OPEN

The ISME Journal (2013), 1–14

www.nature.com/ismej

ORIGINAL ARTICLE

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.**
The ISME Journal advance online publication, 14 March 2013; doi:10.1038/ismej.2013.36
Subject Category: Integrated genomics and post-genomics approaches in microbial ecology
Keywords: anoxia; *Burkholderia*; pathogenicity; persistence; survival; transcriptomics

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

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Received 23 October 2012; revised 25 January 2013; accepted 28 January 2013

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 morphotype-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; Inhülsen *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[a]	*Test condition*	*Control condition (control number)*[b]
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% H_2O_2, 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
[a]Cultures were incubated fully aerated shaking at 150 r.p.m. and at 37 °C if not stated otherwise.
[b]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; Inhülsen *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 of 6%, *B. cenocepacia* cultures were incubated within a CampyGen Compact system following the manufacturer's instructions (Oxoid, Basingstoke, UK). This method of generating a reduced oxygen atmosphere was selected because of its technical simplicity. The stress conditions were chosen to affect growth rate and allow for a change in gene expression, but not to reduce viability. In relation to each condition, growth arrest was defined as a state in which there was no further increase in the culture optical density (OD) or culture viability. Cultivation and processing of bacteria was performed essentially as described previously (Sass *et al.*, 2011) and the details of each growth condition are provided in the Supplementary Information.

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 BCAM0275a to BCAM0323, the resulting mutants were named J2315Δ*lxa* and K56-2Δ*lxa*. 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 5×10^8 for *B. cenocepacia* strain K56-2 and 1×10^9 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, Winooski, 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, Ortholuge, 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 BLASTP (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

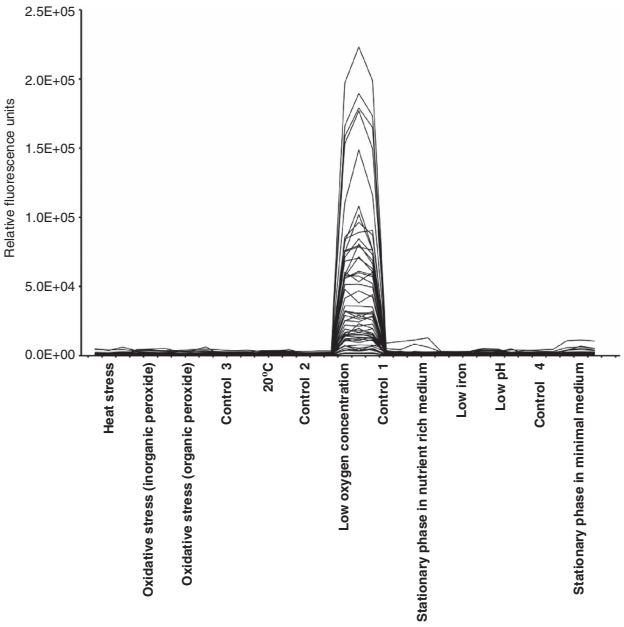

Figure 1 Comparison of *B. cenocepacia* transcription at the *lxa* locus across multiple growth conditions. A plot showing the mean relative fluorescence intensity of microarray probes, as a representation of gene expression across multiple growth conditions for *B. cenocepacia*. Each black line represents 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 (%)*
	Total	*Unknown function*[a] *(%)*	*Total*	*Unknown function*[a] *(%)*	
Stationary phase in nutrient-rich medium	497	29	629	18	15.5
Stationary phase in minimal medium	1053	29	1101	24	29.7
Low oxygen concentration	1046	33	941	22	27.4
Oxidative stress (inorganic peroxide)	360	28	548	24	12.5
Oxidative stress (organic peroxide)	381	31	451	24	11.5
Heat stress	261	37	197	18	6.3
Physiological temperature	118	45	178	26	4.1
Low iron	134	21	25	32	2.2
Low pH	130	27	62	18	2.6

[a]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.

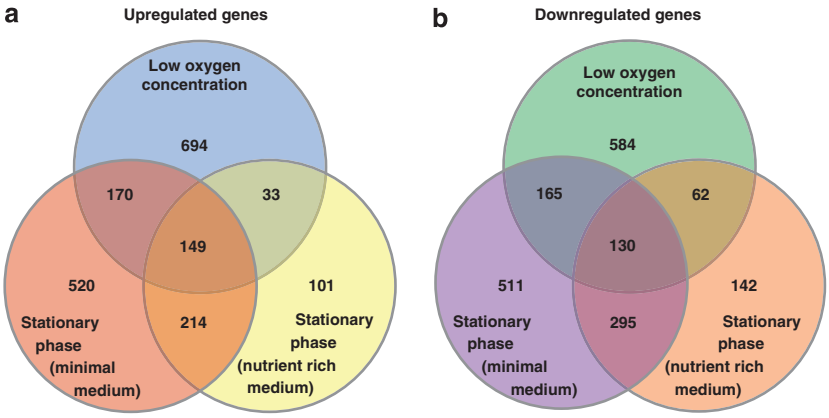

Figure 2 *B. cenocepacia* gene expression during stationary phase and low-oxygen growth conditions. The Venn diagrams illustrate the number of upregulated genes (**a**) and downregulated genes (**b**) observed during stationary phase and low-oxygen growth conditions.

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), thioredoxins (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 $3–4 \times 10^8$ 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

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

Induced virulence gene(s)[a]	*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[b]		+				+	
	BCAM0821–6		+					
Protein secretion								
T1SS	BCAM2140–42	+	+		+			
T3SS	BCAM2020–57				+			
T6SS	BCAL0337–63			+				
Extracellular proteases								
zmpA	BCAS0409	+	+	+	+			
zmpB	BCAM2307	+	+					
Phospholipases	6 Loci	+						
Adhesion								
Fimbriae	BCAL1677–80				+			
	BCAL1826–28		+		+			
	BCAL2634–37	+	+					
flp-type pili	BCAL1520–37	+	+	+	+			
Type IVa 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	BCAL3299					+		+
Catalase	BCAS0635				+	+	+[d]	

[a]Induction: at least twofold increased expression, virulence factor consisting of multiple genes was regarded induced when >50% of the loci were upregulated at least twofold.
[b]Virulence genes as defined by Holden *et al.* (2009).
[c]Induction only during stationary phase in nutrient-rich medium.
[d]Induction only under organic oxidative stress.

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.

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 50 722-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

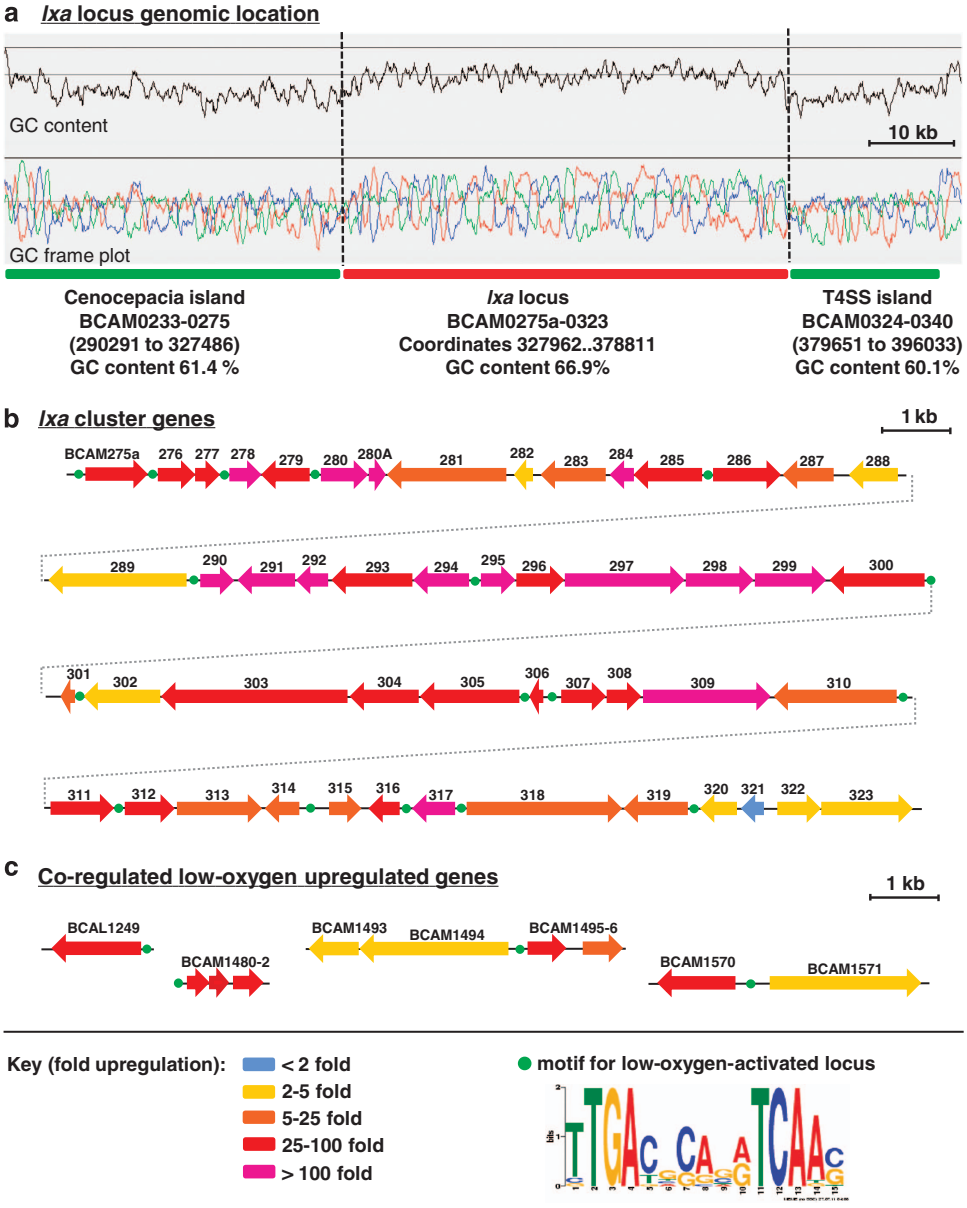

Figure 3 The *B. cenocepacia lxa* locus and low-oxygen co-regulated genes. (**a**) The genomic location of the *lxa* locus between two genomic islands together with a GC content and GC frame plot. (**b**) The 50 genes of the *lxa* cluster with the colour of each gene correlating to the level of upregulation, as provided in the key. (**c**) Four low-oxygen co-regulated *B. cenocepacia* loci. The scale bars for each panel are indicated on the left, with the key for gene expression and *lxa* motif location provided at the bottom. The consensus sequence of the *lxa* motif is shown with the size of the base representative of its degree of conservation in the 21 input sequences analysed.

type IV secretion system genomic island (Holden *et al.*, 2009), on the second largest *B. cenocepacia* genomic replicon (Figure 3a). The *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 *B. cenocepacia* genome (Figure 3a). These data suggest that the *lxa* locus had either originated in an organism with similar genomic characteristics that was closely related to *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 *B. cenocepacia* J2315 genome was compared with other available genomes (Holden *et al.*, 2009).

Induction of genes within the *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 *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

Table 4 *B. cenocepacia lxa* locus and other genes upregulated under low oxygen concentration growth conditions

Gene ID	*Annotation*	*Fold change*
Stress response		
BCAL1648	Universal stress protein	3.5
BCAL2119	Universal stress protein	3.0
BCAM0050	Universal stress protein	16
BCAM0276[a]	Universal stress protein	59
BCAM0278[a]	Small heat shock/α-crystallin protein	240
BCAM0280[a]	Phospholipid-binding protein	221
BCAM0290[a]	Universal stress protein	105
BCAM0291[a]	Universal stress protein	165
BCAM0292[a]	Universal stress protein	202
BCAM0294[a]	Universal stress protein	167
BCAM0307[a]	Phospholipid-binding protein	89
BCAM0319[a]	Universal stress protein	21
BCAM1495[b]	Universal stress protein	55
BCAM1500	Universal stress protein	14
BCAM1829	Universal stress protein	44
Pentose turnover		
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
BCAM0310[a]	Ribonucleotide reductase, class II	15
BCAM0311[a]	Ribokinase	89
Amino-acid turnover		
BCAL1796	Saccharopine dehydrogenase	152
BCAM0283[a]	Lysine decarboxylase	20
BCAM1111	Ornithine decarboxylase	30
BCAM1112	Biodegradative arginine decarboxylase	20
BCAM1113	Basic amino acid/polyamine antiporter	18
Fatty acid, organic acid and alcohol turnover		
BCAM0286[a]	Alcohol dehydrogenase	40
BCAM0293[a]	Acetate kinase	75
BCAM0298[a]	Phosphate acetyl/butyryl transferase	116
BCAM0299[a]	Zinc-binding alcoholdehydrogenase	242
BCAM0312[a]	Polysaccharide deacetylase	46
BCAM1570[b]	Alcohol dehydrogenase	87
BCAM1581	Phosphoenolpyruvate carboxykinase	16
Storage polymer turnover		
BCAM0296[a]	Acetoacetyl-CoA reductase	94
BCAM0297[a]	Polyhydroxyalkanoic acid synthase, class I	224
BCAL1249[b]	Polyhydroxy butyrate depolymerase	42
Electron transfer		
BCAL1830	2-Nitropropane dioxygenase	66
BCAM0279[a]	Nitroreductase	43
BCAM0284[a]	Cytochrome c551/c552	146
BCAM0320[a]	Cytochrome b561	3.5
Membrane/transport		
BCAM0281[a]	Sulphate transporter family protein	19
BCAM0302[a]	ABC transporter protein	3.8
BCAM0303[a]	ABC transporter protein	80
BCAM0304[a]	Transporter system transport protein	36
BCAM0305[a]	Outer membrane transport system protein	58
BCAM0318[a]	Cation-transporting ATPase	15
BCAM1571[b]	Ton-B-dependent receptor	3.6
Transcription and translation		
BCAM0300	Metal-dependent RNase	30
BCAM0048	LysR family regulatory protein	13
BCAM0049	CRP family regulatory protein, Anr-related	77
BCAM0287[a]	CRP family regulatory protein, Anr-related	17
BCAM0288[a]	Two-component regulatory system, response regulator	3.7
BCAM0289[a]	Two-component regulatory system, sensor kinase	2.6
BCAM0322[a]	Two-component regulatory system, response regulator	3.8
BCAM0323[a]	Two-component regulatory system, sensor kinase	2.1
BCAM1114	LysR family regulatory protein	6.5
BCAM1351	Regulatory protein	210
BCAM1483	CRP family regulatory protein, Anr related	3.5
BCAm1484	Two-component regulatory system, response regulator protein	2.5
BCAM1493[b]	Two-component regulatory system, response regulator protein	3.2
BCAM1494[b]	Two-component regulatory system, sensor kinase protein	3.0
Protein turnover		
BCAM0309[a]	ATP-dependent Zn protease	130
Hypothetical		
BCAM1480-2[b]	Conserved hypothetical proteins	27–53
BCAM1496[b]	Conserved hypothetical protein	11

[a]*lxa* locus gene.
[b]Co-regulated gene with a *lxa* motif sequence.

(Figure 3c; Supplementary Table S5). The *lxa* motif occurred at 17 intergenic points within the *lxa* locus and was also associated with four other low-oxygen-induced gene loci (Figure 3c), suggesting that these genes belong to a specific low-oxygen regulon. The level of induction of these co-regulated non-*lxa* gene clusters varied from 3-fold (BCAM1494) to 86-fold (BCAM1570; Supplementary Table S4). They were not substantiatially activated under other growth conditions, however, upregulation greater than twofold was seen for: BCAM1249, BCAM1480–82 and BCAM1495 during stationary phase in minimal medium and BCAM1249 during stationary phase in nutrient-rich medium (Supplementary Table S4).

From the *B. cenocepacia* J2315 genome annotation (Holden *et al*., 2009), the functions of gene products encoded within the *lxa* locus covered predicted roles in metabolism, transport and stress response (Table 4); none of the genes had been previously characterised in *B. cenocepacia*. The region encodes six proteins with one or two universal stress protein (USP) domains and a α-crystallin-related protein (BCAM0278). Other notable *lxa* genes had predicted functions in ribonucleotide transport and metabolism, amino acid, fatty acid and alcohol turnover, storage polymer synthesis,

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 J2315Δ*lxa* and K56-2Δ*lxa,* 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 K56-2Δ*lxa* (Figure 5b) than for J2315Δ*lxa* (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 K56-2Δ*lxa* 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

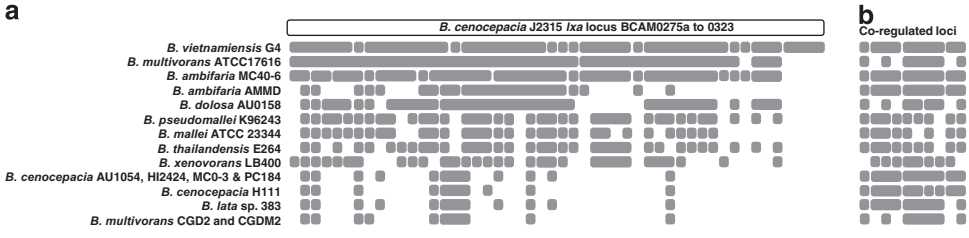

Figure 4 Conservation of the *B. cenocepacia* J2315 *lxa* locus in other *Burkholderia* genomes. Seventeen complete *Burkholderia* species genomes were examined for genes homologous to those in the *B. cenocepacia* J2315 *lxa* locus and an illustration of the conservation and synteny of these genes is 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.

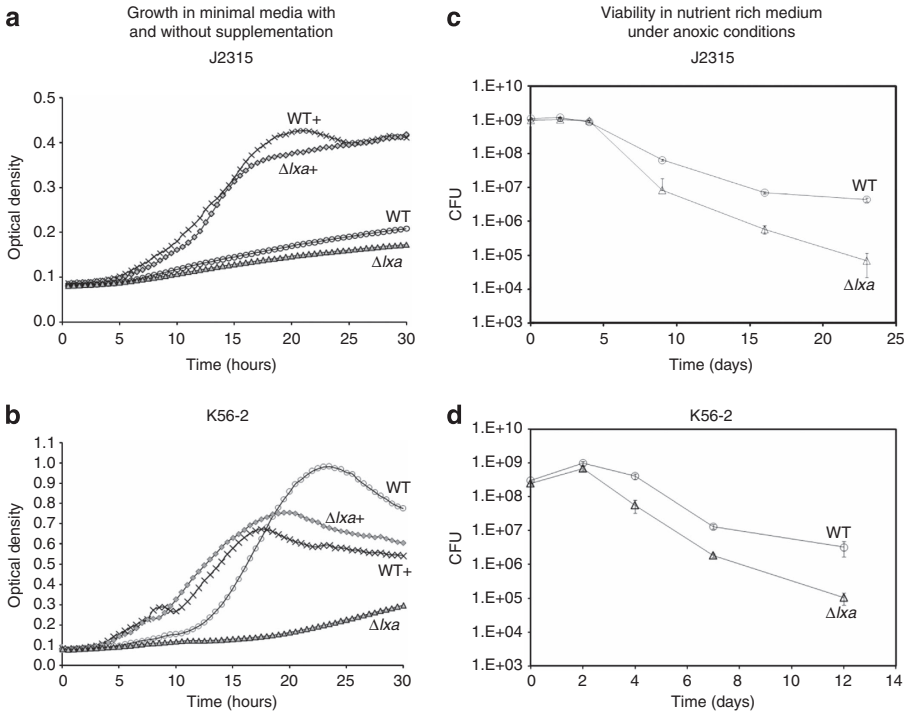

Figure 5 Growth and survival characteristics of *B. cenocepacia lxa* locus deletion mutants. The growth kinetics in minimal medium with and without yeast extract and casamino acid supplementation is shown to the left in **a** and **b**, for strains J2315 and K56-2 respectively, with the mean OD plotted as a growth curve. Each curve is labelled as follows: (**a**) WT+ (crosses), wild-type J2315 and Δ*lxa*+ (shaded diamonds) J2315 *lxa* mutant, both with supplementation; WT (open circles) wild-type J2315 and Δ*lxa* (shaded triangles) J2315 *lxa* mutant, both without supplementation; (**b**) WT+ (crosses), wild-type K56-2 and Δ*lxa*+ (shaded diamonds) K56-2 *lxa* mutant, both with supplementation; WT (open circles) wild-type K56-2 and Δ*lxa* (shaded triangles) K56-2 *lxa* mutant, both without supplementation. The viability of bacteria incubated without oxygen in nutrient-rich medium is shown to the right in **c** and **d**, for strains J2315 and K56-2, respectively, with the WT (open circles) and Δ*lxa* (triangles) labelled for each survival plot. Each data point is the mean of three replicate cultures.

sources on which K56-2Δ*lxa* exhibited reduced growth compared with the parent were purine and pyrimidine nucleosides. In contrast to these negative traits, the K56-2Δ*lxa* 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-Elías 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; Inhülsen *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 lxa 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 *lxa* 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 *lxa* locus deletion mutants.

The regulatory motif associated with *lxa* 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 *lxa* 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 *lxa* 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 *lxa* genes other than USPs have functional or phylogenetic homology to genes of the DosR regulon of *M. tuberculosis* (Voskuil *et al.*, 2003): α-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 oxic 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. Nitroreductase, as well as the cytochromes *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 both oxic 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.

Acknowledgements

We thank Angela Marchbank for excellent technical assistance. AS and EM acknowledge funding from the US Cystic Fibrosis Foundation Therapeutics (CFFT) International *Burkholderia* Array Programme (grant number MAHENT06V0) and UK Cystic Fibrosis Trust (grant PJ551). CS acknowledges postdoctoral fellowship funding from Cystic Fibrosis Canada. LE and KA acknowledge funding by the Swiss National Fund (Project 31003A_122013) to LE. EM acknowledges the European Cooperation in Science and Technology (COST) action BM1003 and Microbiology and Infection Translational Research Group (MITReG) for promoting invaluable networking on microbial pathogenesis in cystic fibrosis.

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