Whole-Genome Sequencing of Three Clonal Clinical Isolates of *B. cenocepacia* from a Patient with Cystic Fibrosis

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Abstract

*Burkholderia cepacia* complex bacteria are amongst the most feared of pathogens in cystic fibrosis (CF). The BCC comprises at least 20 distinct species that can cause chronic and unpredictable lung infections in CF. Historically the species *B. cenocepacia* has been the most prevalent in CF infections and has been associated in some centers with high rates of mortality. Modeling chronic infection by *B. cenocepacia* in the laboratory is challenging and no models exist which effectively recapitulate CF disease caused by BCC bacteria. Therefore our understanding of factors that contribute towards the morbidity and mortality caused by this organism is limited. In this study we used whole-genome sequencing to examine the evolution of 3 clonal clinical isolates of *B. cenocepacia* from a patient with cystic fibrosis. The first isolate was from the beginning of infection, and the second two almost 10 years later during the final year of the patients’ life. These isolates also demonstrated phenotypic heterogeneity, with the first isolate displaying the mucoid phenotype (conferred by the overproduction of exopolysaccharide), while one of the later two was nonmucoid. In addition we also sequenced a nonmucoid derivative of the initial mucoid isolate, acquired in the laboratory by antibiotic pressure. Examination of sequence data revealed that the two late stage isolates shared 20 variant nucleotides in common compared to the early isolate. However, despite their isolation within 10 months of one another, there was also considerable variation between the late stage isolates, including 42 single nucleotide variants and three deletions. Additionally, no sequence differences were identified between the initial mucoid isolate and its laboratory acquired nonmucoid derivative, however transcript analysis indicated at least partial down regulation of genes involved in exopolysaccharide production. Our study examines the progression of *B. cenocepacia* throughout chronic infection, including establishment of sub-populations likely evolved from the original isolate, suggestive of parallel evolution. Additionally, the lack of sequence differences between two of the isolates with differing mucoid phenotypes suggests that other factors, such as gene regulation, come into play in establishing the mucoid phenotype.
Introduction

Bacteria belonging to the *Burkholderia cepacia* complex (BCC) are highly problematic pathogens in people with cystic fibrosis (CF). There are at least 20 distinct species of bacteria in the BCC, and all except for *B. ubonensis* have been isolated from the lungs of people with CF [1–3]. Historically, *B. cenocepacia* has been considered the most problematic species. Epidemic spread of *B. cenocepacia* has previously resulted in high rates of prevalence of this species in CF populations [4–6]. Furthermore, *B. cenocepacia* has been associated with high rates of mortality in some centers as well as exhibiting an elevated risk for death following lung transplantation [7–12], and high levels of intrinsic resistance to antimicrobial agents [6,11,13–16].

Further complicating our understanding of the pathobiology of BCC is their unpredictable infection outcome. Some people will experience severe and rapidly fatal infections, while others will experience a mild infection with little impact on lung function [17,18]. Most seriously, in some instances BCC bacteria can result in a rapidly invasive and typically fatal necrotizing pneumonia known as ‘cepacia syndrome’ [19]. The causes of these disparate outcomes are not understood and there are limited studies on adaptation of BCC bacteria to chronic infection in the CF lung.

We have previously described the prevalence of the mucoid phenotype in clinical sequential clonal isolates of BCC bacteria [20]. Both mucoid and nonmucoid BCC can cause infection in the CF lung, however during chronic infection phenotype switches are predominantly from the mucoid to nonmucoid phenotype. Significantly we have also shown that the degree of mucoidy displayed by clinical isolates of BCC is inversely related to both subsequent lung function decline and survival [17]. Supporting the notion that nonmucoid BCC are more virulent in the CF lung, proteomic and transcriptomic studies on a mucoid/nonmucoid clonal pair of isolates of *B. cenocepacia* from the late stage of a CF infection revealed elevated production of putative virulence determinants in the nonmucoid isolate [21]. Additionally, it was previously found that the nonmucoid variant formed biofilm and produced quorum sensing signaling molecules, while the mucoid variant demonstrated lower interaction with human leukocytes [22].

The genetic determinants of the mucoid phenotype are unknown, however, various genes have been hypothesized to be involved in exopolysaccharide (EPS) production. *bce-I* and *bce-II* are the only genes that have been shown experimentally to be involved in EPS production in *Burkholderia* [23]. Underlining the complexity of EPS production, BCC are thought to be able to produce at least seven different EPS [24], of which the isolates used in this study have been shown to produce at least three [22]. Additional EPS producing clusters that have been putatively identified include the *wcb* cluster, BCAM1330-BCAM1340 [25], as well as others (see Table 3 in: [26])

Next-generation whole-genome sequencing (WGS) now allows high-resolution insight into genomic changes that occur between sequential clonal isolates taken from patients at different time points during infection. This technology has already been employed to examine longitudinal isolates from a large outbreak of *Burkholderia dolosa* in CF patients in Boston [27]. In this study the authors examined sequential isolates from 14 patients and found evidence of 17 genes that were under strong selective pressure during chronic infection. Notably, the mutations they defined included gain of function mutations in antibiotic resistance and O-antigen switching. The only WGS data to date that has examined adaptation of *B. cenocepacia* to chronicity is a study examining experimental evolution of 6 populations across 1,050 generations in a biofilm model of evolution [28]. These data have shown, like the *B. dolosa* study, that there is selection of a limited number of pathways during this model of chronic infection and indeed this study also identified genes involved in LPS modification.
In this study we have used WGS to investigate: i) the evolution of *B. cenocepacia* during a well characterized single case of infection by comparing the initial isolate with two other isolates taken from later during infection and ii) the genetic similarities between exopolysaccharide production variants taken from chronic infection and derived *in-vitro* following antibiotic pressure of the original mucoid isolate from this infection.

**Methods**

**Isolates**

The isolates we investigated in this study have been described previously [17,22]. C3921, C8963 and C9343 are sequential isolates from the same patient. C3921-CTZ32G is an *in-vitro* derived nonmucoid isolate of the mucoid C3921 following exposure to super-MIC levels of cefazidime (chosen because of its use in treating CF lung infections, including this patient’s BCC infection) [17]. Isolates were stored at -80°C in Mueller Hinton broth supplemented with 8% DMSO and have been minimally passaged since their original isolation.

**DNA extraction**

Bacteria were revived from freezer stocks on Columbia Blood Agar plates at 37°C and single colony purified. Overnight cultures were established by inoculating several discrete colonies from the purified plate into 3 ml of Luria Bertani broth (10 g l⁻¹ tryptone, 5 g l⁻¹ yeast extract and 1 g l⁻¹ sodium chloride) in a 15 ml snap top tube and incubating overnight at 37°C on an orbital rotating platform. DNA extraction was then performed exactly as per the Gram-negative bacteria protocol from a Qiagen Gentra Puregene Kit.

**RNA extraction**

RNA was extracted from 16-hour cultures (OD₆₀₀ 1.2) grown in yeast extract mannitol broth (4 g l⁻¹ mannitol and 0.5 g l⁻¹ yeast extract) using the Qiagen RNeasy Mini Kit. RNA was eluted in water and genomic DNA was removed using Turbo DNAse (Life Technologies) and stored at -80°C until use.

**Quantitative RT-PCR**

cDNA synthesized from 10ng of extracted RNA using qScript DNA SuperMix (Quanta BioSciences) and 1:10 aliquots were used for subsequent quantitative PCRs. Primers for transcripts *bce-B, bce-E, bce-G, bce-R*, encoded by the cepacian biosynthesis genes, were used as previously designed by Ferreira *et al.*, as were primers for the reference gene *gyrB* [29]. Reactions were assembled in a final volume of 25 μl containing 12.5 μl of 2X SYBR Green, 10.5 μl nuclease-free H₂O, 1 μl cDNA and 1 μl of a 5 μM stock of primer pairs. Negative controls of nuclease-free H₂O and RNA that had not undergone cDNA synthesis were used. Reactions were performed using a ViiA 7 Real-Time PCR System (Life Technologies). An initial cycle of 50°C for 2 mins was followed by 95°C for 2 mins for denaturation. There were then 40 cycles of 95°C for 15 secs followed by 57°C for 1 min followed by a melting curve stage. The ΔΔCₜ was used to calculated relative gene expression by normalizing all transcripts to the *gyrB* reference. All experiments were performed in triplicate using three separate biological samples and standard errors of the mean calculated.

**DNA sequencing**

Indexed sequencing libraries were created using the Nextera kit (Illumina, San Diego, CA) according to the manufacturer’s instructions. The libraries were sequenced on either the...
Illumina MiSeq to generate paired-end 2x250bp reads or Illumina HiSeq2500 to generate paired-end 2x150bp reads at the McGill University and Genome Quebec Innovation Center, Montreal, Canada. All four sequences have been deposited in the European Nucleotide Archive at the European Bioinformatics Institute under the study accession number PRJEB9630 (http://www.ebi.ac.uk/ena/data/view/PRJEB9630).

**Bioinformatics**

Illumina adapters were removed from raw fastq files using cutadapt [30], after which reference based mapping to the *Burkholderia* J2315 reference [26] was performed, separately for each sample, using bowtie2 [31] with options: —phred33—local—dovetail—maxins 850. Variant positions were called using samtools [32], and to ensure high confidence, were filtered based on five parameters: (i) minimum read depth of five with at least one read in each of the forward and reverse direction; (ii) maximum depth not greater than the highest 2.5% of the distribution for the sample; (iii) minimum root-mean-square read mapping quality of 30; (iv) minimum of 75% of reads supporting the consensus call; (v) calls required to be homozygous under the diploid model assumed by samtools; using a bespoke Python script.

*De novo* assembly was performed on the unmapped reads following assembly with bowtie2, as well as the entire read set, using Velvet [33] in conjunction with the VelvetOptimiser (http://bioinformatics.net.au/software.velvetoptimiser.shtml). All contigs >500bp (mean 200 and 1412 per sample for unmapped and all reads respectively) were used in downstream analyses. To identify large regions varying between samples and not present in J2315, contigs produced from the unmapped reads were entered into Mauve [34] and NUCmer [35] to identify unique regions. Confirmation of any regions identified was then performed using BLAST searches of the region against the Velvet contigs made from the entire read set, and if the region was not present in every sample it was deemed a variant. Larger variants between samples and present in the reference, were identified using a window based approach on the reference based assembly, where any region of 1kb with >500bp different between two samples was identified. BLAST Ring Image Generator (BRIG) was used to generate visual genome comparisons with the J2315 reference [36] with low-confidence calls in the query being assigned the corresponding base from the reference sequence.

**Investigation of selected regions**

Five regions implicated in EPS biosynthesis: *bce-I* (BCAM0854-BCAM0864), *bce-II* (BCAM1003-1011), *wcb* (BCAL3218-3245), BCAM1330-1349 [25] and the BCESM region were examined by extracting their sequence from the J2315 reference and using it as a BLAST query against the *de novo* contigs >1kb produced from the entire read set. The regions from the contigs that matched the query were then aligned and compared. Since BCESM is a longer region, its investigation required manual contig joining.

**Results**

In order to gain insight into changes in *B. cenocepacia* bacteria during chronic infection we sequenced three clinical isolates from a lung infection in a person with cystic fibrosis, as well as one laboratory derived nonmucoid variant (Fig 1). C3921 is the first stored isolate from this infection and displays the mucoid phenotype. C8963 was isolated 9 years and 3 months after C3921, while C9343 was isolated 10 years after C3921 and is also the final isolate we have stored for this patient.

The whole-genome sequence data at the 7 alleles used for MLST in BCC showed that all isolates were ST-33. Comparison of concatenated MLST gene fragments from our isolates to *B.
cenocepacia strains representative of the clonal diversity in the recA subgroup of this species (Fig 2), showed that ST-33 is most closely related to ST-32, which is a trans-continental epidemic strain that has been responsible for infections in Canadian patients and a large outbreak of B. cenocepacia in CF population in the Czech Republic [4,37].

Overview of Whole-Genome Sequencing Results

We chose to map our results against the completed genome sequence for B. cenocepacia J2315 because J2315 is the type strain for the B. cenocepacia species, belongs to the same recA group A evolutionary lineage as the isolates used in our study and because the J2315 genome is complete, very well annotated and actively curated at www.burkholderia.com [26,39]. A mean of 1.2 million reads were produced per sample, of which a mean of 85.5% (range 83.9–86.7%) were mapped to the J2315 reference, resulting in a mean coverage of 89.4% of the reference with median depth of 57. After filtering, calls were made for a median of 81.4% of the reference.

In order to identify any regions in our samples that may not be present in the J2315 reference, de novo assembly was performed on all reads that did not map to J2315. This resulted in 84 contigs. 56 contigs were excluded as either common conserved regions between many bacteria, or contamination if they mapped to species other than Burkholderia that are considered
unlikely to undergo horizontal transfer with *Burkholderia* (*Herminiimonas, Laribacter, Acidovorax, Homo sapiens*). 21/28 remaining contigs could be mapped to 7 different regions, each missing in one isolate. However, all of these contigs were <1200 bp and may have represented repetitive regions that may have been difficult to map. Interestingly 6/7 of the missing regions were in C9343, and one was in C8963.

Comparative analysis of the assembled sequences using BRIG indicated that, compared to J2315, the RAPD01/ST-33 clonal lineage represented by these isolates lacks several regions (Fig 3). Notably, several genomic islands appear to be absent (BcenGI2, BcenGI3 and BcenGI9). It is also notable that several other genomic islands have poor coverage in our sequence results and it is not clear whether these are present or not in the lineage of *B. cenocepacia* represented by these sequences. Additionally, a region on chromosome 1 containing the wcb genes thought to encode a capsular polysaccharide homologous to a capsular polysaccharide characterized in *B. pseudomallei* K96243, was missing from all the isolates we sequenced.

In total we confidently identified 76 single nucleotide variants (SNVs) between our isolates and the J2315 reference genome (Table 1). Of these, 15/76 (20%) were in non-coding regions. Of the 61 SNVs identified in genes, 43/61 (70%) coded for non-synonymous mutations and 18/61 (30%) coded for synonymous mutations. 51 genes contained a single SNV while 5 genes contained 2 SNVs.

**Identification of SNVs In Clinical Isolates Following Evolution in the CF Lung**

Compared to C3921, C8963 has 40 SNVs of which 34 were in coding regions. 24/34 SNVs in coding regions of C8963 were non-synonymous and 15 of these are predicted by *in-silico* analysis to have deleterious effect on the encoded protein. Our data also revealed there were 42 SNVs in C9343 compared to the initial isolate C3921. Of these 42 SNVs, 36 were identified in coding regions of which 24 are predicted to code for non-synonymous mutations and 16 of these predicted to be deleterious mutations (Table 1 and Fig 1). These SNVs were categorized...
by function, which revealed the most commonly mutated genes were those involved in genetic regulation and metabolism both before and after the divergence of C8963 and C9343 (9 SNVs in both categories), while C8963 also acquired a number of SNVs in secretion and transport independent of C9343 (N = 3) (Table 2).

Based on the confidently identified SNVs, these data are suggestive of an average evolutionary rate of $5.3 \times 10^{-7}$ SNVs/bp/year during the course of this CF infection. This rate is similar, albeit slightly higher, to that found in other studies of *Burkholderia* in CF infection, for example $3.28 \times 10^{-7}$ SNVs/bp/year in *Burkholderia dolosa* [27]. Notably, C8963 and C9343 did not share all SNVs in common. We observed 20 SNVs in common that were present in both C8963 and C9343 compared to the initial isolate from this patient, and 20 and 22 SNVs that were unique to C8963 and C9343 respectively (Fig 1 and Table 1). Based on the average SNV rate these data suggest that C8963 and C9343 may have undergone significant parallel evolution, having diverged after approximately four and half years in the CF lung.

There were also an additional number of SNVs or putative SNVs identified. Specifically, there were 5 in either C8963 or C9343 for which there was not a confident identification in the
### Table 1. Summary of SNVs.

| Position in J2315* | Qualifier | Gene Name | Gene Product | J2315 | C3921 | C8963 | C9343 | Amino acid change | SNV effect | Predicted functional effect | Score* * | Transcriptome* | Proteome* |
|------------------|-----------|-----------|--------------|-------|-------|-------|-------|-----------------|------------|-----------------------------|----------|--------------|----------|
| **SNVs in Both C8963 and C9343 compared to C3921** |
| 263702 | BCAL0227 | rpoC | DNA-directed RNA polymerase subunit beta | C | C | T | T | S109L | NS | Deleterious | -5.06 |
| 359602 | BCAL0331 | putative stringent starvation protein A | C | C | T | T | R162C | NS | Deleterious | -7.97 |
| 880552 | BCAL0809 | HPr kinase/ phosphorilase | G | G | A | A | T226L | NS | Deleterious | -5.35 |
| 1767570 | BCAL1609 | binding-protein-dependent transport system inner membrane protein | C | C | T | T | G17D | NS | Deleterious | -5.40 |
| 1874411 | BCAL1700 | ornB | ornibactin receptor | G | G | A | A | G598D | NS | Neutral | 0.96 |
| 2425667 | BCAL2198 | iscS | cysteine desulfurase | G | G | A | A | P338L | NS | Deleterious | -9.51 |
| 3759013 | BCAL3430 | ampD | N-acetyl-anthromuranmyl-L-alanine amidase | G | A | G | G | A82V | NS | Deleterious | -3.85 |
| 3764522 | BCAL3436 | proS | prolyl-tRNA synthetase | G | G | A | A | S | | |
| 4879783 | Non-coding | | | A | A | G | G | | |
| 5360319 | BCAM1342 | putative sigma-54 interacting transcriptional regulator | T | T | C | C | F164L | NS | Deleterious | -5.16 |
| 5385281 | BCAM1362 | putative penicillin-binding protein | G | A | G | G | S | | |
| 5438246 | BCAM1409 | hypothetical protein | C | C | T | T | S | | |
| 5791984 | BCAM1722 | LysR family regulatory protein | A | A | G | G | C242R | NS | Neutral | -1.09 |
| 5791985 | BCAM1722 | LysR family regulatory protein | C | C | G | G | S | | |
| 6304869 | BCAM2177 | hypothetical protein | G | G | A | A | R16K | NS | Neutral | -1.35 |
| 6577710 | Non-coding | | | C | C | T | T | | |
| 6651209 | BCAM2461 | putative inosine-uridine preferring nucleoside hydrolase | T | T | G | G | D289A | NS | Neutral | -0.50 |
| 7095726 | BCAS0007 | TetR family regulatory protein | G | T | G | G | K47N | NS | Deleterious | -4.96 |
| 7423790 | BCAS0302 | hypothetical protein | G | G | T | T | C249F | NS | Deleterious | -7.27 |
| 7625727 | BCAS0471 | outer membrane efflux protein | C | C | T | T | S | | |
| **SNVs only in C8963 versus C3921** |
| 35492 | BCAL0032 | atpF | FOF1 ATP synthase subunit B | G | G | A | G | S | |
| 381576 | BCAL0351 | putative type VI secretion system protein TssM | C | C | T | C | S | |
| 548717 | Non-coding | | | | | | | |
| 1123579 | BCAL1039 | ABC transporter ATP-binding membrane protein | C | C | T | C | | | |
|  |  |  |  |  |  |  |  | (Continued) |
Table 1. (Continued)

| Position in J2315 | Qualifier | Gene Name | Gene Product | J2315 | C3921 | C8963 | C9343 | Amino acid change | SNV effect | Predicted functional effect | Score ** | Transcriptome+ Proteome+ |
|------------------|-----------|-----------|--------------|-------|-------|-------|-------|--------------------|------------|---------------------------|----------|--------------------------|
| 1473608          | BCAL1345  | putative TonB-dependent siderophore receptor | C     | C     | T     | C     | A96V | NS                  | Deleterious | -3.84                    |          |                          |
| 1601953          | BCAL1448  | valS      | putative valyl-tRNA synthetase | G     | G     | C     | G     | T14S               | NS         | Neutral                  | 0.75     |                          |
| 2115945          | BCAL1917  | hypothetical protein | C     | C     | T     | C     | S     |                      |            |                           |          |                          |
| 2544053          | BCAL2292  | putative bipolymer transport protein | C     | C     | T     | C     | A7V   | NS                  | Neutral    | -2.01 0.47               |          |                          |
| 2603160          | Non-coding|           | C8963        | G     | G     | A     | G     |                    |            |                           |          |                          |
| 3580361          | BCAL3271  | thioredoxin | C8963        | C     | C     | T     | C     | S     |                      |            |                           |          |                          |
| 3616073          | BCAL3301 oxyR | oxidative stress regulatory protein | T     | T     | C     | T     | H203R | NS                  | Neutral    | 0.12                    |          |                          |
| 3616430          | BCAL3301 oxyR | oxidative stress regulatory protein | T     | T     | C     | T     | Q84R  | NS                  | Neutral    | -1.29                   |          |                          |
| 3782820          | BCAL3453 secA | preprotein translocase subunit SecA | A     | A     | G     | A     | V207A | NS                  | Deleterious | -3.80                   |          |                          |
| 3934083          | BCAM0059  pcaJ | 3-oxoadipate CoA-transferase subunit B | C     | C     | T     | C     | R6C   | NS                  | Deleterious | -6.62                   |          |                          |
| 5445821          | BCAM1417  | two-component regulatory system sensor kinase | G     | G     | T     | G     | L28M  | NS                  | Neutral    | -1.69 0.43               |          |                          |
| 5825895          | BCAM1745  | putative magnesium-transporting ATPase | G     | G     | A     | G     | A661T | NS                  | Deleterious | -3.94                   |          |                          |
| 5921203          | BCAM1831  | putative cyclophilin | G     | G     | A     | G     | A38T  | NS                  | Deleterious | -2.54                   |          |                          |
| 6286440          | BCAM2168  | putative amylo-1,6-glucosidase | G     | G     | A     | G     | S     |                      |            |                           |          |                          |
| 6837958          | Non-coding|           | C9343        | A     | A     | T     | A     |                    |            |                           |          |                          |
| 7202485          | Non-coding|           | C9343        | C     | C     | T     | C     |                    |            |                           |          |                          |

**SNVs only in C9343 versus C3921**

| Position in J2315 | Qualifier | Gene Name | Gene Product | J2315 | C3921 | C8963 | C9343 | Amino acid change | SNV effect | Predicted functional effect | Score ** | Transcriptome+ Proteome+ |
|------------------|-----------|-----------|--------------|-------|-------|-------|-------|--------------------|------------|---------------------------|----------|--------------------------|
| 405727 | BCAL3074  | prfA   | peptide chain release factor 1 | C     | C     | C     | T     | E161K | NS                  | Deleterious | -4.00                  |          |                          |
| 864111 | Non-coding|           | C9343        | T     | T     | T     | C     |                    |            |                           |          |                          |
| 1894555 | Non-coding|           | C9343        | T     | T     | T     | C     |                    |            |                           |          |                          |
| 2374985 | BCAL2149  | HhH-GPD superfAMILY base excision DNA repair protein | T     | T     | T     | C     | E164G | NS                  | Deleterious | -5.61                  |          |                          |
| 2423968 | BCAL2195  | hscB   | co-chaperone HscB | T     | T     | T     | C     | S     |                      |            |                           |          |                          |
| 2717069 | BCAL2452  | LysR family regulatory protein | T     | T     | T     | C     | L112P | NS                  | Deleterious | -6.18 18                |          |                          |
| 2871548 | Non-coding|           | C9343        | G     | G     | G     | T     |                    |            |                           |          |                          |
| 2873956 | BCAL2615  | putative exported outer membrane porin protein | G     | G     | G     | A     | S     |                      |            |                           |          |                          |
| 3037135 | BCAL2765  | rpsT   | 30S ribosomal protein S20 | C     | C     | C     | T     | S     |                      |            |                           |          |                          |
| 3043868 | BCAL2772  | putative AMP-binding enzyme | T     | T     | T     | G     | V494G | NS                  | Neutral    | -1.75                   |          |                          |

(Continued)
Table 1. (Continued)

| Position in J2315* | Qualifier | Gene Name | Gene Product | J2315 | C3921 | C8963 | C9343 | Amino acid change | SNV effect | Predicted functional effect | Score** | Transcriptome+ | Proteome+ |
|-------------------|-----------|-----------|--------------|-------|-------|-------|-------|-------------------|------------|-----------------------------|---------|--------------|----------|
| 3201111           | BCAL2920  | subfamily M48A metallopeptidase | T      | T      | T      | C      | W185R | NS               | Deleterious | -12.9          |         |              |          |
| 3319069           | BCAL3029  | putative alkane monoxygenase    | T      | T      | T      | C      | F144L | NS               | Neutral    | -1.60          |         |              |          |
| 3601711           | BCAL3287  | putative FAD-binding oxidase    | G      | G      | G      | A      | S      |                 |            |                |         |              |          |
| 3605061           | BCAL3289  | gkE         | G         | G      | G      | A      | G257S | NS               | Neutral    | 0.10           |         |              |          |
| 4114326           | BCAM0207  | putative tyrosine-protein kinase | C      | C      | C      | T      | S      |                 | 62.1       |                |         |              |          |
| 4375146           | BCAM0451  | putative extracellular endonuclease/exonuclease/phosphatase family protein | C      | C      | C      | G      | P39A   | NS               | Neutral    | -0.28          |         |              |          |
| 4807037           | BCAM0851  | hypothetical protein | C      | C      | C      | T      | S      |                 | 0.31       |                |         |              |          |
| 5546631           | Non-coding |          | T      | T      | T      | A      | -      |                 |            |                |         |              |          |
| 6104972           | BCAM2017  | ABC transporter ATP-binding protein | G      | G      | G      | A      | S      |                 |            |                |         |              |          |
| 6117131           | BCAM2027a | hypothetical protein | G      | G      | G      | A      | S53L   | NS               | Deleterious | -3.05          |         |              |          |
| 6283639           | BCAM2165  | putative beta-lactamase         | T      | T      | T      | C      | N155S | NS               | Deleterious | -4.99          |         |              |          |
| 7405450           | BCAS0284  | Major Facilitator Superfamily protein | C      | C      | C      | T      | R70W   | NS               | Deleterious | -5.66          |         |              |          |

**SNVs in at least one of C8963 and C9343 compared to C3921**

| Position in J2315* | Qualifier | Gene Name | Gene Product | J2315 | C3921 | C8963 | C9343 | Amino acid change | SNV effect | Predicted functional effect | Score** | Transcriptome+ | Proteome+ |
|-------------------|-----------|-----------|--------------|-------|-------|-------|-------|-------------------|------------|-----------------------------|---------|--------------|----------|
| 1839129           | BCAL1679  | putative fimbrial chaperone | C      | C      | A      | N      | N175K | NS               | Deleterious | -6.00          |         |              |          |
| 1870872           | BCAL1698  | orbK       | G      | G      | C      | -      | W241C | NS               | Deleterious | -13.0          |         |              |          |
| 3863278           | BCAL3527  | gspD       | T      | T      | A      | N      | Q502L | NS               | Deleterious | -3.44          |         |              |          |
| 5197275           | Non-coding |          | C      | C      | N      | T      | -      |                 |            |                |         |              |          |
| 6283537           | BCAM2165  | penA       | T      | T      | C      | N      | E189G | NS               | Deleterious | -5.61          |         |              |          |

**Additional SNVs in C8963 and C9343 compared to J2315**

| Position in J2315* | Qualifier | Gene Name | Gene Product | J2315 | C3921 | C8963 | C9343 | Amino acid change | SNV effect | Predicted functional effect | Score** | Transcriptome+ | Proteome+ |
|-------------------|-----------|-----------|--------------|-------|-------|-------|-------|-------------------|------------|-----------------------------|---------|--------------|----------|
| 6844295           | Non-coding |          | T      | N      | T      | G      | -      |                 |            |                |         |              |          |
| 3238151           | BCAL2957  | gyrA       | G      | N      | A      | A      | T83I   | NS               | Deleterious | -4.35          |         |              |          |
| 4392099           | BCAM0467  | hypothetical protein | A      | N      | A      | C      | K18Q   | NS               | Deleterious | -3.73          |         |              |          |
| 4392100           | BCAM0467  | hypothetical protein | A      | N      | A      | G      | K18R   | NS               | Neutral    | -2.46          |         |              |          |
| 4393598           | BCAM0468  | hypothetical protein | A      | N      | A      | G      | F339W  | NS               | Neutral    | -1.88          |         |              |          |
| 4393599           | BCAM0468  | hypothetical protein | G      | N      | G      | A      | S      |                 |            |                |         |              |          |
| 4395601           | Non-coding |          | C      | N      | C      | A      | -      |                 |            |                |         |              |          |
| 4393634           | Non-coding |          | A      | N      | A      | C      | -      |                 |            |                |         |              |          |

(Continued)
other late-stage isolate to assign them common to both C8963 and C9343 but not C3921.
Finally there were 9 instances of SNVs where C9343 and or C8963 differed from the J2315 reference sequence, but were too low confidence in C3921. Among these was a SNV in
\textit{gyrA}, which has previously been identified as commonly mutated in \textit{B. dolosa} during chronic infection \cite{27}.
C9343 has previously been shown to have a number of alterations in its pulsed-field gel electrophoresis pattern compared to C8963 following \textit{Xba}I digestion of genomic DNA \cite{22}. Investigation of larger deletions with respect to the reference revealed three regions where C9343 had a deletion compared to the other samples, as well as the reference. The first was a \~{}1600bp region on chromosome 1 corresponding to the genes \textit{orbI}, \textit{orbJ}, \textit{orbK} and \textit{pvdA} and the second a \~{}600bp region corresponding to a family M14 peptidase. Previous PCR and transcriptomic data has suggested that C9343 also has a substantial deletion in the \textit{Burkholderia cepacia} epidemic strain marker (BCESM) \cite{21}. The BCESM region contains a number of putative virulence determinants, including a quorum sensing signaling regulator. The deletion in C9343 can

\begin{table}[h]
\centering
\caption{Functional categorization of non-synonymous SNVs.}
\begin{tabular}{l|c|c|c|c}
\hline
\textbf{Function} $^*$ & \textbf{Both C8963 and C9343} & \textbf{C8963 only} & \textbf{C9343 only} \\
\hline
Genetic regulation & 4 & 3 & 2 \\
Metabolism & 4 & 2 & 3 \\
Iron acquisition & 1 & 0 & 0 \\
Outer membrane & 1 & 0 & 0 \\
Secretion/Transport & 0 & 3 & 1 \\
Antibiotic resistance & 0 & 0 & 1 \\
Unknown & 1 & 1 & 2 \\
Other & 2 & 1 & 2 \\
\hline
\end{tabular}
\end{table}

* = Genes were grouped into functional categories based on their COG annotation in the \textit{Burkholderia.com} database \cite{39}. SNVs included in this analysis were only those for which there was a confident base-pair call in all three isolates (C3921, C8963 and C9343). In addition to these SNVs, 4 others were detected in C8963 for which there was not a confident base-pair call in C9343, these were 2 genes involved in secretion (BCAL1679 and BCAL3527/gspD); one gene involved in iron acquisition (BCAL1698/orbK); and one gene involved in antibiotic resistance (BCAM2615/penA). There were also 4 genes with SNVs in C8963 and C9343 relative to J2315 for which there weren't confident base-pair call. These were 1 gene involved in antibiotic resistance (BCAL2957/gyrA) and, 3 SNVs in 2 genes of unknown function (2 in BCAM0467 and 1 in BCAM0468).
be seen in Fig 3 and is approximately 6 kb ranging from BCAM0211 to BCAM0256 in the J2315 reference.

Fig 4. Quantitative RT-PCR of transcripts encoded by selected genes from the cepacian biosynthesis cluster show upregulation of bce transcripts in mucoid C3921 relative to its corresponding nonmucoid derivative C3921-CTZ32G. RNA extracted from triplicate stationary phase cultures of bacteria (16 hour) grown in yeast extract media was assayed by quantitative RT-PCR in triplicate, using transcripts from the gyrB gene to normalize expression values between experiments. Results are expressed relative to the nonmucoid isolate, C3921-CTZ32G, and error bars represent standard error of the mean. doi:10.1371/journal.pone.0143472.g004

Mucoid C3921 and nonmucoid C3921-CTZ32G

Examination of the data from C3921 and the in-vitro derived nonmucoid isolate C3921-CTZ32G showed there were no confidently identified SNVs or other genomic changes in the sequence data between them. Because we had chosen to assemble our sequences against the somewhat distantly related J2315 reference genome, we also constructed a bespoke reference based upon the sample reads using Medusa [40]. However, we were also unable to identify any differences between C3921 and C3921-CTZ32G using this approach. Furthermore, we were unable to confidently identify any differences between these isolates by reducing the filtering criteria to a minimum depth of 2, minimum root-mean-square read mapping quality of 10, and minimum of 60% of reads supporting the consensus call (data not shown).
To determine if differential expression of genes involved in cepacian biosynthesis is associated with the mucoid phenotype in this strain, we examined data from a study that we have previously published comparing mucoid C9343 with nonmucoid C8963 at the transcriptomic level using a microarray. These data revealed there is differential expression of at least some of the genes involved in the cepacian biosynthesis. bce-F (BCAM0859), bce-G (BCAM0860) and bce-R (BCAM1008) were overexpressed in mucoid C9343 2.9, 4.0 and 2.1 fold respectively [21]. Because there are numerous genetic differences between mucoid C9343 and nonmucoid C8963, we sought to examine transcription of the cepacian biosynthetic operon in C3921 and C3921-CTZ32G. Consistent with our previous data, quantitative RT-PCR of select genes from the bce-I and bce-II clusters showed elevated expression of genes from both clusters in the mucoid isolate (Fig 4).

Discussion

One of the more challenging aspects of the infections caused by BCC bacteria is their chronic nature, with infections lasting years, and sometimes decades, making in-vitro modeling of virulence challenging. Whole-genome sequencing offers the opportunity to understand adaptation at the genomic level 'in-vivo' by examining isolates taken at different stages of chronic infection. The data presented here are one of the first comparative analyses of B. cenocepacia longitudinal clinical isolates taken from a chronic cystic fibrosis infection. The aim of this study was to characterize at the genomic level three sequential clonal isolates of B. cenocepacia taken from the beginning (C3921) and end (C8963 and C9343) of a single and ultimately fatal cystic fibrosis infection (Fig 1). These isolates are of interest because: i) they were part of an epidemic clone responsible for a number of infections in the Vancouver CF population [41]; ii) they are closely related by MLST (differing by a single base pair) to an epidemic clone responsible for a large outbreak in the Czech CF population, where there was significant mortality due to cepacia syndrome [37,42] and iii) they display colony morphology differences, with the initial isolate (C3921) being mucoid while the later isolate (C8963) is nonmucoid and the last isolate (C9343) is mucoid [21,22].

Our data reveal differences between the epidemic lineage represented by the isolates sequenced in this study and the more commonly studied Electrophoretic Type (ET)-12 lineage represented by the J2315 reference sequence (Fig 3). Notably, these isolates are missing several genomic islands encoded in the J2315 genome (BcenGI2, BcenGI3 and BcenGI9) and also a region encoding a putative capsular polysaccharide (Fig 3). This later observation is important in the context of the mucoid phenotype displayed on exopolysaccharide promoting agar plates by these isolates, indicating that these genes are not required for mucoidy. While these isolates were taken from an ultimately fatal CF infection, it is noteworthy that this infection lasted 10 years in a female patient. We have recently published data that showed, in the same population, median survival for females with B. cenocepacia was just 2.28 years [41]. It has also been previously speculated that there may be differences in virulence between the ET-12 and ST-32 lineages, the later of which is closely related to these isolates [4]. Therefore, these regions of difference may represent targets worthy of further investigation for their contribution to heightened virulence in BCC.

Three previous studies have looked at whole-genome sequences of other BCC following evolution in either lung infections caused by an epidemic strain of B. dolosa [27,43] or in an in-vitro model of biofilm evolution in B. cenocepacia H111 [28]. Our data extends these observations to a single CF lung infection caused by an epidemic lineage of B. cenocepacia. There have also been studies examining changes in a closely related bacteria, B. pseudomallei, a primary human pathogen also capable of establishing chronic carriage over decades [44]. In common...
with these studies, we also found mutations in rpoC as well as a number of genes related to central metabolism, iron metabolism and control of transcription, reinforcing these common themes in adaptation to chronic infection in the CF lung (Table 2). Interestingly we also detected SNVs in gyrA in both C8963 and C9343 in amino acid position T83 relative to J2315. Mutations in gyrA can confer resistance to fluoroquinolone antibiotics, which appears to be a common adaptation to growth in the CF lung in both *Burkholderia* and *P. aeruginosa* infections [27,45]. The mutations that we found in common between C8963 and C9343 may indicate features important to initial pathoadaptation to the CF lung. In particular genes involved in genetic regulation (N = 4) and metabolism (N = 4 plus 1 involved in iron acquisition) received the highest numbers of non-synonymous SNVs within each category (Table 2). However, it is important to interpret these results carefully. As with the studies on *B. dolosa* [27,43], this infection is part of an outbreak of *B. cenocepacia* that occurred before effective infection control [41] and therefore it is likely that these isolates were acquired directly from another CF lung infection. Although we do not know for certain if this patient acquired their *B. cenocepacia* from another patient, it is possible that these isolates had already undergone pathoadaptation to the CF lung and any changes that we observed were specific to adaptation in our case. Nonetheless, the genes involved in genetic regulation (BCAL0227/rpoC, BCAM1342, BCAM1722 and BCAS0007), in particular, represent interesting targets for future work to understand factors involved in *B. cenocepacia* evolution in CF lung infections.

Our finding that isolates C8963 and C9343 only shared around 50% of SNVs in common, compared to the initial isolate C3921, implies that these two isolates diverged and co-existed in parallel for almost half of the infection. This finding is consistent with the diverse community model proposed by Lieberman and colleagues, following their sampling of patients infected with an epidemic strain of *B. dolosa* [43]. In this model, an initial infecting strain diverges over time in the CF lung with the establishment of a range of stable sub-populations. Similar findings have also been recently reported for *B. pseudomallei* [46] and *P. aeruginosa* [47]. Therefore, to fully understand BCC infections in the CF lung, it appears that increased sampling is merited.

There was very little overlap between the SNVs identified in this study and the genes and proteins that we have previously described as differentially regulated between C8963 and C9343 [17]. Noteworthy genes that did overlap include the two SNVs in oxyR found in C8963 (Table 1). oxyR is known in other bacteria to be involved in regulating genes in response to oxidative stress [48]. This includes the gene ahpC which we have shown is overexpressed in C8963, therefore it is conceivable that these SNVs are involved in overexpression of ahpC [21]. Another notable SNV is in the gene BCAM0207, annotated as a tyrosine-protein kinase and part of a putative polysaccharide cluster, which contained a SNV in C9343. We previously found this to be overexpressed 62.1 fold in C9343 versus C8963. BCAM0207 is an orthologue of a gene in the cepacian biosynthesis operon, bce-F. bce-F has been previously shown to be required for cepacian biosynthesis [49], as well as for invasion of epithelial cells [50]. While it is not essential for the mucoid phenotype in these isolates (as isolates with both variants of the SNV are mucoid), it is conceivable that this gene is involved in the EPS profile of this isolate and/or the severity of infection in this patient and this merits further study. Finally, in this study we also observed the absence of a significant part of the BCESM in C9343 (Fig 3), this contains a quorum sensor regulator and its loss could in part explain the loss of some of the apparently quorum sensing related phenotypes we have previously observed in C9343.

It is notable that we were unable to detect any genomic differences between the mucoid C3921 isolate and its nonmucoid derivative C3921-CTZ32G. BCC bacteria are known to be capable of producing a number of different exopolysaccharides [22,25,51–53]. However, the main exopolysaccharide produced in most clinical strains of BCC is ‘cepacian’ which is
produced under the control of genes in the bce-I and bce-II loci (annotated on the J2315 genome sequence BCAM0854-BCAM0864 and BCAM1003-1011 respectively) [29]. Our study was unable to find any differences in these regions and in sequences both up and down stream that could account for the difference in morphology. It is, however, clear from these data that another previously described putative exopolysaccharide cluster of genes, wcb, (annotated on the J2315 genome sequence as BCAL3218 to BCAL3245 [25]) are not essential for the mucoid phenotype as they were absent in all strains including those elaborating the mucoid phenotype. Both the results of the RT-PCR (Fig 4) and an analysis of our previous transcriptomic data, show that there is differential expression of at least some genes involved in the cepacian biosynthesis. These data suggest, therefore, that there is genetic control of at least these genes and this may contribute to the lack of exopolysaccharide product. Because our study was not powered to fully complete the genome sequences, it seems likely that the genetic control of these genes is from a region that we have not identified. It is has been shown that the third chromosome (now thought to be a plasmid) does not appear to encode a regulator of exopolysaccharide production in B. cenocepacia H111 [54]. Nonetheless, it remains conceivable that there could be strain specific factors involved in control of exopolysaccharide production and these remain elusive.

One of the limitations of this analysis has been the lack of a reference genome for this lineage of B. cenocepacia which forced us to compare our data against the completed genome sequence for B. cenocepacia J2315. As can be seen from the MLST phylogeny (Fig 2), there is some distance between J2315 and our isolates, which reduces the quality of alignment, including causing a larger percentage of reads to be unmapped. However, we took care to investigate the reads that did not map to J2315 intensively, and were unable to find differences in them between C3921 and C3921-CTZ32G. We used strict filtering criteria of mapping results to prevent identification of false positive variant positions, thus it is possible that additional variation would have been identified if less stringent criteria were used. However, no differences between C3921 and C3921-CTZ32G arose when the filtering criteria were relaxed. The development of additional well annotated reference sequences for lineages of B. cenocepacia would be useful for future studies examining larger numbers of isolates from chronic infections. However, we did also conduct this analysis using a bespoke reference, and were still unable to determine any differences between the nonmucoid C3921-CTZ32G and its mucoid parent. Advances in genome sequencing technologies, which enable longer reads, will in the future enable easier assembly of sequence data.

In conclusion, these data complement existing studies, have identified genes that may be important in the pathobiology of B. cenocepacia in the CF lung, and help clarify our previously published data regarding isolates C8963 and C9343. Specifically, these data support the model of divergent evolution in the CF lung and extend this observation to one of the most common BCC species, B. cenocepacia. Future studies will examine larger numbers of isolates from infection and these will help further illuminate the genetic strategies used by B. cenocepacia to establish and maintain chronic infection in the CF lung.

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Author Contributions
Conceived and designed the experiments: JEAZ RRM PT. Performed the experiments: JEAZ RRM TJH. Analyzed the data: JEAZ RRM. Wrote the paper: JEAZ RRM PT.

References
1. Vandamme P, Dawyndt P. Classification and identification of the Burkholderia cepacia complex: Past, present and future. Syst Appl Microbiol.; 2011; 34: 87–95. doi:10.1016/j.syapm.2010.10.002 PMID: 21257278
2. Peeters C, Zlosnik JEA, Spilker T, Hird TJ, Lipuma JJ, Vandamme P. Burkholderia pseudomultivorans sp. nov., a novel Burkholderia cepacia complex species from human respiratory samples and the rhizosphere. Syst Appl Microbiol. 2013; 36: 48–489. doi:10.1016/j.syapm.2013.06.003
3. De Smet B, Mayo M, Peeters C, Zlosnik JEA, Spilker T, Hird TJ, et al. Burkholderia stagnalis sp. nov. and Burkholderia territiori sp. nov., two novel Burkholderia cepacia complex species from environmental and human sources. Int J Syst Evol Microbiol. 2015; 65: 2265–2271. doi:10.1099/ijs.0.000251 PMID: 25872960
4. Drevinek P, Mahenthiralingam E. Burkholderia cenocepacia in cystic fibrosis: epidemiology and molecular mechanisms of virulence. Clin Microbiol Infect. 2010; 16: 821–30. doi:10.1111/j.1469-0691.2010.03237.x PMID: 20880411
5. Govan JR, Brown AR, Jones AM. Evolving epidemiology of Pseudomonas aeruginosa and the Burkholderia cepacia complex in cystic fibrosis lung infection. Futur Microbiol. 2007; 2: 153–164. doi:10.2217/17460913.2.2.153
6. Lipuma JJ. The changing microbial epidemiology in cystic fibrosis. Clin Microbiol Rev. 2010; 23: 299–323. doi:10.1128/CMR.00068-09 PMID: 20375354
7. Murray S, Charbeneau J, Marshall BC, LiPuma JJ, Hester KLM, Nicholson A, et al. Lung transplantation for patients with cystic fibrosis and Burkholderia cepacia complex infection: a single-center experience. J Heart Lung Transplant. Elsevier Inc.; 2010; 29: 1395–1404. doi:10.1016/j.healun.2010.06.007 PMID: 20810293
8. Govan JR, Brown PH, Maddison J, Doherty CJ, Nelson JW, Dodd M, et al. Evidence for transmission of Pseudomonas cepacia by social contact in cystic fibrosis. Lancet. 1993; 342: 15–19. doi:10.1016/0140-6736(93)91881-L PMID: 7686239
9. Jones AM, Dodd ME, Govan JRW, Barcus V, Doherty CJ, Morris J, et al. Burkholderia cenocepacia and Burkholderia multivorans: influence on survival in cystic fibrosis. Thorax. 2004; 59: 948–51. doi:10.1136/thx.2003.017210 PMID: 15516469
10. Corey M, Farewell V. Determinants of mortality from cystic fibrosis in Canada, 1970–1989. Am J Epidemiol. 1996; 143: 1007–1017. PMID: 8629607
11. LiPuma JJ, Dasen SE, Nielson DW, Stern RC, Stull TL. Person-to-person transmission of Pseudomonas cepacia between patients with cystic fibrosis. Lancet. 1990; 336: 1094–6. doi:10.1016/0140-6736(90)91881-L PMID: 2079781
12. Jones AM, Dodd ME, Govan JRW, Barcus V, Doherty CJ, Morris J, et al. Burkholderia cenocepacia and Burkholderia multivorans: influence on survival in cystic fibrosis. Thorax. 2004; 59: 948–51. doi:10.1136/thx.2003.017210 PMID: 15516469
13. Corey M, Farewell V. Determinants of mortality from cystic fibrosis in Canada, 1970–1989. Am J Epidemiol. 1996; 143: 1007–1017. PMID: 8629607
14. LiPuma JJ, Dasen SE, Nielson DW, Stern RC, Stull TL. Person-to-person transmission of Pseudomonas cepacia between patients with cystic fibrosis. Lancet. 1990; 336: 1094–6. doi:10.1016/0140-6736(90)91881-L PMID: 2079781
15. Nzula S, Vandamme P, Govan JR. Influence of taxonomic status on the in vitro antimicrobial susceptibility of the Burkholderia cepacia complex. J Antimicrob Chemother. 2002; 50: 265–269. doi:10.1093/jac/dkf137 PMID: 12161410
16. Peeters E, Nelis HJ, Coeyne T. In vitro activity of ceftazidime, ciprofloxacin, meropenem, minocycline, tobramycin and trimethoprim/sulfamethoxazole against planktonic and sessile Burkholderia cepacia complex bacteria. J Antimicrob Chemother. 2009; 64: 801–9. doi:10.1093/jac/dkp253 PMID: 19633000
17. Zlosnik JE, Costa PS, Brant R, Mori PYB, Hird TJ, Fraenkel MC, et al. Mucoid and Nonmucoid Burkholderia cepacia Complex Bacteria in Cystic Fibrosis Infections. Am J Respir Crit Care Med. 2011; 183: 67–72. doi:10.1164/rcrm.201002-0203OC PMID: 20798623
18. Coutinho CP, Dos Santos SC, Madeira A, Mira NP, Moreira AS, Sá-Correia I. Long-Term Colonization of the Cystic Fibrosis Lung by Burkholderia cepacia Complex Bacteria: Epidemiology, Clonal Variation, and Genome-Wide Expression Alterations. Front Cell Infect Microbiol. 2011; 1: 12. doi: 10.3389/fcimb.2011.00012 PMID: 22919578

19. Isles A, Maclusky I, Corey M, Gold R, Prober C, Fleming P, et al. Pseudomonas cepacia infection in cystic fibrosis: an emerging problem. J Pediatr. 1984; 104: 206–210. doi: 10.1016/S0022-3476(84)80993-2 PMID: 6420530

20. Zlosnik JE, Hird TJ, Fraenkel MC, Moreira LM, Henry DA, Speert DP. Differential mucoid exopolysaccharide production by members of the Burkholderia cepacia complex. J Clin Microbiol. 2008; 46: 1470–1473. doi: 10.1128/JCM.02273-07 PMID: 18256220

21. Zlosnik JEA, Speert DP. The Role of Mucoidy in Virulence of Bacteria from the Burkholderia cepacia Complex: A Systematic Proteomic and Transcriptomic Analysis. J Infect Dis. 2010; 202: 770–781. doi: 10.1086/655663 PMID: 20670172

22. Conway BA, Chu KK, Bylund J, Altman E, Speert DP. Production of exopolysaccharide by Burkholderia cenocepacia results in altered cell-surface interactions and altered bacterial clearance in mice. J Infect Dis. 2004; 190: 957–966. doi: 10.1086/423141 PMID: 15295701

23. Ferreira AS. Insights into the role of extracellular polysaccharides in Burkholderia adaptation to different environments. Front Cell Infect Microbiol. 2011; 1: 1–9.

24. Dolfi S, Sveronis A, Silipo A, Rizzo R, Cescutti P. A novel rhamno-mannan exopolysaccharide isolated from biofilms of Burkholderia multivorans C1576. Carbohydr Res. 2015; 411: 42–48. doi: 10.1016/j.carres.2015.04.012 PMID: 25974852

25. Bartholdson SJ, Brown AR, Mewburn BR, Clarke DJ, Fry SC, Campopiano DJ, et al. Plant host and sugar alcohol induced exopolysaccharide biosynthesis in the Burkholderia cepacia complex. Microbiology. 2008; 154: 2513–2521. doi: 10.1099/mic.0.2008/019216-0 PMID: 18667584

26. Holden MTG, Seth-Smith HMB, Crossman LC, Sebaihia M, Bentley SD, Cerdeño-Tárraga AM, et al. The genome of Burkholderia cenocepacia J2315, an epidemic pathogen of cystic fibrosis patients. J Bacteriol. 2009; 191: 77–80. doi: 10.1128/JB.01230-08 PMID: 18931103

27. Lieberman TD, Michel J-B, Aingaran M, Potter-Bynoe G, Roux D, Davis MR, et al. Parallel bacterial evolution within multiple patients identifies candidate pathogenicity genes. Nat Genet. Nature Publishing Group; 2011; 43: 1275–80. doi: 10.1038/ng.997 PMID: 22081229

28. Traverse CC, Mayo-Smith LM, Poltak SR, Cooper VS. Tangled bank of experimentally evolved Burkholderia biofilms reflects selection during chronic infections. Proc Natl Acad Sci U S A. 2013; 110:E250–9. doi: 10.1073/pnas.1207025110 PMID: 23271804

29. Ferreira AS, Leitão JH, Silva IN, Pinheiro PF, Sousa SA, Ramos CG, et al. Distribution of cepacian exopolysaccharide synthesis genes among environmental and clinical Burkholderia strains and role of cepacian exopolysaccharide in resistance to stress conditions. Appl Environ Microbiol. 2010; 76: 441–50. doi: 10.1128/AEM.01828-09 PMID: 19948863

30. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBNet. 2011; 17: 10–12. doi: 10.14806/ej.17.1.200

31. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012; 9: 357–359. doi: 10.1038/nmeth.1923 PMID: 22388286

32. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009; 25: 2078–2079. doi: 10.1093/bioinformatics/btp352 PMID: 19505943

33. Zerbino DR, Birney E. Velvet: Algorithms for de novo short read assembly using de Bruijn graphs. Genome Res. 2008; 18: 821–829. doi: 10.1101/gr.074492.107 PMID: 18349386

34. Darling AE, Mau B, Perna NT. Progressivemauve: Multiple genome alignment with gene gain, loss and rearrangement. PLoS One. 2010; 5.

35. Kurtz S, Phillippy A, Delcher AL, Smoot M, Shumway M, Antonescu C, et al. Versatile and open software for comparing large genomes. Genome Biol. 2004; 5: R12. doi: 10.1186/gb-2004-5-2-r12 PMID: 14759262

36. Alikhan N-F, Petty NK, Ben Zakour NL, Beaton S a. BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. BMC Genomics. BioMed Central Ltd; 2011; 12: 402. doi: 10.1186/1471-2164-12-402 PMID: 21824423

37. Drevinek P, Vosahlikova S, Cinek O, Vavrova V, Bartosova J, Pohunek P, et al. Widespread clone of Burkholderia cenocepacia in cystic fibrosis patients in the Czech Republic. J Med Microbiol. 2005; 54: 655–659. doi: 10.1099/jmm.0.46025–0 PMID: 15947430

38. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013; 30: 2725–2729. doi: 10.1093/molbev/mst197 PMID: 24132122
39. Winsor GL, Khaira B, Van Rossum T, Lo R, Whiteside MD, Brinkman FS. The *Burkholderia* Genome Database: facilitating flexible queries and comparative analyses. Bioinformatics. 2008; 24: 2803–2804. doi: 10.1093/bioinformatics/btn524 PMID: 18842600

40. Bosi E, Donati B, Galardini M, Brunetti S, Sagot M-F, Lio P, et al. MEDUSA: a multi-draft based scaffold. Bioinformatics. 2015; 31: 2443–2451. doi: 10.1093/bioinformatics/btv171 PMID: 25810435

41. Zlosnik JEA, Zhou GG, Brant R, Henry DA., Hird TJ, Mahenthiralingam E, et al. *Burkholderia* Species Infections in Patients with Cystic Fibrosis in British Columbia, Canada. 30 Years’ Experience. Ann Am Thorac Soc. 2015; 12: 70–78. doi: 10.1513/AnnalsATS.201408-395OC PMID: 25474359

42. Kalferstova L, Kolar M, Fila L, Vavrova J, Drevinek P. Gene expression profiling of *Burkholderia cenocepacia* at the time of cepacia syndrome: loss of motility as a marker of poor prognosis? J Clin Microbiol. 2015; 53: JCM.03605–14.

43. Lieberman TD, Flett KB, Yelin I, Martin TR, McAdam AJ, Priebe GP, et al. Genetic variation of a bacterial pathogen within individuals with cystic fibrosis provides a record of selective pressures. Nat Genet. Nature Publishing Group; 2013; 46: 1–7. doi: 10.1038/ng.2848

44. Price EPE, Sarovich DSD, Mayo M, Tuanyok A. Within-Host Evolution of *Burkholderia pseudomallei* over a Twelve-Year Chronic Carriage Infection. MBio. 2013; 4: 1–10. doi: 10.1128/mBio.00388-13

45. Marvig RL, Johansen HK, Molin S, Jelsbak L. Genome analysis of a transmissible lineage of *Pseudomonas aeruginosa* reveals pathoadaptive mutations and distinct evolutionary paths of hypermutators. PLoS Genet. 2013; 9: e1003741. doi: 10.1371/journal.pgen.1003741 PMID: 24039595

46. Price EP, Sarovich DS, Viberg L, Mayo M, Kaestli M, Tuanyok A, et al. Whole-Genome Sequencing of *Burkholderia pseudomallei* Isolates from an Unusual Melioidosis Case Identifies a Polyclonal Infection with the Same Multilocus Sequence Type. J Clin Microbiol. 2015; 53: 282–286. doi: 10.1128/JCM.02560-14 PMID: 25339397

47. Jorth P, Staudinger BJ, Wu X, Hisert KB, Hayden H, Garudathri J, et al. Regional Isolation Drives Bacterial Diversification within Cystic Fibrosis Lungs. Cell Host Microbe. Elsevier Inc.; 2015; 18: 1–13.

48. Ochsner UA, Vasil ML, Alsabbagh E, Parvatiyar K, Hassett DJ. Role of the *Pseudomonas aeruginosa* oxyR-recG operon in oxidative stress defense and DNA repair: OxyR-dependent regulation of katB-ankB, ahpB, and ahpC-ahpF. J Bacteriol. 2000; 182: 4533–4544. doi: 10.1128/JB.182.16.4533–4544. 2000 PMID: 10913087

49. Ferreira AS, Leitao JH, Sousa SA, Cosme AM, Sa-Correia I, Moreira LM, et al. Functional analysis of *Burkholderia cepacia* genes bceD and bceF, encoding a phosphotyrosine phosphatase and a tyrosine autokinase, respectively: role in exopolysaccharide biosynthesis and biofilm formation. Appl Environ Microbiol. 2007; 73: 524–534. doi: 10.1128/AEM.01450-06 PMID: 17114319

50. Ferreira AS, Silva IN, Fernandes F, Pilkington R, Callaghan M, McClean S, et al. The Tyrosine Kinase BceF and the Phosphotyrosine Phosphatase BceD of *Burkholderia contaminans* Are Required for Efficient Invasion and Epithelial Disruption of a Cystic Fibrosis Lung Epithelial Cell Line. Infect Immun. 2015; 83: 812–821. doi: 10.1128/IAI.02713-14 PMID: 25486990

51. Cuzzi B, Herasimenka Y, Silipo A, Lanzetta R, Liut G, Rizzo R, et al. Versatility of the *Burkholderia cepacia* Complex for the Biosynthesis of Exopolysaccharides: A Comparative Structural Investigation. PLoS One. 2014; 9: e94372. doi: 10.1371/journal.pone.0094372 PMID: 24722641

52. Chiariini L, Cescutti P, Drigo L, Impallomeni G, Herasimenka Y, Bevivino A, et al. Exopolysaccharides produced by *Burkholderia cenocepacia recA* lineages IIIA and IIIB. J Cyst Fibros. 2004; 3: 165–172. PMID: 15463903

53. Herasimenka Y, Cescutti P, Impallomeni G, Campana S, Taccetti G, Ravanini N, et al. Exopolysaccharides produced by clinical strains belonging to the *Burkholderia cepacia* complex. J Cyst Fibros. 2007; 6: 145–152. doi: 10.1016/j.jcf.2006.06.004 PMID: 16860003

54. Agnoli K, Frauenknecht C, Freitag R, Schwager S, Jenul C, Vergunst A, et al. The third replicon of members of the *Burkholderia cepacia* complex, plasmid pC3, plays a role in stress tolerance. Appl Environ Microbiol. 2014; 80: 1340–1348. doi: 10.1128/AEM.03330-13 PMID: 24334662