Journal of Bacteriology

The Sensor Kinase CbrA Is a Global Regulator That Modulates Metabolism, Virulence, and Antibiotic Resistance in *Pseudomonas aeruginosa*

Amy T. Y. Yeung, Manjeet Bains and Robert E. W. Hancock
J. Bacteriol. **2011, 193(4):918. DOI: 10.1128/JB.00911-10.**
Published Ahead of Print 17 December 2010.

JOURNAL OF BACTERIOLOGY, Feb. 2011, p. 918–931 Vol. 193, No. 4
0021-9193/11/$12.00 doi:10.1128/JB.00911-10

The Sensor Kinase CbrA Is a Global Regulator That Modulates Metabolism, Virulence, and Antibiotic Resistance in *Pseudomonas aeruginosa*▽†

Amy T. Y. Yeung, Manjeet Bains, and Robert E. W. Hancock*

Centre for Microbial Diseases and Immunity Research, University of British Columbia, 2259 Lower Mall, Vancouver, BC, Canada

Received 4 August 2010/Accepted 8 December 2010

Pseudomonas aeruginosa **is an opportunistic pathogen that possesses a large arsenal of virulence factors enabling the pathogen to cause serious infections in immunocompromised patients, burn victims, and cystic fibrosis patients. CbrA is a sensor kinase that has previously been implied to play a role with its cognate response regulator CbrB in the metabolic regulation of carbon and nitrogen utilization in *P. aeruginosa*. Here it is demonstrated that CbrA and CbrB play an important role in various virulence and virulence-related processes of the bacteria, including swarming, biofilm formation, cytotoxicity, and antibiotic resistance. The *cbrA* deletion mutant was completely unable to swarm while exhibiting an increase in biofilm formation, supporting the inverse regulation of swarming and biofilm formation in *P. aeruginosa*. The *cbrA* mutant also exhibited increased cytotoxicity to human lung epithelial cells as early as 4 and 6 h postinfection. Furthermore, the *cbrA* mutant demonstrated increased resistance toward a variety of clinically important antibiotics, including polymyxin B, ciprofloxacin, and tobramycin. Microarray analysis revealed that under swarming conditions, CbrA regulated the expression of many genes, including *phoPQ*, *pmrAB*, *arnBCADTEF*, *dnaK*, and *pvdQ*, consistent with the antibiotic resistance and swarming impairment phenotypes of the *cbrA* mutant. Phenotypic and real-time quantitative PCR (RT-qPCR) analyses of a PA14 *cbrB* mutant suggested that CbrA may be modulating swarming, biofilm formation, and cytotoxicity via CbrB and that the CrcZ small RNA is likely downstream of this two-component regulator. However, as CbrB did not have a resistance phenotype, CbrA likely modulates antibiotic resistance in a manner independent of CbrB.**

Pseudomonas aeruginosa is an important opportunistic human pathogen, causing serious diseases in patients with impaired immunity and mucosal defenses. This Gram-negative bacterium is the dominant pathogen in chronic cystic fibrosis pulmonary infections, persisting in the lungs and inducing serious inflammation that destroys healthy host tissue (1, 39, 42). *P. aeruginosa* infections are particularly difficult to treat due to the bacterium's intrinsic resistance to a broad spectrum of antimicrobial agents and its repertoire of virulence factors (12).

Motility is strongly associated with the virulence of *P. aeruginosa.* It enables the bacterium to colonize different environments, such as those of the lungs of cystic fibrosis patients, and contributes to the ability of the bacterium to attach to and to form biofilms on a variety of biotic and abiotic surfaces (33). *P. aeruginosa* is unusual in that it is capable of three major forms of motility depending on the medium viscosity. *P. aeruginosa* utilizes its single polar flagellum to swim in aqueous environments and at low agar concentrations (<0.3% [wt/vol]). The bacteria also possess type IV pili to enable twitching motility on solid surfaces or interfaces. Besides swimming and twitching, *P. aeruginosa* has recently been observed to swarm on semisolid (viscous) surfaces (0.5 to 0.7% [wt/vol] agar) in a coordinated manner. Swarmer cells are differentiated from vegetative cells in that swarmer cells are elongated and hyperflagellated with two polar flagella (19). This form of motility is induced in *P. aeruginosa* under nitrogen limitation and when certain amino acids, such as glutamate, aspartate, proline, or histidine, are provided as the sole nitrogen source (19). To date, swarming of *P. aeruginosa* has been identified to require the flagella and the type IV pili as well as the production of the biosurfactant rhamnolipids (5, 19, 36, 40, 48).

A recent screen of the *P. aeruginosa* strain PA14 transposon insertion mutant library identified 233 mutants that exhibited alterations in swarming phenotype compared to the wild type (55). The swarming-associated genes functioned not only in flagellum or type IV pilus biosynthesis but also in diverse processes such as transport, secretion, and metabolism. This, together with data demonstrating that hundreds of genes are dysregulated during swarming, including genes encoding most virulence factors, antibiotic resistance loci, and distinct metabolic processes, indicated that swarming is not just a form of motility but rather a complex adaptation and an alternative growth state. Among the mutants with genes that had altered swarming motility were 35 mutants with transposon insertions in genes encoding regulators. Only a few of these regulatory mutants showed significant defects in the production of type IV pili, flagella, or rhamnolipid, each of which is known to be involved in swarming, suggesting that the majority of these regulators control other factors important in swarming (55). Of the 35 transcriptional regulators that were altered in swarming behavior, one interesting mutant with a mutation in the *cbrA* gene was chosen to be investigated in great detail.

* Corresponding author. Mailing address: Centre for Microbial Diseases and Immunity Research, University of British Columbia, 2259 Lower Mall, Vancouver, BC, Canada. Phone: (604) 822-2682. Fax: (604) 827-5566. E-mail: bob@cmdr.ubc.ca.

† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 17 December 2010.

Metabolically versatile pseudomonads effectively utilize a broad range of organic compounds as carbon and/or nitrogen sources. Expression of the components of catabolic pathways involved in utilization of these compounds is subject to catabolite repression (4). In contrast to *Escherichia coli* and *Bacillus subtilis*, succinate and other tricarboxylic acid (TCA) cycle carboxylates are preferable to glucose as carbon sources for *Pseudomonas* spp. Moreover, studies have shown that pseudomonads utilize a different mechanism of catabolite regulation, which involves a Crc protein that binds mRNA and acts as a translational repressor (15, 27, 53). The CbrAB two-component system in *P. aeruginosa* was first identified to be involved in controlling the expression of a number of catabolic pathways involved in carbon and nitrogen utilization (22, 32). Mutations in the sensor kinase CbrA or the response regulator CbrB rendered the bacterium incapable of growing on a variety of organic compounds as the sole carbon source (32). Recently, Sonnleitner et al. discovered that the *P. aeruginosa* genome encodes a small RNA, CrcZ, which binds to and sequesters the Crc protein with high affinity and thus relieves catabolite repression of a variety of degradative genes, such as *amiE* (46). The same group also found that expression of CrcZ is controlled by the CbrAB system.

In addition to its role in metabolism, the CbrAB system has been demonstrated to be involved in the metabolic regulation of the type III secretion system (T3SS) and its effectors, exoenzymes S and T (41). A two-component system, LipQR, was discovered in *Pseudomonas alcaligenes* and demonstrated to be involved in the regulation of lipase expression (20). The LipQR system exhibits significant homology to the CbrAB system in *P. aeruginosa* (20). Analysis of the transcriptome profile of *P. aeruginosa* exposed to sublethal concentrations of tobramycin revealed downregulation of the *cbrA gene* (K. N. Kindrachuk, L. Fernandez, M. Bains, and R. E. W. Hancock, submitted for publication). Furthermore, a previous PA14 screen revealed that a *cbrA* mutant is swarming deficient and exhibits hyperbiofilm formation (55). These results led us to propose that CbrA may be involved in substantially more than just catabolite regulation in *P. aeruginosa.*

In this paper, we demonstrate that CbrA is involved in the regulation of not only carbon and nitrogen metabolism but also various virulence and virulence-related processes in *P. aeruginosa.* We constructed a *cbrA* deletion mutant and showed that this mutant displayed swarming deficiency while exhibiting increased biofilm formation and *in vitro* cytotoxicity toward human bronchial epithelial (HBE) cells. The *cbrA* mutant also demonstrated increased resistance to a variety of clinical antibiotics. Microarray analysis of the *cbrA* mutant provided insight into the basis for these observed phenotypes. Based on detailed phenotypic and genetic studies of the *cbrB* mutant, we propose that CbrA most likely modulates swarming, biofilm formation, and cytotoxicity via the response regulator CbrB while CbrA may cross talk with another regulatory system to modulate antibiotic resistance.

MATERIALS AND METHODS

Tissue culture, bacterial strains, and growth conditions. Bacterial strains and plasmids used in this study are described in Table 1. Cultures were routinely grown in Luria-Bertani (LB) broth, tryptone broth (10 g/liter Bacto tryptone), BM2 minimal medium [62 mM potassium phosphate buffer, pH 7, 7 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 10 μM $FeSO_4$, 0.4% (wt/vol) glucose], or BM2-swarming medium comprising BM2 with 0.1% (wt/vol) Casamino Acids substituted for 7 mM $(NH_4)_2SO_4$. *Escherichia coli* S17-1 λ*pir* was used as the donor strain in bacterial conjugations (45). *P. aeruginosa* competent cells were prepared as previously described (3). For plasmid or transposon selection or maintenance, antibiotics were added to growth media at the indicated concentrations: *E. coli*, 10 μg/ml gentamicin and 100 μg/ml ampicillin; *P. aeruginosa*, 30 μg/ml gentamicin, 100 μg/ml tetracycline, and 500 μg/ml carbenicillin.

The simian virus 40 (SV40)-transformed, immortalized human bronchial epithelial (HBE) cell line 16HBE14o- was a gift from D. Gruenert (University of California, San Francisco, CA). 16HBE14o- cells were cultured in minimum essential medium (MEM) with Earle's salts (Life Technologies Invitrogen), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies Invitrogen) and 2 mM L-glutamine (Life Technologies Invitrogen). 16HBE14o- cells were routinely cultured to 85 to 90% confluence in 100% humidity and 5% CO_2 at 37°C and were used between passages 9 and 15.

General DNA manipulations. Routine genetic manipulations were carried out using standard procedures (43). Primers were synthesized by AlphaDNA Inc. (Montreal, QC, Canada), and their sequences are available from us on request. Plasmid DNA was isolated using QIAprep spin miniprep kits (Qiagen Inc., Mississauga, ON, Canada), and agarose gel fragments were purified using a QIAquick gel extraction kit (Qiagen). Restriction endonucleases were from New England BioLabs (Mississauga, ON, Canada). DNA sequencing was carried out by the UBC NAPS unit.

Recombinant DNA manipulations. For construction of a *cbrA* deletion mutant, an in-frame deletion of *cbrA* was obtained via splicing by an overlap extension PCR strategy (17). Briefly, primers were designed to amplify the gentamicin cassette of pPS858. Primers were also designed to amplify approximately 1-kb fragments located upstream and downstream of *cbrA* from PA14 genomic DNA with additional short sequences of overlap with the gentamicin cassette. Next, the three DNA fragments were fused together and the final product was boosted by a third PCR. The resulting fragment was cloned into pEX18Ap carrying a *sacB* sucrose sensitivity gene (16). This plasmid was transformed into *E. coli* S17-1 λ*pir* and conjugated into *P. aeruginosa* PA14 to generate an in-frame deletion of the *cbrA* gene in the PA14 strain by allelic exchange. Selection for double recombinants was performed on plates containing gentamicin and 5% (wt/vol) sucrose. The deletion was confirmed by PCR and sequencing.

The suicide vector pME9673, obtained from Dieter Haas's laboratory, contained the deleted *crcZ* promoter and the 5′ region of *crcZ* (46). To construct a *crcZ* deletion mutant, a *crcZ* deletion was introduced from plasmid pME9673 into the chromosomal *crcZ* locus of *P. aeruginosa* strain PA14 by gene replacement as described previously (46). Briefly, the plasmid pME9673 was mobilized from *E. coli* DH5α into *P. aeruginosa* PA14 with the help of *E. coli* HB101/pRK2013. PA14 transconjugates carrying a chromosomally integrated copy of pME9673 were selected on tetracycline. Excision of the vector by a second crossover (i.e., tetracycline-sensitive derivatives) was subsequently obtained by enrichment with carbenicillin. The chromosomal *crcZ* deletion was confirmed by PCR and sequencing.

The PA14 *cbrA* deletion mutant and *cbrB* and *crc* transposon mutants were complemented by amplifying *cbrAB*, *cbrB*, or *crc*, including an upstream region of 400 base pairs, from PA14 genomic DNA by PCR and cloning each fragment into the broad-host-range vector pUCP18 or pUCP19 (50). The resulting hybrid plasmids, pUCP18::*cbrAB*$^+$, pUCP19::*cbrB*$^+$, and pUCP18::*crc*$^+$, were transferred into the *cbrA*, *cbrB*, and *crc* mutants, respectively, by electroporation (3).

The *crcZ* deletion mutant was complemented by chromosomal insertion of a mini-Tn*7* carrying the functional *crcZ*$^+$ gene into the Tn*7* attachment site of the mutant. The suicide plasmid, pME9818, which contained the cloned *crcZ*$^+$ gene including the promoter region, was kindly provided by the laboratory of Dieter Haas (L. Abdou and D. Haas, unpublished data). Briefly, pME9818, carried by host *E. coli* S17-1, was transferred into the PA14 *crcZ* mutant by conjugation. Transposition of the mini-Tn*7* carrying the *crcZ*$^+$ gene into the chromosome of the *crcZ* mutant was facilitated by the *E. coli* SM10λ*pir* helper carrying pUXBF-13. The *P. aeruginosa* strain carrying a *crcZ*$^+$ insertion was selected with gentamicin and chloramphenicol and confirmed by PCR.

Motility experiments. Swimming and twitching of *P. aeruginosa* PA14 wild type (WT) and mutants were examined on LB plates with 0.3% (wt/vol) agar and 1% (wt/vol) agar, respectively. Swarming for PA14 strains was examined on BM2-swarming plates containing BM2 with 0.1% (wt/vol) Casamino Acids substituted for 7 mM $(NH_4)_2SO_4$ and 0.5% (wt/vol) agar. To test the effects of the carbon source on the swarming motility of the *cbrA* and *cbrB* mutants, glucose was replaced with 0.4% (wt/vol) glycerol, 0.4% (wt/vol) mannitol, or 20 mM succinate. For iron complementation studies, Fe(II) sulfate was added to the swarm-

TABLE 1. *P. aeruginosa* strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference and/or source
Strains		
P. aeruginosa		
PA14 (WT)	Wild-type *P. aeruginosa* PA14	23
cbrA mutant	PA14 transposon insertion mutant, ID33836	23
Δ*cbrB* mutant	PA14 transposon insertion mutant, ID44074	23
Δ*crc* mutant	PA14 transposon insertion mutant, ID44185	23
Δ*cbrA* mutant	Δ*cbrA* chromosomal deletion mutant of PA14; Gm^r	This study
Δ*crcZ* mutant	Δ*crcZ* chromosomal deletion mutant of PA14	This study
cbrA/B^+ strain	Δ*cbrA* mutant with pUCP18::*cbrAB*$^+$; Cb^r	This study
cbrB$^+$ strain	Δ*cbrB* mutant with pUCP19::*cbrB*$^+$; Cb^r	This study
crc$^+$ strain	Δ*crc* mutant with pUCP18::*crc*$^+$; Cb^r	This study
crcZ$^+$ strain	Δ*crcZ* mutant with *crcZ*$^+$::mini-Tn*7* chromosomal integration; Gm^r	This study
E. coli		
TOP10	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) *recA1 ara*Δ*139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG* ϕ80*lacZ*ΔM15Δ*lacX74*	Invitrogen
DH5α	F^- ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44* λ^- *thi-1 gyrA96 relA*	Invitrogen; 46
S17-1	*galU galK rpsL*(Str^r) *endA1 nupG thi pro hsdR hsdM*$^+$ *recA* (RP4-2Tc::Mu Km::Tn*7*) λ*pir*	45, 46
HB101	*thi-1 hsdS20*(r_B^- m_B^-) *supE44 recA13 ara-14 leuB6 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20*	46
SM10/λ*pir*	*thi-1 thr-1 leu-6 tonA21 lacY1 supE44 recA* chromosomal RP4-2 [Tc^r::Mu Km^r::Tn*7*]λ*pir*	46
Plasmids		
pUCP18, pUCP19	*E. coli-Pseudomonas* shuttle vector, Ap^r Cb^r	50
pUCP18::*cbrAB*$^+$	pUCP18 with *cbrAB* fragment	This study
pUCP19::*cbrB*$^+$	pUCP19 with *cbrB* fragment	This study
pUCP18::*crc*$^+$	pUCP18 with *crc* fragment	This study
pEX18Ap	Suicide plasmid carrying *sacBR*, Ap^r	16
pME3087	Suicide vector, ColE1 replicon, Mob; Tc^r	46
pME9673	pME3087 with a 160-bp deletion in *crcZ*	46
pRK2013	Helper plasmid, ColE1 replicon, Tra; Km^r	46
pME3280a	Chromosomal integration vector, mini-Tn*7*; Gm^r Ap^r	46
pME9818	pME3280a with *crcZ* in mini-Tn*7*	Laetitia Abdou
pUXBF-13	Helper plasmid containing Tn*7* transposition functions, R6K replicon; Ap^r	46

[a] Ap, ampicillin; Cb, carbenicillin; Gm, gentamicin; Km, kanamycin; Tc, tetracycline.

ing medium to a final concentration of 100 μM. Swimming and swarming motility were assayed by spotting 1 μl of mid-logarithmic-growth-phase cultures grown in LB broth or BM2-swarming medium onto the respective motility plates. Twitching motility was assessed by stab-inoculating a single colony grown overnight on LB broth at 37°C. The resultant diameters of the swim or twitch zones were measured after incubation for 20 h and 24 h at 37°C. The resultant swarming colonies were analyzed by measuring the surface coverage on agar plates after 20 h at 37°C. For each form of motility, 3 independent experiments were performed with 3 replicates for each mutant.

Biofilm assays and rapid attachment. Biofilm formation was analyzed using an abiotic solid-surface assay as described elsewhere (9, 34). Overnight cultures were diluted in BM2-biofilm medium [62 mM potassium phosphate buffer, pH 7, 7 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 10 μM $FeSO_4$, 0.4% (wt/vol) glucose, 0.5% (wt/vol) Casamino Acids] in polystyrene microtiter plates (Falcon) and incubated at 8 h or 20 h at 37°C to study the initiation and mature biofilm formation, respectively. To test the effects of the carbon source on biofilm formation of the *cbrA* and *cbrB* mutants, glucose was replaced with 0.4% (wt/vol) glycerol, 0.4% (wt/vol) mannitol, or 20 mM succinate. For iron complementation studies, Fe(II) sulfate was added to the biofilm medium to a final concentration of 100 μM. Biofilms were stained with crystal violet, and absorbance was measured at 600 nm using a microtiter plate reader (Bio-Tek Instruments Inc.).

Rapid attachment was assayed as described previously with modifications (34). Overnight cultures were first diluted 1/100 into fresh BM2-biofilm medium and grown to an optical density at 600 nm (OD_{600}) of 0.5, and 100 μl was added to each well of a 96-well polystyrene microtiter plate. Cells were allowed to attach for 30 min at room temperature prior to staining with crystal violet as described above.

CR assay. Congo red (CR) binding assays were performed as previously described (9). Briefly, tryptone-grown overnight cultures were diluted to OD_{600}s of 0.025 and 1, 5, and 10 μl were spotted onto CR plates (10 g/liter tryptone broth with 10 g/liter agar, 40 μg/ml Congo red, and 20 μg/ml Coomassie brilliant blue). The plates were incubated for 24 h at 37°C, followed by 48 h at room temperature to assess colony morphology.

MIC determination and killing experiments. MICs were measured using standard broth microdilution procedures (26) in BM2-swarming medium. Growth was scored following 24 h of incubation at 37°C. For measuring MICs against polymyxin B, a modified assay was used to prevent artificially high MICs due to aggregation of the antibiotic and binding to polystyrene (52).

To perform killing experiments, cells of *P. aeruginosa* were grown to an OD_{600} of 0.5 in BM2-swarming liquid medium or on BM2-swarming agar plates for 18 h (37). These cultures were diluted into 1× BM2-salts containing 1 μg/ml polymyxin B sulfate (Sigma). Samples were shaken at 37°C, and aliquots were taken at specified times, plated for survivors on LB agar, and grown overnight at 37°C. All experiments were repeated at least 3 times.

Cytotoxicity assays. For the interaction assay, 16HBE14o- cells were seeded in 96-well plates (Corning Life Science, Corning, NY) at a density of 2×10^4 cells/well and grown at 37°C with 5% CO_2 until 100% confluent (~48 h). Bacteria were grown in LB broth until mid-logarithmic phase, washed with phosphate-buffered saline, and resuspended and diluted in MEM containing 1% FBS and 2 mM L-glutamine. The interaction assay was performed at a multi-

plicity of infection (MOI) of 2 bacteria/cell in MEM containing 1% FBS and 2 mM L-glutamine, and the assay mixture was incubated at 37°C with 5% CO_2. At postinfection time points, medium was removed from the wells, placed in microtiter plates, and spun for 10 min at 3,000 rpm to pellet the bacteria and host cell debris. The level of lactate dehydrogenase (LDH) in the supernatant was then assayed in triplicate using a colorimetric cytotoxicity detection kit (Roche, Mannheim, Germany). As a positive control for maximum LDH release, cells were treated with 1% Triton X-100 (Sigma, Oakville, Canada), resulting in complete cell lysis, while untreated cells were used to assess background LDH release.

Growth curves. *P. aeruginosa* mutants and wild type were grown overnight in LB medium, BM2-swarming medium, or BM2-biofilm medium. If necessary, cultures were diluted to obtain equal optical densities. Five-microliter portions of these cultures were added to 195 μl of fresh medium in 96-well microtiter plates. The growth of these cultures at 37°C under shaking conditions was monitored with a Tecan Spectrofluor Plus by determining the absorbance at 620 nm every 20 min for 24 h. Two independent experiments were performed with 3 replicates for each mutant.

Real-time quantitative PCR (RT-qPCR). Total RNAs from the *cbrA*, *cbrB*, *crcZ*, and *crc* mutants were harvested under various conditions as follows: (i) for the swarming condition, cells were obtained from the leading edge of the dendritic swarm colonies of the PA14 wild type and the entire nonswarming colonies of the PA14 *cbrA* transposon mutant (ID33836), the *cbrA* deletion mutant, the *cbrB* transposon mutant, and the *crcZ* deletion mutant; (ii) for the polysaccharide synthesis-inducing condition, cells for RNA isolation were obtained by spotting 10 μl of diluted cultures grown in tryptone broth onto CR plates without Congo red or Coomassie brilliant blue; (iii) for the HBE cell infection condition, 16HBE14o- cells were seeded in tissue culture-treated petri dishes (Corning Life Science, Corning, NY) at a density of 2×10^4 cells/well and grown at 37°C with 5% CO_2 until 100% confluent (~48 h). Bacteria were grown in LB medium until mid-logarithmic phase, washed with phosphate-buffered saline, and resuspended and diluted in MEM containing 1% FBS and 2 mM L-glutamine. The interaction assay was performed at an MOI of 100 bacteria/cell in MEM containing 1% FBS and 2 mM L-glutamine, and the assay mixture was incubated at 37°C with 5% CO_2. At 4 h postinfection, medium was removed from the dishes, placed in sterile Falcon tubes, and spun for 10 min at 3,000 rpm to pellet the bacteria. Subsequently, RNAs were isolated using RNeasy minicolumns (Qiagen) treated with DNase I (Invitrogen) to remove contaminating genomic DNA. Three micrograms of total RNA was combined with 0.5 μM deoxynucleoside triphosphates (dNTPs), 500 U SuperaseIN/ml (Ambion), and 10 μM dithiothreitol (DTT) in 1× reaction buffer and reverse transcribed with Superscript II reverse transcriptase (Invitrogen). The resultant cDNA was used as a template for qPCR. Analysis was carried out in the ABI Prism 7000 sequence detection system (Applied Biosystems) using the two-step RT-qPCR kit with SYBR green detection (Invitrogen). Fold change was determined using the comparative threshold cycle (C_T) method by comparison to the PA1544 housekeeping gene.

DNA microarray experiment. Microarray experiments were performed on three independent cultures. The *cbrA* deletion mutant and wild-type PA14 were grown on a BM2-swarming plate containing 0.5% (wt/vol) agar for 18 h at 37°C. RNA was harvested from the leading edge of the dendritic swarm colonies of the PA14 wild type and of the entire nonswarming colonies of the *cbrA* mutant. As described previously (37), cells were resuspended in BM2-swarming medium supplemented with RNAprotect reagent (Qiagen, Germany). Harvesting of cells, RNA isolation, cDNA synthesis, hybridization to *P. aeruginosa* PAO1 DNA microarray slides (aminosilane coated) from the Institute for Genomic Research (TIGR) Pathogenic Functional Genomics Resource Center, analysis of microarray slides using ArrayPipe version 1.7, and RT-qPCR were performed as described previously.

Pyoverdine assay. Bacterial strains were grown in CAA medium (5 g/liter low-iron Bacto Casamino Acids [Difco], 1.54 g/liter $K_2HPO_4 \cdot 3H_2O$, 0.25 g/liter $MgSO_4 \cdot 7H_2O$) at 37°C for 48 h. The supernatants were diluted 1/75 in 10 mM Tris-HCl, pH 7.5, and excited at 400 nm with a spectrofluorimeter (11).

Microarray accession number. The ArrayExpress accession number is E-FPMI-22.

RESULTS

Construction of a PA14 *cbrA* deletion mutant. Previous studies indicated that the PA14 *cbrA* transposon mutant (ID33836), obtained from Harvard University, was swarming deficient (55). As *cbrA* is located directly upstream of its cognate response regulator gene *cbrB*, it is possible that the swarming defect observed in the *cbrA* transposon mutant was simply due to a polar effect. Therefore, we investigated whether or not the transposon insertion in the PA14 *cbrA* gene affected the expression of *cbrB*. RNA was harvested from the PA14 wild type and the PA14 *cbrA* transposon mutant and then reverse transcribed into cDNA. Changes in the expression levels of *cbrA* and *cbrB* were determined via RT-qPCR. In the PA14 *cbrA* transposon mutant, *cbrB* expression was slightly downregulated (fold change of -1.9 ± 0.1 relative to the wild type), suggesting that the transposon insertion in *cbrA* affected not only the expression of CbrA but also that of CbrB.

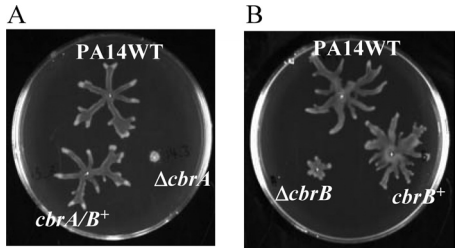

FIG. 1. Swarming motility of the PA14 *cbrA* and *cbrB* mutants. Defective swarming motilities of the *cbrA* deletion mutant (Δ*cbrA*) and the *cbrB* transposon mutant (Δ*cbrB*) were restored to levels similar to that of the wild type, *P. aeruginosa* PA14 (WT), by transforming these mutants with plasmids containing the wild-type *cbrAB* genes or the wild-type *cbrB* gene.

Since the expression of CbrB was affected in the *cbrA* transposon mutant, it was necessary to generate a nonpolar, in-frame deletion of *cbrA* in the PA14 wild type in order to study the role of CbrA in *P. aeruginosa.* To generate a *P. aeruginosa cbrA* deletion mutant, we utilized the *sacB*-based method (16) that involved amplifying three overlapping DNA fragments, splicing these fragments together by overlap extension PCR, and cloning the resultant fragment into a suicide vector, pEX18Ap. The plasmid-borne deletion was then transferred to the *P. aeruginosa* strain PA14 chromosome by homologous recombination and selected on medium containing gentamicin and 5% sucrose. The resultant *cbrA* deletion mutant was verified by sequencing and PCR. RT-qPCR was performed to ensure that the expression of CbrB was not affected in the *cbrA* deletion mutant (data not shown).

The *cbrA* deletion mutant was impaired in swarming motility. The abilities of the *cbrA* deletion mutant to swim, twitch, and swarm on 0.3%, 1%, and 0.5% agar, respectively, were examined. The *cbrA* deletion mutant exhibited minor defects in flagellum-mediated swimming and type IV pilus-mediated twitching motilities (data not shown). In contrast, this mutant's ability to swarm was completely abolished. The swarming-defective phenotype of this mutant could be restored to the wild-type level by introducing the wild-type *cbrAB* genes into the mutant (Fig. 1A). We also investigated whether the inability of the mutant to swarm was influenced by differences in production of rhamnolipids. Using the rhamnolipid agar plate method (6, 7), no difference was observed between the diameters of the halos formed due to rhamnolipid produced from the *cbrA* mutant and the PA14 wild type (data not shown). To investigate whether the inability of the *cbrA* mutant to swarm was due to its poor ability to utilize glucose as the carbon

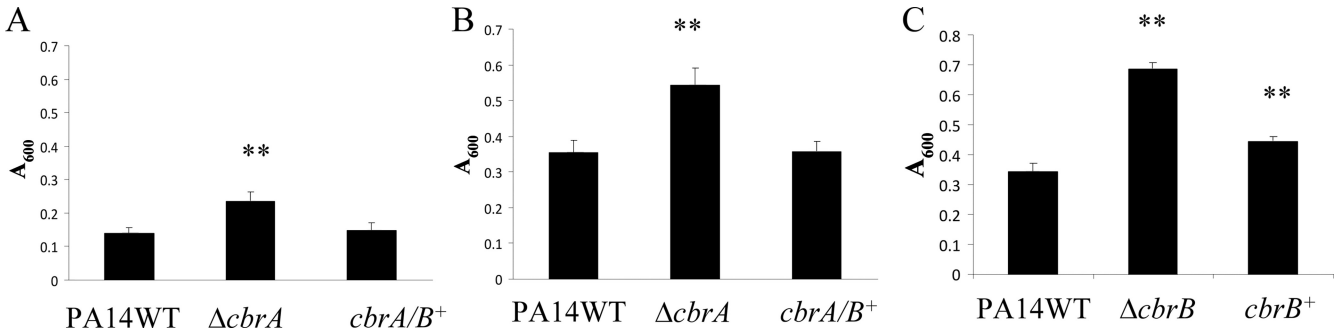

FIG. 2. Biofilm formation of the PA14 *cbrA* and *cbrB* mutants. Cells were incubated in 96-well microtiter plates containing BM2-biofilm medium for 8 h (A) and 20 h (B) at 37°C for the *cbrA* mutant and 20 h at 37°C for the *cbrB* mutant (C). Surface-associated biofilm formation was analyzed by crystal violet staining of the adherent biofilm followed by ethanol solubilization of the crystal violet and quantification (A_{600}) of the stained well. Results shown are means with standard deviations for three biological experiments, each with eight technical repeats. **, statistically significant difference ($P < 0.01$) between the mutants and the wild type as determined by Student's *t* test.

source, we replaced glucose, in the swarming medium, with succinate, a carbon source that had been demonstrated, and confirmed in our growth studies, to sustain the wild-type growth of the *P. aeruginosa cbrA* mutant. Furthermore, swarming of the *cbrA* mutant was tested on other carbon sources, including glycerol and mannitol. Replacement of glucose with other carbon sources did not restore the ability of the *cbrA* mutant to swarm (data not shown).

The *cbrA* mutant exhibited enhanced biofilm formation. *P. aeruginosa* forms biofilms on a number of surfaces, including tissues of the human host. Consequently, biofilm infections are virtually impossible to eradicate due to the biofilm's inherent resistance to conventional antibiotic therapies (28). Therefore, we investigated here the ability of the *cbrA* deletion mutant to form simple biofilms using static microtiter biofilm methods. These experiments demonstrated that the *cbrA* mutant showed a significant ($P < 0.01$ by Student's *t* test) but moderate (~40 to 60%) enhancement in biofilm formation as early as 8 h (Fig. 2A and B). This biofilm phenotype could be successfully complemented by introducing the wild-type *cbrAB* operon into the mutant. To determine whether the biofilm formation phenotype occurred during initial attachment stage or later during biofilm development, a rapid (30-min) attachment assay was performed. No difference in early attachment was observed between the PA14 wild type and the *cbrA* mutant (data not shown).

The excess-biofilm-forming phenotype of the *cbrA* mutant led us to investigate further whether this mutant was altered for other biofilm-related functions. Chemical analyses of *P. aeruginosa* biofilms have suggested that the matrix is comprised of exopolysaccharides (EPS), DNA, RNA, proteins, and ions (47, 51). In *P. aeruginosa*, the *psl* and *pel* loci have been suggested to be involved in the production of the polysaccharide component of the matrix (9, 10). While alginate is a component of the extracellular matrix, studies have suggested that alginate is not a significant component of the extracellular polysaccharide present in the matrix of biofilms formed by *P. aeruginosa* under commonly used laboratory growth conditions (14, 54). Also, while *P. aeruginosa* strain PAO1 has both *pel* and *psl* loci, only the *pel* locus has been identified in the PA14 strain (9). To investigate whether the increased-biofilm-forming phenotype of this mutant was due to increased production of the *pel*-encoded extracellular matrix, we performed Congo red assays, as Congo red has been shown to bind the *pel*-encoded polysaccharide of *P. aeruginosa* PA14. The *cbrA* mutant showed substantially increased binding to Congo red compared to the PA14 wild type (Fig. 3).

Furthermore, biofilm production of the *cbrA* mutant was assessed in different carbon sources, including succinate, mannitol, and glycerol. Interestingly, when glucose was replaced with succinate as the major carbon source, the *cbrA* mutant produced significantly less biofilm than in glucose at 20 h, while no such difference was observed for the wild type (Fig. 4). When mannitol or glycerol was provided as the carbon source, the *cbrA* mutant still produced significantly more biofilm than did the wild type (data not shown).

The *cbrA* mutant displayed enhanced cytotoxicity toward human bronchial epithelial cells. *P. aeruginosa* possesses a large arsenal of virulence factors. One of the major virulence mechanisms employed by *P. aeruginosa* to intoxicate eukaryotic cells is the type III secretion system (T3SS). The T3SS, triggered by cell contact, injects toxins directly into the cytoplasm of the target cell. Moreover, the CbrAB system was previously demonstrated to be involved in the metabolic reg-

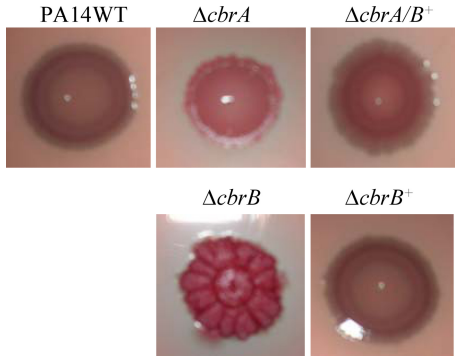

FIG. 3. Congo red binding. Strains were spotted on Congo red and Coomassie brilliant blue plates and incubated for 24 h at 37°C, followed by incubation for 48 h at room temperature. Representative images of the colony morphology of *P. aeruginosa* PA14 (WT), the *cbrA* mutant (Δ*cbrA*), the *cbrB* mutant (Δ*cbrB*), the complemented *cbrA/B*+ strain, and the complemented *cbrB*+ strain are shown.

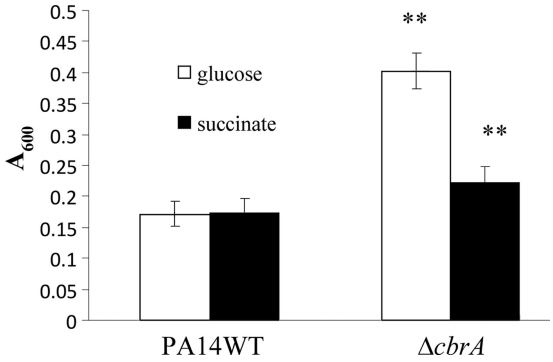

FIG. 4. Influence of carbon source on biofilm formation of the PA14 *cbrA* deletion mutant. Cells were incubated at 37°C for 20 h in 96-well microtiter plates containing BM2-biofilm medium supplemented with 0.4% (wt/vol) glucose (open bars) or 20 mM succinate (filled bars). Surface-associated biofilm formation was analyzed by crystal violet staining of the adherent biofilm followed by ethanol solubilization of the crystal violet and quantification (A_{600}) of the stained well. Results shown are means with standard deviations for three biological experiments, each with eight technical repeats. **, statistically significant difference ($P < 0.01$) between the mutants and the wild type as determined by Student's *t* test.

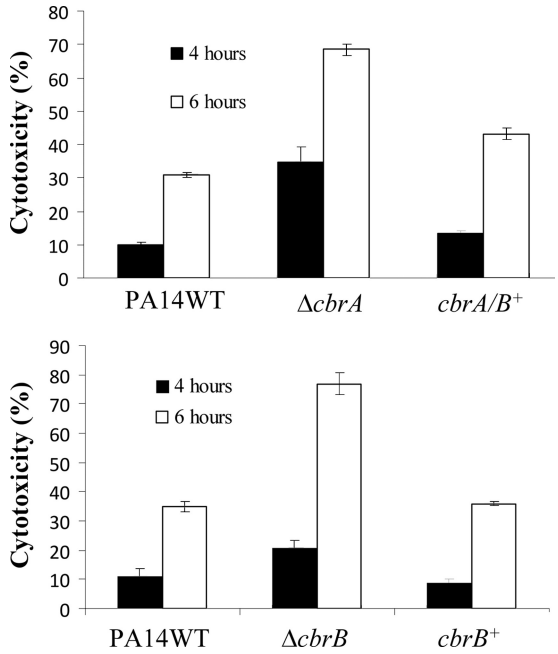

FIG. 5. *In vitro* cytotoxicity toward HBE cells. The abilities of the PA14 wild type (WT), the mutants (*cbrA* mutant [Δ*cbrA*] and *cbrB* mutant [Δ*cbrB*]), and the complemented strains (*cbrA*/B^+ and *cbrB*$^+$) to induce cell damage were determined by monitoring the release of intracellular lactate dehydrogenase (LDH) into the supernatant from HBE cells. Bacteria were cocultured with the cells, and LDH release was monitored at the time points indicated. Each result represents the mean of three independent biological repeats, each assayed in triplicate.

ulation of the T3SS and its effectors (41). Therefore, we examined the ability of the *cbrA* mutant to infect and destroy a monolayer of cultured 16HBE14o- epithelial cells. To measure the cytotoxic effects of the PA14 wild type and *cbrA* mutant on the epithelial cells, the amount of lactate dehydrogenase (LDH) released from the 16HBE14o- cells was quantified using an enzyme assay. The *cbrA* mutant displayed 3.5- and 2.2-fold-greater cytotoxicity than did the wild type at 4 h and 6 h postinfection, respectively (Fig. 5A). Introducing the wild-type *cbrAB* operon into the *cbrA* mutant restored cytotoxicity to wild-type levels at both time points. Furthermore, to investigate whether the *cbrA* mutant was able to infect the epithelial cells better than the wild type was, due to an improved ability to adhere to the epithelial cells, adhesion assays were performed. No significant differences in adherence to 16HBE14o- cells at 1 h were observed between the wild type and the mutant (data not shown).

The *cbrA* mutant demonstrated increased resistance to polymyxins, aminoglycosides, and fluoroquinolones. Recently, a microarray analysis indicated that among the many transcriptional changes, the *cbrA* gene was 2-fold downregulated ($P < 0.05$) in response to sublethal concentrations of tobramycin (Kindrachuk et al., submitted). Therefore, we were interested in investigating the role of CbrA in modulating resistance to a variety of clinical antibiotics, including aminoglycosides, cationic peptides, fluoroquinolones, cephalosporins, and carbapenems (Table 2). Intrinsic resistance of the mutant to these antibiotics was assessed by MIC assay in cells growing in BM2-swarming medium containing high (2 mM) Mg^{2+} to suppress the possibility of induction of genes by limiting Mg^{2+}. Compared to the PA14 wild type, the *cbrA* mutant reproducibly exhibited a 2-fold-increased resistance to polymyxin B and colistin and 4-fold-increased resistance to ciprofloxacin and tobramycin. No difference was observed between the MIC values of the PA14 wild type and the mutant to piperacillin, tetracycline, cefepime, ceftazidime, and imipenem. Although the 2-fold change in MIC observed for the *cbrA* mutant to polymyxin B is often considered within the acceptable range of error of these assays, we confirmed the increased resistance of the *cbrA* mutant to polymyxin B by performing kill curve assays with cells taken from liquid swarm medium and from swarm plates (Fig. 6). The antibiotic susceptibility phenotype could be complemented to wild-type levels by introducing the wild-type *cbrAB* genes into the mutant.

The moderate growth deficiency exhibited by the *cbrA* mutant was insufficient to explain its swarming, biofilm, or cytotoxicity phenotypes. Previous studies identified CbrAB as an important regulatory element for the expression of several

TABLE 2. MICs (μg/ml) of antibiotics toward *P. aeruginosa* grown in swarming medium[a]

Antibiotic	MIC (μg/ml)		
	PA14 (WT)	*cbrA* mutant	*cbrB* mutant
Polymyxin B	1	2	1
Colistin	1	2	1
Tobramycin	2	8	2
Piperacillin	4	4	4
Tetracycline	64	64	64
Ciprofloxacin	0.2	0.8	0.2
Cefepime	2	2	1
Ceftazidime	4	4	4
Imipenem	2	2	2

[a] Results are shown as the mode of 4 to 6 independent experiments.

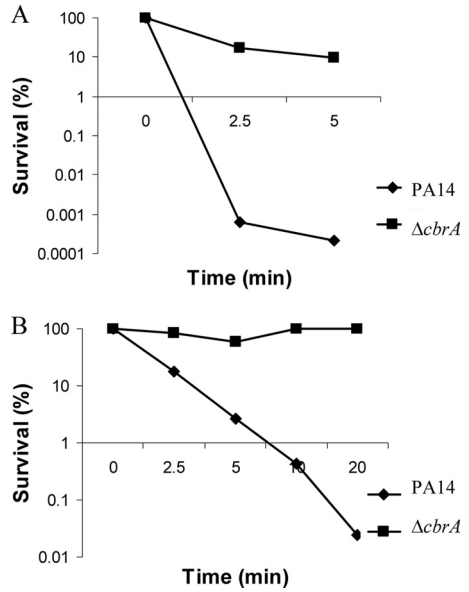

FIG. 6. Polymyxin B resistance in the PA14 *cbrA* deletion mutant. Sensitivity to polymyxin B at 1 μg/ml was analyzed using cells from mid-log phase in swarm medium (A) or using cells directly off swarm plates and then plating diluted aliquots for survivors (B). For each condition, one representative experiment of four independent experiments that produced identical results is shown.

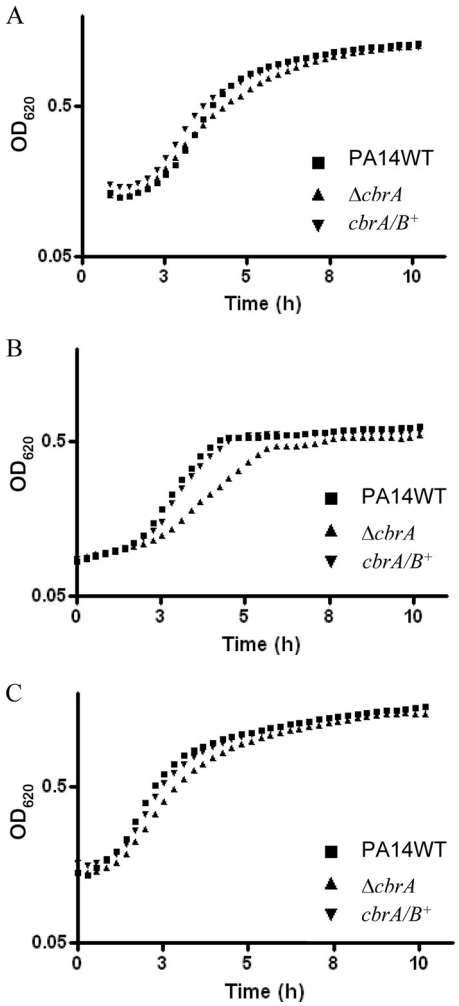

FIG. 7. Growth curves of *P. aeruginosa* PA14 wild type (WT) and *cbrA* deletion mutant. Cells were diluted and grown in LB broth (A), BM2-swarm medium (B), and BM2-biofilm medium (C). Growth was measured at 37°C using a Tecan Spectrofluor Plus.

catabolic pathways and utilization of a variety of organic compounds as the sole carbon source (32). Li and Lu showed that a *cbrAB* mutant displayed weak growth when glucose was used as the sole carbon source, while growth on tricarboxylic acid (TCA) cycle intermediates was sustained (22). Therefore, we investigated whether any of the phenotypes observed for the *cbrA* mutant could be related to growth impairment when glucose was provided as the major carbon source in the swarming, MIC, and biofilm media. The growth of the *cbrA* mutant and the PA14 wild type was measured in the appropriate medium (BM2-swarm medium, LB medium, or BM2-biofilm medium) at 37°C using a Tecan Spectrofluor Plus to measure the absorbance at 620 nm every 20 min for 15 to 20 h under shaking conditions. The growth of the mutant and wild type was also determined by measuring the absorbance at 600 nm every 20 min during infection of 16HBE14o- cells. As shown in Fig. 7A, the *cbrA* mutant exhibited a very minor growth defect in LB medium. A moderate defect in growth under swarming conditions was observed for the mutant (Fig. 7B). The moderate growth defect of the mutant in this medium (~50% change in growth rate), however, seemed insufficient to explain the complete abolition of swarming motility. The *cbrA* mutant also exhibited slight growth defects under biofilm-inducing (Fig. 7C) and HBE infection conditions, but these growth defects also seemed insufficient to explain the increased biofilm production and cytotoxicity of the mutant. Furthermore, we investigated whether changing the carbon source from glucose to succinate would restore the ability of the mutant to swarm and form biofilm to the wild-type level. The *cbrA* mutant indeed showed wild-type growth when glucose was replaced with succinate in the BM2-swarming and BM2-biofilm media, as suggested from previous studies (22), but exhibited the same swarming and biofilm defects as in glucose media.

Microarray analysis of the *cbrA* mutant. To investigate how CbrA contributed to the various phenotypes observed, microarray studies were performed comparing the *cbrA* mutant to the PA14 wild type. For the microarray, RNAs from the PA14 wild type and the *cbrA* mutant were taken directly from BM2-swarm plates that had been incubated at 37°C for 20 h. The microarray revealed 236 genes that were differentially regulated by more than 2.0-fold ($P \leq 0.06$) with 145 transcriptionally upregulated and 91 transcriptionally downregulated genes (see Table S1 in the supplemental material). A selection of these genes is presented in Table 3. Of note, PAO1 DNA microarray slides were used to analyze gene expression of PA14 *cbrA* mutant and PA14 wild type, as there are no PA14-specific microarray slides available. The PA14 genome (6.5 Mbp) is slightly larger than that of PAO1 (6.3 Mbp), but the PA14 and PAO1 genomes are very similar, with greater than 92% of all genes in PA14 also present in PAO1. The additional genes in the PA14 genome that are absent in PAO1 have been

TABLE 3. Selected genes significantly dysregulated in *cbrA* mutant as determined using microarray

Category and gene ID[a]	Name	Fold change[b]	*P* value	Function
Adaptation and protection				
PA1159		−2.36	0.0353	Probable cold shock protein
PA2385	*pvdQ*	−3.01*	0.002	3-Oxo-C_{12}-homoserine lactone acylase and pyoverdine biosynthesis
PA2386	*pvdA*	−2.39*	0.0011	L-Ornithine N_5-oxygenase
PA2397	*pvdE*	−3.44	0.002	Pyoverdine biosynthesis protein
PA2399	*pvdD*	−2.24	0.0061	Pyoverdine synthetase D
PA2920		2.14	0.0068	Probable chemotaxis transducer
PA3349		2.15	0.0013	Probable chemotaxis protein
PA4223	*pchH*	−4.16*	8.59E−05	Probable ATP binding component of ABC transporter
PA4225	*pchF*	−2.53	0.0005	Pyochelin synthetase
PA4231	*pchA*	−2.48	0.0009	Salicylate biosynthesis isochorismate synthase
PA4356	*xenB*	2.27	0.0039	Xenobiotic reductase
PA4468	*sodM*	3.55	8.86E−05	Superoxide dismutase
PA4876	*osmE*	−2.00	0.0161	Osmotically inducible lipoprotein
Chemotaxis; cell wall/LPS/ capsule				
PA1423		2.55	0.0007	Probable chemotaxis transducer
PA2920		2.14	0.0068	Probable chemotaxis transducer
PA3157	*wbpC*	−2.3	0.0186	Probable acetyltransferase
PA3349		2.15	0.0013	Probable chemotaxis protein
PA3545	*algG*	2.08	0.0068	Alginate-C_5-mannuronan-epimerase
Chaperones and heat shock proteins				
PA1596	*htpG*	2.21	0.0016	Heat shock protein 90
PA2830	*htpX*	3.27	0.0001	Heat shock protein
PA3126	*ibpA*	3.26	6.23E−05	Heat shock protein
PA4352		2.65	0.0003	Putative universal stress protein
PA4761	*dnaK*	3.57*	5.33E−05	Molecular chaperone
PA4762	*grpE*	2.95	0.0004	Heat shock protein
PA5053	*hslV*	4.9	9.70E−05	ATP-dependent protease peptidase subunit
PA5054	*hslU*	3.07	0.0054	ATP-dependent protease ATP binding subunit
Antibiotic resistance and susceptibility				
PA1178	*oprH*	9.36*	1.63E−06	PhoP/Q and low-Mg^{2+}-inducible outer membrane protein H1 precursor
PA1170	*phoP*	4.16*	2.74E−05	Two-component response regulator
PA1797		10.37*	1.16E−06	Putative function in adaptive polymyxin resistance
PA2198		3.73	0.001	Putative antibiotic biosynthesis monooxygenase
PA3552	*arnB*	6.16*	6.84E−06	Hypothetical protein
PA3553	*arnC*	3.61	5.17E−05	Probable glycosyl transferase
PA3554	*arnA*	2.44	0.0021	Hypothetical protein
PA3555	*arnD*	4.49	1.57E−05	Hypothetical protein
PA3556	*arnT*	3.13	0.0001	Inner membrane L-Ara4N transferase
PA3557	*arnE*	4.51	2.97E−05	Hypothetical protein
PA3558	*arnF*	2.45	0.0028	Hypothetical protein

[a] ID, identification. Information is according to the *P. aeruginosa* genome website (http://www.pseudomonas.com).
[b] Fold regulation of genes differentially expressed in *cbrA* mutant relative to PA14 wild type. A positive number indicates transcript upregulation and a negative number indicates transcript downregulation in the *cbrA* mutant. An asterisk indicates confirmation of gene regulation by RT-qPCR.

suggested to contribute to its enhanced pathogenicity, as PA14 is more virulent than PAO1 (13).

Analysis of the microarray data revealed dysregulation of genes involved in amino acid biosynthesis and metabolism, carbon compound catabolism, and central intermediary metabolism, consistent with the proposed role of CbrAB in the utilization of a variety of organic compounds as sole carbon source (22, 32). For example, *hutU* (urocanase), part of the *hutUHIG* operon involved in histidine catabolism, was moderately downregulated in the *cbrA* mutant (RT-qPCR revealed a fold change of −6.0 ± 0.6 relative to the wild type).

The *cbrA* microarray results were examined to identify genes that might influence antibiotic resistance of the mutant (since the microarray and MIC experiments utilized similar growth conditions). Moderate (3- to 10-fold) upregulation of the operons encompassing the two-component regulators *oprH-phoPQ* and *pmrAB* and of the downstream *arnBCADTEF* (lipopolysaccharide [LPS] modification) operon was observed in the microarray. To confirm the microarray results, RT-qPCRs were performed and revealed 9.3- ± 1.8-fold upregulation of *oprH*, 3.7- ± 0.5-fold upregulation of *phoP*, 3.1- ± 0.3-fold upregulation of *phoQ*, 10.2- ± 1.9-fold upregulation of *arnB*,

and 4.0- ± 1.4-fold upregulation of *pmrB.* Activation of these operons is known to trigger bacterial resistance to cationic peptides and polymyxins in response to low-Mg^{2+} conditions by controlling the addition of aminoarabinose to lipid A, thereby reducing the net negative charge of LPS and limiting its interaction with polycationic peptides such as polymyxin B (29); however, their link to CbrAB had not been revealed previously. The involvement of the PhoPQ system in aminoglycoside resistance has also been defined (25, 38). Although the details of PhoPQ involvement in aminoglycoside resistance remain to be fully elucidated, PhoPQ appears to mediate resistance to aminoglycosides via a mechanism different from that involved with the polycationic peptides. The *cbrA* microarray also identified the upregulation of several heat shock protein genes, including *htpG*, *ibpA*, *dnaK*, *grpE*, *hslV*, and *hslU*. The upregulation of *dnaK* was confirmed by RT-qPCR, revealing 3.6- ± 0.2-fold upregulation in the mutant relative to the wild type. Recent studies have demonstrated that upregulation of heat shock genes prior to treatment with tobramycin led to increased resistance of *P. aeruginosa* to tobramycin (Kindrachuk et al., submitted). Although the *cbrA* microarray indicated upregulation of the *mexX* gene, known to be involved in involvement in aminoglycoside resistance (38), RT-qPCR failed to detect any significant changes in the transcriptional expression of this gene (fold change of 1.1 ± 0.3) in the *cbrA* mutant compared to the wild type.

Analysis of the microarray data also revealed moderate downregulation of several genes, PA0621, *pvdD*, PA3784, *pchH*, and *pchF*, which were possibly involved in the ciprofloxacin resistance phenotype observed in the *cbrA* mutant. A previous ciprofloxacin screen of the PA14 transposon mutant library by Breidenstein et al. showed that transposon mutants of PA0621, *pvdD*, PA3784, *pchH*, and *pchF* are more resistant than their wild-type parent strain toward ciprofloxacin (2). Flagella and type IV pili play important roles in biofilm and microcolony formation and are also required for swarming (19). Furthermore, studies have suggested that the flagellum secretion system plays a role in *P. aeruginosa* invasion of epithelial cells (8). However, the microarray did not reveal dysregulation in any flagellum- or type IV pilus-related genes. Furthermore, there was no dysregulation of the type III secretion apparatus or effector genes in the *cbrA* mutant.

This transcriptome analysis also highlighted several other interesting genes. These findings included moderate downregulation of the pyoverdine and pyochelin biosynthesis genes, including *pvdA*, *pvdQ*, and *pchF*. RT-qPCR confirmed downregulation of these genes in the mutant with values of −9.9 ± 1.3 for *pvdA*, −16.5 ± 2.4 for *pvdQ*, and −4.2 ± 0.6 for *pchF.* The downregulation of *pvdQ* in the *cbrA* microarray was of interest since a *pvdQ* mutant is swarming deficient (37) and has been suggested to play a role in biofilm formation and virulence of *P. aeruginosa* (18). Consistent with the qPCR data, we grew the *cbrA* mutant and the wild type in a low-iron medium to induce pyoverdine production and observed, using a spectrofluorimeter, that the mutant secreted less pyoverdine into the supernatant than did the wild type (Fig. 8). In a previous study, Jimenez et al. (18) showed that addition of iron in swarm plates restored the swarming of a swarming-impaired *pvdQ* mutant to the wild-type level. Therefore, we also tested whether addition of iron had any effect on swarming motility or the biofilm formation of the *cbrA* mutant. However, addition of iron did not restore swarming or biofilm formation of the *cbrA* mutant to the wild-type level (data not shown).

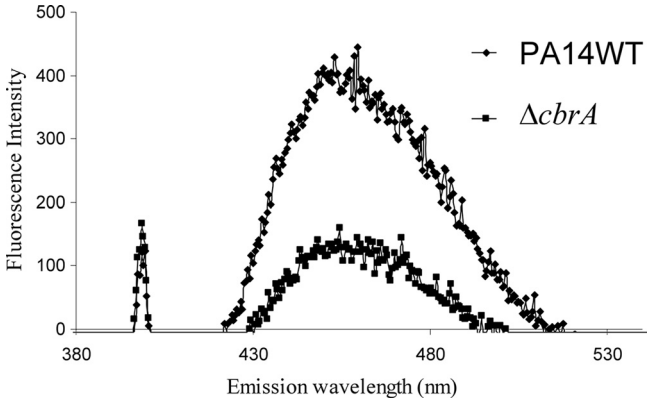

FIG. 8. Pyoverdine production of the PA14 *cbrA* deletion mutant. Cells grown in Casamino Acids medium for 48 h at 37°C were diluted, and the resultant sample was excited at 400 nm with a spectrofluorimeter.

Transcriptional analysis, by RT-qPCR, of the *cbrA* mutant under various growth conditions. The microarray experiment of the *cbrA* mutant versus PA14 wild type did not reveal transcriptional changes in the expression of genes known to be involved in virulence (e.g., the type III secretion system) or genes involved in the production of exopolysaccharides in *P. aeruginosa*. This was not completely unexpected, since the growth conditions used for the microarray experiment and the conditions used to assay exopolysaccharide production or *in vitro* cytotoxicity experiments were different and it is known that CbrAB influences the utilization of many different carbon and nitrogen sources. Therefore, we isolated RNA from bacterial cells during *in vitro* infection of HBE cells and cells growing on Congo red plates. Consistent with the increased binding phenotype observed on the Congo red plates (Fig. 3), RT-qPCR revealed 2.5- to 5-fold upregulation of the exopolysaccharide *pelD* and *pelF* genes in the *cbrA* mutant compared to the PA14 wild type (Table 4). Bacterial cells obtained during HBE cell infection revealed moderate upregulation of the type III secretion apparatus and effectors (*exoT*, *exoY*, *exoU*, *pcrV*, *exsA*, and *popD*) and the type I secretion apparatus, *aprD* (Table 4), consistent with the enhanced cytotoxicity of this mutant.

CbrA mediated regulation of swarming, biofilm formation, and cytotoxicity in conjunction with CbrB. The CbrB response regulator has been identified to play a role along with its cognate sensor kinase CbrA in the global metabolic regulation of carbon and nitrogen utilization in *P. aeruginosa* (22). Therefore, we examined whether CbrB also interacted with CbrA to modulate virulence and virulence-related processes in *P. aeruginosa*. Similarly to the *cbrA* mutant, the *cbrB* mutant displayed impairment in swarming and excessive biofilm formation (Fig. 1B and 2C). These phenotypes could also be complemented by introducing the wild-type *cbrB* allele into the *cbrB* mutant. Congo red assays revealed increased binding of Congo red to the *cbrB* mutant compared to the wild type (Fig. 3). Interestingly, on the Congo red plates, the *cbrB* mutant also

TABLE 4. Dysregulated genes in the *cbrA*, *cbrB*, *crcZ*, and *crc* mutants during human bronchial epithelial (HBE) cell line infection and growth on Congo red plates as determined by RT-qPCR

Expt and gene	Fold change relative to PA14 wild type[a]			
	cbrA	*cbrB*	*crcZ*	*crc*
HBE cell infection				
exoT	2.9 ± 0.3	3.4 ± 1.0	3.8 ± 0.9	−3.8 ± 1.2
exoY	2.7 ± 0.3	2.3 ± 0.6	2.1 ± 0.5	−4.3 ± 0.3
exoU	2.5 ± 0.4	2.7 ± 0.3	2.0 ± 0.4	−9.8 ± 1.0
pcrV	2.6 ± 0.3	2.2 ± 0.1	2.0 ± 0.3	−5.8 ± 0.9
exsA	2.2 ± 0.2	2.7 ± 0.5	1.7 ± 0.6	−2.7 ± 0.9
pcrD	1.4 ± 0.1	1.2 ± 0.5	1.2 ± 0.8	−1.4 ± 0.5
popD	2.9 ± 0.5	2.3 ± 0.5	2.4 ± 0.4	−6.6 ± 0.7
pscF	1.2 ± 0.1	1.4 ± 0.5	1.6 ± 0.9	−2.4 ± 0.3
pcrR	1.3 ± 0.3	1.8 ± 1.1	1.2 ± 0.9	−1.5 ± 0.5
lasB	1.2 ± 0.2	1.1 ± 0.6	1.5 ± 0.8	−1.2 ± 0.5
lipC	1.2 ± 0.1	−1.1 ± 0.3	1.1 ± 0.5	−1.3 ± 0.3
plcB	1.6 ± 0.5	1.3 ± 0.3	1.7 ± 0.6	−1.8 ± 0.6
PA4528	1.5 ± 0.3	1.3 ± 0.1	1.1 ± 0.3	−1.0 ± 0.5
aprD	2.5 ± 0.5	2.9 ± 0.4	2.4 ± 0.3	−6.2 ± 0.8
Congo red				
pelD	2.6 ± 0.5	5.1 ± 0.5	2.7 ± 0.5	−4.1 ± 1.0
pelF	2.5 ± 0.4	4.5 ± 0.4	2.8 ± 0.5	−2.6 ± 0.8

[a] Values are represented as averages of at least 3 biological samples.

showed wrinkled morphology while the *cbrA* mutant remained smooth like the wild type. Furthermore, the *cbrB* mutant exhibited 2-fold-enhanced *in vitro* cytotoxicity toward HBE cells (Fig. 5B). Similarly to the *cbrA* mutant, the *cbrB* mutant also exhibited moderate growth defects in swarming/MIC and biofilm media (data not shown). However, while the *cbrA* mutant showed increased resistance to a variety of antibiotics, the *cbrB* mutant displayed wild-type MIC values for all antibiotics tested (Table 2). Based on the microarray results of the *cbrA* mutant, we examined the expression level of a variety of genes in the *cbrB* mutant under swarming conditions by RT-qPCR. As expected, RT-qPCR revealed no dysregulation in the genes that were thought to explain the altered susceptibility of the *cbrA* mutant; these findings included a lack of dysregulation of the *oprH-phoPQ* operon, the *pmrAB* operon, and the LPS modification operon (data not shown). Similarly to the *cbrA* mutant, upregulation of energy metabolism genes, such as *nirS* and *norC*, was observed in the *cbrB* mutant. The expression of *pvdQ* was also downregulated in the *cbrB* mutant, while other genes involved in pyoverdine biosynthesis, such as *pvdA*, were not dysregulated in the *cbrB* mutant (data not shown). However, similarly to the *cbrA* mutant, pyochelin biosynthesis genes, such as *pchG*, were downregulated in the *cbrB* mutant. In addition, RNA was isolated from the *cbrB* mutant from Congo red plates, and during HBE cell infection, RT-qPCRs were performed. The *cbrB* mutant revealed upregulation of the *pel* operon under the Congo red condition, but to a greater extent than that with the *cbrA* mutant (Table 4). This result was consistent with the wrinkled morphology observed only in the *cbrB* mutant and the increased biofilm formation of the *cbrB* mutant compared to the *cbrA* mutant. RT-qPCR analysis of the *cbrB* mutant during HBE cell infection also revealed upregulation of the type III secretion system during *in vitro* infection (Table 4).

The CbrAB system regulated swarming, biofilm formation, and cytotoxicity through the posttranscriptional regulatory system Crc/CrcZ. Recently, Sonnleitner et al. found that the CbrAB/CrcZ/Crc system enables *P. aeruginosa* to utilize various carbon sources (46). In addition to its role in catabolite repression, O'Toole et al. showed that Crc is required for biofilm formation in *P. aeruginosa* (35). Furthermore, Linares et al. demonstrated that the Crc protein plays a role in the regulation of virulence in *P. aeruginosa* (24). They showed that a *crc* mutant is defective in type III secretion and is less virulent in a *Dictyostelium discoideum* model (24). Thus, these results led us to investigate whether the CbrAB system regulated swarming, biofilm formation, and cytotoxicity through the CrcZ/Crc system. First, we examined the ability of a PA14 *crc* transposon mutant to swarm, form biofilm, and infect HBE cells. As shown in Fig. 9A, the *crc* mutant was able to swarm as well as the wild type but consistently exhibited reduced branching of the swarming tendrils compared to the wild type. The *crc* mutant was also defective in biofilm formation and showed a reduced ability to bind Congo red (Fig. 9B and D). The *crc* mutant also displayed a reduced ability to infect and destroy a monolayer of cultured 16HBE14o- epithelial cells (Fig. 9C). As well, the *crc* mutant showed wild-type growth in swarm and biofilm media (data not shown). These phenotypes could also be complemented by introducing the wild-type *crc* allele into the *crc* mutant. These results suggest that the Crc protein had different effects than did the *cbrA* and *cbrB* mutants on swarming motility (modest effects on branching), while positively regulating biofilm formation and virulence of *P. aeruginosa.* RT-qPCR experiments on the *crc* mutant demonstrated downregulation of the type III secretion system during HBE cell infection and downregulation of the *pel* operon expression from Congo red plates (Table 4).

We also tested the ability of a *crcZ* deletion mutant to swarm, form biofilm, and infect HBE cells. In contrast to the *crc* mutant, the *crcZ* mutant exhibited phenotypes very similar to those of the *cbrA* and *cbrB* mutants, including substantial swarming deficiency, excessive biofilm formation, and enhanced cytotoxicity toward HBE cells (Fig. 10). These phenotypes could be complemented by introducing the wild-type *crcZ* allele into the *crcZ* mutant. In addition, growth studies revealed that the *crcZ* mutant exhibited moderate growth defects in swarming media (data not shown). RT-qPCR analysis of the RNA isolated from the *crcZ* mutant cells grown on Congo red plates revealed upregulation of the *pel* operon and upregulation of the type III secretion system during HBE cell infection (Table 4).

We also tested the susceptibility/resistance of the *crcZ* and *crc* mutants toward the antibiotics tested for the *cbrA* mutant. However, no differences between the mutants and wild type were observed for any of the tested antibiotics (data not shown). These results indicate that the CbrAB system might be regulating swarming, biofilm formation, and cytotoxicity via CrcZ, with a partial inverse regulation by Crc.

DISCUSSION

In this study, we examined the role of the sensor kinase CbrA in motility, biofilm formation, cytotoxicity, and antibiotic resistance. A *cbrA* deletion mutant was swarming de-

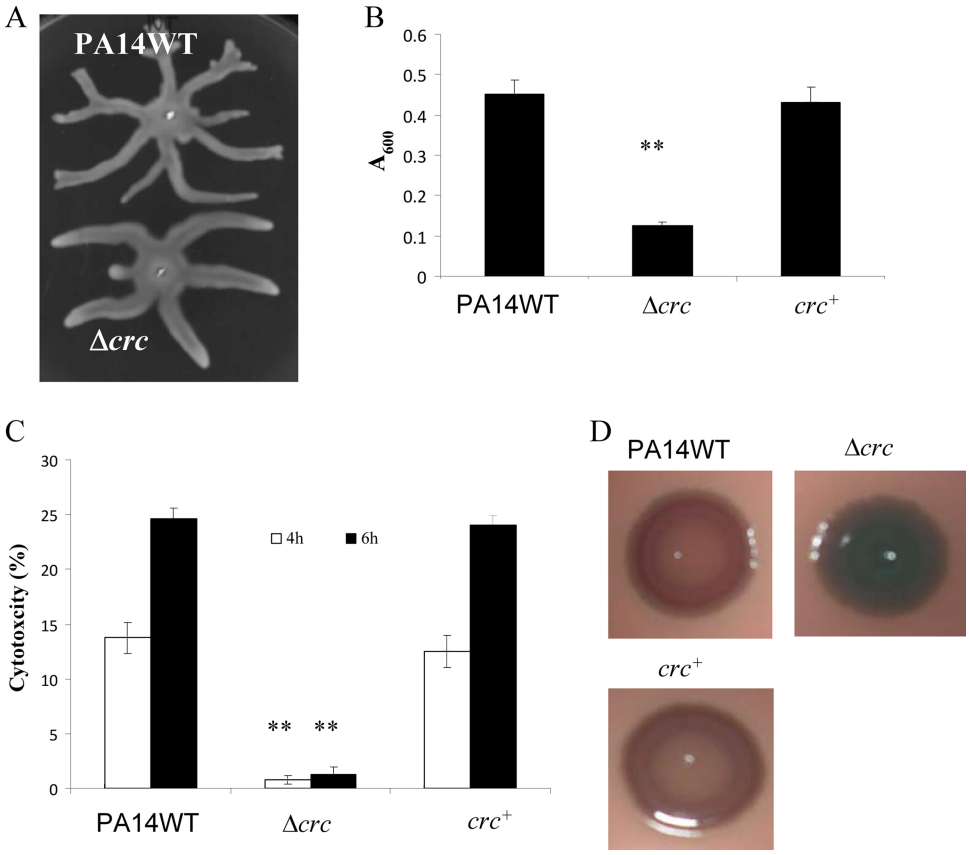

FIG. 9. Swarming motility, biofilm formation, Congo red binding, and *in vitro* cytotoxicity of the PA14 Δ*crc* mutant. (A) Swarming motility was assessed by spotting 1 μl of mid-log cell cultures onto BM2-swarming plates with 0.5% (wt/vol) agar and incubating the plates at 37°C for 20 h. (B) Biofilm formation was assessed by diluting overnight cultures in BM2-biofilm medium and incubating the cultures at 37°C for 20 h. (C) Cytotoxicity toward HBE cells was determined by infecting a monolayer of HBE cells with mid-log bacterial cultures at an MOI of 2. (D) Congo red binding was assessed by spotting 1, 5, or 10 μl of diluted cultures onto Congo red plates and incubating the plates for 24 h at 37°C and for an additional 48 h at room temperature.

fective but exhibited enhanced biofilm formation and *in vitro* cytotoxicity toward human bronchial epithelial cells. Furthermore, the *cbrA* mutant exhibited increased resistance toward several common clinical antibiotics, including polymyxin B, tobramycin, and ciprofloxacin. The sensor kinase CbrA and adjacently encoded response regulator CbrB have been proposed to work together in regulating the utilization of a variety of organic compounds as sole carbon sources. Phenotypic and genetic analyses performed here indicated that CbrA regulated swarming, biofilm formation, and cytotoxicity via its cognate response regulator CbrB. In addition to CbrB, we provided evidence for the involvement of the small RNA CrcZ in the regulation cascade of these virulence and virulence-related phenotypes. In contrast, the antibiotic resistance phenotype observed in the *cbrA* mutant was absent in the *cbrB* and *crcZ* mutants, and we speculate that CbrA may cross talk with other response regulators to regulate antibiotic resistance. A proposed model for the regulation of swarming, biofilm formation, cytotoxicity, and antibiotic resistance by the CbrA/CbrB/CrcZ pathway is shown in Fig. 11. The situation is not as clear with Crc, which is known to be negatively regulated by *crcZ* RNA and to fully participate in regulation of carbon source utilization. If Crc were fully involved, we would expect there to be a reciprocal phenotype in the *crc* mutant. Instead, there was only a partial effect on swarming in our study, but a substantial decrease in biofilm formation and a complete absence of epithelial cell toxicity. Thus, downstream effects on *crc* may be part of the phenotype but do not appear to fully explain the phenotypes observed.

Complementation of the *cbrA* deletion mutant with the wild-type *cbrAB* operon was necessary since complementation with the PA14 wild-type *cbrA* allele alone only moderately but incompletely restored the phenotypes. We speculate that the reason that the *cbrA* mutant was unable to be fully complemented with *cbrA* alone is that in the *cbrA* complemented strain, there were multiple copies of the *cbrA* gene due to the presence of the multicopy vector. Hence, when CbrA was expressed, the disproportionate amount of CbrA relative to the amount of CbrB in the complemented strain, compared with the wild type (confirmed by RT-qPCR), might have changed the functional interaction of these components and even shifted the balance from phosphorylation to dephosphorylation (both functions of sensor kinases).

Several physical factors are currently known to be required for swarming, including flagella, type IV pili, and the production of the biosurfactant rhamnolipids. The ability of the *cbrA*

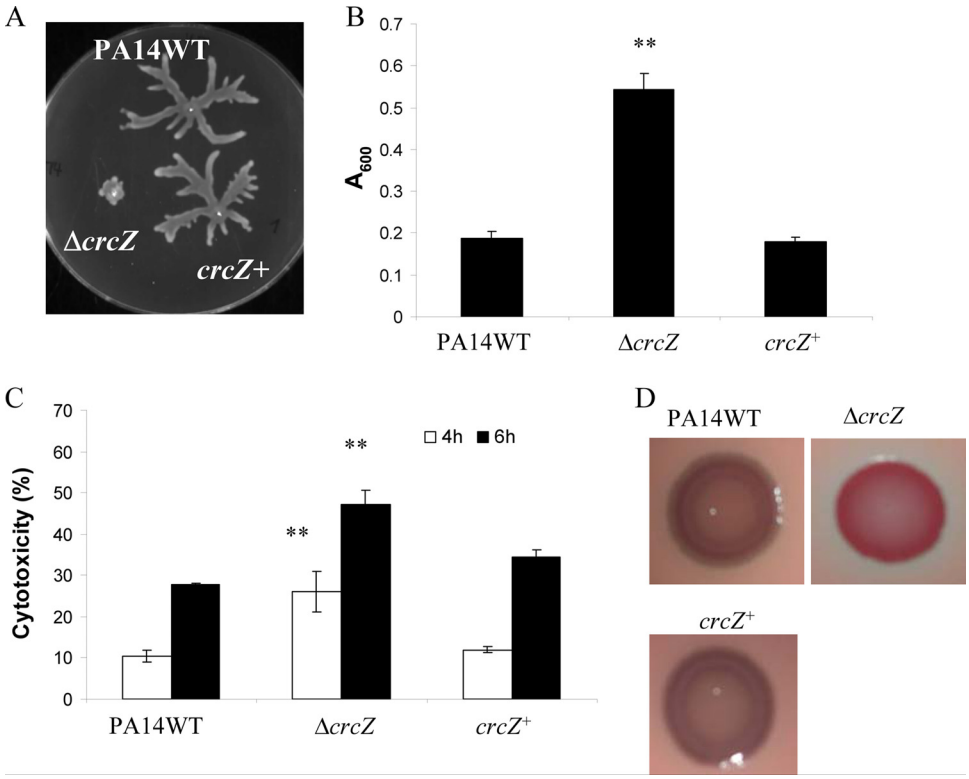

FIG. 10. Swarming motility, biofilm formation, and *in vitro* cytotoxicity of the PA14 Δ*crcZ* mutant. (A) Swarming motility of the PA14 wild type (WT) and *crcZ* mutant was determined by inoculating 1 μl of mid-log cell cultures onto BM2-swarming plates with 0.5% (wt/vol) agar and incubating plates at 37°C for 20 h. (B) Biofilm formation of the PA14 wild type (WT), PA14 *crcZ* mutant, and PA14 *crcZ*$^+$ complemented strain was determined by diluting overnight cultures in BM2-biofilm medium and incubating the cultures at 37°C for 20 h. (C) Cytotoxicity toward HBE cells was determined by infecting a monolayer of HBE cells with mid-log bacterial cultures at an MOI of 2 for 6 h at 37°C with 5% CO_2. **, statistically significant difference ($P < 0.05$) between the mutants and the wild type as determined by Student's *t* test. (D) Congo red binding was assessed by spotting 1, 5, or 10 μl of diluted cultures onto Congo red plates and incubating the plates for 24 h at 37°C and for an additional 48 h at room temperature.

mutant to swim and twitch at close to wild-type levels (55), and in microarrays to express relevant genes at the same level as that for the wild type, suggested that the *cbrA* mutant was not impaired in flagellum or type IV pilus biosynthesis. Furthermore, the *cbrA* mutant produced a wild-type level of rhamnolipids. These results indicate that CbrA normally promotes the expression of additional factors required for swarming in *P. aeruginosa*.

Microarray analysis of the *cbrA* mutant, compared to the PA14 wild type under swarming conditions, revealed the downregulation of a number of genes that have been identified to be upregulated in swarmer cells (37), suggesting that these genes may be regulated through CbrA and involved in the swarming growth state. Among others, these genes included a probable ATP-binding component of the ABC transporter, PA4223; genes involved in pyochelin biosynthesis, *pchG* and *pchF*; genes involved in pyoverdine biosynthesis, *pvdQ*, *pvdE*, and *pvdD*; and a gene encoding an ammonium transporter, *amtB*. Of these genes, only the PA4223 and *pvdQ* mutants displayed swarming deficiencies, consistent with previous studies (37, 55). PvdQ is of particular interest, as this protein plays a dual role in pyoverdine biosynthesis, as well as quorum sensing, with an acylhomoserine lactone acylase activity. Recently, the role of PvdQ in swarming was further studied by Jimenez et al. (18). This group showed that under iron limitation, the *pvdQ* mutant was defective in swarming, and they indicated that swarming could be restored by exogenous addition of iron, suggesting that the role of PvdQ in swarming was closely linked to the pyoverdine/iron pathway (18). In our current study and the study by Overhage et al. (37), we found that, with the exception of *pvdQ*, the genes required for pyoverdine biosynthesis, such as *pvdE* and *pvdD*, exhibited wild-type swarming behavior. Moreover, addition of iron to the swarming medium did not change the swarming phenotype of the *cbrA* mutant. Therefore, we suggest that the role of PvdQ as a quorum signal quencher might be more influential in swarming. As suggested previously, PvdQ may play a role in maintaining the relative concentrations of the two homoserine lactone quorum sensing signals, required for swarming differentiation. Alternatively, the degradation product induced by PvdQ may act a signal during swarmer cell differentiation (37). We are currently investigating these possibilities.

The *cbrA* deletion mutant produced significantly more biofilm than did the wild-type strain in an abiotic biofilm assay (Fig. 2A and B). Congo red assays revealed that the hyperbiofilm phenotype of the *cbrA* mutant might be due to increased production of the *pel*-encoded exopolysaccharide (Fig. 3). CbrA did not appear to play a role during initial

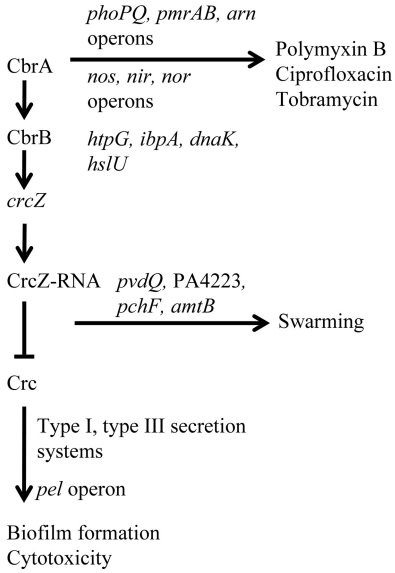

FIG. 11. Proposed model for the involvement of the CbrA/CbrB/CrcZ regulatory cascade in the regulation of swarming, biofilm formation, cytotoxicity, and antibiotic resistance in *P. aeruginosa.* Under conditions that activate the CbrAB two-component system, the phosphorylated response regulator CbrB will activate the expression of CrcZ. In turn, CrcZ has a high affinity for the RNA binding protein Crc. The binding of CrcZ to Crc sequesters Crc, resulting in the inhibition of expression of mRNAs that encode factors to promote biofilm formation and virulence. In addition to sequestration of Crc, CrcZ is involved in the direct or indirect activation of the expression of genes to promote swarming motility. Antibiotic resistance, however, involves only the CbrA sensor kinase.

attachment stage, as a rapid attachment assay revealed no difference in the abilities of the mutant and the wild type to attach to the wells of the polystyrene plates. This result was expected, since the *cbrA* mutant had a functional flagellum and pilus. Overall, our results suggested that CbrA normally negatively regulates biofilm formation. In contrast, a recent screen of the PA14 transposon mutant library by Musken et al. revealed that a *cbrA* mutant exhibited a reduced-biofilm phenotype (31). The reason for the difference in biofilm phenotype may be due to the different media used to grow the biofilms. While we used minimal medium to cultivate formation of biofilm at the air-liquid interface, Musken's group used a rich medium (LB broth) to promote biofilm formation at the bottom of the microtiter plate. The intricate relationship between swarming motility and biofilm formation in *P. aeruginosa* is complex. Although biofilm formation is a surface-associated sessile behavior and swarming is a surface-associated motile behavior, both processes are suggested to involve similar components at certain stages and under specific conditions (49). For example, both swarming motility and the initiation of biofilm formation have been shown to require flagella (33). Moreover, there is evidence that swarming motility can contribute to the early stages of *P. aeruginosa* biofilm formation (44). There are a number of studies that suggest that these surface-associated behaviors are inversely regulated and mediated through the signaling molecule cyclic-di-GMP (c-di-GMP) (21, 30, 49). It has been demonstrated that the intracellular levels of this signaling molecule influence a number of bacterial behaviors, with the common theme being that the accumulation of c-di-GMP promotes sessile behaviors, such as biofilm formation, while the degradation of c-di-GMP favors motile behaviors, such as swarming. Recent studies have shown that BifA, a c-di-GMP phosphodiesterase, participates with SadC, a c-di-GMP diguanylate cyclase, to control the level of cellular c-di-GMP in regulating biofilm formation and swarming motility (21, 30). As the *cbrA* mutant also exhibits a severe swarming defect and a hyperbiofilm phenotype, it will be of interest to examine whether CbrA plays a role in regulating the level of cellular c-di-GMP. Regardless, we previously found numerous other mutations that either exhibited a reciprocal relationship between swarming and biofilm formation or showed similar effects on the two processes (36, 55), so it seems likely that control of these processes is multideterminant.

The antibiotic resistance phenotypes observed for the *cbrA* mutant were not observed for the *cbrB* mutant. Intriguingly, these results mirror what we have seen with other two-component regulators, where the sensor kinase and response regulator have different phenotypes (e.g., *phoQ* is constitutively resistant to polymyxin and aminoglycosides, while *phoP* is null) (25). Using RT-qPCR, we confirmed substantial changes in the *cbrA* mutant, but in contrast in the *cbrB* mutant, we observed no significant changes in the transcriptional expression of the *oprH-phoPQ* operon, the *pmrAB* operon, or the LPS modification operon (*arn* operon). Thus, the upregulation of these operons in the *cbrA* mutant can be concluded to play a major role in the resistance of this mutant to the majority of the antibiotics tested, including polymyxin B, colistin, and tobramycin. We plan to further investigate the mechanism of potential cross talk between CbrA and PhoP.

The involvement of CbrA and CbrB in a number of important adaptation-related processes in *P. aeruginosa* suggests that these regulators contribute in maintaining the overall physiological balance of the bacterium. By enabling the bacteria to utilize a variety of organic compounds as carbon sources and to undergo swarming motility, while sustaining an optimal level of biofilm production, cytotoxicity, and antibiotic resistance, CbrA optimizes the efficiency of the bacteria to adapt to various environments.

ACKNOWLEDGMENTS

We gratefully acknowledge financial support from the Canadian Institutes for Health Research (CIHR) and the Canadian Cystic Fibrosis Foundation (CCFF). A.T.Y.Y. received studentships from the CCFF and the Natural Sciences and Engineering Research Council of Canada (NSERC). R.E.W.H. holds a Canada Research Chair.

We thank Laetitia Abdou and Dieter Haas for providing the necessary plasmids and primers for construction of the PA14 *crcZ* deletion mutant and complementation of the mutant. We thank Shaan Gellatly for her help with the *in vitro* human bronchial epithelial cell infection experiments. We also thank the Institute for Genomic Research (TIGR) for providing the microarray slides.

REFERENCES

1. **Boucher, R. C.** 2004. New concepts of the pathogenesis of cystic fibrosis lung disease. Eur. Respir. J. **23:**146–158.
2. **Breidenstein, E. B., B. K. Khaira, I. Wiegand, J. Overhage, and R. E. W. Hancock.** 2008. Complex ciprofloxacin resistome revealed by screening a *Pseudomonas aeruginosa* mutant library for altered susceptibility. Antimicrob. Agents Chemother. **52:**4486–4491.
3. **Choi, K. H., A. Kumar, and H. P. Schweizer.** 2006. A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. J. Microbiol. Methods **64:**391–397.

4. **Collier, D. N., P. W. Hager, and P. V. Phibbs, Jr.** 1996. Catabolite repression control in the pseudomonads. Res. Microbiol. **147:**551–561.
5. **Deziel, E., F. Lepine, S. Milot, and R. Villemur.** 2003. rhlA is required for the production of a novel biosurfactant promoting swarming motility in *Pseudomonas aeruginosa*: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursors of rhamnolipids. Microbiology **149:**2005–2013.
6. **Deziel, E., G. Paquette, R. Villemur, F. Lepine, and J. Bisaillon.** 1996. Biosurfactant production by a soil *Pseudomonas* strain growing on polycyclic aromatic hydrocarbons. Appl. Environ. Microbiol. **62:**1908–1912.
7. **Deziel, E., et al.** 1999. Liquid chromatography/mass spectrometry analysis of mixtures of rhamnolipids produced by *Pseudomonas aeruginosa* strain 57RP grown on mannitol or naphthalene. Biochim. Biophys. Acta **1440:**244–252.
8. **Fleiszig, S. M., S. K. Arora, R. Van, and R. Ramphal.** 2001. FlhA, a component of the flagellum assembly apparatus of *Pseudomonas aeruginosa*, plays a role in internalization by corneal epithelial cells. Infect. Immun. **69:**4931–4937.
9. **Friedman, L., and R. Kolter.** 2004. Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. Mol. Microbiol. **51:**675–690.
10. **Friedman, L., and R. Kolter.** 2004. Two genetic loci produce distinct carbohydrate-rich structural components of the *Pseudomonas aeruginosa* biofilm matrix. J. Bacteriol. **186:**4457–4465.
11. **Gooderham, W. J., et al.** 2009. The sensor kinase PhoQ mediates virulence in *Pseudomonas aeruginosa*. Microbiology **155:**699–711.
12. **Hancock, R. E. W., and D. P. Speert.** 2000. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and impact on treatment. Drug Resist. Updat. **3:**247–255.
13. **He, J., et al.** 2004. The broad host range pathogen *Pseudomonas aeruginosa* strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes. Proc. Natl. Acad. Sci. U. S. A. **101:**2530–2535.
14. **Hentzer, M., et al.** 2001. Alginate overproduction affects *Pseudomonas aeruginos*a biofilm structure and function. J. Bacteriol. **183:**5395–5401.
15. **Hester, K. L., et al.** 2000. Crc is involved in catabolite repression control of the bkd operons of *Pseudomonas putida* and *Pseudomonas aeruginosa*. J. Bacteriol. **182:**1144–1149.
16. **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.
17. **Horton, R. M., H. D. Hunt, S. N. Ho, J. K. Pullen, and L. R. Pease.** 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene **77:**61–68.
18. **Jimenez, P. N., et al.** 2010. Role of PvdQ in *Pseudomonas aeruginosa* virulence under iron-limiting conditions. Microbiology **156:**49–59.
19. **Kohler, T., L. K. Curty, F. Barja, C. van Delden, and J. C. Pechere.** 2000. Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. J. Bacteriol. **182:**5990–5996.
20. **Krzeslak, J., G. Gerritse, R. van Merkerk, R. H. Cool, and W. J. Quax.** 2008. Lipase expression in *Pseudomonas alcaligenes* is under the control of a two-component regulatory system. Appl. Environ. Microbiol. **74:**1402–1411.
21. **Kuchma, S. L., et al.** 2007. BifA, a cyclic-di-GMP phosphodiesterase, inversely regulates biofilm formation and swarming motility by *Pseudomonas aeruginosa* PA14. J. Bacteriol. **189:**8165–8178.
22. **Li, W., and C. Lu.** 2007. Regulation of carbon and nitrogen utilization by CbrAB and NtrBC two-component systems in *Pseudomonas aeruginosa*. J. Bacteriol. **189:**5413–5420.
23. **Liberati, N. T., et al.** 2006. An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. Proc. Natl. Acad. Sci. U. S. A. **103:**2833–2838.
24. **Linares, J. F., et al.** 2010. The global regulator Crc modulates metabolism, susceptibility to antibiotics and virulence in *Pseudomonas aeruginosa*. Environ. Microbiol. **12:**3196–3212.
25. **Macfarlane, E. L., A. Kwasnicka, and R. E. Hancock.** 2000. Role of *Pseudomonas aeruginosa* PhoP-PhoQ in resistance to antimicrobial cationic peptides and aminoglycosides. Microbiology **146:**2543–2554.
26. **Macfarlane, E. L., A. Kwasnicka, M. M. Ochs, and R. E. Hancock.** 1999. PhoP-PhoQ homologues in *Pseudomonas aeruginosa* regulate expression of the outer-membrane protein OprH and polymyxin B resistance. Mol. Microbiol. **34:**305–316.
27. **MacGregor, C. H., J. A. Wolff, S. K. Arora, and P. V. Phibbs, Jr.** 1991. Cloning of a catabolite repression control (crc) gene from *Pseudomonas aeruginosa*, expression of the gene in *Escherichia coli*, and identification of the gene product in *Pseudomonas aeruginosa*. J. Bacteriol. **173:**7204–7212.
28. **Mah, T. F., et al.** 2003. A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. Nature **426:**306–310.
29. **McPhee, J. B., S. Lewenza, and R. E. Hancock.** 2003. Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in *Pseudomonas aeruginosa*. Mol. Microbiol. **50:**205–217.
30. **Merritt, J. H., K. M. Brothers, S. L. Kuchma, and G. A. O'Toole.** 2007. SadC reciprocally influences biofilm formation and swarming motility via modulation of exopolysaccharide production and flagellar function. J. Bacteriol. **189:**8154–8164.
31. **Musken, M., S. Difiore, A. Dotsch, R. Fischer, and S. Haussler.** 2010. Genetic determinants of *Pseudomonas aeruginosa* biofilm establishment. Microbiology **156:**431–441.
32. **Nishijyo, T., D. Haas, and Y. Itoh.** 2001. The CbrA-CbrB two-component regulatory system controls the utilization of multiple carbon and nitrogen sources in *Pseudomonas aeruginosa*. Mol. Microbiol. **40:**917–931.
33. **O'Toole, G. A., and R. Kolter.** 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol. Microbiol. **30:**295–304.
34. **O'Toole, G. A., and R. Kolter.** 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol. Microbiol. **28:**449–461.
35. **O'Toole, G. A., K. A. Gibbs, P. W. Hager, P. V. Phibbs, Jr., and R. Kolter.** 2000. The global carbon metabolism regulator Crc is a component of a signal transduction pathway required for biofilm development by *Pseudomonas aeruginosa*. J. Bacteriol. **182:**425–431.
36. **Overhage, J., S. Lewenza, A. K. Marr, and R. E. W. Hancock.** 2007. Identification of genes involved in swarming motility using a *Pseudomonas aeruginosa* PAO1 Mini-Tn5-lux mutant library. J. Bacteriol. **189:**2164–2169.
37. **Overhage, J., M. Bains, M. D. Brazas, and R. E. W. Hancock.** 2008. Swarming of *Pseudomonas aeruginosa* is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. J. Bacteriol. **190:**2671–2679.
38. **Poole, K.** 2005. Aminoglycoside resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **49:**479–487.
39. **Rajan, S., and L. Saiman.** 2002. Pulmonary infections in patients with cystic fibrosis. Semin. Respir. Infect. **17:**47–56.
40. **Rashid, M. H., and A. Kornberg.** 2000. Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U. S. A. **97:**4885–4890.
41. **Rietsch, A., M. C. Wolfgang, and J. J. Mekalanos.** 2004. Effect of metabolic imbalance on expression of type III secretion genes in *Pseudomonas aeruginosa*. Infect. Immun. **72:**1383–1390.
42. **Rowe, S. M., S. Miller, and E. J. Sorscher.** 2005. Cystic fibrosis. N. Engl. J. Med. **352:**1992–2001.
43. **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.
44. **Shrout, J. D., et al.** 2006. The impact of quorum sensing and swarming motility on *Pseudomonas aeruginosa* biofilm formation is nutritionally conditional. Mol. Microbiol. **62:**1264–1277.
45. **Simon, R., U. Priefer, and A. Puehler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram negative bacteria. Nat. Biotechnol. **1:**784–791.
46. **Sonnleitner, E., L. Abdou, and D. Haas.** 2009. Small RNA as global regulator of carbon catabolite repression in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U. S. A. **106:**21866–21871.
47. **Sutherland, I. W.** 2001. The biofilm matrix—an immobilized but dynamic microbial environment. Trends Microbiol. **9:**222–227.
48. **Tremblay, J., A. P. Richardson, F. Lepine, and E. Deziel.** 2007. Self-produced extracellular stimuli modulate the *Pseudomonas aeruginosa* swarming motility behaviour. Environ. Microbiol. **9:**2622–2630.
49. **Verstraeten, N., et al.** 2008. Living on a surface: swarming and biofilm formation. Trends Microbiol. **16:**496–506.
50. **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.
51. **Whitchurch, C. B., T. Tolker-Nielsen, P. C. Ragas, and J. S. Mattick.** 2002. Extracellular DNA required for bacterial biofilm formation. Science **295:**1487.
52. **Wiegand, I., K. Hilpert, and R. E. W. Hancock.** 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat. Protoc. **3:**163–175.
53. **Wolff, J. A., C. H. MacGregor, R. C. Eisenberg, and P. V. Phibbs, Jr.** 1991. Isolation and characterization of catabolite repression control mutants of *Pseudomonas aeruginosa* PAO. J. Bacteriol. **173:**4700–4706.
54. **Wozniak, D. J., et al.** 2003. Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms. Proc. Natl. Acad. Sci. U. S. A. **100:**7907–7912.
55. **Yeung, A. T. Y., et al.** 2009. Swarming of *Pseudomonas aeruginosa* is controlled by a broad spectrum of transcriptional regulators, including MetR. J. Bacteriol. **191:**5592–5602.