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**Mannitol promotes adherence of an outbreak strain of *Burkholderia multivorans* via an exopolysaccharide-independent mechanism that is associated with upregulation of newly-identified fimbrial and afimbrial adhesins**

Running title: Adhesins of *Burkholderia multivorans*

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The GenBank accession numbers for the loci encoding the putative fimbrial and afimbrial adhesins of *B. multivorans* C1576 are JX191919 and JX191920 respectively.

## Summary

*Burkholderia multivorans*, a member of the *Burkholderia cepacia* complex (Bcc), is an important pathogen of the cystic fibrosis (CF) lung. Mannitol, approved as an inhaled osmolyte therapy for use in CF patients, promotes exopolysaccharide (EPS) production by the Bcc. In the present study, we investigated the role of mannitol-induced EPS in the adherence of *B. multivorans*. We report that mannitol promoted adherence of two representative *B. multivorans* strains. However, whilst this enhanced adherence was largely EPS-dependent in an environmental isolate, it was EPS-independent within a CF outbreak strain, suggesting strain-to-strain variation in adhesins. Genome sequencing of the outbreak strain enabled the identification of two distinct loci encoding putative fimbrial and afimbrial adhesins. The putative fimbriae-encoding locus was found to be widely distributed amongst clinical and environmental *B. multivorans*. In contrast, the locus encoding the putative afimbrial adhesin (of the filamentous haemagglutinin family, FHA) was restricted to clinical isolates. Both loci contributed to biofilm formation and mucin adherence. Furthermore, we report that mannitol promoted expression of both loci, and that the locus encoding the putative FHA-family adhesin is a key determinant of the enhanced adherence observed following growth in mannitol. Our studies provide the first characterization of *B. multivorans* adhesins, and in so doing highlight the strain-dependent role of EPS in the Bcc, and the difficulties in assigning phenotypic traits to Bcc EPS due to the wider response to mannitol. Our observations also highlight the need to monitor the microbiological impact of inhaled mannitol therapy in Bcc-infected CF patients.

## Introduction

Organisms of the *Burkholderia cepacia* complex (Bcc) are problematic opportunistic bacterial pathogens of the cystic fibrosis (CF) lung. Bcc infection is most commonly associated with chronic infection and a gradual deterioration in lung function, and is consistently identified as an independent risk factor for mortality amongst CF patients (Jones *et al.*, 2004; Kalish *et al.*, 2006; Liou *et al.*, 2001). *B. cenocepacia* and *B. multivorans* are the most common Bcc species associated with CF respiratory infections, together accounting for approximately 90% of Bcc infections. Until recently, *B. cenocepacia* was the most commonly isolated species, being associated with epidemic spread amongst CF patients. As a consequence, *B. cenocepacia* has received the most research attention, resulting in the identification of numerous putative virulence determinants (recently reviewed by (Loutet & Valvano, 2010). However, *B. multivorans* has surpassed *B. cenocepacia* in incidence of respiratory infection amongst CF patients in the United States (LiPuma, 2010) and several European countries (Brisse *et al.*, 2004; Coenye & Vandamme, 2003; Govan *et al.*, 2007; Norskov-Lauritsen *et al.*, 2010), and in comparison to *B. cenocepacia*, very little is known about the virulence mechanisms and strategies used by *B. multivorans* within the CF host.

Amongst the putative virulence determinants most studied within the Bcc is exopolysaccharide (EPS). When grown on certain carbon sources (most notably mannitol), Bcc species produce EPS resulting in a mucoid phenotype (Bartholdson *et al.*, 2008). Whilst clinical *B. cenocepacia* isolates are frequently non-mucoid (EPS-negative) as a result of mutations within EPS biosynthetic or regulatory pathways, clinical isolates of *B. multivorans* frequently retain the capacity for EPS production (Zlosnik *et al.*, 2011). EPS production by the Bcc has been associated with bacterial persistence within the lung (Conway *et al.*, 2004),

biofilm formation (Cunha *et al.*, 2004) and inhibition of neutrophil activity (Bylund *et al.*, 2006). Additionally, it has recently been proposed that an inverse correlation exists between the quantity of EPS production by Bcc organisms and the rate of decline in CF lung function, and that infections with non-mucoid Bcc are associated with reduced patient survival (Zlosnik *et al.*, 2011). The fact that mannitol is a potent inducer of EPS production by the Bcc has gained clinical relevance following the approval of a dry powder preparation of mannitol for use as an inhaled osmolyte in CF patients. Whilst this inhaled mannitol therapy has been shown to enhance lung function in CF patients (Bilton *et al.*, 2011; Daviskas *et al.*, 2010; Jaques *et al.*, 2008; Teper *et al.*, 2011), the potential impact on the course of *Burkholderia* infection within the CF lung is unknown, not least because Bcc-colonization is amongst the listed exclusion criteria that prevents participation in clinical trials.

In the present study, we set out to investigate the role played by mannitol and the associated mucoid phenotype in adherence of *B. multivorans*. As reported herein, we observed that mannitol promoted adherence, but there existed strain-to-strain variation in the extent to which EPS contributed to this phenotype. Specific adhesins have not previously been described in *B. multivorans*, and so we sought to identify and characterise the strain-specific adhesins that contributed to these differing phenotypes. Herein we describe the identification and characterisation of two distinct adhesin-encoding loci in an outbreak strain of *B. multivorans*. These loci, which contribute to biofilm formation and mucin adherence, appear specific to *B. multivorans* and show enhanced expression in the presence of mannitol. These observations provide new insight into *B. multivorans* biology and highlight the potential microbiological impact of inhaled mannitol therapy in CF patients.

## Methods

**Bacterial strains, plasmids, and growth conditions.** Bacteria were routinely cultured at 37 °C in Luria-Bertani (LB) broth (containing 5 g/l NaCl) supplemented with 1.5 % agar as required. Liquid cultures (10 ml volumes in 30 ml culture vessels) were incubated with shaking at 250 rpm unless stated otherwise. Strains and plasmids are detailed in Table 1. For routine culture, media were supplemented with trimethoprim (100 µg/ml for Bcc; 50 µg/ml for *E. coli*), tetracycline (100 µg/ml for Bcc; 25 µg/ml for *E. coli*), gentamicin (50 µg/ml for Bcc), or kanamycin (25 µg/ml for *E. coli*) as required. Sugar media contained 0.2 % yeast extract (Oxoid) supplemented with either 2 % D-mannitol (for EPS-inducing conditions; YEM media), or 2 % D-mannose for non-EPS-inducing conditions (Sage *et al.*, 1990). All strains employed in this study exhibited comparable growth rates within the media used.

**DNA sequencing of *B. multivorans* C1576 and identification of loci encoding putative adhesins.** Genomic DNA from *B. multivorans* C1576 was extracted using the PureLink Genomic DNA kit (Invitrogen) and sequenced on an Illumina platform following library preparation using the TruSeq DNA protocol (Illumina). Reads were initially mapped to the *B. multivorans* ATCC 17616 reference genome, enabling identification of unmapped reads (sequences present in C1576 but absent from ATCC 17616). These unmapped reads were subjected to both *de novo* assembly and re-mapping to alternative Bcc reference genomes. Mapping and *de novo* assembly was performed using CLC Genomics Workbench (CLC bio). Loci encoding putative adhesins were located within the mapped/assembled sequence reads by BLASTN analysis, and the identity of the putative adhesins to known sequences was assessed by BLASTN and BLASTP analysis at NCBI and [www.burkholderia.com](http://www.burkholderia.com) (Winsor *et al.*, 2008).

**Transcriptome analysis by RNA-seq.** Using the RiboPure-Bacteria kit (Ambion), total RNA was isolated from bacterial cells harvested from agar plates containing 0.2 % yeast extract supplemented with either 2 % mannitol (YEM) or 2 % mannose (two biological replicates per culture condition). Following DNase-treatment (10 U DNase, 1 hr), RNA quality was assessed on the Agilent 2100 Bioanalyzer, and only samples with RNA integrity values >7.0 were processed further. Ten micrograms of total RNA were subjected to two successive rounds of rRNA depletion using MicrobeExpress (Ambion). Sequencing libraries were prepared from the resulting mRNA-enriched RNA using the TruSeq RNA protocol (Illumina) according to the manufacturer's instructions prior to sequencing on an Illumina HiSeq2000 platform. Mapping of resulting reads to appropriate reference sequences, calculation of RPKM values (reads per kilobase of transcript per million mapped reads) and subsequent expression analyses to identify differentially-expressed genes (two-group unpaired comparison, with statistical analysis performed on proportions using Baggerley's test with FDR-corrected P-values) was achieved using CLC Genomics Workbench with default parameters.

**Construction of mutants and complemented strains.** Insertional inactivation of target genes was performed using the pGP $\Omega$ Tp suicide vector as described previously (Flannagan *et al.*, 2007). In brief, a 400-500 bp PCR product mapping within the target gene was cloned into pGP $\Omega$ Tp to facilitate homologous recombination and thus insertional inactivation of the target gene. *In trans* complementation of the resulting mutants was achieved using the previously described pDA17 vector (Flannagan *et al.*, 2008), a broad-host range plasmid that drives constitutive expression of the cloned gene from the *dhfr* promoter. All plasmids (for mutagenesis and complementation) were mobilised into the appropriate *B. multivorans* recipient strain by triparental mating with the helper plasmid, pRK2013 (Figurski & Helinski,

1979). Transformants were selected using gentamicin in conjunction with either trimethoprim (to select for pGP $\Omega$ TP integrants) or tetracycline (for pDA17 complemented strains). Mutants and complemented strains were confirmed by appropriate PCR validation. All primer sequences are available upon request.

**Adherence to fibronectin.** Bacterial adherence to fibronectin was assessed as described previously (Mil-Homens *et al.*, 2010), with minor modification. Wells of a 96-well polystyrene plate were coated with 150  $\mu$ l of 10  $\mu$ g/ml fibronectin (Sigma) at 4 °C overnight. Bacteria harvested from fresh overnight LB agar plates were standardised to 10<sup>9</sup> CFU/ml in PBS. Fibronectin-coated wells received either 150  $\mu$ l of bacterial suspension, or 150  $\mu$ l PBS (control), and were incubated at room temperature for 2 h, after which wells were washed 4x with 200  $\mu$ l PBS. Plates were baked (60 °C, 45 min), prior to staining of adhered cells with filter sterilized 0.1 % (w/v) crystal violet for 15 min. Following staining, wells were washed 4x with PBS, before solubilising bound crystal violet with 95 % ethanol (30 min). Absorbance (570 nm) of solubilised crystal violet was measured on a BioRad Microplate reader. There were 16 replicate wells per strain in each individual experiment.

**Adherence to mucin.** Mucin adherence assays were performed as described previously (Ammendolia *et al.*, 2010), with minor modification. In brief, 50  $\mu$ l aliquots of 50  $\mu$ g/ml filter-sterilized porcine mucin protein (Sigma) were applied to wells of a 96-well polystyrene microtiter plate and incubated overnight at 37 °C. Bacteria were harvested from LB agar plates or mannitol/mannose sugar broths following overnight incubation, and standardised to 10<sup>9</sup> CFU/ml in PBS. Mucin-coated wells received either 50  $\mu$ l bacterial suspension, or 50  $\mu$ l PBS, and were incubated at room temperature for 3 h, after which wells were washed 10x with 200  $\mu$ l aliquots of PBS. Bound bacteria were released from mucin with sterile 0.25 %



Triton X-100 (Sigma) and enumerated by plating appropriate dilutions (in triplicate) onto LB agar. Six wells were processed per strain in each individual experiment.

**Biofilm assay.** Biofilm formation was assessed using the 96-well plate and accompanying peg-lid of the MBEC Assay device (Innovotech). Bacteria were harvested from LB agar and standardized to  $10^7$  CFU/ml in tryptone soya broth (TSB) (Oxoid). Wells received either 150  $\mu$ l of bacterial suspension or an equal volume of uninoculated TSB. The peg lid was placed on the plate, and the plate incubated at 37 °C for 24 h with shaking (125 rpm). Following 24 h incubation, the peg lid was transferred to a fresh 96-well plate containing pre-warmed TSB, and incubated for a further 24 h (37 °C, 125 rpm). The peg lid was then transferred to a 96-well plate containing 200  $\mu$ l PBS per well, and incubated at room temperature for 2 min to remove loosely-attached bacteria. The peg lid was baked (60 °C, 20 min) prior to being transferred to a 96-well plate containing 200  $\mu$ l of 0.1 % (w/v) crystal violet per well, and incubated for 30 min at room temperature. Three separate wash plates (200  $\mu$ l of PBS per well) rinsed the pegs following staining, and the bound crystal violet was subsequently solubilised with 95 % ethanol (30 min) prior to measuring absorbance at 570 nm.

***Galleria mellonella* infection model.** Infection of larvae was performed largely as described previously (Seed & Dennis, 2008). Larvae were obtained from LiveFoods UK and stored in woodchips at 14 °C prior to use. Using a 25  $\mu$ l 22s gauge gas-tight Hamilton syringe, larvae were injected in the hindmost proleg with either 10  $\mu$ l PBS (control) or 10  $\mu$ l bacterial suspension ( $10^6$  CFU/ml). Following injection, larvae were incubated at 37 °C, and their survival was monitored for up to 72 hours.

**Dot-blot hybridizations.** Genomic DNA was extracted using the Pure Link Genomic DNA Purification kit (Invitrogen) and was normalised to 85 ng/μl in 0.1 M NaOH. Three microliter aliquots of the resulting DNA were replica-spotted onto Hybond-N+ membranes (GE Healthcare) and air-dried. Membranes were rinsed (4 x SSC, 5 min; 0.5 x SSC, 5 min) prior to baking (80 °C, 2 h). The PCR DIG probe Synthesis kit (Roche) was used to generate probes specific for the genes encoding the fimbrial usher protein and HecB-like protein, with the probe sequences corresponding to nucleotides 2515-2940 of JX191919 and nucleotides 12641-13084 of JX191920 respectively. Subsequent pre-hybridisation, hybridisation, washing and detection was performed using the DIG-Easy Hyb, DIG Wash and Block Buffer Set, and CDP-Star (Roche Applied Science) according to manufacturer's instructions.

**Statistical analysis.** All experiments were performed at least in triplicate, with subsequent statistical analysis by one-way ANOVA followed by relevant comparisons of orthogonal contrasts (IBM SPSS Statistics, v. 20).  $P < 0.05$  was deemed to be statistically significant.

## **Results**

### **The contribution of exopolysaccharide to bacterial adhesion is strain-dependent in *B.***

***multivorans.*** We sought to determine the role played by mannitol and the associated mucoid phenotype in the adherence of *B. multivorans*. Of the putative EPS biosynthetic gene clusters typically found within the Bcc, the *bce* gene cluster is the most highly conserved (Bartholdson *et al.*, 2008). It has previously been shown that disruption of the gene encoding the BceB glycosyltransferase eliminates EPS biosynthesis and thus the mucoid phenotype in *B. ambifaria* (Bartholdson *et al.*, 2008). Consequently, to enable the EPS-dependent and EPS-independent effects of mannitol to be determined, we inactivated *bceB* in two representative strains of *B. multivorans* – the environmental isolate *B. multivorans* ATCC

17616, and the CF isolate *B. multivorans* C1576. This CF isolate is the index case of an outbreak within a paediatric CF unit in Glasgow (UK) that was associated with unusually high mortality (Whiteford *et al.*, 1995). This outbreak strain has previously been designated as ST-27 by multi-locus sequence typing (MLST) (Baldwin *et al.*, 2005), and will be referred to as such within this study. As anticipated, disruption of *bceB* in the *B. multivorans* strains resulted in a complete loss of EPS production, as judged by visual scoring of mucoidy and quantitative analyses (dry weight and sugar content) of EPS extractions from wildtype and *bceB* mutants following growth on YEM media (data not shown). Disruption of *bceB* did not impact on the growth of strains under any culture conditions tested.

We used the mucin adherence assay to assess whether mannitol and/or the associated EPS production influenced adhesion of *B. multivorans* strains ATCC 17616 and C1576. Wildtype and *bceB* mutants of each strain were cultured in the presence of either mannitol (EPS-inducing) or mannose (non-inducing), immediately prior to quantifying adherence to mucin-coated wells. As shown in Fig. 1, within the context of our *in vitro* assay, the environmental ATCC 17616 strain adhered considerably better to mucin than the clinical isolate C1576. However, irrespective of their basal level of adherence, mannitol promoted adherence of both strains to mucin (orthogonal contrast, ATCC 17617:  $t = 8.9$ ,  $df = 51$ ,  $P < 0.0005$ ; orthogonal contrast, C1576:  $t = 4.5$ ,  $df = 24$ ,  $P < 0.0005$ ). Mannose was chosen as a non-EPS-inducing control as (in contrast to other sugars/sugar alcohols tested) mannitol and mannose supported comparable overnight growth of bacteria (when used to supplement 0.2 % yeast extract). Mannose has been suggested to act as an anti-adhesive for certain bacterial species, reducing adherence to epithelial cells (Acord *et al.*, 2005). However, it has previously been reported that mannose does not block mucin-binding by *Pseudomonas (Burkholderia) cepacia* (Sajjan & Forstner, 1992). Consistent with this, mucin adherence of our *B. multivorans* strains of

interest was not reduced by pre-treatment with mannose (data not shown), indicating that the differential adherence observed between the mannitol- and mannose-grown cultures is due to mannitol promoting adherence, rather than mannose reducing adherence.

Whilst mannitol promoted the adherence of both isolates to mucin, comparison of the wildtype and corresponding EPS-deficient *bceB* mutants revealed differing roles for EPS. As shown in Fig. 1(a), inactivation of *bceB* in ATCC 17616 significantly impaired this enhanced adherence in the presence of mannitol (orthogonal contrast:  $t = -6.2$ ,  $df = 51$ ,  $P < 0.0005$ ), indicating that it was largely EPS-dependent. In contrast, the enhanced adherence of C1576 observed following growth in mannitol was unaffected by the inactivation of *bceB* (orthogonal contrast:  $t = -0.2$ ,  $df = 24$ ,  $P > 0.05$ ; Fig. 1b), indicating that it was independent of EPS status. The differing role for EPS in adherence within these strains is not due to different levels of EPS production, as both wild-type strains yielded comparable amounts of EPS (typically 8 – 11 mg dry weight per petri dish). These observations led us to sequence the genome of *B. multivorans* C1576 isolate in an attempt to identify genes encoding strain-specific adhesins that may account for this strain-to-strain variation in adherence phenotype.

### **Identification of loci encoding putative fimbrial and afimbrial adhesins in *B.***

***multivorans* C1576.** Illumina genome sequencing of isolate C1576 with subsequent mapping to the ATCC 17616 reference genome allowed us to identify sequences present within C1576 that were absent from ATCC 17616. Analysis of these unmapped reads resulted in the identification of two distinct loci encoding putative fimbrial and afimbrial adhesins (Fig. 2). The sequences of these loci have been deposited in GenBank under the accession numbers JX191919 (fimbrial) and JX191920 (afimbrial).

The putative fimbriae-encoding locus is predicted to encode three putative fimbrial proteins, a FimC chaperone protein and a fimbrial usher protein. The nucleotide sequence of the locus is >99 % identical to an equivalent fimbriae-encoding locus present in *B. multivorans* CGD1, a recently-sequenced isolate from a chronic granulomatous disease (CGD) patient (Varga *et al.*, 2012). In CGD1, the locus is formed by genes BURMUCGD1\_3349 to BURMUCGD1\_3353. The organisation of the locus is identical in the two isolates, and amino acid identity between the corresponding encoded proteins is 99-100 %. A similar locus with the same gene organisation is observed in two other sequenced CGD *B. multivorans* isolates (CGD2 and CGD2M), although the percentage amino acid identity of the encoded proteins compared to those of C1576 is lower (average 83 %). Outside of *B. multivorans*, the predicted fimbrial proteins of *B. multivorans* C1576 typically exhibit 35-55 % amino acid identity with proteins encoded by comparable loci in other sequenced *Burkholderia* or non-*Burkholderia* species.

The locus encoding the putative afimbrial adhesin of *B. multivorans* C1576 is depicted in Fig. 2(b). Based on sequence similarity to representative proteins (Fig. S1 & S2), the locus is predicted to encode components of a two-partner secretion (TPS) pathway responsible for the secretion of an adhesin of the filamentous haemagglutinin (FHA) family. The locus encodes two putative TpsA proteins (264 kDa and 68 kDa respectively) that belong to the FHA-family of outer membrane proteins, although only the larger of these two proteins has a mass consistent with the large exoproteins of the TpsA family. The locus is also predicted to encode a single protein of the TpsB family that likely facilitates secretion of the FHA family adhesin(s). Phylogenetic analysis confirms relatedness of this protein to known TpsB transporter proteins, particularly the HecB protein of *Erwinia chrysanthemi* (Kim *et al.*, 1998; Rojas *et al.*, 2002) (Fig. S3). Consequently, we propose that the TpsB family protein of

*B. multivorans* C1576 is a HecB-like protein. Consistent with this, comparable phylogenetic analysis of the 264 kDa TpsA family protein of *B. multivorans* C1576 shows relatedness to the HecA protein of *Erwinia chrysanthemi* (Fig. S4), although the phylogenetic distances are greater than that observed between the corresponding TpsB proteins. Immediately downstream of the *hecB*-like gene is a gene encoding a putative PpiC-type peptidyl-prolyl cis-trans isomerase (PPIase) protein, predicted to localise to the cytoplasmic membrane. It is conceivable that this PPIase plays a role in the secretion of the FHA family protein, as has been described for a periplasmic PPIase of *Bordetella pertussis* (Hodak *et al.*, 2008).

Analyses of available *Burkholderia* genomes reveals that the closest FHA-related adhesins to that of *B. multivorans* C1576 are found in the environmental isolate *B. ambifaria* MC40-6 (approximately 83 % amino acid identity between corresponding proteins) and *B. phymatum* STM815 (approximately 65 % amino acid identity between corresponding proteins). Amino acid identity to related proteins of non-*Burkholderia* species (including the prototypic FHA/FhaC of *Bordetella pertussis* and the HecA/HecB of *Erwinia chrysanthemi*) is approximately 28-30 %.

**The loci encoding the putative adhesins of *B. multivorans* C1576 differ in their strain distribution.** The analysis of available genome sequences referred to above suggested that the two loci were not widely distributed within the Bcc and wider *Burkholderia* genus. Using dot-blot hybridization, we assessed the distribution of each locus (based on the presence or absence of a representative gene for each) within a wider panel of *Burkholderia* isolates, with a particular focus on clinical *B. multivorans* isolates. Genomic DNA was isolated from a total of 97 Bcc isolates, comprising 83 clinical *B. multivorans* isolates (including 26 representatives of strain ST-27), six environmental *B. multivorans* isolates, and representatives of other Bcc

species (for strain details, see Table S1). In the course of verifying the *B. multivorans* isolates belonging to the ST-27 outbreak strain, MLST analysis revealed that whilst all shared an identical MLST type, this differed by a single nucleotide within the *lepA* locus from the previously published MLST type for *B. multivorans* C1576 (Baldwin *et al.*, 2005). The corrected MLST profile for the ST-27 outbreak strain (represented by the index case C1576) is thus: *atpD* 13, *gltB* 7, *gyrB* 6, *recA* 10, *lepA* 224, *phaC* 42, and *trpB* 6.

Genomic DNA from the assembled strain panel was probed for the presence of genes encoding the putative fimbrial usher and the HecB-like proteins. Based on the presence of these representative genes, results (summarised in Table 2, and detailed in Table S1) reveal that the locus encoding the putative fimbrial proteins is widely distributed amongst both clinical and environmental *B. multivorans* isolates, being observed in 76/89 isolates tested. In contrast, the locus encoding the FHA-family adhesin is more restricted in distribution, being limited to clinical isolates and particularly the ST-27 outbreak strain (occurring in 100% of ST-27 isolates, compared to < 9% of non-ST-27 isolates). None of the non-*B. multivorans* isolates within the strain panel harboured either gene.

**The fimbrial and FHA-family adhesins of *B. multivorans* C1576 contribute to mucin adherence and biofilm formation.** We next sought to evaluate the role of these putative adhesins in adherence and biofilm formation. Individual mutants were generated by insertional inactivation of the genes encoding the putative HecB-like and fimbrial usher proteins (resulting in strains *hecB* CR and *fim* CR respectively, Table 1). Both mutants were complemented *in trans* using the pDA17 constitutive expression vector. Additionally, RT-PCR analysis confirmed that genes flanking the inactivated genes within each of the mutants were still expressed (data not shown).

To evaluate the role of the adhesins in abiotic adherence, we assessed the ability of the strains to adhere to mucin and to the extracellular matrix protein fibronectin. Neither mutant exhibited a significant reduction in fibronectin binding (orthogonal contrast:  $t = -0.3$ ,  $df = 25$ ,  $P > 0.05$ ). In contrast, both adhesin mutants were significantly reduced in their adherence to mucin relative to the wildtype strain (orthogonal contrast:  $t = -6.7$ ,  $df = 85$ ,  $P < 0.0005$ ; Fig. 3a). Appropriate complementation of the mutants (*fim* CO and *hecB* CO) partially restored mucin adherence ( $P < 0.05$  relative to the corresponding mutant strain), albeit not to wild-type levels (Fig. 3a). This partial complementation has been observed previously with the pDA17 vector, including in a recent study of an adhesin-like gene of *B. cenocepacia* (Mil-Homens *et al.*, 2010), and we believe it reflects sub-optimal gene dosage from the complementation vector.

We assessed the ability of all strains to form biofilm using the MBEC biofilm device (Innovotech). As shown in Fig. 3(b), both mutant strains (*fim* CR and *hecB* CR) exhibited significantly impaired biofilm formation relative to wildtype C1576 (orthogonal contrast:  $t = -6.4$ ,  $df = 115$ ,  $P < 0.0005$ ), indicating that both adhesins are important for biofilm formation. Appropriate complementation of both mutants fully restored biofilm formation (Fig. 3b).

Finally, to investigate the role of these adhesins during infection, all strains were assessed within the *Galleria mellonella* infection model, a model that has been used previously to investigate the role of *Burkholderia* adhesins (Mil-Homens *et al.*, 2010). Neither mutant exhibited altered larval killing compared to wildtype C1576, with all strains killing 100% of larvae within 72 hours at an inoculum of approximately  $10^4$  CFU (data not shown). Larvae injected with PBS exhibited 100% survival for the duration of the experiment. Therefore,



individually, neither the putative fimbrial usher protein nor the HecB-like protein are required for full virulence in the *Galleria* model.

### **Mannitol promotes expression of the putative fimbrial and FHA-family adhesins.**

Having shown that growth of C1576 in mannitol promoted adherence to mucin in an EPS-independent manner (Fig. 1b), we assessed whether growth in mannitol increased the expression of either of the putative adhesin-encoding loci characterized above. As part of a wider on-going study of the genome-wide transcriptional response of *B. multivorans* to mannitol, we have performed RNA-seq analysis on the Illumina platform. The full dataset arising from these studies will be published elsewhere. However, analysis of this transcriptome dataset reveals that growth in mannitol promotes expression of both loci encoding the putative fimbrial and afimbrial adhesins (Fig. 4). This is particularly the case for the putative fimbriae-encoding locus, every gene of which is significantly upregulated by growth in mannitol. We also observed significant upregulation of the cepacian biosynthetic gene clusters *bce-I* and *bce-II* (Ferreira *et al.*, 2010) following growth in YEM (Table S1), consistent with the observed induction of EPS biosynthesis on YEM, and the previous report that growth on YEM upregulates *bceE* expression in *B. ambifaria* AMMD (Bartholdson *et al.*, 2008).

### **The putative FHA-family adhesin of *B. multivorans* C1576 contributes to the enhanced adherence following growth in mannitol.**

Having shown upregulation of both the fimbrial and afimbrial adhesins in response to mannitol, we assessed the extent to which the individual adhesins contribute to the mannitol-promoted adherence phenotype observed previously (Fig. 1b). The wildtype and adhesin mutants were cultured in mannose or mannitol-supplemented broth prior to the mucin adherence assay. Relative to wildtype, both

mutants showed impaired mucin adherence following growth in mannose-containing media (orthogonal contrast:  $t = -2.2$ ,  $df = 112$ ,  $P < 0.05$ ), consistent with the results presented in Fig. 3(a), and consistent with a general defect in adherence following inactivation of either adhesin. Despite this lower basal level of adherence, the adherence of the *fim* CR strain was still elevated 5- to 6-fold following growth in mannitol (orthogonal contrast:  $t = -16.6$ ,  $df = 112$ ,  $P < 0.0005$ ). This is consistent with observations of the wildtype, and suggests that the putative fimbrial adhesin does not play a significant role in the enhanced mucin adherence that is induced by mannitol. In contrast, the adherence of the *hecB* CR strain is not enhanced following growth in mannitol (orthogonal contrast:  $t = -0.7$ ,  $df = 112$ ,  $P > 0.05$ ), indicating that the putative FHA-family adhesin contributes to the mannitol-induced mucin adherence.

## Discussion

In this study we have shown that mannitol can promote adherence of *B. multivorans* in an EPS-independent manner that is associated with the upregulation of genes encoding putative fimbrial and FHA-family adhesins. Whilst it is the fimbrial locus that is most significantly elevated following growth in mannitol, it is the locus encoding the putative FHA-family adhesin that appears to be a key determinant of the enhanced adherence. Although perhaps unexpected, this may reflect the fact that the *hecB* gene is predicted to encode the transporter for the FHA adhesin rather than the adhesin itself, and thus *hecB* expression is not a reliable indicator for the amount of mature adhesin on the cell surface.

Whilst adhesins have been described in other members of the Bcc and within the wider *Burkholderia* genus (Balder *et al.*, 2010; Mil-Homens *et al.*, 2010; Mil-Homens & Fialho, 2012; Urban *et al.*, 2005), the present study represents the first investigation of putative fimbrial and FHA-family adhesins within the Bcc, and the first characterisation of any

specific adhesins within *B. multivorans*. Whilst fimbrial and FHA-family adhesins are common within the *Burkholderia* genus, there is considerable diversity at the amino acid level between the adhesins of different species. Such sequence variation within adhesins can profoundly alter their binding specificities and affinities, thus impacting on host tropism and capacity for virulence (Sokurenko *et al.*, 1998; Weissman *et al.*, 2006). *Burkholderia* species are an extremely versatile group of organisms, having been reported as human and animal pathogens, plant pathogens, plant growth promoters and endosymbionts of insects and fungi (for review, see (Vial *et al.*, 2011). The role played by species-specific and strain-specific adhesins in conferring such diverse host- and niche-adaptations of *Burkholderia* remains to be elucidated. Within the present study, we observed that the environmental isolate (ATCC 17616) adhered significantly better to mucin than the clinical isolate (C1576), and further studies are required to identify the molecular basis for this. A heightened capacity for adherence to mucin may promote colonisation of the airways. Epidemiological data suggests that the majority of new cases of *B. multivorans* infection in CF patients are due to acquisition from environmental sources (Brisse *et al.*, 2004; Coenye & Vandamme, 2003; Govan *et al.*, 2007; Norskov-Lauritsen *et al.*, 2010), and it is notable that CF isolates of *B. multivorans* that share the same MLST profile as the environmental ATCC 17616 isolate have been recovered from CF patients (Baldwin *et al.*, 2008).

In contrast to the widely-distributed fimbrial-encoding locus, it is striking that the locus encoding the putative FHA-family adhesin reported herein was found only in clinical isolates. Clearly further environmental isolates need to be studied to test this association more rigorously – the low number of environmental *B. multivorans* included in the assembled strain panel reflect the infrequent isolation of this species from the natural environment (Baldwin *et al.*, 2008). Furthermore, all but two of the clinical isolates detailed in Table S1

that were found to harbour the *hecB*-like gene (representative of the FHA-encoding locus) belong to sequence types known to be associated with patient-to-patient transmission, notably ST-27, ST-25 and ST-179 (Baldwin *et al.*, 2008). Putative transmissibility factors have been identified in other Bcc species (Clode *et al.*, 2000), and whilst their predictive value has been questioned (Govan *et al.*, 2007), our observations justify further studies to investigate the strength of the association of the FHA-encoding locus with transmissible strains, and its potential as a marker for transmissibility amongst clinical *B. multivorans*.

The rationale for this study of adhesins within the *B. multivorans* ST-27 strain stemmed from our observations that whilst mannitol promoted bacterial adherence to mucin, the role played by EPS was strain-dependent. There remains considerable debate about the significance of EPS production as a Bcc virulence factor, fuelled by the fact that arguably one of the most virulent Bcc strains (*B. cenocepacia* ET-12) does not produce EPS and is consistently non-mucoid (Bartholdson *et al.*, 2008; Zlosnik *et al.*, 2008). Our observations from the present study emphasize the difficulty in establishing the role of EPS in virulence, as the growth conditions commonly used to induce and study EPS (the mannitol-containing media, YEM) clearly elicit a response within the bacterium that is wider than the EPS biosynthetic pathway. Consequently, attempts to identify the role of EPS through phenotypic observations of whole organisms are prone to being skewed by the wider EPS-independent response to mannitol, potentially resulting in phenotypic traits being wrongly assigned to the mucoid phenotype. We suggest that the approach undertaken in the present study (parallel studies of the wildtype and an isogenic EPS-deficient mutant) is best-suited for identifying phenotypes truly associated with EPS production, although clearly such an approach depends on having sufficient knowledge of the genetic basis of EPS biosynthesis. Our observation that EPS plays a significant role in the mucin adherence of ATCC 17616 but not the C1576 strain also

highlights the multi-factorial aspect of Bcc virulence. The contribution of individual virulence determinants can vary from strain-to-strain and can be influenced by the presence or absence of other complementary factors. With specific reference to EPS, one consequence of this is that the biological significance of the documented mucoid to non-mucoid transition that is frequently observed in sequential Bcc isolates from individual CF patients (Zlosnik *et al.*, 2008) may be strain-dependent. This may also be influenced by differences within the EPS itself, as the differing roles attributed to the EPS of the two strains studied herein may reflect differences in EPS composition and structure. Whilst beyond the scope of the current study, there is clearly merit in performing a structure-function analysis of *Burkholderia* EPS.

During early pilot studies of inhaled mannitol therapy, it was acknowledged that the ability to utilise mannitol as a carbon and energy source was common amongst both Bcc and *Pseudomonas aeruginosa* (the dominant CF pathogen) (Robinson *et al.*, 1999). However, it was considered unlikely that this would significantly affect bacterial burden in the lung due to the abundance of alternative nutrient sources within respiratory secretions. This appears to have been borne out by clinical trials that have found no difference in sputum microbiology between treatment and control groups (Jaques *et al.*, 2008; Teper *et al.*, 2011). However, the fact that mannitol promotes EPS production, together with our observations that mannitol promotes expression of adhesin-encoding loci that contribute to abiotic adherence and biofilm formation, indicate that administration of mannitol is likely to have profound phenotypic consequences on Bcc within the lung. Furthermore, by enhancing expression of the adhesins, it is conceivable that mannitol may promote the initial colonisation of the airways with Bcc. The full impact of mannitol on relevant virulence traits of the Bcc (and indeed other microbial species commonly found in the CF lung) has yet to be established. However, based solely on observations to date, the phenotypic impact of mannitol on the Bcc

is unequivocal, and close microbiological monitoring of patients receiving inhaled mannitol therapy would appear prudent.

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**Table 1.** Strains and plasmids used in this study.

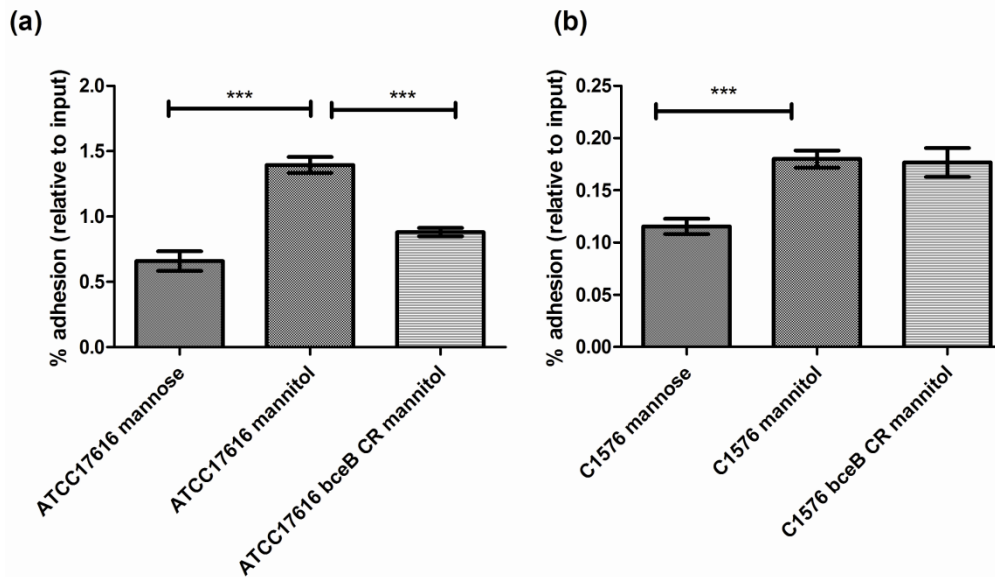
Strain or plasmid	Relevant characteristics	Source and/or reference
<i>B. multivorans</i> strains		
C1576	CF clinical isolate; Index case of outbreak strain, ST-27	(Mahenthalingam <i>et al.</i> , 2000)
<i>fim</i> CR	C1576, pGPΩTp:: <i>fim</i> , Tp <sup>R</sup>	This study
<i>hecB</i> CR	C1576, pGPΩTp:: <i>hecB</i> , Tp <sup>R</sup>	This study
<i>fim</i> CO	C1576, pGPΩTp:: <i>fim</i> , pDA17:: <i>fim</i> , Tp <sup>R</sup> Tet <sup>R</sup>	This study
<i>hecB</i> CO	C1576, pGPΩTp:: <i>hecB</i> , pDA17:: <i>hecB</i> , Tp <sup>R</sup> Tet <sup>R</sup>	This study
C1576 <i>bceB</i> CR	C1576, pGPΩTp:: <i>bceB</i> , Tp <sup>R</sup>	This study
ATCC 17616	Environmental isolate from soil	(Mahenthalingam <i>et al.</i> , 2000)
ATCC <i>bceB</i> CR	ATCC 17616, pGPΩTp:: <i>bceB</i> , Tp <sup>R</sup>	This study
<i>E. coli</i> strains		
TOP10	<i>F mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (Str<sup>R</sup>) endA1 nupG λ<sup>-</sup></i>	Invitrogen
GT115	<i>F mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 rspL (Str<sup>A</sup>) endA1 Δdcm uidA(ΔMluI)::pir-116 ΔsbcC-sbcD</i>	InvivoGen
Plasmids		
pGPΩTp	<i>Ori<sub>R6K</sub></i> , ΩTp <sup>R</sup> cassette, <i>mob</i> <sup>+</sup>	(Flannagan <i>et al.</i> , 2007)
pRK2013	<i>Ori<sub>colE1</sub></i> , RK2 derivative, Kan <sup>R</sup> , <i>mob</i> <sup>+</sup> <i>tra</i> <sup>+</sup>	(Figurski & Helinski, 1979)
pDA17	<i>Ori<sub>pBBR1</sub></i> , Tet <sup>R</sup> , <i>mob</i> <sup>+</sup> , <i>P<sub>dhfr</sub></i>	(Flannagan <i>et al.</i> , 2008)
pGPΩTp:: <i>hecB</i>	pGPΩTp; 444 bp internal fragment from C1576 <i>hecB</i>	This study
pGPΩTp:: <i>fim</i>	pGPΩTp; 426 bp internal fragment from C1576 gene encoding fimbrial usher protein	This study
pDA17:: <i>hecB</i>	pDA17; 1.8 kb fragment encoding the C1576 HecB-like protein	This study
pDA17:: <i>fim</i>	pDA17; 2.7 kb fragment encoding the C1576 fimbrial usher protein	This study

The *fim* and *hecB* nomenclature relates to the genes encoding the fimbrial usher protein and the HecB-like protein (respectively) of *B. multivorans* C1576.

Tp<sup>R</sup>, trimethoprim resistance; Tet<sup>R</sup>, tetracycline resistance; Kan<sup>R</sup>, kanamycin resistance.

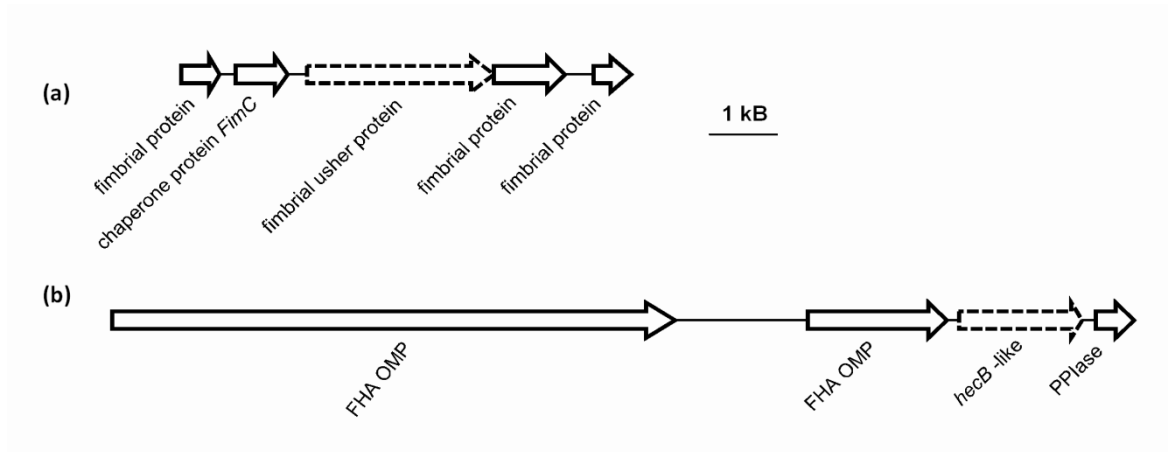
**Table 2.** Distribution of the genes encoding the putative HecB-like and fimbrial usher proteins amongst clinical and environmental *B. multivorans* isolates, as determined by dot-blot analysis. Clinical isolates are sub-divided into ST-27 and non-ST-27.

	Clinical (ST-27)	Clinical (non-ST-27)	Environ.
<i>hecB</i> -like	26/26	5/57	0/6
<i>fim</i>	26/26	47/57	3/6

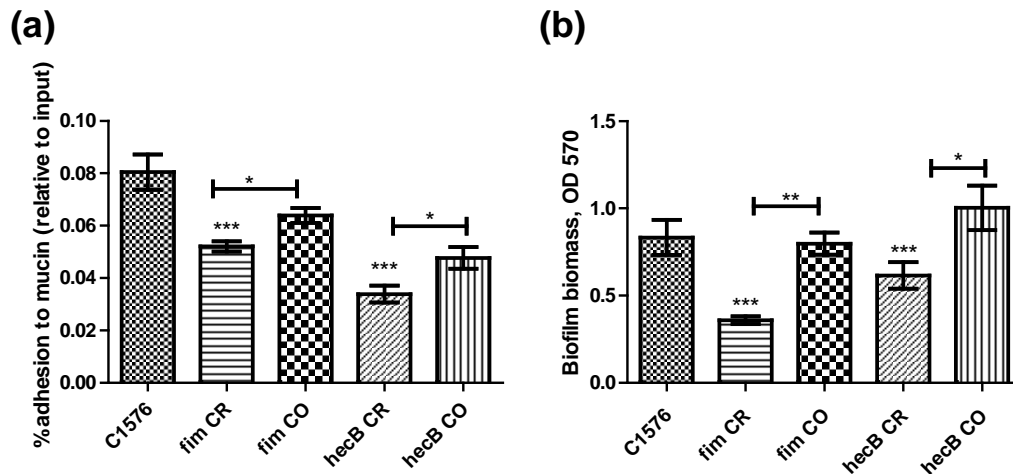


**Fig. 1.** The role of exopolysaccharides (EPS) in promoting the adherence of *B. multivorans* to mucin is strain-dependent.

*B. multivorans* strains ATCC 17616 and C1576 (wildtype and *bceB* mutant of each) were grown overnight in either mannitol-containing media (YEM) or mannose-containing media, prior to assessing adherence to mucin-coated wells. The ATCC 17616 strain adhered significantly better to mucin than the C1576 strain (note the differing scales on the y-axes). However, irrespective of this difference, growth in mannitol promoted the adherence of both strains (relative to growth in mannose). Disruption of the *bceB* gene in ATCC 17616 abolished the enhanced adherence observed following growth in mannitol, indicating it to be EPS-dependent (a). In contrast, inactivation of *bceB* in C1576 did not affect the enhanced adherence, indicating it to be EPS-independent (b). Data correspond to mean of three independent experiments, each in triplicate. Error bars indicate SEM. \*\*\*  $P < 0.0005$ .



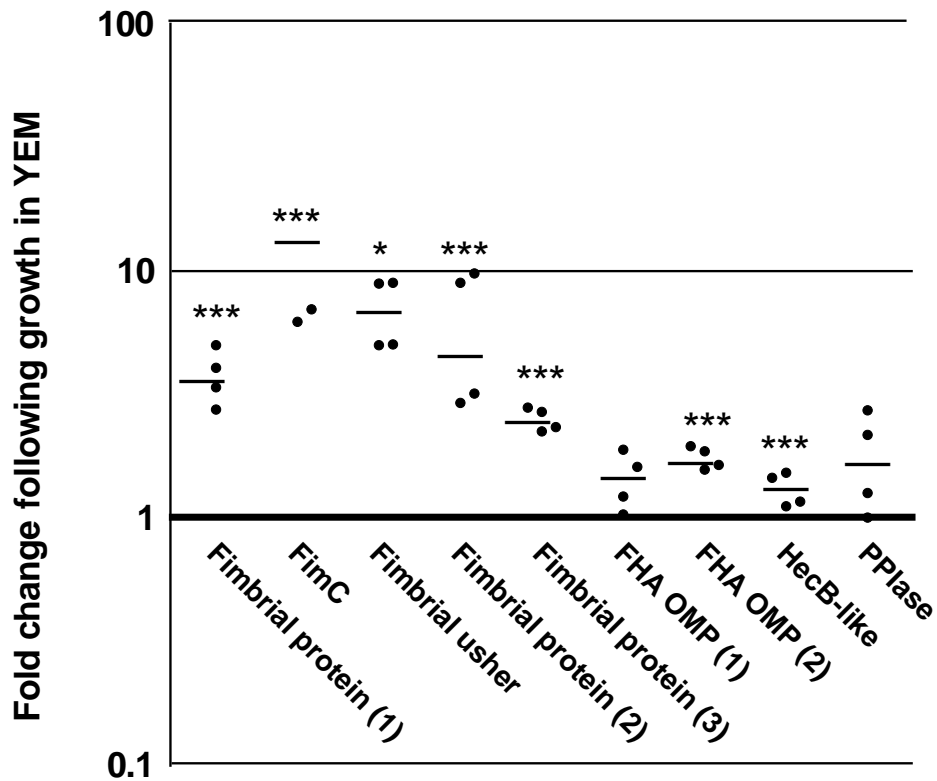
**Fig. 2.** Schematic representation of the *B. multivorans* C1576 loci encoding the putative fimbrial adhesins (a) and the putative FHA family adhesins (b). Refer to text for details. FHA OMP, Filamentous haemagglutinin outer membrane protein; PPIase, PpiC-type peptidyl-prolyl cis-trans isomerase. Dashed outline denotes genes targeted by insertional inactivation.



**Fig. 3.** The putative fimbrial and HecB-like adhesins of *B. multivorans* C1576 contribute to mucin adherence and biofilm formation.

- (a) Both adhesin mutants (*fim* CR and *hecB* CR) are significantly impaired in adherence to mucin relative to wildtype C1576. Complementation of the *fim* and *hecB* mutants partially-restored adherence (*fim* CO and *hecB* CO respectively).
- (b) Both adhesin mutants show reduced biofilm formation relative to WT, with complementation fully restoring biofilm formation.

Graphs show representative data from at least three independent experiments. Mucin adherence results are expressed as percentage adherence relative to input from the mean of six wells per strain. Asterisks alone indicate statistical significance relative to wildtype C1576, whilst asterisks with bracket bar directly beneath compare the adhesin mutant (CR) to the corresponding complemented strain (CO). \*  $P < 0.05$ , \*\*  $P < 0.005$ , \*\*\*  $P < 0.0005$ . Error bars represent SEM.



**Fig 4.** RNA-seq analysis reveals elevated expression of both putative adhesin-encoding loci following growth in mannitol.

The relative expression of the genes of the putative adhesin-encoding loci of *B. multivorans* C1576 was assessed by RNA-seq. The graph shows the fold change in gene expression observed following growth in the mannitol-containing YEM media, relative to the expression level observed in equivalent mannose-containing media. For each gene, data are depicted in two ways: (1) The horizontal bar indicates the fold change that was calculated from the combined analysis of the two biological replicates per condition; (2) the variance in the fold change for each gene is shown by the individual data points (filled circles) that each represent analysis of the biological replicates individually (i.e. a single pairwise mannitol-vs-mannose comparison). The fold change of the *fimC* gene could not be calculated for two of the four pairwise comparisons as one of the mannose-grown samples had an RPKM value of 0. Asterisks denote FDR-adjusted P-values (\*  $P < 0.05$ , \*\*\*  $P < 0.0005$ ), and relate to the



combined analysis of the biological replicates. All genes of the putative fimbriae-encoding locus were significantly upregulated following growth in YEM, with 2.5- to 13-fold increases in expression relative to that observed in mannose-grown cultures. Upregulation of the locus encoding the putative FHA-family adhesins was also observed, albeit to a lesser extent (1.5- to 2-fold). Of those, only the genes encoding the putative 68 kDa FHA-like protein (FHA OMP 2) and HecB-like protein were deemed to be significantly upregulated.