Burkholderia cenocepacia differential gene expression during host–pathogen interactions and adaptation to the host environment

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Members of the Burkholderia cepacia complex (Bcc) are important in medical, biotechnological, and agricultural disciplines. These bacteria naturally occur in soil and water environments and have adapted to survive in association with plants and animals including humans. All Bcc species are opportunistic pathogens including Burkholderia cenocepacia that causes infections in cystic fibrosis and chronic granulomatous disease patients. The adaptation of B. cenocepacia to the host environment was assessed in a rat chronic respiratory infection model and compared to that of high cell-density in vitro grown cultures using transcriptomics. The distribution of genes differentially expressed on chromosomes 1, 2, and 3 was relatively proportional to the size of each genomic element, whereas the proportion of plasmid-encoded genes differentially expressed was much higher relative to its size and most genes were induced in vivo. The majority of genes encoding known virulence factors, components of types II and III secretion systems and chromosome 2-encoded type IV secretion system were similarly expressed between in vitro and in vivo environments. Lower expression in vivo was detected for genes encoding N-acylhomoserine lactone synthase CepI, orphan LuxR homolog CepR2, zinc metalloproteases ZmpA and ZmpB, LysR-type transcriptional regulator ShvR, nematocidal protein AidA, and genes associated with flagellar motility, Flp type pilus formation, and type VI secretion. Plasmid-encoded type IV secretion genes were markedly induced in vivo. Additional genes induced in vivo included genes predicted to be involved in osmotic stress adaptation or intracellular survival, metal ion, and nutrient transport, as well as those encoding outer membrane proteins. Genes identified in this study are potentially important for virulence during host–pathogen interactions and may be associated with survival and adaptation to the host environment during chronic lung infections.

**Keywords:** Burkholderia cenocepacia, Burkholderia cepacia complex, microarray, lung infection, rat chronic respiratory infection model, in vitro, in vivo

INTRODUCTION

Members of the Burkholderia cepacia complex (Bcc) are commonly found in soil and aquatic environments (LiPuma, 2010; Loutet and Valvano, 2010). Seventeen Bcc species have been identified, all of which have the potential to be opportunistic pathogens, although Burkholderia cenocepacia is the most clinically significant. B. cenocepacia causes lung infections resulting in significantly decreased survival rates in cystic fibrosis and chronic granulomatous disease patients (Mahenthiralingam et al., 2005). The organism is intrinsically multidrug resistant and can persist in the lungs of CF patients for many years (Mahenthiralingam et al., 2008). In some patients, infection with B. cenocepacia can progress to what is termed “cepacia syndrome.” Cepacia syndrome is associated with a rapid deterioration in lung function associated with necrotizing pneumonia, bacteremia and sepsis that can result in death (Isles et al., 1984). Many virulence factors have been identified in B. cenocepacia including extracellular enzymes, toxins, secretions systems, iron acquisition systems, cell–cell communication (quorum sensing, QS) systems, regulatory proteins as well as genes contributing to motility, biofilm formation, adhesion, cell invasion, intracellular survival, and bacterial protection from host factors (for review see Loutet and Valvano, 2010). Several infection models have been employed to identify and characterize the contribution of numerous genes to pathogenesis (Uehlinger et al., 2009). B. cenocepacia exhibits virulence against Caenorhabditis elegans (Kothe et al., 2003), Galleria mellonella (Seed and Denn, 2008), Acanthamoeba (Marolda et al., 1999), Dictyostelium discoideum (Aubert et al., 2008), Danio rerio (Vergunst et al., 2009), Drosophila melanogaster (Castonguay-Vanier et al., 2010), and alfalfa seedlings (Bernier et al., 2003). Chronic respiratory infection models have been developed in mice and rats to investigate pathogenesis of Bcc species. The rat chronic respiratory infection model described by Cash et al. (1979) involves transtracheal delivery of agar-embedded bacteria directly into the lung allowing for bacterial persistence and pathology to be measured.
This chronic infection model has been used to identify Bcc species and bacterial strains that persisted or caused lung pathology from less virulent strains such as mutants in ornithinobactin biosynthesis, uptake and utilization, zinc metalloproteases, and genes encoding other enzymes, transcriptional regulators, and lipopolysaccharide (Sokol et al., 1999, 2006; Bernier et al., 2003, 2008; Corbett et al., 2003; Baldwin et al., 2004; Bernier and Sokol, 2005; Kooi et al., 2006; Loutet et al., 2006; Flanagan et al., 2007). These studies have revealed the importance of individual genes or systems to virulence but have not assessed bacterial gene expression during infection.

Transcriptional profiling using custom B. cenocepacia microarrays and RNA sequencing technology have enabled in vitro gene expression studies to be performed at a genome level. Transcriptional profiling has been used to examine gene expression in different environmental conditions such as those mimicking CF sputum or soil, or in response to antimicrobials (Drevinek et al., 2008; Yoder-Himes et al., 2009, 2010; Peeters et al., 2010; Bazzini et al., 2011; Coenye et al., 2011; Sass et al., 2011). In addition to further characterizing genes previously known to be important in virulence, these studies have also identified many genes with potential importance in virulence. Our current understanding of B. cenocepacia physiology, pathogenesis, and survival is incomplete since the B. cenocepacia genome, which is over 8 Mb, contains genes encoding many uncharacterized proteins. Identifying such proteins and determining their functional significance will improve our abilities to target such proteins for therapeutic purposes. To date, no studies have profiled B. cenocepacia gene expression at the whole genome level directly from lung samples, K56-2 cultures were grown at 37°C, in 10 ml Miller’s Luria broth (LB; Invitrogen, Burlington, ON, Canada) with shaking in 125 ml Erlenmeyer flasks to stationary phase (16 h) as previously described (O’Grady et al., 2009). Bacterial growth was assessed by determining the optical density (OD) at 600 nm.

**MATERIALS AND METHODS**

**BACTERIAL STRAINS AND GROWTH CONDITIONS FOR IN VITRO SAMPLES**

*Burkholderia cenocepacia* K56-2 is a CF isolate that belongs to the ET12 lineage (RAPD type 2) and is clonally related to the sequenced strain J2315 (Mahenthiralingam et al., 2000; Baldwin et al., 2004; Holden et al., 2009). To generate in vitro samples, K56-2 cultures were grown at 37°C, in 10 ml Miller’s Luria broth (LB; Invitrogen, Burlington, ON, Canada) with shaking in 125 ml Erlenmeyer flasks to stationary phase (16 h) as previously described (O’Grady et al., 2009). Bacterial growth was assessed by determining the optical density (OD) at 600 nm.

**ANIMAL STUDIES**

Animal infections were performed using the rat agar bead respiratory infection model (Cash et al., 1979). Adult male Sprague-Dawley rats (150–180 g; Charles River, QC, Canada) were inoculated transtracheally with approximately 10^7 CFU of K56-2. At 3 days postinfection, infected lungs were aseptically removed, stored at 4°C overnight in 15 ml of RNA later (Ambion, Streetsville, ON, Canada), and subsequently maintained at −70°C to prevent RNA degradation. Animal experiments were conducted according to the guidelines of the Canadian Council of Animal Care for the care and use of experimental animals under protocol M08089 approved by the University of Calgary Animal Care Committee.

**RNA MANIPULATIONS**

Total RNA from in vitro samples was prepared as previously described (O’Grady et al., 2009) using a RiboPure bacterial RNA isolation kit according to manufacturer’s instructions (Ambion). For in vivo samples, total RNA from infected lungs was isolated using Tri Reagent (Invitrogen) as recommended by the manufacturer. Total RNA samples were enriched for bacterial RNA using a MicrobEnrich kit (Ambion) and purified using a MegaClear kit (Ambion). Enriched and purified bacterial RNA was depleted of 16S and 23S rRNAs using a MicroExpress kit (Ambion) to isolate mRNA according to manufacturer’s instructions to provide enhanced sensitivity for microarray experiments. DNase treatment was performed on all RNA samples using DNA-Free (Ambion), and samples were confirmed by PCR using Taq polymerase (Invitrogen) to be free of DNA prior to cDNA synthesis.

**MICROARRAY ANALYSIS**

In vitro-derived total RNA and in vivo-derived mRNA samples were indirectly labeled with the CyScribe Post-Labeling Kit (GE Healthcare) and cDNA synthesis performed as described by Sass et al. (2011) with the following modifications. Three independent RNA samples were used for in vitro samples and two mRNA samples (each consisting of an mRNA pool isolated from two infected rats to reduce variability between animals) were used for in vivo samples. Approximately 10 µg total RNA was labeled for each in vitro sample and 8 µg mRNA was labeled for each in vivo sample. The reference pool for microarray experiments consisted of B. cenocepacia J2315 genomic DNA isolated and labeled as described (Sass et al., 2011). The B. cenocepacia J2315 custom microarray, with each probe printed four times using the Agilent Sure Print 4×44 microarray platform, was used (Drevinek et al., 2008; Sass et al., 2011). Approximately 700–1000 ng labeled cDNA from the in vitro and in vivo samples and 55 ng labeled control genomic DNA was used per microarray. Hybridization, washing, and scanning were performed as described using the Two Color Microarray Based Gene Expression Analysis Protocol (Agilent) and the data analyzed using GeneSpring GX version 7.3.1. All labeling, hybridization, and scanning were performed by the Mahenthiralingam Laboratory, Cardiff University, Wales. Initial data were preprocessed by employing the enhanced Agilent FE import method. Probes specific to J2315 were filtered on a 1.5-fold change in expression between conditions to identify clusters of differentially regulated genes related to specific functions or potentially organized in operons. To eliminate potential differences in RNA between samples, data were normalized to control samples and mean log2 ratios (in vivo/in vitro) calculated from replicates were used and reported as expression ratios. Mean log2 ratios were also filtered on twofold changes in expression between in vivo and in vitro conditions to identify more stringently differentially regulated genes. The in vitro- or in vivo-derived K56-2
cDNA produced a signal that was detected by at least 94% of the probes on the microarray. Operon prediction and gene annotation or predicted protein function were retrieved from the B. cenocepacia J2315 genome at http://www.burkholderia.com (Winston et al., 2008) or http://www.microbesonline.org (Dehal et al., 2008). The entire microarray data set has been deposited in the Array Express database http://www.ebi.ac.uk/arrayexpress under accession number E-MEXP-3367.

**QUANTITATIVE RT-PCR**

RNA for quantitative RT-PCR (qRT-PCR) was derived independently of that used for microarray analysis. Briefly, total RNA was isolated from three independent in vitro cultures prepared as described above. In a separate animal experiment to that used to prepare the microarray samples, enriched and purified total RNA was isolated as described above from three infected rats yielding three independent in vivo samples. Oligonucleotide primers (Table 1) were designed with Primer3 (Rozen and Skaletsky, 2000) and were synthesized by the University of Calgary Core DNA Services (Calgary, AB, Canada). BCAL0421 (gyrB) encoding DNA gyrase subunit B, previously used as a housekeeping gene in the Bcc multilocus sequence typing scheme (Baldwin et al., 2005) was used as a control as described previously (Peeters et al., 2010). Expression of gyrB was not significantly altered according to microarray analysis (data not shown). RT-PCR was performed using an iScript Select cDNA synthesis kit (Bio-Rad, Mississauga, ON, Canada). Quantification and melting curve analyses were performed with SsoFast Evagreen supermix with low ROX on an iCycler (Bio-Rad) according to manufacturer’s instructions. For each of the three in vitro and in vivo cDNA samples, qRT-PCRs were performed in triplicate, normalized to the control gene, gyrB. Data were calculated as previously described (Schmittgen and Livak, 2008) and represented as fold change of the in vivo samples relative to the in vitro samples.

### RESULTS

**GENES ON ALL GENOMIC ELEMENTS ARE INDUCED IN RESPONSE TO THE HOST ENVIRONMENT**

Global gene expression profiles were generated using microarrays from B. cenocepacia cultures recovered from rat lungs 3 days postinfection using a chronic respiratory infection model and compared to those of B. cenocepacia cultures grown to high cell-density in vitro. Using a fold change cut off of ≥ 1.5, we identified 366 genes that were induced in vitro and 304 genes that were induced in vivo (Table 2). The B. cenocepacia J2315 genome is comprised of four genetic elements: chromosome 1, 3.87 Mb; chromosome 2, 3.22 Mb; chromosome 3, 0.88 Mb; and a plasmid, 0.09 Mb (Holden et al., 2009). Differential expression was observed for genes present on the three chromosomes as well as the plasmid. The number of genes induced in vitro or induced in vivo on each genomic element and the percentage of the total number of genes induced in vitro or in vivo located on each genomic element is shown in Table 2. For in vitro induced genes, the distribution of changes across the genome was relatively proportional to the size of each genomic element, i.e., a decreasing percentage of genes showed altered expression from chromosomes 1 through 3 and to the plasmid. Interestingly, more than 20% of genes induced in vivo were plasmid genes indicating this group of genes was highly overrepresented (Table 2). Consistent with this observation, for chromosomes 1 through 3, the percentage of genes on each repli- con induced in vivo was similar and ranged from 2.9 to 4.8%, in marked contrast to the plasmid where 66% of plasmid-encoded genes were induced in vivo (Table 2).

**A MAJORITY OF CHARACTERIZED VIRULENCE GENES ARE SIMILARLY EXPRESSED BETWEEN **IN VITRO** AND **IN VIVO** ENVIRONMENTS**

At least 28 genes have been characterized in B. cenocepacia that are known to be important for virulence and belong to functional groups including stress resistance, extracellular enzymes or

| Primer         | Sequence (5’–3’)                        | Product size (bp) | Reference        |
|---------------|-----------------------------------------|-------------------|-----------------|
| L0114fliCRTfor1 | GCGTGTCTGATGATTCAACGGCAT                | 159              | O’Grady et al. (2009) |
| L0114fliCRTrev1  | TCACCTCCTGATCCTGCTGGAAA                 | 120              | This study       |
| L0343hpcRfor1   | ACCTGTTCTGAGAAGCTGACG                 | 120              | This study       |
| L0343hpcRrev1   | CGCGTAGGTCTTGCTGGTCT                | 87               | This study       |
| L1525qRTfor1   | AGCAATCATCAAGCGTTCCC                 | 195              | This study       |
| L1525qRTrev1   | AGAGCGACTGCGATAAGTCC                | 195              | This study       |
| M2194mmsAqRTfor1 | TCAACGCTGATGATTCAACGGCAT                | 159              | O’Grady et al. (2009) |
| M2194mmsAqRTrev1 | CGCGTAGGTCTTGCTGGTCT                | 87               | This study       |
| M2702pprCqRTfor1 | ACAAATCAGCAGCGATGAG                | 164              | This study       |
| M2702pprCqRTrev1 | CCGTAGCTGAGCAGCGAT                 | 83               | This study       |
| pBCA025traFqRfor1 | TCAACGCTGATGATTCAACGGCAT                | 159              | O’Grady et al. (2009) |
| pBCA025traFqRrev1 | CGCGTAGGTCTTGCTGGTCT                | 87               | This study       |
| pBCA045traKqRTfor1 | TCAACGCTGATGATTCAACGGCAT                | 159              | O’Grady et al. (2009) |
| pBCA045traKqRTrev1 | CGCGTAGGTCTTGCTGGTCT                | 87               | This study       |
| pBCA065qRTfor1  | SGCGATGATGATTCAACGGCAT                | 164              | This study       |
| pBCA065qRTrev1  | CGGGTGCGGACGATGAG                     | 83               | This study       |
| L0421gyrBqRTfor1 | TCAACGCTGATGATTCAACGGCAT                | 159              | O’Grady et al. (2009) |
| L0421gyrBqRTrev1 | CGCGTAGGTCTTGCTGGTCT                | 87               | This study       |
Table 2 | Microarray analysis of *B. cenocepacia* genes induced *in vitro* or induced *in vivo*.

| Genomic element | Number of genes induced *in vitro* | Number of genes induced *in vivo* | Percentage of total genes induced *in vitro* (%) | Percentage of total genes induced *in vivo* (%) | Percentage of genes on each replicon induced *in vitro* (%) | Percentage of genes on each replicon induced *in vivo* (%) |
|-----------------|-----------------------------------|-----------------------------------|-----------------------------------------------|-----------------------------------------------|----------------------------------------------------------|----------------------------------------------------------|
| Chr 1<sup>a</sup> | 182<sup>b</sup>                  | 104                               | 49.7<sup>c</sup>                             | 34.2                                          | 5.1<sup>c</sup>                                         | 2.9                                                      |
| Chr 2           | 139                               | 102                               | 38.0                                          | 33.6                                          | 4.9                                                      | 3.6                                                      |
| Chr 3           | 44                                | 36                                | 12.0                                          | 11.8                                          | 6.0                                                      | 4.8                                                      |
| Plasmid         | 1                                 | 62                                | 0.3                                           | 20.4                                          | 1.1                                                      | 66.0                                                     |

<sup>a</sup>Chr, chromosomes 1, 2, or 3 of *B. cenocepacia* J2315.

<sup>b</sup>Number or percentage of *B. cenocepacia* genes or percentage of a total of 7210 *B. cenocepacia* genes exhibiting changes (≥ 1.5-fold) in expression in RNA recovered from rat lungs (*in vivo*), relative to RNA isolated from *in vitro* grown cultures as determined by microarray analysis.

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**FIGURE 1** | *In vivo* expression of characterized virulence genes. Expression ratio of RNA recovered from rat lungs (*in vivo*) relative to RNA isolated from *in vitro* grown cultures as determined by microarray analysis. The “BCA” designation has been removed from names of genes encoded on chromosomes 1, 2, and 3 for image clarity.

secreted toxins, QS, transcriptional regulation, as well as genes involved in heme uptake, iron acquisition, and the synthesis of structural components such as lipopolysaccharide, porins, and lectins (Loutet and Valvano, 2010). Analysis of these virulence genes showed that expression of the majority of these genes was similar between *in vitro* and *in vivo* conditions (Figure 1). The expression of *cepI*, encoding an N-acyl-homoserine lactone (AHL) synthase, was somewhat lower *in vivo* and this observation was consistent with lower expression of CepIR-regulated genes including those encoding extracellular zinc metalloproteases ZmpA and ZmpB, the orphan LuxR homolog CepR2 and the LysR-type transcriptional regulator ShvR (Figure 1). Two other genes known to be influenced by CepIR such as the major catalase/peroxidase encoded by *katB* and an acyl-CoA dehydrogenase encoded by BCAS0208 were similarly expressed in the *in vitro* and *in vivo* environments (Figure 1). The BCAS0208 mutant caused less lung pathology than wild type in the rat chronic respiratory infection model (Subramoni et al., 2011).

Limited iron availability in mammals is circumvented by infectious pathogens by the production of iron binding and transport complexes such as heme binding proteins and siderophores. Although genes involved in heme transport (*huvA* and *hmuS*) were not differentially expressed between *in vivo* and *in vitro* environments (Figure 1), *huvA* mutants exhibited survival defects in the rat chronic respiratory infection model (Hunt et al., 2004). Genes involved in ornibactin biosynthesis and transport were also expressed at similar levels in both environments, although ornibactin mediated iron uptake is required for persistence in the rat chronic respiratory infection model (Visser et al., 2004). Among the characterized virulence genes, the lowest *in vivo* expression ratio (0.04) was observed for BCAS0293 (*aidA*; Figure 1). The *aidA* gene encodes a protein that significantly contributes to virulence against *C. elegans* (Huber et al., 2004), but an *aidA* mutation had no effect on virulence in the rat chronic respiratory infection model (Uehlinger et al., 2009).
SECRETION SYSTEMS ARE SELECTIVELY REGULATED BETWEEN IN VITRO AND IN VIVO ENVIRONMENTS

*Burkholderia cenocepacia* has one type II, type III, and type VI protein secretion systems (T2SS, T3SS, and T6SS, respectively) that contribute to pathogenesis, and two type IV secretion systems (T4SS), one of which has been shown to be important in virulence. Expression of genes encoding components of each of these systems varied between *in vitro* and *in vivo* environments.

The T2SS is composed at least 12 ORFs on three gsp operons and is involved in secretion of extracellular zinc metalloproteases ZmpA, ZmpB, and other extracellular proteins that have enzymatic activity such as phospholipase C, hemolysin, lipase, and polygalacturonase (Fehlner-Gardiner et al., 2002; Kothe et al., 2003; Gingues et al., 2005; Somvanshi et al., 2010). Expression of the three gsp operons encoding the T2SS was similar between *in vitro* and *in vivo* conditions (Figure 2A). Apart from the lower expression of *zmpA* and *zmpB* in *in vivo* (Figure 1), expression of other genes encoding enzymes secreted by the T2SS described above was not different between *in vitro* and *in vivo* conditions (data not shown). The *B. cenocepacia* T3SS genes are organized in two operons on chromosome 2 thought to be responsible for secretion of effector proteins that have yet to be identified (Tomich et al., 2003; Glendinning et al., 2004). Mutation of *bescN*, encoding an ATP-binding protein, reduced bacterial survival, and lung inflammation in a mouse agar bead infection model (Tomich et al., 2003). In our study, the mean expression ratio of genes in the *bescQ* and *bescV* operons was 1.03 and 0.99, respectively, in the *in vivo* compared to *in vitro* conditions (Figure 2B) indicating that there was no difference in expression.

Two gene clusters located on chromosome 2 and the plasmid have been identified to encode components of T4SS. Interestingly, the plasmid-encoded T4SS was induced *in vivo*. The bc-VirB/D4 T4SS on chromosome 2 shares homology with the *Agrobacterium tumefaciens* T4SS and is involved in plasmid mobilization.

![FIGURE 2 | In vivo expression of genes encoding secretion systems. Expression ratio of RNA recovered from rat lungs (*in vivo*) relative to RNA isolated from *in vitro* grown cultures as determined by microarray analysis. (A) T2SS, (B) T3SS, (C) T4SS, (D) T6SS. Inset in (C) is chromosome 2-encoded T4SS genes with expanded y-axis. The "BCA" designation has been removed from names of genes encoded on chromosomes 1, 2, and 3 for image clarity. Putative operons are indicated by arrows.](image-url)
The second T4SS gene cluster exists on a 92.7-kb plasmid that is found in relatively few *B. cenocepacia* strains including J2315 and K56-2 (Engledow et al., 2004) but not AU1054 or MCO-3 (Winsor et al., 2008). This plasmid-encoded T4SS contributes to the plant tissue watersoaking (ptw) phenotype and disease symptoms in onion tissue (Engledow et al., 2004) and increased survival of *B. cenocepacia* in macrophages and airway epithelial cells (Sajjan et al., 2008). Expression of genes on the chromosome 2-encoded T4SS were similar in the *in vitro* and *in vivo* conditions (Figure 2C). In contrast, several genes that are part of the plasmid-encoded T4SS were markedly induced *in vivo* at levels ranging from 3- to 46.1-fold (Figure 2C). Higher *in vivo* expression of pBCA025 encoding the putative conjugative transfer protein TraF and pBCA045 encoding the putative exported protein TraK was confirmed using qRT-PCR (Table 3). These data indicated differential regulation of chromosome 2- and plasmid-encoded T4SS between *in vitro* and *in vivo* conditions.

The *B. cenocepacia* T6SS comprises 16 genes organized in three adjacent operons on chromosome 1. The T6SS contributes to survival of *B. cenocepacia* in the rat chronic respiratory infection model (Hunt et al., 2004) and influences infection of macrophages (Aubert et al., 2008). Expression of BCAL0339 and BCAL0346 was lower in *B. cenocepacia* growing in medium supplemented with CF sputum compared to control cultures (Drevinek et al., 2008). In our study, expression of six T6SS genes was lower *in vivo* compared to *in vitro* conditions. The BCAL0340–0348 operon exhibited the lowest expression in *in vivo* (0.66) compared to the other two T6SS operons (Figure 2D). The BCAL0340 operon includes genes encoding the ClpV-like chaperone (BCAL0347) and the hemolysin-coregulated protein (Hcp) (BCAL0343; Aubert et al., 2008). The ClpV-like chaperone is required for secretion of Hcp in *Pseudomonas aeruginosa* (Mougous et al., 2006). The hcp gene showed the lowest in *vivo* expression (0.54) of any T6SS gene and the low hcp expression *in vivo* was confirmed using qRT-PCR (Figure 2D; Table 3).

**MOTILITY AND Flp TYPE PILUS-ENCODING GENES ARE INDUCED IN VITRO**

Bacterial motility, attachment, and invasion via flagellar- and pilus-encoding genes are known to be important in virulence (Tomich et al., 2002; Urban et al., 2004). Expression of 24 flagellar-associated genes from eight different operons distributed across chromosome 1 was lower *in vivo*, with the lowest *in vivo/in vitro* expression ratio being 0.05 for the BCAL0340–0348 operon (Figure 3A). In contrast, the Flp type pilus-encoding genes were induced *in vivo* compared to *in vitro* conditions (Figure 3B). The highest *in vivo* expression of these genes was observed for the BCAL0300–0302 operon (Figure 3B). The BCAL0300 operon includes genes encoding the ClpV-like chaperone (BCAL0301) and the hemolysin-coregulated protein (Hcp) (BCAL0302). The ClpV-like chaperone is required for secretion of Hcp in *Pseudomonas aeruginosa* (Mougous et al., 2006). The hcp gene showed the lowest in *vivo* expression (0.54) of any T6SS gene and the low hcp expression *in vivo* was confirmed using qRT-PCR (Figure 2D; Table 3).

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### Table 3 | Microarray and qRT-PCR analysis of selected genes showing differential expression from *in vivo* compared to *in vitro* grown cultures.

| Gene     | Annotation or predicted function | Fold change<sup>b</sup> | microarray | qRT-PCR |
|----------|----------------------------------|--------------------------|------------|---------|
| BCAL014  | fliC, type II flagellin protein   | −8.29                    | −28.00     |         |
| BCAL0343 | Hcp, hemolysin-coregulated protein | −1.86                    | −7.82      |         |
| BCAL1525 | Flp type pilus subunit           | −11.75                   | −12.95     |         |
| BCAM2194 | mmsA, methylmalonate-semialdehyde dehydrogenase | 2.26 | 1.74 |         |
| BCAM2702 | prpC, 2-methylcitrate synthase   | 5.88                     | 8.29       |         |
| pBCA025  | traF, putative conjugative transfer protein | 7.10 | 344.86 |         |
| pBCA045  | traK, putative exported protein  | 12.43                    | 33.26      |         |
| pBCA053  | Putative extracellular solute-binding protein | 480.70 | 10.44 |         |

<sup>a</sup> Derived from *B. cenocepacia* J2315 (Holden et al., 2009) at http://www.burkholderia.com (Winsor et al., 2008) or http://www.microbesonline.org (Dehal et al., 2009).

<sup>b</sup> Fold change of RNA recovered from rat lungs (*in vivo*) relative to RNA isolated from *in vitro* grown cultures as determined by microarray or qRT-PCR analysis.
expression ratio (0.23) observed for fliC, encoding type II flagellin (Figure 3A). Lower expression of fliC in vivo compared to in vitro conditions was independently confirmed using qRT-PCR (Table 3).

The genomic locus from BCAL1520–1537 encodes components of a subclass of type IVb piliplins, called a Flp type pilus, that is similar to the flp–tad–rcp locus that is involved in adherence and biofilm formation in Actinobacillus actinomycetemcomitans (Kachlany et al., 2001; Inoue et al., 2003) and aggregation and biofilm formation in P. aeruginosa (de Bentzmann et al., 2006). Ten genes encoding components of the chromosome 1-encoded Flp type pilus had lower in vivo expression. The lowest expression was observed for BCAL1525 encoding a Flp type pilus subunit and this trend was confirmed using qRT-PCR (Figure 3B; Table 3).

**IDENTIFICATION OF GENES POTENTIALLY IMPORTANT IN THE HOST ENVIRONMENT**

Approximately 300 genes were identified with at least a 1.5-fold change in expression in vivo compared to in vitro grown cultures (Table A1 in Appendix). Selected genes and their fold change differences are shown in Table 4. Many of these genes have not been previously characterized in B. cenocepacia. The most common putative functions of these in vivo induced genes were related to adaptation to stress or a host environment, metabolism, or nutrient acquisition (Table 4).

**NOVEL GENES INDUCED IN VIVO**

A four gene operon (BCAM2703–2700) containing genes involved in the methylcitrate cycle, required for propionyl-CoA metabolism, and fatty-acid utilization, were markedly induced in vivo (Table 4). Induced in vivo expression of BCAM2702 (prpC) encoding 2-methylcitrate synthase was confirmed using qRT-PCR (Table 3). Genes involved in the methylcitrate and glyoxylate cycles are required for virulence in Mycobacterium tuberculosis, which relies more on fatty acids than carbohydrates during infection (Munoz-Elias and McKinney, 2005). Genes involved in the methylcitrate cycle are upregulated in M. tuberculosis isolated from murine macrophages (Schnappinger et al., 2003) and are important for growth in macrophages but not for intracellular survival (Munoz-Elias et al., 2006). It is unknown whether the methylcitrate cycle plays a role in B. cenocepacia intracellular survival in macrophages. An uncharacterized seven gene operon (BCAM2196–BCAM2191) containing genes putatively involved in lipid metabolism was also induced in vivo (Table 4), suggesting that fatty-acid metabolism or utilization may be important in B. cenocepacia lung infections. Using qRT-PCR we confirmed expression of BCAM2194 (mmsA) encoding methylmalonate-semialdehyde dehydrogenase was induced in vivo (Table 3). A four gene operon (BCAL1212–1215) induced in vivo encodes genes for a 2-oxo acid dehydrogenase complex (Table 4). The dihydrodipamide dehydrogenase gene component of a similar complex was shown to be important for persistence and virulence in Streptococcus pneumoniae infection models likely due to having a role in capsule synthesis rather than metabolism of 2-oxo acids (Smith et al., 2002).

BCAM0415 encodes a betaine aldehyde dehydrogenase (BADH; Table 4). In P. aeruginosa, BADH has been shown to

| Table 4 | Selected genes induced during chronic lung infection. |
|---------|--------------------------------------------------------|
| Gene    | Annotation or predicted function                        | Fold change  |
|---------|--------------------------------------------------------|--------------|
| **OSMOTIC STRESS AND ADAPTATION**                                          |              |
| BCAL1103| Putative OsmB-like lipoprotein                          | 2.1          |
| BCAL2044| LdcA LD-carboxypeptidase A                              | 1.5          |
| BCAL2558| Pyridine nucleotide-disulfide oxidoreductase            | 2.1          |
| BCAL3297| DPS-family DNA-binding ferritin like protein            | 1.7          |
| BCAL3310| Ycel family protein, osmotic, and acid stress adaptation| 1.7          |
| BCAL3311| Ycel family protein, osmotic, and acid stress adaptation| 1.6          |
| BCAL3314| PqiA parquat inducible protein A                         | 2.4          |
| BCAL3362| Putative oxidoreductase                                 | 1.8          |
| BCAM0027| PadR family regulatory protein, phenolic acid induced stress response | 1.5 |
| BCAM0414| Conserved hypothetical protein                          | 2.0          |
| BCAM0415| Putative betaine aldehyde dehydrogenase                 | 1.5          |
| BCAM2700| propF putative membrane protein                         | 1.8          |
| BCAM2701| acnA, aconitate hydratase 1                             | 2.7          |
| BCAM2702| propC, 2-methylcitrate synthase                         | 5.9          |
| BCAM2703| propB, probable methylisocitrate lyase                   | 2.8          |
| **METAL ION TRANSPORT OR METABOLISM**                                      |              |
| BCAL0269| Oxidoreductase, molybdopterin-binding domain            | 1.6          |
| BCAL0366| Nitroreductase family protein, metal ion oxidation       | 1.6          |
| BCAL0580| Putative chromate transport protein                      | 1.6          |
| BCAL1789| ExbB, iron-transport protein                            | 1.7          |
| BCAL2485| Putative iron–sulfur cluster-binding electron           | 2.1          |
| BCAL2486| Putative iron–sulfur oxidoreductase                     | 2.1          |
| BCAM0447| Putative exported multicopper oxidase                   | 13.0         |
| BCAM1187| TonB-dependent siderophore receptor                     | 1.7          |
| BCAM1527| Putative cation efflux protein                          | 1.8          |
| BCAM2007| TonB-dependent siderophore receptor                     | 1.6          |
| BCAS0028| Succinylglutamate                                       | 2.8          |
| BCAS0449| Nickle ion binding-protein-dependent transport           | 1.6          |
| **CARBOHYDRATE TRANSPORT AND METABOLISM**                                  |              |
| BCAL0804| N-acetylglucosamine transferase                         | 1.5          |
| BCAL1657| Putative ribose transport system                        | 1.8          |
| BCAL1658| Putative ribose ABC transporter                         | 1.5          |
| BCAL1754| Major facilitator superfamily protein, carbohydrate transport | 3.5 |
| BCAL2040| Polysaccharide deacetylase, carbohydrate transport      | 1.5          |
| BCAM3038| ABC transporter ATP-binding component, carbohydrate ABC transporter | 1.6 |
| BCAM3039| ABC transporter, membrane permease                      | 1.5          |
| BCAM3040| ABC transporter, membrane permease                      | 1.7          |

(Continued)
### Table 4 | Continued

| Gene          | Annotation or predicted function<sup>a</sup>               | Fold change<sup>b</sup> |
|---------------|-------------------------------------------------------------|-------------------------|
| BCAL3041      | MalE, maltose-binding protein                               | 2.1                     |
| BCAL3364      | Putative gluconokinase                                     | 1.7                     |
| BCAM0094      | Xylulose kinase                                             | 1.7                     |
| BCAM1330      | Cellulose polysaccharide export protein                     | 1.7                     |
| BCAM1333      | Cellulose exopolysaccharide acyltransferase                | 1.6                     |
| BCAM1390      | Putative aldolase                                           | 3.0                     |
| BCAM2260      | Major facilitator superfamily protein                      | 1.6                     |
| BCAS0230      | Putative sugar ABC transporter ATP-binding                 | 1.6                     |

**AMINO ACID TRANSPORT AND METABOLISM**

| Gene          | Annotation or predicted function<sup>a</sup>               | Fold change<sup>b</sup> |
|---------------|-------------------------------------------------------------|-------------------------|
| BCAL0446      | Putative aminotransferase                                   | 2.9                     |
| BCAL1212      | bkdA1, 2-oxoisovalerate dehydrogenase alpha subunit         | 3.0                     |
| BCAL1213      | bkdA2, 2-oxoisovalerate dehydrogenase beta subunit          | 2.9                     |
| BCAL1214      | bhdB, lipamidate acyltransferase                            | 3.7                     |
| BCAL1215      | IpdV, dihydrolipoamide dehydrogenase                       | 2.2                     |
| BCAL1749      | Putative CoA-transferase                                    | 2.4                     |
| BCAL1750      | Conserved hypothetical protein, pyruvate decarboxylase      | 2.4                     |
| BCAL1751      | Glyoxalase/bleomycin resistance, amino acid transport      | 1.7                     |
| BCAM0047      | Lysine exporter – LysE/YggA                                 | 2.6                     |
| BCAM0178      | ABC transporter periplasmal solute-binding protein          | 2.7                     |
| BCAM0368      | Putative branched-chain amino acid transport                | 1.5                     |
| BCAM0459      | Cysteine desulfurase                                        | 3.6                     |
| BCAM0983      | leuC1, 3-isopropylmalate dehydratase large subunit          | 2.9                     |
| BCAM0983A     | Putative entericidin B-like bacteriolytic toxin             | 2.0                     |
| BCAM0984      | leuD1, 3-isopropylmalate dehydratase small subunit          | 2.1                     |
| BCAM1150      | 3-Hydroxyisobutyrate dehydrogenase                          | 1.6                     |
| BCAM1151      | Methylmalonate-semialdehyde dehydrogenase                  | 2.4                     |
| BCAM1427      | Lyse family transporter                                     | 3.7                     |
| BCAM1487      | Putative ABC transporter, substrate-binding                 | 3.1                     |
| BCAM1488      | Putative proline racemase                                   | 1.9                     |
| BCAM2095      | Putative HTH transcriptional regulator                     | 1.6                     |
| BCAM2096      | puuB gamma-glutamylputrescine oxidoreductase                | 1.9                     |
| BCAM2191      | Enol-CoA hydratase/isomerase family                         | 1.9                     |
| BCAM2192      | Enol-CoA hydratase/isomerase family protein                 | 2.4                     |
| BCAM2193      | mmsB, 3-hydroxyisobutyrate dehydrogenase                   | 2.4                     |
| BCAM2194      | mmsA, methylmalonate-semialdehyde dehydrogenase            | 2.3                     |

**MEMBRANE PROTEINS**

| Gene          | Annotation or predicted function<sup>a</sup>               | Fold change<sup>b</sup> |
|---------------|-------------------------------------------------------------|-------------------------|
| BCAL0403      | Putative outer membrane-bound lytic murine                  | 1.5                     |
| BCAL0624      | Putative OmpC, outer membrane porin protein precursor       | 1.6                     |
| BCAL1678      | Putative outer membrane usher protein precursor, fimD pilin biogenesis | 2.4                     |
| BCAL2083      | YaeT, Outer membrane protein assembly factor               | 1.5                     |
| BCAL2191      | Putative 17 kDa membrane protein surface antigen           | 3.1                     |
| BCAL2468      | Putative membrane protein                                   | 1.9                     |
| BCAL2482      | Putative OmpC outer membrane protein                       | 3.1                     |
| BCAL2505      | Putative membrane protein                                   | 1.5                     |
| BCAL2552      | Putative membrane protein                                   | 1.5                     |
| BCAL2553      | Putative membrane protein                                   | 1.8                     |
| BCAL3003      | Probable outer membrane lipoprotein carrier                 | 1.5                     |
| BCAL3203      | Putative periplasmic TolB protein                           | 1.6                     |
| BCAL3204      | Putative OmpA family lipoprotein/PAL                        | 1.7                     |
| BCAL3205      | YbgF Tol-PAL system protein                                 | 1.6                     |
| BCAL3473      | Putative OmpC-like outer membrane porin                     | 1.9                     |
| BCAM0926      | Multidrug efflux system transporter protein                 | 5.9                     |
| BCAM1207      | ABC transporter ATP-binding membrane protein               | 1.5                     |
| BCAM1341      | Acyltransferase like protein                                | 3.2                     |
| BCAM1425      | Putative membrane protein                                   | 2.9                     |
| BCAM1563      | ABC transporter ATP-binding membrane protein               | 1.7                     |
| BCAM1946      | Putative quinoxaline efflux system transporter              | 1.6                     |
| BCAM1957      | ABC transporter ATP-binding protein                         | 1.6                     |
| BCAM2647      | Putative membrane protein                                   | 1.7                     |
| BCAM2648      | NAD dependent epimerase/dehydratase family, outer membrane biogenesis | 1.6                     |
| BCAS0308      | Putative flp type pilus assembly protein, TadG-like pilus   | 2.4                     |
| BCAS0463      | Putative membrane protein                                   | 1.6                     |
| pBCA010       | Putative membrane protein                                   | 3.2                     |

(Continued)
Table 4 | Continued

| Gene       | Annotation or predicted functiona | Fold changeb |
|------------|-----------------------------------|--------------|
| pBCA014    | Putative membrane protein          | 3.3          |
| pBCA019    | Putative membrane protein          | 2.4          |
| pBCA026    | Putative membrane protein          | 10.6         |
| pBCA029    | Putative membrane protein          | 8.6          |
| pBCA034    | Putative membrane protein          | 6.0          |
| pBCA036    | Putative membrane protein          | 13.8         |
| pBCA037    | Putative membrane protein          | 7.3          |
| pBCA048    | Putative membrane protein          | 55.6         |

**EXPORTED PROTEINS**

| Protein     | Annotation or predicted functiona | Fold changeb |
|-------------|-----------------------------------|--------------|
| BCAL0305    | Putative exported protein          | 2.2          |
| BCAL0623    | Putative exported protein          | 1.7          |
| BCAL1279    | Putative exported protein          | 1.6          |
| BCAL1499    | Putative exported protein          | 1.8          |
| BCAL1539    | Putative exported protein          | 2.3          |
| BCAL1798    | Putative exported protein          | 1.9          |
| BCAL1961    | Putative exported protein          | 1.9          |
| BCAL2187    | Putative exported protein          | 1.6          |
| BCAL2607    | Putative exported outer membrane porin protein | 2.7          |
| BCAL2615    | Putative exported outer membrane porin protein | 2.2          |
| BCAL2911    | Proline-rich exported protein      | 1.6          |
| BCAL2966    | Putative exported protein          | 1.5          |
| BCAL3024    | Putative exported protein          | 1.6          |
| BCAL3490    | Putative exported protein          | 2.0          |
| BCAL3492    | Putative exported protein          | 1.6          |
| BCAM0676    | Putative exported protein          | 1.8          |
| BCAM1726    | Putative exported protein          | 2.0          |
| BCAM1742    | Putative exported protein          | 1.9          |
| BCAM1964    | Putative exported protein          | 1.6          |
| BCAM2073    | Putative exported protein          | 3.0          |
| pBCA013     | Putative exported protein          | 6.3          |

**REGULATORY PROTEINS**

| Protein     | Annotation or predicted functiona | Fold changeb |
|-------------|-----------------------------------|--------------|
| BCAL2488    | LysR family regulatory protein     | 2.0          |
| BCAL2529    | LysR family regulatory protein     | 1.5          |
| BCAL3486    | ecfM, RNA polymerase sigma factor, sigma-70 | 1.8          |
| BCAM0422    | LuxR superfamily regulatory protein | 1.9          |
| BCAM0595    | LysR family regulatory protein     | 2.6          |
| BCAM2025    | Sigma-54 interacting regulatory protein | 1.9          |
| BCAM2162    | MarR family regulatory protein     | 2.0          |
| BCAS0436    | AraC family regulatory protein     | 1.7          |
| pBCA035     | GntR family regulatory protein     | 18.9         |

aDerived from B. cenocepacia J2315 (Holden et al., 2009) at http://www.burkholderia.com (Winsor et al., 2008) or http://www.microbesonline.org (Dahal et al., 2009).
bFold change of RNA recovered from rat lungs (in vivo) relative to RNA isolated from in vitro grown cultures as determined by microarray analysis.

be induced by choline and choline precursors (Velasco-Garcia et al., 2006a) which are abundant in infected lung tissues (Wright and Clements, 1987). In addition to playing a role in assimilating carbon and nitrogen from choline, BADH produces glycerol betaine which can protect bacteria from high osmolarity stress and oxidative stress in infected tissues. BADH has been proposed as a therapeutic target for P. aeruginosa since inactivation of this enzyme leads to intracellular accumulation of betaine aldehyde, which is toxic, and the inability to grow in medium with choline (Velasco-Garcia et al., 2006b; Zaldívar-Machorro et al., 2011). Homologs of other genes induced by osmotic stress in bacteria were also identified as being induced in vivo (Table 4). BCAL1103, encodes an OsmB-like protein. OsmB is induced by osmotic stress and stationary phase growth conditions in E. coli (Jung et al., 1990; Boulanger et al., 2005). BCAL3310 and BCAL3311 are predicted to be co-transcribed YceI family proteins, homologs of which have been shown to be induced in response to osmotic stress in E. coli (Weber et al., 2006) and acid stress in Helicobacter pylori (Sisinni et al., 2010). BCAL2358, a putative pyridine nucleotide-disulfide oxidoreductase with some similarity to TrxB (thioredoxin reductase) homologs, was induced twofold in vivo. TrxB genes are involved in cellular redox processes and defense against oxidative stress and are important in intracellular survival in some pathogens (Bjur et al., 2006; Potter et al., 2009). BCAL3314 encodes a homolog of PqiA-like proteins, which are induced by paraquat and other superoxide generators in E. coli (Koh and Roe, 1995). BCAL3297 encodes a DPS-family DNA-binding ferritin. Homologs of these proteins are involved in resistance as well as iron sequestration (Calhoun and Kwon, 2011).

Although many of the in vivo induced outer membrane protein encoding genes are uncharacterized, a few have homology to proteins with predicted functions. BCAL3203, L3204, and L3205 form part of the Tol-PAL system membrane complex that is required for membrane integrity and has been implicated in the pathogenesis of several Gram-negative bacteria (Bowen et al., 1998; Godlewksa et al., 2009; Paterson et al., 2009). TolB (BCAL3203) is a periplasmic protein involved in biopolymer transport. BCAL3205 is a YbgF homolog which is the last gene of the Tol-PAL complex and interacts with TolA (Krachler et al., 2010). BCAL3204 has been annotated as OmpA/PAL. PAL has been shown to contribute to virulence in several Gram-negative bacteria and in E. coli has been shown to be released into the bloodstream contributing to septic shock (Hellman et al., 2002; Liang et al., 2005). A 17 kDa OmpA-like protein has recently been shown to be an immunodominant antigen following intranasal immunization with a B. cenocepacia outer membrane protein preparation in mice (Makidon et al., 2010). Although the immunoreactive protein reported to be an OmpA-like protein was not conclusively identified, the partial amino acid sequence determined from a peptide of this molecular mass isolated from SDS-polyacrylamide gels, has 95.8% identity to BCAL3204. There are at least six other OmpA-like proteins in B. cenocepacia with varying degrees of sequence identity; however, PAL has been shown to highly immunogenic in other bacteria (Godlewksa et al., 2009). Therefore it is possible that the immunodominant antigen identified by Makidon et al. (2010) is PAL. BCAL2191, which was increased threefold in vivo (Table 4) is predicted to be an outer membrane lipoprotein with similarity to 17 kDa surface antigens in other species and therefore it is also possible that this protein contributed to the observed reaction with antiserum on Western blots in the study by Makidon et al. (2010). Several other proteins involved in biogenesis of membrane and other cell surface components were also identified (Table 4) including BCAL2083, a YaeT homolog, which in E. coli is an essential gene required for outer membrane assembly
shown to be involved in the oxidation of ferrous to ferric iron and expressed at similar levels in the in vitro

Although ornibactin biosynthesis and uptake genes were expressed at similar levels in the in vitro and in vivo conditions used in this study, a number of other genes potentially involved in metal ion transport and metabolism were identified as being induced in vivo (Table 4). These included exbB, genes coding for iron–sulfur proteins and receptors for unknown siderophores.

One of the most highly induced genes in vivo was BCAM0447 which encodes a putative multicopper oxidase (MCO). MCO genes are found in a number of genomes but have only recently been characterized. The MCO protein of P. aeruginosa has been shown to be involved in the oxidation of ferrous to ferric iron and may be important in iron acquisition (Huston et al., 2002). MCO homologs are also involved in copper resistance and dissemination in mice in S. typhimurium (Achard et al., 2010) and copper tolerance in Campylobacter jejuni (Hall et al., 2008).

Genes encoding proteins of unknown function induced in vivo are shown in Table 4 and Table A1 in Appendix. Many of the expressed genes encode outer membrane proteins (11) and exported proteins (24) that could contribute to cell surface alterations or virulence. Genes encoding six hypothetical proteins were conserved in one or more members of the Bcc, of which 11 were also conserved in Burkholderia pseudomallei (Table A1 in Appendix). It is possible that these proteins are involved in adaptation, survival, or virulence in lung infections although further studies are required to determine their potential importance.

PLASMID-ASSOCIATED GENES

Interestingly, the most highly induced genes in vivo were located on the plasmid where the vast majority of the genes were expressed at much higher levels in vivo than in vitro (Figure 4). Of the plasmid genes annotated in the J2315 sequence (Winsor et al., 2008), 62 genes had higher expression in the lung infection model. Only one gene, pBCA055, had higher expression levels in vitro, and the following genes had similar expression: pBCA003–007, 061, 063, 064, 066–075, 078–081, 083–086, 091–094.

Many of the highly induced genes are part of the plasmid-encoded T4SS, which has been shown to play a role in both plant pathogenesis and survival in eukaryotic cells (Engledow et al., 2004; Sajjan et al., 2008). Expression ratios of genes known or predicted to be a part of the T4SS are shown in Figure 2C and described above. The presence of the plasmid-encoded T4SS in the B. cenocepacia ET12 lineage strains J2315 and K56-2 but not AU1054 or MCO-3 that entirely lack a plasmid is an interesting characteristic. Gene expression of pBCA054 encoding a LuxR family regulatory protein was higher in vivo. Interestingly, the most closely related pBCA054 orthologs are found in B. pseudomallei and Burkholderia mallei, rather than in other members of the Bcc. pBCA001–002 are parAB-like homologs that are putatively involved in chromosome partitioning. pBCA017 is similar to the zeta toxin family of toxin–antitoxin complexes which are involved in programmed cell death to prevent proliferation of plasmid free cells (Gerdes et al., 2005). In addition to plasmid maintenance, toxin–antitoxin pairs can also be involved in responding to nutrient stress. Zeta toxins have recently been shown to target peptidoglycan synthesis triggering autolysis (Mutschler et al., 2011). Zeta toxins are typically paired with epsilon antigens; however, there does not appear to be an epsilon homolog adjacent to pBCA017. In some cases, a chromosomal antitoxin can neutralize the plasmid toxin, but in this case toxin expression would not favor plasmid maintenance (Van Melder and Saavedra De Bast, 2009). Alternatively the toxin can be integrated into other regulatory networks or serve to reduce the overall population to increase nutrient availability for the survivors. Three genes forming an operon (pBCA053–051) exhibited the highest induction of any group of genes in vivo (Figure 4). pBCA053 encodes an extracellular solute-binding protein involved in dicarboxylate transporter carbohydrate metabolism and we confirmed higher in vivo expression of this gene using qRT-PCR (Table 3). The second and third genes in the operon encode an exported protein and a protein with homology to LamB/YcsF family proteins, respectively. In addition to the hypothetical proteins noted above, four putative exported proteins, nine putative membrane proteins, 12 conserved hypothetical proteins and 10 hypothetical proteins encoded on the plasmid were induced in vivo (Table A1 in Appendix). Few genes on this plasmid have been studied in detail opening the possibility for identifying proteins with potentially novel functions.

DISCUSSION

In this study, we have identified the gene expression signature of B. cenocepacia during lung infections. To the best of our knowledge, this is the first study to apply transcriptomics for any member of the Bcc to study gene expression during infection of a susceptible host. Differential gene expression was observed for characterized virulence genes as well as potential novel virulence genes between in vitro and in vivo environments.

Altered in vivo gene expression was observed for genes encoding enzymes, regulators, structural appendages as well as those contributing to ornibactin biosynthesis, and quorum sensing systems. Lower in vivo expression was observed for AHL-dependent QS controlled genes that are directly (e.g., aidA) and indirectly (e.g., shvR) regulated at the transcriptional level by CepR (Weingart et al., 2005; O’Grady et al., 2011). These observations suggest that
more favorable conditions exist for CepIR-dependent regulation of selected genes in high cell-density (∼10^9) laboratory-grown cultures compared to the lower cell-density (∼10^6) in the lung infections, although it is possible that higher expression of QS regulated genes occurs in selected locations in the lungs where bacteria are present in high cell-density biofilms. Since cepI and CepR-regulated genes including zmpA, zmpB, and shvR have been shown to be important for virulence in the rat chronic respiratory infection model (Corbett et al., 2003; Sokol et al., 2003; Kooi et al., 2006; Bernier et al., 2008), it is clear that these genes are expressed at sufficient levels to play a role in infection. The majority of characterized virulence genes were similarly expressed in the in vivo and in vitro conditions. This suggests that expression of these genes is just as important in high cell-density cultures and during lung infections. The contribution of these individual genes has been characterized in one or more infection models highlighting their importance in B. cenocepacia pathogenesis. Similar expression of characterized virulence genes on chromosome 2. A mutation in the chromosome specific environmental signal(s) in the lung including a gene encoding the secreted effector Hcp. Previous work has previously been observed for B. pseudomallei (Tuanyok et al., 2006).

Increased expression of some genes belonging to the T3SS was observed in the closely related pathogens B. mallei and B. pseudomallei during infection of mice and hamsters, respectively (Kim et al., 2005; Tuanyok et al., 2006). In the present study, expression of T2SS and T3SS genes was similar between in vitro and in vivo environments. Genes in these secretion systems appear to be expressed at moderate levels in both in vitro and in vivo environments. We previously showed expression of the T2SS genes gspC and gspG was influenced by growth medium composition (O’Grady et al., 2011). A previous study was not able to identify growth conditions that altered expression of T3SS genes suggesting these genes are constitutively high (Engledow et al., 2004). The in vivo growth conditions provided a stimulus for expression of genes in the plasmid-encoded T4SS but did not affect expression of the T4SS genes on chromosome 2. A mutation in the chromosome 2-encoded T4SS was shown not to contribute to bacterial persistence or histopathology in the rat chronic respiratory infection model (Bernier and Sokol, 2005). To date, no studies have observed such a dramatic increase in expression of plasmid-encoded T4SS genes suggesting that specific environmental signal(s) in the lung environment enabled increased expression of these genes to be detected. It was shown that the plasmid-encoded T4SS contributed to organism tissue decimation through secretion of one or more effectors (Engledow et al., 2004). Whether this plasmid-encoded T4SS or its effectors have a role in mammalian cell/tissue damage has yet to be determined. We observed some T6SS genes had lower in vivo expression, in particular those genes on the BCAL0340 operon that includes a gene encoding the secreted effector Hcp. Previous work identified a transposon insertion in each of the three operons of the T6SS locus affected survival of B. cenocepacia in the rat chronic respiratory infection model (Hunt et al., 2004).

Using a mouse agar bead infection model, a flagellin mutant failed to cause mortality compared to wild type (Urban et al., 2004). It was also shown that motility mutants were less able to invade epithelial cells (Tomich et al., 2002). Recent work showed expression of flagellar- and chemotaxis-associated genes and motility was reduced in B. cenocepacia strains of the ET12 lineage that were isolated from CF patients (Sass et al., 2011). However, a previous study showed transcription of flagellar-associated genes was increased in B. cenocepacia J2315 cultured in medium supplemented with CF sputum (Drevinek et al., 2008). Conflicting data regarding expression of flagellar-associated genes in these two studies likely reflect the experimental conditions employed where increased expression of flagellar-associated genes was detected in rapidly growing cultures (Drevinek et al., 2008). The phenotypic characteristics of the B. cenocepacia non-motile CF isolates are similar to P. aeruginosa clinical isolates which often acquire loss-of-function mutations associated with motility during chronic lung infection (Mahenthiralingam et al., 1994). It has also been shown that P. aeruginosa exhibited decreased transcription of flagellar-associated genes when cultured in CF sputum (Wolfgang et al., 2004). In our study, we detected lower in vivo expression of genes involved in motility and Flp type pilus formation. This result was likely due to differences in culture conditions between in vitro and in vivo environments. The agar bead infection model bypasses the colonization step during infection (Cash et al., 1979). Our data suggest expression of these genes is not required in an established infection taking place in the lower respiratory tract. Therefore, decreased expression of these genes was expected since expression of these genes is an energy-expensive process and is more likely associated with rapidly growing cultures than cultures recovered from chronic lung infection.

We identified numerous genes that were induced during lung infections. Many of these genes encode proteins with functions related to metabolism, physiology, or adaptation to a stressful environment. While homologs of some of these proteins have been studied in other pathogens, these proteins have not been specifically studied in B. cenocepacia. Several B. cenocepacia ET12 lineage strains contain at least a 45-kb fragment of the plasmid found in K56-2 and J2315 (Engledow et al., 2004) while strains AU1054 and MCO-3 lack a plasmid (Winsor et al., 2008). While plasmid-minus derivatives of B. cenocepacia J2315 or K56-2 have not been reported, it would be interesting to determine what influence absence of the plasmid may have on infection considering the vast majority of plasmid-encoded genes were induced in vivo. Further confirmatory experiments are required to substantiate trends for additional genes that exhibited altered expression in the in vivo environmental conditions. Revealing the changes in gene expression that occur in bacterial cells during infection is a first step in understanding the response of bacterial cells to the host environment. Increased expression of genes during infection suggests these genes promote bacterial survival and adaptation in the lungs and potentially influence virulence. The identification of potential novel virulence genes among these in vivo induced genes provides an opportunity to characterize these genes in more detail in future studies. Determining what growth conditions alter the expression of these genes and how they are regulated in B. cenocepacia will shed light on their expression pattern. Increased expression of genes during lung infection could be due to a change in environmental cues that enable transcriptional activation by a positive regulator(s) or derepression by a negative regulator(s). For potentially novel virulence genes, it will be important to construct mutations and examine their influence on virulence-related
phenotypes and pathogenesis in one or more infection models. This study provides an insight into *B. cenocepacia* gene expression in vivo and may provide opportunities to devise strategies to reduce or control *B. cenocepacia* lung infections.

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# APPENDIX

## Table A1 | *Burkholderia cenocepacia* genes induced during chronic lung infection.

| Gene       | Annotation or predicted function | Fold change |
|------------|----------------------------------|-------------|
| BCAL0123   | Putative glycosyltransferase      | 2.17        |
| BCAL0194   | Putative oxidoreductase           | 1.62        |
| BCAL0206A  | Putative outer membrane protein   | 2.36        |
| BCAL0226   | DNA-directed RNA polymerase beta  | 1.73        |
| BCAL0227   | DNA-directed RNA polymerase beta' chain | 1.56 |
| BCAL0269   | Putative oxidoreductase           | 1.59        |
| BCAL0278   | Putative type IV pili secretin    | 1.58        |
| BCAL0290   | Glutamate synthase small subunit  | 1.78        |
| BCAL0292   | 2',5' RNA ligase family protein   | 1.66        |
| BCAL0305   | Putative exported protein         | 2.21        |
| BCAL0366   | Nitroreductase family protein     | 1.60        |
| BCAL0403   | Putative outer membrane-bound lytic murein | 1.54 |
| BCAL0466   | Putative aminotransferase         | 2.86        |
| BCAL0580   | Putative chromatase transport     | 1.62        |
| BCAL0623   | Putative export protein           | 1.72        |
| BCAL0624   | Putative outer membrane porin protein precursor | 1.62 |
| BCAL0658   | Allophanate hydrolase subunit 2   | 1.56        |
| BCAL0668   | Serine peptidase, family S9 unassigned | 1.52 |
| BCAL0704   | d-alanyl-d-alanine carboxypeptidase | 1.64 |
| BCAL0804   | Putative membrane protein         | 1.53        |
| BCAL1103   | Putative Osmb-like lipoprotein    | 2.13        |
| BCAL1211   | Hypothetical protein              | 1.65        |
| BCAL1212   | 2-Oxoisovalerate dehydrogenase alpha subunit | 3.02 |
| BCAL1213   | 2-Oxoisovalerate dehydrogenase beta subunit | 2.91 |
| BCAL1214   | Lipoamide acyltransferase component of | 3.68 |
| BCAL1215   | Dihydrioloapamide dehydrogenase   | 2.22        |
| BCAL1226   | Major facilitator superfamily protein | 1.52 |
| BCAL1279   | Putative exported protein         | 1.64        |
| BCAL1468   | Putative electron transport protein | 1.51 |
| BCAL1499   | Putative exported protein         | 1.79        |
| BCAL1539   | Putative exported protein         | 2.30        |
| BCAL1657   | Putative ribose transport system  | 1.77        |
| BCAL1658   | Putative ribose ABC transporter   | 1.56        |
| BCAL1671   | Metallo peptidase, subfamily M23B | 1.61 |
| BCAL1678   | Putative outer membrane usher protein precursor | 2.40 |
| BCAL1699   | Putative l-ornithine 5-monooxygenase | 1.61 |
| BCAL1715   | Conserved hypothetical protein     | 1.53        |
| BCAL1749   | Putative CoA-transferase          | 2.39        |
| BCAL1750   | Conserved hypothetical protein     | 2.39        |
| BCAL1751   | Glyoxalase/bleomycin resistance   | 1.70        |
| BCAL1754   | Major facilitator superfamily protein | 3.50 |
| BCAL1783_i0 | TonB-dependent receptor (pseudogene) | 1.59 |

| Gene       | Annotation or predicted function | Fold change |
|------------|----------------------------------|-------------|
| BCAL1789   | Putative iron-transport protein   | 1.73        |
| BCAL1798   | Putative exported protein         | 1.95        |
| BCAL1961   | Putative exported protein         | 1.94        |
| BCAL1980   | Putative acyl-CoA synthetase      | 1.54        |
| BCAL1992   | Putative acyl-CoA thioesterase precursor | 2.00 |
| BCAL2037   | Putative ureidoglycolate hydrolase | 1.61        |
| BCAL2038   | Putative allantoicase             | 1.53        |
| BCAL2039   | Putative uricase                  | 1.72        |
| BCAL2040   | Polysaccharide deacetylase        | 1.54        |
| BCAL2044   | Muramoyltetraepptide carboxypeptidase | 1.51 |
| BCAL2083   | Outer membrane protein assembly factor YaeT | 1.53 |
| BCAL2155   | Putative serine acetyltransferase | 1.61        |
| BCAL2179   | Enolase                          | 1.51        |
| BCAL2187   | Putative exported protein         | 1.56        |
| BCAL2191   | Putative membrane protein         | 3.09        |
| BCAL2272   | Conserved hypothetical protein     | 1.57        |
| BCAL2357   | Ketol-acid reductoisomerase       | 1.59        |
| BCAL2467   | Putative lipoprotein              | 2.10        |
| BCAL2468   | Putative membrane protein         | 1.91        |
| BCAL2475a  | Conserved hypothetical protein     | 1.63        |
| BCAL2476   | Hypothetical protein              | 1.73        |
| BCAL2482   | Putative outer membrane protein   | 3.15        |
| BCAL2485   | Putative iron–sulfur cluster-binding electron | 2.12 |
| BCAL2486   | Putative iron–sulfur oxidoreductase | 2.11 |
| BCAL2488   | Lys family regulatory protein     | 2.07        |
| BCAL2500   | Hypothetical protein              | 1.67        |
| BCAL2505   | Putative membrane protein         | 1.55        |
| BCAL2507   | Conserved hypothetical protein     | 1.93        |
| BCAL2516   | Hypothetical protein              | 1.70        |
| BCAL2529   | Putative transcriptional regulator | 1.53 |
| BCAL2541   | Putative hydrolase                | 1.53        |
| BCAL2552   | Putative membrane protein         | 1.53        |
| BCAL2553   | Putative membrane protein         | 1.85        |
| BCAL2558   | Putative thioredoxin/FAD-dependent pyridine | 2.09 |
| BCAL2588   | Putative transposase (fragment)   | 1.81        |
| BCAL2607   | Putative exported protein         | 2.70        |
| BCAL2615   | Putative exported outer membrane porin protein | 2.16 |
| BCAL2777   | Putative N-acetylmuramoyl-l-alanine amidase | 1.58 |
| BCAL2819   | Putative permease protein         | 1.61        |
| BCAL2911   | Proline-rich exported protein     | 1.58        |
| BCAL2956   | Putative exported protein         | 1.52        |
| BCAL3024   | Putative exported protein         | 1.57        |
| BCAL3033   | Probable outer membrane lipoproteins carrier | 1.53 |

(Continued)
Table A1 | Continued

| Gene          | Annotation or predicted function                        | Fold change | Gene          | Annotation or predicted function                        | Fold change |
|---------------|--------------------------------------------------------|-------------|---------------|--------------------------------------------------------|-------------|
| BCAL3038      | ABC transporter ATP-binding component                   | 1.61        | BCAM0502      | Conserved hypothetical protein                           | 1.78e       |
| BCAL3039      | ABC transporter, membrane permease                      | 1.54        | BCAM0595      | LysR family regulatory protein                          | 2.56        |
| BCAL3040      | ABC transporter, membrane permease                      | 1.71        | BCAM0630      | Putative dehydrogenase                                  | 1.73        |
| BCAL3041      | Maltose-binding protein                                 | 2.09        | BCAM0676      | Putative exported protein                               | 1.84        |
| BCAL3163      | Putative nucleotidytransferase                         | 1.68        | BCAM0880      | Putative methyltransferase                              | 7.10        |
| BCAL3203      | Putative periplasmic TolB protein                       | 1.64        | BCAM0895      | Conserved hypothetical protein                           | 1.56e       |
| BCAL3204      | Putative OmpA family lipoprotein                       | 1.68        | BCAM0926      | Multidrug efflux system transporter protein             | 5.89        |
| BCAL3205      | Putative exported protein                               | 1.62        | BCAM0944      | Putative lipoprotein                                    | 1.58        |
| BCAL3289      | Putative glycolate oxidase subunit GlcE                | 1.64        | BCAM0983      | 3-Isopropylmalate dehydrogenase large subunit           | 2.87        |
| BCAL3297      | Putative ferrin DPS-family                              | 1.67        | BCAM0983A     | Putative entericidin B-like bacteriolytic toxin          | 2.01        |
| BCAM0303      | ABC transporter ATP-binding membrane protein            | 1.52        | BCAM1150      | 3-Hydroxyisobutyrate dehydrogenase                      | 1.64        |
| BCAM0347      | Putative exported protein                               | 1.74        | BCAM1151      | Methylmalonate-semialdehyde dehydrogenase               | 2.40        |
| BCAM0396      | Putative exported protein                               | 1.60        | BCAM1171      | Major facilitator superfamily protein                   | 1.55        |
| BCAM0402      | Putative membrane protein                               | 2.43        | BCAM1187      | TonB-dependent siderophore receptor                     | 1.71        |
| BCAM0403      | Putative oxidoreductase                                 | 1.77        | BCAM1207      | ABC transporter ATP-binding membrane protein            | 1.52        |
| BCAM0474      | Putative gluconokinase                                  | 1.66        | BCAM1263      | Putative malate/l-lactate dehydrogenase                 | 1.79        |
| BCAM0493      | Putative exported protein                               | 1.65        | BCAM1279      | Conserved hypothetical protein                           | 1.54d       |
| BCAM0499      | Putative RNA polymerase sigma factor, sigma-70          | 1.84        | BCAM1313      | Putative amidase accessory protein                      | 1.60        |
| BCAM0502      | Conserved hypothetical protein                           | 1.66d       | BCAM1315      | Aliphatic amidase (acylamide amidohydrolyase)           | 1.55        |
| BCAM0503      | ABC transporter ATP-binding membrane protein            | 1.62        | BCAM1330      | Putative polysaccharide export protein                  | 1.73        |
| BCAM0535      | Putative sodium bile acid symporter family              | 1.51        | BCAM1333      | Putative expopolysaccharide                             | 1.56        |
| BCAM0575      | Conserved hypothetical protein                           | 1.60d       | BCAM1341      | Conserved hypothetical protein                           | 3.22e       |
| BCAM0576      | Hypothetical protein                                    | 1.95        | BCAM1374      | Conserved hypothetical protein                           | 1.87e       |
| BCAM0577      | Conserved hypothetical protein                           | 1.60d       | BCAM1390      | Putative aldolase                                       | 3.00        |
| BCAM0578      | Conserved hypothetical protein                           | 1.72e       | BCAM1425      | Putative membrane protein                               | 2.88        |
| BCAM0589      | Conserved hypothetical protein                           | 1.70d       | BCAM1427      | LysE family transporter                                 | 3.76        |
| BCAM0590      | Conserved hypothetical protein                           | 1.69d       | BCAM1487      | Putative ABC transporter, substrate-binding             | 3.14        |
| BCAM0596      | Conserved hypothetical protein                           | 1.66d       | BCAM1488      | Putative proline racemase                               | 1.90        |
| BCAM0621      | Putative branched-chain amino acid transport            | 1.52        | BCAM1527      | Putative cation efflux protein                          | 1.82        |
| BCAM0622      | LuxR superfamily regulatory protein                     | 1.89        | BCAM1563      | ABC transporter ATP-binding membrane protein            | 1.70        |
| BCAM0647      | Putative exported multicopper oxidase                   | 13.01       | BCAM1679      | Putative lysophosphatidylglycerol synthetase            | 1.62        |
| BCAM0649      | Cysteine desulfurase                                    | 3.60        | BCAM1726      | Putative exported protein                               | 2.01        |
| BCAM0678      | Glucosamine – fructose-6-phosphate                      | 1.52        | BCAM1742      | Putative exported protein                               | 1.87        |
| BCAM0679      | ABC transporter ATP-binding membrane protein            | 1.52        | BCAM1775      | Putative transglycosylase associated protein            | 1.76        |

(Continued)
### Table A1 | Continued

| Gene          | Annotation or predicted functiona | Fold changeb |
|---------------|-----------------------------------|--------------|
| BCAM1823      | Putative methyltransferase         | 1.52         |
| BCAM1901      | Hypothetical phage protein         | 1.65         |
| BCAM1904      | Hypothetical phage protein         | 1.58         |
| BCAM1911      | Hypothetical phage protein         | 1.65         |
| BCAM1946      | Putative quinoxaline efflux system transporter | 1.61 |
| BCAM1957      | ABC transporter ATP-binding protein | 1.56         |
| BCAM1964      | Putative exported protein          | 1.57         |
| BCAM2007      | TonB-dependent siderophore receptor | 1.58         |
| BCAM2025      | Sigma-54 interacting regulatory protein | 1.87 |
| BCAM2051      | Type III secretion system protein  | 1.73         |
| BCAM2073      | Putative exported protein          | 2.98         |
| BCAM2095      | Putative HTH transcriptional regulator | 1.57         |
| BCAM2096      | Putative gamma-glutamylputrescine  | 1.87         |
| BCAM2119      | Carboxylesterase                   | 1.81         |
| BCAM2162      | MarR family regulatory protein     | 1.99         |
| BCAM2191      | Enoyl-CoA hydratase/isomerase family | 1.94         |
| BCAM2192      | Enoyl-CoA hydratase/isomerase family protein | 2.37 |
| BCAM2193      | Putative 3-hydroxyisobutyrate dehydrogenase | 2.39 |
| BCAM2194      | Methylmalonate-semialdehyde dehydrogenase | 2.26 |
| BCAM2195      | Putative AMP-binding enzyme        | 2.51         |
| BCAM2196      | Putative acyl-CoA dehydrogenase    | 2.10         |
| BCAM2237      | Putative 2,2-dialkylglycine decarboxylase | 2.41 |
| BCAM2260      | Major facilitator superfamily protein | 1.61         |
| BCAM2338      | Putative glycosyltransferase       | 1.53         |
| BCAM2356      | Conserved hypothetical protein     | 1.63d        |
| BCAM2453      | Putative redoxin protein           | 1.69         |
| BCAM2479      | Putative transporter – LysE family | 1.54         |
| BCAM2488      | Putative phosphoglycerate/bisphosphoglycerate | 1.56 |
| BCAM2504      | Conserved hypothetical protein     | 1.84d        |
| BCAM2542      | Fenitrothion hydrolase protein FedA | 1.57         |
| BCAM2618      | Putative periplasmic                | 1.64         |
| BCAM2623      | Conserved hypothetical protein     | 2.06d        |
| BCAM2647      | Putative membrane protein          | 1.71         |
| BCAM2648      | NAD dependent                      | 1.61         |
| BCAM2685      | Conserved hypothetical protein     | 2.11d        |
| BCAM2700      | Putative membrane protein          | 1.81         |
| BCAM2701      | Aconitate hydratase 1              | 2.66         |
| BCAM2702      | 2-Methylcitrate synthase           | 5.88         |
| BCAM2703      | Probable methylisocitrate lyase    | 2.78         |
| BCAM2730      | Putative tripeptide permease       | 1.54         |
| BCAS0028      | Succinylglutamate                  | 2.80         |
| BCAS0043      | Putative l-lysine 6-monoxygenase   | 3.11         |
| BCAS0050      | Putative amido hydrolase           | 1.53         |
| BCAS0053      | FMN reductase                      | 2.34         |

| Gene          | Annotation or predicted functiona | Fold changeb |
|---------------|-----------------------------------|--------------|
| BCAS0097      | Putative cobalamin synthesis protein | 1.66         |
| BCAS0100      | Putative ribokinase                | 1.52         |
| BCAS0230      | Putative sugar ABC transporter     | 1.58         |
| BCAS0251      | Putative lipoprotein               | 1.61         |
| BCAS0260      | Conserved hypothetical protein     | 2.20d        |
| BCAS0278      | Tartrate dehydrogenase             | 1.66         |
| BCAS0308      | Putative flp type pilus assembly protein | 2.44 |
| BCAS0362      | Putative ketopantoate reductase    | 1.58         |
| BCAS0397      | Metallo peptidase, subfamily M20D  | 2.01         |
| BCAS0436      | AraC family regulatory protein     | 1.66         |
| BCAS0443      | Putative binding-protein-dependent transport | 5.32 |
| BCAS0449      | Putative binding-protein-dependent transport | 1.62 |
| BCAS0461      | Putative lipoprotein               | 3.69         |
| BCAS0463      | Putative membrane protein          | 1.64         |
| BCAS0477      | Putative lipoprotein               | 2.07         |
| BCAS0482      | Conserved hypothetical protein     | 4.80d        |
| BCAS0513      | Putative phage tail protein        | 1.54         |
| BCAS0519      | Hypothetical phage protein         | 1.64         |
| BCAS0543      | Putative phage transcriptional regulator | 1.84 |
| BCAS0545      | Hypothetical phage protein         | 1.55         |
| BCAS0547      | Putative phage DNA-binding protein | 1.54         |
| BCAS0552      | Hypothetical phage protein         | 1.72         |
| BCAS0569      | Conserved hypothetical protein     | 2.31d        |
| BCAS0574      | Amino acid ABC transporter         | 3.67         |
| BCAS0575      | Putative binding-protein-dependent transport | 2.02 |
| BCAS0577      | Periplasmic solute-binding protein | 1.54         |
| BCAS0587_1_0  | Aminopyrrolnitrin oxidase PrnD (fragment) | 2.33 |
| BCAS0588      | Putative membrane protein (fragment) | 1.52         |
| BCAS0672      | Hypothetical protein               | 1.91         |
| BCAS0713      | Putative short-chain oxidoreductase | 1.66 |
| BCAS0730      | Putative Na+ dependent nucleoside transporter | 2.13 |
| BCAS0750      | Putative exported protein          | 1.82         |
| pBCA001       | Putative partition protein         | 1.93         |
| pBCA002       | Putative partitioning protein      | 1.52         |
| pBCA008       | Conserved hypothetical protein     | 2.49d        |
| pBCA009       | Conserved hypothetical protein     | 1.74d        |
| pBCA010       | Putative membrane protein          | 3.19         |
| pBCA012       | Hypothetical protein               | 3.34         |
| pBCA013       | Putative exported protein          | 6.32         |
| pBCA014       | Putative membrane protein          | 3.28         |
| pBCA015       | Hypothetical protein               | 2.71         |
| pBCA016       | Conserved hypothetical protein     | 6.53d        |
| pBCA017       | Conserved hypothetical protein     | 3.24d        |
| pBCA018       | Hypothetical protein               | 8.91         |
| pBCA019       | Putative membrane protein          | 2.40         |

(Continued)
| Gene     | Annotation or predicted function                        | Fold changeb |
|----------|--------------------------------------------------------|--------------|
| pBCA020  | Putative TraG conjugative transfer protein             | 5.51         |
| pBCA021  | Putative TraH conjugative transfer protein             | 13.21        |
| pBCA022  | Conserved hypothetical protein                         | 8.09         |
| pBCA023  | Conserved hypothetical protein                         | 5.09         |
| pBCA024  | Conserved hypothetical protein                         | 10.16        |
| pBCA025  | Putative TraF conjugative transfer protein             | 7.10         |
| pBCA026  | Putative membrane protein                              | 10.57        |
| pBCA027  | Putative conjugative transfer protein                  | 14.73        |
| pBCA028  | Conserved hypothetical protein                         | 5.03         |
| pBCA029  | Putative membrane protein                              | 8.60         |
| pBCA030  | Putative conjugative transfer protein                  | 6.06         |
| pBCA031  | Putative TraU conjugative transfer protein             | 6.92         |
| pBCA032  | Putative TraV conjugative transfer protein             | 8.96         |
| pBCA033  | Putative peptidase protein                             | 4.97         |
| pBCA034  | Putative membrane protein                              | 6.01         |
| pBCA035  | GntR family regulatory protein                         | 18.91        |
| pBCA036  | Putative membrane protein                              | 13.82        |
| pBCA037  | Putative membrane protein                              | 7.33         |
| pBCA037a | Hypothetical protein                                   | 11.90        |
| pBCA038  | Hypothetical protein                                   | 9.54         |
| pBCA039  | Hypothetical protein                                   | 1.98         |
| pBCA040  | Hypothetical protein                                   | 2.04         |
| pBCA041  | Putative TraC conjugative transfer protein             | 9.20         |
| pBCA042  | Type IV secretion system TraV                         | 19.71        |
| pBCA043  | Thiol:disulfide interchange protein DsbC               | 7.91         |
| pBCA044  | Putative TraB conjugative transfer protein             | 3.00         |

Gene Annotation or predicted function\(^a\) Fold change\(^b\)

| Gene     | Annotation or predicted function                        | Fold changeb |
|----------|--------------------------------------------------------|--------------|
| pBCA045  | Putative exported protein TraK                         | 12.43        |
| pBCA046  | Putative TraE conjugative transfer protein             | 16.87        |
| pBCA047  | Type IV conjugative transfer system protein TraL       | 46.07        |
| pBCA048  | Putative membrane protein                              | 55.79        |
| pBCA049  | Putative transglycosylase protein                      | 4.97         |
| pBCA050  | Hypothetical protein                                   | 8.74         |
| pBCA051  | LamB/YcsF family protein                               | 159.40       |
| pBCA052  | Putative exported protein                              | 789.20       |
| pBCA053  | Putative extracellular solute-binding protein          | 480.70       |
| pBCA054  | LuxR family regulatory protein                         | 3.90         |
| pBCA055  | Hypothetical protein                                   | 4.34         |
| pBCA056  | Putative conjugative transfer protein                  | 4.80         |
| pBCA057  | Thiol:disulfide interchange protein DsbD               | 7.43         |
| pBCA058  | Putative membrane protein                              | 55.79        |
| pBCA059  | Putative membrane protein                              | 7.33         |
| pBCA060  | Hypothetical protein                                   | 1.98         |
| pBCA061  | Conserved hypothetical protein                         | 2.52         |
| pBCA062  | Conserved hypothetical protein                         | 1.53         |
| pBCA063  | Conserved hypothetical protein                         | 1.55         |
| pBCA064  | Conserved hypothetical protein                         | 1.66         |
| pBCA065  | NUDIX hydrolase family protein                        | 1.53         |
| pBCA066  | Amidohydrolase family protein                          | 1.64         |
| pBCA067  | Putative integrase                                     | 1.59         |

\(^a\)Derived from *B. cenocepacia* J2315 (Holden et al., 2009) at http://www.burkholderia.com (Winsor et al., 2008) or http://www.microbesonline.org (Dehal et al., 2009).

\(^b\)Fold change of RNA recovered from rat lungs (in vivo) relative to RNA isolated from in vitro grown cultures as determined by microarray analysis.

\(^c\)Conserved hypothetical protein in one or more members of the Bcc and in B. pseudomallei.

\(^d\)Conserved hypothetical protein in one or more members of the Bcc.
| Gene         | Annotation or predicted function | Fold change |
|--------------|----------------------------------|-------------|
| BCAL0046     | Putative fatty-acid CoA ligase    | 1.56        |
| BCAL0057     | Putative membrane protein         | 2.17        |
| BCAL0112     | Conserved hypothetical protein    | 1.82        |
| BCAL0113     | B-type flagellar hook-associated protein 2 | 2.71 |
| BCAL0114     | Flagellin (type II)               | 8.29        |
| BCAL0121     | Aquaporin Z                       | 3.29        |
| BCAL0126     | Chemotaxis protein MotA           | 2.19        |
| BCAL0127     | Chemotaxis protein MotB           | 2.03        |
| BCAL0128     | Chemotaxis two-component response regulator | 2.96 |
| BCAL0129     | Chemotaxis two-component sensor kinase CheA | 2.38 |
| BCAL0130     | Chemotaxis protein CheW           | 1.63        |
| BCAL0132     | Chemotaxis protein methyltransferase | 2.52 |
| BCAL0133     | Putative chemoreceptor glutamine deamidase cheD | 2.47 |
| BCAL0134     | Chemotaxis response regulator protein-glutamate | 2.04 |
| BCAL0135     | Chemotaxis protein CheY           | 1.52        |
| BCAL0136     | Chemotaxis protein CheZ           | 2.09        |
| BCAL0140     | Flagellar biosynthetic protein FlhB | 2.46 |
| BCAL0143     | Putative flagellar biosynthesis protein | 1.71 |
| BCAL0147     | 5,10-Methylenetetrahydrofolate reductase | 2.17 |
| BCAL0154     | Histone-like nucleoid-structuring (H-NS) | 1.97 |
| BCAL0168     | Hypothetical protein              | 2.50        |
| BCAL0169     | Conserved hypothetical protein    | 2.42        |
| BCAL0179     | Hypothetical protein              | 1.87        |
| BCAL0203     | Phosphatidylethanolamine-binding protein | 1.56 |
| BCAL0212     | Putative phenylactic acid degradation NADH | 1.63 |
| BCAL0233     | 30s Ribosomal protein S10         | 1.59        |
| BCAL0339     | Putative lipoprotein              | 1.60        |
| BCAL0341     | Conserved hypothetical protein    | 1.75        |
| BCAL0342     | Conserved hypothetical protein    | 1.68        |
| BCAL0343     | Conserved hypothetical protein    | 1.86        |
| BCAL0344     | Conserved hypothetical protein    | 1.58        |
| BCAL0345     | Conserved hypothetical protein    | 1.78        |
| BCAL0356     | Putative quinone oxidoreductase   | 1.51        |
| BCAL0404     | Phenylacetate-coenzyme A ligase   | 1.59        |
| BCAL0406     | Probable enoyl-CoA hydratase PaaG | 1.56 |
| BCAL0412     | Conserved hypothetical protein (pseudogene) | 2.11 |
| BCAL0413     | Conserved hypothetical protein    | 1.67        |
| BCAL0431     | Conserved hypothetical protein    | 1.86        |
| BCAL0432     | Putative membrane protein         | 1.61        |
| BCAL0434     | Putative exported protein         | 2.13        |
| BCAL0505     | Integrase/recombinase             | 1.71        |
| BCAL0511     | Putative deoxyxygenases           | 1.60        |

(Continued)
| Gene     | Annotation or predicted function | Fold change |
|----------|----------------------------------|-------------|
| BCAL1155 | Putative maleate cis–trans isomerase | 3.29        |
| BCAL1159 | Putative 2,3-dihydroxybenzoate-AMP ligase | 1.52        |
| BCAL1167 | Putative exported protein | 1.74        |
| BCAL1168 | Conserved hypothetical protein | 1.71        |
| BCAL1221 | Putative porin | 1.54        |
| BCAL1233 | Putative heat shock Hsp20-related protein | 1.65        |
| BCAL1273 | Phosphate ABC transporter ATP-binding protein | 1.55        |
| BCAL1282 | Putative membrane protein | 2.39        |
| BCAL1291 | Putative membrane protein | 1.54        |
| BCAL1292 | Putative membrane protein | 1.75        |
| BCAL1299 | Conserved hypothetical protein | 1.51        |
| BCAL1300 | Conserved hypothetical protein | 1.98        |
| BCAL1316 | Conserved hypothetical protein | 1.56        |
| BCAL1326 | Conserved hypothetical protein | 8.68        |
| BCAL1357 | Putative exported protein | 1.56        |
| BCAL1359 | Conserved hypothetical protein | 1.54        |
| BCAL1360 | Hypothetical protein | 1.85        |
| BCAL1373 | LysR family regulatory protein | 1.94        |
| BCAL1390 | Endoglucanase precursor | 2.00        |
| BCAL1394 | Putative exported protein | 1.51        |
| BCAL1396 | Putative membrane protein | 1.72        |
| BCAL1418 | Major facilitator superfamily protein | 2.31        |
| BCAL1435 | Inositol 2-dehydrogenase | 2.41        |
| BCAL1452 | Putative methyl-accepting chemotaxis protein | 1.75        |
| BCAL1525 | Hip type pilus subunit | 12.95       |
| BCAL1525a | Putative Hip type pilus leader peptidase | 4.62        |
| BCAL1526 | Putative Hip type pilus assembly protein | 2.62        |
| BCAL1527 | Hip type pilus assembly protein | 2.05        |
| BCAL1528 | Hip type pilus assembly protein | 2.87        |
| BCAL1529 | Hip type pilus assembly-related protein | 1.93        |
| BCAL1530 | Hip type pilus assembly protein | 3.56        |
| BCAL1531 | Hip type pilus assembly protein | 2.02        |
| BCAL1532 | Hip type pilus assembly protein | 2.40        |
| BCAL1533 | Putative lipoprotein | 2.15        |
| BCAL1534 | Putative exported protein | 2.81        |
| BCAL1535 | Putative membrane protein | 1.71        |
| BCAL1573 | Hypothetical phage protein | 1.52        |
| BCAL1574 | Hypothetical phage protein | 1.56        |
| BCAL1577 | Hypothetical phage protein | 2.26        |
| BCAL1596 | Hypothetical phage protein | 1.66        |
| BCAL1597 | Hypothetical phage protein | 1.78        |
| BCAL1610 | Periplasmic cystine-binding protein | 1.59        |
| BCAL1640 | Major facilitator superfamily protein | 3.38        |
| BCAL1668 | Periplasmic solute-binding protein | 2.02        |
| BCAL1677 | Putative type-1 fimbrial protein | 1.74        |
| BCAL1730 | Precorrin-4 C11-methyltransferase | 1.71        |
| BCAL1775 | Putative demethylase oxidoreductase | 1.85        |
| BCAL1791 | Conserved hypothetical protein | 2.23        |

(Continued)
| Gene         | Annotation or predicted function | Fold change |
|-------------|----------------------------------|-------------|
| BCAL3211    | Conserved hypothetical protein    | 1.66        |
| BCAL3227    | Conserved hypothetical protein    | 2.10        |
| BCAL3231    | Hypothetical protein              | 1.63        |
| BCAL3234    | Glycosyltransferase               | 1.69        |
| BCAL3239    | Glucosyltransferase               | 1.84        |
| BCAL3368    | Putative regulatory protein       | 1.85        |
| BCAL3427    | Histone H1-like protein           | 2.68        |
| BCAL3428    | Ribonucleoside-diphosphate reductase | 1.58   |
| BCAL3457    | Cell division protein FtsZ        | 1.71        |
| BCAM0010    | 2-Amino-3-ketobutyrate coenzyme A ligase | 2.03 |
| BCAM0011    | Threonine 3-dehydrogenase         | 1.71        |
| BCAM0028    | Putative FHA-domain protein       | 1.58        |
| BCAM0030    | Conserved hypothetical protein     | 8.45        |
| BCAM0031    | Conserved hypothetical protein     | 5.26        |
| BCAM0032    | Conserved hypothetical protein     | 1.71        |
| BCAM0064    | Conserved hypothetical protein     | 1.89        |
| BCAM0067    | Putative short-chain dehydrogenase | 2.24        |
| BCAM0069    | Conserved hypothetical protein     | 1.57        |
| BCAM0070    | Putative hydrolyase                | 1.66        |
| BCAM0096    | ABC transporter ATP-binding protein | 2.32    |
| BCAM0103    | Major facilitator superfamily protein | 1.65 |
| BCAM0186    | Lectin                            | 2.64        |
| BCAM0188    | N-acetyl-homoserine lactone dependent regulatory | 1.57 |
| BCAM0190    | Putative aminotransferase – class III | 2.44 |
| BCAM0191    | Putative non-ribosomal peptide synthetase | 2.05 |
| BCAM0192    | Conserved hypothetical protein     | 1.65        |
| BCAM0194    | Conserved hypothetical protein     | 1.94        |
| BCAM0210    | Putative transerase                | 1.71        |
| BCAM0288    | Two-component regulatory system, response | 1.52 |
| BCAM0446    | Outer membrane efflux protein     | 187.90      |
| BCAM0486    | LacI family regulatory protein    | 4.99        |
| BCAM0487    | Conserved hypothetical             | 1.53        |
| BCAM0504    | CsbD-like protein                  | 2.24        |
| BCAM0505    | Putative membrane-attached protein | 1.67        |
| BCAM0507    | CsbD-like protein                  | 2.40        |
| BCAM0521    | Putative IstB-like ATP-binding protein | 2.85  |
| BCAM0522    | Putative integrase                 | 1.76        |
| BCAM0589    | Conserved hypothetical protein     | 1.68        |
| BCAM0622    | Two-component regulatory system, sensor kinase | 1.58 |
| BCAM0623    | Two-component regulatory system, response | 1.62   |
| BCAM0633    | Conserved hypothetical protein     | 2.67        |
| BCAM0634    | Hypothetical protein               | 10.80       |
| BCAM0717    | Putative Gram-negative porin       | 2.44        |
| BCAM0753    | Putative membrane protein          | 2.18        |
| BCAM0780    | Putative helicase                  | 1.59        |

Table A2 | Continued

| Gene         | Annotation or predicted function | Fold change |
|-------------|----------------------------------|-------------|
| BCAM0851    | Conserved hypothetical protein    | 1.83        |
| BCAM0917    | Putative DNA primase              | 1.64        |
| BCAM0918    | RNA polymerase sigma factor RpoD | 1.52        |
| BCAM0942    | Putative exported protein         | 1.59        |
| BCAM0953    | Extracellular solute-binding protein | 1.80   |
| BCAM0957    | Putative pepstatin-insensitive carboxyl | 1.64 |
| BCAM1041    | Putative phage coiled coil domain protein | 2.06 |
| BCAM1123    | ABC transporter ATP-binding protein | 1.52     |
| BCAM1138    | Major facilitator superfamily protein | 1.77 |
| BCAM1140    | Putative aldehyde oxidase/xanthine | 1.52 |
| BCAM1141    | Putative isochorismatase          | 1.81        |
| BCAM1142    | Conserved hypothetical protein     | 1.76        |
| BCAM1143    | Putative hydrolase                | 1.86        |
| BCAM1144    | Putative Asp/Glu/Hydantoin racemase | 2.22  |
| BCAM1146    | Putative flavoprotein monooxygenase | 2.33     |
| BCAM1147    | Isoquinoline 1-oxidoreductase alpha subunit | 1.98 |
| BCAM1164    | Conserved hypothetical protein     | 1.87        |
| BCAM1175    | Putative iron-sulfur cluster protein | 1.60  |
| BCAM1213    | Putative membrane protein          | 2.19        |
| BCAM1255    | Putative exported protein          | 1.88        |
| BCAM1265    | Putative amino acid permease       | 1.80        |
| BCAM1316a   | Conserved hypothetical protein     | 2.00        |
| BCAM1316b   | Conserved hypothetical protein     | 1.54        |
| BCAM1335    | Glycosyltransferase               | 1.52        |
| BCAM1358    | Gluconate 2-dehydrogenase          | 1.52        |
| BCAM1411    | Putative short-chain dehydrogenase | 1.53     |
| BCAM1412    | Conserved hypothetical protein     | 10.28       |
| BCAM1413A   | Conserved hypothetical protein     | 24.61       |
| BCAM1414    | Conserved hypothetical protein     | 3.86        |
| BCAM1424    | Methyl-accepting chemotaxis protein | 1.68    |
| BCAM1443    | Putative exported protein          | 2.64        |
| BCAM1473    | Putative di-haem cytochrome c peroxidase | 1.67   |
| BCAM1491    | Putative exported protein          | 1.56        |
| BCAM1572    | Methyl-accepting chemotaxis protein | 1.93  |
| BCAM1573    | Alpha, alpha-trehalose-phosphate synthase | 1.64 |
| BCAM1588    | Putative lyase                     | 1.74        |
| BCAM1602    | Conserved hypothetical protein     | 1.59        |
| BCAM1623    | Thiolase                          | 2.75        |
| BCAM1643    | AMP-binding protein                | 1.76        |
| BCAM1704    | 2,3-Butanediol dehydrogenase      | 1.79        |
| BCAM1710    | Putative enoyl-CoA hydratase/isomerase | 1.58  |
| BCAM1711    | Phenylacetate-coczyme A ligase     | 1.57        |
| BCAM1733    | Putative membrane protein          | 2.36        |
| BCAM1734    | Putative cytochrome c              | 1.73        |
| BCAM1735    | Putative oxidoreductase            | 1.89        |
| BCAM1736    | Conserved hypothetical protein     | 1.84        |
| Gene     | Annotation or predicted function | Fold change |
|----------|----------------------------------|-------------|
| BCAM1744 | Serine peptidase, family S9     | 1.67        |
| BCAM1777A| Putative exported protein       | 4.61        |
| BCAM1804 | Methyl-accepting chemotaxis protein | 2.10     |
| BCAM1869 | Conserved hypothetical protein  | 1.85        |
| BCAM1871 | Conserved hypothetical protein  | 2.64        |
| BCAM1881 | Hypothetical phage protein      | 1.86        |
| BCAM1882 | Hypothetical phage protein      | 1.80        |
| BCAM1912 | Hypothetical phage protein      | 1.90        |
| BCAM1927 | Putative exported protein       | 1.94        |
| BCAM2021 | Methyl-accepting chemotaxis protein | 1.94    |
| BCAM2024 | Putative membrane protein       | 2.65        |
| BCAM2048 | Type III secretion system protein | 1.69      |
| BCAM2052 | Putative type III secretion system protein | 1.85 |
| BCAM2053 | Putative type III secretion system protein | 1.98 |
| BCAM2067 | Putative undecaprenyl pyrophosphate synthetase | 1.54 |
| BCAM2087 | Putative lipoprotein            | 2.24        |
| BCAM2105 | MerR family regulatory protein  | 1.64        |
| BCAM2106 | Non-heme chloroperoxidase       | 1.64        |
| BCAM2167 | Conserved hypothetical protein  | 1.51        |
| BCAM2169 | Putative outer membrane auto transporter | 1.73 |
| BCAM2198 | Serine peptidase, family S49   | 2.78        |
| BCAM2199 | Putative membrane protein       | 2.03        |
| BCAM2207 | Conserved hypothetical protein  | 1.90        |
| BCAM2210 | Putative membrane protein       | 2.59        |
| BCAM2215 | Putative copper resistance protein C precursor | 1.55 |
| BCAM2307 | Zinc metalloprotease ZmpB       | 2.28        |
| BCAM2312 | Putative ABC-type glycine betaine transport | 2.59 |
| BCAM2321 | Putative electron transfer flavoprotein alpha | 1.74 |
| BCAM2325 | Putative dipeptidase            | 1.75        |
| BCAM2333 | Putative glutathione-independent formaldehyde | 1.73 |
| BCAM2366 | Putative proline iminopeptidase | 1.57        |
| BCAM2374 | Putative methyl-accepting chemotaxis protein | 2.01 |
| BCAM2377 | Putative exported protein       | 3.99        |
| BCAM2378 | Putative Xaa-Pro dipeptidyl-peptidase | 1.63 |
| BCAM2403 | Conserved hypothetical protein  | 1.97        |
| BCAM2419 | Putative outer membrane protein A precursor | 1.79 |
| BCAM2444 | Putative exported protein       | 2.52        |
| BCAM2523 | Conserved hypothetical protein  | 2.31        |
| BCAM2545 | Major facilitator superfamily protein | 1.72 |
| BCAM2563 | Methyl-accepting chemotaxis protein | 1.62 |
| BCAM2564 | Putative aerotaxis receptor     | 3.44        |

(Continued)
Table A2 | Continued

| Gene     | Annotation or predicted function     | Fold change |
|----------|--------------------------------------|-------------|
| BCAS0481 | Putative lipoprotein                 | 1.86        |
| BCAS0510 | Hypothetical phage protein           | 2.29        |
| BCAS0540 | Hypothetical phage protein           | 1.72        |
| BCAS0548 | Hypothetical phage protein           | 1.69        |
| BCAS0572 | Putative exported protein            | 1.70        |
| BCAS0573 | Putative exported protein            | 1.72        |
| BCAS0576 | Putative binding-protein-dependent   | 1.52        |
|          | transport                            |             |
| BCAS0579 | Putative exported protein            | 2.01        |
| BCAS0595 | Putative sugar efflux transporter    | 1.53        |
| BCAS0596 | Conserved hypothetical protein       | 1.58        |
|          |                                      |             |
| BCAS0661C| Hypothetical protein                 | 1.83        |
| BCAS0662 | Conserved hypothetical protein       | 1.91        |
| BCAS0669 | Hypothetical protein                 | 1.90        |
| BCAS0700 | Putative oxygen-insensitive NAD(P)H  | 1.52        |
| BCAS0717 | Hypothetical protein                 | 2.26        |
| BCAS0773 | Putative exported protein            | 1.64        |
| pBCA055  | Putative membrane protein            | 18.16       |

*a Derived from B. cenocepacia J2315 (Holden et al., 2009) at http://www.burkholderia.com (Winsor et al., 2008) or http://www.microbesonline.org (Dehal et al., 2009).

*b Fold change of RNA isolated from in vitro grown cultures relative to RNA recovered from rat lungs (in vivo) as determined by microarray analysis.