The unexpected discovery of a novel low-oxygen-activated locus for the anoxic persistence of Burkholderia cenocepacia

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Burkholderia cenocepacia is a Gram-negative aerobic bacterium that belongs to a group of opportunistic pathogens displaying diverse environmental and pathogenic lifestyles. B. cenocepacia is known for its ability to cause lung infections in people with cystic fibrosis and it possesses a large 8 Mb multireplicon genome encoding a wide array of pathogenicity and fitness genes. Transcriptomic profiling across nine growth conditions was performed to identify the global gene expression changes made when B. cenocepacia changes niches from an environmental lifestyle to infection. In comparison to exponential growth, the results demonstrated that B. cenocepacia changes expression of over one-quarter of its genome during conditions of growth arrest, stationary phase and surprisingly, under reduced oxygen concentrations (6% instead of 20.9% normal atmospheric conditions). Multiple virulence factors are upregulated during these growth arrest conditions. A unique discovery from the comparative expression analysis was the identification of a distinct, co-regulated 50-gene cluster that was significantly upregulated during growth under low oxygen conditions. This gene cluster was designated the low-oxygen-activated (lxa) locus and encodes six universal stress proteins and proteins predicted to be involved in metabolism, transport, electron transfer and regulation. Deletion of the lxa locus resulted in B. cenocepacia mutants with aerobic growth deficiencies in minimal medium and compromised viability after prolonged incubation in the absence of oxygen. In summary, transcriptomic profiling of B. cenocepacia revealed an unexpected ability of aerobic Burkholderia to persist in the absence of oxygen and identified the novel lxa locus as key determinant of this important ecophysiological trait.

Subject Category: Integrated genomics and post-genomics approaches in microbial ecology
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Introduction

Burkholderia cenocepacia is a member of the Burkholderia cepacia complex, a group of Gram-negative environmental bacteria of clinical importance as opportunistic pathogens. B. cenocepacia causes problematic lung infections in people with cystic fibrosis (CF) because it is multi-drug resistant, transmissible and associated with poor clinical outcome (Drevinek and Mahenthiralingam, 2010). Clonal strains of B. cenocepacia have been isolated from the environment and infected patients, indicating that in the absence of nosocomial or patient-to-patient acquisition, the environment is a reservoir for infection (LiPuma et al., 2002; Baldwin et al., 2007). Burkholderia bacteria are prevalent in various terrestrial environments, with B. cenocepacia strains from the recA group B phylogenetic lineage (Vandamme et al., 2003) being found within the rhizosphere of crops such as maize (Dalmastri et al., 2007) and onions (LiPuma et al., 2002). Environmental sources for B. cenocepacia strains of recA group A lineage that are particularly virulent in CF patients (Vandamme et al., 2003; Drevinek and Mahenthiralingam, 2010) remain poorly defined.

Environmental bacteria are exposed to dramatic changes in growth conditions when they enter the respiratory tract and establish infection. The temperature rises to a stable 37 °C, the concentration
and composition of nutrients change, and when engulfed by macrophages bacteria are subject to low pH, severe nutrient limitation and oxidative stress. In particular, alterations that occur under reduced oxygen availability are an area of emerging interest in microbial infection research (Marteyn et al., 2011; Dietz et al., 2012). The CF lung environment is characterised by steep oxygen gradients, with low oxygen concentrations prevailing within the mucus (Yang et al., 2011). Oxygen concentrations within the infected CF lung can vary from atmospheric (20.9%) to zero and this is in part attested by the isolation of strict anaerobes from CF sputum at cell densities equivalent to that of well-characterised pathogens such as Pseudomonas aeruginosa (Tunney et al., 2008). P. aeruginosa can adapt to this environment because it is facultatively anaerobic (Yang et al., 2011). Certain Burkholderia species can fix nitrogen, an oxygen-sensitive process, suggesting they can survive anoxic environments and may be able to grow without oxygen (Menard et al., 2007; Suarez-Moreno et al., 2012). However, profiling data from moist soil environments where oxygen is limited demonstrates that the diversity of Burkholderia is considerably reduced under anoxic conditions (Pett-Ridge and Firestone, 2005). Burkholderia pseudomallei can survive, but not grow under anoxic conditions, with colony morphology-switching increasing as a result of anoxic exposure (Tandhavanant et al., 2010). B. cenocepacia is conventionally thought of as an obligate aerobic non-fermenting microorganism (Vandamme et al., 2003) and we know little about its ability to grow or survive without oxygen.

The discovery of gene function in B. cenocepacia is challenging because of its large genome size (>8 Mb and 7000 coding sequences), unusual multireplicon structure (Holden et al., 2009; Agnoli et al., 2012) and presence of multiple paralogous gene pathways (Chain et al., 2006). The availability of a genome sequence for B. cenocepacia J2315 (Holden et al., 2009) has enabled the design of a custom microarray and multiple investigations of global gene expression as a means to explore the encoded phenotypic diversity of these bacteria. Transcriptomic analysis of B. cenocepacia has provided new insights into regulatory RNAs (Coenye et al., 2007), gene expression in CF sputum (Drevinek et al., 2008) and in artificial sputum versus soil-based medium (Yoder-Himes et al., 2009); it has also contributed to mapping of multiple gene pathways involved in quorum sensing (O’Grady et al., 2009; Inhulsen et al., 2012), antibiotic resistance (Bazzini et al., 2011; Sass et al., 2011), biofilm formation and resistance (Peeters et al., 2010), and the adaptive evolution of isolates during chronic CF infection (Mira et al., 2011; Sass et al., 2011).

Although these studies have considerably increased our understanding of Burkholderia biology, the global gene expression data sets from these independent microarray experiments are not directly comparable. Here we provide for the first time for B. cenocepacia, a comparable reference data set of global gene expression representing nine growth conditions pertinent to survival in the natural environment and the CF lung. Of the conditions tested in this study, cellular stress caused by growth arrest induced the largest number of annotated virulence factors (Holden et al., 2009) in this opportunistic pathogen. An unexpected finding from the transcriptomic data set was that reduced oxygen concentration specifically triggered increased transcription of a novel gene regulon, which we designated as the low-oxygen-activated (lxa) locus. Our study shows that low oxygen concentration has a massive impact on gene expression in B. cenocepacia and raises major questions about the conventional practice of modelling aerobic bacteria at atmospheric oxygen concentrations.

Methods

Cultivation of B. cenocepacia J2315 for microarray experiments

B. cenocepacia J2315 was grown under nine different growth conditions modelling physiological temperature, low pH, low iron and nutrient availability, increased cell density, oxidative stress and reduced oxygen concentration, each compared with a specific control (Table 1). All experiments were performed in triplicate with planktonic cultures

| Table 1 Growth conditions for B. cenocepacia transcriptomic reference set |
|------------------------|-----------------|------------------|
| Experiment* | Test condition | Control condition (control number)$^a$ |
| Stationary phase in minimal medium | BSM | Log phase in BSM (4) |
| Stationary phase in nutrient-rich medium | LB | Log phase in LB (1) |
| Low oxygen concentration | LB, atmosphere with 6% oxygen | Fully aerated LB (2) |
| Oxidative stress (inorganic peroxide) | LB, 0.15% H2O2, 15 min | LB without added peroxide (3) |
| Oxidative stress (organic peroxide) | LB, 0.001% tertiary butyl hydroperoxide, 15 min | LB without added peroxide (3) |
| Heat stress | LB, 42.5 °C, 1 h | LB at 37 °C (3) |
| Physiological temperature | LB, 20 °C | LB at 37 °C (2) |
| Low iron concentration | BSM, Fe content 0.064 p.p.m. (1.2 μM) | BSM, Fe content 2.45 p.p.m. = 44 μM (4) |
| Low pH | BSM, pH 5.5 | BSM, pH 7.0 (4) |

Abbreviations: BSM, basal salts medium; LB, Luria Bertani broth
* Cultures were incubated fully aerated shaking at 150 r.p.m. and at 37 °C if not stated otherwise.
* Controls we run for each condition as indicated by the control number; this number also corresponds to the control conditions shown in Figure 1.
providing gene expression data from homogenous cultures with all cells in the same physiological state and exposed to the same concentration/perturbation of the respective effector (Table 1); *B. cenocepacia* biofilm-mediated global gene expression is described elsewhere (Peeters *et al.*, 2010; Inhultsen *et al.*, 2012). For low oxygen, oxidative and heat stress conditions, bacteria were first grown under standard conditions and then exposed to the stress factor. To generate the low oxygen atmosphere under standard conditions and then exposed to the heat stress conditions, bacteria were first grown on R2A agar plates and inoculated into the plates according to manufacturer’s instructions. Plates were incubated without agitation at 37 °C for 24 h for fully aerated, or for 36 h for reduced oxygen conditions. Reduced oxygen concentration conditions were generated using the CampyGen (Oxoid, Basingstoke, UK) as described above. The OD_{600} was measured using a plate reader (BioTek Instruments, Winoski, VT, USA). OD readings of > 50% difference between wild type and mutant were classed as indicative of a phenotypic difference, where at least one value > 0.4 (Agnoli *et al.*, 2012).

**Genetic manipulation**

Unmarked deletion mutants were constructed in gentamicin-sensitive derivatives of *B. cenocepacia* strains J2315 and K56-2 (MH1J and MH1K; Hamad *et al.*, 2010) using the homing endonuclease Sce-I (Flannagan *et al.*, 2008). The sensitive derivatives differ from their parent strains by an unmarked deletion of efflux pump BCAL1674–1676. Genes deleted for this study extend from BCAM0275 to BCAM0323, the resulting mutants were named J2315Δlxa and K56-2Δlxa. Primers used for deletion mutation are listed in Supplementary Table S1.

**Phenotypic characterisation of *B. cenocepacia* strains**

Growth rates were measured with a Bioscreen Automated Microbial Growth Analyzer (Bioscreen C, Oy Growth Curves AB, Helsinki, Finland; Sass *et al.*, 2011). OD was measured every 5 min after shaking for 10 s. To achieve a reduced oxygen concentration, cultures in the wells were overlaid with mineral oil and incubated without shaking. Intracellular survival assays were performed with murine RAW 264.7 macrophages (Hamad *et al.*, 2010). Nitrate reduction was determined with the nitrate reduction test kit (Sigma-Aldrich, Buchs, Switzerland), cells were grown in Luria Bertani broth before adding sodium nitrate to 10 mM final concentration.

To assess survival under anoxia, bacteria were grown aerobically in a basal salt medium with 0.4% glucose to an OD at 600 nm of 1 unit, reflecting a colony-forming unit count of $5 \times 10^8$ for *B. cenocepacia* strain K56-2 and $1 \times 10^9$ for strain J2315, before the cultures were transferred into 15-ml screw cap tubes. The tubes were further incubated in an anaerobic chamber (Don Whitley Scientific, Shipley, UK) at ambient temperature without shaking and sub-sampled at appropriate intervals for drop counts. Carbon and nitrogen source utilisation was determined using Biolog plates PM1, PM2a and PM3b (Biolog, Hayward, CA, USA). Cells were grown on R2A agar plates and inoculated into the plates according to manufacturer’s instructions. Plates were incubated without agitation at 37 °C for 24 h for fully aerated, or for 36 h for reduced oxygen conditions. Reduced oxygen concentration conditions were generated using the CampyGen (Oxoid, Basingstoke, UK) as described above. The OD_{600} was measured using a plate reader (BioTek Instruments, Winoski, VT, USA). OD readings of > 50% difference between wild type and mutant were classed as indicative of a phenotypic difference, where at least one value > 0.4 (Agnoli *et al.*, 2012).

**Microarray analysis and bioinformatics**

All microarray experiments were performed with genomic DNA as a common reference. Processing of test RNA and reference DNA, microarray hybridisations, array scanning and analysis were performed as described previously (Sass *et al.*, 2011); additional details on the global gene expression analysis are provided in the Supplementary Information file methods section. To determine differential gene expression, the scanned microarray images were analysed with the Feature Extraction Version 9.5.1 software (Agilent, Santa Clara, CA, USA) using the FE protocol GE2_v5_95. The raw fluorescence intensity data were then imported into GeneSpring GX version 7.3.1. (Agilent). Normalisation was performed as follows: first every spot of the signal channel was divided by the control channel and then each chip was normalised to the 50th percentile of all measurements of that chip followed by normalisation of each gene to its median.

For analysis of differential gene expression, a preliminary filter of 1.5-fold change in expression during the test condition compared with the respective control condition was applied. A one-way analysis of variance was carried out on the resulting gene lists using the Wech *t*-test with 5% false discovery rate. Values for all four replica spots for each probe were averaged during this process. Differences in gene expression were calculated based on mean values from three biological replicates and *P*-values resulting from analysis of variance obtained as a measure of variation between replicates. Differential gene expression across the growth conditions (Table 1) were reported using a standard cutoff of twofold change, with statistically significant (*P*<0.05) gene expression changes as low as 1.5-fold evaluated when appropriate (Sass *et al.*, 2011).

Quantitative real-time PCR was used to validate the microarray results and was performed as
described previously (Sass et al., 2011); quantitative real-time PCR primers (Supplementary Table S1) and additional details of the microarray analysis are provided in the Supplementary Information file. Putative orthologous genes within the *Burkholderia* genus were identified using the *Burkholderia* Genome Database (BDG; http://www.burkholderia.com; Winsor et al., 2008). The BDG uses a high-throughput computational method, Orthologue, to predict orthologues and avoid false positives that may be obtained with genome-scale reciprocal best BLAST hits (RBBH) approaches (Winsor et al., 2008). For *Burkholderia* strains not available on the BDG, manual prediction of orthologues was performed using BLAST (Altschul et al., 1997) with 40% identity across the entire amino-acid sequence set as the analysis threshold. Regulatory motifs were predicted with a two-step MEME search (Chambers et al., 2006). Toxin–antitoxin systems were searched for using the TA-Dec database (http://tadec.ulb.ac.be) and genomic islands were predicted with the Island Viewer (Langille and Brinkman, 2009). Raw microarray data were deposited at ArrayExpress, under accession numbers E-MEXP-2754, -2762, -2772, -2787, -2789, -2790 and -2791. The expression of selected genes altered under each environmental condition is provided in Supplementary Table S3 and the complete gene expression data set in Supplementary Table S4.

**Results**

*Transcriptomic plasticity of B. cenocepacia J2315*

The reference design of our microarray experiments, with all samples hybridised against genomic DNA as a common reference, allowed reproducible monitoring and comparison of global gene expression across all nine growth conditions (Figure 1 and Table 1). The performance of the *B. cenocepacia* J2315 microarray was validated by quantitative real-time PCR (Supplementary Table S2) and in all cases the data supported the microarray analysis. The greatest changes in gene expression were observed when comparing stationary phase to logarithmic growth, and growth under reduced oxygen concentration (6%) to fully aerated cultures (Table 2 and Figure 2). Low oxygen restricted the growth of *B. cenocepacia* as fully aerated control cultures achieved 80% higher OD during the incubation period. Approximately 30% of the differentially regulated genes were found under both stationary phase and low-oxygen conditions (Figure 2), demonstrating common adaptations irrespective of the cause of growth arrest. The genes downregulated in both stationary phase and under low-oxygen conditions included ATP synthase (BCAL0029–0037) and NADH dehydrogenase genes (BCAL2331–2344), and those encoding components of the transcription and translation apparatus (multiple genes; Supplementary Table S3). Commonly upregulated in stationary phase cultures and under low oxygen concentration were genes involved in fatty acid metabolism (for example, BCAL0046–0047), the

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**Figure 1** Comparison of *B. cenocepacia* transcription at the lxa locus across multiple growth conditions. A plot showing the mean relative fluorescence intensity of microarray probes, as a representation of gene expression across multiple growth conditions for *B. cenocepacia*. Each black line represents the profile of one of 50 CDSs from the lxa locus (BCAM275a to BCAM323) and demonstrates the unique and acute upregulation of this region under low-oxygen growth conditions.

**Table 2** Total number of *B. cenocepacia* coding sequences (CDS) with expression changes greater than twofold

| Growth condition                                                                 | Upregulated CDS | Downregulated CDS | Regulated CDS in proportion to total on array (%) |
|----------------------------------------------------------------------------------|-----------------|-------------------|--------------------------------------------------|
| Stationary phase in nutrient-rich medium                                          | 497             | 629               | 15.5                                             |
| Stationary phase in minimal medium                                               | 1053            | 1101              | 29.7                                             |
| Low oxygen concentration                                                         | 1046            | 941               | 22                                               |
| Oxidative stress (inorganic peroxide)                                            | 360             | 548               | 12.5                                             |
| Oxidative stress (organic peroxide)                                              | 381             | 451               | 11.5                                             |
| Heat stress                                                                       | 261             | 197               | 6.3                                              |
| Physiological temperature                                                        | 118             | 178               | 4.1                                              |
| Low iron                                                                         | 134             | 25                | 2.2                                              |
| Low pH                                                                           | 130             | 62                | 2.6                                              |

*Genes designated as unknown function were those with annotations indicative of poorly characterised function such as: hypothetical, conserved hypothetical, membrane associated, or exported protein encoding genes.*
glyoxylate pathway (isocitrate lyases (ICLs); BCAL2118 and BCAM1588) and genes for storage polymer turnover (BCAL0831–0833). The quorum sensing-regulated extracellular protease ZmpA (BCAS0409) and nematocidal protein AidA (BCAS0293) were highly upregulated under growth arrest. Genes involved in coordinate regulation, such as sigma factors (for example, BCAL0812 and BCAM1259), a cyclic-di-GMP signalling protein (BCAM0580) and diguanylate cyclase (BCAL1975), as well as one of the B. cenocepacia J2315 acyl homoserine lactone synthases (BCAM1870; cepI; O’Grady et al., 2009), were induced in these conditions. Putative toxin–antitoxin genes were also conspicuously upregulated upon growth arrest (Supplementary Table S3). Genes upregulated specifically in stationary phase relate to changes in metabolism and substrate availability, such as induction of acetoin (for example, BCAL1910–1914) and homogentisate degradation (for example, BCAL3183–3187).

Gene expression under oxidative stress displayed features in common with growth arrest, such as downregulation of transcription and energy production (Supplementary Table S1). Exposure to inorganic (hydrogen peroxide) or organic peroxide (tert-butyl hydrogen peroxide; Table 1) was characterised by upregulation of organic hydroperoxide resistance-related genes (BCAM0896, BCAS0085), hydroperoxide reductases (BCAM1216–1217), thiorodoxins (BCAL2780), proteases, chaperones (BCAL0500–0501) and other known stress-related protein genes encoding the LexA repressor (BCAL1651) and DNA polymerases (for example, BCAL2214, BCAL0422).

An up shift in temperature from 20°C to 37°C, characteristic of human infection, increased transcript levels of various chaperones and heat-shock proteins (for example, BCAS0638–0638, BCAL1233–1234), as did short-term exposure to 42.5°C (Supplementary Table S3). These genes were induced under the majority of the conditions tested and constituted a more general stress response.

Growth at 20°C specifically induced cold-shock genes (for example, BCAL0368) and those linked to maintaining transcription/translation (for example, BCAS0245, BCAM1618–1619) at lower temperature. Low iron concentration induced genes for iron uptake mechanisms like siderophores (for example, BCAL1689–1702) and TonB-dependent receptors (for example, BCAM2007), and reduced pH caused adaptive changes for maintenance of intracellular pH (for example, urease BCAL3104–3109). The latter two conditions did not affect growth rates compared with controls (data not shown) demonstrating the ability of B. cenocepacia to adapt well to moderately low-pH and low-iron environments.

Genes and pathways relevant for pathogenicity in CF infection
The stress conditions of stationary phase, low oxygen concentration and elevated temperature globally activated transcription of the largest number of known virulence factor genes compared with the other conditions tested in this study (Table 3; Supplementary Table S3). The CepI/R quorum sensing system with the major hierarchical responsibility for positive gene regulation (Sokol et al., 2007; O’Grady et al., 2009) was upregulated during stationary phase and temperature stress (up shift to physiological temperature and heat shock). Interestingly, low-oxygen growth led to a cepI upregulation level equivalent to that seen in stationary phase cultures (Table 3; Supplementary Table S3), even though the cells were harvested at the same cell density as the log phase controls (approximately 3–4×10⁸ colony-forming units per ml; stationary phase cultures had a cell density that was 1 log greater).

Flagella and nearly all genes relating to chemotaxis were upregulated at low oxygen concentration, with an aerotaxis receptor (BCAM2564) induced more than 20-fold. The cable pilus operon, a well-characterised and strain-specific B. cenocepacia virulence factor (Drevinek and Mahenthiralingam, 2010), was specifically upregulated upon growth
Temperature shift from 20°C to 37°C. Upregulation of zmpA (BCAS0409), lectins (BCAM0184–0186), Flp-type pili (BCAL1520–1537) and a type I secretion system (BCAM2140–242) with adjacent large surface protein (BCAM2143, bapA or adhA) was most pronounced in stress conditions of stationary phase, low oxygen and heat exposure (Table 3).

In addition to known virulence factors, a number of hypothetical genes and gene clusters were among the most regulated genes under conditions of growth arrest. The description and significance of these genes, and the regulation of transposases is included in the Supplementary Information file.

**Table 3** Induction of major virulence genes and genes clusters in *B. cenocepacia* J2315

| Induced virulence gene(s) | Loci | Stationary phase (minimal medium and nutrient-rich medium unless indicated) | Low oxygen concentration | Physiological temperature | Heat stress | Low iron concentration | Oxidative stress (organic or inorganic peroxide unless indicated) | Low pH |
|---------------------------|------|--------------------------------------------------------------------------|--------------------------|----------------------------|-------------|-----------------------|-----------------------------------------------------------------|--------|
| Motility                  |      |                                                                          |                          |                            |             |                       |                                                                 |        |
| Flagella                  | 6 Loci |                                                                          |                          |                            |             |                       |                                                                 |        |
| Chemotaxis BCAL0126–36    |      | +                                                                       |                          |                            |             |                       |                                                                 |        |
| BCAM0821–6                |      | +                                                                       |                          |                            |             |                       |                                                                 |        |
| Protein secretion         |      |                                                                          |                          |                            |             |                       |                                                                 |        |
| T1SS BCAM2140–42          |      | +                                                                       |                          |                            |             |                       |                                                                 |        |
| T6SS BCAM2020–57          |      | +                                                                       |                          |                            |             |                       |                                                                 |        |
| T6SS BCAM0337–63          |      | +                                                                       |                          |                            |             |                       |                                                                 |        |
| Extracellular proteases   |      |                                                                          |                          |                            |             |                       |                                                                 |        |
| zmpA BCAS0409             |      | +                                                                       |                          |                            |             |                       |                                                                 |        |
| zmpB BCAM2307             |      | +                                                                       |                          |                            |             |                       |                                                                 |        |
| Phospholipases            |      |                                                                          |                          |                            |             |                       |                                                                 |        |
| 6 Loci                    |      | +                                                                       |                          |                            |             |                       |                                                                 |        |
| Adhesion                  |      |                                                                          |                          |                            |             |                       |                                                                 |        |
| Fimbriae BCAL1677–80      |      | +                                                                       |                          |                            |             |                       |                                                                 |        |
| BCAL1826–28               |      | +                                                                       |                          |                            |             |                       |                                                                 |        |
| BCAL2634–37               |      | +                                                                       |                          |                            |             |                       |                                                                 |        |
| Flp-type pili BCAL1520–37 |      | +                                                                       |                          |                            |             |                       |                                                                 |        |
| Type Ia pili BCAL0276–78  |      | +                                                                       |                          |                            |             |                       |                                                                 |        |
| Lectin BCAM0184–86        |      | +                                                                       |                          |                            |             |                       |                                                                 |        |
| Cable pili BCAM2759–62    |      | +                                                                       |                          |                            |             |                       |                                                                 |        |
| bapA or adhA BCAM2143     |      | +                                                                       |                          |                            |             |                       |                                                                 |        |
| Surface polysaccharides   |      |                                                                          |                          |                            |             |                       |                                                                 |        |
| BCAM0854–64              |      | +                                                                       |                          |                            |             |                       |                                                                 |        |
| BCAM1003–11              |      | +                                                                       |                          |                            |             |                       |                                                                 |        |
| BCAS0294–97              |      | +                                                                       |                          |                            |             |                       |                                                                 |        |
| Quorum sensing            |      |                                                                          |                          |                            |             |                       |                                                                 |        |
| cepI BCAM1870             |      | +                                                                       |                          |                            |             |                       |                                                                 |        |
| Siderophores              |      |                                                                          |                          |                            |             |                       |                                                                 |        |
| Ornibactin BCAL1688–1702  |      | + c                                                                      |                          |                            |             |                       |                                                                 |        |
| Pyochelin BCAM2224–35     |      | +                                                                       |                          |                            |             |                       |                                                                 |        |
| Reactive oxygen species   |      |                                                                          |                          |                            |             |                       |                                                                 |        |
| detoxification             |      |                                                                          |                          |                            |             |                       |                                                                 |        |
| ahr BCAM1216–17           |      | + c                                                                      |                          |                            |             |                       |                                                                 |        |
| sodB BCAL2757             |      | +                                                                       |                          |                            |             |                       |                                                                 |        |
| katB BCAM3299             |      | +                                                                       |                          |                            |             |                       |                                                                 |        |
| Catalase BCAS0635         |      | +                                                                       |                          |                            |             |                       |                                                                 |        |

aInduction: at least twofold increased expression, virulence factor consisting of multiple genes was regarded induced when >50% of the loci were upregulated at least twofold.
bVirulence genes as defined by Holden et al. (2009).
cInduction only during stationary phase in nutrient-rich medium.
dInduction only under organic oxidative stress.

A specific transcriptomic response to low oxygen concentration

Over one-quarter of the genomic capacity of *B. cenocepacia* J2315 (1987 CDS; 27% of the genome capacity) was differentially regulated when it was grown at a low oxygen concentration (Table 2). In particular, we identified a 50722-bp cluster of 50 genes, BCAM0275a to BCAM0323 (Figures 1 and 3; Supplementary Table S3), which was specifically highly upregulated under low oxygen and designated as the low-oxygen-activated (lxa) locus. This locus located between the *B. cenocepacia* pathogenicity island (cci; Baldwin et al., 2004) and the
type IV secretion system genomic island (Holden et al., 2009), on the second largest \textit{B. cenocepacia} genomic replicon (Figure 3a). The \textit{lxa} locus was not predicted to be a genomic island by computational methods and possessed a G+C content, 66.9%, and GC frame plot analysis typical of the \textit{B. cenocepacia} genome (Figure 3a). These data suggest that the \textit{lxa} locus had either originated in an organism with similar genomic characteristics that was closely related to \textit{B. cenocepacia} or that it had not been recently acquired by horizontal gene transfer. Previous analysis had indicated that it was a strain-specific region of difference when the \textit{B. cenocepacia} J2315 genome was compared with other available genomes (Holden et al., 2009).

Induction of genes within the \textit{lxa} locus ranged from 2-fold to more than 240-fold change (Table 4, Figure 3b). Examination of the raw microarray data (Figure 1) demonstrated that the majority of these genes were not expressed during growth under normal atmospheric oxygen concentration. A motif search using 21 upstream sequences for genes within \textit{lxa} locus and low-oxygen co-regulated CDSs elsewhere in the genome, revealed a regulatory motif with a 4-bp inverted repeat separated by 6 bp of nonconserved sequence.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{The \textit{B. cenocepacia lxa} locus and low-oxygen co-regulated genes. (a) The genomic location of the \textit{lxa} locus between two genomic islands together with a GC content and GC frame plot. (b) The 50 genes of the \textit{lxa} cluster with the colour of each gene correlating to the level of upregulation, as provided in the key. (c) Four low-oxygen co-regulated \textit{B. cenocepacia} loci. The scale bars for each panel are indicated on the left, with the key for gene expression and \textit{lxa} motif location provided at the bottom. The consensus sequence of the \textit{lxa} motif is shown with the size of the base representative of its degree of conservation in the 21 input sequences analysed.}
\end{figure}
Table 4. B. cenocepacia lxa locus and other genes upregulated under low oxygen concentration growth conditions

| Gene ID | Annotation | Fold change |
|---------|------------|-------------|
| BCAL1657 | Ribose transport substrate-binding protein | 7.5 |
| BCAL1658 | Ribose ABC transporter ATP-binding protein | 8.8 |
| BCAL1659 | Ribose transport system, permease protein | 2.9 |
| BCAL1660 | Ribose operon repressor | 3.1 |
| BCAL1661 | Ribokinase | 8.9 |
| BCM0310 | Ribonucleotide reductase class II | 15 |
| BCM0311 | Ribokinase | 89 |
| BCCAL1796 | Saccharopine dehydrogenase | 152 |
| BCCAL1111 | Ornithine decarboxylase | 30 |
| BCCAL1112 | Biodegradative arginine decarboxylase | 20 |
| BCCAL1113 | Basic amino acid/polyamine antiporter | 18 |
| BCCAL1249 | Alcohol dehydrogenase | 40 |
| BCCAL1249 | Acetate kinase | 75 |
| BCCAL1249 | Phosphate acetylbutyryl transferase | 116 |
| BCCAL1249 | Zinc-binding alcohol dehydrogenase | 242 |
| CCM01570 | Alcohol dehydrogenase | 87 |
| BCCAL1581 | Phosphoenoxyruvate carboxykinase | 16 |
| BCCAL1249 | Acetoacetyl-CoA reductase | 94 |
| BCCAL1249 | Polyhydroxyalkanoic acid synthase, class I | 224 |
| BCCAL1249 | Polyafrhyth butyrate dehydrogenase | 42 |
| BCCAL1830 | 2-Nitropropane dioxygenase | 66 |
| BCCAL2079 | Nitroreductase | 43 |
| BCCAL2084 | Cytochrome c551/c552 | 146 |
| BCCAL2084 | Cytochrome b561 | 3.5 |
| BCCAL2084 | Sulphate transporter family protein | 19 |
| BCCAL2084 | ABC transporter protein | 3.8 |
| BCCAL2084 | ABC transporter protein | 80 |
| BCCAL2084 | Transporter system transport protein | 58 |
| BCCAL2084 | Outer membrane transport system protein | 15 |
| BCCAL2084 | Ton-B-dependent receptor | 3.6 |

Table 4 (Continued)

| Gene ID | Annotation | Fold change |
|---------|------------|-------------|
| BCCAL2084 | Two-component regulatory system, response regulator | 3.7 |
| BCCAL2084 | Two-component regulatory system, sensor kinase | 2.6 |
| BCCAL2084 | Two-component regulatory system, sensor kinase | 3.8 |
| BCCAL2084 | Two-component regulatory system, sensor kinase | 2.1 |
| BCCAL2084 | Two-component regulatory system, response regulator | 2.5 |
| BCCAL2084 | Two-component regulatory system, response regulator | 3.2 |
| BCCAL2084 | Two-component regulatory system, sensor kinase | 3.0 |

Protein turnover

| Gene ID | Annotation | Fold change |
|---------|------------|-------------|
| BCCAL2084 | ATP-dependent Zn protease | 130 |

Hypothetical

| Gene ID | Annotation | Fold change |
|---------|------------|-------------|
| BCCAL2084 | Conserved hypothetical proteins | 27–53 |
| BCCAL2084 | Conserved hypothetical protein | 11 |

aLxa locus gene.

bCo-regulated gene with a lxa motif sequence.

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electron transfer, membrane and transport functions, signal transduction and transcriptional regulation. Genes upregulated under low-oxygen conditions that were not part of the lxa locus but contain a lxa motif in their promoter regions included an additional USP, regulatory proteins and metabolic enzymes.

Examination of 17 other available Burkholderia species genomes demonstrated that the lxa locus is highly syntenous with B. vietnamiensis G4, B. multivorans ATCC17616 and B. ambifaria MC40-6 genomes, but none of the genomes examined showed complete synteny (Figure 4, Supplementary Table S6). Most strains, including the pathogen B. pseudomallei, contained fewer lxa homologues and the majority of these were encoded in a different order compared with B. cenocepacia J2315. All Burkholderia genomes analysed contained homologues of the majority of non-lxa genes that were induced when B. cenocepacia was grown under low oxygen concentration (Figure 4 and Table 4).

**Phenotypic characterisation of lxa locus deletion mutants**

To establish a functional role for the lxa locus and corroborate its considerable upregulation by low oxygen, deletion mutants spanning BCAM0275a through BCAM0323 were constructed in two B. cenocepacia strains (Hamad et al., 2010); the resulting mutants were designated J2315Δlxa and K56-2Δlxa, respectively. Both mutants were viable and demonstrated indistinguishable aerobic growth on nutrient-rich media when compared with their respective parental strains. Overlaying the cultures with mineral oil to restrict oxygen availability did not reveal growth rate differences between the parental strains and lxa mutants during experiments with up to 5 days incubation. Survival of mutant strains within macrophages after 24 h of infection was indistinguishable from survival of the parent strains (data not shown). However, in minimal medium, growth of lxa mutants was impaired compared with their parent strains. The impaired aerobic growth in minimal medium was more apparent for K56-2Δlxa (Figure 5b) than for J2315Δlxa (Figure 5a), which is probably due to faster growth of strain K56-2 compared with J2315, the growth of which is already reduced in the absence of organic supplements. These data suggest that the lxa region provides a growth fitness advantage for B. cenocepacia under aerobic conditions.

The growth and survival phenotype of B. cenocepacia J2315 and K56-2 lxa mutants were further evaluated under anoxic conditions. Neither strain grew in the absence of oxygen in nutrient-rich medium or in a minimal medium with glucose as a carbon source. Adding nitrate as electron donor under anoxic growth conditions did not promote growth and neither B. cenocepacia J2315 nor K56-2 could produce nitrite from nitrate, in accordance with the absence of genes for dissimilatory nitrate reduction in the B. cenocepacia genome (data not shown). It was clear from these results that B. cenocepacia is an obligate aerobe and cannot grow without oxygen; therefore, the ability to survive without oxygen was evaluated as a phenotype. Once grown aerobically to high cell density (> 10^8 colony-forming units per ml) and transferred to an oxygen-free environment, viability loss of mutant strains was greater than of parental strains (Figures 5c and d). These data suggest that the lxa locus has a key role in maintaining the viability of B. cenocepacia once oxygen has been depleted in an environment.

Phenotypic profiling of the deletion mutants using the Biolog assay (Agnoli et al., 2012) permitted exploration of the growth deficiencies seen in minimal media for the lxa mutants (Figures 5a and b). The slow growth of B. cenocepacia J2315 in minimal media (Figure 5a) prevented this mutant from being studied due to a lack of sufficient yield in the Biolog assay. Carbon source profiling of the K56-2Δlxa mutant demonstrated it was impaired in utilisation of: ribose, deoxyribose, the amino acids L-valine, L-leucine, L-isoleucine, L-ornithine and L-glycine, the nucleoside thymidine and the organic acids formic acid and quinic acid (Supplementary Table S7). The wild type but not the mutant could use nitrate as nitrogen source, which could explain the reduced growth rate of the mutant in un-supplemented minimal medium. Other nitrogen

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**Figure 4** Conservation of the B. cenocepacia J2315 lxa locus in other Burkholderia genomes. Seventeen complete Burkholderia species genomes were examined for genes homologous to those in the B. cenocepacia J2315 lxa locus and an illustration of the conservation and synteny of these genes is shown above. The genomes and strains analysed are listed on the left; conservation and synteny with: (a) the lxa locus and (b) other genes co-regulated under low-oxygen growth (see Figure 3) are shown by the continuous grey blocks in the respective panels. The gaps between the blocks represent a lack of a continuous gene order with the lxa as no other Burkholderia genome encoded a complete lxa gene cluster. The identity of each orthologous gene is provided in Supplementary Table S6.
sources on which K56-2Δlxa exhibited reduced growth compared with the parent were purine and pyrimidine nucleosides. In contrast to these negative traits, the K56-2Δlxa mutant reproducibly grew to a greater OD on β-hydroxybutyric and capric acid than the parental strain (Supplementary Table S7). Overall, the diversity of phenotypic alterations resulting from lxa deletion cannot be fully explained by its gene content and suggests that the region has a key role in pleiotropic regulation of the low-oxygen response.

Discussion

Transcriptomic profiling of B. cenocepacia has for the first time provided a comprehensive global gene expression data set comparable across multiple growth conditions representative of its ecology in disease and the natural environment. It has also led to the discovery of the lxa, a unique, large co-regulated gene cluster required for survival of an aerobic bacterial species at low oxygen concentrations. Burkholderia are classically considered aerobic bacteria and, hence, in the laboratory, like many other aerobic bacteria, the majority of researchers grow them under atmospheric oxygen conditions. The lxa locus and massive impact of low-oxygen growth on gene regulation in B. cenocepacia owe their discovery to the unique combination of transcriptomics and growth modelling, which we have applied to this aerobic bacterium.

Stress and metabolic responses occurring under low oxygen and growth arrest

Bacterial stress resistance and adaptation to growth arrest are important for survival under changing environmental conditions, whether in soil or in a human host. Genes induced in both stationary phase and low-oxygen conditions include a large number of signalling proteins, sigma factors and toxin–antitoxin genes. This underscored the complexity of a regulatory network involved in growth arrest that is active in response to nutrient starvation as well as oxygen limitation. Some genes commonly induced under stationary phase and oxygen limitation rank among genes with the highest fold changes, for example, ICLs and three clusters of genes with unknown function, which points to common metabolic and stress responses under growth-limiting conditions.

The increased transcription of the two ICL genes of B. cenocepacia J2315 during growth arrest
indicates an increased activity of the glyoxylate cycle, putatively enabling carbohydrate synthesis and growth on fatty acids as sole carbon source. In *Ralstonia eutropha*, ICL has been implicated in the synthesis of polyhydroxy alkanoates (PHA; Wang *et al.*, 2003), which have a role in carbon and energy storage by bacteria. ICL is necessary for the establishment of chronic infection by *B. pseudomallei* (Van Schaik *et al.*, 2009), *Mycobacterium tuberculosis* (Muñoz-Elias and McKinney, 2005) and *P. aeruginosa* (Lindsey *et al.*, 2008) and ICL transcription in a *B. cenocepacia* isolate from late stage infection was increased compared with an early-stage isolate (Mira *et al.*, 2011). The exact role of ICL in growth arrest in *B. cenocepacia* J2315 is unknown. Its induction pattern and the co-expression with storage polymer turnover genes in stationary phase and low-oxygen conditions support an involvement in PHA synthesis by diverting metabolic fluxes.

**Infection and pathogenesis**

Upregulation of extracellular virulence factor genes was most pronounced under stationary phase and low oxygen concentration, conditions associated with growth arrest, and growth at increased temperature. Nutrient and energy limitation as well as temperature adaptation during host infection might therefore have a major role in *B. cenocepacia* pathogenesis. The N-acyl homoserine lactone synthase, *cepI*, was induced under conditions that also induced most other virulence factors. This is consistent with findings that the CepI/R quorum sensing system of *B. cenocepacia* regulates expression of many of these virulence factors (Chambers *et al.*, 2006; O’Grady *et al.*, 2009; Inhulsen *et al.*, 2012). Remarkably, upregulation of *B. cenocepacia* J2315 *cepI* in response to low oxygen concentration was independent of cell density. Cells had, however, been cultivated for a longer period of time for experiments under low oxygen. Whether the increase in *cepI* expression is independent of autoinducer accumulation is therefore unknown. Oxygen concentration is a factor in virulence and quorum sensing regulation in *P. aeruginosa*. Autoinducer synthase *lasI* was upregulated under low-oxygen and anoxic conditions (Alvarez-Ortega and Harwood, 2007), independently of cell density; low-oxygen conditions were shown to induce type III secretion system (T3SS), and regulation of T3SS expression depends among others on the oxygen-sensing Anr regulatory protein (O’Callaghan *et al.*, 2012). Oxygen sensing or energy limitation due to lack of oxygen could also have a role in regulation of virulence factor expression in *B. cenocepacia*, and requires further investigation.

**Function, regulation and prevalence of the *Ixa* locus**

From our analysis, it is clear that *B. cenocepacia* J2315 is an obligate aerobe unable to grow anaerobically by fermentation or reduction of alternative electron acceptors. It is, however, likely to encounter oxygen-depleted conditions in the natural environment as well as in the CF lung, and therefore, must be able to survive under temporary anoxia. The novel *Ixa* locus clearly offers *B. cenocepacia* a fitness advantage under anoxic conditions as shown by its regulation pattern and confirmed by the reduced survival of the *Ixa* locus deletion mutants.

The regulatory motif associated with *Ixa* genes shares the common sequence TGA-N$_6$-TCA with anaerobe nitrate regulator, Anr,- and fumarate/nitrate regulator, Fnr,-binding motifs of facultative anaerobic bacteria like *P. aeruginosa* and *Escherichia coli* (Trunk *et al.*, 2010), suggesting that expression of the *Ixa* locus could be under a similar regulatory control in *B. cenocepacia*. Anr- and Fnr-regulons are activated under anoxic conditions, and include functions such as nitrate and fumarate reduction for energy production under anoxic conditions. USPs in *P. aeruginosa* are also under regulatory control by Anr (Trunk *et al.*, 2010). Potential candidates for oxygen-sensing regulators in *B. cenocepacia* J2315 are low-oxygen-induced genes encoding regulatory proteins BCAM0049, BCAM0287 and BCAM1483, with 43%, 44% and 42% protein sequence similarity to Anr of *P. aeruginosa* PAO1 (PA1544), respectively. A gene encoding an Anr-like regulatory protein in *Burkholderia dolosa* (BDAG_041800) was among the most mutated genes found in a study of bacterial evolution during CF infection (Lieberman *et al.*, 2011). This points to a deregulation of pathways normally regulated via oxygen concentration during long-term infection and provides a possible link between oxygen availability and host adaptation.

There is remarkable redundancy of USPs among the *Ixa* genes. USPs were first described in *E. coli*, where they are induced by a number of stress conditions, including stationary phase, and facilitate adaptation to growth arrest (Kvint *et al.*, 2003). In *B. cenocepacia*, however, these genes are almost exclusively upregulated under low-oxygen conditions. USPs usually occur in multiple copies, as is the case in *P. aeruginosa*, where they are induced under oxygen depletion (Alvarez-Ortega and Harwood, 2007), and are necessary for survival under anoxic conditions (Trunk *et al.*, 2010). In *M. tuberculosis*, multiple USP-encoding genes are part of the DosR or dormancy regulon. The DosR regulon consists of at least 48 genes induced by hypoxic conditions and low, non-lethal concentrations of NO and CO, and is necessary for long-term survival of anoxic conditions and non-replicative states (Voskuil *et al.*, 2003; Gerasimova *et al.*, 2011). Several *Ixa* genes other than USPs have functional or phylogenetic homology to genes of the DosR regulon of *M. tuberculosis* (Voskuil *et al.*, 2003): γ-crystallin (BCAM0278), nitroreductase (BCAM0279), sulphate...
transport protein (BCAM0281), ribonucleotide reductase (BCAM0312), phosphofructokinase (BCAM0311) and cation-transporting ATPase (BCAM0318). This overlap suggests similarities in the metabolic pathways employed by both strictly aerobic bacterial species during adaptation to oxygen-depleted conditions.

BCAM0311, annotated as phosphofructokinase, belongs to the ribokinase-phosphofructokinase-2 group of sugar kinases. The lxa deletion mutant is deficient in ribose utilisation, which suggests that BCAM0311 encodes a ribokinase, and that BCAM0311 might have been misannotated. BCAM0312 encodes a class II ribonucleotide reductase, which can function under anoxic as well as anoxic conditions. In contrast, the other ribonucleotide reductase encoded on the B. cenocepacia J2315 large replicon (BCAL2348), belongs to class I and would therefore only function under oxic conditions. Upregulation of BCAM0312 under low oxygen concentration could therefore compensate for loss of function of the class I ribonucleotide reductase. The association of ribokinase with ribonucleotide reductase seems to point to a role for both enzymes in nucleoside synthesis, as ribose phosphate can feed via phosphoribosyl pyrophosphate into that pathway.

The lxa locus contains genes for PHA synthesis. This provides an explanation for the faster growth of the lxa deletion mutant on fatty acid compounds such as β-hydroxybutyrate, as these compounds would not be channelled into PHA synthesis in the mutant, and would therefore be available as carbon sources. PHA synthesis can act as an electron sink during low-oxygen conditions in E. coli (Nikel et al., 2006). This process restores the oxidized form of nicotinamide adenine dinucleotide (NAD +) and is important for the redox balance within the E. coli cell. Other genes like those encoding acetate kinase (BCAM0293) and phosphate acetyl/butyryl transferase (BCAM0298) could be involved in fermentative pathways or in feeding acetate into PHA synthesis. Nitrouriductase, as well as the cytobchromes b and c encoded by the lxa locus (BCAM0320, BCAM0284), could be involved in electron transport processes. Low-oxygen-upregulated genes outside the lxa locus and potentially involved in fermentative pathways include phosphoenolpyruvate carboxykinase (BCAM1581), arginine decarboxylase and ornithine decarboxylase (BCAM1111–1112). Consequently, proteins encoded by low-oxygen-activated genes could be involved in energy production via fermentation and reduction of electron acceptors other than oxygen, although enzymes typically involved in this, such as fumarate reductase are not among them. Even a limited capacity to generate ATP via incomplete anaerobic pathways could contribute to a delay in energy starvation once oxygen is depleted. A role of lxa genes in maintaining redox balance is equally possible. Although the low-oxygen-induced genes do not enable the obligate aerobic B. cenocepacia to grow under anoxia, they support survival of growth arrest caused by anoxia.

The prevalence of two highly regulated genes within the lxa locus, one encoding a small heat-shock protein BCAM0278 (240-fold upregulation; Table 4) and the other a phospholipid-binding protein BCAM0280 (221-fold upregulation; Table 4), had been examined as part of previous work characterising the cci (Baldwin et al., 2004). Southern hybridisation of these gene probes under stringent conditions had shown that among the genomic DNA from 241 B. cepacia complex strains, close orthologues of BCAM0278 were exclusive to B. cenocepacia recA group A strains such as J2315 (Baldwin et al., 2004). Within B. cenocepacia, close orthologues of BCAM0280 were exclusive to the recA group A lineage, however, hybridisation signals were also detected in 7 out of 19 B. ambifaria strains and 1 of the B. stabilis strains examined (Baldwin et al., 2004). Genetically, B. cenocepacia recA group A strains are very closely related (Vandamme et al., 2003; Drevinek and Mahenthiralingam, 2010). Our current bioinformatic analysis of orthologues within the available B. cenocepacia genomes (Figure 4) and past mapping of BCAM0278 and BCAM0280 (Baldwin et al., 2004), suggest that a complete lxa locus is exclusive to B. cenocepacia recA group A strains.

Conclusions

We have shown that B. cenocepacia J2315 is well adapted to growth in multiple harsh environmental conditions and can draw on a multitude of gene pathways to cope with stress. In particular, growth arrest caused by a reduction in oxygen concentration or organic nutrients, and increased temperature, appears to trigger virulence factor expression in this opportunistic pathogen. We have discovered a new ecological fitness regulon specifically induced by growth under a low oxygen concentration of 6%. Our preliminary analysis of the lxa locus indicates that it increases survival of B. cenocepacia under anoxic conditions, possibly by delaying energy starvation and maintaining redox balance. However, the lxa locus also appears to have a role in increasing the fitness of B. cenocepacia under aerobic conditions where its loss leads to growth impairment on minimal medium. The full impact and function of the lxa locus during bothoxic and anoxic growth of B. cenocepacia will be fascinating to explore. Overall, our data indicate a need for a paradigm shift in the way we model the ecology of aerobic microorganisms in the laboratory. Oxygen is likely one of the most regulatory and restricting growth factors for aerobic microorganisms in the natural environment and during infection, and we should strive to model this important survival parameter more closely in future research.
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