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Identification of Genes Regulated by the *cepIR* Quorum-Sensing System in *Burkholderia cenocepacia* by High-Throughput Screening of a Random Promoter Library[▽]

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The *Burkholderia cenocepacia cepIR* quorum-sensing system regulates expression of extracellular proteases, chitinase, and genes involved in ornibactin biosynthesis, biofilm formation, and motility. In a genome-wide screen we identified *cepIR*-regulated genes by screening a random promoter library of *B. cenocepacia* K56-2 constructed in a luminescence reporter detection plasmid for differential expression in response to *N*-octanoyl-L-homoserine lactone (OHL). Eighty-nine clones were identified; in 58 of these clones expression was positively regulated by *cepIR*, and in 31 expression was negatively regulated by *cepIR*. The expression profiles of the 89 promoter clones were compared in the *cepI* mutant K56-dI2 in medium supplemented with 30 pM OHL and K56-2 to confirm that the presence of OHL restored expression to wild-type levels. To validate the promoter library observations and to determine the effect of a *cepR* mutation on expression of selected genes, the mRNA levels of nine genes whose promoters were predicted to be regulated by *cepR* were quantitated by quantitative reverse transcription-PCR in the wild type and *cepI* and *cepR* mutants. The expression levels of all nine genes were similar in the *cepI* and *cepR* mutants and consistent with the promoter-*lux* reporter activity. The expression of four selected *cepIR*-regulated gene promoters was examined in a *cciIR* mutant, and two of these promoters were also regulated by *cciIR*. This study extends our understanding of genes whose expression is influenced by *cepIR* and indicates the global regulatory effect of the *cepIR* system in *B. cenocepacia*.

Strains of the *Burkholderia cepacia* complex (Bcc) have emerged as important opportunistic pathogens in patients with cystic fibrosis (CF) and chronic granulomatous disease, and infections often result in significant mortality rates (10, 27, 29, 41). The Bcc is comprised of nine species, all of which have been isolated from environmental and clinical sources (10). *Burkholderia cenocepacia* is the most prevalent Bcc species in CF respiratory infections (35, 41). There have been numerous cases of transmission of Bcc strains or clones between CF patients, resulting in rigorous infection control measures (6, 29, 30, 32, 41). The majority of transmissible clones belong to *B. cenocepacia*, which has also been reported to replace other Bcc species in CF respiratory infections (6, 30, 32).

The quorum-sensing (QS) system is a form of cell-cell communication that involves the production of small signaling molecules produced in a cell density-dependent manner. In gram-negative bacteria these signaling molecules are usually *N*-acyl-homoserine lactones (AHLs) (for reviews, see references 44 and 46). Two sets of QS genes, *cepIR* and *cciIR*, have been identified in *B. cenocepacia* (5, 23). CepI synthesizes two AHL molecules, *N*-octanoyl-L-homoserine lactone (OHL) and *N*-hexanoyl-L-homoserine lactone (23, 24). OHL is the predominant AHL produced by Bcc strains (15, 26). CepR is a transcriptional regulator that responds to the AHL signals and either positively or negatively regulates target genes (13, 24, 44). CciI produces predominantly *N*-hexanoyl-L-homoserine lactone and minor amounts of OHL (31). The *cepIR* quorum-sensing genes have been reported to be present in all Bcc species (15, 26), whereas the *cciIR* system is present only in epidemic *B. cenocepacia* strains containing the cenocepacia island (*cci*) (5). The *cepIR* system appears to be the ancestral system, and the *cciIR* system is incorporated into the *cepIR* regulatory network (31). CepR is required for expression of the *cciIR* genes, which are cotranscribed (12). Both QS systems have been implicated in virulence. Murine respiratory, nematode, and onion infection models have shown that *cepIR* contributes to the virulence of *B. cenocepacia* and *B. cepacia* (2, 21, 40). A *B. cenocepacia cciI* mutant was shown to have attenuated virulence in a rat chronic respiratory infection model (5).

QS systems regulate genes involved in the virulence of many bacterial species (11, 46, 48). The *cepIR* QS system was first identified in strains of *B. cenocepacia* because of its involvement in regulation of biosynthesis of the siderophore ornibactin (23) and its requirement for biofilm formation on abiotic surfaces (17). *B. cenocepacia cepI* or *cepR* mutants have been reported to be altered in extracellular protease production, chitinase activity, swarming motility, and mature biofilm development (17, 18, 20, 23, 24, 40, 43). Expression of the *pvdA* gene involved in ornibactin biosysthesis (24) and expression of the zinc metalloprotease genes, *zmpA* (40) and *zmpB* (20), have been shown to be negatively and positively regulated, respectively, in *cepI* and *cepR* mutants, suggesting that the expression of these genes is directly regulated by CepR. Expression of *aidA*, a protein having an unknown function that is important for virulence in nematodes, is also positively regulated by *cepR* in both *B. cenocepacia* and *B. cepacia* (3, 16, 36, 47). Little is currently known about *cepR* regulation of other genes that might

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
E. coli strains		
DH5α	F⁻ ϕ80*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 recA1 endA gyrA96 thi-1 hsdR17 supE44 relA1 deoR*	Invitrogen
TOP10	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*Δ*M15* Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
B. cenocepacia strains		
K56-2	Cystic fibrosis respiratory isolate	28
K56-R2	*cepR*::Tn*5*-OT182 derivative of K56-2, Tc^r	23
K56-dI2	Δ*cepI* derivative of K56-2	31
Plasmids		
pCR2.1TOPO	Cloning vector for PCR products, Ap^r Km^r	Invitrogen
pBS7	pCR2.1TOPO containing 1.3-kb *ndh* fragment	14
pMS402	*lux*-based promoter reporter plasmid, Km^r Tp^r	12
pCC1	pMS402 containing a *zmpA*::*luxCDABE* fusion, Km^r Tp^r	40
pSLS225	pUCP26 with 1.5-kb SphI-KpnI fragment containing *cepI*, Tc^r	25
pRM445	pMS402 containing a *cepR*::*luxCDABE* transcriptional fusion, Km^r Tp^r	31
pCP300	pMS402 containing a *cepI*::*luxCDABE* transcriptional fusion, Km^r Tp^r	31
pMVbcscV	pMS402 containing a *bcscV*::*luxCDABE* transcriptional fusion, Km^r Tp^r	This study
pBS18	pMS402 containing a *katB*::*luxCDBAE* transcriptional fusion, Km^r Tp^r	
pVDA01	pMS402 containing a *pvdA*::*luxCDBAE* transcriptional fusion, Km^r Tp^r	This study
pVDO301	pMS402 containing an *orbI*::*luxCDBAE* transcriptional fusion, Km^r Tp^r	This study

[a] Ap, ampicillin; Km, kanamycin; Tp, trimethoprim; Tc, tetracycline.

influence the phenotypes described above or the virulence of *B. cenocepacia*.

Several approaches have been used to identify genes regulated by the *cepIR* QS system. Aguilar et al. identified positively regulated OHL-CepR-dependent promoters in *B. cepacia* ATCC 25416 using a library of ATCC 25416 fragments cloned into a vector containing *cepR* and a promoterless *lacZ* gene downstream from a multiple cloning site (3). The library was screened in *Escherichia coli* DH5α, and 28 putative gene promoters that exhibited CepR-dependent expression in the presence of OHL were identified (3). Proteomic analysis was used to compare *B. cenocepacia* H111 to its *cepI* mutant (36). Two-dimensional gel electrophoresis of the protein profiles revealed that there was differential expression of 55 of 985 detectable spots. Approximately 5% of the *B. cenocepacia* H111 proteome was downregulated and 1% was upregulated in the *cepI* mutant. Nineteen peptides from 11 proteins were identified by N-terminal amino acid sequencing, and these peptides included peptides from AidA, FimA, SodB, RpsD, a thermolysin metallopeptidase, and some hypothetical proteins (28). The thermolysin metallopeptidase was subsequently determined to be ZmpB and was shown to be *cepIR* regulated in *B. cenocepacia* K56-2 using *zmpB*::*lux* reporter fusions (20). Genes regulated by the *cepIR* QS system have also been identified using transposon mutagenesis. Seven genes, including *cepI* and *aidA*, were identified using a transposon that incorporates a promoterless *lacZ* reporter gene and screening for induction of β-galactosidase activity in the presence of OHL (47). Purified CepR was shown to directly bind to promoters upstream of *cepI* and *aidA*, confirming that the CepIR system directly regulates expression of these genes (47).

Although several *cepIR*-regulated genes have been identified using molecular and proteomic methods, our understanding of the *cepIR* regulon is far from complete. To identify additional *cepIR*-regulated genes in *B. cenocepacia* K56-2, we employed a random promoter library using a sensitive luminescent reporter system that provided a high-throughput method for measurement of real-time gene expression (7, 12). A random promoter library of *B. cenocepacia* K56-2 *luxCDABE* transcriptional fusions was generated and screened in a *cepI* deletion mutant grown in medium with or without OHL to identify OHL-responsive promoters. In addition, real-time reverse transcription (RT)-PCR was performed with representative genes to confirm that there was differential gene expression in K56-2 and *cepI* and *cepR* mutants of this strain. The results of this study increase our understanding of *cepIR*-mediated gene regulation in *B. cenocepacia* K56-2.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Strains and plasmids used in this study are listed in Table 1. *E. coli* and *B. cenocepacia* were cultured aerobically at 37°C in Luria-Bertani (LB) medium (Invitrogen Canada Inc., Burlington, ON, Canada). For analysis of the expression of *pvdA* and *orbI* promoters, cultures were grown in TSB-DC medium (33). When appropriate, antibiotics were used at the following concentrations: for *B. cenocepacia*, 100 μg/ml trimethroprim and 200 μg/ml tetracycline; and for *E. coli*, 25 μg/ml kanamycin and 1.5 mg/ml trimethroprim. Antibiotics were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

Construction of the *B. cenocepacia* K56-2 random promoter library. Molecular biology procedures were generally performed as described by Sambrook et al. (38). K56-2 genomic DNA was isolated as described by Ausubel et al. (4) and was partially digested with Sau3AI (Invitrogen Canada Inc.). The digested DNA fragments were separated by sucrose density gradient centrifugation. Fragments in the range from 0.5 to 3 kb long were ligated into pMS402, which contains a promoterless *luxCDABE* operon (12), previously digested with BamHI (New England BioLabs, Mississauga, ON, Canada) and dephosphorylated with shrimp alkaline phosphatase (Roche, Mannheim, Germany). After enzyme inactivation, the dephosphorylated plasmid was purified using a QIAgen PCR purification kit (QIAGEN, Mississauga, ON, Canada). Ten independent ligation reactions were performed using various vector/insert ratios, and the products were electroporated into ElectroMAX DH10B cells (Invitrogen Canada Inc.) using a Gene Pulser (Bio-Rad Laboratories Inc., Hercules, CA). The efficiency of ligation was determined for 12 randomly selected transformants from each ligation using colony PCR performed with primers pZE05 (5′-CCAGCTGGCAATTCCGA-

TABLE 2. Oligonucleotide primer sequences used in real-time RT-PCR[a]

Oligonucleotide	Open reading frame	Homolog	Forward primer	Reverse primer
F/RBS-B02	BCAL2732	Cold shock	GGGCGGTGAGGATCTGTTT	GCCTTCCTTCAGCGTCTTGA
F/RBS-B29	BCAL3010	*spoT*	CGCGAACATCGTCCATATTG	CGCTGACCTGGATCACGAA
F/RBS-B34	BCAL3506	*fliM*	GGAATGCGGCTACGGAATT	GCTCGCGCCGATCATCT
F/RBS-C53	BCAL3524	*gspC*	GGCCAGCTCGACAAGA	CGAGAATGCCGAACAG
F/RBS-E13	BCAL1699	*pvdA*	TGCAGATCTCGTTTCTGAAGGA	TTCGAACAGGTAGTTGATGAACGT
F/RBS-E62	BCAS0409	*zmpA*	CGCGGCAGACATCGACTAC	AGATGCCGTTGCGGTTGT
F/RBS-F105	BCAM2041	*bcscR*	CGTGCTGGTGCCAAGCTT	CGACGATGAACACGATGTAGATC
F/RBS-F135	BCAM0931	*katB*	CTGGAACATGATGACGAACGA	AGGTCCCACTTCGGGAAATC
F/RBS-E51	pBCA054	Regulator	GCCACCTCGCGATCCA	ATTCGGCGAACGCTGTCA
F/Rndh	BCAM0166	*ndh*	GCGATCGGGCTGTACAAGTT	AGTGGCTCAGCGACTGGAA

[a] Oligonucleotide primers were designed based on the unpublished sequence of *B. cenocepacia* J2315 (http://www.sanger.ac.uk/Projects/B_cenocepacia/).

3′) and pZE06 (5′-AATCATCACTTTCGGGAA-3′) (12) corresponding to sequences that flank the BamHI site of pMS402. The pooled plasmid extracts from five high-efficiency ligation mixtures that contained approximately 60% inserts were subsequently electroporated into the *cepI* deletion mutant, K56-dI2.

Screening the random promoter library. OHL was purified from *B. cenocepacia* K56-2(pSLS225) as previously described (9). The optimum concentration of OHL used for screening the library was determined by comparing the luminescence values for known quorum-sensing-regulated gene fusions, *zmpA*::*lux*, *cepI*::*lux*, and *cepR*::*lux*, in medium containing 0, 300 pM, 30 pM, 3 pM, 300 fM, and 30 fM OHL. The method used for screening the random promoter library was adapted from the method of Bjarnason et al. (7). Transformants containing the random promoter library clones were inoculated into clear 384-well plates (Costar; Corning Incorporated, Corning, NY) containing 70 μl of LB medium using a colony-picking robot (Norgren Systems, Palo Alto, CA) and were incubated overnight at 37°C. These cultures were subcultured into black 384-well plates (3710 Costar; Corning Incorporated, Corning, NY) containing LB medium and LB medium supplemented with 300 pM or 3 pM OHL using a 384-pin manual plate replicator (V&P Scientific Inc., San Diego, CA) and incubated at 37°C. Luminescence was measured with a Wallac Victor2 1420 multilabel counter (Perkin-Elmer Life Sciences, Boston, MA) after 16 h of incubation. Clones for which there were differences in promoter activity between the medium with OHL and the medium without OHL were rearrayed (using Norgren Systems software) and used in subsequent experiments.

A second screen was performed to reduce the incidence of false-responsive promoters. Clones with at least twofold differences in luminescence between the medium with OHL and the medium without OHL were rearrayed into clear 96-well plates (531172; Falcon, Franklin Lakes, NJ) containing 150 μl LB medium. The overnight cultures of clones exhibiting promoter activity were diluted to an optical density at 600 nm (OD_{600}) of 0.05 in LB medium supplemented with 0, 3 pM, or 30 pM OHL in 96-well clear-bottom black plates (9520 Costar; Corning Incorporated, Corning, NY), and the luminescence and absorbance (OD_{600}) were determined at 16 h. The expression levels were normalized to the OD_{600} to determine the relative ratio of number of cps to absorbance. Clones exhibiting at least a twofold difference in expression between the medium with 3 pM or 30 pM OHL and the medium without OHL were rearrayed and subsequently analyzed by DNA sequencing.

DNA sequencing and sequence analysis. Plasmids containing OHL-responsive promoters were transformed into *E. coli* DH5α by heat shock at 42°C (38) to isolate plasmids that were pure enough for DNA sequencing. The size of the fragment inserted was determined by PCR using primers pZE05 and pZE06. Purified plasmids containing a <3-kb insert were sequenced at Macrogen Inc. (Seoul, Korea) using primers pZE05 and pZE06. The DNA sequences obtained were compared with the *B. cenocepacia* J2315 genome-sequencing project data (http://www.sanger.ac.uk/Projects/B_cenocepacia/) using BLASTN. The sequences obtained were compared to the unpublished annotation file for *B. cenocepacia* J2315 to identify identical sequence locations on the chromosomes. Artemis software (37) was used to view the annotation files and to identify open reading frames downstream of predicted promoters.

Potential *cep* box-like sequences were identified using the consensus sequence CTGTAAAAGTTACCAGTT based on the promoter sequences of six *B. cenocepacia* K56-2 genes positively regulated by CepR, including *cepI*, *zmpA*, *aidA*, and *phuR* (9a). Sequences 300 bp upstream and 50 bp downstream of the predicted start codon for the open reading frame identified in the promoter fusion were analyzed on both the sense and antisense strands, and sequences with at least 50% identity to the consensus were considered putative *cep* boxes.

Construction of *luxCDBAE* transcriptional fusions. A 439-bp fragment containing the predicted promoter region of *bcscV* (BCAM 2057) was amplified using primers 5′-GGCCCTCGAGTCGGCGACGAGCAGCAGCAG-3′ and 5′-GGCCGGATCCGCCATCGTGCCGTCGTCCTG-3′ (the underlined sequences are XhoI and BamHI restriction sites, respectively). A 1.3-kb PCR fragment containing the *katB* upstream region was amplified with primers 5′-GGGCCTCGAGGCAGCGGTCGATGCCGATCT-3′ and 5′-CCGGGGATCCGGCCGTCTGCGAATTCTGGTTG-3′. These products were cloned into the XhoI-BamHI sites of pMS402.

A 329-bp fragment containing the *orbI* (BCAM1696) promoter region was amplified using primers 5′-GGAAGAAGGCTCGTGGC-3′ and 5′-GATGCAGCGCAGTCGGG-3′. A 471-bp fragment containing the region upstream of *pvdA* was amplified using primers 5′-CGCATTCGCGGACGGC-3′and 5′CAGCGTGAAGCGCACGC-3′. These PCR products were cloned into pCR2.1 TOPO (Invitrogen). An EcoRI fragment was excised from each of these constructs and cloned into pMS402. All pMS402 constructs were verified by sequencing with primers pZEO5 and pZEO6.

Real-time quantitative RT-PCR (qRT-PCR). *B. cenocepacia* K56-2, K56-dI2, and K56-R2 were cultured, harvested after 8, 12, and 16 h, and used for extraction of total RNA. Total RNA was isolated from these strains using a Ribopure bacterial kit (Ambion, Austin TX), followed by DNase I treatment with 8 U of TURBO DNase and inactivation by DNase inactivation reagent (Ambion, Austin, TX). Oligonucleotide primers were designed with the Primer Express software (version 2; Applied Biosystems, Foster City, CA) and synthesized by University of Calgary Core DNA and Protein Services, Calgary, Alberta, Canada. They are listed in Table 2.

The NADH dehydrogenase gene *ndh* (BCAM0166) was determined not to be regulated by *cepIR* and was used as a reference standard. The *ndh* RNA was generated and purified from pBS7 as previously described (14). The purified *ndh* RNA (20 fg to 200 pg) was used to construct a standard curve for quantification using primers ndhF and ndhR to amplify the internal sequence.

Quantification was performed with a TaqMan 7500 instrument and SYBR green RT-PCR reagents (Applied Biosystems). The primer amplification efficiency for each primer pair was within 10% of the efficiency determined for *ndh*, which indicated the validity of using relative gene expression analysis. The cDNA from genes homologous to BCAS0409 (*zmpA*), BCAL3506 (*fliM*), pBCA054 (regulator), BCAL3010 (*spoT*), BCAM2041 (*bcscR*), BCAL1699 (*pvdA*), BCAL2732 (cold shock), BCAL3524 (*gspC*), and BCAM0931 (*katB*) were generated by using gene-specific primers listed in Table 2. Reverse transcription reactions were performed in 10-μl mixtures (1× TaqMan RT buffer, 2.5 μM forward primer, 2.5 μM reverse primer, 5.5 mM $MgCl_2$, mixture containing each deoxynucleoside triphosphate at a concentration of 500 μM, 0.4 U/μl RNase inhibitor, 1.25 U/μl MultiScribe reverse transcriptase, 20 ng total RNA template), and 5 μl of cDNA diluted 1:20 was subjected to real-time PCR in 25-μl reaction mixtures (1× SYBR green PCR master mix, 50 nM forward primer, 50 nM reverse primer). The parameters for RT were 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min, followed by 45 PCR cycles consisting of 95°C for 15 s and 60°C for 1 min. The concentrations of target mRNA in samples were normalized to concentrations of *ndh* mRNA. All quantitative real-time RT-PCR assays were repeated three times, and similar results were obtained.

RESULTS

Development of an assay to identify CepR-regulated promoter fusions. We developed an assay to identify both positively and negatively regulated promoters under control of the *cepIR* QS system by looking for differences in expression of genes between cultures in medium supplemented with OHL and cultures in medium not supplemented with OHL. Strain K56-dI2 has a deletion in *cepI*; therefore, the expression of positively regulated genes and the repression of negatively regulated genes by *cepIR* require addition of exogenous OHL. The appropriate concentrations of OHL to use in the assay were determined by comparing the levels of expression of *cepI* (pCP300), *cepR* (pRM432), and *zmpA* (pCC1) promoter-*luxCDABE* transcriptional fusions in K56-2 and K56-dI2. The culture medium for K56-dI2 was supplemented with 0, 300 pM, 30 pM, 3 pM, 300 fM, or 30 fM OHL, and the expression profiles of the promoter-*lux* fusions were compared for each condition. Luminescence and growth were measured hourly between 9 and 20 h (Fig. 1). The expression levels of the *cepI*::*lux* and *zmpA*::*lux* fusions in K56-dI2 were higher in medium supplemented with OHL than in medium without OHL. For example, at 16 h the levels of expression of *cepI*::*lux* and *zmpA*::*lux* were 8.6-fold and 18.6-fold higher, respectively, in medium with 30 pM OHL than in medium with no OHL added (Fig. 1A and B). The expression levels of *cepR*::*lux* in K56-2 and K56-dI2 in medium with 30 fM to 300 pM OHL were lower than the expression levels in K56-dI2 in medium without OHL at times between 9 and 16 h (Fig. 1C). At 16 h, the expression of the *cepR*::*lux* fusion in K56-dI2 was 2.4-fold lower in medium with 30 fM OHL than in medium with no OHL. These results are similar to previously reported observations that *cepI* and *zmpA* are positively regulated by *cepIR* and *cepR* is negatively regulated by *cepIR* (24, 40). Little difference in expression of the *cepI* or *zmpA* fusions was observed in media supplemented with between 3 and 300 pM OHL, and therefore OHL concentrations in this range were considered to be sufficient for identification of promoters positively regulated by CepR. OHL at these concentrations had no effect on the growth of these strains (data not shown).

The levels of expression of the *cepR*::*lux* fusion decreased to wild-type levels in K56-dI2 grown in medium with 300 fM to 3 pM OHL during the late log and early stationary phases of growth; however, expression of this fusion increased over time, possibly because the culture either used up or broke down the OHL added to the medium (Fig. 1C). These data suggest that it would be possible to identify genes negatively regulated by *cepR* at 16 h but that it might not be possible to identify negatively regulated genes at later times. Although the optimum time for identification of negatively regulated genes in cultures was after 10 to 12 h of incubation, there is considerable variation in the growth rates of K56-dI2 in 384-well plates, making analysis of expression of the promoter fusions difficult at these times. Therefore, K56-dI2 containing the promoter clone library was screened at 16 h in medium with 0, 3, or 300 pM OHL. Two OHL concentrations were used since although there was little difference in the responses of the three test promoters to medium with 3 and medium with 300 pM OHL, it is possible that CepR might have different affinities for promoters depending on OHL availability.

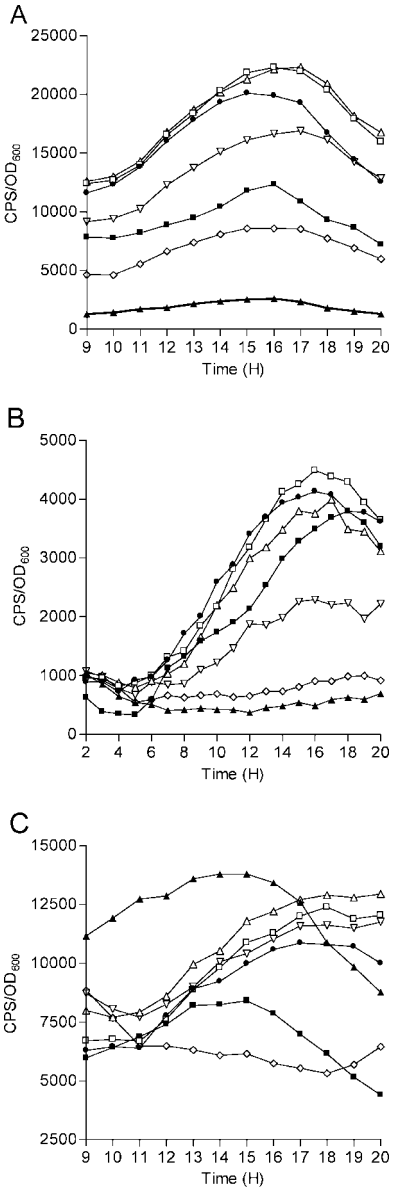

FIG. 1. Effects of various concentrations of OHL on expression of *cepI*::*luxCDABE*, *zmpA*::*luxCDABE*, and *cepR*::*luxCDBAE* fusions. Expression of promoter-*luxCDABE* fusions was measured throughout growth in LB medium containing 100 μg/ml trimethoprim supplemented with different concentrations of OHL. The values are the means for triplicate cultures. (A) *cepI*::*luxCDABE*. (B) *zmpA*::*luxCDABE*. (C) *cepR*::*luxCDABE*. ■, K56-2; ▲, K56-dI2; ●, K56-dI2 with 300 pM OHL; □, K56-dI2 with 30 pM OHL; △, K56-dI2 with 3 pM OHL; ▽, K56-dI2 with 300 fM OHL; ◇, K56-dI2 with 30 fM OHL.

Construction and screening of a random promoter library. To identify genes controlled by the *cepIR* QS system, a random promoter library of *B. cenocepacia* K56-2 was generated using DNA fragments up to 3 kb long cloned upstream of *luxCDABE* in pMS402 in K56-dI2, a *cepI* mutant. The insertion efficiency of the random promoter library was estimated by averaging the efficiencies of the individual ligation reactions. Therefore, in a total of 36,096 clones,

21,952 clones (60.82%) were predicted to contain inserted fragments.

Of the 36,096 potential clones initially screened, 12,676 clones had luminescent activity either in medium with OHL or in medium without OHL. These data suggest that 57% of the potential clones contained promoters. The genome size of *B. cenocepacia* J2315 is 8,056 kb (http://www.sanger.ac.uk/Projects/B_cenocepacia/). If 2 kb of DNA were predicted to contain one promoter, this random promoter library should exhibit approximately threefold coverage of the predicted promoters in the genome. Of the 12,676 clones with luminescence activity in either medium, 2,282 clones produced luminescence with at least a twofold difference in expression between the medium with OHL and the medium without OHL after subtraction of the background luminescence activity obtained for K56-dI2 with the control vector pMS402. In order to rule out false-positive results potentially due to variations in growth or cross talk between wells in a 384-well plate, the 2,282 clones were rearrayed into black 96-well plates and rescreened in triplicate in medium with 0, 3 pM, or 30 pM OHL. Clones that showed at least a twofold difference between expression in media with the two OHL concentrations and expression in medium without OHL were selected for sequence analysis.

Identification and classification of genes. A total of 440 plasmids containing promoter gene fragments were extracted, and the sizes of the insert fragments were determined by PCR using primers pZE05 and pZE06. Of the 440 plasmid clones, 200 clones had insert fragments smaller than 1.5 kb (45%), 140 clones had insert fragments between 1.5 and 3 kb long (32%), and 100 clones had insert fragments larger than 3 kb (23%). The nucleotide sequences of clones containing insert fragments that were <3 kb long (340 clones) were obtained using primers pZE05 and pZE06. The sequences were compared to the sequenced genome of *B. cenocepacia* J2315 (http://www.sanger.ac.uk/Project/B_cenocepacia/) using the BLASTN program to determine the chromosomal location and orientation of each clone. The location of each sequence was used to identify open reading frames downstream from predicted promoters using the unpublished annotation file of the *B. cenocepacia* J2315 genome. Many clones had identical or overlapping DNA sequences, suggesting that the screen was reliable since the same promoters were often identified more than once. A total of 134 unique promoter clones were differentially expressed in the presence or absence of OHL and therefore predicted to be regulated by *cepIR*; 91 were positively regulated, and 43 were negatively regulated.

Upon further analysis of the sequences of several promoter clones, we discovered that these clones contained sequences that were not adjacent on the J2315 genome and therefore were hybrid plasmids containing multiple inserts. To eliminate clones containing multiple fragments and possibly multiple promoters, the complete sequence of each clone was determined, which revealed that 45 (34%) of the 134 promoter clones contained inserts consisting of nonadjacent sequences in the genome or were hybrid clones.

Of the remaining 89 promoter clones, expression of 58 was positively influenced by OHL in the medium, whereas expression of 31 clones was negatively influenced by OHL (Table 3). Five clones showed >5-fold differential *luxCDABE* expression in medium with OHL and medium without OHL, 57 clones showed between 2- and 5 fold differential expression, and 27 showed 2-fold differential expression in response to OHL (Table 4). These 89 clones were introduced into K56-2, and the levels of expression were compared to levels of expression in K56-dI2 (Table 3). For 47 clones the difference in expression of luminescence between K56-dI2 and K56-2 was 2- to 5-fold, for 12 clones the difference in expression was >5-fold, and for 10 clones the difference in expression was 2-fold; however, for 20 clones the difference in expression was <2-fold. The differences in the expression profiles of promoter clones between K56-dI2 in medium with 30 pM OHL and K56-dI2 in medium without OHL were generally similar to the differences between the promoter activities of the clones expressed in K56-dI2 and the promoter activities of the clones expressed in K56-2. For 14 clones with an approximately twofold difference between expression in K56-dI2 in medium with OHL and expression in K56-dI2 in medium without OHL the differences between expression in K56-2 and expression in K56-dI2 were between 1.4- and 1.9-fold.

Genes were identified based on the unpublished annotation file for *B. cenocepacia* J2315 (Table 3). Genes expressed under the control of *cepIR* are involved in adaptation or resistance, type II and type III secretion systems, metabolism, membrane or surface structures, regulatory genes, and transport, and there are also genes with unknown functions. OHL-responsive promoters were identified on all three chromosomes and the plasmid.

Clone PBS-E62, which contained part of the *zmpA* gene and its promoter region promoter, had 4.6-fold-higher activity in K56-dI2 in medium with 30 pM OHL than in K56-dI2 in medium without OHL and 2.6-fold-higher activity in K56-2 than in K56-dI2. These results are similar to those previously reported for *zmpA*::*lacZ* fusions (40). Two clones containing the type II secretion system genes *gspG* (PBS-B40) and *gspC* (PBS-C53) were identified and showed twofold negative regulation. We obtained 20 copies of clone PBS-E51, which contains a promoter for the plasmid-encoded gene pBCA054, which exhibits similarity to genes encoding response regulator proteins of the LuxR family. This promoter was positively regulated; there was an 8.6-fold increase in expression in K56-dI2 in medium with OHL, and the level of expression was approximately 15-fold higher in K56-2 than in K56-dI2 (Table 3).

Clone PBS-F24 contained part of *cepI* and the *cepI* promoter region. This clone had 16-fold-higher *lux* activity in K56-dI2 grown in medium supplemented with 30 pM OHL than in medium without OHL and 3.6-fold-higher activity in K56-2 than in K56-dI2. These data are similar to data obtained in previous studies that demonstrated that *cepI* expression is positively regulated by *cepR* and is significantly decreased in a *cepI* mutant without OHL supplementation (24). This clone was determined to contain multiple fragments of the *cepI* promoter region, as well as a nonadjacent fragment, and therefore was not included in Table 3.

The sequence upstream of the *pvdA* gene was found in two identical clones, which unfortunately contained nonadjacent DNA fragments, and is also not listed in Table 3. The *pvdA* gene (BCAL1699) encodes L-ornithine 5-monooxygenase and has previously been shown to be negatively regulated by *cepIR* (24). Interestingly, the promoter clones that contained *pvdA*

TABLE 3. *B. cenocepacia* K56-2 genes with OHL-responsive promoters

J2315 open reading frame[a]	Clone (no. of times found)[b]	Gene homolog and/or predicted function[c]	Fold induction with OHL[d]	K56-2 vs K56-dI2[e]	Insert size (kb)[f]	Possible *cep* box[g]
BCAL0010	PBS-B14 (1)	*phhA*, phenylalanine-4-hydroxylase	−3.4	−3.3	0.46	
BCAL0111	PBS-B30 (1)	Putative TPR domain protein	2.5	3.7	0.68	
BCAL0153	PBS-C39 (1)	Putative flavin mononucleotide flavoprotein, unknown	−2.1	−2.1	0.3	
BCAL0170	PBS-C17 (1)	Hypothetical protein	−2.3	−4.1	0.82	
BCAL0305	PBS-B24 (1)	Putative exported protein	2.5	2.5	0.75	GCGCGTAAGCTACCTGCT (+, −55)
BCAL0353	PBS-F53 (1)	Putative membrane protein	−2	−2	1.72	CGGTGAATTAAACCAGGA (+, −35)
BCAL0380	PBS-F106 (1)	Putative ABC transporter ATP-binding subunit	6.3	2.7	2.01	GTGTGAAAGTCAGAAGTG (+, −6)
BCAL0554	PBS-B33 (1)	5-Formyltetrahydrofolate cyclo-ligase family protein	2.2	7.9	0.51	
BCAL0685	PBS-B37 (1)	IclR family regulatory protein	2	2.5	0.89	
BCAL0723	PBS-C55 (1)	*dctB*, C_4-dicarboxylate transport sensor protein	−2	−1.8	0.83	
BCAL0793	PBS-C46 (1)	Putative cell division protein	2	2.5	0.09	
BCAL0812	PBS-E21 (2)	Sigma 54 modulation protein	2	2	0.21	
BCAL0854	PBS-F38 (1)	GntR family regulatory protein	3.5	3.5	1.97	
BCAL1040	PBS-C01 (1)	Glycosyl transferase group 1 protein	2.2	5.2	0.94	
BCAL1182	PBS-E27 (2)	Putative transcriptional regulator	5.2	2.2	0.75	GTGAAAAAGGTGTAAGCA (−, −17)
BCAL1374	PBS-C44 (1)	Glyoxalase/bleomycin resistance protein	2.3	1.6	1.1	
BCAL1446	PBS-B48 (2)	Putative lipoprotein	2	1.8	0.23	
BCAL1513	PBS-C20 (1)	Putative transcriptional regulatory protein	3.4	3.5	2.47	
BCAL1550	PBS-E46 (1)	Putative sugar ABC transport system	2.8	1.4	0.1	
BCAL1625	PBS-C48 (1)	*acoR*, acetoin catabolism regulatory protein	−2.2	−4.4	0.32	
BCAL1651	PBS-C47 (1)	*lexA*, LexA repressor	2.7	1.8	0.82	CTGTATAAAAATACAGCT (−, −42)
BCAL1814	PBS-D11 (1)	Putative MerR family transcriptional regulator	−2.1	−2.2	1	ACGTTCGAGACTCCAGTG (+, −39)
BCAL1889	PBS-F50 (2)	Putative 23S rRNA (uracil-5-)-methyltransferase/orthologue, *ygcA*	2.5	2.5	1.27	CTTCAACGTTTGTCAGGA (−, −25)
BCAL1914	PBS-E02 (2)	Unknown	2.6	2.6	0.7	
BCAL1990	PBS-F40 (1)	*pgi*, glucose-6-phosphate isomerase	4.4	2.2	2.93	
BCAL2037	PBS-C30 (4)	Putative membrane protein	8.4	2.4	1.17	
BCAL2068	PBS-E56 (1)	Putative membrane protein	9.6	9.7	0.9	
BCAL2170	PBS-C51 (1)	Putative membrane protein	−2.1	−1.6	0.48	
BCAL2244	PBS-C10 (1)	*hutU*, urocanate hydratase	2	2.2	0.25	
BCAL2301	PBS-C50 (1)	Putative exported protein	2.1	3.3	0.26	
BCAL2302	PBS-C09 (1)	*uvrB*, excinuclease ABC subunit B	−2.3	−2	0.66	
BCAL2321	PBS-E09 (1)	Putative glutathione *S*-transferase-related protein	2	2.6	0.8	
BCAL2373	PBS-C03 (1)	Putative globin	2.1	2.5	0.74	
BCAL2464	PBS-B13 (1)	Short-chain dehydrogenase	2	4.4	0.78	CACAGAATGTGGCAAGTT (+, −34)
BCAL2630	PBS-C21 (1)	*hemC*, putative porphobilinogen deaminase protein	−2.5	−2.3	0.82	
BCAL2644	PBS-C04 (1)	Putative ATP-binding protein	−2	−1.9	0.62	
BCAL2652	PBS-B27 (1)	*panD*, putative aspartate 1-decarboxylase	2.2	1.8	0.15	CTGGAAATCTGACGGGCG (+, −55)
BCAL2732	PBS-B02 (1)	Cold shock protein, DNA binding	−2.5	−2.5	0.9	
BCAL2799	PBS-C24 (1)	*dalK*, putative carbohydrate kinase	2	2.1	0.51	
BCAL2818	PBS-E40 (1)	Putative sugar kinase protein	2.5	7.4	0.56	
BCAL2931	PBS-E12 (1)	*acpD*, acyl carrier protein phosphodiesterase	−2.3	−3.2	1.17	CTGTATAAATGTACAGTA (−, −43)
BCAM3006	PBS-F44 (1)	*cspA*, cold shock-like protein	2.9	1.9	1.51	CTGTCAATATTACCGGGA (+, −239)
BCAL3010	PBS-B29 (1)	*spoT*, guanosine-3′,5′-bis(diphosphate) 3′-pyrophosphohydrolase	2.2	6.3	1.21	CTGAAGAAGGTGCCGGTG (−, −83)
BCAL3051	PBS-E67 (1)	*ribE*, riboflavin synthase alpha chain	−2.2	−1.4	0.58	
BCAL3103	PBS-E33 (1)	*ureD*, UreD family accessory protein	2	1.6	0.26	GCTTCCTCGTTTCCCGTT (+, −25)
BCAL3130	PBS-F48 (1)	*wtz*, ABC transporter, ATP-binding component	2	3	1.7	
BCAL3443	PBS-C35 (2)	*ispB*, octaprenyl-diphosphate synthase	2.3	5.6	0.63	CTGTCAGACGTACCGGCA (+, −73)
BCAL3506	PBS-B34 (3)	*fliM*, flagellar motor switch protein FliM	2.9	2.3	0.97	
BCAL3523	PBS-B40 (1)	*gspG*, general secretory pathway protein G	−2	−2	0.2	ATGTGAAATTTGCGTTGA (−, −155)
BCAL3524	PBS-C53 (1)	*gspC*, putative general secretory pathway protein	−2	−1.9	0.22	
BCAM0003	PBS-E08 (1)	Putative partition protein ParA	2.8	1.4	0.54	CTGTAGAATTCCAACGAC (+, −34)
BCAM0010	PBS-E44 (1)	*klb*, 2-amino-3-ketobutyrate coenzyme A ligase	2.5	6.9	1.15	CTGTTATAGTCGTCGTTC (−, −67)

Continued on following page

TABLE 3—*Continued*

J2315 open reading frame[a]	Clone (no. of times found)[b]	Gene homolog and/or predicted function[c]	Fold induction with OHL[d]	K56-2 vs K56-dI2[e]	Insert size (kb)[f]	Possible *cep* box[g]
BCAM0038	PBS-D03 (1)	*feaR*, AraC family regulatory protein	3.3	6.1	2	
BCAM0057	PBS-C33 (1)	*p*-Hydroxybenzoate hydroxylase	−2	−2.4	0.28	GTTTTGAAGATACCGGCC (−, −94)
BCAM0130	PBS-C37 (1)	Putative acyl-coenzyme A dehydrogenase	2	2	0.58	TGGTATGTATTACTAGAT (+, −80)
BCAM0300	PBS-C41 (1)	Putative metallo-beta-lactamase family protein	2.2	3.5	1	
BCAM0341	PBS-F130 (1)	Unknown	−2	−9.3	3	
BCAM0677	PBS-E10 (1)	Putative transcriptional regulator, *araC* family	−2	−1.7	0.6	CGGTAGAATTCGCCATTC (−, −41)
BCAM0722	PBS-B45 (1)	Putative *O*-methyltransferase-like protein	−2	−4.2	0.25	
BCAM0832	PBS-F37 (1)	Putative dyd-type peroxidase conserved hypothetical protein	−2.3	−2.1	2.52	
BCAM0860	PBS-D06 (1)	Putative glycosyltransferase	−2.9	−11.5	1.2	CTGAGCGCGTGACCGGGT (+, −73)
BCAM0881	PBS-F108 (1)	Putative trehalose trehalohydrolase alpha-amylase	−3.9	−3.6	1.61	GCGTGACGGTCGCCCGTT (+, −190)
BCAM0901	PBS-E28 (1)	*amn*, AMP nucleosidase	2.7	1.7	0.23	
BCAM0931	PBS-F135 (1)	*katB*, catalase precursor	−2.2	−2	2.18	
BCAM1110	PBS-B01 (1)	Unknown putative sugar transporter	−3.3	−1.6	0.7	
BCAM1142	PBS-C32 (1)	Unknown	2.1	2.2	0.9	
BCAM1165	PBS-D01 (1)	Putative membrane protein	2.7	3.9	2	GTGTTAGATTTCGCCGTC (+, −48)
BCAM1189	PBS-F70 (1)	Putative transcriptional regulator, LysR family	2.3	2.1	1.75	
BCAM1227	PBS-D09 (1)	*pgaB*, polysaccharide deacetylase	−2	−2.3	2.7	
BCAM1453	PBS-E68 (1)	LysR family regulatory protein	2.1	3.2	0.35	
BCAM1501	PBS-B06 (1)	Unknown	−2.5	−2.3	0.19	TGGTTCATGTGATCAGTA (+, −72),
BCAM1515	PBS-F110 (1)	Conserved hypothetical protein	2.1	2.7	2.19	CTGTACAAATGTACAGTG (+, −27)
BCAM1573	PBS-B22 (1)	*otsA*, glycosyltransferase family	2	1.9	0.99	
BCAM1650	PBS-E32 (1)	LysR transcriptional regulator	3	3	0.45	
BCAM2155	PBS-E05 (1)	Putative cytochrome P450 oxidoreductase	2	2	0.63	CTGGCAGATCAGCCAGTC (+, −238)
BCAM2158	PBS-B39 (1)	Putative DNA-binding protein	2.6	2.7	1.46	
BCAM2162	PBS-E24 (2)	Putative MarR transcriptional regulator family	2	2	0.75	GTGGACAACCTTCCATTA (+, −276)
BCAM2176	PBS-E37 (1)	*glsA*, thermolabile glutaminase	−2	−2	0.45	
BCAM2507	PBS-E43 (1)	Putative sugar transport-related membrane protein	−2.1	−1.5	1.19	
BCAM2583	PBS-F119 (1)	Putative amidohydrolase	−2.2	−2.3	2.42	
BCAM2615	PBS-A13 (1)	Unknown	2.4	2	0.5	
BCAM2805	PBS-F112 (1)	Periplasmic binding protein	2.3	2.6	2	
BCAM2835	PBS-E20 (2)	*estC*, putative esterase	2.2	2.6	0.45	
BCAS0161	PBS-C15 (1)	Putative transcriptional regulator, LysR family	−2.3	−1.8	0.54	ACATAATATTTACAACTT (−, −36)
BCAS0162	PBS-C16 (1)	Putative periplasmic solute-binding protein	3.2	6.5	0.82	
BCAS0221	PBS-C42 (1)	Putative ABC transporter (pseudogene)	2	1.7	0.39	
BCAS0409	PBS-E62 (2)	*zmpA*, extracellular zinc metalloprotease	2.3	2.6	1.3	GTTTAAAAGTCATCACTT (+, −60)
BCAS0730	PBS-E30 (2)	Putative Na^+-dependent nucleoside transporter	2	2.9	0.26	
pBCA054	PBS-E51 (20)	LuxR family regulatory protein	8.6	15	1.68	CAATACGAGTCGCCCGTC (+, −69)

[a] Open reading frame predicted with the unpublished annotation file of the *B. cenocepacia* J2315 genome (http://www.sanger.ac.uk/Projects/B_cenocepacia/).
[b] Promoter clone designation. The number in parentheses indicates the number of times that the identical clone was found in the library.
[c] Predicted gene or function based on the unpublished annotation of the *B. cenocepacia* J2315 genome (http://www.sanger.ac.uk/projects/B_cenocepacia/).
[d] Fold difference in expression between K56-dI2 in LB medium with 30 pM OHL and K56-dI2 in LB medium without OHL.
[e] Fold difference in expression between K56-2 and K56-dI2.
[f] Size of the fragment inserted in pMS402.
[g] Potential *cep* box. For the data in parentheses, a plus sign indicates that the box is on the coding strand, and a minus sign indicates that the box is on the antisense strand. The values indicate the direction and distance from the start codon (position 1).

appeared to be positively regulated by *cepIR*. To investigate this discrepancy, a 300-bp fragment upstream of *pvdA* was cloned into pMS402 and designated pVDA01. The *pvdA* gene has recently been shown to be transcribed as part of a large operon from a promoter upstream of BCAL1696 or *orbI* (1). The promoter region upstream of BCAL1696 was cloned into pMS402 and designated pVDO301. The levels of expression of these two promoter-*lux* fusions in K56-2, K56-dI2, and K56-R2, the *cepR* mutant, were compared (Fig. 2). The expression of the *lux* fusion in pVDA01 was twofold higher in K56-2 than in the *cepI* or *cepR* mutant, indicating that this fragment did contain a promoter that was positively regulated by CepR. The levels of expression of the promoter in pVDO301, however, were approximately fivefold higher in the *cepI* and *cepR* mutants than in K56-2, indicating that this promoter was negatively regulated by CepR (Fig. 2). Expression of *lux* in pVDO301 was also repressed by addition of iron to the medium (data not shown). These data suggest that expression of

TABLE 4. Relative differential expression of promoter clones in response to OHL

Regulation	No. of promoters for[a]:	
	K56-dI2 with OHL vs K56-dI2	K56-2 vs K56-dI2
Positive		
2- to 5-fold	37	32
>5-fold	5	10
2-fold	16	5
<2-fold	0	11
Total	58	58
Negative		
2- to 5-fold	20	15
>5-fold	0	2
2-fold	11	5
<2-fold	0	9
Total	31	31

[a] Numbers of promoters with fold differences indicated after subtraction of the background.

pvdA may be influenced by two promoters that are regulated differently by CepR but the major iron-regulated promoter of this ornibactin biosynthesis operon is negatively regulated.

Clone PBS-F105 contained the promoter region upstream of *bcscR* (BCAM2041), a putative type III secretion system gene. The expression of *luxCDABE* was positively regulated 2.4-fold (data not shown); however, this clone also contained multiple inserts. The positive regulation of *bcscR* was confirmed by qRT-PCR as described below. Two clusters of type III secretion genes have been identified in *B. cenocepacia* (34, 42). The *bcscQ* and *bcscR* genes are located upstream of *virB1* and are predicted to be in the same operon. A second cluster of 12 genes is separated from *bcscQR* by approximately 10 kb and is transcribed in the opposite orientation. The first gene in this cluster is *bcscV* (BCAM2057). This cluster also includes *bcscN*, which has been shown to be important in virulence in an acute mouse respiratory infection model (42). To determine if genes in the *bcscV* cluster are also regulated by *cepIR*, the *bcscV*

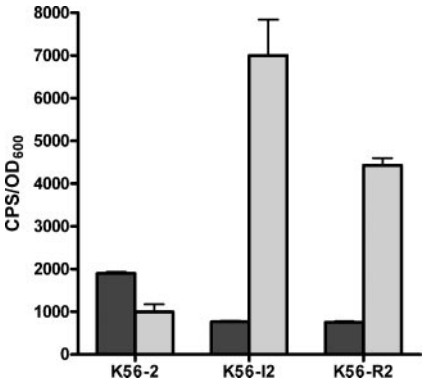

FIG. 2. Comparison of the levels of expression of *pvdA* (pVDA01) and *orbI*::*luxCDBAE* (pVDO301) fusions in K56-2, K56-dI2, and K56-R2. Cultures were grown in TSB-DC medium containing 100 μg/ml trimethoprim. Dark gray bars, pVDA01; light gray bars, pVDO301. The values are means ± standard deviations for data obtained at 24 h for triplicate cultures and are representative of three experiments. Similar results were obtained at other times in the stationary phase.

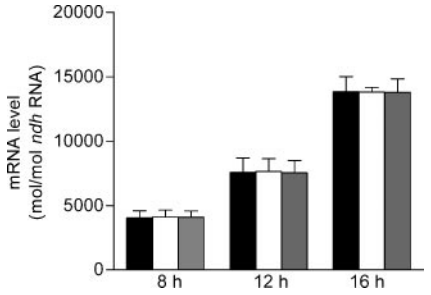

FIG. 3. Expression of the *ndh* gene in *B. cenocepacia* K56-2, K56-dI2, and K56-R2. Total RNA was extracted from 8, 12, and 16 cultures and subjected to real-time RT-PCR. The absolute expression levels were determined using an *ndh* standard curve. The values are means ± standard deviations for triplicate assays. Solid bars, K56-2; open bars, K56-dI2; gray bars, K56-R2.

predicted promoter region was cloned into pMS402 to create a *bcscV*::*luxCDABE* reporter (pMVbcscV) and was introduced into K56, K56-dI2, and K56-R2. The luminescence activity was monitored over time. The level of expression of the *bcscV*::*lux* fusion was significantly higher in K56-2 than in either K56-dI2 or K56-R2 ($P < 0.05$, as determined by analysis of variance). For example, after growth for 16 h the cps/OD_{600} ratios were 9,345 ± 3,403 for K56-2, 2,465 ± 34 for K56-dI2, and 514 ± 92 for K56-R2 (means ± standard deviations). Therefore, *bcscV* is also positively regulated by the *cepIR* QS system.

Confirmation of the promoter gene expression analysis by quantitative RT-PCR. To confirm expression results obtained with the random promoter library and to determine the requirement for CepR for expression of genes responsive to OHL in the *cepI* mutant, nine genes downstream of the predicted promoters for BCAS0409 (*zmpA*), BCAL3506 (*fliM*), pBCA054 (regulator), BCAL3010 (*spoT*), BCAM2041 (*bcscR*), BCAL1699 (*pvdA*), BCAL2732 (cold shock), BCAL3524 (*gspC*), and BCAM0931(*katB*) that were differentially expressed in the random promoter library were selected for quantitative analysis of mRNA by real-time qRT-PCR. Since the two promoters cloned upstream of *pvdA* were regulated differently on the multicopy plasmid pMS402, *pvdA* (BCAL1699) was included in the analysis to determine the regulation of a single copy of this gene in the genome. BCAM2041 (*bcscR*) was included in the analysis even though the promoter clone contained multiple inserts to confirm that *bcscR* was regulated by *cepIR*, since the promoter for the second type III gene cluster upstream of *bcscV* was shown using the promoter *lux* fusions to be positively regulated by *cepIR*. Total RNA of K56-2, K56-dI2, and K56-R2 were extracted and used as templates in qRT-PCR. Quantitative expression levels of each gene were determined using sequence-specific primers for each target gene (Table 2). The *ndh* gene, encoding NADH dehydrogenase, was used as a reference gene for relative quantification in real-time RT-PCR. We first determined if expression of *ndh* was influenced by the *cepIR* QS system. Although the level of expression of *ndh* increased over time, the levels of expression were the same in all three strains, indicating that *cepIR* does not regulate *ndh* expression (Fig. 3). Therefore, in subsequent assays the *ndh* gene was used as a reference to

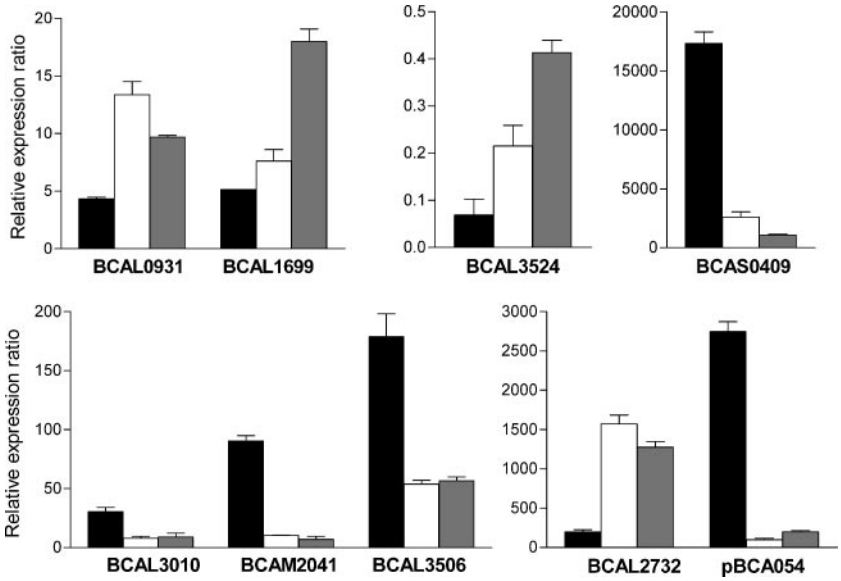

FIG. 4. Quantitative RT-PCR of genes expressed from the random promoter library. The mean relative expression levels of the target gene were normalized to the levels of *ndh* and multiplied by 1,000. Solid bars, K56-2; open bars, K56-dI2; gray bars, K56-R2. The values are means ± standard deviations for six replicates.

compare the expression of the other genes predicted to be regulated by CepR.

The results of the real-time RT-PCR demonstrated that the levels of expression of BCAS0409 (*zmpA*), BCAL3506 (*fliM*), pBCA054 (regulator), BCAL3010 (*spoT*), and BCAM2041 (*bcscR*) were 6.7-, 3.3-, 28-, 3.8-, 8.9-fold higher, respectively, and the levels of expression of BCAL1699 (*pvdA*), BCAL2732 (cold shock protein), BCAL3524 (*gspC*), and BCAM0931 (*katB*) were 1.5-, 8-, 3-, and 3-fold lower, respectively, in K56-2 than in K56-dI2 (Fig. 4). These results confirmed the expression data obtained in the random promoter luminescence assays, with the exception of the data for BCAL1699 (*pvdA*). The qRT-PCR data for *pvdA* are consistent with the *lux* expression of the promoter upstream of *orbI*, a previous report that expression of a chromosomally encoded *pvdA*::*lacZ* fusion was negatively regulated by *cepIR* (24), and the results of a transcriptional analysis indicating that *pvdA* is cotranscribed with *orbI* (1). Although the original clone (PBS-F105) which harbored *bcscR* contained additional fragments, the qRT-PCR data confirmed that the expression of this gene was positively influenced by *cepI*.

We also used qRT-PCR to quantitate the expression of these genes in the *cepR* mutant. The relative mRNA levels of the nine genes in K56-R2 were similar to the relative mRNA levels in K56-dI2.The levels of expression of BCAS0409 (*zmpA*), BCAL3506 (*fliM*), pBCA054 (regulator), BCAL3010 (*spoT*), and BCAM2041 (*bcscR*) were 16.2-, 3.2-, 13.8-, 3.4-, 13-fold higher, respectively, and the levels of expression of BCAL1699 (*pvdA*), BCAL2732 (cold shock protein), BCAL3524 (*gspC*), and BCAM0931 (*katB*) were 3.5-, 6.5-, 6-, and 2.2-fold lower, respectively, in K56-2 than in K56-R2 (Fig. 4). These results indicate that CepR and the AHLs synthesized by CepI are required for regulation of these genes.

Influence of *cciIR* on expression of selected genes. Since K56-2 also contains the CciIR quorum-sensing system, we decided to examine the expression of a few genes identified in the promoter library to see if they are also regulated by CciIR. Plasmids pBSC01 (BCAL1040::*luxCDABE*), pVDO301, pBS34 (*fliM*::*luxCDABE*), and pBS18 (*katB*::*luxCDABE*) were introduced into K56-2*cciIR*, which has a deletion of the *cciIR* promoter and therefore does not express either *cciI* or *cciR* (31). The expression of BCAL1040 and *orbI* was influenced by the *cciIR* mutation throughout growth in a manner similar to the expression in the *cepI* mutant. BCAL1040 expression was positively regulated by *cciIR* (Fig. 5A), and *orbI* expression was negatively regulated by *cciIR* (Fig. 5B). There was no difference in expression of either *fliM* or *katB* between K56-2 and K56-2*cciIR*. For example, the cps/OD_{600} ratios in these strains containing pBS-B34 (*fliM*) were $1.1 \times 10^4 \pm 206$ and $1.4 \times 10^4 \pm$ 1,009, respectively. The cps/OD_{600} ratios for pBS18 (*katB*) were 670 ± 61and 620 ± 2 4, respectively. Therefore, some genes are regulated by both quorum-sensing systems, whereas others are likely regulated independently.

Identification of potential *cep* boxes upstream of CepR-regulated genes. To determine which promoters might be directly regulated by *cepIR*, a *cep* box consensus sequence was used to search the region upstream of the open reading frames identified in the promoter clones. Thirty genes had a potential *cep* box with at least 50% identity (Table 3). If more than one potential *cep* box was identified, the box with the highest levels of homology or closest to the start codon was selected. A *cep* box was also identified upstream of BCAL1696 (*orbI*) (CCGTTCGTCACACCAGTG) at position −42 bp relative to the start codon on the coding strand. These data suggest that most of the genes identified are likely to be indirectly regulated by CepR. Six potential regulatory genes that had putative *cep* boxes were identified. It is possible that these genes are intermediate regulators in the CepR regulatory cascade.

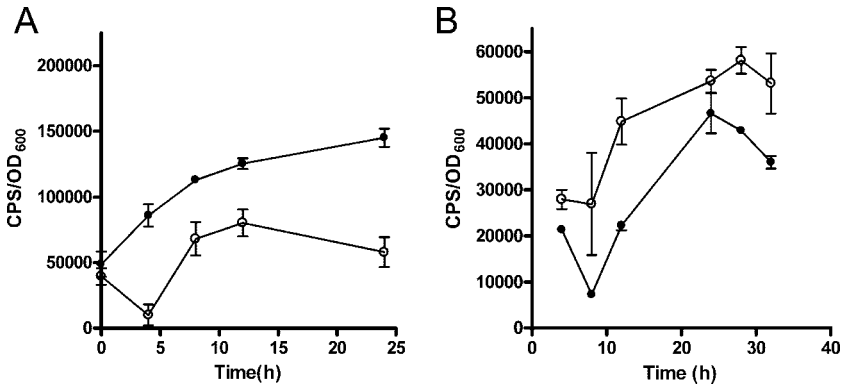

FIG. 5. Comparison of the levels of expression of pBSCO1 (BCAL1040::*luxCDABE*) and pVDO301 (BCAL1696::*luxCDABE*) in K56-2 and K56*cciIR*. (A) pBSC01. Cultures were grown in LB medium containing 100 μg/ml trimethoprim. (B) pVDO301. Cultures were grown in TSB-DC medium containing 100 μg/ml trimethoprim. The values are means ± standard deviations for triplicate cultures.

DISCUSSION

A random promoter library with a *lux*-based reporter was used to identify genes with promoters responsive to OHL. This approach provided a sensitive quantitative method to identify differentially expressed genes in high-throughput conditions. Eighty-nine promoter clones were differentially expressed at least twofold in the *cepI* mutant in the presence or absence of exogenous OHL. The majority of these clones were also differentially expressed at least twofold in the wild type and the *cepI* mutant. Both positively and negatively regulated promoters were identified, and the majority of these promoters have not previously been shown to be regulated by the *cepIR* QS system.

Several approaches have been used to identify *cepIR*-regulated genes in *B. cenocepacia* or *B. cepacia*. A promoter library approach employing a *lacZ* reporter plasmid that also contained *cepR* was used to identify *B. cepacia cepIR*-regulated genes expressed in *E. coli* (3). The vector used in our study enabled screening in the natural host rather than *E. coli*, which should make it possible to identify clones that require additional genes for expression that are present in *B. cenocepacia* but not present in *E. coli*. A promoterless *lacZ*-based transposon approach was used to identify OHL-induced genes in a *cepI* mutant of *B. cenocepacia* (47). In the studies using the *lacZ* reporter systems (3, 47), the initial screens were performed using blue-white selection, which is less sensitive for determining differences in expression than the *luxCDBAE* reporter, and this resulted in identification of genes whose expression was detectable only in the presence of OHL. The pMS402 *lux* reporter system has an advantage over the two *lacZ* reporter systems, since it is possible to detect genes that are expressed in the absence of *cepI* and OHL but exhibit increased expression in the presence of *cepI* and OHL. It was also possible to use this system to identify negatively regulated genes as well as positively regulated genes. Although we screened only for promoters expressed in stationary phase, the library could be used for detection of promoters expressed at other stages of growth. A proteomics approach was used to identify 11 differentially expressed proteins in *B. cenocepacia* H111 (36). Interestingly, each of the approaches used to identify genes influenced by *cepI* has led to identification of a different set of genes (2, 36, 47). This is not surprising since three different strains and two different species were used in these studies. One gene consistently identified in the other three studies but not in our study was the *aidA* gene (2, 36, 47), although we have determined that this gene is regulated by *cepIR* in *B. cenocepacia* K56-2 (E. Lutter and P. A. Sokol, unpublished observations). The *cepI* gene was identified in both our study and the study of Weingart et al. (47). The lack of an annotated genome sequence makes it difficult to compare the CepR-regulated genes identified in different studies; however, the transcriptional regulator pBCA54, shown to be positively regulated in our study, appears to be the gene designated *bqiD* that was identified by Weingart et al. (47).

The genome of *B. cenocepacia* J2315 contains 8.056 Mb in three chromosomes (3.870, 3.217, and 0.876 Mb) and a 92.7-kb plasmid containing approximately 7,200 coding sequences (http://www.sanger.ac.uk/Projects/B_cenocepacia/). The 89 promoter clones characterized were randomly distributed throughout the genome; 50 clones contained genes from the large chromosome, 33 clones contained genes from the medium chromosome, 5 clones contained genes from the small chromosome, and 1 clone contained genes from the plasmid. This indicates that there was a lack of bias in cloning of the promoters and suggests that good coverage of the genome was obtained. Although we estimated that our random promoter library should have threefold coverage of the genome, it clearly did not contain promoters for many of the previously identified genes regulated by *cepIR*, including *zmpB* (20), *cepR* (24), *cciIR* (31), and *aidA* (2, 36, 47). The conditions used to construct a library with a high insertion efficiency in pMS402 resulted in a very significant number of clones with multiple inserts, and 34% of our unique sequenced clones contained nonadjacent DNA sequences. The frequency of hybrid clones likely masked identification of some potential promoters. Careful sequencing of all promoter clones is clearly necessary when random promoter libraries are used due to the frequent occurrence of hybrid clones. Environmental factors such as medium composition and oxygen availability, as well as the growth phase, can also affect the transcription of many genes that are QS regulated. Thus, a number of *cepIR*-regulated genes may not have been detected under the conditions employed. All of the strategies

used to date have led to identification of a portion of the *cepIR* QS regulon, but additional assay conditions and methods must be employed before this global regulatory network is fully described.

The random promoter library has led to identification of a number of novel genes in *B. cenocepacia* that are influenced by OHL concentrations and a functional *cepI* gene; these genes include *katB*, *gspC*, *gspG*, *bcscR*, *spoT*, and *cspA*. Expression of the *katB* (PBS-F135) gene was negatively regulated by the *cepIR* QS system. *B. cenocepacia* strain C5452 contains two catalase/peroxidase genes, *katA* and *katB* (22). KatB is the major catalase/peroxidase that plays a role in cellular protection against oxidative stress. A *katB* mutant exhibited reduced growth and hypersensitivity to hydrogen peroxide (22).

Cold shock proteins are small proteins (~7 kDa) that are involved in mRNA folding, protein synthesis, and/or freeze protection. It is believed that they can function as RNA chaperones to reduce the secondary structure of RNA at low temperatures (49). Expression of the *cspA* gene (PBS-F44), which encodes a putative cold shock protein, was induced 2.9-fold in medium with OHL, indicating that it is positively regulated by the *cepIR* system. A cold shock protein transcriptional regulator, *cspC*, was identified using signature-tagged mutagenesis and was determined to be important for survival of *B. cenocepacia* in a chronic respiratory infection model (19).

Two clones containing different promoters for general secretion pathway genes were identified. Expression of both *gspG* (PBS-B40) and *gspC* (PBS-C53) was negatively influenced by the presence of OHL, and there was twofold negative induction in medium with OHL or when the parent and the *cepI* mutant were compared. It is interesting that expression of genes encoding the type II secretion machinery proteins are negatively regulated by *cepIR*; however, expression of at least two genes encoding proteins predicted to be secreted using this pathway, *zmpA* and *zmpB*, is positively influenced by *cepI* and *cepR*. Expression of *bcscR* and *bcscV*, the first genes in the two major type III secretion gene clusters, was shown to be positively influenced by OHL and *cepI*, indicating that genes in both type II and type III secretion systems are regulated by the *cepIR* QS system but there is an opposite effect on the expression of these secretion genes. Genes involved in type II and type III secretion have previously been shown to be regulated by the *lasRI* or *rhlRI* QS systems in *Pseudomonas aeruginosa* (8, 39, 45). Control of type II and III secretion system gene expression differs in *P. aeruginosa* and *B. cenocepacia*, however, since type II secretion genes are positively regulated and type III genes are negatively regulated in *P. aeruginosa*. Although quorum sensing may be a common mechanism of gene regulation in bacterial pathogens, clearly the targets of these regulatory genes are different in different pathogens.

To validate the results obtained for the promoter library, qRT-PCR was used to confirm expression of genes downstream of nine *cepIR*-regulated promoters. The expression levels of eight genes were consistent with the promoter library observations and similar for the *cepI* and *cepR* mutants. BCAS0409 (*zmpA*), BCAL3506 (*fliM*), pBCA054 (regulator), BCAL3010 (*spoT*), and BCAM2041 (*bcsCR*) were positively regulated, and BCAL2732 (cold shock), BCAL3524 (*gspC*), and BCAM0931 (*katB*) were negatively regulated. BCAL1699 (*pvdA*) was negatively regulated, which was consistent with the promoter upstream of the operon but not the putative promoter directly upstream of the *pvdA*. It is not clear why the sequence upstream of *pvdA* functions as a promoter in pMS402. There are no predicted promoter elements in the 300 bp upstream of *pvdA*, such as −35 or −10 sequences; however, at least when this sequence is present on a multicopy plasmid, it appears to have promoter activity.

Although we showed that the *cepIR* QS system influences expression of the genes identified using the random promoter library, we did not determine whether CepR directly regulates transcription of these genes. It is possible that CepR influences expression indirectly by regulating expression of an intermediate regulatory gene that subsequently alters the expression of other promoters. Several regulatory genes were identified in the promoter library and may be candidates for intermediate regulators in the quorum-sensing network. Inactivation of *cepI* might also influence expression of some promoters regulated by other regulators. We identified possible *cep* boxes upstream of 30 of 89 genes that we identified. These data should be interpreted with caution since direct binding of CepR has not been demonstrated and in most cases the putative *cep* box is not highly conserved. The *cep* boxes upstream of *zmpA*, *spoT*, *cspA*, and BCAL0380 were among the *cep* boxes with the greatest similarity. The 59 genes for which *cep* boxes were not identified are likely to be indirectly regulated by the *cepIR* system. It should also be noted that we measured only gene expression, not protein levels or any effects of posttranslational modification.

Recently, our laboratory has demonstrated that the *cciIR* QS system interacts with the *cepIR* QS system in *B. cenocepacia* K56-2 (31). Expression of some genes is influenced by both systems, whereas other genes appear to be regulated by only one QS system. For example, expression of *zmpA* (31, 40), BCAL1040, and BCAL1696 (*orbI*) is regulated in similar ways by *cepIR* and *cciIR*. Expression of *zmpB* is markedly reduced in *cepI*, *cepR*, and *cciR* mutants but is significantly enhanced in a *cciI* mutant (20). The *cepIR* genes, but not the *cciIR* genes, influence the expression of *katB* and *fliM*. It is not known whether CciR binds to the same binding site as CepR or whether it has a different specificity for target genes. Further studies are under way to investigate the influence of *cciIR* on gene expression and to further elucidate the QS gene regulatory network in *B. cenocepacia*.

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