculated with a 5 µl drop of a culture at an $OD_{640}$ of 1.0 and were incubated for 24 h at 37°C. The diameter of the swimming zone was then measured. Results are the means of data from at least ten replicates of three independent experiments.

**Growth Rate Determination**

Isolates were grown overnight in LB medium at 37°C. A volume was then centrifuged (2 min, 8 rpm), and the pellet was washed with saline solution (0.9% NaCl) and used to inoculate a flask with 50 mL of liquid synthetic cystic fibrosis medium (SCFM)[24], generating an initial optical density at 640 nm ($OD_{640}$) of 0.1. Flasks were incubated at 37°C with agitation at 250 rpm, and growth rates were measured by monitoring the $OD_{640}$ for 24 h. Growth rates were calculated from the exponential phase of growth from two independent experiments.

**Detection of single-nucleotide polymorphism (SNP) and indel mutations**

Genomic DNA from the *B. multivorans* clinical isolates was extracted and purified using the DNeasy blood and tissue kit (Qiagen). DNA samples were processed according to Illumina's instructions for generating paired-end libraries of the 37 *B. multivorans* isolates, which were sequenced at least to 38-fold coverage using an Illumina MiSeq system at the Instituto Gulbenkian de Ciência (Portugal). The genome of the first bacterial isolate of each patient was also sequenced using a PacBio system at Icahn School of Medicine at Mount Sinai, New York (USA) and polished using the reads generated by the Illumina MiSeq sequencing system, after *de novo* assembly. To obtain these corrected assemblies, the program MeDuSa v1.6[25] was used to align the assemblies to a reference (*Burkholderia multivorans* ATCC 17616) to reorder contigs; all the Illumina reads were trimmed using Trimmomatic[26]; the trimmed reads were aligned to MeDuSa-assemblies with BWA v0.7.15[27]; the mapped reads were used to predict and correct misassemblies with Pilon v1.19[28]; and genes were predicted and annotated using Prokka v1.11[29]. This polished assembly resulted in 5 contigs for the genome of the first isolate retrieved from patient P213 and 7 contigs for the genome of the first isolate retrieved from patient P426, which were used as the reference genomes for the subsequent detection of SNP and indel mutations. To identify which contig corresponded to each *B. multivorans* chromosome, Basic Local Alignment Search (BLAST)[30] was used to align each contig to the genome of *B. multivorans* ATCC 17616. A standard nucleotide BLAST optimized for highly similar sequences (megablast) was used. To detect SNP and indel mutations, raw paired-end reads from the 37 *B. multivorans* isolates generated with Illumina MiSeq were filtered based on Phred quality scores, followed by trimming off of adapter contamination and ambiguous nucleotides using the fastq-mcf tool[31]. Only reads with Phred scores higher than 30 (99.9% accuracy) were considered for further analysis, while maintaining at least 70% of the initial coverage. The resulting filtered paired-end data sets were aligned against the reference genome sequences of the first *B. multivorans* isolates of each patient using BWA-MEM[32] and NovoAlign v.3.02.13 (Novocraft; website last accessed January 2017) to determine a first list of putative mutations. Only SNPs and indels detected in alignments using both BWA-MEM and NovoAlign and occurring in at least three forward and three reverse reads were further analysed. Additionally, a visual inspection of the

alignments using Geneious v.6.1.8[33] allowed the confirmation of each indel mutation and the identification of large deletions that were also included in the final lists of mutations.

**Table 1** - Date of isolation, ID and mucoidy of each *B. multivorans* isolate recovered from CF patients P213 and P426. Mucoidy was assessed in solid yeast extract mannitol (YEM) medium and isolates were classified as highly mucoid (++), mucoid (+) or non-mucoid (-) by visual inspection.

| Isolate | ID | Date of isolation | Mucoidy |
|---|---|---|---|
| P213-1 | VC7495 | 13-02-1996 | ++ |
| P213-2 | VC7704 | 04-06-1996 | ++ |
| P213-3 | VC7804 | 13-08-1996 | ++ |
| P213-4 | VC8896 | 06-05-1998 | ++ |
| P213-5 | VC9452 | 20-01-1999 | ++ |
| P213-6 | VC10037 | 18-01-2000 | ++ |
| P213-7 | VC10458 | 07-11-2000 | ++ |
| P213-8 | VC11002 | 23-10-2001 | + |
| P213-9 | VC11446 | 23-07-2002 | + |
| P213-10 | VC12063 | 26-09-2003 | - |
| P213-11 | VC12556 | 30-09-2004 | + |
| P213-12 | VC13750 | 11-04-2007 | - |
| P213-13 | VC13748 | 11-04-2007 | + |
| P213-14 | VC13833 | 20-06-2007 | + |
| P213-15 | VC13834 | 20-06-2007 | - |
| P213-16 | VC15071 | 11-01-2010 | + |
| P426-1 | VC8086 | 11-02-1997 | ++ |
| P426-2 | VC8136 | 17-03-1997 | ++ |
| P426-3 | VC8585 | 20-01-1998 | ++ |
| P426-4 | VC9177 | 25-09-1998 | ++ |
| P426-5 | VC9783 | 12-08-1999 | ++ |
| P426-6 | VC10411 | 12-10-2000 | ++ |
| P426-7 | VC11369 | 24-05-2002 | ++ |
| P426-8 | VC11982 | 17-07-2003 | ++ |
| P426-9 | VC12458 | 22-07-2004 | - |
| P426-10 | VC13030 | 22-09-2005 | ++ |
| P426-11 | VC13616 | 12-01-2007 | ++ |
| P426-12 | VC13617 | 12-01-2007 | ++ |
| P426-13 | VC13777 | 02-05-2007 | ++ |
| P426-14 | VC13778 | 02-05-2007 | ++ |
| P426-15 | VC14363 | 06-07-2008 | ++ |
| P426-16 | VC14863 | 11-08-2009 | ++ |
| P426-17 | VC15667 | 23-06-2011 | + |
| P426-18 | VC15867 | 17-11-2011 | + |
| P426-19 | VC16979 | 04-12-2013 | - |
| P426-20 | VC17389 | 22-09-2014 | + |
| P426-21 | VC17390 | 22-09-2014 | + |

### 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 v.7.03. Kaplan-Meier survival curves were also performed with GraphPad Prism software v.7.03. Differences were considered statistically significant for P-values lower than 0.05.

## Results and Discussion

### Detection of single-nucleotide polymorphism (SNP) and indel mutations

Detection of mutations that could underlie phenotypic variation was performed by comparing the genomes of the isolates with the first isolate for each patient, after sequencing and *de novo* assembly of the first isolates. Comparing the genomes of the 16 *B. multivorans* isolates recovered from patient P213 led to the identification of a total of 291 mutations, comprising 240 SNPs, 35 indels and 16 large deletions. Interestingly, a 48-bp deletion in the mismatch repair gene *mutL* was found in isolate P213-8, which could explain the increased number of mutations identified for this isolate (total of 98 mutations). Mutations in the *mutL* gene lead to a hypermutator phenotype, characterized by an increased mutation rate. Hypermutator phenotypes have been frequently reported for *P. aeruginosa* and Bcc isolates retrieved from CF patients[6,20,34–36]. The comparative genomics analysis of the 21 *B. multivorans* isolates recovered from patient P426 identified a total of 337 mutations, including 275 SNPs, 40 indels and 22 large deletions. When evaluating the rate of accumulated SNPs per year, two distinct patterns were observed in both patient series. While isolates 1 to 11 (except for hypermutator P213-8) seem to have acquired mutations at constant rates, the sets of latter isolates showed significantly less SNPs than expected if the linear tendency of the first set were followed. Mutation rates of 8.2 and 5.4 SNPs per year were observed for the first groups of isolates of patients P213 and

P426, respectively, presenting higher values than the rate of about 2 SNPs per year observed for other long-term infections with *B. dolosa* and *B. multivorans* in CF patients[18,20]. A search for polymorphic genes identified a Fis family transcriptional regulator with five different mutations among the isolates collected from both CF patients. This gene conserved in the *Burkholderia* genus has unknown regulatory targets and it was recently found to have six mutations in *B. multivorans* isolates recovered from another chronically infected CF patient over 20 years[20]. Relevant mutated genes distinguishing the first sets of isolates from the latter ones of each patient series may be involved in antibiotic resistance (genes encoding MFS and multidrug efflux transporters), lipid metabolism (*accA* and *accC* genes, encoding the acetyl-CoA carboxylase subunits alpha and biotin, respectively) and regulation of transcription targeting biofilm formation, motility and virulence (genes encoding a *fixL* homolog and a c-di-GMP phosphodiesterase). Four mutations were found in the *accA* gene in a similar study of *B. multivorans* isolates recovered from a chronically infected CF patient over 20 years[20]. Also, a reduction of the degree of saturation of fatty acids produced by *B. cenocepacia* isolates infecting a CF patient had previously been associated with lung function deterioration[16]. Furthermore, homologs of *fixL* were suggested as important regulators in studies with *B. dolosa* and *B. multivorans* clinical isolates recovered from CF patients[18,20], indicating that this gene could play a role in the adaptation of Bcc isolates to the CF environment.

**Exopolysaccharide production**

Mucoidy was analysed by visual inspection in YEM medium and isolates were classified as highly mucoid, mucoid and non-mucoid (table 1; figure 1). Regarding patient P213, the first seven isolates displayed a higher mucoidy than the remaining ones, and isolates 10, 12 and 15 were non-mucoid. For patient P426, the first 16 isolates, except for 9, displayed a higher mucoidy than the final 5 isolates. P426 isolates 9 and 19 were non-mucoid. EPS is considered a virulence factor of Bcc bacteria, as it has been demonstrated to inhibit neutrophil chemotaxis, to neutralize reactive oxygen species *in vitro*[37], to affect the phagocytosis of bacteria by human neutrophils and to facilitate persistent bacterial infection in mice[38]. Bcc isolates sequentially sampled from CF patients have been shown to undergo a transition from a mucoid phenotype to a non-mucoid one[10,11]. The results for mucoidy analysis in solid medium for the two patients studied in this work are in agreement with a decrease in mucoidy over time.

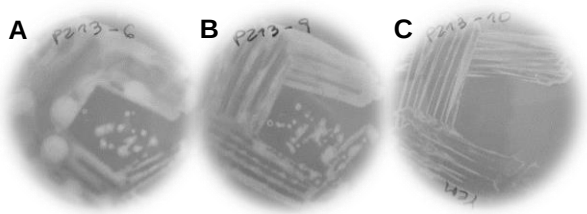


**Figure 1** - Mucoid phenotype assessment in yeast extract medium (YEM) after 48 h of incubation at 37°C for *B. multivorans* isolates 6 (**A**; highly mucoid, ++), 9 (**B**; mucoid, +) and 10 (**C**; non-mucoid, -) of patient P213.

**Antimicrobial Susceptibility**

Susceptibility to the β-lactam aztreonam was evaluated, with results showing that the first two to three isolates of each patient presented increased susceptibility to this antimicrobial than the latter isolates (figure 2). During long-term chronic CF infections, bacteria commonly become more resistant to antibiotics[16,18,20], as frequent and prolonged exposure to antimicrobials promotes the emergence of strains with improved ability to survive in the challenging CF environment. The comparative genomics analysis led to the identification of mutations distinguishing the earlier isolates of each patient from the latter ones

in genes encoding major facilitator superfamily (MFS) and multidrug efflux transporters, which could underlie the increase in resistance to antibiotics observed for the two CF patients.

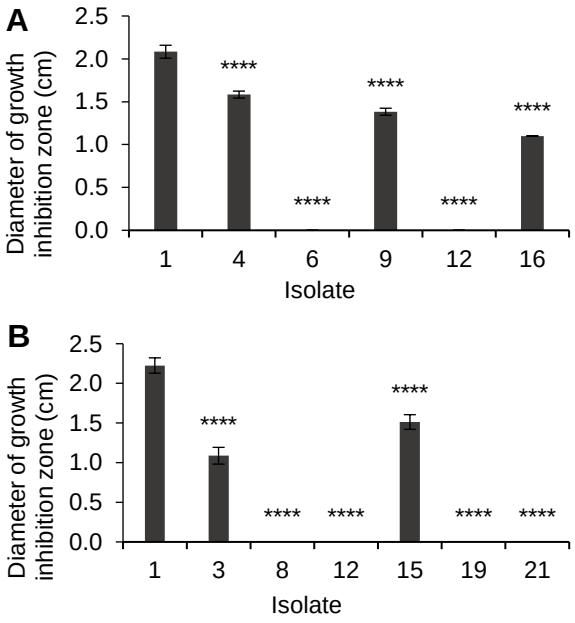


**Figure 2** - Susceptibility of *B. multivorans* isolates sequentially retrieved from CF patients P213 (**A**) and P426 (**B**) to antibiotic aztreonam, measured as the diameter of cell growth inhibition, after growth for 24h at 37ºC. Error bars represent the standard deviations of the mean values for three independent experiments (statistical significance of differences between the first isolate and the subsequent ones was determined: ****, $P < 0.0001$ by ANOVA followed by Dunnett's multiple comparisons test).

### Biofilm formation

Concerning biofilm formation, the first two to three isolates of both CF patients produced a reduced amount of biofilm in comparison with the subsequent isolates (except for isolates P213-6, P213-7 and P426-18, for which no statistically significant differences were found; figure 3). Living within biofilms, bacteria are protected from environmental stresses, such as the host immune response and the presence of antibiotics, which are common stress sources in the CF airways. It was demonstrated that bacteria within biofilms are more resistant to antibiotics[15] and the observations that isolates that produced more biofilm also showed increased resistance to the antimicrobial aztreonam is in agreement with these findings. A non-synonymous SNP in a *fixL* homolog was identified in the latter isolates of patient P213. In *B. dolosa*, *fixL* encodes a sensor histidine kinase of a two-component regulatory system (fixLJ) that is activated by low oxygen levels and regulates biofilm formation, motility, virulence and intracellular invasion[39]. Also, a non-synonymous mutation in a gene encoding a cyclic diguanosine monophosphate (c-di-GMP) phosphodiesterase was found in 18 isolates of patient P426. This signaling molecule is related to regulation of biofilm formation, motility, virulence and adhesion[40–42].

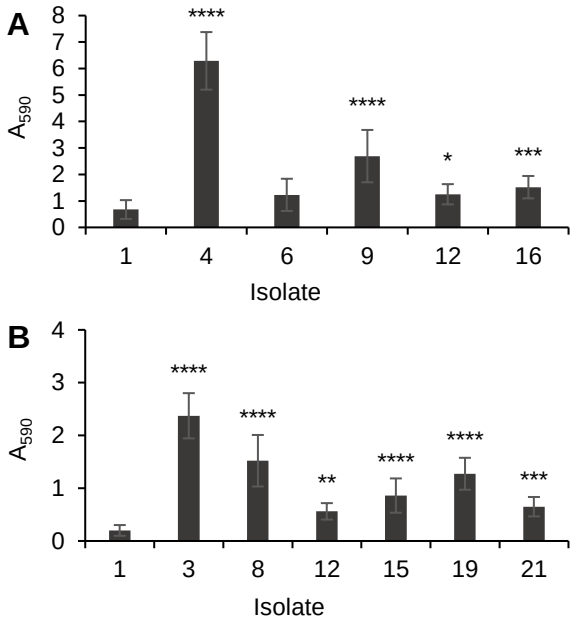


**Figure 3** - Biofilm formation of *B. multivorans* isolates sequentially retrieved from CF patients P213 (**A**) and P426 (**B**), after growth in polystyrene microplates for 48h at 37ºC. Error bars represent the standard deviations of the mean values for at least three independent experiments (statistical significance of differences between the first isolate and the subsequent ones was determined: **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ by ANOVA followed by Dunnett's multiple comparisons test).

### Motility

Differences in motility were evaluated in swimming and swarming agar plates. For both patient series, results show that the first isolates display increased motility when compared with the subsequent ones (figure 4). This decrease in motility may be a method of bacteria to adapt to

the respiratory tract environment of patients with CF and has been frequently reported in members of the Bcc[16,20]. Relevant mutations possibly underlying this phenotypic transition were previously referred for biofilm formation, as sensor histidine kinase *fixL* and c-di-GMP phosphodiesterase have been associated with regulation of motility as well as biofilm production[39,40]. Moreover, several mutations in genes associated with motility were identified in isolates of both patients, such as genes encoding flagellar proteins (*fliI*, *fliJ*, *fliL*, *flgE*, *flgI*), a flagellar transcriptional activator (*flhC*) and proteins related to chemotaxis (*cheA*, *cheB*), supporting the hypothesis that alterations in this phenotypic trait are involved in adaptation of Bcc bacteria to the airways of CF patients.

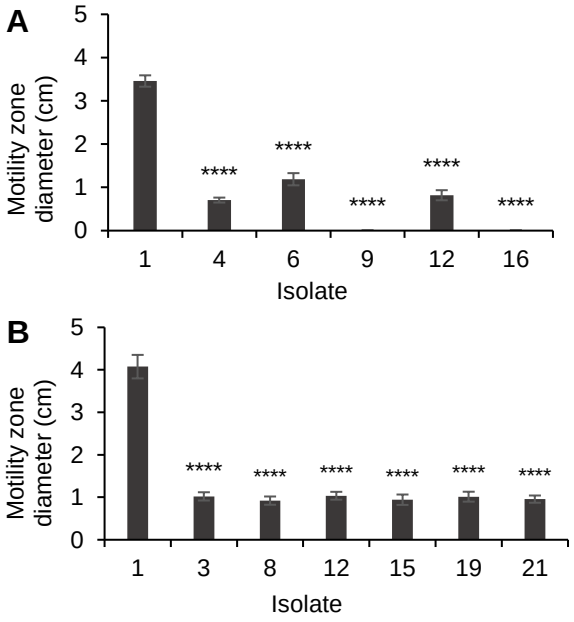


**Figure 4** - Swimming motility of *B. multivorans* isolates sequentially retrieved from CF patients P213 (**A**) and P426 (**B**), after growth in swimming agar plates for 24 h at 37ºC. Error bars represent the standard deviations of the mean values for three independent experiments (statistical significance of differences between the first isolate and the subsequent ones was determined: ****, $P < 0.0001$ by ANOVA followed by Dunnett's multiple comparisons test).

### Growth rate determination

Growth rate of each bacterial strain was measured in synthetic cystic fibrosis medium (SCFM)[24]. For both CF patients, the first isolates showed decreased doubling times when compared with the latter ones (table 2). These findings suggest a possible role for decreasing growth rate in adaptation of *B. multivorans* isolates to the airways of CF patients during infection. This tendency to reduce growth rates over time during long-term chronic infections in CF patients has been reported both for *P. aeruginosa* and Bcc bacteria[5,20,21], and it might occur in response to the different nutrient composition of the CF airways or as a consequence of a greater commitment to biofilm production[20], with proven benefits to bacteria living in that environment. Mutations in genes involved in amino acid biosynthesis were identified in isolates of both patients, but no mutations that could explain the growth rate variations in all the isolates were found.

**Table 2** - Doubling times (in hours) calculated for *B. multivorans* isolates sampled from CF patients P213 and P426, based on growth rates measured from cultures grown in synthetic cystic fibrosis medium (SCFM). Results are the means of data from two independent experiments.

| Isolate | Doubling time (h) | Isolate | Doubling time (h) |
|---|---|---|---|
| **P213-1** | 1.6 | **P426-1** | 1.3 |
| **P213-9** | 2.8 | **P426-5** | 1.8 |
| **P213-12** | 2.0 | **P426-12** | 2.1 |
| **P213-16** | 2.7 | **P426-21** | 2.2 |

## Conclusions and future prospects

In conclusion, the latter sets of isolates longitudinally collected from the CF patients displayed increased resistance to antibiotics, increased biofilm formation, decreased motility and decreased growth rates, shifting the bacteria toward a 'chronic infection phenotype' that is well established for *P. aeruginosa* infections and seems to apply to infections caused by Bcc bacteria as well. Unlike *P. aeruginosa* isolates, which undergo a non-mucoid to mucoid transition, Bcc isolates often convert from a mucoid morphotype into a non-mucoid one, with results of mucoidy in solid medium reported here supporting this decrease in mucoidy. Mutations in genes

related to resistance to antimicrobials, lipid metabolism and regulation of transcription likely drive the transition to the 'chronic infection phenotype', and regulatory genes might play important roles in adaptation of Bcc bacteria to the CF environment. For *P. aeruginosa*, genes *mucA* and *lasR* have been identified as important global regulators, with *mucA* showing particularly extensive pleiotropic effects on gene expression and leading to changes in central metabolism, motility, production of virulence factors and membrane permeability[5,6]. In the present work, the *fixL* gene seems to be a possible candidate for regulating traits that impact Bcc bacteria adaptation to the host. This gene has been identified as an important regulator in similar studies with *B. multivorans* and *B. dolosa*[18,20], supporting this hypothesis. Furthermore, a Fis family transcriptional regulator conserved in the *Burkholderia* genus was found to be polymorphic in isolates from both patients, suggesting a possible role for this regulator in adaptation of *B. multivorans* to the CF airways. The study of evolutionary paths of bacteria in specific environments is being increasingly used as an investigative strategy, as this type of experiments offers knowledge about adaptive changes that take place during evolution and allows the comparison of different evolutionary trajectories. It is expected that the combination of phenotypic assessments with information provided by comparative genomics analysis of Bcc isolates sampled from CF patients will help elucidate which mutations may underlie adaptation to the CF airways. The identification of adaptive strategies during bacterial colonization of the airways of individuals with CF may point to potential therapeutic targets for the development of improved treatments against Bcc bacteria.

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