Reciprocal regulation by the CepIR and CciIR quorum sensing systems in \textit{Burkholderia cenocepacia}

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Abstract

\textbf{Background:} \textit{Burkholderia cenocepacia} belongs to a group of closely related organisms called the \textit{B. cepacia} complex (Bcc) which are important opportunistic human pathogens. \textit{B. cenocepacia} utilizes a mechanism of cell-cell communication called quorum sensing to control gene expression including genes involved in virulence. The \textit{B. cenocepacia} quorum sensing network includes the CepIR and CciIR regulatory systems.

\textbf{Results:} Global gene expression profiles during growth in stationary phase were generated using microarrays of \textit{B. cenocepacia} cepR, cciR and cepRcciIR mutants. This is the first time CciR was shown to be a global regulator of quorum sensing gene expression. CepR was primarily responsible for positive regulation of gene expression while CciR generally exerted negative gene regulation. Many of the genes that were regulated by both quorum sensing systems were reciprocally regulated by CepR and CciR. Microarray analysis of the cepRcciIR mutant suggested that CepR is positioned upstream of CciR in the quorum sensing hierarchy in \textit{B. cenocepacia}. A comparison of CepIR-regulated genes identified in previous studies and in the current study showed a substantial amount of overlap validating the microarray approach. Several novel quorum sensing-controlled genes were confirmed using qRT-PCR or promoter::lux fusions. CepR and CciR inversely regulated flagellar-associated genes, the nematocidal protein AidA and a large gene cluster on Chromosome 3. CepR and CciR also regulated genes required for iron transport, synthesis of extracellular enzymes and surface appendages, resistance to oxidative stress, and phage-related genes.

\textbf{Conclusion:} For the first time, the influence of CciIR on global gene regulation in \textit{B. cenocepacia} has been elucidated. Novel genes under the control of the CepIR and CciIR quorum sensing systems in \textit{B. cenocepacia} have been identified. The two quorum sensing systems exert reciprocal regulation of many genes likely enabling fine-tuned control of quorum sensing gene expression in \textit{B. cenocepacia} strains carrying the cenocepacia island.

Background

\textit{Burkholderia cenocepacia} is a member of a group of closely related organisms called the \textit{B. cepacia} complex (Bcc), which are important opportunistic pathogens in individuals with cystic fibrosis (CF) or chronic granulomatous disease [1-5]. \textit{B. cenocepacia} and \textit{B. multivorans} are the...
most common members of the Bcc isolated from lungs of CF patients [6,7]. Infections with B. cenocepacia can lead to what is termed "cepacia syndrome", a rapid decline in lung function associated with necrotizing pneumonia, bacteremia and sepsis which can result in death [8]. B. cenocepacia is intrinsically resistant to antibiotic therapy and often impossible to eradicate from lungs of infected CF patients [9].

Quorum sensing (QS) is an intricate cell-cell signaling system used by a diverse range of microbial species to communicate with neighbouring cells to regulate gene expression. In Gram-negative bacteria, homologs of the LuxR protein family synthesize signaling molecules termed N-acyl-homoserine lactones (AHLs) that are bound by homologs of the LuxR protein family which act as transcriptional regulators (for reviews see [10] and [11]). B. cenocepacia has two pairs of QS systems, the CepIR system which is present in all species of the Bcc [12-15] and the CciIR system, which is only present in B. cenocepacia containing the cenocepacia island (cci) found in highly transmissible ET12 strains [13]. CepI is primarily responsible for the synthesis of C8-HSL [14] and minor amounts C6-HSL [15]. CciI primarily synthesizes C6-HSL with lesser amounts of C8-HSL produced [16]. At the genomic level, cepI and cepR are divergently transcribed from each other while cciI and cciR form a transcriptional unit [16]. The QS systems are arranged in a hierarchical fashion as CepR is required for the transcription of the cciI operon [16]. However, CciR negatively regulates the expression of cepI thus allowing negative regulatory feedback on the CepIR system [16]. Additionally, CepR activity can be inhibited by excess amounts of C6-HSL [17]. B. cenocepacia also contains a third LuxR homolog, CepR2, that lacks an associated AHL synthase gene [18]. CepR2 negatively regulates its own expression and is negatively regulated by CciR. We have recently identified several CepR2-regulated genes, including virulence factors, and demonstrated that CepR2 can influence gene expression in the absence of AHLs [18].

The CepIR system in B. cenocepacia and B. cepacia positively influences virulence in murine, nematode, wax moth, alfalfa and onion infection models [19-22]. B. cenocepacia CepIR negatively regulates genes involved in the biosynthesis of the siderophore ornibactin [14], but positively regulates expression of the zmpA and zmpB extracellular zinc metalloprotease genes [14,19,20]. CepIR also positively influences swarming motility [21], biofilm formation and maturation [21,23]. Several studies have shown that CepR positively regulates the expression of AidA, a protein involved in nematode virulence [17,24-26].

Several global approaches have been used to identify the CepIR regulon. A B. cepacia ATCC 25416 random promoter library screened in E. coli identified 20 ORFs that were positively regulated by CepR in the presence of C8-HSL including a malate synthase gene and oxidative stress induced genes [24]. A random promoter library approach was employed for B. cenocepacia K56-2, using a K56-2 cepI mutant as a host, that identified 58 or 31 genes with increased or decreased expression, respectively, in the presence of C8-HSL [27], including genes involved in type II and type III secretion systems, catalase activity, cold shock proteins and genes with regulatory functions [27]. Transposon mutagenesis strategies in a B. cenocepacia cepI mutant identified seven [17] and six genes [28], differentially regulated by CepR, including the huv (phu) heme uptake system, a TonB-dependent siderophore receptor, and aidA [17,28]. Bioinformatic analysis of known CepR-regulated gene promoters was used to predict a consensus cep box motif sequence. An in silico screen of the B. cenocepacia genome identified promoters containing the cep box motif that are potentially regulated by CepIR including genes involved in type V secretion and lipopolysaccharide biosynthesis. CepR was shown to regulate promoters containing a cep box upstream of genes now known to encode proteins of the B. cenocepacia type VI secretion system (T6SS) [28,29] as well as several other genes including transcriptional regulators [28]. Additionally, a proteomics approach in B. cenocepacia H1111, which lacks cciIR, identified differential expression of 11 proteins, including AidA, FimA, and SodB, when C8-HSL was added to cultures of a H1111cepI mutant [26]. These combined approaches have facilitated the delineation of genes regulated by the CepIR system; however, there has been relatively little overlap in the genes identified using different approaches which suggests that our knowledge of the CepIR QS regulon is not complete.

Considerably less is known about the genes regulated by the CciIR QS system. A B. cenocepacia cciI mutant exhibited reduced virulence in a murine chronic respiratory infection model [13]. Reduced expression of zmpA was observed in B. cenocepacia cciI and cciR mutants [16]. Expression of zmpB was shown to be increased and decreased, respectively, in B. cenocepacia cciI and cciR mutants although a cciR mutant had similar levels to the parent strain [19]. Swarming motility was decreased in a cciI mutant but unchanged in a cciR mutant [16]. Both the CepIR and CciIR systems regulate orbI which is involved in ornibactin synthesis [27].

Studies of expression of individual genes in cepIR or cciIR mutants have suggested that some genes are co-regulated by these two QS systems, but that other genes are independently regulated. A genome-wide investigation of the contribution of the CciIR system to gene regulation in B.
cenocepacia has not yet been undertaken. Furthermore, the identification of CepIR-regulated genes is incomplete since differential expression of genes that would account for some observed phenotypes of cepI or cepR mutants have not yet been reported. In this study, global gene expression profiling was performed using B. cenocepacia microarrays in order to more fully comprehend the extent of gene regulation exerted by both CepR and CciR. These data provide evidence of the individual, overlapping and opposing roles played by each of the QS systems in the regulation of global gene expression in B. cenocepacia.

Results

Identification of genes differentially expressed in cepR, cciR and cepRcciIR mutants

Transcriptional profiling using a custom B. cenocepacia oligonucleotide microarray was used to identify genes differentially expressed in stationary phase cultures of strain K56-2 cepR, cciR and cepRcciIR mutants compared to wildtype K56-2. Both CepR and CciR were determined to positively and negatively regulate gene expression, and both QS systems influenced gene expression on all three chromosomes as well as the plasmid (Fig. 1A). Using a 2-fold difference in expression as a cut-off, 646 open reading frames (ORFs) were identified that were positively regulated by CepR and 214 ORFs were identified that were negatively regulated by CepR. CciR positively influenced the expression of 100 ORFs and negatively regulated the expression of 495 ORFs. In the cepRcciIR mutant, 313 ORFs exhibited decreased expression and 176 ORFs showed increased expression compared to wildtype (See Additional File 1: Genes with increased or decreased expression in cepR, cciR or cepRcciIR mutants compared to K56-2). Both QS systems regulated genes involved in a range of biological processes including virulence, surface structures, transport or secretion, metabolism and regulation.

Subsets of unique ORFs independently regulated by CepR or CciR were identified in the microarray data (Fig. 1B). CepR was primarily a positive regulator while CciR generally was a negative regulator of gene expression. Although the majority of differentially expressed genes appeared to be independently regulated by either CepR or CciR, 196 ORFs were identified that were regulated by both CepR and CciR (Fig. 1C). Of these 196 ORFs, 167 were positively regulated by CepR and negatively regulated by CciR. Other patterns of co-regulation were observed including negative regulation by CepR and positive regulation by CciR, positive regulation by both QS systems or negative regulation by both QS systems. More than one quarter of the unique ORFs that were reciprocally regulated by CepR and CciR showed the same trend in regulation in the cepR and cepRcciIR mutants. Other ORFs showed the same regulation pattern in the cepR and cepRcciIR mutants but no change in the cciR mutant. Together, these data indicate that positive regulation is more frequently performed by CepR compared to CciR, that the majority (92%) of co-regulated genes were inversely regulated by CepR and CciR and that CepR regulation can be dominant over CciR regulation.

CepR gene regulation

To validate the transcriptome data we first compared genes differentially regulated between K56-2 and the cepR mutant to data from previous studies on CepRI regulation.
in this strain. Previously we used a random promoter library to identify promoter::lux fusions differentially expressed between K56-2 and a cepI mutant [27]. Comparison of transcriptome data of K56-2 and the cepR mutant to the promoter fusion data for K56-2 and the cepI mutant revealed that there was an overlap of at least 10 genes or operons identified using the two approaches (Table 1). Regulation of these genes by CepR was usually in the same direction and fold change was generally similar between the two studies. In some cases, genes were identified as differentially regulated in the transcriptome analysis that are predicted to be in the same operon as genes previously identified using the promoter fusion approach also suggesting a correlation between the approaches (data not shown). Two genes were identified for which opposite regulation by the CepIR system was observed between the promoter::lux fusions and the transcriptome analysis. For example, opposite results between the promoter fusion and microarray data were observed for BCAL1814 expression in the cepR mutant (Table 1). Subsequent investigation using qRT-PCR confirmed the microarray data showing reduced BCAL1814 expression in both the cepR and cciR mutants compared to K56-2 indicating co-regulation (Table 2).

In another previous study, we used a bioinformatics approach to search the B. cenocepacia J2315 genome with a cep box consensus sequence to identify potential CepIR-regulated genes [28]. Twenty-nine ORFs identified to be CepR-regulated by microarray analysis were previously shown to have a cep box motif in their predicted promoter region or were located in a putative operon downstream from an ORF with a cep box motif (Table 3). These genes included cepI, zmpA, aidA, and genes of the T6SS. Taken together, the data suggest the three different experimental approaches were complementary and that the data obtained by transcriptome analysis are valid since many of the genes identified as CepR-regulated using microarray analysis have been confirmed by other approaches.

Regulation of genes adjacent to cepI and cepR

The genomic location of cepI and the downstream ORF (BCAM1871) suggests they are part of the same operon. Expression of cepI and BCAM1871 was reduced in the cepR and cepRcciR mutants compared to K56-2 (Tables 3 and 4). BCAM1869 is located between cepR and cepI and is divergently transcribed from cepR. Expression of BCAM1869 was reduced in the cepR mutant compared to K56-2 (Table 4). While the function of these proteins is not yet known, genomic locations are conserved for

Table 1: Comparison of genes (or operons) showing differential expression in microarray analysis and previously determined to be CepIR regulated using transcriptional fusions.

| Gene     | Functiona                                      | Change (fold) for K56-di2 (cepI) vs K56-2 | Change (fold) for K56-R2 (cepR) vs K56-2 | Change (fold) for K56-2cciR vs K56-2 | Change (fold) for K56-2cepRcciIR vs K56-2 |
|----------|------------------------------------------------|------------------------------------------|----------------------------------------|--------------------------------------|-------------------------------------------|
|          |                                               | Promoter fusionb | Microarrayc | Microarrayc | Microarrayc |
| BCAL0111 | putative TPR domain                           | -3.7          | -2.2        | NC          | -2.2        |
| BCAL0380 | ABC transporter ATP-binding subunit           | -2.7          | -2.3        | NC          | NC          |
| BCAL0812 | sigma 54 modulation protein                   | -2.0          | -2.8        | NC          | NC          |
| BCAL1814 | MerR family regulatory protein                | 2.2           | -2.9        | -2.7        | NC          |
| BCAL1990 | glucose-6-phosphate isomerase Pgi             | -2.2          | -2.6        | NC          | NC          |
| BCAL2244 | urocate hydratase HutU                        | -2.2          | -2.1        | NC          | NC          |
| BCAL2931 | radical SAM superfamily protein              | 3.2           | 2.0         | NC          | NC          |
| BCAL3006 | cold shock-like protein CspA                  | -1.9          | -5.6        | 2.8         | -4.9        |
| BCAS0221 | ABC transporter ATP-binding protein AfcB (pseudogene) | -1.7          | -3.6        | 2.9         | NC          |
| BCAS0409 | zinc metalloprotease ZmpA                     | -2.6          | -5.3        | 4.0         | -4.5        |

aFunction derived from B. cenocepacia J2315 [34].
bChange in 16-h cultures of cepI mutant compared to 16-h cultures of K56-2 as determined by promoter fusion Subsin et al. 2007 [27].
cChange in 16-h cultures of cepR, cciR or cepRcciIR mutants compared to 16-h cultures of K56-2 as determined by microarray analysis with at least two biological replicates (NC, no change).
dPresence of a cep box motif was identified by Subsin et al. 2007 [27] for this gene.
orthologs of BCAM1869 and BCAM1871 in many *B. cenocepacia* and other *Burkholderia* strains [30].

**CciR exerts global gene regulation**

The percentage of genes showing differential expression in the *cciR* mutant was 8.3% of the genome compared to 12.0% for the *cepR* mutant (See Additional File 1). The proportion of CciR-regulated genes was similar on each chromosome (7.9 to 8.6%) while 3 genes (3.1%) were regulated on the plasmid indicating CciR regulation is global in *B. cenocepacia*.

A large number of genes independently regulated by CciR were identified (See Additional File 1). CciR negatively regulated expression of 25 unique ORFs encoding ribosomal proteins only two of which were positively regulated by CepR. The majority of these ribosomal proteins form a large cluster from BCAL0233-0261. Expression of ORFs forming part of the putative AmrA-AmrB-BCAL1676 efflux pump was increased in the *cciR* mutant compared to K56-2. Expression of some of the genes encoding for components of this pump was also increased during growth of *B. cenocepacia* in CF sputum [31].

CciR reciprocally regulated many genes previously reported to be CepR-regulated [27,28] (Tables 1 & 3). Examples include increased expression of the cold shock-like protein CspA and the majority of genes in the BCAL0340-0348 T6SS operon in the *cciR* mutant compared to the *cepR* mutant. Additionally, CciR negatively regulated expression of pBCA055 (*bqiC*) which encodes a hypothetical protein with GGDEF and EAL domains (Table 4). Weingart et al. [17] reported CepR positively regulated expression of pBCA055 which was confirmed in the *cepR* and *cepRcciIR* mutants (Table 4).

**Regulation of genes on the cenocepacia island**

The *cciR* genes are located on a genomic island only found in strains of the ET12 lineage [13]. Mutations in two additional genes located on this island, *amil*
Table 3: Comparison of genes (or operons) showing differential expression in microarray analysis and identified to have a cep box motif.

| Gene       | Function                           | cep box motif name or genomic context of downstream gene | Change (fold) for K56-R2 (cepR) vs K56-2<sup>c</sup> | Change (fold) for K56-2cciR vs K56-2<sup>c</sup> | Change (fold) for K56-2cepRcciR vs K56-2<sup>c</sup> |
|------------|------------------------------------|--------------------------------------------------------|------------------------------------------------|------------------------------------------------|------------------------------------------------|
| BCAL0051   | periplasmic solute-binding protein | MST2001                                               | -2.1                                          | 2.5                                           | NC                                              |
| BCAL0052   | putative oxidoreductase            | downstream from MST2001                               | -2.2                                          | 2.7                                           | NC                                              |
| BCAL0232   | elongation factor Tu               | MST2002                                               | -2.1                                          | NC                                           | NC                                              |
| BCAL0340   | putative lipoprotein of T6SS       | MST005 & MST2004                                      | -2.8                                          | NC                                           | NC                                              |
| BCAL0341   | putative type VI secretion system protein TssB | downstream from MST005 & MST2004 | -2.5                                          | NC                                           | -2.1                                           |
| BCAL0342   | putative type VI secretion system protein TssG | downstream from MST005 & MST2004 | -3.1                                          | NC                                           | -2.3                                           |
| BCAL0343   | putative type VI secretion system protein TssD | downstream from MST005 & MST2004 | -3.2                                          | 2.1                                          | NC                                              |
| BCAL0344   | putative type VI secretion system protein TssE | downstream from MST005 & MST2004 | -2.7                                          | NC                                           | NC                                              |
| BCAL0345   | putative type VI secretion system protein TssF | downstream from MST005 & MST2004 | -5.1                                          | 2.8                                          | -2.1                                           |
| BCAL0346   | putative type VI secretion system protein TssG | downstream from MST005 & MST2004 | -2.9                                          | 2.1                                          | -2.1                                           |
| BCAL0347   | putative type VI secretion system protein TssH (ClpB) | downstream from MST005 & MST2004 | NC                                           | 2.1                                          | NC                                              |
| BCAL0348   | putative type VI secretion system protein TssA | downstream from MST005 & MST2004 | -2.7                                          | 2.1                                          | -2.2                                           |
| BCAL0831   | putative storage protein            | MST2009                                               | -5.2                                          | NC                                           | -4.0                                           |
| BCAL0832   | poly-beta-hydroxybutyrate storage protein PhaA | downstream from MST2009 | -2.4                                          | NC                                           | NC                                              |
| BCAL0833   | putative acetoacetyl-CoA reductase PhbB | downstream from MST2009 | -2.6                                          | NC                                           | -3.6                                           |
| BCAL0834   | hypothetical protein                | downstream from MST2009                               | -2.4                                          | NC                                           | NC                                              |
| BCAL0999   | sigma-E factor negative regulatory protein 2 RseA2 | MST2013                                          | NC                                           | NC                                           | -3.4                                           |
| BCAL1124   | conserved hypothetical protein      | MST2014                                               | 2.0                                           | NC                                           | NC                                              |
| BCAL1354   | conserved hypothetical protein      | MST2020                                               | NC                                           | NC                                           | -2.2                                           |
| BCAL1562   | hypothetical phage protein          | MST2024                                               | NC                                           | 2.7                                          | NC                                              |
| BCAL2871   | sigma-E factor negative regulatory protein 1 RseA1 | MST2036                                          | NC                                           | NC                                           | NC                                              |
| BCAL2870   | sigma-E factor regulatory protein RseB precursor 1 MucB1 | downstream from MST2036 | NC                                           | NC                                           | -3.5                                           |
| BCAL3190   | IclR family regulatory protein     | MST2039                                               | NC                                           | 2.3                                          | 2.6                                              |
| BCAL3205<sup>e</sup> | hypothetical protein        | MST2040                                               | -2.0                                          | NC                                           | NC                                              |
| BCAL3419   | 3-dehydroquinate dehydratase AroQ1 | MST2043                                          | -2.0                                          | NC                                           | NC                                              |
| BCAM1015   | putative porin                     | MST2050                                               | -2.3                                          | NC                                           | NC                                              |
| BCAM1405   | levansucrase                       | MST2055                                               | -2.9                                          | NC                                           | NC                                              |
| BCAM1502   | hypothetical protein                | MST2056                                               | -2.8                                          | NC                                           | NC                                              |
| BCAM1870   | N-acylhomoserine lactone synthase Cepl | MST2059                                          | -67.0                                         | NC                                           | -6.9                                           |
| BCAM1871   | hypothetical protein                | downstream from MST2059                               | -37.6                                         | NC                                           | -20.2                                          |
| BCAM2502   | 3-dehydroquinate dehydratase AroQ (similar to BCAL3419) | MST2064                                          | -2.1                                          | NC                                           | NC                                              |
BCAM2626  putative heme receptor protein HnuA  MST2066  3.4  -2.2  2.7
BCAM2627  putative hemin ABC transport system protein HmuS downstream from MST2066  3.1  NC  2.9
BCAS0293  nematocidal protein AidA  MST2069  -88.7  NC  -214.9
BCAS0292  hypothetical protein  downstream from MST2069  -137.3  NC  -278.2
BCAS0409  zinc metalloprotease ZmpA  MST2070  -5.3  4.0  -4.5

\*Function derived from *B. cenocepacia* J2315 [34].
\*cep box motif name from Chambers et al. 2006 [28] or genomic context of downstream.
\*Change in 16-h cultures of cepR, cciR or cepRcciIR mutants compared to 16-h cultures of K56-2 as determined by microarray analysis with at least two biological replicates (NC, no change).

Microarray analysis showed CciR negatively regulated its own expression, and that of cepR, as has previously been reported using cci::lux promoter fusions [16]. Although the cepR and cciR genes were shown to be co-transcribed [16], cepR expression was markedly more increased than the expression of cciR in the cciR mutant (Table 4). Microarray analysis showed cciR expression was decreased in the cepRcciIR mutant compared to K56-2 (Table 4). Expression of neither cepR nor cciR was changed in the cepR mutant grown in LB although we have previously demonstrated that CepR was required for cepR expression in PTSB medium using cepR::lux promoter fusions [16].

**Regulation of genes encoding extracellular enzymes by CepR and CciR**

We have previously shown decreased expression of zmpA in the cepR mutant compared to K56-2 [20,27]. Microarray and qRT-PCR analysis confirmed that zmpA expression was reduced in the cepR mutant (Table 1 &2) and demonstrated that zmpA expression was reduced in the cepRcciIR mutant (Tables 1 &2) confirming phenotypic data for these mutants [16]. Decreased expression of zmpA in the cciR mutant compared to K56-2 was previously demonstrated in PTSB medium using cciR::lacZ fusions [16]. In the current study, zmpB expression was increased in the cciR mutant compared to K56-2 in cultures grown in LB medium for 16 h, by both microarray and qRT-PCR analysis (Tables 1 &2).

Expression of zmpB was previously shown to be decreased in cepR, cciR and cepRcciIR mutants but increased in a cciR mutant compared to K56-2 using promoter::lux fusions [19]. Positive regulation of zmpB, was confirmed in the cepR and cepRcciIR mutants by microarray analysis (Table 4); however, zmpB expression was increased in the cciR mutant compared to K56-2. Although LB medium was used in both cases, data from the promoter::lux fusions was generated at 20 h growth while data from the microarray analysis was performed on cultures grown for 16 h. Several attempts were made to quantify zmpB expression levels by qRT-PCR but a high degree of variability was observed for this weakly expressed transcript. In fact, zmpB was the most weakly expressed transcript in the majority of the microarray samples. Together, these data indicate zmpA and zmpB expression is positively influenced by CepR under all conditions examined and that growth medium and phase of growth influence regulation by CciR.

Genes encoding the exported lipase LipA (BCAM0949) and the lipase chaperone LipB (BCAM0950, previously called limA) are required for lipase production [32]. Expression of both lipA and lipB was decreased in the cepR mutant compared to K56-2, but was unchanged in the cciR or cepRcciIR mutants (Table 4). Lipase activity was previously shown to be reduced in a cepR mutant compared to K56-2 [14].

BCAL1722 encoding a putative exported chitinase showed decreased expression in the cepR mutant compared to K56-2 but no change in expression in the cciR or cepRcciIR mutants (Table 4). Chitinase activity has been shown to be CepR-regulated in *B. cenocepacia* H111, with lower activity reported in *B. cenocepacia* H111 cepI and cepR mutants [21].

**CepR and CciR regulate genes adjacent to cepR2**

We have recently shown that the *B. cenocepacia* orphan LuxR homolog CepR2 is involved in negative regulation of genes adjacent to itself [18]. Additionally, cepR2 expression is increased in the cciR mutant [18]. Several CepR2-regulated genes and operons also showed differential
Table 4: Microarray analysis of selected genes showing differential expression in cepR, cciR or cepRcciIR mutants compared to K56-2.

| Gene          | Function                         | Change (fold) for K56-R2 (cepR) vs K56-2 | Change (fold) for K56-2cciR vs K56-2 | Change (fold) for K56-2cepRcciIR vs K56-2 |
|---------------|----------------------------------|----------------------------------------|-------------------------------------|------------------------------------------|
|               |                                  | microarrayb                            | microarrayb                         | microarrayb                             |
| BCAL1369      | Sigma factor 70 EcfC (FecI)      | 4.6                                    | NC                                  | 3.0                                      |
| BCAL1370      | Iron uptake regulatory protein FecR | 2.7                                    | NC                                  | 2.1                                      |
| BCAL1520      | Putative lipoprotein             | -2.1                                   | NC                                  | NC                                       |
| BCAL1528      | Flp type pilus assembly protein  | -2.1                                   | NC                                  | NC                                       |
| BCAL1530      | Flp type pilus assembly protein  | -3.2                                   | NC                                  | -2.0                                     |
| BCAL1531      | Flp type pilus assembly protein  | -2.2                                   | NC                                  | NC                                       |
| BCAL1532      | Flp type pilus assembly protein  | NC                                     | NC                                  | -2.2                                     |
| BCAL1533      | Putative lipoprotein             | -2.1                                   | 2.3                                 | NC                                       |
| BCAL1534      | Putative exported protein        | -3.3                                   | 2.6                                 | NC                                       |
| BCAL1368      | Probable porin                  | -2.4                                   | 2.5                                 | -2.1                                     |
| BCAL1677      | Putative type-I fimbrial protein | -2.3                                   | 3.1                                 | NC                                       |
| BCAL1688      | Sigma factor 70 EcfI (OrbS)     | NC                                     | -2.5                                | NC                                       |
| BCAL1689      | MbtH-like protein OrbH           | NC                                     | -2.2                                | NC                                       |
| BCAL1690      | Putative dioxygenase OrbG        | NC                                     | NC                                  | 2.5                                      |
| BCAL1692      | Iron transport-related membrane protein OrbD | NC                                  | NC                                  | 3.0                                      |
| BCAL1693      | Iron transport-related membrane protein OrbF | NC                                  | NC                                  | 2.1                                      |
| BCAL1694      | Iron transport-related exported protein OrbB | NC                                  | NC                                  | 2.4                                      |
| BCAL1696      | Ornibactin biosynthesis non-ribosomal peptide synthase OrbI | NC                                  | -2.4                                | NC                                       |
| BCAL1697      | Ornibactin biosynthesis non-ribosomal peptide synthase OrbJ | NC                                  | NC                                  | 2.4                                      |
| BCAL1698      | Ornibactin biosynthesis protein OrbK | NC                                  | -2.8                                | NC                                       |
| BCAL1699      | L-ornithine 5-monoxygenase PvdA  | NC                                     | -2.8                                | NC                                       |
| BCAL1700      | Ornibactin receptor precursor OrbA | NC                                  | -2.2                                | 2.2                                      |
| BCAL1701      | Ornibactin synthetase OrbF       | NC                                     | NC                                  | 2.2                                      |
| BCAL1702      | Ornibactin biosynthesis protein OrbL | NC                                  | NC                                  | 2.5                                      |
| BCAL1722      | Putative exported chitinase      | -2.8                                   | NC                                  | NC                                       |
| BCAL2757      | Superoxide dismutase SodB        | -2.3                                   | NC                                  | NC                                       |
| BCAL3297      | Ferretin DPS-family DNA-binding protein | -2.8                                 | NC                                  | -3.8                                     |
| BCAL3298      | Conserved hypothetical protein   | -2.1                                   | NC                                  | -2.8                                     |
| BCAL3299      | Peroxidase/catalase KatB         | -2.3                                   | NC                                  | -4.0                                     |
| BCAM0184      | Lectin                           | NC                                     | 2.0                                 | -2.1                                     |
| BCAM0186      | Lectin BclA                      | -7.5                                   | 3.1                                 | -3.2                                     |
| BCAM0233      | ArsR family regulatory protein   | NC                                     | -2.0                                | NC                                       |
| BCAM0238      | Putative iron transporter        | NC                                     | 2.0                                 | NC                                       |
| BCAM0239a     | N-acylhomoserine lactone synthase CciI | NC                                  | 52.1                                | NC                                       |
| BCAM0240      | N-acylhomoserine lactone dependent regulatory protein CciR | NC                                  | 4.0                                 | -23.5                                    |
| BCAM0949      | Exported lipase LipA             | -3.1                                   | NC                                  | NC                                       |
| BCAM0950      | Lipase chaperone LipB            | -2.5                                   | NC                                  | NC                                       |
| BCAM1869      | Conserved hypothetical protein   | -5.9                                   | NC                                  | NC                                       |
| BCAM1871      | Conserved hypothetical protein   | -37.6                                  | NC                                  | -20.2                                    |
| BCAM2307      | Zinc metalloprotease ZmpB        | -2.3                                   | 2.1                                 | -2.4                                     |
| pBCA055       | Putative membrane protein        | -12.0                                  | 2.4                                 | -9.1                                     |

*Function derived from B. cenocepacia J2315 [34].

*aChange in 16-h cultures of cepR, cciR or cepRcciIR mutants compared to 16-h cultures of K56-2 as determined by microarray analysis with at least two biological replicates (NC, no change).
expression in the current study (See Additional File 1). These genes included BCAM0189 (AraC family regulatory protein), BCAM0191 (putative non-ribosomal peptide synthetase) and BCAM0199 (outer membrane efflux protein). Expression of BCAM0189 and BCAM0191 were decreased but expression of BCAM0199 was increased in the cepR mutant compared to K56-2 (Table 2). This trend in regulation was confirmed for all three genes using qRT-PCR (Table 2). BCAM0189, BCAM0191 and BCAM0199 expression levels were similar between the cciR and cepRcciIR mutants compared to K56-2 by microarray; however, qRT-PCR analysis indicated that expression of these genes was decreased in the cciR mutant, but the expression pattern was similar between the cepRcciIR and cepR mutants (Table 2). CepR2 negatively regulates expression of the lectin-encoding gene, bclA, [33] which lies adjacent to two other co-transcribed lectin-encoding genes (BCAM0185-0184) [18]. Expression of bclA was decreased in the cepR mutant (Table 4). Expression of both bclA and BCAM0184 was increased in the cciR mutant and decreased in the cepRcciIR mutant compared to K56-2 (Table 4).

CciR negatively regulates aidA expression

Several studies illustrated that CepR positively regulates the expression of the nematocidal protein, AidA [17,24-26]. Expression levels of aidA and BCAS0292 were reduced in the cepR and cepRcciIR mutants (Table 2). Measurement of aidA expression using qRT-PCR confirmed reduced expression in the cepR and cepRcciIR mutants and indicated increased expression in the cciR mutant compared to K56-2 (Table 2). Negative regulation of aidA expression by CciR was also demonstrated using a promoter::lux fusion which showed significantly increased aidA expression in the cciR mutant between 12 and 16 h of growth (P < 0.05, unpaired t-test, Welch corrected) (Fig. 2).

QS regulation of flagellar and motility genes

Previously it was demonstrated that the cepR mutant exhibits reduced swimming motility while the cciR mutant has similar swimming motility compared to K56-2 [16]. Expression levels of 31 genes in nine operons involved in flagellar motility were analyzed. The overall trend showed decreased expression of these genes in the cepR and cepRcciIR mutants but increased expression in cciR mutant compared to K56-2 (Fig. 3A). Investigation of fliC (BCAL0114) expression using qRT-PCR confirmed this trend in regulation (Table 2). Separately, activity of a fliC promoter::lux fusion confirmed fliC expression was positively regulated by CepR and negatively regulated by CciR (data not shown). No obvious difference in flagellin protein expression was detected by Western blot in the QS mutants compared to wildtype (data not shown). Mutations in cepI or cepR were previously reported not to alter swimming motility of B. cenocepacia H111 growing at 37°C [21]. Strain K56-2 cepR and cepRcciIR mutants exhibited significantly reduced swimming motility at 22°C and 28°C compared to K56-2 (P < 0.001, two-way ANOVA) (Fig. 3BC), whereas swimming motility was significantly increased in the cciR mutant at 22°C and 28°C compared to K56-2 (P < 0.001, two-way ANOVA) (Fig. 3BC).

Flp type pilus and fimbrial proteins

Bacterial pili and fimbriae are frequently involved in binding eukaryotic cells and have been shown to play a role in infection in many pathogenic bacteria. A Flp type pilus cluster containing two putative operons from BCAL1524-1520 and BCAL1525-1537 has been identified [34]. Several genes in these operons showed decreased expression in the cepR and cepRcciIR mutants while two genes showed increased expression in the cciR mutant (Table 4) suggesting reciprocal regulation of this cluster. It is likely that the BCAL1525 operon is similar to that of clone P15 which was positively regulated by CepR in B. cepacia [24]. BCAL1677, which was positively regulated by CepR and negatively regulated by CciR (Table 4), encodes a putative type-1 fimbrial protein and was shown to be CepR-regulated in B. cenocepacia H111 [26].

QS controls a regulator involved in virulence (ShvR) and proteins of unknown function

Many ORFs located in a genomic region of approximately 27 kb on Chromosome 3 were differentially expressed in the QS mutants compared to K56-2 (Fig. 4A). Part of this QS-regulated gene cluster included BCAS0225, which regulates the rough-shiny morphotype and contributes to vir-
Regulation of flagellar-associated genes and swimming motility. (A) Cluster analysis of flagellar-associated genes with decreased (green), increased (red) or no change (black) in expression in each mutant compared to K56-2 according to microarray analysis. Arrows indicate putative (dashed lines) or experimentally-determined (solid lines) transcriptional units. Gene name and function are derived from *B. cenocepacia* [34]. Mean change (fold) in expression is indicated for the displayed group of genes for each mutant. Swimming motility was assessed by measuring zones of growth of cultures at (B) 22°C or (C) 28°C. Significantly different swimming motilities were observed for the QS mutants compared to K56-2 at 22°C and 28°C (***, *P* < 0.001, two-way ANOVA) (Fig. 3). All values are the means ± SEM of triplicate cultures and are representative of two individual trials.
Figure 4
Regulation of a 27 kb gene cluster on Chromosome 3. (A) Cluster analysis of genes in the *afrA* and *shvR* genomic regions with decreased (green), increased (red) or no change (black) in expression in each mutant compared to K56-2 according to microarray analysis. Arrows indicated putative (dashed lines) or experimentally-determined (solid lines) transcriptional units. Gene name and function are derived from *B. cenocepacia* [34]. Mean change (fold) in expression is indicated for the displayed group of genes for each mutant. (B) Expression of *afrA* was monitored throughout growth in K56-2ΔcepR and K56-2cciIR mutants compared to K56-2 in PTSB plus 100 μg/ml of Tp. The expression of *afrA::lux* (pAfrA) is significantly greater in K56-2cciIR than K56-2 from 6 to 10 h along the time course (P < 0.05, unpaired t-test, Welch corrected). All values are the means ± SD of triplicate cultures and are representative of two individual trials.
ulence in K56-2 [35]. Expression of BCAS0225 (which we now refer to as shuR for shiny variant regulator) was decreased in the cepR and cepRcciIR mutants compared to K56-2 (Table 2). Lower expression of shuR was confirmed using qRT-PCR in the cepR and cepRcciIR mutants while increased expression was observed in the cciR mutant (Table 2).

In B. cepacia BC11, AfcA and AfcCD are responsible for production of an antifungal compound [36]. Orthologs of these genes are present in B. cenocepacia as part of two putative transcriptional units; afcA (BCAS0222) to BCAS0202 and afcCD [30]. In the afcA genomic region, expression of the majority of ORFs was decreased in the cepR and cepRcciIR mutants but increased in the cciR mutant compared to K56-2 (Fig. 4A). Furthermore, qRT-PCR analysis of BCAS0204 and BCAS0220 confirmed this trend in the cepR, cciR and cepRcciIR mutants (Table 2). Although a difference in afcA expression was not detectable in these mutants by microarray analysis, an afcA::lux promoter fusion had lower expression in the cepR mutant and significantly higher expression in the cciR mutant compared to K56-2 between 6 and 10 h of growth ($P < 0.05$, unpaired t-test, Welch corrected) (Fig. 4B). Previously, CepR was also shown to positively regulate an afcB::lux reporter fusion [27] (Table 1).

**Transcriptional control of a resistance-nodulation division family efflux pump**

*B. cenocepacia* is known to exhibit high levels of intrinsic resistance to antimicrobials [9]. At least 14 potential resistance-nodulation division (RND) family efflux pumps [37] have been identified in *B. cenocepacia* [37,38]. We recently demonstrated CepR2 positively regulates expression of BCAM1420, part of a putative RND efflux pump [18]. Expression of BCAM1420 and several adjacent regulatory genes was lower in the cepR and cepRcciIR mutants but increased in the cciR mutant compared to K56-2 (Table 2) (See Additional File 1). The expression patterns of BCAM1420 and BCAM1418 (two-component regulatory system, response regulator) were confirmed by qRT-PCR (Table 2). Antibiotic susceptibility is not significantly different in peg-formed biofilms of cepR or cciR mutants compared to K56-2 [23]. No differences in resistance to a selection of heavy metals were observed between the QS mutants and K56-2 (data not shown).

**Reciprocal QS regulation of iron transport genes**

We have shown CepR negatively regulates ornibactin synthesis in *B. cenocepacia* strains K56-2 and H111 [15, 18]. No change in expression was detected for any gene in the orbl (BCAL1696) or orcf (orbs, BCAL1688) operons in the cepR mutant (Table 4); however, expression of several genes in these operons was decreased in the cciR mutant and increased in the cepRcciIR mutant compared to K56-2 (Table 4). This indicates that CciR positively regulates ornibactin synthesis and transport genes, and suggests CepIR is dominant over CciIR, since these genes are negatively regulated in the cepRcciIR mutant as has previously been reported in the cepR mutant.

*B. cenocepacia* contains a FecIR-like system potentially involved in iron uptake [39]. Increased expression of fecI (ecfC, BCAL1369) and fecR (BCAL1370) was detected in cepR and cepRcciIR mutants compared to K56-2 indicating that CepR also negatively regulates ferric citrate transport (Table 4). Divergently transcribed from ecfC is a probable porin gene that showed lower expression in cepR and cepRcciIR mutants compared to K56-2 (Table 4). This appears to be similar to clone P57 identified by Aguilar et al. 2003 [24] that was positively regulated by CepR in *B. cepacia*. Expression of a putative oxidoreductase (BCAL0269) and a ferric reductase-like transmembrane component (BCAL0270) was decreased in the cepRcciIR mutant compared to K56-2 (Table 4). It was recently reported that expression of BCAL0270, was increased during growth of *B. cenocepacia* in CF sputum [31].

We previously demonstrated that genes involved in heme transport are positively regulated by the CepR QS system and that huvA (BCAM2626) contained a cep box in its promoter region [28] (Table 3). In this study huvA expression was increased in the cepR and cepRcciIR mutants and decreased in the cciR mutant compared to wildtype. This trend in regulation for huvA, which contradicts the data obtained previously with huvA transcriptional fusions was independently confirmed using qRT-PCR (Table 2).

**CepR regulation of oxidative stress genes**

*B. cenocepacia* contains a major catalase/peroxidase protein, KatB (BCAL3299), important for resistance to hydrogen peroxide [40]. Expression of a putative three-gene operon consisting of katB and two downstream ORFs was decreased in the cepR and cepRcciIR mutants compared to K56-2 (Table 4). Expression of superoxide dismutase sodB (BCAL2757), previously shown to be CepR-regulated in *B. cenocepacia* H111 [26], was also reduced in the cepR mutant compared to K56-2 (Table 4).

**QS regulation of phage-related genes**

Recently, an epidemic strain of *Pseudomonas aeruginosa* with mutations in prophage and genomic island sequences was shown to have reduced ability to compete with the parent strain in a rat lung infection model [41]. *B. cenocepacia* J2315 possesses 5 prophages on 14 genomic islands (termed BcenGI) [34]. Twenty-four differentially regulated phage-related genes were identified in the cepR mutant, twenty genes were differentially expressed in the cepRcciIR mutant, whereas only 5 phage-
related genes showed changes in expression in the \textit{cciR} mutant (See Additional File 1).

Discussion

In this study we characterized the contributions of CepR and CciR to global gene regulation in \textit{B. cenocepacia}. Elucidation of the CciR regulon indicates that it is a global regulator of QS gene expression in \textit{B. cenocepacia}. Many CepR-regulated genes identified in prior studies were also identified as CepR-regulated using microarrays. This approach facilitated the identification of novel genes regulated by the CepIR and CciIR QS systems. Genes independently regulated by CepR or CciR, as well as co-regulated genes, were identified. Importantly, the majority of co-regulated genes were reciprocally regulated by CepR and CciR. This pattern of regulation was independently confirmed for a number of these genes using qRT-PCR or promoter::\textit{lux} fusions.

CepR-regulation of AidA has been consistently identified in previous studies [17,24-26] suggesting tight regulation by CepR. The putative two-gene operon comprising \textit{aidA} and the downstream ORF (BCAS0292) contained the most highly regulated genes in the \textit{cep} and \textit{cepRcciIR} mutants compared to K56-2. Promoter::\textit{lux} fusions showed that \textit{aidA} expression was negatively regulated by the CciIR system. Other genes, including \textit{cepI}, \textit{cciI}, pBCA055 and BCAM1871 also showed high levels of QS regulation (12- to 67-fold) using microarrays. The majority of genes showed low levels of QS regulation consistent with previous studies in \textit{B. cenocepacia} K56-2 [27,28]. A 2-fold change in expression was selected as a cut-off for microarray analysis which was consistent with the degree of differential expression of a number of previously-identified CepR-regulated genes in strain K56-2 [17,27,28]. These changes in gene expression have also been correlated with changes in some phenotypes such as protease activity and siderophore production [15,19,20]. Furthermore, some CepR-regulated genes identified in \textit{B. cenocepacia} H111 or \textit{B. cepacia} ATCC 25416 were shown to be CepR regulated in strain K56-2, suggesting that QS-mediated regulation of many genes is conserved in the Bcc strains [24,26].

The interrelationship between the CepIR and CciIR systems is complex. Although we previously reported that CciR negatively regulated \textit{cepI} in PTSB medium [16], no change in \textit{cepI} expression was detectable in the transcriptome analysis of the \textit{cciR} mutant grown in LB medium; however, consistent with previous data, CciR negatively regulated expression of the \textit{cciR} operon. We previously demonstrated that CepR was required for expression of a \textit{cciR} promoter::\textit{lux} fusion in PTSB medium [16], but similar results were not obtained in this study with cultures grown in LB medium. These data suggest that the regulatory relationship between the two QS systems varies depending on growth conditions and nutrient availability.

Presence of cci has been associated with several transmissible \textit{B. cenocepacia} strains [13]. The cci can be found in strains belonging to the ET12 lineage [13], but is absent from genomes of other \textit{B. cenocepacia} strains, including representatives of the transmissible PHDC lineage (AU1054, HI2424) and strain H111 [26,34]. Our study now shows that presence of CciIR in certain \textit{B. cenocepacia} strains has major implications for QS-regulated genes across the genome; most notably genes reciprocally regulated by CepIR and CciIR. Three master regulators of CepR have been identified in \textit{B. cenocepacia} H111 [42]. It is unknown if other regulatory factors (other than CciR, SuhB, YciL and YciR) exist which can limit the influence of CepR and thus provide balance to QS-mediated gene expression. This may have important consequences during infection to fine tune expression of virulence factors in response to particular environmental cues.

It has previously been shown that LasR, RhlR and QscR, components of the \textit{Pseudomonas aeruginosa} QS network, have overlapping but distinct regulons [43]. The presence of \textit{las-rhl} box-like sequences in the promoter regions of many QS-regulated genes has been demonstrated [44]. A subsequent study showed a consensus motif could be more easily identified for genes regulated by RhlR rather than LasR and that a single conserved motif could not be identified for QS-regulated promoters [45]. Recently, it was shown that \textit{rhlR} is expressed in the absence of LasR in \textit{P. aeruginosa} [46] and that RhlR exerts regulation over genes that were thought to be specifically LasR-regulated including \textit{lasI}.

In our study we confirmed CepR regulation for a number of genes which contain a \textit{cep} box motif in their promoters [28]. We do not know if CepR and CciR bind the same promoter motif. There were no genes with a \textit{cep} box in their promoters that showed only CciR regulation. Negative regulation by CciR occurs in genes with or without a \textit{cep} box in their promoters suggesting this motif is not required for regulation by CciR and opens the possibility that CciR recognizes a distinct motif. Gene regulation in the \textit{cepRcciIR} mutant more closely resembled gene regulation in the \textit{cep} mutant than the \textit{cciR} mutant. This suggests CepR is the more dominant regulator and very likely acts upstream from CciR. Direct binding of CepR to two promoters has been demonstrated [17]. CepR and CciR promoter binding studies might provide insight into the reciprocal regulation of co-regulated genes. It is possible that CciR could inactivate CepR by forming heterodimers as has been suggested as a mechanism for negative regulation by QscR [47]. The majority of C8-HSL or C6-HSL is
produced by CepI and CciI, respectively [14-16]. Therefore, QS-regulation by CepR and CciR may also be strictly dependent on the relative abundance of the signaling molecules.

Flagella are recognized as important virulence factors for human and plant pathogens [48]. A clear change in expression was observed at the transcriptional level for many flagellar-associated genes in the QS mutants compared to wildtype. Differences in swimming motility were found when cultures were grown at 22°C and 28°C confirming the reciprocal regulation of flagellar-associated genes and swimming motility by the CepR and CciR systems. A change in flagellin protein expression was not apparent in the QS mutants grown at 37°C. Positive control of flagella formation by QS is observed in B. glumae. However, lack of flagella formation in a B. glumae tofI mutant can be overcome by incubation at 28°C indicating other factors are involved [49]. Reduced biosurfactant production, as opposed to improper flagella formation, is responsible for reduced swarming in an AHL-deficient mutant of Serratia liquefaciens [50]. Reduced swarming observed in the B. cenocepacia H111CepR mutant can be restored by addition of exogenous surfactants. An endogenously-produced biosurfactant has not yet been described in B. cenocepacia. From an evolutionary perspective, flagella share common features with the type 3 secretion system (T3SS) [51] which we have also shown to be positively regulated by CepIR [27]. The expression of flagellar-associated and T3SS genes was increased in B. cenocepacia grown in CF sputum indicating conditions promoting their expression may be present during infection [31].

The majority of genes in the afcA genomic region have not been studied in detail in Burkholderia species, although some of these genes in B. cepacia BC11 are responsible for production of an antifungal with inhibitory activity against Rhizoctonia solani [36]. Antifungal production was highest in stationary phase and under low aeration growth conditions [36]. Expression of three putative operons containing afcA-BCAS0202, afcCD and shvR-BCAS0226 is induced in a B. cenocepacia agricultural field isolate (strain H12424) growing under soil-like conditions compared to CF-like conditions but was unchanged in a B. cenocepacia CF isolate (strain A1U1054) in these conditions [52]. We have now clearly shown that both CepR and CciR regulate many genes in the afcA genomic region.

Our recent characterization of the B. cenocepacia orphan LuxR homolog CepR2 [18] facilitated the incorporation of its regulon into the current study. A number of CepR2-regulated genes were also regulated by CepR and CciR, including genes in the genomic region adjacent to cepR2. A larger number of co-regulated genes may have been identified if the experiments were conducted at the same time in growth as opposed when CepR2 expression is maximal (mid-log phase) and when CepR and CciR expression is maximal (late stationary phase).

Intrinsic antibiotic resistance due to efflux pumps is a major problem associated with the treatment of B. cenocepacia infections. Presence of multiple efflux pumps with redundant functions is suggested by the lack of difference in cepR or cciR mutants compared to wildtype in resistance to antibiotics and heavy metals [23] (this study). However, QS regulation of efflux pumps may have other consequences. Efflux pumps are also involved in the efflux of QS molecules in P. aeruginosa [53] and B. pseudomallei [54]. QS regulation of efflux pumps may be more important in certain environments than in others. Growth of B. cenocepacia in CF sputum was shown to increase expression of a component of the BCAM0199-0201 putative multi-drug efflux pump [31] which we showed was negatively regulated by CepR and positively regulated by CciR and CepR2 [18] (this study).

Iron is an essential cofactor in many metabolic pathways however iron availability is usually limited in the host [55]. B. cenocepacia produce the siderophores ornibactin and pyochelin to sequester iron. CepR2 and indirectly CepR positively regulate pyochelin biosynthesis in B. cenocepacia H111 [18]. Pyochelin biosynthesis does not occur in B. cenocepacia K56-2 because a point mutation exists in the pyochelin synthetase gene pchF [34]. In this study we demonstrate that the CepR and CciR systems inversely regulate genes involved in ornibactin biosynthesis and uptake, as well as regulate genes involved in other iron transport systems including heme and the FecIR uptake systems. QS regulation of multiple iron transport mechanisms may facilitate growth in specific environmental niches where resources are limited. Some ornibactin genes were poorly expressed, most likely due to the fact cultures for microarray analysis were grown in LB medium compared to cultures with promoter::lux fusions which were grown in low-iron TSB-DC medium [56]. The difference in media may explain the difference in regulation for some of these iron acquisition genes between the current and previous studies [28], since we also demonstrated that the expression of cepI and cciI varied depending on the media. Different media are also known to influence lasIR, rhlIR and QS-regulated genes in P. aeruginosa [57].

Evidence of a link between oxidative stress and QS was shown in the regulation of katA, sodA and sodB by the las and rhl systems in P. aeruginosa [58]. In B. pseudomallei, the response to oxidative stress occurs through QS regulation of dpsA expression [59]. In this study we demonstrated that CepR regulates katB (BCAL3299) and a downstream gene BCAL3297 encoding a putative ferritin DPS-family.
DNA binding protein in *B. cenocepacia*. Genomic analysis suggests that BCAL3297 is a homolog of the ORF designated *dpsA* positioned downstream from *katG* (clone P80), which is CepR-regulated in *B. cepacia* [24].

QS-regulated phage-related genes included a large proportion of genes contained on prophage BcenGI12. We have not investigated the consequences of QS regulation on phage activity in *B. cenocepacia*; however, mutation of phage components in *P. aeruginosa* has important consequences for bacterial competition in vivo [41]. It is enticing to consider that unidentified virulence factors may be carried on genomic islands/prophages and that expression of these is under QS control. It is also possible that cell density-dependent regulation of phage elements facilitates genomic rearrangement within a species or horizontal gene transfer between mixed bacterial populations.

**Conclusion**

The CepIR and CciIR QS systems regulate expression of multiple genes at the transcriptional level in *B. cenocepacia*, including potential virulence genes. QS-regulated genes involved in motility, biofilm formation/adhesion, extracellular enzymes, secretion systems, iron transport, stress response and antibiotic resistance are summarized in Fig. 5. The CepIR system is primarily responsible for positive regulation while the opposite is true for the CcciIR system. The majority of the co-regulated QS-controlled genes are subject to reciprocal regulation by CepR and CcciR. Until now the scale of this inverse regulation was not fully appreciated. The antagonistic influence of CepR and CcciR ensures that QS-regulated gene expression in *B. cenocepacia* is tightly regulated. Novel gene clusters, not previously shown to be QS-regulated, were identified, facilitating the future examination of these genes in relation to pathogenesis. This work provides significant advances for understanding QS-mediated regulation of virulence genes in *B. cenocepacia*. A detailed picture of the QS network is required to facilitate the development of therapies aimed at interfering with cell-cell communication systems to control bacterial infection.

**Methods**

**Strains and growth conditions**

The bacterial strains used in this study are listed in Table 2. Cultures were routinely grown at 37°C in Miller’s Luria broth (LB) (Invitrogen, Burlington, ON) with shaking or on 1.5% Lennox LB agar plates. For promoter::lux assays, strains were grown in 0.25% tryptcase soy broth (Difco, Franklin Lakes, NJ) with 5% Bacto-Peptone (Difco) (PTSB). Swimming motility assays were performed as previously described [35] except that overnight cultures were normalized to an OD_{600} of 0.4 prior to inoculation and growth was assessed at 22°C or 28°C. When appropriate, the following concentrations of antibiotics were used: 100 µg/ml of trimethoprim (Tp) and 200 µg/ml of tetracycline (Tc). Antibiotics were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON).

**DNA manipulations**

DNA manipulations were performed using standard techniques as described by Sambrook et al. [60]. Genomic DNA was isolated as described by Ausubel et al. [61] or Walsh et al. [62]. Oligonucleotide primers (See Additional File 2: Oligonucleotide primers used in this study) were designed with Primer3 [63] and were synthesized by the University of Calgary Core DNA and Protein Services (Calgary, Alberta, Canada). Plasmids were introduced into *B. cenocepacia* by electroporation [64].

**Construction of the K56-2 ΔcepR mutant**

An unmarked cepR mutant was constructed in K56-2 following the procedure outlined by Flannagan et al. [65]. Briefly, two regions of homology flanking cepR were amplified using primers F1-M1868-UP-EcoRI, R1-M1868-UP-Clal, F2-M1868-DW-Clal and R2-M1868-DW-Xbal. The amplified products were digested with Clal to remove an internal portion of cepR and ligated into pCR2.1TOPO, giving rise to plasmid TPCR2.1::H12. Plasmid TPCR2.1::H12 was digested with XbaI and EcoRI and the fragment was inserted into pGPI-Scl, giving rise to mutagenesis plasmid pGPI-Scl::H12. pGPI-Scl::H12 was introduced into *B. cenocepacia* by conjugation. A single crossover event in K56-2 was confirmed by PCR in Tp resistant clones. To these clones, pDAI-Scl was introduced by conjugation to obtain the double crossover event. The pDAI-Scl plasmid was resolved by curing the exconjugants in LB broth. PCR confirmed that the deletion had occurred.

**RNA manipulations**

*B. cenocepacia* was subcultured to obtain an initial optical density at 600 nm (OD_{600}) of 0.02 and grown for the time indicated below for each experiment without selection. Total RNA was isolated using a Ribopure bacterial RNA isolation kit (Ambion, Streetsville, Ontario, Canada). DNase treatment was performed, and samples were confirmed by PCR using Taq polymerase (Invitrogen) to be free of DNA prior to cDNA synthesis.

**Microarray sample preparation**

Three independent RNA samples from *B. cenocepacia* strains grown for 16 h were used in microarray experiments. Gene expression profiles were generated using custom *B. cenocepacia* J2315 microarrays (Agilent, Santa Clara, CA) [34,66]. A reference pool of K56-2 cDNA was fluorescently labelled with Cy3 while the test cDNA samples (K56-2, K56-R2, K56-cciIR and K56-cepRcciIR) were fluorescently labelled with Cy5. cDNA generation and labelling was performed using the CyScribe Post-Label-
ling kit (GE Healthcare, Wales) according to the manufacturer’s protocol. Spike-in controls (Agilent) were included into the labelling procedure for quality control purposes. cDNA purification was performed by ethanol precipitation and the labelled cDNA was purified using a CyScribe GFX purification kit and eluted with water. Hybridization and washing of arrays was performed according to the two-colour microarray based gene expression analysis protocol (Agilent) with minor modifications where the 25× fragmentation buffer was omitted and the cDNA mix and 10× blocking agent were heat-denatured for 3 min at 98°C and cooled to room temperature before adding the hybridization buffer. Washing of microarrays was performed including acetonitrile as well as stabilization and

Figure 5
Hierarchical organization of the CepIR, CciIR and CepR2 quorum sensing systems and traits under their control in B. cenocepacia K56-2. Summary diagram of the regulatory interrelationship between CepIR and CciR, and CciR and CepR. AHLs are required to activate CepR and CciR but not CepR2. All three regulators negatively control their own expression. The three QS systems positively and negatively influence gene expression. +, positive regulation; -, negative regulation.
under accession number E-MEXP-2303. The entire microarray data set has been deposited in the Microarray accession number with differentially regulated genes. They appeared to be in transcriptional units associated with microarray analysis, certain genes were noted because a fold cutoff was used for comparison. Subsequent to filtering on flags (present/marginal versus absent), genes were selected on the basis of changes, for which a 2-

After filtering on flags (present/marginal versus absent), genes (added prior to hybridization) (data not shown). 16 arrays of the signal intensities of spike-in control genes were formed for all arrays. Some variation was noted across the arrays. Signal intensities were adjusted to 61 × 21.6 mm. The extended dynamic range of the scanning solution (Agilent). A G2565 BA microarray scanner and the scan control software (Agilent) were used. Scanning resolution was set to 5 μm and the scan region was adjusted to 61 × 21.6 mm. The extended dynamic range function was switched on with 100% and 10% photomultiplier gain settings. Images were analysed with the feature extraction software (Agilent). Labelling, hybridization and scanning were performed by the Mahenthiralingam Laboratory, Cardiff University, Wales.

Microarray data analysis
Microarray data analysis was performed using GeneSpring GX 7.3.1 software (Agilent). Initial data were preprocessed by employing the enhanced Agilent FE import method, and then per-spot and per-chip normalizations were performed for all arrays. Some variation was noted across the 16 arrays of the signal intensities of spike-in control genes (added prior to cDNA synthesis) and prelabeled control genes (added prior to hybridization) (data not shown). After filtering on flags (present/marginal versus absent), genes were selected on the basis of changes, for which a 2-fold cutoff was used for comparison. Subsequent to microarray analysis, certain genes were noted because they appeared to be in transcriptional units associated with differentially regulated genes.

Microarray accession number
The entire microarray data set has been deposited in the ArrayExpress database http://www.ebi.ac.uk/arrayexpress under accession number E-MEXP-2303.

| Strain or plasmid | Description | Reference |
|-------------------|-------------|-----------|
| **B. cenocepacia** |             |           |
| K56-2             | CF isolate, BCESM +, cepR::Tn5-OT18 derivative of K56-2, Tc<sup>+</sup> | [70] |
| K56-R2            | cepR::Tn5-OT18 derivative of K56-2, Tc<sup>+</sup> | [14] |
| K56-2cepR         | cepR derivative of K56-2 | This study |
| K56-2ccIR         | cciR::Tp derivative of K56-2, Tp<sup>R</sup> | [16] |
| K56-2ccIR         | cciIR derivative of K56-2 | [16] |
| K56-2cepFccIR     | cepR::Tp<sup>R</sup>, cciIR derivative of K56-2, Tp<sup>R</sup> | [16] |
| **E. coli**       |             |           |
| DH5α              | F−, mcrA::(mrn-hsdRMS-mcrBC) ψ80lacZΔM15 ΔlacX74 recA1 endA1 araD139Δ(ara, leu)7697 galU galK λ− rpsL<sup>N</sup> nupG<sup>+</sup> | Invitrogen |
| TOP10             | F−, mcrA::(mrn-hsdRMS-mcrBC) ψ80lacZΔM15 ΔlacX74 recA1 endA1 araD139Δ(ara-leu)7697 galU galK rpsL<sup>N</sup> (Str<sup>R</sup>) nupG<sup>+</sup> λ− | Invitrogen |
| SY327             | araD, Δ(lac pro) argE(Am) recA56 rif<sup>N</sup> nolaA<sup>+</sup>, pir | [71] |

**Plasmids**

|   |   |   |
|---|---|---|
| pGPI-Scl | ori<sub>SMC</sub>, Tp<sup>R</sup>, mob<sup>+</sup>, carries l-Scl cut site | [65] |
| pDAI-Scl | pDAI7 carrying the l-Scl gene | [65] |
| TPCR2.1::H12 | pCR2.1TOPO containing amplified homologous regions flanking cepR | This study |
| pGPI-Scl::H12 | pCR2.1TOPO containing amplified homologous regions flanking cepR | This study |
| pAidA301 | aidA::lux transcriptional fusion constructed in pMS402 Km<sup>R</sup> Tp<sup>R</sup> | [28] |
| pAfA | afaA::lux transcriptional fusion constructed in pMS402 Km<sup>R</sup> Tp<sup>R</sup> | This study |

Cluster analysis and operon prediction
Microarray data was analyzed with Cluster [67] and visualized using Treeview to allow a visual comparison of expression levels for each gene in an operon. Operon prediction was performed by analysis of the B. cenocepacia J2315 genome at http://www.burkholderia.com[30]. Adjacent genes on the same coding strand with less than 300 bp intergenic space between them were arranged in putative operons.

Quantitative RT-PCR
The sigma factor gene sigE [39] (previously termed sigA [18]) (BCAM0918) was used as a reference standard as described previously [18]. Expression of sigE was not significantly altered according to microarray analysis (data not shown). RT-PCR was performed using an iScript Select cDNA synthesis kit (Bio-Rad). For quantitative RT-PCR (qRT-PCR), quantification and melting curve analyses were performed with an iCycler and iQ SYBR green Supermix (Bio-Rad) according to manufacturer's instructions. qRT-PCRs were performed in triplicate, and the data shown below represent data from at least two independent experiments. Relative expression values for each gene were calculated using the ΔΔCt equation [68].

Transcriptional fusions to luxCDABE (lux)
The 371 bp afaA promoter region was amplified using primers AfAPromfor1 and AfAPromrev1 and cloned into the Xhol-BamHI site upstream of lux in pMS402 [69]. The aidA transcriptional fusion was previously described.
[28]. Luminescence assays were carried out as previously described [18,28]. The level of promoter activity is expressed below as the ratio of luminescence to turbidity (CPS/OD<sub>600</sub>).

**Authors’ contributions**

EPO prepared samples for microarray experiments and performed qRT-PCR experiments. EPO and PAS analyzed and interpreted microarray data. DFR constructed the unmarked mutant. EPO, DFR and RJM performed promoter::lux reporter experiments. EPO and PAS conceived and designed the experiments. EPO and PAS wrote the manuscript. All authors read and approved the final manuscript.

**Additional material**

**Additional file 1**

Genes with increased or decreased expression in cepR, cciR or cepRcciIR mutants compared to K56-2. Microarray analysis of selected genes showing differential expression in cepR, cciR or cepRcciIR mutants compared to K56-2.

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[http://www.biomedcentral.com/content supplementary/1471-2164-10-441-S1.DOC](http://www.biomedcentral.com/content supplementary/1471-2164-10-441-S1.DOC)

**Additional file 2**

Oligonucleotide primers used in this study. Complete list of oligonucleotide primers used in this study.

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[http://www.biomedcentral.com/content supplementary/1471-2164-10-441-S2.DOC](http://www.biomedcentral.com/content supplementary/1471-2164-10-441-S2.DOC)

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