Growth on mannitol-rich media elicits a genome-wide transcriptional response in *Burkholderia multivorans* that impacts on multiple virulence traits in an exopolysaccharide-independent manner

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In common with other members of the *Burkholderia cepacia* complex (BCC), *Burkholderia multivorans* is capable of producing exopolysaccharide (EPS) when grown on certain mannitol-rich media. The significance of the resulting mucoid phenotype and the genome-wide response to mannitol has never been characterized despite its clinical relevance following the approval of a dried-powder preparation of mannitol as an inhaled osmolyte therapy for cystic fibrosis (CF) patients. In the present study we defined the transcriptional response of *B. multivorans* ATCC 17616, a model genome-sequenced strain of environmental origin, to growth on mannitol-rich yeast extract media (MYEM). EPS-dependent and -independent impact of MYEM on virulence-associated traits was assessed in both strain ATCC 17616 and the CF isolate *B. multivorans* C1576. Our studies revealed a significant transcriptional response to MYEM encompassing approximately 23% of predicted genes within the genome. Strikingly, this transcriptional response identified that EPS induction occurs in ATCC 17616 without the upregulation of the *bce-I* and *bce-II* EPS gene clusters, despite their pivotal role in EPS biosynthesis. Of approximately 20 differentially expressed putative virulence factors, 16 exhibited upregulation including flagella, ornibactin, oxidative stress proteins and phospholipases. MYEM-grown *B. multivorans* also exhibited enhanced motility, biofilm formation and epithelial cell invasion. In contrast to these potential virulence enhancements, MYEM-grown *B. multivorans* C1576 showed attenuated virulence in the *Galleria mellonella* infection model. All of the observed phenotypic responses occurred independently of EPS production, highlighting the profound impact that mannitol-based growth has on the physiology and virulence of *B. multivorans*.

INTRODUCTION

*Burkholderia multivorans* is a member of the *Burkholderia cepacia* complex (BCC), a group of at least 18 closely related Gram-negative bacterial species that are widely distributed in the natural environment, including soil, water and the plant rhizosphere (Vial et al., 2011). The BCC is also established as a frequent cause of chronic respiratory infection in patients with cystic fibrosis (CF), and is consistently identified as an independent risk factor for mortality within this patient group (Liou et al., 2001; Jones et al., 2004; Kalish et al., 2006). Although most commonly causing a gradual deterioration in lung function like other CF pathogens, BCC can also uniquely cause systemic CF infection termed cepacia syndrome, characterized by necrotizing pneumonia, bacteraemia and sepsis (Isles et al., 1984). The majority of BCC infections in CF patients (typically at least 70%) are caused by *Burkholderia cenocepacia* and *Burkholderia multivorans*, and whilst *B. cenocepacia* previously accounted for the majority of cases, in recent years the incidence of *B. multivorans* infection has surpassed that of *B. cenocepacia* (Govan et al., 2007; LiPuma, 2010). *B. cenocepacia* typically has a greater impact on patient survival than *B. multivorans* (Courtney et al., 2004; Jones et al., 2004), although certain strains of *B.
*multivorans* have been associated with high morbidity and mortality (Whiteford et al., 1995), and both species have been associated with cepacia syndrome (Zahariadis et al., 2003; Jones et al., 2004; Shafiq et al., 2011). In addition to their recognition as CF pathogens, both *B. multivorans* and *B. cenocepacia* have also been responsible for outbreaks amongst hospitalized non-CF patients, often being associated with poor patient outcomes (Woods et al., 2004; Mann et al., 2010; Graindorge et al., 2010; Hanulik et al., 2013).

The present study focuses on defining the genome-wide response of *B. multivorans* to growth on mannitol-rich yeast extract medium, MYEM (Sage et al., 1990). Mannitol is one of several sugars and sugar alcohols that can induce the overproduction of exopolysaccharide (EPS) by members of the BCC, resulting in a profoundly mucoid phenotype (Bartholdson et al., 2008), and MYEM has been widely used in studies defining the biological and structural properties of this EPS. The most common EPS produced by members of the BCC is cepacian (Herasimenka et al., 2007), the biosynthetic enzymes for which are encoded by the *bce*-I and *bce*-II gene clusters (Moreira et al., 2003; Ferreira et al., 2010). Whilst EPS has been identified as a putative virulence factor within the BCC (Conway et al., 2004; Cunha et al., 2004; Bylund et al., 2006), it has been reported that an inverse correlation exists between the amount of EPS produced and the rate of lung function decline in CF patients, with the presence of nonmucoid isolates linked to the greatest decline in the patients studied (Zlosnik et al., 2011). However, the precise role played by EPS during infection of the CF lung remains unclear, and direct evidence for EPS production by BCC within the lung is currently lacking. The fact that mannitol promotes EPS biosynthesis has also gained further clinical relevance following the approval of a dry-powder preparation of mannitol for use as an inhaled osmolyte in CF patients (Jaques et al., 2008; Daviskas et al., 2010; Teper et al., 2011; Bilton et al., 2011). Since colonization with BCC was amongst the exclusion criteria for participation in the mannitol powder clinical trials, the impact of this osmolyte therapy on the course of infection in *Burkholderia*-infected patients has yet to be determined.

Irrespective of the role played by EPS during BCC infection of the CF lung, it is now apparent that growth on MYEM can have EPS-independent effects on *B. multivorans* that can impact on virulence-associated traits. A comparison of the ‗mannitol response‘ of isogenic EPS-proficient and EPS-deficient strains recently revealed that the adherence of a clinical isolate of *B. multivorans* to mucin was enhanced following growth in MYEM (Denman & Brown, 2013). This enhanced adherence was independent of EPS production and was instead associated with the upregulation of newly identified fimbrial and afimbrial adhesins. In the present study, we adopted an expanded experimental approach to assess the EPS-dependent and EPS-independent effects of MYEM on a wider range of virulence traits in *B. multivorans*. In addition, microarray analysis was performed to define the genome-wide transcriptional response to growth on mannitol. Our observations reveal that growth of *B. multivorans* in the mannitol-containing MYEM has a profound effect on the transcriptome and resulting phenotype of the bacterium, influencing biofilm formation and resistance, epithelial cell invasion, motility, and virulence in the *Galleria mellonella* infection model. This phenotypic response appears largely independent of EPS production, highlighting that EPS production and the resulting mucoid phenotype is only one of many markers worthy of further study in the extensive genome-wide response to growth on mannitol.

**METHODS**

**Bacterial strains and growth conditions.** The study used two representative isolates of *B. multivorans*, namely the environmental isolate ATCC 17616 and the CF isolate C1576, both of which belong to the previously published experimental strain panel (Mahenthiralingam et al., 2000). To enable discrimination between EPS-dependent and EPS-independent effects, EPS-deficient mutants of each strain were studied alongside the relevant parental strain. These EPS-deficient mutants, C1576(*ΔbceB*) and ATCC 17616(*ΔbceB*), were generated previously through the insertional inactivation of the *bceB* gene of the *bce*-I gene cluster (Denman & Brown, 2013).

Bacteria were routinely cultured at 37 °C in Luria–Bertani (LB) broth (containing 5 g l⁻¹ NaCl) supplemented with 1.5 % (w/v) agar as required. Liquid cultures were incubated with shaking (250 r.p.m.). For routine culture of the *bceB* mutants, media was supplemented with 100 μg ml⁻¹ trimethoprim. Sugar media, based on that described previously (Sage et al., 1990), contained 0.2 % w/v yeast extract (Oxoid) supplemented with either 2 % w/v D-mannitol (for EPS-inducing conditions, MYEM media), or 2 % w/v D-mannose for non-EPS-inducing conditions, defined as non-mannitol (mannose equivalent) yeast extract media for the purpose of this study (NM-YEM). All strains employed in this study exhibited comparable growth rates within the sugar media used.

**Biofilm assays.** Biofilm formation was assessed using the 96-well plate and accompanying pegged lid of the minimum biofilm eradication concentration (MBEC) Assay device (Innovotech). This was performed as described previously (Denman & Brown, 2013), with the exception that biofilms were grown in MYEM or NM-YEM, and biofilm biomass was assessed after 24 h. Biofilm formation was assessed in at least three independent experiments, with 16 replicate wells per strain per experiment.

To assess the resistance of the resulting biofilms to tobramycin, pegs harbouring biofilms were rinsed twice in PBS before transfer to a fresh 96-well plate containing 200 μl of 8 μg ml⁻¹ tobramycin sulphate (in PBS) per well, or PBS alone (control wells). Following 24 h incubation at 37 °C, cells were removed from pegs using an ultrasonic cleaner (Branson, Bransonic Model 2510) into a fresh 96-well plate containing 200 μl PBS per well. Surviving c.f.u. were quantified by plating appropriate dilutions onto LB agar, with percentage survival calculated relative to the untreated biofilms.

**Galleria mellonella infection model.** Following growth of bacteria in MYEM or NM-YEM, infection of larvae with 10⁷ c.f.u. was performed as described previously (Denman & Brown, 2013) and larval survival monitored over a 72 h period. For each strain and culture condition, ten larvae were inoculated per experiment in at least three independent experiments.
Hydrogen peroxide resistance assay. Resistance to hydrogen peroxide (H₂O₂) exposure was assessed as described previously (Lefebre & Valvano, 2001). In brief, cells from overnight cultures were harvested, washed and resuspended in PBS, and diluted into LB broth to a density of 10⁸ c.f.u. ml⁻¹. Hydrogen peroxide was added to a final concentration of 5 mM, with an equivalent volume of water added to controls. Following 30 min incubation at 25 °C, serial dilutions were plated on LB agar to enable quantification of c.f.u. and calculation of percentage survival relative to untreated controls.

Swimming motility. Overnight cultures of bacteria were harvested, standardized to an OD₅₉₀ of 1.0 in PBS, and adjusted to approximately 5 × 10⁶ c.f.u. ml⁻¹. This bacterial suspension was used to inoculate (via a sterile pipette tip) mannose- or mannitol-containing sugar media supplemented with 0.3 % w/v agar. Plates were incubated at 37 °C for 48 h prior to measuring motility.

A549 epithelial cell invasion assay. A549 human alveolar epithelial cells (ATCC CCL-185) were cultured according to ATCC guidelines. The method for evaluating invasion of A549 cells by B. multivorans was based on that described previously (Burns et al., 1996). Bacteria grown overnight in MYEM or NM-YEM were harvested, washed and resuspended in pre-warmed L-15 medium, prior to addition to A549 monolayers (m.o.i. 50). Bacteria were allowed to invade for 2 h (37 °C, static incubation), following which the epithelial cells were washed and overlaid with L-15 medium supplemented with cetazidime (1 mg ml⁻¹) and kanamycin (500 µg ml⁻¹). After 2 h, epithelial cells were washed and lysed with 0.25% (v/v) Triton X-100. Appropriate dilutions of the resulting lysate were plated onto LB agar for enumeration of c.f.u., and invasion frequency was calculated relative to the input.

Transcriptional profiling of the mannitol response. Microarray analysis was performed using custom-made 4 × 44 K microarrays based on the genome of B. multivorans ATCC 17616, using genomic DNA as a common reference. Two test conditions were evaluated: (i) growth on MYEM as the specific mannitol-containing medium that induces EPS production in B. multivorans (Bartholdson et al., 2008) and (ii) growth on NM-YEM as the equivalent mannose-containing agar which does not induce EPS production in B. multivorans (Bartholdson et al., 2008). In both cases, the RiboPure-Bacteria Kit (Ambion) was used to extract total RNA from B. multivorans ATCC 17616 following 16 h growth. Additional information on culture conditions is available in Microbiology Online. RNA was DNase-treated prior to analysis on the Agilent Bioanalyzer with RNA 6000 Nano chip (Agilent) to ensure RNA integrity. The processing of test RNA and reference DNA, microarray hybridizations, array scanning and analysis were performed as described previously (Sass et al., 2011). All experiments were carried out with three biological replicates. Differential gene expression (defined as ≥1.5-fold change in MYEM relative to NM-YEM) was determined from the scanned microarray images according to the protocol described previously (Sass et al., 2013). Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MEXP-3971. Bioinformatic analysis of differentially expressed genes and identification of orthologues was performed using the Burkholderia Genome Database (http://www.burkholderia.com; Winsor et al., 2008).

Statistical analysis. A Kaplan–Meier survival plot (GraphPad Prism 6) with log-rank (Mantel–Cox) test and Bonferroni correction was used to compare larval survival within the Galleria mellonella infection model. For all other assays, statistical analyses were performed by one-way ANOVA with Tukey post hoc testing (IBM SPSS Statistics, v. 20). P<0.05 was deemed statistically significant. All experiments were performed at least in triplicate. Only genes with significant differential expression (P<0.05) were reported for the global gene expression analysis of MYEM versus NM-YEM.

RESULTS AND DISCUSSION

Transcriptome analysis of B. multivorans ATCC 17616 reveals a genome-wide response to growth on MYEM

The fact that mannitol induces EPS in B. multivorans whilst mannose does not is striking given that D-mannose and D-mannitol are each a single reaction away from D-fructose, which itself can promote EPS production (Bartholdson et al., 2008) through the action of D-mannose isomerase and D-mannitol 2-dehydrogenase respectively. Both enzymic activities have previously been reported in Burkholderia (Pseudomonas) cepacia (Allenza et al., 1982; Allenza et al., 1990). In the present study, a custom-made microarray was used to assess the transcriptional response of B. multivorans ATCC 17616 to growth on the EPS-inducing, mannitol-containing MYEM, relative to equivalent mannose-containing, non-EPS-inducing NM-YEM medium. Both media supported comparable growth of B. multivorans. (Denman & Brown, 2013), and microarray analysis was used to shed light on the enigma of differential EPS induction, as well as assessing the genome-wide response to growth on MYEM.

Considering the metabolic proximity of mannitol and mannose, we anticipated limited differential gene expression between the two growth conditions. However, unexpectedly, 1460 genes were differentially expressed ≥1.5-fold (625 downregulated, 835 upregulated) in MYEM relative to NM-YEM, representing approximately 23% of the predicted genes within the genome of ATCC 17616. Table S1 (available in Microbiology Online) details selected differentially expressed genes that are discussed in the following sections, whilst Table S2 lists all differentially expressed genes within the dataset. Of the 1460 genes exhibiting differential expression, 885 could be assigned to well-characterized COG (Cluster of Orthologous Groups) categories (Tatusov et al., 2000). The remaining genes (575/1460) were poorly characterized, and are either unassigned (n=365, mostly encoding hypothetical proteins) or categorized as ‘function unknown’ (n=110) or ‘general function prediction only’ (n=100). Table S3 details the distribution of differentially expressed genes within COG categories. The 885 differentially expressed genes assigned to well-characterized COG categories are represented in Fig. 1, which shows the number of genes belonging to each category (in parentheses), and also indicates the proportion of upregulated and downregulated genes within each category. The number of differentially expressed genes that fall within each of the COG categories correlates with the differing proportions of those categories within the entire genome (R²=0.9199; Fig. S1) and did not indicate a transcriptional bias towards particular functional categories.

Consistent with the predicted metabolic requirements of growth on mannitol, the ‘carbohydrate transport and
metabolism’ response included the significant upregulation of genes that are predicted to facilitate the active transport of mannitol and its subsequent conversion to fructose-6-phosphate (which is then reportedly degraded via the Entner-Duodoroff pathway; Allenza et al., 1982). The upregulated genes Bmul_0702 (4.3-fold), Bmul_0703 (4.8-fold), Bmul_0704 (4.5-fold) and Bmul_0706 (1.9-fold) encode proteins that display between 55% and 66% amino acid identity with the MtlE, MtlF, MtlG and MtlK proteins (respectively) of Pseudomonas fluorescens that comprise an ABC maltose/mannitol transporter (Brüninger et al., 1998). We also observed significant upregulation (6.8-fold) of Bmul_0712, an orthologue of mtlD of P. fluorescens (Brüninger et al., 1998) that encodes a mannitol 2-dehydrogenase responsible for the oxidation of mannitol to fructose. In addition, Bmul_0700 (part of the same predicted operon as the mtlEFGK orthologues) was upregulated 5.2-fold; this gene encoded a ribokinase domain and was predicted to be involved in fructose and mannose metabolism, and hence may encode the fructokinase responsible for the phosphorylation of fructose to fructose-6-phosphate.

It has previously been reported that growth in mannitol strongly induces the expression of all genes within the bce-I and bce-II gene clusters of B. multivorans C1576 (Denman & Brown, 2013). Unexpectedly, microarray analysis revealed that the same is not true in B. multivorans ATCC 17616. In ATCC 17616, the bce-I cluster corresponds to genes Bmul_4910 to Bmul_4920, and the bce-II cluster corresponds to Bmul_4604 to Bmul_4613. With the exception of Bmul_4920 (bceA), all genes within the bce-I and bce-II clusters exhibited comparable expression levels on both the mannose- and mannitol-containing media. Bmul_4920 was significantly upregulated on MYEM relative to NM-YEM, but only 2.4-fold. By comparison, in B. multivorans C1576, all genes of the bce-I and bce-II gene clusters were significantly upregulated (≥62-fold) on MYEM when an RNA-seq strategy was used to assess gene expression, with bceA being upregulated >300-fold.

**Fig. 1.** Overview of the transcriptional response of B. multivorans ATCC 17616 to growth on MYEM. The transcriptional response of ATCC 17616 to growth on mannitol-containing MYEM (relative to equivalent mannose-containing media, NM-YEM) was determined by microarray analysis. The figure shows the distribution of the 885 differentially expressed genes (≥1.5-fold) that could be assigned to well-characterized COG categories. The number of genes within each category is indicated in parentheses. Shading indicates the proportion of genes within each category that are upregulated (light grey) or downregulated (dark grey).
(Denman & Brown, 2013). This differing bce induction between strains was confirmed independently by quantitative reverse transcriptase PCR (RT-PCR) analysis of bceB, confirming it to be significantly upregulated in C1576 (193-fold) but not elevated in ATCC 17616. This observation was striking, given that both C1576 and ATCC 17616 are known to produce cepacian upon growth on MYEM (P. Cescutti, Personal Communication; Ferreira et al., 2010), and disruption of the bceB gene in both strains abolishes detectable EPS production, highlighting the pivotal role of the bce gene cluster in the EPS biosynthesis of both strains. However, the role of the bce gene clusters in ATCC 17616 EPS production did not appear to arise from significant changes in their transcriptional activation. No other known EPS biosynthesis-related genes were upregulated in ATCC 17616 following growth on MYEM, and thus the molecular basis for the induction of EPS in this strain remains unknown. It is conceivable that regulation of EPS biosynthesis within ATCC 17616 may occur at a post-translational level, perhaps through the actions of the BceF tyrosine kinase encoded within the bce-I cluster. It has been proposed that phosphorylation of BceF is necessary for the biosynthesis of cepacian (Ferreira et al., 2007).

Defining the EPS-dependent and EPS-independent consequences of the transcriptional response to growth on MYEM

It was clear from the transcriptome analysis of ATCC 17616 that growth on MYEM elicits a very significant genome-wide response when compared to growth on NM-YEM. In order to define the impact of this mannitol response on relevant phenotypes, and to ascertain the extent to which the EPS production contributes to that response, we assessed the impact of the mannitol response on relevant virulence-associated traits of wild-type and corresponding EPS-deficient mutants of B. multivorans ATCC 17616 and B. multivorans C1576. As reported previously (Denman & Brown, 2013), these EPS-deficient mutants lack detectable EPS production following growth on MYEM, as judged by visual scoring of mucoidy and quantitative analysis of EPS extractions (dry weight and Dubois test for sugar content; Dubois et al., 1951). These strains therefore enabled us to determine the contribution of EPS to individual virulence traits following growth on MYEM, as EPS-dependent responses would only be apparent in the wild-type (EPS-producing) strains, whilst EPS-independent responses would be apparent in both the wild-type and corresponding EPS-deficient mutants.

Growth in mannitol enhances motility and invasion of A549 epithelial cells in an EPS-independent manner

As highlighted in Fig. 1, the transcriptional response of ATCC 17616 to MYEM included a significant upregulation of genes associated with cell motility and secretion. There was profound upregulation of genes belonging to both the flagellar assembly and bacterial chemotaxis KEGG pathways, detailed in Table S1. Upregulated genes included representatives of the flaLMNOPQR operon (Bmul_0038 to Bmul_0044, 2.5- to 10.0-fold), the flfEFGHIJK operon (Bmul_3060 to Bmul_3066, 2.7- to 7.2-fold), the flgABCDEFGHIJ operon (Bmul_3012 to Bmul_3021, 2.4- to 14.9-fold) and the motABcheAW-containing chemotaxis operon (Bmul_0162 to Bmul_0172, 1.6- to 10.0-fold). Quantitative RT-PCR analysis on a representative motility-associated gene (motA) confirmed the observed upregulation (9.3-fold and 8.0-fold by microarray and RT-PCR, respectively). Consistent with this transcriptional response, mannitol significantly enhanced the swimming motility of all strains relative to mannose (Fig. 2). This was observed in both wild-type isolates and the corresponding EPS-deficient mutants, indicating it to be independent of EPS status.

As in many bacterial species, the flagella of Burkholderia species are considered virulence factors. The flagella of B. cenocepacia have been shown to be required for full virulence within a murine infection model (Urban et al., 2004) and for efficient invasion of respiratory epithelial cells in vitro (Tomich et al., 2002). Given the enhanced motility of B. multivorans observed in MYEM, we examined whether growth in mannitol influenced the ability to invade A549 alveolar epithelial cells. Growth of ATCC 17616 in MYEM did not significantly influence the ability to invade epithelial cells relative to that observed for mannose-grown bacteria (P>0.05; data not shown). In contrast, the invasion of the CF strain C1576 was significantly enhanced following growth in MYEM (Fig. 3). As with the motility phenotype, this was observed with both the wild-type and EPS-deficient mutant, indicating it to be independent of EPS status. The observed strain difference suggests that the enhanced invasiveness observed following growth of C1576 in MYEM is not attributable to enhanced motility, as this was a common mannitol-induced phenotype in both strains.

**Fig. 2.** MYEM promotes motility of B. multivorans in an EPS-independent manner. Enhanced swimming motility of both B. multivorans strains (C1576 and ATCC 17616) was observed on MYEM supplemented with 0.3 % (w/v) agar. This was observed in the wild-type strains and the corresponding EPS-deficient mutants (ΔbceB), indicating it to be independent of EPS production. ***P<0.0005.
bacterial species, including initiation and/or development of biofilms in numerous Flagellum-mediated motility is also required for the in an EPS-independent manner and enhances tobramycin resistance of biofilms Growth in mannitol promotes biofilm formation epithelial cells, promoting subsequent invasion. These C1576 following growth in MYEM (Denman & Brown, 2013) enhances bacterial attachment of this strain to C1576 mannitol, the bceB mutant, indicating it to be independent of EPS status. *P<0.05; **P<0.005.

One alternative explanation is that the increased expression of fimbrial and afimbrial adhesins previously observed in C1576 following growth in MYEM (Denman & Brown, 2013) enhances bacterial attachment of this strain to epithelial cells, promoting subsequent invasion. These adhesins are not present in ATCC 17616.

Growth in mannitol promotes biofilm formation and enhances tobramycin resistance of biofilms in an EPS-independent manner

Flagellum-mediated motility is also required for the initiation and/or development of biofilms in numerous bacterial species, including Burkholderia pseudomallei (Tunpiboonsak et al., 2010). Given the profound upregulation of flagellar biosynthetic pathways and the enhanced motility observed in MYEM, we assessed whether MYEM also influenced biofilm formation. Consistent with previous literature (Rose et al., 2009), we observed that biofilm formation by B. multivorans C1576 was minimal when compared with ATCC 17616. This was particularly pronounced when using MYEM and NM-YEM media, in which C1576 biofilm formation was negligible (data not shown). As a result, only the ATCC 17616 strain and its corresponding EPS-deficient mutant were evaluated in the biofilm assays.

B. multivorans ATCC 17616 biofilm formation was significantly enhanced in MYEM, relative to NM-YEM (Fig. 4a; P<0.0001). Whilst cepacian has been reported to contribute to the development of thick BCC biofilms (Cunha et al., 2004), we observed that MYEM enhanced biofilm formation in both the wild-type and EPS-deficient mutant, indicating that MYEM can itself promote biofilm formation in an EPS-independent manner. ATCC 17616 exhibited comparable growth in MYEM and NM-YEM (Fig. 52); thus the observed increase in biofilm biomass was not attributable to differing growth rates.

We went on to assess whether mannitol- or mannose-grown biofilms differed in their ability to resist tobramycin treatment. Tobramycin, a positively charged aminoglycoside antibiotic that is frequently used in the treatment of CF lung infections, has been reported as an effective treatment option in cases of BCC infection (Etherington et al., 2003; Middleton et al., 2005). EPS constituents of the BCC biofilm matrix have recently been implicated in tobramycin resistance (Messiaen et al., 2013). Perhaps consistent with this, we observed that MYEM-grown biofilms of wild-type ATCC 17616 were considerably more resistant to tobramycin, displaying a 2.5- to 3-log increase in survival post-tobramycin-treatment relative to mannose-grown biofilms (P<0.05; Fig. 4b). However, our studies suggest that this heightened tobramycin resistance of MYEM-grown ATCC 17616 biofilms was largely independent of EPS production, as a similar trend was apparent in the EPS-deficient bceB mutant (Fig. 4b), although it did not reach statistical significance (P=0.078). Potential differences in the apparent role of EPS between our study and that of Messiaen and colleagues likely reflect the differing strains and growth media used, which may result in differing EPS constituents within biofilms. It is worth noting that the tobramycin concentration used to challenge the biofilms in the present study (8 μg ml⁻¹ tobramycin sulphate in PBS) was considerably lower than the tobramycin MIC for this strain (MIC 64 μg ml⁻¹; based on triplicate MIC determination by agar incorporation method in Mueller–Hinton agar). However, a concentration of 8 μg ml⁻¹ is within the range of tobramycin concentrations achieved within the airways of CF patients (Baran et al., 1990), and we observed that stationary phase planktomic cells of ATCC 17616 exposed to 8 μg ml⁻¹ tobramycin sulphate in PBS for 24 h (the same conditions as used to challenge the biofilms) exhibit a 2.0 log reduction in viable c.f.u. compared with untreated controls (P<0.005).

In Burkholderia species, a major determinant of aminoglycoside resistance is the RND-family efflux pump, AmrAB-OprA (Moore et al., 1999; Hamad et al., 2010). However, the genes encoding the equivalent pump in B. multivorans ATCC 17616 (genes Bmul1614 to Bmul1616) showed no differential expression in the present study, being equally expressed in both growth conditions (data not shown). It is possible that the increased tobramycin resistance of the MYEM-grown biofilms simply reflects the greater biofilm biomass that occurs in MYEM relative to that following growth in equivalent mannose media. It has recently been reported that the extracellular matrix of Pseudomonas
aeruginosa biofilms limits the penetration of tobramycin, such that tobramycin is sequestered by the biofilm periphery (Tseng et al., 2013). If the same is true of B. multivorans biofilms, a greater proportion of biofilm-associated cells would be expected to survive tobramycin challenge in growth conditions that promote thicker biofilms.

Growth of B. multivorans in mannitol results in differential expression of putative virulence genes and attenuation in the Galleria mellonella infection model

In addition to the upregulation of the flagellar assembly pathway referred to above, the genome-wide response to growth on MYEM included the altered expression of approximately 20 other putative virulence determinants, the majority of which (~80%) were upregulated. These virulence determinants, listed in Table S1, were identified on the basis of their being orthologues of putative virulence factors of other Burkholderia species (primarily B. cenocepacia and B. pseudomallei). Upregulated genes included Bmul_3969 (3.8-fold), which encodes the CepI autoinducer synthetase of the CepIR quorum sensing (QS) system responsible for the synthesis of N-octanoyl homoserine lactone (OHL) and N-hexanoyl homoserine lactone (HHL) (Lewenza et al., 1999; Gotschlich et al., 2001). The QS regulon of B. multivorans has not been defined. However, based on extrapolation from literature describing the QS regulon of B. cenocepacia, there is no evidence of significant QS involvement in the response of B. multivorans ATCC 17616 to mannitol-rich media. For example, of 89 OHL-responsive genes reported in B. cenocepacia (Subsin et al., 2007), 72 have orthologues in B. multivorans ATCC 17616 based on annotations within the Burkholderia Genome Database (Winsor et al., 2008). However, of those 72 genes, only 22 were differentially expressed in the present study, and only 11 of those were altered in the same direction as that observed in B. cenocepacia in response to OHL.

Amongst the other virulence factors showing significant upregulation were genes predicted to encode components of the ornibactin biosynthesis and transport pathway (orbA, orbB, orbE, orbG, orbH, orbS and pvdA; 2.1- to 4.1-fold upregulation), which is required for iron uptake and full virulence in B. cenocepacia (Sokol et al., 1999, 2000). There was also modest upregulation of one gene from each of a putative type III and type VI secretion system (1.9- and 1.5-fold, respectively), although in the absence of coordinated upregulation of more components of the secretion system(s), biological significance is considered unlikely. Growth on MYEM also resulted in modest upregulation (1.6 to 2.7-fold) of genes associated with an oxidative stress response, namely those encoding putative SodB and SodC enzymes (Bmul_0753 and Bmul_0860 respectively), a bifunctional catalase/peroxidase enzyme (Bmul_2660) and a monofunctional catalase enzyme (Bmul_2003). None of these genes has been specifically studied within B. multivorans, although orthologues of two

Fig. 4. MYEM promotes biofilm formation and resistance of biofilms to tobramycin in an EPS-independent manner. (a) Both wild-type B. multivorans ATCC 17616 and the corresponding bceB mutant exhibited enhanced biofilm formation in mannitol-containing MYEM relative to the equivalent mannose media. (b) MYEM-grown biofilms exhibited greater resistance to tobramycin than equivalent mannose-grown biofilms. Whilst this increased resistance was statistically significant for wild-type ATCC 17616 (P<0.05), it failed to reach significance for the corresponding bceB mutant (P=0.078). *P<0.05; ***P<0.0005.
of the genes (encoding SodC and the bifunctional catalase/peroxidase) have been shown to protect against oxidative stress in *B. cenocepacia* (Keith & Valvano, 2007). This prompted us to assess whether MYEM-grown *B. multivorans* ATCC 17616 exhibited heightened resistance to hydrogen peroxide killing, but no significant difference was observed relative to NM-YEM-grown bacteria (*P* > 0.05; data not shown). Amongst the genes exhibiting the greatest degree of upregulation within the entire transcriptome dataset were two genes that encode putative phospholipase C enzymes (Bmul_2139 and Bmul_2951, upregulated 10.1- and 17.2-fold, respectively). Bmul_2139 encodes an orthologue of Plc-1 phospholipase of *B. pseudomallei* (~83 % amino acid identity), whilst Bmul_2951 encodes an orthologue of *B. pseudomallei* Plc-2 (~85 % amino acid identity). Both Plc-1 and Plc-2, which are non-haemolytic towards human erythrocytes, are suggested to contribute to *B. pseudomallei* virulence, with Plc-2 playing a particular role in host cell cytotoxicity (Korbsrisate et al., 2007).

Downregulated virulence factors included genes within the phenylacetic acid catabolic pathway (2.5- to 3.3-fold), which were reported to be required for full virulence of *B. cenocepacia in Caenorhabditis elegans* (Law et al., 2008). An orthologue of the hppD gene (Bmul_0223) that is required for the synthesis of a pigment that protects *B. cenocepacia* from *in vitro* and *in vivo* sources of oxidative stress (Keith et al., 2007) was also downregulated (1.7-fold). One of the most downregulated genes within the dataset was Bmul_0682 (80-fold), which is an orthologue of the HtrA protease-encoding gene of *B. cenocepacia* that is required for *in vivo* survival within the rat agar bead model of chronic infection (Flannagan et al., 2007).

Given the altered expression of several putative virulence determinants, we used the *G. mellonella* infection model to assess the overall impact of EPS and/or growth in mannitol on the virulence of *B. multivorans*. Whilst it has been proposed that BCC isolates that are phenotypically non-mucoid in colony morphology on mannitol agar are more virulent than their mucoid counterparts (Zlosnik et al., 2011), there have only been limited studies directly assessing the role of secreted EPS in BCC virulence. In *B. cepacia IST408*, disruption of either bceE or bceF leads to loss of EPS production, but only the bceF mutant exhibits altered virulence, being attenuated within the *G. mellonella* infection model (Ferreira et al., 2013). This attenuation is believed to be due to the wider role of BceF, which encodes a tyrosine kinase, rather than the loss of EPS biosynthesis itself (Ferreira et al., 2013). To date there have been no comparable studies assessing the virulence of isogenic EPS mutants in *B. multivorans* or *B. cenocepacia*, the more clinically widespread BCC species.

In agreement with a recent report by Schmerk & Valvano (2013), we observed that *B. multivorans* ATCC 17616 is avirulent within the *Galleria* model, preventing us from drawing any conclusions about the impact of culture conditions or bceB mutagenesis on the virulence of this strain. Irrespective of the bacterial growth conditions, larvae inoculated with 10^6–10^7 c.f.u. of ATCC 17616 (or its derivatives) exhibited 70–100 % survival 72 h post-infection. In contrast, inoculation of larvae with 10^6 c.f.u. of *B. multivorans* C1576 resulted in consistent killing of all larvae within 72 h post-infection irrespective of the bacterial growth media used (data not shown). Consequently, studies to define the impact of EPS production and/or growth on mannitol on virulence in *Galleria* were only performed with *B. multivorans* C1576.

Despite the potential enhancements of virulence gene expression and relevant virulence traits observed in MYEM-grown bacteria described above, both wild-type and EPS-deficient C1576 exhibited reduced virulence (extended time to larval killing) following growth on mannitol relative to that observed following growth on mannose (Fig. 5; log-rank test and Bonferroni correction; *P* < 0.005). Thus, within the *Galleria* model, growth of *B. multivorans* C1576 in mannitol results in attenuated virulence independent of EPS production. Further studies are required to define the cause of this attenuation. It is also evident that, when grown in the same media, the wild-type and bceB mutant do not differ in their virulence (*P* > 0.05), indicating that the bceB gene and MYEM-induced EPS production do not play a role in the virulence of *B. multivorans* C1576 within the *Galleria* infection model.

**CONCLUSIONS**

Our studies have revealed an extensive whole genome response following the growth of *B. multivorans* on mannitol-containing MYEM, which impacts on numerous virulence-associated traits in an EPS-independent manner. Whilst many of these traits might be individually associated with increased virulence, the overall impact on virulence (based on our observations of *B. multivorans* C1576 in the *G. mellonella* infection model) appears to be one of attenuation. Whilst further studies that encompass a wider representative panel of *B. multivorans* isolates are required to determine how universal this phenomenon is, our observations to date highlight the profound impact that alternative carbon sources such as mannitol can have on virulence traits. In addition, our studies have highlighted that EPS itself is not required for full virulence of *B. multivorans* C1576 within the *Galleria* infection model, consistent with the previous observations made with *B. cepacia IST408* (Ferreira et al., 2013). Furthermore, it is clear from our studies that the transcriptional response of different *B. multivorans* isolates to growth on MYEM is not uniform, and the complex molecular events that underlie the induction of EPS biosynthesis remain to be defined.

The significance of the present study lies primarily in the dissection of the EPS-dependent and EPS-independent response to MYEM media, further enforcing the fact that attempts to define the role of mucoid/EPS production in the virulence of BCC must take into account the wider...
response of the organism to growth on mannitol-containing media. Whilst a dry-powder preparation of mannitol is an approved osmolyte therapy for use in CF patients, it is important to exercise caution when considering the clinical significance of the present study, as clearly the nutritional cues present in the CF airways are not equivalent to those present in MYEM. We have observed that supplementation of synthetic CF sputum medium (SCFM; Palmer et al., 2007) with mannitol supports EPS biosynthesis (C. Denman & A. Brown, unpublished observations). Whether the genome-wide EPS-independent response that we have observed in MYEM occurs to the same extent in mannitol-supplemented SCFM requires investigation at the transcriptomic and phenotypic level. However, on the basis of studies to date, it is clear that alternative carbon sources can profoundly influence virulence-associated traits of *B. multivorans*, as well as a multitude of other differentially regulated genes. In addition to its application as an osmolyte, mannitol and other simple sugars (and their derivatives) are being investigated as antimicrobial therapies. For example, mannitol can potentiate the activity of aminoglycosides, enhancing persister-cell eradication *in vitro* and the treatment of a chronic murine infection model (Allison et al., 2011). Similarly, mannose (in conjunction with other simple sugars) diminishes lung damage and potentiates standard antibiotic therapy in a murine model of *P. aeruginosa* pneumonia (Bucior et al., 2013). In the continued development of such therapies, it is important to consider the wider impact of alternative carbon sources on the bacterial cell, and the linkage between bacterial physiology and virulence.

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**Fig. 5.** MYEM-grown *B. multivorans* C1576 is attenuated within the *Galleria mellonella* infection model in an EPS-independent manner. Larvae infected with MYEM-grown wild-type C1576 (a) and the corresponding bceB mutant (b) exhibited an extended time to death relative to larvae infected with equivalent mannose-grown bacteria (log-rank (Mantel–Cox) test and Bonferroni correction; *P*<0.005). Inactivation of *bceB* did not result in altered virulence relative to the wild-type C1576 (*P*>0.05).


Response of Burkholderia multivorans to mannitol


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