JOURNAL OF BACTERIOLOGY, Apr. 2001, p. 2212–2218 Vol. 183, No. 7
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.7.2212–2218.2001

Regulation of Ornibactin Biosynthesis and *N*-Acyl-L-Homoserine Lactone Production by CepR in *Burkholderia cepacia*

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Received 13 September 2000/Accepted 14 January 2001

The CepR-CepI quorum-sensing system has been shown to regulate production of the siderophore ornibactin, extracellular proteases, and *N*-octanoyl-homoserine-L-lactone (OHL) in *Burkholderia cepacia* strain K56-2. To examine the effect of *cepIR* on production of other siderophores, *cepR* mutants were constructed in strains that produce pyochelin in addition to salicylic acid and ornibactins. Pc715j-R1 (*cepR*::tp) hyperproduced ornibactin but produced parental levels of pyochelin and salicylic acid, suggesting that CepR is a negative regulator of ornibactin synthesis but not pyochelin or salicylic acid. Pc715j-R1 was also protease deficient and OHL negative. The effects of *cepR* on ornibactin biosynthetic genes were examined by constructing *cepR pvdA-lacZ* and *cepR pvdD-lacZ* mutants and monitoring β-galactosidase activity. There was an increase in expression of *pvdA* in the *cepR* mutant compared to the level in its parent strain in both low- and high-iron media during stationary phase. When the outer membrane protein profiles of a *cepR* mutant and the wild-type strain were compared on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, there did not appear to be any difference in levels of expression of the ornibactin receptor. Experiments with *cepI-lacZ* and *cepR-lacZ* transcriptional fusions indicated that *cepI* was not expressed in the *cepR* mutant and that *cepR* acts as a negative regulator of its own expression. By a thin-layer chromatography assay for *N*-acyl homoserine lactones, OHL and *N*-hexanoyl-L-homoserine lactone (HHL) were detectable in K56-2 and Pc715j, both wild-type strains. OHL was not detectable and HHL was only weakly detectable in the *cepI* and *cepR* mutants. These results suggest that CepR is both a positive and negative transcriptional regulator and that CepR may influence the expression of ornibactin biosynthetic genes in addition to the expression of the *cepIR* quorum-sensing system.

Burkholderia cepacia is an opportunistic pathogen that primarily infects immunocompromised patients with cystic fibrosis (CF), chronic granulomatous disease, cancer, or burns or patients with indwelling devices (12). *B. cepacia* infections are a particular cause for concern in CF patients. First, although *B. cepacia* infections have varied outcomes, a significant number of *B. cepacia*-infected patients (20 to 35%) experience cepacia syndrome, which often culminates in death (14, 42). Second, there is increasing epidemiological evidence for patient-to-patient transmission (18). Since several epidemics have been shown to be caused by a single lineage, there is a possibility that some strains have an increased capacity for virulence and transmission (41). Last, the inherent multidrug resistance *of B. cepacia* (27) makes treatment very difficult and eradication almost impossible.

Strains of *B. cepacia* have recently been divided into a group of five distinct genomovars, referred to as the *B. cepacia* complex (43). The term genomovar denotes a group of strains with phenotypic similarity but genotypic uniqueness. Of the genomovars (numbered I through V), genomovars II, IV, and V have been renamed *Burkholderia multivorans, Burkholderia stabilis*, and *Burkholderia vietnamiensis*, respectively (43, 44). The majority of CF-transmissible or epidemic strains belong to *B. cepacia* genomovar III (43, 44).

Quorum sensing is a mechanism for regulating virulence factor production that involves the production and detection of signaling molecules (*N*-acyl homoserine lactones [AHLs]) (for reviews, see references 10, 15, and 28). The *luxI* gene family encodes an autoinducer synthase responsible for generation of the signaling molecule, which accumulates until a threshold concentration is attained. At this point, *luxR*-type transcriptional regulators bind the autoinducer and subsequently activate or repress the expression of target virulence genes. We have recently described a quorum-sensing system consisting of the *cepI* and *cepR* genes in a *B. cepacia* genomovar III strain (17). An *N*-acyl homoserine lactone (HSL) was purified from wild-type but not *cepI* mutant culture supernatants and identified as *N*-octanoyl-L-HSL (OHL). The initial characterization of *cepI* and *cepR* mutants revealed a lack of protease and OHL production, suggesting that protease and OHL production are positively regulated, and increased production of the siderophore ornibactin, suggesting that ornibactin is negatively regulated (17). A 20-bp *lux* box-like sequence that partially overlaps the −35 region of the putative *cepI* promoter was identified, suggesting that CepR binds to the *cepI* promoter to activate *cepI* expression (17). The majority of quorum-sensing systems are known to act as positive activators of target genes, although it is now recognized that LuxR-type proteins can act as transcriptional repressors (6, 45, 46). CepR appears to function as both a positive and a negative regulator of *B. cepacia* genes.

B. cepacia produces at least four types of siderophores: sal-

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icylic acid, pyochelin, cepabactin, and ornibactins (21, 22, 35, 38, 40), with salicylic acid and ornibactins being predominant in clinical isolates (3). Ornibactin-mediated iron acquisition has been shown to be important in both chronic and acute models of respiratory infection (36, 37). Pyochelin production was associated with 62% of clinical isolates, and its production correlated with severe pulmonary disease in CF patients (3, 35). Cepabactin is produced by few clinical isolates (11%) (3). Strain K56-2 produces ornibactins, salicylic acid, and negligible amounts of pyochelin (3). K56-2 *cepR* or *cepI* mutations resulted in hyperproduction of ornibactin but not salicylic acid.

The objectives of the present study were (i) to determine the role of *cepR* in the regulation of other *B. cepacia* siderophores and (ii) to further study the mechanisms of positive and negative regulation of *cepI* and *cepR* target genes in *B. cepacia*. Since the protease gene(s) has not been cloned and characterized, we have examined the role of *cepR* in the regulation of ornibactin biosynthesis and uptake genes and in the regulation of the *cepI* and *cepR* quorum-sensing genes.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. For genetic manipulations, *Escherichia coli* DH5α and *B. cepacia* K56-2 were grown at 37°C in Luria-Bertani (LB) broth (Life Technologies) or Bacto-Terrific broth (1.2% tryptone, 2.4% yeast extract, 0.4% glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4) or on 1.5% LB agar plates. The following concentrations of antibiotics were used when necessary: 100 μg of ampicillin, 15 μg of tetracycline, 20 μg of gentamicin, and 1.5 mg of trimethoprim per ml for *E. coli* and 300 μg of tetracycline and 100 μg of trimethoprim per ml for *B. cepacia*. For ornibactin production and protease and Chrome Azurol S (CAS) assays, cultures were grown in succinate medium supplemented with ornithine (10 mM) (23) at 37°C. For pyochelin and salicylic acid assays, cultures were grown in deferrated CAA medium (35) at 37°C. For β-galactosidase assays of *cepI-lacZ* or *cepR-lacZ* fusions, cultures were grown in tryptic soy broth medium at 37°C. For β-galactosidase assays of *pvdA-lacZ* or *pvdD-lacZ* fusions, cultures were grown in TSBD-C medium (low iron) or TSBD-C supplemented with 5 μM $FeCl_3$ (37). All medium components were products of Difco (Detroit, Mich.) unless otherwise stated.

Construction of *cepR* mutants by allelic exchange. The *cepR* gene in pSLR101 was insertionally inactivated by the introduction of the trimethoprim cassette from p34E-Tp into an internal *Pst*I site (pSLR101-T). The inactivated *cepR* fragment was amplified by PCR using the primers pNOT19-1 (5′-GGCATGCG CAAGGCGATTAAGTTGG-3′) and pNOT19-2 (5′-GGCATGCCTTTATGCT TCCGGCTCG-3′), which incorporate *Sph*I linkers, and cloned into the *Sph*I site within the suicide vector pEX18Tc (13). This construct, designated pEXCEPR, was transferred to *B. cepacia* Pc224c or Pc715j by conjugation using either triparental matings with pRK2013 (7) as the mobilizing plasmid or biparental matings with *E. coli* SM10 as the donor strain (34). Transconjugants were plated onto *Pseudomonas* isolation agar plates containing 100 μg of trimethoprim per ml to select for single-crossover events in *B. cepacia*. Tp^r transconjugants were streaked for isolated colonies on LB agar plates containing 100 μg of trimethoprim per ml and 5% sucrose to select for the loss of the vector sequence and screened to confirm tetracycline sensitivity. The insertional inactivation of *cepR* was confirmed with Southern hybridization analysis.

DNA manipulations. Molecular biology techniques were performed as generally described by Sambrook et al. (29). Restriction enzymes and oligonucleotide primers were purchased from Life Technologies. T4 DNA ligase was purchased from Promega Corp. (Madison, Wis.). Genomic DNA was isolated as described by Ausubel et al. (1). PCR products were cloned using the $pCR^R2.1$ TOPO cloning system as recommended by the manufacturer (Invitrogen, Carlsbad, Calif.). Plasmids were introduced by electroporation using a Gene Pulser (Bio-Rad, Richmond, Calif.) according to the manufacturer's recommendations into *E. coli* and into *B. cepacia* as previously described (4).

Siderophore, protease, and autoinducer bioassays. Siderophore activity was measured using CAS assays (32). On CAS agar, siderophores remove iron from the CAS dye complex, resulting in a blue-to-orange color change in zones surrounding the colonies. The same dye complex was used to quantitate siderophore activity in culture supernatant fluid by measuring the increase in orange color at an optical density at 630 nm (OD_{630}) as previously described (17). Ornibactins were purified by Sephadex LH-20 chromatography and quantitated using the CAS assay as previously described (3, 17). Pyochelin and salicylic acid were isolated and quantitated by thin-layer chromatography (TLC) as previously described (35, 38).

For protease assays, cultures were grown overnight, normalized to a turbidity of 0.3 at 600 nm, and spotted (3 μl) onto dialyzed brain heart infusion (D-BHI) agar containing 1.5% skim milk (39). The plates were incubated for 2 days at 37°C and examined for clear zones surrounding the colonies.

The *cepI* reporter strain K56-I2 does not produce autoinducer (OHL) or protease but responds to OHL produced by test strains inoculated at right angles to the reporter. The ability of test strains to produce autoinducers was detected by the restoration of protease production at the junction of the test and reporter strains on D-BHI–skim milk agar.

Agrobacterium tumefaciens A136 (pCF218)(pCF372) was used as a reporter to detect AHLs with 3-oxo-, 3-hydroxy-, and 3-unsubstituted side chains as previously described (33). Plasmid pCF218 contains *traR*, and pCF372 contains a *traI-lacZ* fusion (9, 49). AHLs were extracted from 20 ml of culture twice with equal volumes of acidified ethyl acetate (0.1 ml of glacial acetic acid per liter). Ethyl acetate was removed by rotary evaporation. The residue was resuspended in 2 ml of ethyl acetate, dried over N_2 gas, and resuspended in 100 μl of acidified ethyl acetate. TLC bioassays were performed as described previously (33) with minor modifications. Samples were spotted onto a 20- by 20-cm C_{18} reversed-phase TLC plate (Whatman) and chromatographed using methanol-water (60:40, vol/vol) as a solvent. The plates were overlaid with a 20-ml *A. tumefaciens* A136 culture grown as follows. A 3-ml overnight culture was subcultured at a 1/100 dilution into 30 ml of LB broth and grown to log phase. Cells were pelleted by centrifugation, resuspended in 20 ml of AT medium (10.7g of KH_2PO_4, 160 mg of $MgSO_4 \cdot 7H_2O$, 10 mg of $CaCl_2 \cdot 2H_2O$, 5 mg of $FeSO_4 \cdot 7H_2O$, 2 mg of $MnSO_4 \cdot 7H_2O$, 2 g of $(NH_4)_2SO_4$, 0.5% glucose, 1 liter of H_20), and incubated for 30 min. This culture was then added to 150 ml of 0.7% AT agar and 60 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside per ml. TLC plates were incubated for 24 to 48 h at 30°C. Synthetic *N*-hexanoyl-HSL (HHL) and OHL (Sigma-Aldrich, Oakville, Ontario, Canada) were used as reference standards.

Construction of *cepR-lacZ* and *cepI-lacZ* transcriptional fusions. The *lacZ*-Gm^r fragment from pZ1918G was ligated into the *Pst*I site of pSLS200, which contains a 2.3-kb *Sph*I-*Pst*I fragment that encodes the entire *cepI* gene and the 5′ end of *cepR*, resulting in a transcriptional *cepR-lacZ* fusion (pSLR111). The *cepI* and *lac* vector promoters are in the orientation opposite to that of the *cepR* promoter, which is solely responsible for *lacZ* expression. A 769-bp fragment containing the *cepI* promoter was PCR amplified using the primers IN-CEPI (5′-GCGGATCCACCAGACGCCCATCTACCTGCTTCG-3′) and EX-CEPI (5′-GCCTGCAGGGCACAACGACGCCTATCATGC-3′), which incorporate *Bam*HI and *Pst*I linkers, respectively. The PCR product was cloned as a *Pst*I-*Bam*HI fragment into pUCP28T (pSLS210). The *lacZ*-Gm^r fragment was ligated into the *Bam*HI site of pSLS210, resulting in the *cepI-lacZ* transcriptional fusion (pSLS222). The *lac* vector promoter is in the opposite direction of the *cepI* promoter, which is solely responsible for *lacZ* expression.

β-Galactosidase activity was measured as previously described (26). Overnight cultures were subcultured in 50 ml of medium containing 100 μg of trimethoprim per ml to an OD_{600} of 0.01. One-milliliter aliquots were removed throughout growth and assayed for enzyme activity as previously described (26). β-Galactosidase activity is expressed in Miller units.

Isolation of outer membrane proteins. Outer membrane proteins were isolated as previously described (33) from cultures grown for 16 to 24 h (stationary phase) after overnight cultures were subcultured in fresh medium to an OD_{600} of 0.01. Protein samples (10 μg) were electrophoresed by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (16).

RESULTS

CepR regulates ornibactin but not pyochelin or salicylic acid production in *B. cepacia*. Previously, the effects of a *cepR*::Tn5-OT182 insertion were determined in strain K56-2, which produces ornibactin and salicylic acid, negligible amounts of pyochelin, and no cepabactin (17). We attempted to construct *cepR* mutants by allelic exchange in other strains that produce pyochelin and cepabactin as well as ornibactin and salicylic acid in order to determine if pyochelin or cepabactin is regulated by CepR. *CepR*::tp mutants were successfully constructed with strains Pc715j and Pc224c, which produce

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
Strains		
E. coli		
DH5α	ϕ80d*lacZ*ΔM15 (*lacZYA-argF*) *recA1 endA gyrA96 thi-1 hsdR17 supE44 relA1 deoR U169*	Life Technologies
SM10	Mobilizing strain, RP4 integrated in chromosome, Km^r	34
HB101	*supE44 hsdS20* (r_B m_B) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	29
B. cepacia		
K56-2	CF respiratory isolate, genomovar III, $BCESM^+$ $cblA^+$	17, 19
K56-R2	*cepR*::Tn*5*-OT182 derivative of K56-2, Tc^r	17
K56-I2	*cepI*::*tp* derivative of K56-2, Tp^r	17
Pc224c	CF respiratory isolate (Cleveland, Ohio)	3
Pc224c-R1	*cepR*::*tp* derivative of Pc224c, Tp^r	This study
Pc715j	CF respiratory isolate, genomovar III	3, 20
Pc715j-R1	*cepR*::*tp* derivative of Pc715j, Tp^r	This study
ATCC 17759	Soil isolate, genomovar I	19
ATCC 25416	Onion isolate, genomovar I	19
P109	CF respiratory isolate (Seattle, Wash.)	3
Pc22-20	Plant isolate	3
34192	CF respiratory isolate (Calgary, Alberta, Canada)	3
34930	CF respiratory isolate (Calgary)	3
T10	*pvdD*::Tn*5*OT182 derivative of K56-2, Tc^r	37
T10-R10	*pvdD*::Tn*5*OT182, *cepR*::*tp* Tc^r Tp^r	This study
I117	*pvdA*::Tn*5*OT182 derivative of K56-2, Tc^r	37
I117-R21	*pvdA*::Tn*5*OT182, *cepR*::*tp* Tc^r Tp^r	This study
A. tumefaciens A136	Ti plasmidless host	C. Fuqua
Plasmids		
pNOT19	Modified pUC19 cloning vector, Ap^r	30
pUCP28T	Broad-host-range vector, IncP OriT, pRO1600 ori, Tp^r	31
p34E-Tp	Source of trimethoprim cassette, Tp^r	5
pRK2013	ColE1 Tra $(RK2)^+$ Km^r	7
pEX18Tc	Suicide vector, *sacB* Tc^r	13
pUCP26	Broad-host-range vector, IncP pRO1600 ori, Tc^r	47
pZ1918G	Source of *lacZ* reporter, Ap^r Gm^r	H. P. Schweizer
pCR^R2.1TOPO	Cloning vector for PCR products	Invitrogen
pSLA3.2	pUCP28T with 3.2-kb *Sph*I fragment containing *cepIR*, Tp^r	17
pSLR101	pNOT19 with 1.6-kb *Kpn*I-*Sph*I fragment from pSLA3.2 containing *cepR*, Ap^r	This study
pSLR101-T	pSLR101 with *tp* cassette cloned in *Pst*I site, Ap^r Tp^r	This study
pSLS200	pUCP28T with 2.3-kb *Sph*I-*Pst*I fragment from pSLA3.2 containing *cepI* and a part of *cepR*, Tp^r	This study
pSLR111	*cepR-lacZ* transcriptional fusion with *lacZ*-Gm^r cassette cloned in *Pst*I site of pSLS200, Tp^r	This study
pSLS210	pUCP28T with 0.8-kb *Pst*I-*BamH*I fragment containing *cepI* promoter, Tp^r	This study
pSLS222	*cepI-lacZ* transcriptional fusion with *lacZ*-Gm^r cassette cloned in *Bam*HI site of pSLS210, Tp^r	This study
pEXCEPR	pEX18Tc with *tp*-inactivated *cepR* fragment from pSLR101-T, Tp^r Ap^r	This study
pCF218	IncP *traR*	49
pCF372	*traI-lacZ*	9

[a] BCESM, *B. cepacia* epidemic strain marker.

ornibactin, salicylic acid, and pyochelin (3), and designated Pc715j-R1 and Pc224c-R1. It was not possible to obtain double-crossover *cepR*::tp recombinants using the following strains: ATCC 25416, ATCC 17759, P109, Pc22-20, 34192, and 34930, which also produce cepabactin (3).

The siderophore, protease, and autoinducer phenotypes of Pc715j-R1 were characterized (i) to confirm the role of CepR in autoinducer and protease production and (ii) to determine if pyochelin synthesis is regulated by CepR. CAS activity was measured in culture fluids throughout the growth of Pc715j and Pc715j-R1 (Fig. 1). Siderophore production is growth phase dependent in that siderophore activity is not observed until late log phase and is maximal in stationary phase. The *cepR* mutant had significantly greater CAS activity than the respective parent strain during stationary phase, indicating that one or more siderophores were being hyperproduced (Fig. 1).

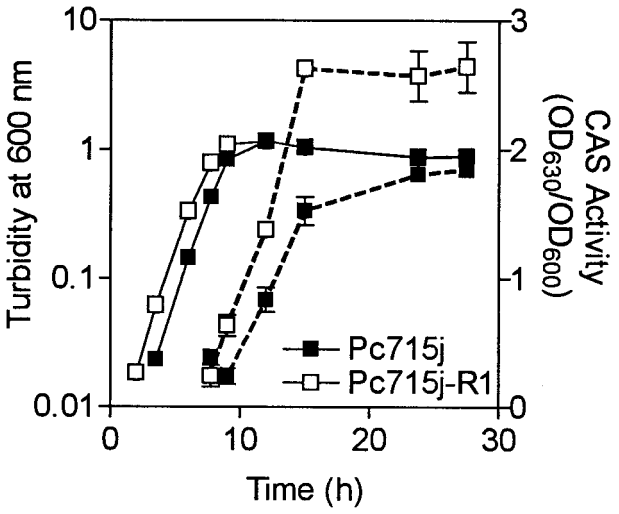

FIG. 1. Effect of *cepR* on CAS activity in *B. cepacia*. CAS activity was monitored throughout growth in succinate medium. ■, Pc715j (wild type); □, Pc715j-R1 (*cepR*). Growth (turbidity at 600 nm) is shown as solid lines, and CAS activity (OD_{630}/OD_{600}) is shown as dashed lines. All values shown are the means ± standard deviations of values from triplicate experiments. The difference between Pc715j and Pc715jR1 is significant at all time points between 9 and 30 h ($P < 0.05$; *t* test for unpaired observations).

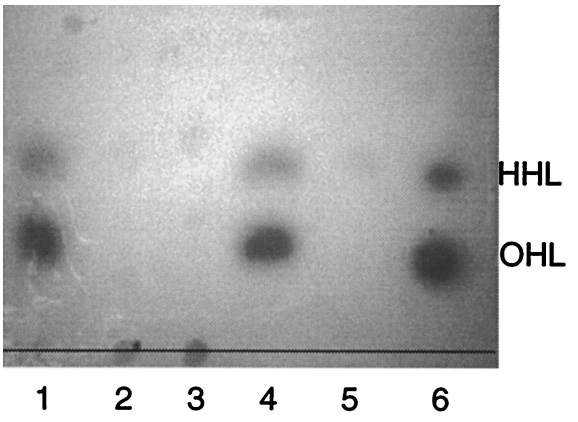

FIG. 2. TLC of acyl-HSLs. Ethyl acetate extracts were chromatographed on C_{18} reversed-phase TLC plates developed with methanol-water (60:40, vol/vol). The spots were visualized using the *A. tumefaciens* reporter strain. Lane 1, K56-2; lane 2, K56-I2; lane 3, K56-R2; lane 4, Pc715j; lane 5, Pc715j-R1; lane 6, synthetic OHL and HHL standards.

Consistent with this observation, Pc715j-R1 produced 50%-larger zones on CAS agar than those of Pc715j (Table 2). Since CAS activity is a measure of total siderophore activity, the yields of individual siderophores in culture supernatants were also quantitated. Pc715j-R1 produced parental levels of pyochelin and salicylic acid but nearly 100% more ornibactin than Pc715j (Table 2). These data suggest that CepR is a negative regulator of ornibactin synthesis but not of pyochelin or salicylic acid synthesis.

Interestingly, Pc715j-R1 produced significantly less protease activity than that of the parent strain Pc715j (Table 2), whereas K56-R2 had no detectable protease in this assay. It is possible that Pc715j produces more than one protease, only one of which is regulated by CepR. Pc715j did not produce detectable autoinducer activity in the bioassay with the *cepI* reporter K56-12 (Table 2), indicating that *cepR* expression is required for OHL production. The phenotypes observed in the *cepR* mutant Pc224c-R1 were similar to those of Pc715j-R1 (data not shown).

Using an *A. tumefaciens traI*::*lacZ* reporter assay, K56-2 and Pc715j produced detectable AHLs that comigrated on TLC plates with synthetic OHL and HHL (Fig. 2). The *cepR* mutants, K56-R2 and Pc715j-R1, and the *cepI* mutant, K56-I2, produced very small amounts of HHL, and OHL was only weakly detectable if the plates were incubated for several days. These data indicate that both the *cepI* and *cepR* genes are required for OHL synthesis.

The effect of a *cepR* mutation on genes involved in ornibactin synthesis and uptake. Since ornibactin yields are increased in *cepR* mutants, we hypothesized that ornibactin biosynthetic genes are negatively regulated by CepR at the level of transcription. If our hypothesis is true, the hyperproduction of ornibactin would be a result of the increased expression of ornibactin biosynthetic genes in *cepR* mutants. Previously we described two Tn5-OT182 mutants, I117 and T10, which contain transposon insertions in ornibactin biosynthetic genes (37). Strain I117 has a Tn5-OT182 insertion in the *pvdA* gene that encodes L-ornithine N^5-oxygenase, which catalyzes the hydroxylation of L-ornithine, forming the hydroxamate ligand in the peptide moiety of ornibactin. T10 contains an insertion in the *pvdD* gene that shares homology with nonribosomal peptide synthetases (37). The Tn5-OT182 mutants I117 and T10 contain chromosomally borne transcriptional *lacZ* fusions

TABLE 2. Effect of a *cepR* mutation on siderophore, protease, and autoinducer production[a]

Strain	Genotype	Length of CAS agar zones[b] (mm)	Ornibactin[c] (μg/ml/OD_{600} unit)	Pyochelin[d] (μg/ml/OD_{600} unit)	Salicylic acid[d] (μg/ml/OD_{600} unit)	Radius of protease zone[e] (mm)	*cepI* bioassay result[f]
Pc715j	Wild type	7.3 ± 0.3	53.9 ± 20.5	5.4 ± 0.8	0.5 ± 0.2	8.3 ± 0.3	+
Pc715j-R1	*cepR*::*tp*	10.8 ± 0.3*	99.1 ± 19.7	4.1 ± 2.0	0.3 ± 0.2	6.8 ± 0.3*	−

[a] All values are the means of results of triplicate experiments ± standard deviations, unless indicated otherwise. *, value is significantly different from that for the parent Pc715j in an unpaired *t* test ($P < 0.05$).
[b] The CAS zone is the distance from the edge of the orange zone to the edge of the colony after 48 h.
[c] Values are means ± standard deviations of results of duplicate experiments.
[d] Amount of siderophore in CAA cultures grown for 24 h.
[e] The protease zone radius is the distance from the edge of the colony to the edge of the zone of clearance on D-BHI plus 1.5% skim milk agar at 48 h.
[f] Cross-feeds the *cepI* reporter K56-I2 and restores protease production.

TABLE 3. Effect of *cepR* on *pvdA* and *pvdD* expression in *B. cepacia*

Strain	β-Galactosidase activity (Miller units)[a] in:	
	TSBD-C medium	TSBD-C + $FeCl_3$ medium
I117	11,324 ± 548	270 ± 37
I117-R21	13,526 ± 330[b]	439 ± 69[b]
T10	8,290 ± 113	1,469 ± 200
T10-R10	9,678 ± 357	1,986 ± 112[c]

[a] β-Galactosidase activity was determined from stationary-phase cultures (22 h) normalized for absorbance at A_{600}. Values are the means ± standard deviations of results of triplicate assays.
[b] Significantly different than the value for I17 ($P < 0.02$; *t* test for unpaired observations).
[c] Significantly different than the value for T10 ($P < 0.02$; *t* test for unpaired observations).

due to the presence of a promoterless *lacZ* gene within the transposon (37).

We constructed *cepR* mutants by allelic exchange in I117 and T10, which resulted in *cepR pvdA* and *cepR pvdD* mutants, respectively. β-Galactosidase activity was monitored throughout growth in low-iron medium and iron-supplemented medium (5 μM $FeCl_3$) to examine the effects of *cepR* on *pvdA* and *pvdD* expression. During stationary phase there was a 20% increase in *pvdA* expression in I117-R21 in low-iron medium and a 62% increase in *pvdA* expression in iron-supplemented medium compared to levels in I117 ($P < 0.02$, *t* test for unpaired observations) (Table 3). There was a 35% increase in *pvdD* expression in T10-R10 compared to that in T10 in iron-supplemented medium during stationary phase ($P < 0.02$, *t* test for unpaired observations), but there was no significant difference between these strains in low-iron medium. The increase in *pvdA* expression correlates with the increased levels of CAS activity in the *cepR* mutants, K56-R2 and Pc715j-R1 (Table 2). (17).

The *orbA* gene encoding the 78.5-kDa ornibactin outer membrane receptor protein is located downstream of the *pvdA* gene (36). To determine if other genes involved in ornibactin-mediated iron acquisition were regulated by *cepIR*, outer membrane proteins were isolated from K56-I2 (*cepI*::tp) and K56-R2 and compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. There did not appear to be any difference in levels of expression of the ornibactin receptor in the *cepR* and *cepI* mutants (data not shown).

The effect of a *cepR* mutation on *cepI* and *cepR* expression. To determine the effects of CepR on *cepI* and *cepR* expression, transcriptional *cepR-lacZ* and *cepI-lacZ* fusions were constructed in pUCP28T and introduced into K56-2 and K56-R2. β-Galactosidase activity was monitored throughout growth (Fig. 3). In K56-2, the levels of expression of both *cepR* and *cepI* were low in early log phase but increased rapidly during late log and stationary phase (Fig. 3). This expression pattern is typical of quorum-sensing-regulated genes. Two important observations were made regarding *cepIR* expression in a *cepR* mutant background. In K56-R2, *cepR* expression increased twofold in the absence of functional *cepR*, indicating that *cepR* is autoregulated. *cepI* expression was abolished in the absence of functional *cepR*, indicating that *cepI* expression is positively regulated by CepR at the level of transcription (Fig. 3). Therefore, *cepR* is involved in regulation of both its own and *cepI* expression, in addition to that of genes involved in ornibactin biosynthesis.

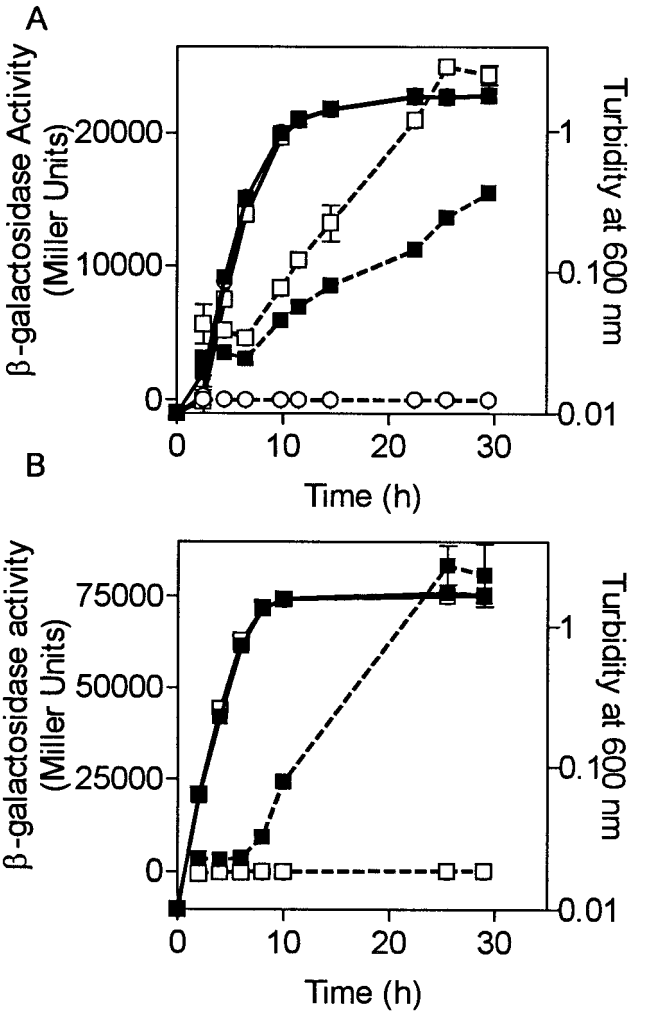

FIG. 3. Effect of *cepR* on *cepR* and *cepI* expression. β-Galactosidase activity was monitored throughout growth in tryptic soy broth plus 100 μg of trimethoprim per ml. (A) ■, K56-2 (pSLR111, *cepR-lacZ*); □, K56-R2 (pSLR111, *cepR-lacZ*); ○, K56-2 (pUCP28T). The difference between K56-2 and K56-R2 is significant at all time points between 4.5 and 29.5 h ($P < 0.02$; *t* test for unpaired observations). (B) ■, K56-2 (pSLS222, *cepI-lacZ*); □, K56-R2 (pSLS222, *cepI-lacZ*). The difference between K56-2 and K56-R2 is significantly different at all time points between 4.5 and 29.5 h ($P < 0.02$; *t* test for unpaired observations). Growth (turbidity at 600 nm) is indicated by solid lines, and β-galactosidase activity (Miller units) is indicated by dashed lines. All values are the means ± standard deviations of values from triplicate experiments.

The expression of the *cepR-lacZ* fusion in K56-2 was examined in cultures grown for 19 h in medium with and without iron supplementation to determine the effect of iron on *cepR* expression. There was 6,956 ± 1,276, 9,644 ± 472, and 10,178 ± 557 Miller units of β-galactosidase activity (values are means ± standard deviations) in cultures of K56-2 (pSLR211) grown in medium with 0, 5, and 10 μM $FeCl_3$, respectively. The level of *cepR* expression was significantly higher in cultures supplemented with iron ($P < 0.05$). This result suggests that

iron increases *cepR* expression, which may lead to higher levels of *pvdA* and *pvdD* repression in the parent strain. This result agrees with the observation that there is a greater effect of a *cepR* mutation on *pvdA* and *pvdD* expression in the presence of iron in the culture medium than in the absence of iron.

DISCUSSION

Analysis of multiple strains and their respective *cepR* mutants has shown that *cepI* and *cepR* comprise a functional quorum-sensing system that consistently regulates protease, ornibactin, OHL, and HHL production. Construction of gene replacement mutants in pyochelin-producing strains made it possible to determine that pyochelin biosynthesis is not regulated by *cepIR*. The effect of *cepIR* on siderophore production appears to be specific for ornibactin biosynthesis. Attempts to construct *cepR*::*tp* mutants with several strains previously reported to produce cepabactin were unsuccessful. Therefore, it was not possible to determine if cepabactin is regulated by *cepIR*. Many strains of the *B. cepacia* complex are not very amenable to genetic manipulations (19). K56-2 was the only previously reported strain to be successfully used for gene replacement studies (17, 19, 37). In this study, we have identified two additional *B. cepacia* complex strains that may be used for gene replacement studies.

We have determined that the hyperproduction of ornibactins in a *cepR* mutant is due to the increased expression of *pvdA* and possibly *pvdD*. These experiments suggest that *cepR* directly regulates ornibactin production at the level of transcription of ornibactin biosynthetic genes, although it is possible that this regulation occurs through an intermediate regulator. Since there are so few examples of negative regulation by quorum-sensing transcriptional regulators, it is difficult to understand the significance of a modest level of repression of ornibactin production by CepR. The negative regulation of ornibactin biosynthesis may serve as a fine-tuning mechanism to reduce energy expended synthesizing ornibactin at high cell densities in an environment in which the concentration of ornibactin is probably sufficient for iron acquisition.

Alternatively, cell density may serve as an additional environmental signal to limit the uptake of iron. The accumulation of intracellular iron can be deleterious due to the formation of hydroxyl radicals in the Haber-Weiss and Fenton reactions (2). When present in high concentrations, iron is the predominant environmental signal leading to the Fur-mediated repression of iron acquisition pathways. It is known that other conditions lead to the downregulation of iron acquisition pathways such as oxidative stress mediated by OxyR and SoxRS (48). The negative regulation of ornibactin production by *cepR*, when cells are growing at high cell densities, regardless of the iron concentration in the medium, may provide an additional level of control of siderophore-mediated uptake.

The roles of *cepR* in the control of the autoinducer synthase gene *cepI* and in its own expression were examined. Previously, we had been unable to detect OHL activity in stationary-phase culture supernatants of the *cepR* mutant K56-R2 (17). In this study using a different reporter assay, we determined that K56-2 and Pc715j produce both OHL and HHL. Production of these two AHLs has also been detected in another strain of *B. cepacia* using a *Chromobacterium violaceum* biosensor TLC assay (11). K56-2 and Pc715j *cepI* and *cepR* mutants produced little if any OHL and HHL, suggesting that *cepI* directs the synthesis of both AHLs and that *cepI* is positively regulated by CepR. This possibility was confirmed by demonstrating that there was no expression of a plasmid-borne *cepI-lacZ* fusion in a *cepR* mutant. Transcriptional fusions on high-copy-number plasmids were used for this study; however, Pesci et al. (24) have shown that *Pseudomonas aeruginosa lasR* expression patterns did not vary when *lasR-lacZ* fusions were expressed on high- or low-copy-number plasmids. This type of positive feedback control of autoinducer production is thought to provide a mechanism of signal amplification in which AHL signals are upregulated, allowing for more activation of the cognate R protein and subsequent activation of virulence genes. Although commonly employed, the positive control of autoinducer production is not an absolute requirement since several *luxI* homologs are not controlled by their respective R proteins (8).

Interestingly, the *cepR* gene was shown to negatively regulate its own expression, but the mechanism of repression is unknown. The repression effect of CepR on *cepR* expression would possibly be greater if expression was monitored from a single-copy chromosomal *cepR-lacZ* reporter since the effects of CepR would not be diluted by additional copies of the *cepR* promoter. *Pantoea stewartii* subsp. *stewartii* (formerly *Erwinia stewartii*) *esaR* is autoregulated in a similar pattern (45). von Bodman and Farrand proposed that the *lux* box overlapping the −10 region in the *esaR* promoter serves as an EsaR binding site that might occlude the RNA polymerase binding site and prevent its own expression (45). In fact, extracts of *E. coli* expressing *esaR* retard the mobility of a 61-bp oligonucleotide containing this *lux* box element (25). Although the significance of autoregulation of LuxR proteins is unknown, it is a possibility that the negative autoregulation of *luxR* genes may function to turn off the quorum-sensing response.

Using transcriptional *lacZ* fusions, we have provided evidence that *cepR* regulates *pvdA* and *cepIR* at the transcriptional level. To prove conclusively that CepR directly regulates these genes, further studies are required to purify CepR and examine its ability to bind to promoter regions of CepR-regulated genes.

ACKNOWLEDGMENTS

This study was supported by a grant from the Canadian Cystic Fibrosis Foundation. S.L. is the recipient of a studentship award from the Alberta Heritage Foundation for Medical Research.

We thank M. B. Visser for excellent technical assistance and D. Storey and D. Erickson for advice on the TLC autoinducer bioassays.

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