Mucoid morphotype variation of *Burkholderia multivorans* during chronic cystic fibrosis lung infection is correlated with changes in metabolism, motility, biofilm formation and virulence

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Burkholderia cepacia complex (*Bcc*) bacteria are opportunistic pathogens infecting hosts such as cystic fibrosis (CF) patients. Long-term *Bcc* infection of CF patients' airways has been associated with emergence of phenotypic variation. Here we studied two *Burkholderia multivorans* clonal isolates displaying different morphotypes from a chronically infected CF patient to evaluate trait development during lung infection. Expression profiling of mucoid D2095 and non-mucoid D2214 isolates revealed decreased expression of genes encoding products related to virulence-associated traits and metabolism in D2214. Furthermore, D2214 showed no exopolysaccharide production, lower motility and chemotaxis, and more biofilm formation, particularly under microaerophilic conditions, than the clonal mucoid isolate D2095. When *Galleria mellonella* was used as acute infection model, D2214 at a cell number of approximately 7×10^6 c.f.u. caused a higher survival rate than D2095, although 6 days post-infection most of the larvae were dead. Infection with the same number of cells by mucoid D2095 caused larval death by day 4. The decreased expression of genes involved in carbon and nitrogen metabolism may reflect lower metabolic needs of D2214 caused by lack of exopolysaccharide, but also by the attenuation of pathways not required for survival. As a result, D2214 showed higher survival than D2095 in minimal medium for 28 days under aerobic conditions. Overall, adaptation during *Bcc* chronic lung infections gave rise to genotypic and phenotypic variation among isolates, contributing to their fitness while maintaining their capacity for survival in this opportunistic human niche.

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Abbreviations: *Bcc*, *Burkholderia cepacia* complex; CF, cystic fibrosis; CHP, cumene hydroperoxide; COGs, clusters of orthologous groups; DOC, sodium deoxycholate; EPS, exopolysaccharide; qRT-PCR, quantitative RT-PCR.

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Two supplementary figures and four supplementary tables are available with the online version of this paper.

INTRODUCTION

Burkholderia cepacia complex (*Bcc*) comprises Gram-negative bacteria found in water, soil and associated with plants (Baldwin *et al.*, 2007). They have large genomes (between 7 and 9 Mbp) and a number of genes ranging from 5500 to 7900. Due to this high gene content, *Bcc* strains have numerous metabolic enzymes, transporters, regulatory genes and putative virulence determinants, providing them with a great competitive capacity to move

between different niches (Holden *et al.*, 2009). *Bcc* bacteria have attracted considerable interest due to infections caused in the airways of cystic fibrosis (CF) patients (Govan & Nelson, 1992) and the difficulty of being eradicated from lung infections due to their intrinsic antibiotic resistance (Nzula *et al.*, 2002). The outcome of CF lung infection by *Bcc* ranges from mild asymptomatic carriage to a rapid and fatal decline in lung function (Isles *et al.*, 1984).

Many studies have been undertaken to understand *Bcc* pathogenicity, and several putative virulence factors have been identified (Leitão *et al.*, 2010; Mahenthiralingam *et al.*, 2005). One such factor is the exopolysaccharide (EPS) cepacian produced by a majority of *Bcc* strains (Zlosnik *et al.*, 2008). *In vitro* studies have shown the ability of EPS to inhibit neutrophil chemotaxis and to scavenge reactive oxygen species (Bylund *et al.*, 2006). Furthermore, the EPSs produced by a *Burkholderia cenocepacia* clinical isolate interfered with phagocytosis of bacteria by human neutrophils and facilitated persistent bacterial infection in the BALB/c mouse model of infection (Conway *et al.*, 2004). In the gp91^{phox-/-} mouse model of infection, EPS-producing strains of *Burkholderia cepacia* were more virulent than non-mucoid isogenic mutants (Sousa *et al.*, 2007). This suggests that EPS enhances bacterial virulence, but does not rule out the possibility that non-EPS-producers can cause severe infections. That is the case for *B. cenocepacia* and *Burkholderia multivorans* isolates, which, despite their lack of EPS biosynthesis ability, are highly infectious (Govan *et al.*, 1993; Zlosnik *et al.*, 2011). A hypothesis derived from these observations is that mucoid strains would be favoured in chronic lung infections while non-mucoid strains would be more prone to virulence in acute infections (Zlosnik *et al.*, 2011).

Despite the evidence gained from infection models showing the importance of EPS in *Bcc* virulence, there is no experimental demonstration of *Bcc* bacteria producing it within the lung environment. By contrast, in *Pseudomonas aeruginosa*, the major pathogen of the CF lung, the initial colonization is made by environmental strains that during the course of infection develop the mucoid phenotype caused by production of alginate (Pedersen *et al.*, 1992). This mucoid phenotypic conversion has also been reported for *Bcc* isolates recovered from CF patients chronically infected but, opposite to *P. aeruginosa*, most transitions were from mucoid to non-mucoid morphotypes (Zlosnik *et al.*, 2008). Characterization of two *B. cenocepacia* sequential isolates displaying different morphotypes (mucoid vs non-mucoid) isolated from a CF patient showed differential expression of other phenotypic traits besides EPS (Conway *et al.*, 2004; Zlosnik & Speert, 2010). In contrast to *P. aeruginosa* CF lung colonization, where genetic adaptations leading to phenotypic variation are characterized (Smith *et al.*, 2006), the diversity generated by *Burkholderia* persistence in the lungs is mostly unknown. It is therefore of interest to understand trait developments during *Bcc* persistence in CF lungs and to evaluate whether there is a typical phenotypic profile for

mucoid and non-mucoid isolates during chronic infections. With that aim, the approach followed in this study was to combine transcriptional profiling using custom *Burkholderia* Affymetrix DNA arrays with phenotypic characterization to compare a pair of clonal *B. multivorans* sequential isolates recovered from a chronically infected CF patient where a mucoid to non-mucoid morphotypic transition had occurred.

METHODS

Bacterial strains and growth conditions. The *Burkholderia* isolates used were D2095 and D2214 (Table 1). These isolates are from a patient attending a Vancouver CF clinic as previously described (Zlosnik *et al.*, 2008, 2011). Isolates were grown in Lennox broth (LB) or in EPS-producing medium (SM) at 37 °C (Ferreira *et al.*, 2010). Growth under microaerophilic conditions was carried out using microaerophilic generators (GENbox microaer, bioMérieux) contained within a sealed 2 l jar. For expression profiling, overnight cultures of the isolates were grown in SM medium and were diluted to an initial OD₆₄₀ of 0.1 into the same medium. Triplicate 250 ml Erlenmeyer flasks containing 100 ml medium were cultured at 37 °C with 250 r.p.m. agitation for 17 h.

Survival to long-term nutrient deprivation. Liquid cultures of *B. multivorans* D2095 and D2214 grown overnight in LB at 37 °C, were harvested, washed with saline solution and added to 100 ml M63 minimal medium without a carbon source (Sambrook & Russell, 2001), to a final OD₆₄₀ of 1.0. Cultures were incubated under aerobic conditions for 28 days at 37 °C with agitation. The number of surviving bacteria was assessed by quantification of c.f.u. on LB plates after 48 h incubation at 37 °C.

Phenotypic assays. EPS production was assessed based on the ethanol-precipitated polysaccharide dry weight recovered from 100 ml bacterial cultures grown in liquid SM over 3 days at 37 °C, as described previously (Ferreira *et al.*, 2007). Antimicrobial

Table 1. Mucoid phenotype displayed by *B. multivorans* sequential isolates recovered from a chronically infected CF patient

Isolate	Date of isolation	Source	EPS score*
C5568	30 November 1993	Sputum	+++
C6558	26 May 1995	Throat swab	++
C7148	14 June 1996	Sputum	+++
C7149	14 June 1996	Sputum	++
C7637	06 June 1997	Sputum	+
C8179	20 June 1998	Sputum	++
C9326	23 September 2000	Sputum	++
D0089	29 March 2002	Throat swab	++
D1782	03 October 2005	Throat swab	+++
D2094	01 June 2006	Throat swab	+++
D2095	01 June 2006	Throat swab	+++
D2214	09 November 2006	Sputum	–

*Classification according to Zlosnik *et al.* (2008): very mucoid isolates are scored as +++ or ++; slightly mucoid are scored as ++; non-mucoid isolates are scored as –.

susceptibility tests were based on the agar disc diffusion method (Bauer *et al.*, 1966) against piperacillin (100 µg), piperacillin (75 µg) plus tazobactam (10 µg), ciprofloxacin (5 µg), ceftazidime (30 µg) and amikacin (30 µg). The discs were applied onto the surface of Mueller–Hinton (Difco Laboratories) agar plates previously inoculated with 100 µl of bacterial cultures grown overnight in SM, at 37 °C and diluted to OD₆₄₀ 0.1. Growth inhibition diameter was measured after 24 h incubation at 37 °C. For zone inhibition assays, bacteria were grown in SM medium and 100 µl of a culture of OD₆₄₀ 1.0 were spread onto SM plates. Sterile paper discs 6 mm in diameter were placed on the agar surface. A total of 20 µl SDS (10% w/v), sodium deoxycholate (DOC) (5% w/v), cumene hydroperoxide (CHP) (10% v/v) and H₂O₂ (30% v/v) was pipetted onto separate discs. The plates were incubated for 24 h at 37 °C and the zone of growth inhibition measured. Biofilm formation assays were performed as previously described (Ferreira *et al.*, 2007). Bacteria were grown in SM medium at 37 °C to mid-exponential phase, diluted to OD₆₄₀ 0.05, and 200 µl of these cell suspensions were inoculated into wells of a 96-well polystyrene microtitre plate. Plates were incubated at 37 °C without agitation under either normal atmosphere or microaerophilic conditions inside anaerobic jars. The biofilm was stained with crystal violet solution, followed by dye solubilization with ethanol and measurement of the solution's A₅₉₀ using a microplate reader as previously described (Ferreira *et al.*, 2007). Swimming plates with 0.3% (w/v) Bacto agar (Difco) and swarming plates with 0.5% (w/v) Noble agar (Difco) were prepared using Broomfield medium [0.04% (w/v) tryptone; 0.01% (w/v) yeast extract; and 0.0067% (w/v) CaCl₂] and LB with 0.5% (w/v) glucose, respectively. For estimation of motility, overnight SM bacterial cultures (5 µl) were inoculated onto the agar surface and incubated at 37 °C for 48 h followed by measurement of colony diameter.

Virulence determination in *Galleria mellonella*. Killing assays were performed as described by Seed & Dennis (2008). Larvae were given injections of an increasing number of bacteria ranging from 7 × 10³ to 7 × 10⁶ c.f.u. diluted in 10 mM MgSO₄ with 1.2 mg ampicillin ml⁻¹ and the survival rate was evaluated for 6 days post-infection. As a negative control, 10 mM MgSO₄ with 1.2 mg ampicillin ml⁻¹ was used. Triplicates of 10 larvae were used in each experiment.

Bacterial genotyping. Sample preparation and PFGE were performed as previously described (Moreira *et al.*, 1997). Prior to PFGE, immobilized DNA was digested with 20 U *SpeI* restriction endonuclease before being loaded onto a 1% (w/v) agarose gel in 0.5 × Tris/borate/EDTA buffer. PFGE was carried out with a Gene Navigator apparatus (Pharmacia-LKB) at 180 V, using 5–120 s pulse times for 22 h.

recA gene amplification and RFLP analysis was performed to confirm that the two isolates belonged to *B. multivorans*. The primers used for *recA* amplification were BCR1 (TGACCGCCGAGAAGAGCAA) and BCR2 (CTCTTCTTCGTCCATCGCCTC), followed by *HaeIII* digestion of the 1043 bp amplification product.

Custom *Burkholderia* microarray design. The nucleotide sequences used for microarray design were from *B. multivorans* ATCC 17616 and *B. cenocepacia* J2315 genomes present in GenBank. Sequences corresponding to coding regions from the two genomes were aligned against each other based on BLASTN (Altschul *et al.*, 1997). Using the web tools available through the Personal BLAST Navigator (PLAN) (He *et al.*, 2007) system, genes with >90% identity at the nucleotide level were targeted and exported in tabular format for common probe selection. The parameters chosen for the custom array design were as follows: 11 µm feature size, standard 12.8 mm array format, prokaryotic antisense target type, perfect-match probes only, full-length sequence for probe selection, and eight

as the minimum number of acceptable probes per sequence. The types of probe sets represented are: 10 032 unique; 3342 gene (_a); 247 identical (_s); and 1203 mixed (_x). *B. cenocepacia* J2315 genes are represented by 4790 unique probes; 3210 gene (_a); 136 identical (_s); and 676 mixed (_x). *B. multivorans* ATCC 17616 genes are represented by 5099 unique probes; 3268 gene (_a); 158 identical (_s); and 766 mixed (_x). A total of 280 probe sets representing 213 regions of the *B. cenocepacia* J2315 genome encoding putative non-coding small RNAs as predicted elsewhere (Coenye *et al.*, 2007) were also included. Probe gene coverage is 99.0 and 99.8% for *B. cenocepacia* J2315 and *B. multivorans* ATCC 17616, respectively.

Isolation and processing of RNA and DNA samples. For RNA analysis bacterial cells were resuspended in RNeasy Protect Bacteria reagent (Qiagen), and total RNA extraction was carried out using the RNeasy MiniKit (Qiagen) by following the manufacturer's recommendations. RNA integrity was checked on an Agilent 2100 Bioanalyser using an RNA Nano assay. RNA was processed for use on Affymetrix custom dual-species *Burkholderia* arrays, according to the manufacturer's Prokaryotic Target Preparation Assay. Briefly, 10 µg total RNA containing spiked-in poly(A) RNA controls (GeneChip Poly-A RNA Control kit; Affymetrix) was used in a reverse transcription reaction with random primers (Invitrogen Life Technologies) to generate first-strand cDNA. After removal of RNA, 2 µg cDNA was fragmented with DNase and end-labelled with biotin using terminal polynucleotidyltransferase (GeneChip WT Terminal Labelling kit, Affymetrix). Size distribution of the fragmented and end-labelled cDNA was assessed using an Agilent 2100 Bioanalyser. Samples (2 µg) of end-labelled fragmented cDNA were used in a 200 µl hybridization cocktail containing added hybridization controls and hybridized on arrays for 16 h at 50 °C. Modified post-hybridization wash and double-stain protocols (FLEX450_0005; GeneChip HWS kit, Affymetrix) were used on an Affymetrix GeneChip Fluidics Station 450. Arrays were scanned on an Affymetrix GeneChip scanner 3000 7G. Biological triplicates of RNA from each bacterial culture were processed and analysed.

For DNA analysis bacterial cells were grown in liquid LB for 15 h, followed by DNA extraction using the DNeasy blood and tissue kit (Qiagen). A total of 1.5 µg genomic DNA per sample was labelled using the Bioprime DNA labelling System (Invitrogen) following a strategy for genomic DNA hybridizations to GeneChips developed by Hammond *et al.*, (2005). Cleanup was performed using MinElute PCR Purification kit (Qiagen) and quality was checked on an Agilent 2100 Bioanalyser using a DNA 1000 assay. Five micrograms of each sample was analysed on Affymetrix custom dual-species *Burkholderia* arrays following the protocol described above for RNA samples. Duplicates of DNA from each bacterial culture were processed and analysed.

Microarray analysis. For RNA analysis scanned arrays were analysed with Affymetrix Expression Console software to assure that all quality parameters were in the recommended range. Subsequent analysis was carried out with DNA-Chip Analyser 2008. First a digital mask was applied, leaving for analysis only the 9291 probe sets representing *B. multivorans* ATCC 17616 transcripts. Then the six arrays were normalized to a baseline array with median CEL intensity by applying an Invariant Set Normalization Method (Li & Wong, 2001a). Normalized CEL intensities of the arrays were used to obtain model-based gene expression indices based on a Perfect Match (PM)-only model (Li & Wong, 2001b). Replicate data (triplicates) for each bacterial isolate were weighted gene-wise by using inverse squared standard error as weights. All genes compared were considered to be differentially expressed if the 90% lower confidence bound of the fold change (LCB) between experiment and baseline was above 1.2, resulting in 392 differentially expressed transcripts with a median false discovery rate of 4.7%.

For DNA a separate analysis was performed for the four arrays following the steps described above for RNA. An LCB cut-off of 1.5 in combination with a consistent hybridization signal above 10 in the two replicates of at least one strain was chosen, resulting in 52 genomic loci showing significantly different hybridization signals.

Quantitative real-time RT-PCR (qRT-PCR). DNA microarray data were validated by qRT-PCR as previously described (Ferreira *et al.*, 2010). Total RNA was used in a reverse transcription reaction with TaqMan Reverse Transcription Reagents (Applied Biosystems). qRT-PCR amplification of each gene (for primer sequences see Supplementary Table S1, available with the online version of this paper) was performed with a model 7500 thermocycler (Applied Biosystems). The expression ratio of the target genes relative to the reference gene *trpB*, which showed no variation in the transcription abundance under the conditions tested, was determined. Relative quantification of gene expression by real-time qRT-PCR was determined using the $\Delta\Delta C_t$ method (Pfaffl, 2001).

RESULTS

Mucoid phenotype assessment in sequential isolates of *B. multivorans*

During a 13-year period 12 isolates were recovered from a CF patient persistently infected with *Burkholderia* (Table 1). This patient, who is still alive, had simultaneously yielded cultures of other bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*, but at the times when the last six isolates were obtained only *B. multivorans* was present. These isolates were analysed by randomly amplified polymorphic DNA (RAPD) and all of them belonged to RAPD group BM-019, corresponding to the same *B. multivorans* strain (Zlosnik *et al.*, 2008). The assessment of EPS production in yeast extract mannitol (YEM) agar showed that isolates recovered from November 1993 until June 2006 were mucoid, although some variation in the amount of EPS was observed (Table 1). Interestingly, the last isolate (D2214) was completely non-mucoid and represents a mucoid to non-mucoid transition. Additional confirmation that mucoid D2095 and non-mucoid D2214 are indeed clonal came from PFGE of genomic DNA digested with *SpeI*, since the DNA fragment pattern obtained was almost identical (see Supplementary Fig. S1). Specific primers to amplify the *recA* gene from *Burkholderia* followed by restriction analysis with *HaeIII* also confirmed that both isolates are indeed from the species *B. multivorans* (results not shown).

Isolates D2095 and D2214 were chosen here to gain insights into phenotypic variation during *Bcc* chronic lung infection and in particular to assess differences between the mucoid and non-mucoid morphotypes. Two of the analysed features were growth behaviour under aerobic conditions and production of EPS in SM liquid medium. The two isolates displayed identical growth rates (Fig. 1a), but assessment of EPS biosynthesis confirmed that D2095 produces approximately 10 g EPS l⁻¹ after 72 h of growth, while no EPS was recovered from the supernatant of D2214 (Fig. 1b), confirming the morphotypes obtained previously in YEM (Zlosnik *et al.*, 2008).

Fig. 1. Growth curves (a) and EPS production (b) for *B. multivorans* D2095 under aerobic (□) or microaerophilic (■) conditions and D2214 under aerobic (◆) or microaerophilic (◇) conditions, grown in SM medium at 37 °C. The standard deviation for both panels was below 5 % of the mean. The data are based on mean values from the results of at least three independent cell cultivations.

Expression profiling among sequential mucoid and non-mucoid isolates of *B. multivorans*

To understand the differences between isolates D2095 and D2214, transcriptional profiling was performed and data mapped to cellular processes and metabolic pathways. For that purpose, a dual-species custom *Burkholderia* microarray (Bcc1sa520656F) was designed and produced by Affymetrix as described in Methods. One of the species represented in the array is *B. multivorans* ATCC 17616 and its coding regions are represented by 5099 unique probes, 3268 gene (_a), 158 identical (_s) and 766 mixed (_x), with gene coverage of 99.8 %. To determine which probes from the array hybridize differentially between the genomes of *B. multivorans* D2095 and D2214, both genomic DNAs were extracted, biotin-labelled and hybridized in duplicate to the microarray Bcc1sa520656F. Genomic DNA hybridization intensities from D2214 were compared to those of D2095 using dChip (≥ 1.5 -fold change lower confidence bound and hybridization signal above 10), resulting in 52 genes

with differential hybridization (see Supplementary Table S2). The majority of these genes are located very close to each other on chromosome 2. Genes Bmul_4779 to Bmul_4788 seem to be absent from the genome of isolate D2214 as shown by the image of the probe set intensities in both isolates for gene Bmul_4779 (Supplementary Fig. S2a). These genes encode putative ABC-transporter-related proteins and a transcriptional regulator of the MarR family. Another region most likely absent from D2214 comprises genes Bmul_4804 to Bmul_4812 with the exception of Bmul_4807, putatively involved in the degradation pathway of phthalate. Finally, the largest group of genes, comprising Bmul_4834 to Bmul_4874 (with a few exceptions), seem to be duplicated in the D2214 genome. Since the probe sets corresponding to these genes showed intense hybridization signals (for an example see Supplementary Fig. S2b), it is unlikely that it corresponds to a deletion in the D2095 genome. All of this last group of genes encode proteins putatively related to bacteriophages. From this genomic hybridization experiment we demonstrate that indeed some genetic alterations took place during lung colonization by these isolates.

To carry out transcription profiling, RNA was extracted from the mucoid D2095 and the non-mucoid D2214 isolates grown in SM medium until early stationary phase, corresponding to a period where high-molecular-mass EPS is not yet recovered from the culture supernatant. The expression profile of D2214 when compared with D2095 using dChip (≥ 1.2 -fold change lower confidence bound with a resulting false discovery rate of $\leq 4.7\%$) showed 113 genes with significantly increased expression (see Supplementary Table S3) and 279 genes with significantly decreased expression (see Supplementary Table S4). The search for common genes between the expression data and the genomic hybridization data showed 22 genes, 8

increased in both datasets and 14 decreased (Fig. 2). These genes were excluded from our expression analysis, leaving 105 genes with significantly increased expression and 265 with significantly decreased expression (Fig. 2). The differentially expressed genes were assigned to clusters of orthologous groups (COGs) as shown in Fig. 3. No particular categories with a large number of genes with significantly increased expression were obtained (Fig. 3, grey bars). Categories with a large number of genes with significantly decreased expression were cell wall/membrane/envelope biogenesis and cell motility (Fig. 3, black bars). There was no preferential distribution of differentially expressed genes within the three replicons, with 6.8, 6.8 and 3.5% of the genes from chromosome 1, 2 and 3, respectively, being differentially expressed.

To confirm the data obtained by microarray analysis, expression of seven representative genes from COGs K, M, N and T was analysed by qRT-PCR. The results obtained were in good agreement with the microarray data although the fold change for most of the genes was higher when expression was analysed by qRT-PCR (Table 2).

Genes involved in virulence traits

In general, a decrease in transcripts of genes encoding virulence-associated traits, such as motility, chemotaxis, type VI secretion and antibiotic resistance, was observed in D2214 (Table 3, Supplementary Table S4). Concerning motility and chemotaxis, nearly all *flh*, *flg*, *mot*, *fli* and *che* genes showed decreased expression in D2214 when compared with D2095 (Table 3). The sole exceptions were the filament-encoding genes *fliCD* and *fliOPQR*, whose gene products are involved in flagellar assembly, with no significant change. Besides the decreased expression of several genes encoding methyl-accepting chemotaxis proteins, the D2214 isolate also

Fig. 2. Diagram representing the intersection between the differentially expressed genes and the genomic DNA differential hybridization when D2214 was compared with D2095. Genes excluded from our expression dataset are indicated.

Fig. 3. Functional distribution into COGs of genes with altered expression when the non-mucoid *B. multivorans* D2214 isolate was compared with the mucoid isolate D2095. A total of 370 genes with statistically significantly altered expression were obtained by using a custom Affymetrix *Burkholderia* GeneChip.

displayed decreased expression of the *aer* gene encoding a protein homologous to the aerotaxis (oxygen-sensing) receptor Aer from other bacteria. Through control of the flagellar motor, Aer sensing enables motile bacteria to avoid niches where carbon deficiency, hypoxia or other insults would limit energy production (Taylor, 2007). To confirm the microarray data, swimming and swarming motilities were assessed by quantitative plate assays. Under the conditions tested, D2214 showed a reduction of 15 % of swimming motility and 45 % of swarming motility when the size of the colonies formed was compared with those of the D2095 isolate (Fig. 4a).

Another set of genes with significantly decreased expression in isolate D2214 were those encoding products required for type VI secretion: 9 of the 18 genes annotated as being involved in type VI secretion showed differential expression (Table 3). Although genes encoding type VI secretion system components are poorly expressed *in vitro*, this

type of transport was discovered as a novel factor of *B. cenocepacia* survival in a rat model of chronic lung infection (Aubert *et al.*, 2010; Hunt *et al.*, 2004). In a recent study, Schwarz *et al.* (2010) proposed a broader physiological significance of type VI secretion in providing defence against simple eukaryotic cells and other bacteria in the environment. Co-inoculation of D2095 and D2214 isolates in SM liquid medium and incubation for 3 days at 37 °C followed by mucoid versus non-mucoid c.f.u. determination indicated that neither of the strains predominated over the other (data not shown). As controls, single cultures were grown under the same tested conditions to demonstrate that no morphotype variation occurred during that period of time.

The expression of genes Bmul_1515 and Bmul_6008, encoding two putative β -lactamases, was significantly decreased in isolate D2214 (Table 3). This was consistent with the intrinsic antibiotic resistance pattern obtained by using the antibiotic disc assay. The inhibition haloes obtained in the presence of ciprofloxacin, ceftazidime and amikacin were very similar for both isolates, but for the β -lactam piperacillin alone or with tazobactam, isolate D2095 was significantly more resistant than D2214 (Fig. 4b). The same result was obtained in liquid cultures supplemented with each of the antibiotics under test (results not shown).

Other genes encoding putative virulence factors which had decreased expression in D2214 were Bmul_3709, Bmul_3710 and Bmul_3712, directing the synthesis of a haemolysin-type calcium-binding protein and a type-I transporter from the HlyD family (Table 3), and the acid-phosphatase-encoding gene Bmul_4180. The protein encoded by Bmul_4180 is homologous to AcpA from *Burkholderia pseudomallei* and, although this protein is

Table 2. Quantitative real-time RT-PCR analysis performed in *B. multivorans* D2095 and D2214 cells

Gene	Microarray lower bound of fold change	Real-time fold change $\pm$ SD
Bmul_0161 (<i>flhC</i>)	-2.2	-3.5 ± 1.6
Bmul_0195 (<i>rlpA</i>)	1.8	1.2 ± 0.1
Bmul_3013 (<i>flgI</i>)	-2.3	-3.3 ± 0.2
Bmul_4264	-1.5	-3.1 ± 0.5
Bmul_4265	-1.5	-3.5 ± 1.2
Bmul_4613 (<i>bceM</i>)	-3.4	-6.2 ± 1.3
Bmul_4914 (<i>bceG</i>)	-18.3	-8.8 ± 0.1

Table 3. Selection of a set of differentially expressed genes when comparing the *B. multivorans* D2214 transcriptome with that of D2095, separated by functional groups

Functional class	Gene identifier	LB-FC*	Gene name	Description
Flagellar synthesis, motility and chemotaxis†	Bmul_0043	−1.8	<i>fliM</i>	Flagellar motor switch protein FliM
	Bmul_0165	−1.9	<i>cheA</i>	CheA signal transduction histidine kinase
	Bmul_0168	−1.8	<i>cheR</i>	MCP methyltransferase, CheR-type
	Bmul_0169	−2.1	<i>cheD</i>	CheD
	Bmul_0177	−1.8	<i>flhF</i>	GTP-binding signal recognition particle SRP54 G-domain
	Bmul_0178	−1.9	<i>flhG</i>	Flagellar biosynthesis protein, FlhG
	Bmul_3010	−1.9	<i>flgK</i>	Flagellar hook-associated protein FlgK
	Bmul_3013	−2.3	<i>flgI</i>	Flagellar basal body P-ring protein
	Bmul_3014	−1.9	<i>flgH</i>	Flagellar basal body L-ring protein
	Bmul_3015	−1.9	<i>flgG</i>	Flagellar basal-body rod protein FlgG
	Bmul_3018	−1.9	<i>flgD</i>	Flagellar hook capping protein
	Bmul_3020	−1.7	<i>flgB</i>	Flagellar basal-body rod protein FlgB
	Bmul_3023	−2.0	<i>flgN</i>	FlgN family protein
	Bmul_3059	−1.8	<i>fliS</i>	Flagellar protein FliS
	Bmul_3061	−1.9	<i>fliF</i>	Flagellar M-ring protein FliF
	Bmul_3062	−1.9	<i>fliG</i>	Flagellar motor switch protein FliG
	Bmul_3362	−1.7	<i>aer</i>	Methyl-accepting chemotaxis sensory transducer
Virulence and pathogenesis	Bmul_1515	−2.2	–	β-Lactamase domain protein
	Bmul_2854	−1.3	<i>paaI</i>	Phenylacetic acid degradation protein PaaD
	Bmul_2923	−1.3	<i>bcsE</i>	Type VI secretion-associated protein, ImpA family
	Bmul_2924	−1.2	<i>bcsF</i>	Type VI secretion ATPase, ClpV1 family
	Bmul_2925	−1.4	<i>bcsG</i>	Type VI secretion protein, VC_A0111 family
	Bmul_2927	−1.6	<i>bcsI</i>	Type VI secretion system lysozyme-related protein
	Bmul_2928	−1.3	<i>bcsJ</i>	Type VI secretion system effector, Hcp1 family
	Bmul_2929	−1.4	<i>bcsK</i>	Type VI secretion protein, EvpB/VC_A0108 family
	Bmul_2930	−1.2	<i>bcsL</i>	Type VI secretion protein
	Bmul_2931	−1.3	<i>bcsM</i>	TPR repeat-containing protein
	Bmul_2934	−1.2	<i>bcsP</i>	Hypothetical protein (type VI)
	Bmul_3709	−1.4	<i>adh</i>	Haemolysin-type calcium-binding region
	Bmul_3710	−1.7	–	Outer membrane efflux protein
	Bmul_3712	−1.2	–	Type I secretion membrane fusion protein, HlyD family
	Bmul_6008	−1.4	<i>ampC</i>	Beta-lactamase
	Bmul_0303	−1.4	<i>ompW</i>	OmpW family protein
Cell wall/membrane biogenesis‡	Bmul_4605	−10.1	<i>bceT</i>	UTP-glucose-1-phosphate uridylyltransferase
	Bmul_4612	−6.3	<i>bceN</i>	GDP-mannose 4,6-dehydratase
	Bmul_4913	−8.2	<i>bceH</i>	Glycosyltransferase-like protein
	Bmul_4914	−18.3	<i>bceG</i>	Glycosyltransferase family 2
	Bmul_4915	−8.0	<i>bceF</i>	Phosphotyrosine autokinase
	Bmul_4916	−8.4	<i>bceE</i>	Polysaccharide biosynthesis protein
	Bmul_4918	−10.2	<i>bceC</i>	Nucleotide sugar dehydrogenase
	Bmul_4919	−4.2	<i>bceB</i>	Undecaprenyl-phosphate glucose phosphotransferase
	Bmul_4920	−8.1	<i>bceA</i>	Mannose-1P guanylyltransferase/mannose-6P isomerase
	Bmul_0179	−1.9	<i>fliA</i>	RNA polymerase, sigma 28 subunit, FliA/WhiG
Transcription regulators	Bmul_2557	−2.1	–	Transcriptional regulator, LysR family
	Bmul_3022	−1.7	<i>flgM</i>	Anti-sigma-28 factor, FlgM
	Bmul_3720	2.9	–	Transcriptional regulator, XRE family
	Bmul_3782	−1.8	–	Transcriptional regulator, AraC family
Regulatory/signal transduction	Bmul_0160	−1.5	<i>flhD</i>	Flagellar transcriptional activator
	Bmul_0161	−2.2	<i>flhC</i>	Flagellar transcriptional activator FlhC
	Bmul_0171	−1.7	<i>cheY</i>	Response regulator receiver protein
	Bmul_2116	−1.5	–	Diguanylate cyclase/phosphodiesterase with PAS/PAC sensor
	Bmul_3168	−1.5	–	Response regulator receiver protein
	Bmul_4264	−1.5	–	Response regulator receiver protein
	Bmul_4265	−1.5	<i>fnr</i>	Transcriptional regulator, Crp/Fnr family
	Bmul_5122	−1.2	–	Diguanylate cyclase/phosphodiesterase with PAS/PAC sensor

Table 3. cont.

Functional class	Gene identifier	LB-FC*	Gene name	Description
Energy production and conversion	Bmul_1434	-2.1	<i>pdhA</i>	Dehydrogenase E1 component
	Bmul_1435	-1.9	<i>pdhB</i>	Transketolase central region
	Bmul_1436	-1.3	–	Branched-chain alpha-keto acid dehydrogenase subunit E2
	Bmul_2651	-1.7	<i>dld</i>	FAD linked oxidase domain protein (lactate dehydrogenase)
	Bmul_2652	-1.6	<i>glcD</i>	FAD linked oxidase domain protein (glycolate oxidase)
	Bmul_5383	-1.5	<i>narG</i>	Nitrate reductase, alpha subunit
	Bmul_5384	-1.4	<i>narH</i>	Nitrate reductase, beta subunit
	Bmul_5385	-1.7	<i>narJ</i>	Nitrate reductase molybdenum cofactor assembly chaperone
	Bmul_5386	-1.3	<i>narI</i>	Respiratory nitrate reductase, gamma subunit
	Bmul_5388	-1.3	<i>narK</i>	Major facilitator superfamily MFS_1
Carbohydrate transport and metabolism	Bmul_0700	-1.3	–	Pfk domain protein
	Bmul_0702	-1.5	<i>smoE</i>	Extracellular solute binding protein
	Bmul_0706	-1.5	<i>smoG</i>	Sorbitol/mannitol transport system ATP-binding protein
	Bmul_0712	-1.4	<i>mtlK</i>	Mannitol dehydrogenase domain
	Bmul_0897	1.3	<i>otsA</i>	α,α -Trehalose-phosphate synthase (UDP-forming)
	Bmul_3783	-1.7	–	Periplasmic-binding protein
	Bmul_3784	-1.3	–	Monosaccharide-transporting ATPase
	Bmul_3785	-1.5	–	Monosaccharide-transporting ATPase
Amino acid transport and metabolism	Bmul_0305	-1.4	<i>gltB</i>	Glutamate synthase (ferredoxin)
	Bmul_0306	-1.4	<i>gltD</i>	Glutamate synthase, NADH/NADPH, small subunit
	Bmul_1255	-1.2	<i>glnD</i>	UTP-GlnB uridylyltransferase
	Bmul_2715	-1.4	<i>gdhA</i>	Glu-Leu-Phe-Val dehydrogenase
	Bmul_3721	1.5	<i>glnA</i>	Glutamine synthetase catalytic region

*LB-FC, lower bound of fold-change;

†Only genes with a lower bound of fold-change above 1.7 are shown.

‡The *bce* genes shown are the ones with a lower bound of fold-change above 4.0.

considered a putative virulence factor, the disruption of the *acpA* gene from *B. pseudomallei* did not significantly reduce the virulence of this organism (Burtneck *et al.*, 2001).

Bcc bacteria are most probably subjected to oxidative stress in the lung, as caused by the presence of abundant neutrophils (Downey *et al.*, 2009). Although none of the genes usually found to be involved in combating oxidative stress were differentially expressed in our dataset, the behaviour of both isolates under this type of stress was evaluated. We compared the resistance of isolates D2095 and D2214 grown in the presence of cumene hydroperoxide (CHP) and H_2O_2 (Fig. 4c); the results confirmed that the isolates react with the same magnitude to oxidative stress. Stress induced by SDS and DOC was also tested and the results showed a slight increase in susceptibility by D2214 (Fig. 4c).

Biofilm formation contributes to infection by protecting the bacteria from the host immune defence (Costerton *et al.*, 1999). To assess biofilm formation on an abiotic surface by D2095 and D2214, a quantitative assay using crystal violet was used. After 48 h incubation under aerophilic conditions, the adherence level by isolate D2214 was slightly greater than that of D2095, as determined from the absorbance of the solubilized dye (Fig. 5a). Interestingly, when the biofilm was formed under microaerophilic

conditions, this difference became much more prominent, suggesting that a reduction of the oxygen level favoured D2214 biofilm formation (Fig. 5a).

Galleria mellonella was used as an acute model of infection to compare the virulence of the mucoid isolate D2095 and the non-mucoid isolate D2214. Several inocula ranging from 7×10^3 to 7×10^6 c.f.u. were used for both isolates. For the lower number of bacteria (approximately 7×10^3 and 7×10^4 c.f.u.), none of the larvae was dead at 6 days post-infection (data not shown). When approximately 7×10^5 c.f.u. were used virulence attenuation by non-mucoid D2214 was observed, with more than 80 % of the larvae still being alive at 4 days post-infection while in D2095 only 10 % remained alive (Fig. 5b, open symbols). A larger inoculum size (approximately 7×10^6 c.f.u.) also showed virulence attenuation for D2214 when compared with D2095, but in this case larval survival rate was less pronounced in the presence of both isolates (Fig. 5b, filled symbols). These data showing higher survival rates in the presence of D2214 were consistent with the decreased expression of many transcripts encoding proteins putatively involved in virulence that we observed in our microarray data. Nevertheless, with prolonged incubation time and a large inoculum size, both strains display a fully virulent phenotype.

Fig. 4. Phenotypic properties displayed by isolates D2095 and D2214. (a) Swimming and swarming motilities assayed in 0.3 % or 0.5 % agar plates spotted with 5 μ l bacterial cultures and incubated at 37 °C for 48 h, followed by measurement of colony diameter. (b) Susceptibility to antibiotics determined at 37 °C after 24 h incubation determined by measuring the diameter of cell growth inhibition (Pip, piperacillin; Taz, tazobactam; Cip, ciprofloxacin; Cef, ceftazidime; Ami, amikacin). (c) Sensitivity of isolates to stress-inducing agents (10 % SDS, 5 % DOC, 10 % CHP and 30 % H₂O₂) determined by measuring growth inhibition haloes after incubation of plates at 37 °C for 24 h. The data are means $\pm$ SD from the results of at least three independent cell cultivations. The *t*-test was performed using GraphPad Prism 5.0 software. A *P*-value of <0.013 was considered significant compared to D2095 (*).

Genes involved in cell wall biogenesis

In the category of cell wall biogenesis, the largest group of genes differentially expressed comprises the *bce* genes

Fig. 5. Comparison between *B. multivorans* isolates D2095 and D2214 relative to (a) biofilm formation and (b) virulence in *Galleria mellonella*. The biomass of the biofilm was assessed after 48 h incubation at 37 °C in aerophilic and microaerophilic conditions in polystyrene microtitre plates containing SM medium. Virulence tests were performed by injecting *G. mellonella* with *B. multivorans* D2095 using approximately 7×10⁵ (□) and 7×10⁶ (■) cells or with *B. multivorans* D2214 using approximately 7×10⁵ (◇) and 7×10⁶ (◆) cells, and estimation of larvae survival rates over 6 days. The control experiment without bacteria is also shown (△). The data are means $\pm$ SD from the results of at least three independent experiments. A *P*-value of <0.01 was considered significant compared with D2095 (*).

involved in cepacian biosynthesis (Table 3). All genes from both *bce-I* and *bce-II* gene clusters (Ferreira *et al.*, 2010; Moreira *et al.*, 2003) showed strongly decreased expression in D2214 in comparison to D2095. In fact, 15 out of 19 genes had more than twofold decreased expression. This result partially confirms the non-mucoid phenotype observed for D2214 and suggests that regulation of cepacian biosynthesis in this isolate is probably at the transcriptional level, but post-transcriptional regulation may also account for that phenotype.

Some differences in the outer-membrane composition of the two isolates may also be present since many genes encoding outer-membrane porins showed decreased expression in D2214. These genes are *ompW*, Bmul_0880, Bmul_2395, Bmul_2847, Bmul_3710 and Bmul_4600. The decrease in the expression levels for porin-encoding genes could indicate a less permeable outer membrane in this isolate.

Genes involved in transcription and signal transduction

In the category of transcriptional regulators, the expression of five genes encoding products involved in motility and chemotaxis regulation was found to be decreased in D2214 (Table 3). These genes encode the master regulators FlhCD, the sigma 28 subunit of RNA polymerase FliA, the anti-sigma 28 FlgM and CheY protein, which when phosphorylated binds to FliM and changes flagellar motor rotation. As described before, most of the genes under control of these regulators showed decreased expression in D2214.

Genes Bmul_4264 and Bmul_4265, encoding a response regulator receiver protein and a transcriptional regulator of the Crp/Fnr family, respectively, showed decreased expression in the D2214 isolate (Table 3). The Crp (cAMP receptor protein)-like and Fnr (fumarate and nitrate reductase regulatory protein)-like transcription factors comprise proteins which respond directly or indirectly to a broad spectrum of intracellular and exogenous signals (cAMP, oxygen or redox state, oxidative and nitrosative stress, nitric oxide, carbon monoxide, 2-oxoglutarate or temperature) (Körner *et al.*, 2003). Dufour *et al.* (2010) analysed the Crp/Fnr conserved regulons within α -proteobacteria and found that the core Fnr regulon encodes enzymes for microaerobic or anaerobic respiratory growth, including synthesis of haem, the high-affinity cytochrome *cbh3*-type oxidase, metal cation transporters, the OmpW porin and the universal stress protein UspA. To our knowledge none of the *Burkholderia* Crp/Fnr regulators has been characterized, but it is interesting to observe that genes Bmul_2345, encoding a heavy-metal-translocating P-type ATPase, Bmul_5381, encoding a UspA protein, Bmul_0982, encoding a coproporphyrinogen oxidase, *ompW* and *narGHJI*, encoding nitrate reductase, all showed decreased expression in D2214. The decreased expression of genes encoding products such as the Fnr-type regulator and nitrate reductase in D2214 suggests that this isolate is less adapted to survive under low oxygen tension. To test this hypothesis, isolates D2095 and D2214 were grown in SM medium at 37 °C under microaerophilic conditions. Growth curves (Fig. 1a) confirmed the slower growth rate and lower biomass formed by D2214 when compared with D2095 during the 48 h of growth.

Genes involved in energy and central intermediary metabolism

We found decreased expression of several genes involved in central metabolism and energy production in D2214, suggesting lower energetic needs of this isolate. For instance, a decrease of the expression of some *smoEFGK* genes involved in the uptake of mannitol, the carbon source provided in our experiment, was observed. Similarly, *mtlK*, encoding a mannitol dehydrogenase converting D-mannitol into D-fructose, and the fructokinase-encoding gene Bmul_0700, converting D-fructose into D-fructose 6-phosphate, showed decreased expression in

D2214 (Table 3). Another set of genes encoding a putative monosaccharide transporter encoded by Bmul_3783 to Bmul_3785 (Table 3) showed decreased expression in D2214, reinforcing the idea of reduced sugar uptake needs.

Concerning other carbohydrate metabolism reactions, a decrease in expression of many genes encoding enzymes leading to the cepacian nucleotide sugar precursors was observed. Besides decreased expression of *bceACMNT*, the expression of genes Bmul_2501 and Bmul_2506, encoding putative UDP-glucose epimerases converting UDP-glucose into UDP-galactose, was decreased in D2214. A set of genes with increased expression in D2214 are putatively involved in osmotic stress response and encode an ABC transporter (Bmul_3511 and Bmul_3512) and a protein putatively involved in the synthesis of the osmoprotectant α -trehalose (*otsA*) (Table 3). Regarding energy production and conversion, three genes (Bmul_1434–Bmul_1436) encoding products involved in the formation of acetyl-CoA from pyruvate showed decreased expression in D2214 (Table 3). That may indicate a lower availability of acetyl-CoA for the tricarboxylic acid cycle and consequently a reduction of anabolic pathways and ATP synthesis.

Many genes involved in nitrogen metabolism had decreased expression in D2214. That is the case for a cluster of six genes probably involved in anaerobic nitrate respiration. Those genes seem to be an operonic structure composed of *narGHJI* encoding nitrate reductase, gene Bmul_5387 encoding a peptidyl-prolyl *cis-trans* isomerase and *narK* encoding a nitrate/nitrite antiporter. Since gene expression was measured under aerobic conditions, the decreased expression of genes involved in nitrate respiration may suggest that D2214 has a tighter control on the expression of genes whose products are not necessary for bacterial survival, but may also reflect the lower ability to survive under low oxygen as we have shown. Other genes involved in nitrogen metabolism with decreased expression are required for biosynthesis of glutamate, a central metabolite providing nitrogen for the synthesis of all other nitrogen-containing components. These were the glutamate synthase-encoding genes *gltBD* as well as glutamate dehydrogenase *gdhA*. Although not to a great extent, the differential expression of genes encoding enzymes for amino acid biosynthesis was primarily decreased in D2214. Decreased expression in D2214 of Bmul_1255, encoding the primary nitrogen sensor GlnD, was also observed (Table 3). D2214 probably senses an excess of nitrogen, and therefore many genes related to its metabolism have decreased expression. Together, these results may reflect the lower need for nitrogen and carbon compounds by D2214. This may reflect the lack of EPS biosynthesis burden, although cells were collected several hours before high-molecular-mass EPS was detected in the supernatant, or a broader adaptation mechanism to save energy.

To evaluate whether the metabolic differences observed at the transcriptional level between D2095 and D2214 could give rise to different survival ability, both cultures were

aerobically incubated for a prolonged period (28 days) under nutrient starvation in minimal medium without a carbon source. C.f.u. counts showed a higher survival rate of D2214 over the full incubation period (Fig. 6).

DISCUSSION

One of the conclusions derived from our analysis is the confirmation that during the course of chronic lung colonization by *Burkholderia*, genetic or epigenetic changes leading to development of new bacterial phenotypes arise. These different phenotypes are much more than just mucoid variation and have repercussions in other virulence traits and metabolism. The combined analysis of transcriptional profiling and phenotypic assays allowed us to observe a general reduction of the expression of several virulence factors in the non-mucoid D2214 isolate when compared with the clonal mucoid isolate D2095. This was observed for swimming and swarming motility, type VI secretion, haemolysin-type protein secretion and EPS production. To evaluate whether these changes had an effect on *Burkholderia* virulence, we chose *G. mellonella* as an animal model for acute infection assessment (Seed & Dennis, 2008). Our results showed that D2214 displayed reduced acute virulence when compared with D2095 but it was still able to kill approximately 90 % of the larvae within the time period of the experiment and for the highest cell density tested. Another phenotype displayed by D2214 was a decrease in resistance to some β -lactam antibiotics. This is an unusual result since due to the antibiotic therapies administered to CF patients bacteria tend to develop higher resistance. The lower resistance level of D2214 isolate against some β -lactam antibiotics may be due to pleiotropic effects of the mutation(s) that have occurred in this isolate and is most likely not relevant clinically. Isolate

D2214 forms a biofilm with more biomass than D2095 and, despite a lower growth rate under microaerophilic conditions, this effect is more pronounced under this low oxygen tension. This result of biofilm formation ability is in accordance with the lower motility and chemotactic responses displayed by D2214 and may indicate a preference of this bacterium to live a sessile lifestyle, especially if areas of low oxygen tension are available within the lungs and/or chemotactic gradients of nutrients are absent. Similarly, biofilm formation by the non-mucoid *B. cenocepacia* C8963 isolate was shown to be higher than in the highly mucoid C9343, both sequential clonal isolates from a CF patient (Conway *et al.*, 2004). Nevertheless, our result was unexpected due to previous studies showing the positive effect on biofilm formation of the presence of EPS production in *B. cepacia* IST408 CF isolate (Cunha *et al.*, 2004; Ferreira *et al.*, 2007). It is possible that in D2214, especially under microaerophilic conditions, other determinants of biofilm formation may become relevant.

When growing aerobically in rich medium, D2095 and D2214 showed a similar growth rate. In addition, no significant differences in metabolizing 95 sources of carbon and nitrogen present on Microlog GN2 panels were detected for the two isolates (data not shown). Nevertheless, expression data from cells growing under the same conditions showed decreased expression of many genes involved in carbon and nitrogen metabolism in isolate D2214. This shutdown of unnecessary transcripts and proteins may reflect lower energetic needs since less energy has to be expended on motility and polysaccharide production, two very energetically demanding processes. It is also possible that the decreased expression of many genes involved in metabolism is an adaptation to thrive in an environment, such as the CF lung, where the availability of nutrients varies and other bacterial competitors are present. That could explain the improved survival of D2214 under nutrient starvation for a prolonged period of time.

The first pair of sequential isolates of *B. cenocepacia* that have been characterized were recovered within 10 months of each other from a chronically infected CF patient (Conway *et al.*, 2004; Zlosnik & Speert, 2010). The earliest isolate, C8963, was non-mucoid and the latest, C9343, was very mucoid. The comparison of C8963 with C9343 by PFGE showed several *SpeI*-digested chromosomal fragments of a different size (Conway *et al.*, 2004) and C9343 had mutations in the gene *cepR*, encoding a quorum-sensing regulator, and deletion of a region from the pathogenicity island present mostly in *B. cenocepacia* (McKeon *et al.*, 2011; Zlosnik & Speert, 2010). Our data from the differential genomic DNA hybridization of D2095 and D2214 against the *B. multivorans* ATCC 17616 genome also demonstrated genetic variation within a region of chromosome 2, resulting in deletion and duplication of genes, confirming that genotypic variation occurs during the course of chronic infection by bacteria. Results from transcriptomic and proteomic analyses in those *B. cenocepacia* isolates showed that the non-mucoid isolate,

Fig. 6. Ability of *B. multivorans* isolates D2095 and D2214 to survive starvation in M63 minimal medium without a carbon source. Survival was assessed by c.f.u. determination on LB agar incubated at 37 °C for 48 h under aerobic conditions. Error bars indicate SD. A *P*-value of <0.034 was considered significant compared with D2095 (*).

C8963, had increased expression of virulence factors such as the nematocidal protein AidA and the zinc metalloprotease ZmpA, and was more resistant to oxidative stress (Zlosnik & Speert, 2010). The genes and proteins with lower expression in C8963 were related to metabolism and transport (Zlosnik & Speert, 2010). In addition, non-mucoid C8963 bound more efficiently to cells of the immune system and displayed a higher clearance rate from the lungs of BALB/c mice when compared with the mucoid C9343 isolate (Conway *et al.*, 2004). In our pair of *B. multivorans* isolates D2095 and D2214 we observed the same general decreased expression of genes involved in metabolism and transport functions, but also a clear shutdown of the expression of some genes involved in virulence traits. This probably explains the slower mortality rate that we observed for *G. mellonella*. Since two different infection models were used to assess virulence of C8963/C9343 (Conway *et al.*, 2004) and D2095/D2214, it is not possible to compare the results obtained in the two cases.

Considering the mucoid phenotype displayed by isolates *B. multivorans* C5568 to D2095, it is likely that during the 13 years of lung colonization some fitness-improving mutations may have occurred in these bacterial genomes, although they did not impair the bacteria's ability to produce EPS. By contrast, in isolate D2214, additional changes must have occurred so that not only was polysaccharide production ability suppressed but many other phenotypic traits also showed considerable variation. What does this mean in terms of bacterial fitness to persist in the CF lung? Considering that the main characteristics of the CF lung environment are the host immune system, antibiotic therapies and substrate composition, isolate D2214, by its ability to decrease the expression of several virulence traits and perhaps better escape from the immune system, to form more biofilm, and a better management of nutrient resources, may have increased fitness under all those circumstances. These findings are in agreement with the observed inverse correlation between the ability of *Bcc* bacteria to produce EPS and rate of decline in CF lung function (Zlosnik *et al.*, 2011). Nevertheless, other pairs of mucoid/non-mucoid clonal isolates from other *Bcc* species should be tested to evaluate the possibly increased fitness of the non-mucoid variants.

Data obtained from the transcriptional analysis do not allow us to suggest any specific mechanism behind the morphotype variation observed between D2095 and D2214. It could result from the additive effects of several mutations, or from a mutation in a particular regulatory gene with profound pleiotropic effects. Since hypermutable strains of *P. aeruginosa* are common in persistently infected CF patients (Oliver *et al.*, 2000), we sequenced the *mutS* and *mutL* genes, involved in DNA mismatch repair, of D2095 and D2214, but no nucleotide differences were found. Therefore, full DNA sequence determination of both genomes is required. Some other mechanisms responsible for genotypic and phenotypic variation within CF isolates of *P. aeruginosa* obtained from colonized lungs are well known

(Huse *et al.*, 2010; Smith *et al.*, 2006). Genome sequence determination of two *P. aeruginosa* isolates from one CF patient, recovered with a time offset of 90 months, showed several mutations in regulators and virulence factors in the last isolate, including O-antigen biosynthesis, type III secretion, motility, multidrug efflux, osmotic balance, phenazine biosynthesis, quorum sensing and iron acquisition (Smith *et al.*, 2006). Analysis of such mutations in a large collection of isolates showed that the most frequently mutated genes were *mexZ*, a negative regulator of an efflux pump, and *lasR*, the primary regulator of quorum sensing (Smith *et al.*, 2006). Another mutation that has stronger implications in disease progression is in the regulatory gene *mucA* that increases alginate production (Martin *et al.*, 1993; Rau *et al.*, 2010). No protein homologue to anti-sigma factor MucA can be found in *Burkholderia* genomes and the player(s) directly regulating EPS production in these micro-organisms remain(s) unknown. Although the differences at the genetic or epigenetic level giving rise to phenotypic variability in CF isolates are not yet known, our results point to some mechanisms used by *Bcc* bacteria for persistence in the lung and will hopefully help to identify vulnerabilities and potential targets for future antimicrobial agent development against these micro-organisms.

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