JOURNAL OF BACTERIOLOGY, Apr. 2007, p. 3006–3016 Vol. 189, No. 8
0021-9193/07/$08.00+0 doi:10.1128/JB.01544-06

Expression of the *bviIR* and *cepIR* Quorum-Sensing Systems of *Burkholderia vietnamiensis*▽

Rebecca J. Malott and Pamela A. Sokol*

Department of Microbiology and Infectious Diseases, University of Calgary Health Sciences Center, Calgary, Alberta, Canada T2N 4N1

Received 3 October 2006/Accepted 29 January 2007

Burkholderia vietnamiensis **has both the *cepIR* quorum-sensing system that is widely distributed among the *Burkholderia cepacia* complex (BCC) and the *bviIR* system. Comparison of the expression of *cepI*, *cepR*, *bviI*, and *bviR-luxCDABE* fusions in *B. vietnamiensis* G4 and the G4 *cepR* and *bviR* mutants determined that the expression of *bviI* requires both a functional cognate regulator, BviR, and functional CepR. The *cepIR* system, however, is not regulated by BviR. Unlike the *cepIR* genes in other BCC species, the *cepIR* genes are not autoregulated in G4. *N*-Acyl-homoserine lactone (AHL) production profiles in G4 *cepI*, *cepR*, *bviI*, and *bviR* mutants confirmed the regulatory organization of the G4 quorum-sensing systems. The regulatory network in strain PC259 is similar to that in G4, except that CepR positively regulates *cepI* and negatively regulates *cepR*. AHL production and the *bviI* expression levels in seven *B. vietnamiensis* isolates were compared. All strains produced *N*-octanoyl-homoserine lactone and *N*-hexanoyl-homoserine lactone; however, only one of four clinical strains but all three environmental strains produced the BviI synthase product, *N*-decanoyl-homoserine lactone (DHL). The three strains that did not produce DHL expressed *bviR* but not *bviI*. Heterologous expression of *bviR* restored DHL production in these strains. The *bviIR* loci of the non-DHL-producing strains were sequenced to confirm that *bviR* encodes a functional transcriptional regulator. Lack of expression of G4 *bviI* in these three strains indicated that an additional regulatory element may be involved in the regulation of *bviIR* expression in certain strains of *B. vietnamiensis*.**

Burkholderia vietnamiensis is a part of the *B. cepacia* complex (BCC), a group of nine closely related bacterial species with extreme metabolic versatility. The beneficial metabolic properties of *B. vietnamiensis*, including bioremediation and plant growth promotion, have been well characterized (38, 45). *B. vietnamiensis* G4 produces *ortho*-monooxygenase that degrades the groundwater contaminant trichloroethylene. *B. vietnamiensis* strains are also able to fix atmospheric nitrogen and enhance crop yields, presumably by increasing the available nitrogen in the rhizosphere (24). Inoculation of rice with *B. vietnamiensis* significantly increases grain yield and is potentially an economic alternative to nitrogen-based fertilizers (60).

In addition to having biocontrol, bioremediation, and plant growth-enhancing applications (38, 45), BCC species are also opportunistic pathogens of particular importance for people with cystic fibrosis (CF) and chronic granulomatous disease (41). Each BCC species has been isolated from CF patients; however, the species vary in frequency of colonization, transmissibility, and geographic distribution. *Burkholderia cenocepacia* is the most commonly reported BCC species isolated from CF patients in North America, where 80 percent of BCC isolates recovered from CF patients in Canada and 50 percent of BCC isolates recovered from patients in the United States are *B. cenocepacia* (35, 56). There is a low incidence of *B. vietnamiensis* infections in North American CF patients; such infections comprise 1.6 percent of Canadian and 5.1 percent of U.S. CF patient BCC isolates (47, 57). An epidemiological study conducted in Brazil with 11 patients, however, reported equal incidences of *B. cenocepacia* and *B. vietnamiensis* isolates from CF patients, illustrating the potential of *B. vietnamiensis* as a CF pathogen (16). The pathogenic potential of *B. vietnamiensis* has delayed further research on its biotechnological applications (9).

BCC species utilize *N*-acyl-homoserine lactone (AHL)-based quorum-sensing systems for the regulation of diverse physiological processes, including those involved in virulence. Quorum sensing is a form of genetic regulation typically mediated by the accumulation of self-produced signal compounds in the environment. AHL-mediated quorum-sensing systems are comprised of a *luxI* homologue, which encodes an AHL synthase that catalyzes the synthesis of an AHL signal molecule(s), and a *luxR* homologue that encodes a transcriptional regulator that mediates gene expression in its active, AHL-bound form (22).

The *cepIR* quorum-sensing system is widely distributed among BCC strains (26, 36). CepI directs the synthesis of *N*-octanoyl-homoserine lactone (OHL) and *N*-hexanoyl-homoserine lactone (HHL) (32, 33). In *B. cepacia*, the *cepIR* quorum-sensing system is autoregulated in that CepR positively regulates the expression of *cepI* (2). Transcriptional analysis of a *B. cepacia* promoter library identified 28 genes that are positively regulated by CepR (2). The *B. cepacia cepIR* system also negatively regulates the stationary-phase sigma factor *rpoS*, positively regulates protease production, and contributes to onion maceration by positively regulating polygalacturonase production (1).

In *B. cenocepacia*, CepR positively regulates *cepI* expression and negatively controls its own expression (33). The *cepIR*

* Corresponding author. Mailing address: Department of Microbiology and Infectious Diseases, University of Calgary Health Sciences Centre, 3330 Hospital Dr. N.W., Calgary, Alberta, Canada T2N 4N1. Phone: (403) 220-6037. Fax: (403) 270-2772. E-mail: psokol@ucalgary.ca.

▽ Published ahead of print on 2 February 2007.

quorum-sensing system in *B. cenocepacia* is involved in the regulation of swarming motility, mature biofilm development, chitinase production, extracellular protease production, and the biosynthesis of the siderophore ornibactin (29, 32–34). Proteomic analysis of *B. cenocepacia cepI* mutant revealed that 55 of 985 examined proteins are differentially expressed in the quorum-sensing mutant and the wild type (48). Animal and nematode infection models have demonstrated a role for the *cepIR* quorum-sensing system in *B. cenocepacia* virulence (30, 55).

In epidemic strains of *B. cenocepacia* that possess the *B. cenocepacia* pathogenicity island (*cci*), CepR is required for the expression of an additional quorum-sensing system, *cciIR* (5, 39). The predominant AHL produced by the AHL synthase, CciI, is HHL with minor amounts of OHL (39). The *cciI* and *cciR* genes are cotranscribed, and CciR negatively regulates the expression of the *cciIR* operon. CciR is involved in the negative regulation of *cepI* as well as in the regulation of extracellular protease production and swarming motility (39).

B. vietnamiensis strains possess an additional quorum-sensing system, *bviIR*. PCR amplification and Southern hybridization analysis revealed that *B. vietnamiensis* is the only BCC species to contain the *bviIR* system (10, 36). In addition to OHL and HHL, *B. vietnamiensis* strains produce *N*-decanoyl-homoserine lactone (DHL), *N*-dodecanoyl-homoserine lactone, and *N*-(3oxodecanoyl)-homoserine lactone (10, 11, 44). Mutations constructed in *bviI* and *bviR* of *B. vietnamiensis* strain G4 ($bviI_{G4}$ and $bviR_{G4}$, respectively) indicated that BviI is responsible for the synthesis of all AHLs produced by G4 in that the *bviI* mutant did not produce detectable levels of any AHL except minor amounts of OHL (10). Although *B. vietnamiensis* possesses the genes for two quorum-sensing systems, its AHL production has been shown to be strain dependent with regard to type and quantity of AHL produced (11, 26, 72).

Few phenotypes are known to be regulated by the *bviIR* system. One study suggested that a *B. vietnamiensis* G4 transposon mutant with decreased AHL production showed reduced antibiotic production, although this antibiotic has yet to be characterized (44). In the same study, it was determined that the degradation of toluene and thus the expression of toluene *ortho*-monooxygenase is not regulated by quorum sensing (44). There is also evidence that the *bviIR* system is not involved in siderophore production (10).

The objectives of this study were to further characterize the *bviIR* and *cepIR* quorum-sensing systems of *B. vietnamiensis* G4, as well as the regulatory relationship between the two quorum-sensing systems. In this study we also investigate the basis for the variations in AHL production in clinical and environmental *B. vietnamiensis* strains at a molecular level.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. For genetic manipulations, cultures were routinely grown at 37°C in Luria-Bertani broth (Invitrogen, Burlington, Ontario, Canada) with shaking (200 rpm) or on 1.5% LB agar plates. When appropriate, the following concentrations of antibiotics were used: 100 μg/ml of trimethoprim (Tp), 30 μg/ml of tetracycline (Tc), 20 μg/ml of gentamicin (Gm), and 80 μg/ml of chloramphenicol (Cm) for *B. vietnamiensis* G4; 100 μg/ml of Tp, 250 μg/ml of Tc, and 400 μg/ml of kanamycin (Km) for *B. vietnamiensis* PC259; and 1.5 mg/ml Tp, 15 μg/ml of Tc, 25 μg/ml Gm, 35 μg/ml of Cm, and 50 μg/ml of Km for *Escherichia coli*. Antibiotics were purchased from Sigma-Aldrich Canada, Ltd. (Oakville, Ontario, Canada). For RNA isolation, luminescence assays, biofilm assays, the alfalfa model of infection, and AHL extractions, cultures were grown in Trypticase soy broth (Difco, Franklin Lakes, NJ) at 30°C. For chrome azurol S assays, cultures were grown at 32°C in succinate medium supplemented with ornithine (10 mM) (40). For examination of swarming motility, cultures were grown in nutrient broth (Difco) supplemented with 0.5% glucose.

DNA manipulations. DNA manipulations were performed using standard techniques as described by Sambrook et al. (49). Genomic DNA was isolated as described by Ausubel et al. (4). Genomic DNA for PCR was isolated as described by Walsh et al. (66). Restriction endonucleases and T4 DNA polymerase were purchased from Invitrogen. T4 DNA ligase was purchased from New England Biolabs (Mississauga, Ontario, Canada) or Invitrogen. Shrimp alkaline phosphatase was purchased from Roche (Mannheim, Germany). Oligonucleotide primers (Table 2) were synthesized at Invitrogen or at the University of Calgary Core DNA and Protein Services (Calgary, Alberta, Canada). PCR was performed with either Platinum *Taq* polymerase (Invitrogen) or Phusion *Taq* polymerase (Finnzymes, Espoo, Finland) according to the manufacturer-recommended protocol. DNA fragments used in cloning procedures were purified with a QIAquick gel extraction kit (QIAGEN, Mississauga, Ontario, Canada). Plasmids were introduced into *B. vietnamiensis* G4 by electroporation using a Gene Pulser (Bio-Rad, Richmond, CA) as previously described (13). Plasmids were transferred into all other *B. vietnamiensis* strains by conjugation employing pRK2013 (21) as the mobilization vector. Nucleotide sequencing was performed by University of Calgary Core DNA and Protein Services. Sequence analysis was performed with DNAMAN sequence analysis software (Lynnon Biosoft, Vandreuil, Quebec, Canada). The sequence data for *B. vietnamiensis* G4 was provided by the U.S. Department of Energy Joint Genome Institute (JGI) (http://www.jgi.doe.gov/).

Cloning of quorum-sensing genes from G4. The *cepR*, *bviI*, and *bviR* genes and open reading frame 7350 were PCR amplified from G4 and cloned into the broad-host-range vector pUCP28T (51) as follows. The *cepR* clone pRM282 was constructed by amplifying a 1,547-bp fragment with the G4cepRKOF and G4cepRHind primers. The *bviI* clone pRM285 was constructed by amplifying a 997-bp fragment with the bviIKOF and bviIKOR primers. The *bviR* clone pRM284 was constructed by amplifying an 1,108-bp fragment with the bviRKOF and bviRKOR primers. The open reading frame 7350 clone pRM292 was constructed by amplifying a 1,426-bp fragment with the 7350F and 7350R primers. A 6.8-kb BamHI fragment from PC259 containing *cepIR* was shotgun cloned into pUCP26 (70). A positive clone was identified by colony hybridization (71) with a 627-bp *cepI* probe amplified using the cepIF and cepIR primers and designated pRM6.8.

Analysis of quorum-sensing genes in *B. vietnamiensis* strains. The presence of the *bviIR* genes in seven *B. vietnamiensis* strains was determined by PCR with primers RTbviIF and RTbviIR for *bviI* and RTbviRF and RTbviRR for *bviR*. The *bviIR* loci from strains FC466, FC441, C2822, and G4 were cloned by PCR amplification of a 2.6-kb fragment from genomic DNA using the bviIKOR and bviRKOR primers. The nucleotide sequence for the G4 *bviIR* locus was confirmed with the incomplete *B. vietnamiensis* G4 sequencing project (http://www.jgi.doe.gov/), which contained only 1.2 kb of the G4 *bviIR* locus. Since the complete $cepI_{G4}$ sequence was not available through the JGI sequencing project, the *cepI* gene was amplified on an 826-bp fragment with the G4cepIF and G4cepIR primers and sequenced. The presence of the open reading frame 7349 and 7350 LuxRI homologue genes in seven *B. vietnamiensis* strains was determined by PCR with primers IN7349F and IN7349R and IN7350F and IN7350R, respectively.

Construction of *cepI* and *cepR* mutants. To construct a *cepI*::*cat* mutant of *B. vietnamiensis* G4, a 2.2-kb PstI fragment from pRM6.8 containing *cepI* was blunt ended and cloned into the EcoRV site of pCR2.1Topo (Invitrogen). A SmaI fragment from p35S-Cm (14) harboring a Cm resistance cassette was inserted into the MluI site of *cepI*, and the resulting fragment was subcloned into pEX18Tc (27) with BamHI and XbaI, resulting in pRM2X2-Cm, which was transferred into G4 by conjugation. Transconjugants were plated onto *Pseudomonas* isolation agar (Difco) plates with Cm to select for single-crossover events. Attempts to identify a *cepI*::*cat* mutant by screening Cm-resistant colonies for Tc sensitivity and loss of the plasmid were unsuccessful. Attempts were made to construct the *cepI* mutant with pEXCEPI (32) and other vectors with various amounts of flanking DNA and different resistance cassettes. None were successful; therefore, the G4 *cepI* mutant (G4*cepI*) is a merodiploid. The mutation was confirmed by PCR with the internal *cepI* primers UnicepIF and UnicepIR.

To construct a *cepR*::*cat* mutant of G4, a 2.4-kb fragment containing *cepR* was PCR amplified using the G4cepRKOF and G4cepRKOR primers and cloned into pEX18Tc (27), resulting in pRM2X1. The *cepR* open reading frame was disrupted at the PstI site by the Cm resistance cassette from p34S-Cm (14),

TABLE 1. Bacterial strains and plasmids used in this study

Strain (alternate name) or plasmid	Relevant characteristic(s)[a]	Source or reference
Strains		
E. coli		
DH5α	F$^-$ ϕ80*lacZ*Δ*M15* Δ(*lacZYA-argF*) *recA1 endA gyrA96 thi-1 hsdR17 supE44 relAl deoR U169*	Invitrogen
TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*Δ*M15* Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (St^r) *endA1 nupG*	Invitrogen
HB101	*supE44 hsdS20*(r_B m_B) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	49
A. tumefaciens		
A136	Ti plasmidless host	C. Fuqua
B. vietnamiensis		
PC259 (LMG18835)	Isolated from a patient with CF in Washington State	31
FC466 (LMG18836)	Isolated from a patient with CF in Sweden	64
FC441 (LMG16232)	Isolated from a patient with septic granulomatous disease in British Columbia, Canada	37
C2822	Isolated from a patient with CF in British Columbia, Canada	E. Mahenthiralingam
FC369^T (LMG10929T)	Isolated from a rice rhizosphere in Vietnam	24
DBO1 (LMG17830)	Environmental isolate	67
G4 (R1808)	Isolated from a water treatment facility in Florida	43
IMT-61	*bviI*::*aacCI* derivative of G4, Gm^r	10
RMT-14	*bviR*::*aacCI* derivative of G4, Gm^r	10
G4*cepI*	*cepI*::*cat* derivative of G4, Cm^r	This study
G4*cepR*	*cepR*::*cat* derivative of G4, Cm^r	This study
G4*cepI*GSV	*cepI*::pRMSVI derivative of G4, Gm^r	This study
G4*cepR*GSV	*cepR*::pRMSVR derivative of G4, Gm^r	This study
PC259*cepR*	*cepR*::*dhfRII* derivative of PC259, Tp^r	This study
M. luteus		
ATCC 9341	Soil isolate	T. Louie
Plasmids		
pEX18Tc	Suicide vector, *sacB* Tc^r	27
pUCP26	Broad-host-range vector, Tc^r	70
pUCP28T	Broad-host-range vector, Tp^r	51
pCR2.1Topo	Cloning vector for PCR products, Ap^r Km^r	Invitrogen
pRK2013	ColE1 Tra (RK2)$^+$ Km^r	21
pCF218	IncP plasmid expressing TraR, Tc^r	73
pCF372	pUCD2 with a *traI-lacZ* fusion, Sp^r	23
p34S-Cm	Source of the Cm resistance cassette	14
pRM6.8	pUCP26 with a 6.8-kb BamHI fragment containing the *cepIR* locus from PC259	This study
pRM282	pUCP28T with a 1.5-kb PCR-amplified *cepR* fragment from G4, Tp^r	This study
pRM284	pUCP28T with a 1.1-kb PCR-amplified *bviR* fragment from G4, Tp^r	This study
pRM285	pUCP28T with a 997-bp PCR-amplified *bviI* fragment from G4, Tp^r	This study
pRM292	pUCP28T with a 1,426-bp PCR-amplified 7350 fragment from G4, Tp^r	This study
pRM2X1	pEX18Tc with a 2.3-kb PCR-amplified *cepR* fragment from G4, Tc^r	This study
pRM2X1-Cm	PRM2X2 with the *cepR* gene disrupted by a Cm resistance cassette at the PstI site, Tc^r Cm^r	This study
pRM2X2-Cm	pEX18Tc with the disrupted *cepI* fragment from pRM2T2-Cm, Tc^r Cm^r	This study
pEXCEPI	pEX18Tc containing the *cepI* gene disrupted by the Tp resistance cassette, Tp^r Tc^r	32
pEXCEPR	pEX18Tc containing the *cepR* gene disrupted by the Tp resistance cassette, Tp^r Tc^r	33
pMS402	*luxCDABE*-based promoter reporter plasmid, Km^r Tp^r	18
pRM452	pMS402 containing the 1.8-kb PC259 *cepR* promoter fragment, Km^r Tp^r	This study
pRM453	pMS402 containing the 769-bp PC259 *cepI* promoter fragment, Km^r Tp^r	This study
pRM455	pMS402 containing the 983-bp PC259 *bviI* promoter fragment, Km^r Tp^r	This study
pRM462	pMS402 containing the 266-bp G4 *cepR* promoter fragment, Km^r Tp^r	This study
pRM463	pMS402 containing the 266-bp G4 *cepI* promoter fragment, Km^r Tp^r	This study
pRM464	pMS402 containing the 695-bp G4 *bviR* promoter fragment, Km^r Tp^r	This study
pRM465	pMS402 containing the 983-bp G4 *bviI* promoter fragment, Km^r Tp^r	This study
pRM475	pMS402 containing the 983-bp FC466 *bviI* promoter fragment	This study
pRM485	pMS402 containing the 983-bp FC441 *bviI* promoter fragment	This study
pRM495	pMS402 containing the 983-bp FC369^T *bviI* promoter fragment	This study
pRM415	pMS402 containing the 983-bp DBO1 *bviI* promoter fragment	This study
pGSV3	Mobilizable suicide vector, Gm^r	15
pRMSVI	pGSV3 containing the EcoRI fragment from pRM2T5 from G4, Gm^r	This study
pRMSVR	pGSV3 containing the EcoRI fragment from pRM2T4, Gm^r	This study

[a] Ap, ampicillin; St, streptomycin; Sp, spectinomycin.

TABLE 2. Oligonucleotide primers used in this study

Name	Sequence (5′–3′)[f]	Restriction site	Reference
7350F	GAGCTGCGCAAGGAACTCA[a]		This study
7350R	TGGGTAAAGGACGCGGATAAC[a]		This study
bviIKOF	GTCCGAGGATCCAGAGCG[b]	BamHI	This study
bviIKOR	GCACGCGAAGCTTCACGG[b]	HindIII	This study
bviIproB	CGAATTGGATCCATTATCGG[b]	BamHI	This study
bviIproX	AATAGCCCTCGAGTGGCC[b]	XhoI	This study
bviRKOF	CACGGTCGTCTAGACGAGG[b]	XbaI	This study
bviRKOR	GGCCGAAGCTTGATGAATCG[b]	HindIII	This study
bviRUF	GACCAGCTCGAGGTAGCCG3[b]	XhoI	This study
bviRUR	CAGTGGTCGGATCCGAGCG[b]	BamHI	This study
cepIF	CAGGCGGCGATAGCTTG[c]		This study
cepIR	CACAGATCCGAGGACATCCA[c]		This study
cepRF	GAGAAAGAATGGAACTGCGC[c]		This study
cepRProF	GGCCGCTCGCGACATGGT[c]		This study
cepRProR	CCGCGGCGCTGAATTGTTGG[c]		This study
cepRR	TTGTTCACGTGGAAGTTGAC[c]		This study
ExcepI	GCCTGCAGGGCACAACGACGCCTATCATGC[c]		33
G4cepIF	TCAATCCCGCCGATCAAG[e]		This study
G4cepIR	GCGCGAAAGACCTGAGACTG[e]		This study
G4cepRHind	CATTTCAAGCTTGAGCTGGACC[a]	HindIII	This study
G4cepRKOF	CGGATCGGTACCTTGGGATG[a]	KpnI	This study
G4cepRKOR	CCGCAAGCTTCCCGTTTCAC[a]	HindIII	This study
IN7349F	TCAAGACCCAGCATCTCAATG[a]		This study
IN7349R	ACCCGACAGGTTGATGAGC[a]		This study
IN7350F	TCCAACGGACCGGTATGTG[a]		This study
IN7350R	AGGCCACGAACGGAGAGGTA[a]		This study
INcepI	GCGGATCCACCAGACGCCCATCTACCTGCTTCG[c]		33
R2Cla3RE	GAACGAAGGTCTGCATGGATG[c]		This study
RTbviIF	CACGGAGAACGCAATGAGG[b]		This study
RTbviIR	CACGCGGATACCCTTTACGTC[b]		This study
RTbviRF	CAGACGTGGGTCGAACGCTA[b]		This study
RTbviRR	ATAGTTGGCCGTGTTGGCG[b]		This study
RTsigAF	AATGACCGAGGCGAACCTG[a]		This study
RTsigAR	TCTTGTCTTCCGGCATCTCC[a]		This study
UnicepIF	GACCTTCGTTCACGAGGAAG[d]		This study
UnicepIR	CGTCACGCCGATCAGCTGC[d]		This study

[a] Primer designed based on the sequence from the incomplete *B. vietnamiensis* G4 sequencing project (http://genome.jgi-psf.org/draft_microbes/bur08/bur08.home.html).
[b] Primer designed based on the *B. vietnamiensis* DBO1 *bviIR* sequence (accession no. AF296284) (36).
[c] Primer designed based on the *B. cenocepacia* K56-2 *cepIR* sequence (accession no. AF019654) (32).
[d] Primer designed based on the *B. vietnamiensis* PC259 *cepIR* sequence (accession no. AF337814) (36).
[e] Primer designed based on the *B. vietnamiensis* PC259 *cepIR* sequence (accession no. AF337814) (36).
[f] Restriction endonuclease sites incorporated into primers are underlined.

resulting in pRM2X1-Cm. Attempts to construct a *cepR*::*cat* mutant were carried out as outlined above, again resulting in the merodiploid, G4*cepR*. The mutation was confirmed by PCR with the primers cepRF and cepRR. The *cepR* mutant of PC259 (PC259*cepR*) was constructed using the allelic-exchange vector pEXCEPR (33). The mutation was confirmed by PCR with the cepRF and cepRR primers and Southern hybridization using a 650-bp *cepR* probe PCR amplified with the same primers.

Construction of *cepI* and *cepR* insertion mutants of G4. The *cepI* and *cepR* genes in *B. vietnamiensis* G4 were inactivated by insertion of pGSV3 (15). A 426-bp internal *cepI* fragment was PCR amplified using the UnicepIF and UnicepRR primers, and the resulting fragment was cloned into pCR2.1Topo. An EcoRI fragment containing the *cepI* fragment was cloned into pGSV3 (15) to construct pRMSVI. The *cepR* insertional activation construct pRMSVR was constructed in a similar manner following PCR amplification of a 647-bp *cepR* fragment with the cepRF and cepRR primers. The pRMSVR and pRMSVI constructs were mobilized into G4 by conjugation using pRK2013 (21). Transconjugants were plated onto *Pseudomonas* isolation agar (Difco) plates containing Gm. Insertions were confirmed by PCR with the G4cepIF and G4cepIR primers for the *cepI* mutant (G4*cepI*GSV) and with the G4cepRKOF and G4cepRKOR primers for the *cepR* mutant (G4*cepR*GSV).

Construction of *luxCDABE* transcriptional fusions. Promoter regions were predicted in silico using SoftBerry BPROM. Promoter fragments were amplified by PCR and cloned upstream of the *luxCDABE* operon in the XhoI/BamHI promoter cloning site of pMS402 (18) as follows. The *cepI-luxCDABE* transcriptional fusion plasmid pRM463 was constructed by amplifying a 266-bp promoter fragment using the primers R2Cla3RE and ExcepI (33). The *cepR-luxCDABE* transcriptional fusion plasmid pRM462 was constructed by amplifying a 227-bp *cepR* promoter fragment with the cepRProF and cepRProR primers. The *bviR-luxCDABE* transcriptional fusion plasmid pRM464 was constructed by amplifying a 695-bp promoter fragment with the bviRUF and bviRUR primers. The *bviI-luxCDABE* transcriptional fusion plasmid pRM465 was constructed by amplifying a 983-bp fragment with the bviIproX and bviIproB primers. The *bviI-luxCDABE* transcriptional fusions pRM455, pRM475, pRM485, pRM495, and pRM415 were constructed using the respective genomic DNA (PC259, FC466, FC441, FC369^T, and DBO1) as a template.

The *cepI-luxCDABE* transcriptional fusion plasmid pRM453 was constructed by amplifying a 769-bp promoter fragment from PC259 DNA with the INcepI and ExcepI primers (33) The *cepR-luxCDABE* transcriptional fusion plasmid pRM452 was constructed as previously described (39), using PC259 genomic DNA as the template.

Luminescence assays. Overnight cultures were subcultured to an initial optical density at 600 nm (OD_{600}) of 0.02 in 20 ml medium. At selected times, 100-μl aliquots were removed and the luminescence in counts per second and turbidity at an OD_{600} or OD_{620} were measured using a Wallac Victor^2 multilabel counter (Perkin Elmer Life Sciences, Woodbridge, Ontario, Canada) or a MicroBeta TriLux microplate scintillation and luminescence counter (Perkin Elmer Life Sciences). The samples were read in black, clear-bottom, 96-well microtiter plates (Corning, Inc., Corning, NY). The level of promoter expression is reported as the ratio of luminescence to turbidity or relative luminescence. Luminescence assays for screening for gene expression in a 96-well plate format were

performed as described above, with overnight cultures being subcultured (1/100) into 150 μl of medium.

RT-PCR. Overnight cultures were subcultured (1/100) into 20 ml medium and grown for 24 h. Total RNA was isolated from approximately 1×10^9 cells with the RiboPure-Bacteria RNA isolation kit (Ambion, Austin, TX). RNA was treated with amplification-grade DNase I (Invitrogen) before use. Reverse transcription (RT)-PCR was performed using a Titan one-tube RT-PCR kit (Roche) according to the manufacturer's instructions. For each reaction, 50 ng of RNA was used. cDNA was synthesized by RT at 50°C for 40 min. Denaturation was performed for 2 min at 96°C, followed by 35 cycles of PCR as suggested by the manufacturer. A final elongation step at 68°C for 7 min was conducted.

The *sigA* gene encodes the principal sigma factor (8) and was used as a control gene. A homologue of *B. cepacia sigA* was identified in the *B. vietnamiensis* G4 sequencing project (http://www.jgi.doe.gov/). *B. vietnamiensis* G4 SigA is 89.93% identical to SigA of *B. cepacia* (accession no. AAD03549) (8).

Three primer sets were designed to internally amplify *bviI*, *bviR*, and *sigA* to yield 297-bp, 297-bp, and 347-bp products, respectively. The annealing temperatures and primers used are as follows: for *bviI*, 58°C with RTbviIF and RTbviIR; for *bviR*, 64°C with RTbviRF and RTbviRR; and for *sigA*, 62°C with RTsigAF and RTsigAR. To ensure that there was no DNA contamination in the RNA samples, PCR was performed on the RNA samples using the RTsigAF and RTsigAR primers with Platinum *Taq* polymerase (Invitrogen). RTbviRF and RTbviRR primers yielded an approximately 745-bp nonspecific contaminating band (data not shown). This band was sequenced, and a BLAST (3) search indicated that the fragment has homology to *B. vietnamiensis* LMG10929T 23S rRNA and is not related to *bviR*.

TLC-AHL bioassays. AHLs were extracted from 40 ml of culture supernatants with equal volumes of acidified ethyl acetate as described elsewhere (33). Thin-layer chromatography and AHL extraction (TLC-AHL) bioassays were performed as described previously using *Agrobacterium tumefaciens* A136(pCF218)(pCF372) as a reporter strain (33). This reporter strain is able to identify AHLs with 3-oxo, 3-hydroxy, and 3-unsubstituted side chains ranging from 6 to 12 carbons in length (53). Synthetic HHL, OHL, and DHL (Sigma-Aldrich) were used as reference standards.

Phenotypic characterization. Siderophore activity present in the culture supernatant fluid was measured by chrome azurol S assays (32, 52). Biofilm formation was determined by staining the cellular matter with crystal violet as described by Tomlin et al. (59), except that biofilms were formed on the polystyrene pegs of a 96-peg replica plate lid inserted in a 96-well plate containing the culture (Nalge Nunc International, Rochester, NY). Swarming motility was evaluated using semisolid agar (0.5%) motility assays as previously described (34). Virulence studies with the alfalfa infection model were performed as previously described (6, 7), with 40 sprouts per group and incubation of the sprouts at 30°C for 7 days. The API 20NE (bioMerieux, St. Laurent, Quebec, Canada) were used for testing basic biochemical attributes as per the instructions of the manufacturer. The ability to fix atmospheric nitrogen was tested by determining growth in the nitrogen-free medium BAz (20) after 48 h of growth at 30°C under anaerobic conditions. Antibiotic production was determined by disk diffusion as previously described (44) on lawns of *Micrococcus luteus* ATCC 9341.

Statistics. Statistical analyses, including unpaired *t* tests and analyses of variance, were performed with INSTAT software (GraphPad Software, San Diego, CA). A P value of <0.05 was considered significant.

Nucleotide sequence accession numbers. The G4, FC466, FC441, and C2822 *bviIR* sequences were deposited into the NCBI database with the respective accession nos. EF032807, EF032808, EF032809, and EF032810. In addition, the *cepI* gene sequence was deposited into the NCBI database with the accession no. EF212890.

RESULTS

Transcriptional analysis of *bviIR* and *cepIR*. To determine whether BviR is involved in the regulation of the *bviIR* system, the levels of expression of *bviI* and *bviR-luxCDABE* transcriptional fusion constructs were compared between G4 and a G4 *bviR* mutant. The expression of *bviI* was reduced to almost background levels in the *bviR* mutant, indicating that BviR is required for the expression of *bviI* (Fig. 1a). The levels of expression of *bviR* were similar in G4 and the G4 *bviR* mutant, demonstrating that BviR is not involved in autoregulation (data not shown).

To determine whether CepR is involved in the regulation of *cepIR* in *B. vietnamiensis*, transcriptional analysis of *cepI* and *cepR-luxCDABE* fusions in G4 and a G4 *cepR* mutant was performed. Unexpectedly, the levels of expression of *cepI* and *cepR* were similar in both the parent and the *cepR* mutant (Fig. 1B and C), indicating that CepR does not regulate either *cepI* or *cepR* in *B. vietnamiensis* G4. To determine whether the lack of CepR autoregulation was common in *B. vietnamiensis*, similar comparative expression studies were performed with strain PC259. The expression of *cepR* was significantly greater in the *cepR* mutant than in the parent strain from hours 3 to 12 ($P < 0.05$, *t* test) (Fig. 1D), indicating that CepR is involved in negative autoregulation. The expression of *cepI* was reduced to almost background levels in the PC259 *cepR* mutant (Fig. 1E), indicating that CepR is required for the expression of *cepI*.

To determine whether there is a regulatory relationship between the *cepIR* and *bviIR* systems, comparative transcriptional analysis of *cepI* and *cepR-luxCDABE* transcriptional fusions in G4 and the G4 *bviR* mutant as well as analysis of the expression of *bviI* and *bviR-luxCDABE* in G4 and G4*cepR* were performed. The levels of expression of *cepR* and *cepI* were similar in G4 and the G4 *bviR* mutant (data not shown), indicating that BviR is not involved in the regulation of the *cepIR* system. The levels of expression of *bviR* were also similar between the parent and the *cepR* mutant (data not shown); however, the expression of *bviI* in the G4 *cepR* mutant was reduced to almost background levels (Fig. 1F), indicating that CepR positively regulates *bviI* in G4. To determine whether CepR positively regulates *bviI* in PC259, RT-PCR was performed to amplify *bviI* and *bviR* from PC259 and the PC259 *cepR* mutant total RNA (Fig. 2). RT-PCR was employed since *bviI* expression was not detected in PC259 using the *bviI-luxCDABE* fusion plasmid pRM455 (Table 3). There was a smaller amount of the *bviR* product in the PC259 *cepR* mutant than in PC259 (Fig. 2, compare lanes 3a and 3b) and no detectable *bviI* product in the PC259 *cepR* mutant (Fig. 2, lane 2b), indicating that CepR positively regulates the *bviIR* system in PC259. The *sigA* gene was used as a control, and there was no observable difference in expression between PC259 and the PC259 *cepR* mutant (Fig. 2, compare lanes 4a and 4b).

Phenotypic characterization of *B. vietnamiensis* quorum-sensing mutants. TLC-AHL bioassays of the G4 *bviI* and *bviR* mutants were performed (Fig. 3A). Ethyl acetate extracts of spent culture supernatants were chromatographed and AHLs were visualized using an *A. tumefaciens* A136(pCF218)(pCF372) AHL reporter strain agar overlay. G4 produced detectable amounts of HHL, OHL, and DHL (Fig. 3A, lane 1). DHL production was not detected for the G4 *bviI* or *bviR* mutant (Fig. 3A, lanes 2 and 4). The production of DHL was restored when *bviI* and *bviR* were present in *trans* (Fig. 3A, lanes 3 and 5), thus corroborating the role of BviR in the positive regulation of *bviI*. Considerable amounts of HHL and OHL remained in the AHL production profiles of the *bviIR* mutants (Fig. 3A, lanes 2 and 4), thereby confirming that the *bviIR* system is not required for the expression of *cepIR*.

The G4 *cepR* mutant produces marginally less HHL and OHL than the parent but no detectable amounts of DHL (Fig. 3B, compare lanes 1 and 2), confirming the role of CepR in the positive regulation of *bviI* and in the negative regulation of *cepI*. The AHL production profile of the G4 *cepI* mutant has considerably less HHL and OHL than the parent (Fig. 3B, compare lanes 1 and 3) and no detectable

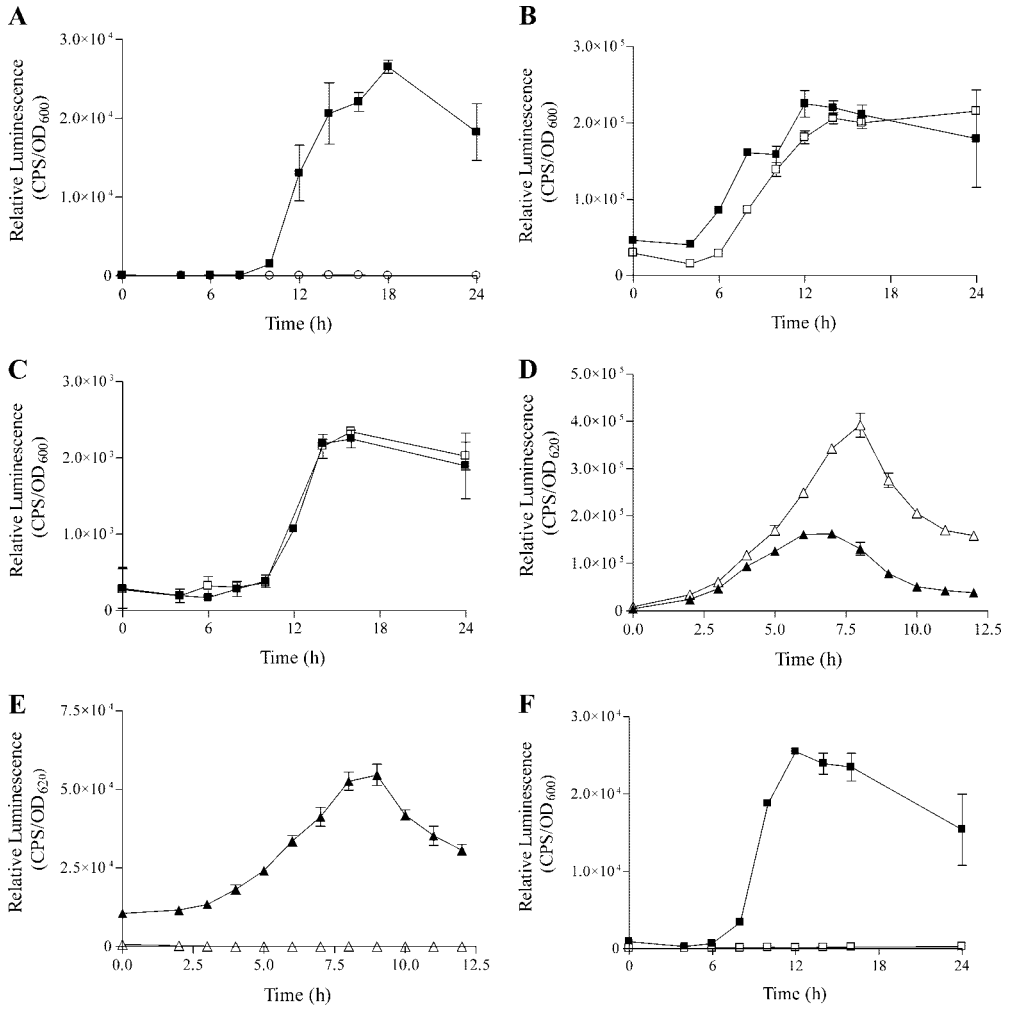

FIG. 1. Transcriptional analysis of *B. vietnamiensis* quorum-sensing genes. All values are means ± standard deviations of the results of triplicate assays. ■, G4; ○, G4 *bviR* mutant (RMT-14); □, G4*cepR* (PC259); ▲, PC259; △, PC259*cepR*. (A) Effect of BviR on *bviI* expression (*bviI-luxCDABE*, pRM465). (B) Effect of CepR on *cepI* expression (*cepI-luxCDABE*, pRM463). (C) Effect of CepR on *cepR* expression (*cepR-luxCDABE*, pRM462). (D) Effect of CepR on *cepR* expression (*cepR-luxCDABE*, pRM452). The expression of pRM452 was significantly greater in PC259*cepR* from 3 to 12 h ($P < 0.05$, *t* test). (E) Effect of CepR on *cepI* expression (*cepI-luxCDABE*, pRM453). (F) Effect of CepR on *bviI* expression (*bviI-luxCDABE*, pRM465). CPS, counts per second.

DHL, indicating that *cepI* is expressed and encodes a functional AHL synthase in G4. The AHLs that remain in the G4 *cepI* mutant supernatant are presumed to be due to the functional BviI synthase. PC259 produces detectable amounts of HHL, OHL, and DHL (Fig. 3B, lane 4). The AHL production profile for the PC259 *cepR* mutant was devoid of detectable AHL (Fig. 3B, lane 5), confirming that CepR is involved in the positive regulation of the AHL synthase genes *cepI* and *bviI* in this strain.

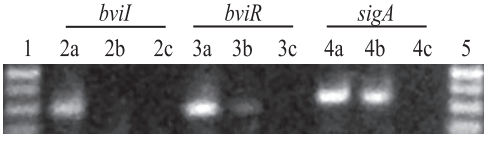

FIG. 2. Effect of CepR on *bviIR* expression in PC259 by RT-PCR. Products were electrophoresed on a 1.0% gel. Lane 1, ladder; lanes 2a to 2c, product amplified by RTbviIF and RTbviIR; lanes 3a to 3c, product amplified by RTbviRF and RTbviRR; lanes 4a to 4c, product amplified by RTsigAF and RTsigAR; lane 5, ladder. Lanes a, PC259 RNA; lanes b, PC259*cepR* RNA; lanes c, no-template control.

The discrepancies between the G4 *cepR* merodiploid and the PC259 *cepR* haploid mutant, with regard to the regulation of *cepIR* and AHL production, prompted a second mutant construction strategy for G4. Mutant construction has proven successful in *Burkholderia mallei* and *Burkholderia thailandensis* by insertion of pGSV3 (15, 42). G4 *cepI* and *cepR* mutants were constructed with this approach and were designated G4*cepI*GSV and G4*cepR*GSV, respectively. PCR analysis indicated that the mutant cultures had a mixture of wild-type and mutated *cepI* or *cepR* genes (data not shown), indicating that the mutation was not stable. The presence of bacteria with wild-type genes decreased with increasing Gm concentrations (data not shown), although there were always revertants in the population. The AHL production profiles of G4*cepI*GSV and G4*cepR*GSV were

TABLE 3. Summary of AHL production, *bviIR* gene presence, and *bviIR* expression in clinical and environmental *B. vietnamiensis* strains[i]

Strain	Source of isolate	HHL production[a]	OHL production[a]	DHL production[a]	Presence of *bviIR* genes[b]	Expression of *bviI*	Expression of *bviR*[g]	Presence of 7349 and 7350[h]
PC259	Clinical	+	+	+	+	+[c]/−[d,e]	+	−
FC466	Clinical	(+)	(+)	−	+	−[c,d,e]	(+)	+
FC441	Clinical	+	+	−	+	−[c,d,e]	(+)	+
C2822	Clinical	+	+	−	+	−[c,e]	(+)	−
FC369[T]	Environmental	+	+	+	+	+[c,d,f]	ND	+
DBO1	Environmental	+	+	+	+	+[d,f]	ND	+
G4	Environmental	+	+	+	+	+[c,d,f]	+	+

[a] Production of AHLs was determined by AHL-TLC bioassays using *A. tumefaciens* A136(pCF218)(pCF372) as the reporter strain.
[b] The presence of the *bviI* and *bviR* genes was determined by PCR.
[c] The expression of *bviI* was assessed by RT-PCR.
[d] The expression of *bviI* was analyzed by transcriptional analysis of *bviI-luxCDABE* fusion constructs (pRM455, pRM465, pRM475, pRM485, pRM495, pRM415) in their respective host backgrounds.
[e] The expression of $bviI_{G4}$ was analyzed by transcriptional analysis of the $bviI_{G4}$-*luxCDABE* fusion construct (pRM465).
[f] The expression of $bviI_{PC259}$ was analyzed by transcriptional analysis of the $bviI_{PC259}$-*luxCDABE* fusion construct (pRM465).
[g] The expression of *bviR* was assessed by RT-PCR with primers internal to *bviR*.
[h] The presence of the 7349 and 7350 genes was determined by PCR.
[i] +, positive; (+), weakly positive; −, negative; ND, not determined.

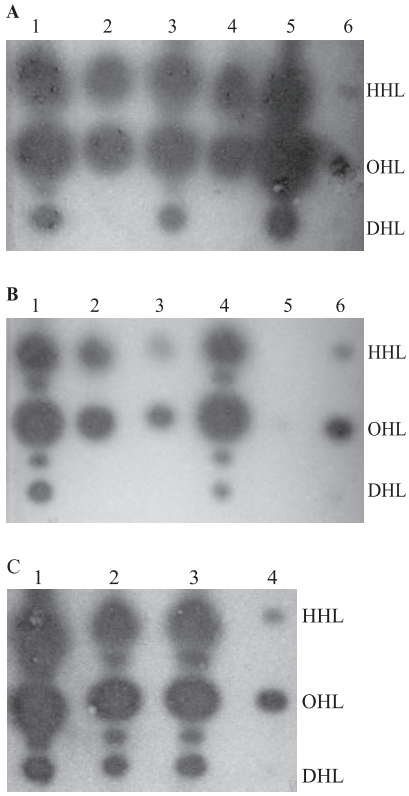

FIG. 3. AHL production profiles of the quorum-sensing mutants using TLC-AHL bioassays with the *A. tumefaciens*(pCF218)(pCF372) reporter strain. (A) AHL production profile of G4 *bviI* and *bviR* mutants. Lane 1, G4(pUCP28T); lane 2, G4 *bviR*(pUCP28T); lane 3, G4 *bviR* (pRM612) containing the *bviR* gene; lane 4, G4 *bviI*(pUCP28T); lane 5, G4 *bviI*(pRM611) containing the *bviI* gene; lane 6, synthetic standards. (B) AHL production profiles of *cepIR* mutants. Lane 1, G4; lane 2, G4*cepR*; lane 3, G4*cepI*; lane 4, PC259; lane 5, PC259*cepR*; lane 6, synthetic standards. (C) AHL production profiles of the G4 *cepI* and G4 *cepR* insertion mutants. Lane 1, G4; lane 2, G4*cepI*GSV; lane 3, G4*cepR*GSV; lane 4, synthetic standards.

unaffected (Fig. 3C, compare lanes 2 and 3 with lane 1); therefore, these mutants were not studied further.

Assays, including siderophore biosynthesis, biofilm formation, antibiotic production, nitrogen fixation, and swarming motility assays (12, 29, 32–34, 44), were performed to determine whether the *bviIR* quorum-sensing system is involved in phenotypes known to be quorum-sensing regulated. Extracellular protease production and expression of the AHL-dependent gene *aidA* were not investigated, since *B. vietnamiensis* does not produce proteases and G4 does not contain *aidA* (25, 26, 72). G4 did not exhibit swarming motility, was not virulent in the alfalfa model of infection, and was unable to grow on nitrogen-free media. Previously, G4 AHL production was correlated with antibacterial activity that inhibited the growth of *M. luteus* (44); however, we found that *M. luteus* was not sensitive to G4-secreted products. The levels of siderophore biosynthesis in the G4 *bviI* and *bviR* mutants were indistinguishable from that in wild-type G4 (data not shown). The *bviI* and *bviR* mutants exhibited marginally less biofilm formation; however, this decrease was not restored when *bviI* and *bviR* were added in *trans* (data not shown). The *bviI* and *bviR* mutants did not exhibit a phenotype distinguishable from that of the wild type in any of the API 20 NE biochemical tests (data not shown).

Analysis of differences in AHL production in clinical and environmental strains of *B. vietnamiensis*. AHL production in the BCC is strain dependent with respect to quantity and type of AHL produced (11, 26, 72). Since the regulation of the quorum-sensing systems appeared to be strain dependent, further investigation into the differences in AHL production by *B. vietnamiensis* strains was pursued. The AHL production profiles of seven *B. vietnamiensis* strains were determined by TLC-AHL bioassays (Table 3). Only one of four clinical strains but all three environmental strains produced DHL, suggesting that the *bviIR* genes may be less expressed in clinical strains than in environmental strains, since OHL and HHL could be produced by CepI in these strains.

The presence of the *bviI* and *bviR* genes in the seven *B. vietnamiensis* strains was confirmed by PCR analysis with primers that amplified DNA fragments internal to *bviI* and *bviR*

(Table 3). To determine whether *bviI* and *bviR* are expressed in the clinical strains that do not produce DHL, RT-PCR was performed (Table 3). There was a decrease in amplification of the *bviR* RT-PCR products and no detectable *bviI* RT-PCR products from the non-DHL-producing strains, indicating that the *bviI* and *bviR* genes are poorly expressed in these three strains. The *bviI* RT-PCR data were confirmed for the majority of strains by transcriptional analysis of *bviI-luxCDABE* transcriptional fusions (Table 3). The only discrepancy was that the expression of *bviI* in PC259 was almost at background levels, whereas a *bviI* RT-PCR product was obviously amplified.

Given that BviR is implicated in the positive regulation of *bviI* and that the expression of *bviR* was lower in the three non-DHL-producing clinical strains, heterologous expression of $bviR_{G4}$ was performed to determine whether the absence of *bviI* expression in these strains was due to a lack of induction by BviR (Fig. 4A). The presence of $bviR_{G4}$ in *trans* restored DHL production in each of the strains (Fig. 4A, lanes 3, 5, and 7), suggesting that the absence of *bviI* expression in the non-DHL-producing strains is due to a hindered induction of *bviI* by BviR.

The *bviIR* loci of the three non-DHL-producing strains and G4 were sequenced to determine whether there were any sequence differences in the non-DHL-producing strains that could lead to a lack of DHL synthesis. The sequence of *bviIR* from DBO1 (accession no. AF296284) (36) was included in the comparison. The BviI sequences were identical in all six strains (data not shown), demonstrating that each strain encodes a functional AHL synthase. The BviR sequences were identical at all but residue 106 (data not shown). This difference was not related to the ability to produce DHL, since C2822 and G4 contained a glycine and FC466 and FC411 contained aspartate at this position. Therefore, *bviR* encodes a functional transcriptional regulator in all strains. There are nucleotide differences in the intergenic region proximal to the *bviI* start codon. The −10 and −35 promoter elements did not contain sequence differences that correlate with *bviI* expression levels. A *lux* box-type consensus sequence, the same in all five strains, was predicted to be centered 56 bp upstream of the *bviI* start codon. A *B. cenocepacia cep* box sequence (69) was predicted to be centered 337 bp upstream of the *bviI* start codon. The predicted *cep* boxes were identical in all strains except for G4.

To determine whether the absence of *bviI* expression in the non-DHL-producing strains is due to sequence differences upstream of *bviI*, expression of $bviI_{G4}$-*luxCDABE* in PC259, FC466, FC441, and C2822 and expression of $bviI_{PC259}$-*luxCDABE* in FC369^T, DBO1, and G4 were examined (Table 3). There was expression of the $bviI_{PC259}$-*luxCDABE* fusion in FC369^T, DBO1, and G4, but there was no detectable $bviI_{G4}$-*luxCDABE* fusion expression in FC466, FC441, and C2822, indicating that the lack of *bviI* expression in these three clinical strains is not due to mutations in the *bviI* promoter region.

It is possible that the lack of DHL production in these three clinical strains may be due to CepR not inducing *bviI* expression, since CepR positively regulates *bviI* in both G4 and PC259. Heterologous expression of $cepR_{G4}$ in FC466, FC441, and C2822 was performed to determine whether CepR is able to induce *bviI* (Fig. 4B). The presence of $cepR_{G4}$ increased the production of HHL and OHL in FC466 (Fig. 4B, compare lanes 1 and 2), but it did not restore DHL production in FC466, FC441, or C2822 (Fig. 4B, lanes 1, 3, and 5), indicating that the lack of DHL is not due to a lack of CepR functioning as a positive regulator of *bviI* in these strains.

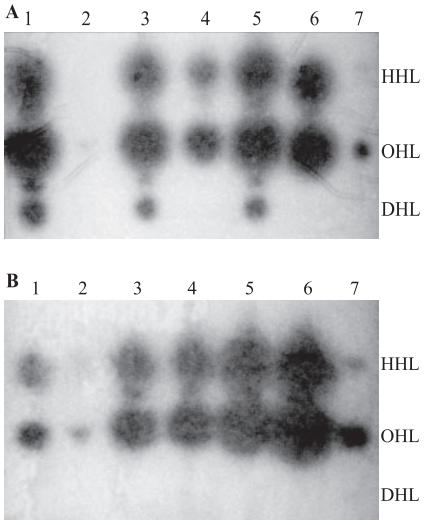

FIG. 4. Heterologous expression of $bviR_{G4}$ and $cepR_{G4}$ in non-DHL-producing clinical strains using TLC-AHL bioassays with the *A. tumefaciens*(pCF218)(pCF372) reporter strain. (A) AHL production profile of non-DHL-producing strains complemented with $bviR_{G4}$ in *trans.* Lane 1, FC466(pRM284); lane 2, FC466(pUCP28T); lane 3, FC411(pRM284); lane 4, FC441(pUCP28T); lane 5, C2822(pRM284); lane 6, C2822(pUCP28T); lane 7, synthetic standards. (B) AHL production profile of non-DHL-producing strains with $cepR_{G4}$ in *trans.* Lane 1, FC466(pRM282); lane 2, FC466(pUCP28T); lane 3, FC411(pRM282); lane 4, FC441(pUCP28T); lane 5, C2822(pRM282); lane 6, C2822(pUCP28T); lane 7, synthetic standards.

Additional LuxI and LuxR homologues were identified in silico by a BLAST search (3) of the incomplete draft G4 genome sequence (http://www.jgi.doe.gov/) with *Vibrio fischeri* LuxI (accession no. 1403259B) (17), LuxR of *V. fischeri* (accession no. 1403259A) (17), *B. vietnamiensis* PC259 CepI (accession no. AAK70355) (36), and *B. vietnamiensis* PC259 CepR (accession no. AAK70352) (36). There are at least three LuxI homologues and five LuxR homologues present in the G4 genome draft sequence. There are three sets of AHL synthase/transcriptional regulator pairs, including *cepIR*, *bviIR*, and an additional system designated open reading frames 7349 and 7350 (http://www.jgi.doe.gov/). There are two LuxR homologues that do not have a proximal AHL synthase and are designated open reading frames 6095 and 3039 (http://genome.ornl.gov/microbial/bcep_1808/).

To determine whether open reading frame 7350 encodes a functional AHL synthase, the 7350 open reading frame was cloned and expressed in *E. coli*. There were no AHLs detected in the culture supernatant of *E. coli*(pRM292), containing 7350, when the *A. tumefaciens* TLC-AHL bioassay was performed under the conditions used to visualize AHLs produced by both *cepI* and *bviI* (data not shown). The presence of the 7349/7350 *luxIR* homologues in other *B. vietnamiensis* strains was determined by PCR with primers that amplified DNA fragments internal to 7349 and 7350. All of the strains except for PC259 and C2822 contained these genes (Table 3).

DISCUSSION

AHL-mediated quorum-sensing systems have complex regulatory organizations (68). The quorum-sensing systems of the BCC species are no exception, since the CepIR system is required for *cciIR* expression in *B. cenocepacia* (39) and we have now demonstrated that CepR is required for the expression of *bviIR* in *B. vietnamiensis*. The initiation of *cepI* expression at least 6 h before *bviI* expression is consistent with the idea that the CepIR system is required for the expression of *bviI*. Phylogenetic analysis established that the *cepIR* system is the ancestral quorum-sensing system for the BCC and that the *bviIR* system is distinct (39); therefore, the *bviIR* system is presumed to have been acquired independently and subsequently incorporated into the CepIR regulatory network. A principal role for the *cepIR* system in *B. vietnamiensis* quorum sensing is contrary to the previous hypotheses that indicated a role for *bviIR* as the principal system based on the AHL production profiles of the G4 *bviI* and *bviR* mutants (10). The differences in results and interpretation may be due to the difference in AHL reporters used to characterize the AHL production profile of the G4 *bviI* and *bviR* mutants in the two studies. Conway and Greenberg (10) used an AHL radiotracer assay and an *E. coli*-based AHL bioassay to examine the AHL production profiles of the *bviI* and *bviR* mutants. The radiotracer assay quantifies the amount of radiolabeled methionine incorporated into AHLs during synthesis and distinguishes the AHLs present by high-performance liquid chromatography (54). The *E. coli* biosensor (pHV200I$^-$) is based on the *V. fischeri luxIR* system and is most sensitive to 3-oxo-HHL (46). The *A. tumefaciens* reporter used in the current study is most sensitive to 3-oxoacyl-homoserine lactones with side chains of 10 or 12 carbons and is able to detect unsubstituted homoserine lactones with side chains ranging from 6 to 12 carbons in length (53). If the assay employed by Conway and Greenberg (10) were as sensitive to HHL and OHL, it is likely that they would have detected greater AHL production by the *bviIR* mutants and a greater role for CepI in AHL production. The Conway and Greenberg study (10) and the current study are in agreement regarding the role of *bviR* in the positive regulation of *bviI*. Autoregulation is common in AHL-mediated quorum-sensing systems to achieve exponential activation of the system (68). Feedback also occurs for the *cepIR* system in PC259, since CepR positively regulates *cepI* and negatively regulates itself. The G4 *cepIR* system, however, did not exhibit autoregulation. There was a marked difference in the AHL production profiles for the *cepR* mutants in PC259 and G4 that is likely due to the differences in the roles of CepR in autoregulation. The difference in the AHL production profiles of the *cepR* mutants could also be due to the 7349/7350 system, which is present in G4 but not present in PC259. Although the 7350 AHL synthase was not shown to produce AHLs under the set of conditions tested, this system could be involved in the quorum-sensing regulatory hierarchy in G4. Another possibility is that G4*cepR* is a merodiploid and therefore could still produce functional copies of CepR that would obscure the effect of a *cepR* mutation.

The AHL production profiles of the quorum-sensing mutants confirmed the transcriptional-analysis experiments. The G4 *cepI* mutant AHL production profile exhibits less HHL and OHL than the wild type, presumably due to the lack of synthesis of these AHLs by CepI; however, the G4 *cepI* mutant did not produce detectable levels of DHL. With less AHL present in the environment of the *cepI* mutant, there would be less of the active AHL-bound form of CepR, leading to a decrease in *bviI* expression and the synthesis of DHL.

It is curious that all of the environmental isolates tested produced DHL but that all of the clinical isolates examined either did not produce DHL or had less *bviI* expression than G4. The ability of BCC strains to produce AHLs has been extensively surveyed, and a clear correlation between the quantity or the type of AHL produced and the origin of the strain has not been demonstrated (11, 26, 72). These three studies employed different methodologies but agree with the current study of the AHLs produced by all strains except FC441, for which one study reported DHL production (72). All 3 environmental isolates collectively assayed in four independent studies produced DHL, whereas 4 out of the 10 clinical isolates either did not produce DHL or did not produce DHL as their predominant AHL (11, 26, 72), suggesting less DHL production in *B. vietnamiensis* clinical strains.

It has been suggested that differences between environmental strains and those which cause infections may occur at the level of the regulation of genes rather than in their presence or absence (45). Heterologous expression of *bviR* from G4 in the clinical non-DHL-producing strains restored DHL production, implicating inefficient induction of *bviI* by BviR as the reason for the absence of DHL production in these three strains. Transcriptional analysis of the G4 *bviI* promoter in the non-DHL-producing strains and of the PC259 *bviI* promoter in the DHL-producing strains determined that the lack of *bviI* expression is not due to mutations in the *bviI* promoter region, suggesting that an unknown upstream regulatory element influences the expression of *bviI*. Heterologous expression of *cepR* from G4 in the clinical non-DHL-producing strains did not restore DHL production; therefore, CepR is not the affected upstream regulatory element. The distribution of the *luxR* homologue 7349 is inconsistent with that of the non-DHL-producing strains, and 7349 is not likely the involved upstream regulator. Like *B. vietnamiensis* G4, *B. pseudomallei* and *B. thailandensis* possess three complete AHL-mediated quorum-sensing systems and two additional *luxR* homologues (61, 63). *B. mallei* possesses two *luxI* and four *luxR* homologues (62). The regulatory networks of these systems have yet to be characterized, but each gene may play a role in the coordinate expression of quorum-controlled genes.

Environmental conditions influence the expression of quorum-sensing networks (50) and could be a factor in the expression of *bviI* and the production of DHL in the clinical non-DHL-producing strains. Expression of *bviI* and DHL production were greater when cultures were grown at 30°C than at 37°C (data not shown). Efforts were made to identify culture conditions for *bviI* expression in the non-DHL-producing strains, yet none were found. However, the possibility that *bviI* can be expressed in these strains under specific environmental conditions should be acknowledged.

This study has resulted in further characterization of the *B. vietnamiensis* quorum-sensing regulatory network, yet little is known about what genes are regulated by these systems. Since the G4 *bviIR* mutants retain the ability to produce HHL and OHL, it is believed that the *cepIR* system and potentially the

7349/7350 system are still functional in these mutants and compensate for mutations in *bviIR*, making it difficult to detect phenotypic differences in the mutants. It might be possible to detect quorum-sensing-regulated phenotypes by constructing double or triple mutants; however, the construction of such mutants of *B. vietnamiensis* proves difficult, as this species is less amenable to genetic manipulation than other BCC species. There is a possibility of analyzing the effect of the absence of AHLs in *B. vietnamiensis* strains by employing a quorum-quenching approach, as suggested by Wopperer et al. (72). With this approach, Wopperer et al. (72) determined that *aidA*, a *cepIR*-regulated gene of unknown function that is involved in nematode virulence (28), is also regulated by quorum sensing in *B. vietnamiensis* strains. The quorum-quenching approach, however, does not make it possible to distinguish which quorum-sensing system is involved in the regulation of *aidA* in *B. vietnamiensis*.

Quorum-sensing systems in *B. cepacia* and *B. cenocepacia* regulate many physiological processes (19, 65). Proteomic (48) and transcriptional (58) analyses of quorum-sensing mutants of *B. cenocepacia* have been successful in identifying numerous quorum-sensing-regulated genes. With further development of molecular tools for *B. vietnamiensis*, including a complete genome sequence, the role of quorum sensing in regulating environmentally beneficial phenotypes as well as pathogenic traits may be elucidated.

ACKNOWLEDGMENTS

This study was supported by a grant from the Canadian Cystic Fibrosis Foundation to P. A. Sokol. R. J. Malott is the recipient of a studentship from the Alberta Heritage Foundation for Medical Research.

We thank T. Louie for providing *M. luteus*.

REFERENCES

1. **Aguilar, C., I. Bertani, and V. Venturi.** 2003. Quorum-sensing system and stationary-phase sigma factor (*rpoS*) of the onion pathogen *Burkholderia cepacia* genomovar I type strain, ATCC 25416. Appl. Environ. Microbiol. **69:**1739–1747.
2. **Aguilar, C., A. Friscina, G. Devescovi, M. Kojic, and V. Venturi.** 2003. Identification of quorum-sensing-regulated genes of *Burkholderia cepacia*. J. Bacteriol. **185:**6456–6462.
3. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
4. **Ausubel, F., R. Brent, R. Kingston, D. Moore, J. Seidman, J. Smith, and K. Struhl.** 1989. Current protocols in molecular biology, vol. 1. John Wiley & Sons, Inc., New York, NY.
5. **Baldwin, A., P. A. Sokol, J. Parkhill, and E. Mahenthiralingam.** 2004. The *Burkholderia cepacia* epidemic strain marker is part of a novel genomic island encoding both virulence and metabolism-associated genes in *Burkholderia cenocepacia*. Infect. Immun. **72:**1537–1547.
6. **Bernier, S. P., L. Silo-Suh, D. E. Woods, D. E. Ohman, and P. A. Sokol.** 2003. Comparative analysis of plant and animal models for characterization of *Burkholderia cepacia* virulence. Infect. Immun. **71:**5306–5313.
7. **Bernier, S. P., and P. A. Sokol.** 2005. Use of suppression-subtractive hybridization to identify genes in the *Burkholderia cepacia* complex that are unique to *Burkholderia cenocepacia*. J. Bacteriol. **187:**5278–5291.
8. **Chang, H.-K., and G. J. Zylstra.** 1998. Novel organization of the genes for phthalate degradation from *Burkholderia cepacia* DBO1. J. Bacteriol. **180:** 6529–6537.
9. **Chiarini, L., A. Bevivino, C. Dalmastri, S. Tabacchioni, and P. Visca.** 2006. *Burkholderia cepacia* complex species: health hazards and biotechnological potential. Trends Microbiol. **14:**277–286.
10. **Conway, B.-A., and E. P. Greenberg.** 2002. Quorum-sensing signals and quorum-sensing genes in *Burkholderia vietnamiensis*. J. Bacteriol. **184:**1187–1191.
11. **Conway, B.-A. D., V. Venu, and D. P. Speert.** 2002. Biofilm formation and acyl homoserine lactone production in the *Burkholderia cepacia* complex. J. Bacteriol. **184:**5678–5685.
12. **Daniels, R., D. E. De Vos, J. Desair, G. Raedschelders, E. Luyten, V. Rosemeyer, C. Verreth, E. Schoeters, J. Vanderleyden, and J. Michiels.** 2002. The *cin* quorum sensing locus of *Rhizobium etli* CNPAF512 affects growth and symbiotic nitrogen fixation. J. Biol. Chem. **277:**462–468.
13. **Dennis, J. J., and P. A. Sokol.** 1995. Electrotransformation of *Pseudomonas*. Methods Mol. Biol. **47:**125–133.
14. **Dennis, J. J., and G. J. Zylstra.** 1998. Plasposons: modular self-cloning minitransposon derivatives for rapid genetic analysis of gram-negative bacterial genomes. Appl. Environ. Microbiol. **64:**2710–2715.
15. **DeShazer, D., D. M. Waag, D. L. Fritz, and D. E. Woods.** 2001. Identification of a *Burkholderia mallei* polysaccharide gene cluster by subtractive hybridization and demonstration that the encoded capsule is an essential virulence determinant. Microb. Pathog. **30:**253–269.
16. **Detsika, M. G., J. E. Corkill, M. Magalhães, K. J. Glendinning, C. A. Hart, and C. Winstanley.** 2003. Molecular typing of, and distribution of genetic markers among, *Burkholderia cepacia* complex isolates from Brazil. J. Clin. Microbiol. **41:**4148–4153.
17. **Devine, J. H., C. Countryman, and T. Baldwin.** 1988. Nucleotide sequence of the *luxI* and *luxR* genes and structure of the primary regulatory region of the *lux* regulon of *Vibrio fischeri* ATCC 7744. Biochemistry **27:**837–842.
18. **Duan, K., C. Dammel, J. Stein, H. Rabin, and M. G. Surette.** 2003. Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication. Mol. Microbiol. **50:**1477–1491.
19. **Eberl, L.** 2006. Quorum sensing in the genus *Burkholderia*. Int. J. Med. Microbiol. **296:**103–110.
20. **Estrada-De Los Santos, P., R. Bustillos-Cristales, and J. Caballero-Mellado.** 2001. *Burkholderia*, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. Appl. Environ. Microbiol. **67:** 2790–2798.
21. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
22. **Fuqua, C., and E. P. Greenberg.** 2002. Listening in on bacteria: acyl-homoserine lactone signalling. Nat. Rev. Mol. Cell Biol. **3:**685–695.
23. **Fuqua, C., and S. C. Winans.** 1996. Conserved *cis*-acting promoter elements are required for density-dependent transcription of *Agrobacterium tumefaciens* conjugal transfer genes. J. Bacteriol. **178:**435–440.
24. **Gillis, M., T. Van Van, R. Bardin, M. Goor, P. Hebbar, A. Willems, P. Segers, K. Kersters, T. Heulin, and M. P. Fernandez.** 1995. Polyphasic taxonomy in the genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamiensis* sp. nov. for N_2-fixing isolates from rice in Vietnam. Int. J. Syst. Bacteriol. **45:**274–289.
25. **Gingues, S., C. Kooi, M. B. Visser, B. Subsin, and P. A. Sokol.** 2005. Distribution and expression of the ZmpA metalloprotease in the *Burkholderia cepacia* complex. J. Bacteriol. **187:**8247–8255.
26. **Gotschlich, A., B. Huber, O. Geisenberger, A. Togl, A. Steidle, K. Riedel, P. Hill, B. Tummler, P. Vandamme, B. Middleton, M. Camara, P. Williams, A. Hardman, and L. Eberl.** 2001. Synthesis of multiple *N*-acylhomoserine lactones is wide-spread among the members of the *Burkholderia cepacia* complex. Syst. Appl. Microbiol. **24:**1–14.
27. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77–86.
28. **Huber, B., F. Feldmann, M. Köthe, P. Vandamme, J. Wopperer, K. Riedel, and L. Eberl.** 2004. Identification of a novel virulence factor in *Burkholderia cenocepacia* H111 required for efficient slow killing of *Caenorhabditis elegans*. Infect. Immun. **72:**7220–7230.
29. **Huber, B., K. Riedel, M. Hentzer, A. Heydorn, A. Gotschlich, M. Givskov, S. Molin, and L. Eberl.** 2001. The *cep* quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility. Microbiology **147:**2517–2528.
30. **Kothe, M., M. Antl, B. Huber, K. Stoecker, D. Ebrecht, I. Steinmetz, and L. Eberl.** 2003. Killing of *Caenorhabditis elegans* by *Burkholderia cepacia* is controlled by the *cep* quorum-sensing system. Cell. Microbiol. **5:**343–351.
31. **Larsen, G. Y., T. L. Stull, and J. L. Burns.** 1993. Marked phenotypic variability in *Pseudomonas cepacia* isolated from a patient with cystic fibrosis. J. Clin. Microbiol. **31:**788–792.
32. **Lewenza, S., B. Conway, E. P. Greenberg, and P. A. Sokol.** 1999. Quorum sensing in *Burkholderia cepacia*: identification of the LuxRI homologs CepRI. J. Bacteriol. **181:**748–756.
33. **Lewenza, S., and P. A. Sokol.** 2001. Regulation of ornibactin biosynthesis and *N*-acyl-L-homoserine lactone production by CepR in *Burkholderia cepacia*. J. Bacteriol. **183:**2212–2218.
34. **Lewenza, S., M. B. Visser, and P. A. Sokol.** 2002. Interspecies communication between *Burkholderia cepacia* and *Pseudomonas aeruginosa*. Can. J. Microbiol. **48:**707–716.
35. **LiPuma, J. J., T. Spilker, L. H. Gill, P. W. Campbell III, L. Liu, and E. Mahenthiralingam.** 2001. Disproportionate distribution of *Burkholderia cepacia* complex species and transmissibility markers in cystic fibrosis. Am. J. Respir. Crit. Care Med. **164:**92–96.
36. **Lutter, E., S. Lewenza, J. J. Dennis, M. B. Visser, and P. A. Sokol.** 2001.

Distribution of quorum-sensing genes in the *Burkholderia cepacia* complex. Infect. Immun. **69:**4661–4666.
37. **Mahenthiralingam, E., T. Coenye, J. W. Chung, D. P. Speert, J. R. W. Govan, P. Taylor, and P. Vandamme.** 2000. Diagnostically and experimentally useful panel of strains from the *Burkholderia cepacia* complex. J. Clin. Microbiol. **38:**910–913.
38. **Mahenthiralingam, E., T. A. Urban, and J. B. Goldberg.** 2005. The multifarious, multireplicon *Burkholderia cepacia* complex. Nat. Rev. Microbiol. **3:**144–156.
39. **Malott, R. J., A. Baldwin, E. Mahenthiralingam, and P. A. Sokol.** 2005. Characterization of the *cciIR* quorum-sensing system in *Burkholderia cenocepacia*. Infect. Immun. **73:**4982–4992.
40. **Meyer, J. M., and M. A. Abdallah.** 1978. The fluorescent pigment of *Pseudomonas fluorescens:* biosynthesis, purification and physico-chemical properties. J. Gen. Microbiol. **107:**319–328.
41. **Mohr, C. D., M. Tomich, and C. A. Herfst.** 2001. Cellular aspects of *Burkholderia cepacia* infection. Microbes Infect. **3:**425–435.
42. **Moore, R. A., S. Reckseidler-Zenteno, H. Kim, W. Nierman, Y. Yu, A. Tuanyok, J. Warawa, D. DeShazer, and D. E. Woods.** 2004. Contribution of gene loss to the pathogenic evolution of *Burkholderia pseudomallei* and *Burkholderia mallei*. Infect. Immun. **72:**4172–4187.
43. **Nelson, M. J. K., S. O. Montgomery, W. R. Mahaffey, and P. H. Pritchard.** 1987. Biodegradation of trichloroethylene and involvement of an aromatic biodegradative pathway. Appl. Environ. Microbiol. **53:**949–954.
44. **Park, J., I. Hwang, J. Kim, S. Lee, B. Conway, E. P. Greenberg, and K. Lee.** 2001. Characterization of quorum-sensing signaling molecules produced by *Burkholderia cepacia* G4. J. Microbiol. Biotechnol. **11:**804–811.
45. **Parke, J. L., and D. Gurian-Sherman.** 2001. Diversity of the *Burkholderia cepacia* complex and implications for risk assessment of biological control strains. Annu. Rev. Phytopathol. **39:**225–258.
46. **Pearson, J. P., K. M. Gray, L. Passador, K. D. Tucker, A. Eberhard, B. H. Iglewski, and E. P. Greenberg.** 1994. Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. Proc. Natl. Acad. Sci. USA **91:**197–201.
47. **Reik, R., T. Spilker, and J. J. LiPuma.** 2005. Distribution of *Burkholderia cepacia* complex species among isolates recovered from persons with or without cystic fibrosis. J. Clin. Microbiol. **43:**2926–2928.
48. **Riedel, K., C. Arevalo-Ferro, G. Reil, A. Gorg, F. Lottspeich, and L. Eberl.** 2003. Analysis of the quorum-sensing regulon of the opportunistic pathogen *Burkholderia cepacia* H111 by proteomics. Electrophoresis **24:**740–750.
49. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
50. **Schuster, M., and E. P. Greenberg.** 2006. A network of networks: quorum-sensing gene regulation in *Pseudomonas aeruginosa*. Int. J. Med. Microbiol. **296:**73–81.
51. **Schweizer, H. P.** 1992. Allelic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable *oriT* and the counter-selectable *Bacillus subtilis sacB* marker. Mol. Microbiol. **6:**1195–1204.
52. **Schwyn, B., and J. B. Neilands.** 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160:**47–56.
53. **Shaw, P. D., G. Ping, S. L. Daly, C. Cha, J. E. Cronan, Jr., K. L. Rinehart, and S. K. Farrand.** 1997. Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer chromatography. Proc. Natl. Acad. Sci. USA **94:**6036–6041.
54. **Singh, P. K., A. L. Schaefer, M. R. Parsek, T. O. Moninger, M. J. Welsh, and E. P. Greenberg.** 2000. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature **407:**762–764.
55. **Sokol, P. A., U. Sajjan, M. B. Visser, S. Gingues, J. Forstner, and C. Kooi.** 2003. The CepIR quorum-sensing system contributes to the virulence of *Burkholderia cenocepacia* respiratory infections. Microbiology **149:**3649–3658.
56. **Speert, D. P., M. E. Campbell, D. A. Henry, R. Milner, F. Taha, A. Gravelle, A. G. Davidson, L. T. Wong, and E. Mahenthiralingam.** 2002. Epidemiology of *Pseudomonas aeruginosa* in cystic fibrosis in British Columbia, Canada. Am. J. Respir. Crit. Care Med. **166:**988–993.
57. **Speert, D. P., D. Henry, P. Vandamme, M. Corey, and E. Mahenthiralingam.** 2002. Epidemiology of *Burkholderia cepacia* complex in patients with cystic fibrosis, Canada. Emerg. Infect. Dis. **8:**181–187.
58. **Subsin, B., C. E. Chambers, M. B. Visser, and P. A. Sokol.** 22 November 2006. Identification of genes regulated by the *cepIR* quorum-sensing system in *Burkholderia cenocepacia* by high-throughput screening of a random promoter library. J. Bacteriol. doi:10.1128/JB.01201-06. (Subsequently published, J. Bacteriol. **189:**968–979, 2007.)
59. **Tomlin, K. L., R. J. Malott, G. Ramage, D. G. Storey, P. A. Sokol, and H. Ceri.** 2005. Quorum-sensing mutations affect attachment and stability of *Burkholderia cenocepacia* biofilms. Appl. Environ. Microbiol. **71:**5208–5218.
60. **Tran Van, V., O. Berge, S. Ngo, J. Balandreau, and T. Heulin.** 2000. Repeated beneficial effects of rice inoculation with a strain of *Burkholderia vietnamiensis* on early and late yield components in low fertility sulphate acid soils of Vietnam. Plant Soil **218:**273–284.
61. **Ulrich, R. L., D. Deshazer, E. E. Brueggemann, H. B. Hines, P. C. Oyston, and J. A. Jeddeloh.** 2004. Role of quorum sensing in the pathogenicity of *Burkholderia pseudomallei*. J. Med. Microbiol. **53:**1053–1064.
62. **Ulrich, R. L., D. DeShazer, H. B. Hines, and J. A. Jeddeloh.** 2004. Quorum sensing: a transcriptional regulatory system involved in the pathogenicity of *Burkholderia mallei*. Infect. Immun. **72:**6589–6596.
63. **Ulrich, R. L., H. B. Hines, N. Parthasarathy, and J. A. Jeddeloh.** 2004. Mutational analysis and biochemical characterization of the *Burkholderia thailandensis* DW503 quorum-sensing network. J. Bacteriol. **186:**4350–4360.
64. **Vandamme, P., B. Holmes, M. Vancanneyt, T. Coenye, B. Hoste, R. Coopman, H. Revets, S. Lauwers, M. Gillis, K. Kersters, and J. R. Govan.** 1997. Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients and proposal of *Burkholderia multivorans* sp. nov. Int. J. Syst. Bacteriol. **47:**1188–1200.
65. **Venturi, V., A. Friscina, I. Bertani, G. Devescovi, and C. Aguilar.** 2004. Quorum sensing in the *Burkholderia cepacia* complex. Res. Microbiol. **155:** 238–244.
66. **Walsh, P. S., D. A. Metzger, and R. Higuchi.** 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques **10:**506–513.
67. **Walsh, T. A., and D. P. Ballou.** 1983. Halogenated protocatechuates as substrates for protocatechuate dioxygenase from *Pseudomonas cepacia*. J. Biol. Chem. **258:**14413–14421.
68. **Waters, C. M., and B. L. Bassler.** 2005. Quorum sensing: cell-to-cell communication in bacteria. Annu. Rev. Cell Dev. Biol. **21:**319–346.
69. **Weingart, C. L., C. E. White, S. Liu, Y. Chai, H. Cho, C. S. Tsai, Y. Wei, N. R. Delay, M. R. Gronquist, A. Eberhard, and S. C. Winans.** 2005. Direct binding of the quorum sensing regulator CepR of *Burkholderia cenocepacia* to two target promoters *in vitro*. Mol. Microbiol. **57:**452–467.
70. **West, S. E., H. P. Schweizer, C. Dall, A. K. Sample, and L. J. Runyen-Janecky.** 1994. Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. Gene **148:**81–86.
71. **Woods, D.** 1984. Oligonucleotide screening of cDNA libraries. Focus **6:**1–2.
72. **Wopperer, J., S. T. Cardona, B. Huber, C. A. Jacobi, M. A. Valvano, and L. Eberl.** 2006. A quorum-quenching approach to investigate the conservation of quorum-sensing-regulated functions within the *Burkholderia cepacia* complex. Appl. Environ. Microbiol. **72:**1579–1587.
73. **Zhu, J., J. W. Beaber, M. I. Moré, C. Fuqua, A. Eberhard, and S. C. Winans.** 1998. Analogs of the autoinducer 3-oxooctanoyl-homoserine lactone strongly inhibit activity of the TraR protein of *Agrobacterium tumefaciens*. J. Bacteriol. **180:**5398–5405.