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Requirement of the *Pseudomonas aeruginosa* CbrA Sensor Kinase for Full Virulence in a Murine Acute Lung Infection Model

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***Pseudomonas aeruginosa* is an opportunistic pathogen that is a major cause of respiratory tract and other nosocomial infections. The sensor kinase CbrA is a central regulator of carbon and nitrogen metabolism and *in vitro* also regulates virulence-related processes in *P. aeruginosa*. Here, we investigated the role of CbrA in two murine models of infection. In both peritoneal infections in leukopenic mice and lung infection models, the *cbrA* mutant was less virulent since substantially larger numbers of *cbrA* mutant bacteria were required to cause the same level of infection as wild-type or complemented bacteria. In contrast, in the chronic rat lung model the *cbrA* mutant grew and persisted as well as the wild type, indicating that the decrease of *in vivo* virulence of the *cbrA* mutant did not result from growth deficiencies on particular carbon substrates observed *in vitro*. In addition, a mutant in the cognate response regulator CbrB showed no defect in virulence in the peritoneal infection model, ruling out the involvement of certain alterations of virulence properties in the *cbrA* mutant including defective swarming motility, increased biofilm formation, and cytotoxicity, since these alterations are controlled through CbrB. Further investigations indicated that the mutant was more susceptible to uptake by phagocytes *in vitro*, resulting in greater overall bacterial killing. Consistent with the virulence defect, it took a smaller number of *Dictyostelium discoideum* amoebae to kill the *cbrA* mutant than to kill the wild type. Transcriptional analysis of the *cbrA* mutant during *D. discoideum* infection led to the conclusion that CbrA played an important role in the iron metabolism, protection of *P. aeruginosa* against oxidative stress, and the regulation of certain virulence factors.**

The early fate of a bacterial pathogen during infections is controlled by its interaction with the innate immune system, which normally acts rapidly to mount a first line of defense to keep the pathogen in check (1). Although defense mechanisms against different pathogens are diverse, many of them are evolutionarily conserved, including the production of an array of antimicrobial enzymes and peptides, the activation of phagocytic cells, and the production of antimicrobial metabolites (1). *Pseudomonas aeruginosa* is an important Gram-negative opportunistic human pathogen that is responsible for a number of serious acute and chronic infections in patients with impaired immunity and mucosal defenses (2, 3). *P. aeruginosa* has evolved various strategies to circumvent host immune defenses. This includes the expression of multiple virulence factors that work in concert either to inactivate components of the host immune response or to facilitate the bacteria's evasion of the host response (4). *P. aeruginosa* uses its type III and type VI secretion systems to inject virulence factors into the host cells to modulate host immune response (5, 6). Secreted components of *P. aeruginosa*, including exotoxins, phospholipases, proteases, and phenazines, inactivate effectors and cells of the innate and adaptive immune systems and contribute to host tissue damage (7). During chronic infections, *P. aeruginosa* forms biofilms that protect the bacteria from host defense mechanisms, including phagocytosis (8). Virulence and the avoidance of host defense mechanisms are intricately regulated, with several regulators having very broad influences.

P. aeruginosa has one of the largest sets of two-component systems (TCSs) known in bacteria (64 sensor kinases and 72 response regulators). The capability of TCSs to sense diverse stimuli and elicit rapid adaptive response that can involve the altered transcription of a number of genes contributes to the ability of *P. aeruginosa* to adapt to a wide range of environments, including the human host (9). Several well-studied TCS regulators, including GacAS, PhoQ, RetS, LadS, and AlgR, are known to regulate virulence and virulence-associated processes in *P. aeruginosa*, contributing to the ability of this bacterium to cause infections in multiple hosts (9).

The CbrAB TCS in *P. aeruginosa* was originally shown to be involved in controlling the expression of a number of catabolic pathways involved in carbon and nitrogen utilization (10, 11). Mutations in this system impair the ability of the bacterium to grow on specific organic compounds, including glucose, as the sole carbon source. Previously, we demonstrated that the sensor kinase CbrA regulates various virulence-related processes in *P. aeruginosa* (12). A mutant in the *cbrA* gene was completely unable to swarm and impaired in pyoverdine production but exhibited increased biofilm production. Furthermore, *in vitro* cytotoxicity experiments revealed that the *cbrA* mutant exhibited increased cytotoxicity toward human bronchial epithelial (HBE) cells (12).

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The *cbrA* mutant also exhibited increased resistance to a variety of clinical antibiotics, including polymyxin B, ciprofloxacin, and tobramycin. Similarly, a mutant in the adjacent *cbrB* gene encoding the cognate response regulator exhibited impaired swarming motility, increased biofilm formation, and increased cytotoxicity toward HBE cells. In contrast, the antibiotic resistance and impaired pyoverdine phenotypes observed in the *cbrA* mutant were absent in the *cbrB* mutant (12).

In this study, we examined the *in vivo* virulence of the *cbrA* mutant in two mouse models, the acute lung infection model and the leukopenic mouse peritoneal infection model, and demonstrated a 10-fold and a 10^4-fold reduction in virulence, respectively. This was not due to an *in vivo* growth defect reflecting any known metabolic deficiencies, since there was no significant difference in the ability of the wild type (WT) and the *cbrA* mutant to grow and persist *in vivo* in a chronic rat lung persistence model and no alteration in virulence of the *cbrB* mutant. By investigating the role of CbrA in phagocytosis, we found that there was significantly enhanced uptake of the *cbrA* mutant compared to the WT by both macrophages and neutrophils *in vitro*. Moreover, transcriptional analysis of the *cbrA* mutant during *Dictyostelium discoideum* infection provided additional cues as to the underlying mechanism(s). Intriguingly, CbrA-regulated *in vivo* virulence of *P. aeruginosa* was independent of CbrB.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The WT strain PA14, its *cbrA* deletion mutant, the *cbrA*/pUCP18::*cbrAB*$^+$ complemented strain, and transposon mutants (12) were routinely grown in Luria-Bertani (LB) broth. For phenotypic characterization, the following transposon mutants of the PA14 Harvard mutant library (13) were used: *cbrB* (ID44074), *pcrH* (ID48586), *pvdA* (ID30448), *pvdQ* (ID27758), *osmC* (ID5655), *sodM* (ID36454), *katE* (ID40227), *rhlA* (ID23291), *rhlB* (ID27130), *lasB* (ID31938), *aprA* (ID23768), *phzA* (ID37612), and *phzB* (ID24980) mutants. For plasmid or transposon maintenance, antibiotics were added to the growth medium at concentrations of 15 μg/ml gentamicin and/or 500 μg/ml carbenicillin.

Tissue culture and phagocyte experiments. Venous blood was collected from healthy adult donors in Vacutainer collection tubes containing sodium heparin as an anticoagulant (BD Biosciences) in accordance with the ethical approval and guidelines of the UBC Research Ethics Board. Peripheral blood mononuclear cells (PBMC) were isolated as previously described (14, 15). To obtain human monocyte-derived macrophages (MDM), isolated PBMC were resuspended in serum-free RPMI 1640 medium and 2×10^6 cells/well were seeded into 24-well tissue culture plates (Corning Life Science, Corning, NY) and allowed to rest at 37°C in 100% humidity and 5% CO_2 for 1 h, and then the medium was replaced with complete RPMI medium, i.e., RPMI 1640 medium (Life Technologies Invitrogen) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 1 mM sodium pyruvate. Cells were then cultured for 7 days in 100% humidity and 5% CO_2 at 37°C. On days 2 and 6, cells were washed and medium was replaced with complete RPMI containing 10 ng/ml macrophage colony-stimulating factor (M-CSF) (Research Diagnostic, Concord, MA).

Murine bone marrow-derived macrophages were prepared by culturing bone marrow cells (10^6 cells/ml) of C57BL/6 mice for 7 days in high-glucose Dulbecco's modified Eagle medium (DMEM) with 20% fetal calf serum (FCS), 2 mM L-glutamine, and 1 mM sodium pyruvate (all from Invitrogen) and supplemented with 25 ng/ml recombinant M-CSF (eBioscience). On day 7, after incubation with phosphate-buffered saline (PBS) for 30 min at 4°C, cells were detached with a cell scraper, washed by centrifugation, and counted.

Human neutrophils and serum were isolated as previously described (14, 16), resuspended in Hanks' buffered salt solution (HBSS; Invitrogen), and used immediately for opsonization or treatments.

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

Animals. All mouse experiments were conducted in accordance with the Animal Care Ethics Approval and Guidelines of the University of British Columbia (UBC). C57BL/6 and CD-1 female mice (from the Center for Modeling Disease, UBC) were maintained under specific-pathogen-free conditions. Mice were 6 weeks of age and weight matched in all experiments. Male Sprague-Dawley rats of approximately the same weight were used according to the ethics committee for animal treatment of Laval University.

Mouse model of peritoneal infection. Peritoneal infections were performed as described previously with modifications (17). C57BL/6 mice were injected with cyclophosphamide (Sigma) intraperitoneally (i.p.) at 150 mg/kg of body weight at 4 and 2 days prior to infection. The level of leukopenia was assessed by cytometry of leukocytes from 50 μl blood collected from the tail vein the day prior to initiation of the infection. Cell staining with Ly6G phycoerythrin (PE) and CD11b PercP (eBioscience) showed a reduction of 85% in neutrophils and 87% in the monocyte population. Infections were initiated by injection by the i.p. route with fresh cultures of *P. aeruginosa*, either 60 CFU (in 200 μl PBS) of the WT, the *cbrA* complemented strain, or the *cbrB* mutant or 60, 500, 10^3, 10^4, and 10^6 CFU of these strains in 200 μl of PBS. At 18 h postinfection, mice were euthanized by CO_2 inhalation. Blood was collected by cardiac puncture, and the peritoneal lavage fluid was collected by installation and withdrawal of 3 ml of sterile saline. Bacterial numbers in the blood and peritoneal lavage fluid were measured by serial dilution and growth at 37°C overnight after plating on LB agar plates.

Mouse model of acute lung infection. In the acute lung infection model, CD-1 mice were anesthetized via inhalation of aerosolized isoflurane (2 to 5%) mixed with oxygen and infected by intranasal administration with a fresh culture of *P. aeruginosa* resuspended at 10^6 CFU in 20 μl PBS for WT and the *cbrA* complemented strains and at 10^6 or 10^7 CFU in 20 μl PBS for the *cbrA* mutant. At 18 h postinfection, mice were euthanized by lethal pentobarbital injection (120 mg/kg, i.p.). The bronchoalveolar lavage (BAL) fluid was collected by cannulating the trachea and washing the lung 3 times with 600 μl of saline solution. Bacterial numbers in the BAL fluid were measured by serial dilution and plating for single colonies after overnight growth on LB agar plates.

Rat model of chronic lung infection. Agar beads were prepared by a modification of a previously described method (18, 19). The *cbrA* mutant (tagged with gentamicin resistance) and the PA14 strain were grown separately in tryptic soy broth (TSB). Overnight cultures were sedimented by centrifugation at $4.8 \times 10^3 \times g$ for 2 min, washed twice with 500 μl of PBS, resuspended in 1 ml PBS, and added to 9 ml of 2% agar, prewarmed to 48°C. A mixture of equal counts of WT and mutant was added into 200 ml heavy mineral oil at 48°C with rapid stirring on a magnetic stirrer in a water bath for 5 min at room temperature, followed by a 10-min period without stirring. The oil-agar mixture was centrifuged at 9,300 × *g* for 20 min to sediment the beads and washed twice with PBS. The preparations, containing beads of 100 μm to 200 μm in diameter, were used as inocula for animal experiments. The number of bacteria in the beads was determined by homogenizing the bacterial bead suspension and plating 10-fold serial dilutions on Mueller-Hinton agar (MHA) and MHA supplemented with 35 μg/ml gentamicin.

Sprague-Dawley rats were anesthetized using isoflurane (2% of respiratory volume) and inoculated by intubation using a venous catheter (18G) and syringe (1-ml tuberculin) with 120 μl of a suspension of agar bead-embedded bacteria containing approximately 5×10^7 CFU/injec-

tion. After 7 days, the lungs were removed from sacrificed rats, and homogenized tissues were plated in triplicates on appropriate media.

The *in vivo* competitive index (CI) was determined as the CFU output (*in vivo*) ratio of the *cbrA* mutant in comparison to the WT strain, divided by the CFU input ratio of *cbrA* mutant to WT (20, 21). The injections of approximately 120 μl of each bacterial mixture were administered to 6 animals. After 7 days of infection, the bacterial counts were performed from infected rat lungs using MHA to assess total bacterial numbers of the WT and mutant or MHA with 35 μg/ml gentamicin for mutant selection. The presented CIs were calculated as the geometric means for animals in the same group.

Amoeba model of infection. *Dictyostelium discoideum* Ax2 was provided by M. Steinert (TU Branschweig) and routinely grown in HL-5 medium in cell culture flasks at 22.5°C (22). To analyze the virulence of the *cbrA* mutant using the amoeba host, the assay was performed according to a method described previously (23). Briefly, 50 μl of overnight cultures grown in LB medium was mixed with 200 μl PBS buffer and plated on M9 agar plates (6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 10 ml 100 mM $MgSO_4$, 10 ml 10 mM $CaCl_2$, 10 ml 40% glucose, and 15 g agar, per liter). Plates were dried under a laminar flow bench for 1 h to obtain a dry and even bacterial lawn. Amoebae grown for 2 to 4 days in HL5 medium were harvested by centrifugation at 1,600 × *g* for 10 min, washed, and resuspended in PBS buffer. Cells were adjusted to 8×10^6 cells/ml and kept on ice. This stock solution was used to prepare droplets of 5 μl containing between 5 and 20,000 amoebae, which were subsequently spotted onto the bacterial lawn. The plates were incubated at 22.5°C for 5 days. The highest dilution at which growth of the amoebae in form of a plaque was visible was reported. Six independent experiments were performed for each bacterial strain.

Cytotoxicity assays. To examine the effect of carbon source on cytotoxicity of the WT and its *cbrA* mutant, interaction assays of HBE cells with *P. aeruginosa* were performed as previously described (12). Mid-log *P. aeruginosa* isolates were added to cultured HBE cells, at a multiplicity of infection of 2 bacteria per HBE cell, in glucose-free DMEM supplemented with 0.1% (wt/vol) of succinate or glycerol (enabling growth of the *cbrA* mutant at rates equivalent to that of the WT) or glucose or mannitol (carbon sources in which the *cbrA* mutant grows poorly). The assay mixture was incubated at 37°C with 5% CO_2, and at various postinfection time points, the medium was removed from the wells, placed in microtiter plates, and centrifuged for 10 min at 800 × *g* to pellet the bacteria and host cell debris. Cytotoxicity was determined by measuring the release of the cytosolic enzyme lactate dehydrogenase (LDH) into the supernatants using a colorimetric cytotoxicity detection kit (Roche, Mannheim, Germany), with controls as described previously.

Cytotoxicity toward human isolated PBMC was examined by incubating PBMC at 1×10^6 cells/well with *P. aeruginosa* (multiplicity of infection [MOI], 1) for 4 h at 37°C. At various postinfection time points, supernatants from the samples were obtained and tested for LDH release. Three independent experiments were performed for each assay.

Growth curves. *P. aeruginosa* PA14 and mutants were grown overnight in glucose-free DMEM supplemented with 0.1% (wt/vol) of glucose or succinate. Five-microliter portions of these cultures were added to 195 μl of the same medium in a 96-well microtiter plate and incubated at 37°C with 5% CO_2. Growth of these cultures was measured by determining the optical density at 600 nm (OD_{600}) every hour for 9 h.

***In vitro* phagocytosis assays (gentamicin protection assay).** Assays of *in vitro* phagocytosis of PA14 and mutants by MDM were performed as previously described (24) with modifications. Briefly, mid-log cultures of *P. aeruginosa* were washed with complete RPMI medium and resuspended in 1 ml of the medium. MDM at 1.5×10^5 cells/ml were mixed with the resuspended bacteria at an MOI of 10 and incubated for 1 h at 37°C (at which time minimal intracellular killing had occurred). Subsequently, cells were washed with complete RPMI and incubated with 400 μg/ml gentamicin for 30 min at 37°C to kill the extracellular and attached bacteria. After the gentamicin treatment, MDM cells were washed and lysed with 0.1% Triton X-100. Lysates were plated onto LB agar and incubated overnight at 37°C. The next day, colonies were counted and relative phagocytosis was determined by CFU counts. Three independent experiments with duplicates in each experiment were performed for each bacterial strain.

***In vitro* macrophage and neutrophil killing assays.** *In vitro* killing of PA14 and mutants by stimulated MDM was performed as previously described (25) with minor changes. To activate MDM, gamma interferon (IFN-γ) (20 ng/ml), in addition to M-CSF, was added on day 6 of culturing. Briefly, mid-logarithmic-phase cultures of *P. aeruginosa* were washed with complete RPMI medium and resuspended in 1 ml of the medium. MDM at 1.5×10^5 cells/ml were incubated with the resuspended bacteria at an MOI of 1 for 1 h at 37°C. Subsequently, MDM were washed with complete RPMI and incubated with 400 μg/ml gentamicin for 2 h at 37°C to kill extracellular bacteria. After the gentamicin treatment, either MDM were lysed with 0.1% Triton X-100 and plated for viable intracellular bacteria (T0) or the medium was replaced with fresh medium and the mixture was incubated for an additional 3 h (T3) and 6 h (T6) at 37°C. At T3 and T6, MDM were lysed with 0.1% Triton X-100. Lysates were plated onto LB agar and incubated overnight at 37°C, and residual bacterial colonies were counted. Three independent experiments with duplicates for each experiment were performed for each bacterial strain.

An *in vitro* neutrophil killing assay was adapted from the protocol of Mishra et al. (26). Briefly, mid-log cultures of *P. aeruginosa* were washed and opsonized with 10% fresh human serum for 20 min at 37°C while the samples were rotated end over end (27). Opsonized bacteria were added to freshly isolated neutrophils (2×10^6 cell/ml) at an MOI of 10 followed by incubation at 37°C for 30 min, rotating end over end. Subsequently, neutrophils were washed with HBSS and incubated with 400 μg/ml gentamicin for 30 min at 37°C to kill bacteria. After 30 min, either neutrophils were lysed and plated for viable intracellular bacteria (T0) or the medium was replaced with fresh medium and the neutrophils were incubated for an additional 0.5 h (T0.5) and 1 h (T1) at 37°C. At T0.5 and T1, neutrophils were lysed with 0.1% Triton X-100. Lysates were plated onto LB agar and incubated overnight at 37°C. The next day, colonies were counted and relative phagocytosis was determined by CFU counts. At least 3 independent experiments with duplicates in each experiment were performed for each bacterial strain.

ELISA. Cytokine levels were measured by enzyme-linked immunosorbent assay (ELISA) using anti-mouse interleukin-6 (IL-6) (clone MP5-20F3 and MP5-32C11) and anti-mouse tumor necrosis factor alpha (TNF-α) (clone 1F3F3D4 and MP6-XT22) (eBioscience), all followed by avidin horseradish peroxidase (HRP; eBioscience) as per the manufacturer's protocols. The Duokit RD System (DY453) was used to assay the concentration of the chemokine KC (also known as CXCL1).

Microarrays. The transcriptional responses of *P. aeruginosa* WT and the *cbrA* mutant in cocultivation with *D. discoideum* were analyzed using the *P. aeruginosa* Affymetrix GeneChips (Affymetrix Inc., USA). Cells of the WT and *cbrA* mutant were grown in the presence of *D. discoideum* for 48 h at 22.5°C on M9 agar plates prior to RNA isolation and microarray analyses. Specifically, overnight cultures of *P. aeruginosa* WT and *cbrA* mutant were washed twice and resuspended in PBS buffer and adjusted to an OD_{600} of 2.0. Of these bacterial cell suspensions, 10 μl was mixed with washed amoeba cells of 2-day-old *D. discoideum* cultures in a ratio of 3:1, bacteria to amoebae. These mixtures were subsequently plated on M9 agar plates. After incubation for 48 h at 22.5°C, cells were harvested from the agar plate surface using an inoculation loop and resuspended in M9 medium supplemented with RNAprotect reagent (Qiagen, Germany). To separate cells of *D. discoideum* from the bacterial cells, the mixtures were centrifuged for 1 min at 100 × *g* and the supernatants containing the bacterial cells were used for RNA extraction. Six independent cocultures each with 5 agar plates of PA14 or the *cbrA* mutant were pooled. RNA isolation, cDNA synthesis, hybridizations, and analysis were performed as described previously (28). Two independent microarray experiments were performed, and changes of ≥2-fold were used as the cutoffs for

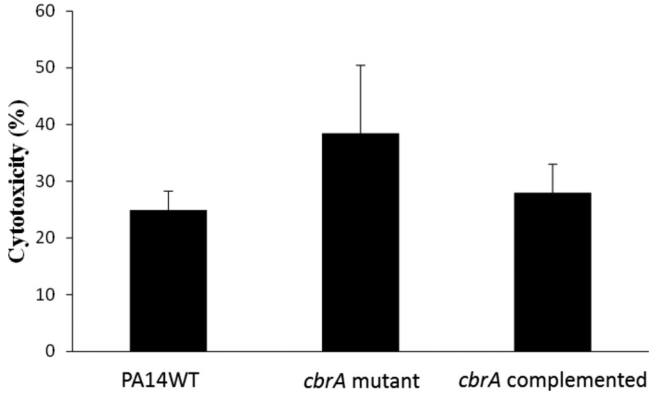

FIG 1 *In vitro* cytotoxicity toward human PBMC. The abilities of wild-type strain PA14 and the *cbrA* mutant to induce cell damage were determined by monitoring the release of intracellular LDH into the supernatant from the PBMC. Bacteria were cocultured with 1×10^6 PBMC at an MOI of 1, and LDH release was monitored at 4 h postinfection. The percentage of cytotoxicity was calculated as follows: % cytotoxicity = [(*A* of experimental sample − *A* of background control)/(*A* of positive control − *A* of background control)] × 100, where *A* is absorbance. Each result represents the mean of 3 independent biological repeats, each assayed in triplicate. The moderate differences observed were not statistically significant ($P > 0.05$ by Student's *t* test).

reporting expression changes with average changes in expression reported. For the validation of microarray results, quantitative real-time PCR (RT-qPCR) was performed as described previously (29) using the Power SYBR green PCR Master Mix in an ABI 7300 Real Time PCR System (Applied Biosystems). All reactions were normalized to the housekeeping sigma factor gene, *rpoD*.

Microarray accession numbers. The microarrays have been deposited in ArrayExpress under accession number E-MTAB-2160.

Statistical analyses. Statistical analyses were performed with GraphPad Prism software using the *F* test or Student's *t* test.

RESULTS

Cytotoxicity of the *cbrA* mutant. Previously (12), we showed that the *cbrA* mutant exhibited increased cytotoxicity toward HBE cells compared to the wild-type strain in MEM, a commercial medium that contains glucose. Here we investigated whether the ability of the *cbrA* mutant to induce damage to HBE cells was associated with its known growth deficiencies on certain carbon sources such as glucose. Indeed, we confirmed here that the *cbrA* mutant grew more weakly than the WT in glucose and mannitol but grew like the WT in succinate and glycerol. Supplementation of DMEM with glucose, succinate, mannitol, or glycerol did not affect the increased cytotoxicity phenotype of the *cbrA* mutant observed previously with glucose in MEM (data not shown). These results suggest that the ability of the *cbrA* mutant to kill HBE cells was not dependent on the carbon source and that impaired growth of the mutant did not contribute to its toxicity toward HBE cells.

We investigated whether the *cbrA* mutant exhibited an enhanced ability to kill other host cell types, namely, human PBMC. As shown in Fig. 1, after 4 h of incubation at 37°C, the *cbrA* mutant was slightly but not significantly ($P > 0.05$) more cytotoxic toward PBMC. This indicated that the enhanced cytotoxicity of the *cbrA* mutant, observed with HBE cells, might be somewhat cell-type specific.

Reduced virulence of the *cbrA* mutant in a mouse model of acute lung infection. The acute lung infection model is typically used to mimic pneumonia caused by *P. aeruginosa* (30). To examine the role of the CbrA sensor kinase in virulence of *P. aeruginosa* strain PA14 *in vivo*, we inoculated strain PA14 or its *cbrA* mutant into the lungs of mice. Intranasal administration of 10^6 CFU of either PA14 or the *cbrA* mutant gave significantly different results in symptoms and the bacterial load in the BAL fluid (Fig. 2A). Mice infected with the WT strain exhibited rapid breathing, indicating respiratory distress, and after 18 h the average number of bacteria recovered from the BAL fluid was 10^6 CFU/ml. In contrast, mice instilled with the *cbrA* mutant showed no signs of altered respiration and the mutant was not detectable in the BAL fluid. Acute lung infection by *P. aeruginosa* is known to induce strong neutrophil influx to the site of the infection (31). The levels of leukocytes and chemokine KC (known to induce migration of neutrophils) in the BAL fluid were strongly reduced in mice infected with the *cbrA* mutant compared to those infected with PA14 (Fig. 2B and C). These results are consistent with enhanced clearance from the lungs of the *cbrA* mutant compared to PA14. When the experiment was repeated by increasing the inoculum of the *cbrA* mutant to 10^7 CFU, we observed that mice showed signs of respiratory distress and levels of bacterial recovery from the BAL fluid and lungs similar to those of mice infected with 10^6 CFU of PA14. These results showed that the *cbrA* mutant was less virulent than PA14 in a mouse model of acute lung infection in that 10 times more of the *cbrA* mutant was required to cause a level of infection in the lungs of mice similar to that caused by PA14.

Reduced virulence of the *cbrA* mutant in a mouse model of peritoneal infection. Nosocomial peritonitis is caused by exogenous pathogenic bacteria, including *P. aeruginosa*, that gain access to the abdominal cavity through a variety of routes, including during abdominal surgery (32). In this study, we examined the ability of PA14 and the *cbrA* mutant to grow in the peritoneum as well as spread to disseminated sites (i.e., the blood), using a mouse model of peritoneal infection. Since the *P. aeruginosa* WT strain is readily cleared by healthy mice, mice were rendered leukopenic with the chemotherapeutic agent cyclophosphamide. As shown in Fig. 3A, mice injected with 60 CFU of the PA14 showed bacterial loads of approximately 10^3 and 10^2 CFU/ml in the blood and peritoneal lavage fluid, respectively, whereas animals infected with the *cbrA* mutant completely cleared the pathogen by 18 h postinfection. When the experiment was repeated with higher input doses, mice were able to clear the pathogen effectively even at concentrations of the *cbrA* mutant as high as 10^4 CFU (Fig. 3B; only this dose is shown, but similar results were observed with input doses of 5×10^2 and 10^3 bacteria). However, at an input dosage of 10^6 CFU of the *cbrA* mutant, the number of bacteria recovered was similar to that for mice infected with 60 CFU of the strain PA14 (Fig. 3B). The infectivity of the *cbrA* mutant could be restored to the WT level by complementation (Fig. 3B). Thus, the *cbrA* mutant was up to 10^4-fold less virulent than strain PA14 in a mouse model of peritoneal infection, indicating that CbrA most likely played a crucial role in the ability of strain PA14 to grow in the peritoneum and/or disseminate.

Previously, we showed that CbrA mediated *in vitro* cytotoxicity toward HBE cells via its cognate response regulator CbrB (12). In contrast, a *cbrA* mutant demonstrated attenuated *in vivo* virulence, and this did not occur for a *cbrB* mutant. As shown in Fig. 3A, mice infected with 60 CFU of the *cbrB* mutant showed a bacterial recovery in the peritoneal lavage and blood (and similar levels of distress as judged by ruffling of fur, hunching, altered breathing, and reduced movement) similar to that of mice in-

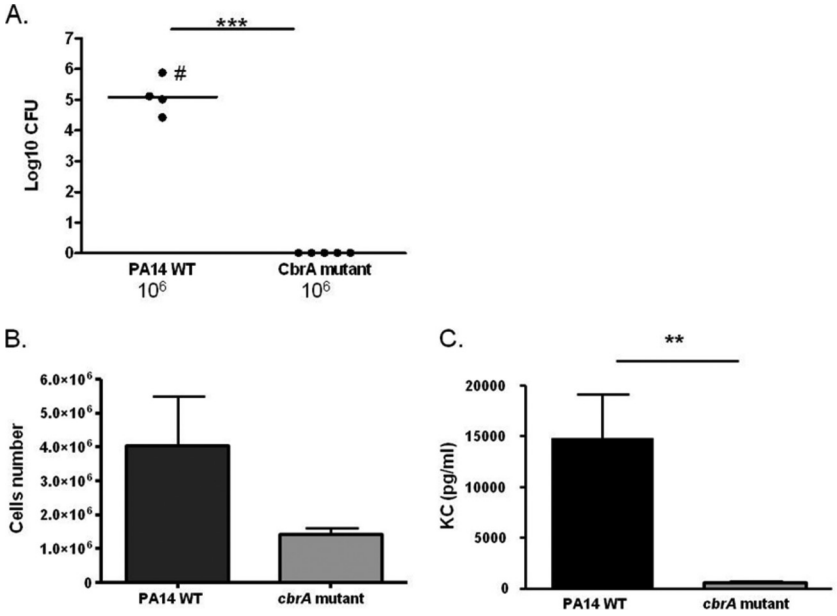

FIG 2 Lung infection model: bacterial load and immune cell recruitment in the BAL fluid of mice infected with *P. aeruginosa*. CD-1 mice were inoculated via the intranasal route with 10^6 CFU PA14 or 10^6 CFU of the *cbrA* mutant. Eighteen hours postinfection, the mice were sacrificed and their broncheoalveolar lavage fluids were collected for CFU determination (A) and assessment of leukocyte recruitment (B) and chemokine KC induction (C). In panel A, each point represents a single mouse with the one dead mouse indicated by # (it was assigned the CFU value recovered from the most heavily infected live mouse). Statistically significant differences, shown by asterisks (**, $P < 0.01$; ***, $P < 0.001$), were determined by paired Student's *t* test.

fected with 60 CFU of PA14. This result was consistent with previous conclusions that CbrA is able to modulate bacterial phenotypes and the expression of certain genes independently of CbrB (12).

Lack of effect of the *cbrA* mutant on *in vivo* competitive growth. The significant reduction in virulence for the *cbrA* mutant compared to strain PA14 in both mouse models of acute lung infection and peritoneal infection led to an investigation of whether the attenuated virulence observed for the *cbrA* mutant might be due to the mutant's poor ability to grow *in vivo*. To study this, we examined the *in vivo* competitive growth of 1:1 mixtures of strains PA14 and the *cbrA* mutant in a rat model of chronic lung infection. In this model, *P. aeruginosa* is embedded in agar beads to retain the bacteria physically in the airways and avoid physical elimination by the host, e.g., through mucociliary clearance. As shown in Fig. 4, after 7 days postinfection, the *cbrA* mutant showed no major difference in bacterial counts from the lung, with a mean competitive index of 0.66 (compare, for example, another sensor kinase mutant in *phoQ* that showed a competitive index of 0.00019 [9]). These results suggested that the *cbrA* mutant was able to grow and persist in the lungs as well as the wild-type PA14 isolate. These studies were performed in rats, while the virulence studies were performed in mice. However, taken together with the fact that the *cbrB* mutant, like the *cbrA* mutant, exhibits a growth defect in glucose *in vitro* but unlike *cbrA* does not have a virulence defect (Fig. 3), we can conclude that the influence of *cbrA* on virulence was likely not due to a poor ability to grow *in vivo*.

Enhanced uptake of the *cbrA* mutant by macrophages. One possible explanation for the difference observed for the *cbrA* mutant between *in vitro* and *in vivo* virulence models is the reduced effectiveness of host defense mechanisms, such as phagocytosis. To investigate whether the *cbrA* mutant was more susceptible to uptake by phagocytes than the WT strain, we utilized a gentamicin survival assay by incubating strain PA14 and its *cbrA* mutant with human macrophages and determining the number of bacteria taken up after killing of extracellular bacteria with gentamicin. Compared to strain PA14, there was an average 3.6-fold increase in uptake of the *cbrA* mutant by human macrophages (Fig. 5). The level of uptake for the *cbrA* mutant could be restored to the WT level by complementation of the mutant with the WT *cbrAB* genes. Similarly, significant increases were observed in uptake by murine macrophages of the *cbrA* mutant compared to the WT strain (data not shown). Consistent with its unaltered virulence, a *cbrB* mutant exhibited a WT level of uptake by macrophages.

Enhanced clearance of the *cbrA* mutant by macrophages and neutrophils. The enhanced uptake of the *cbrA* mutant compared to its WT strain led us to examine whether the *cbrA* mutant was also more sensitive to macrophage-mediated killing than strain PA14. Data were analyzed by assessing the average intracellular bacterial counts for strain PA14 and the *cbrA* mutant; Fig. 6A shows the percentage of viable bacteria recovered from the macrophages relative to the initial inoculum at each time point. At T0, after 1 h of incubation of bacteria with macrophages and gentamicin killing of extracellular bacteria, the intracellular bacterial count for the *cbrA* mutant was on average 9.5-fold higher than that of strain PA14, consistent with the above-described phagocytosis results. When cells were incubated for a further 3 h (between T0 and T3), there was little to no bacterial killing, with similar bacterial counts at T0 and T3 for both the WT and mutant strains. A reduction in bacterial counts for both strains after 6 h of incubation (T6) indicated that both strains were killed by the macrophages. About 10% of the initial number of *cbrA* mutant bacteria added to the macrophages were cleared at T6 relative to T0, while

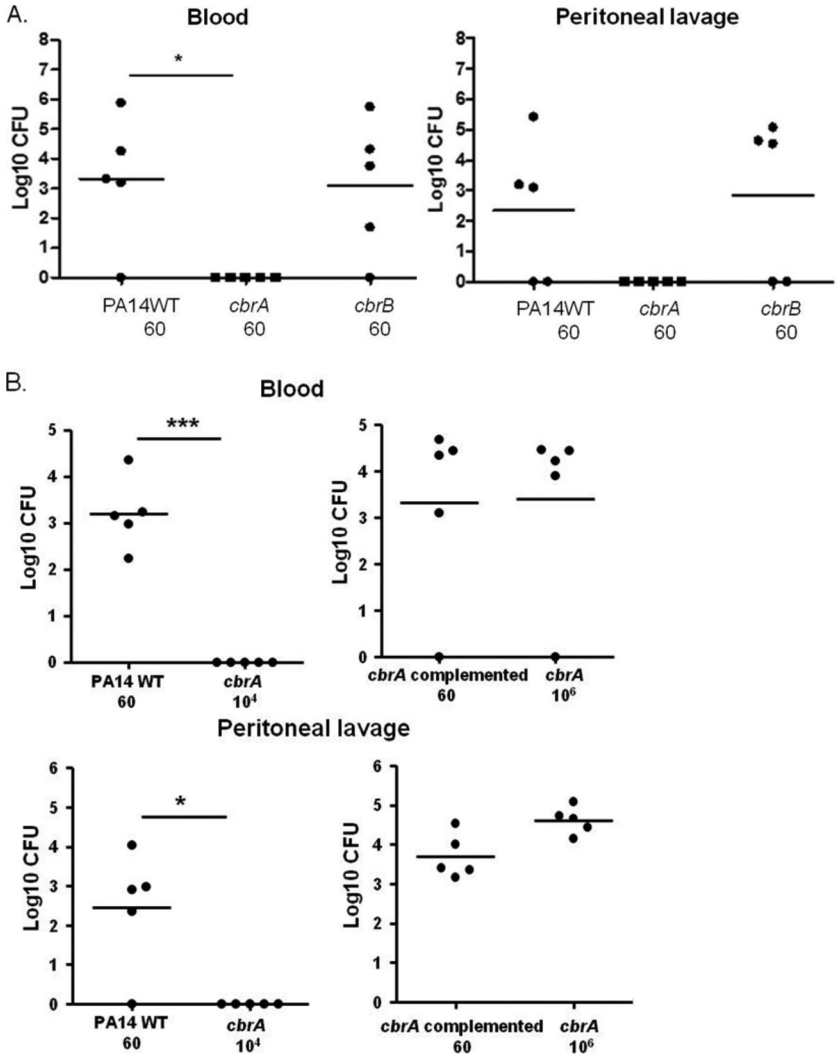

FIG 3 Bacterial load in the peritoneal lavage fluid and blood of leukopenic mice infected with *P. aeruginosa* via the intraperitoneal route. C57BL/6 mice were injected with cyclophosphamide 4 and 2 days prior to the infection to render them leukopenic and thus more susceptible to infection. On the day of the infection, the mice were injected via the i.p. route with 60 CFU PA14 or the *cbrA* complemented strains or with various amounts (60, 10^4, or 10^6 CFU) of the *cbrA* mutant. Eighteen hours postinfection, the mice were sacrificed and their blood and peritoneal lavage fluid were collected for CFU determinations. Each point represents a single mouse. Statistically significant differences, signified by asterisks (*, $P < 0.05$; ***, $P < 0.001$), were determined by analysis of variance (ANOVA).

only about 1% of strain PA14 bacteria were cleared. Extending the incubation period beyond 6 h led to an increase in the bacterial CFU counts, indicating that *P. aeruginosa* either was replicating intracellularly, perhaps due to inactivation of the macrophages, or had exited the macrophages to replicate extracellularly. The bacterial counts in the *cbrA* complemented strain at all of the time points were the same as the WT level. Similar results were observed using murine macrophages (data not shown).

Neutrophils represent one of the primary cell-mediated defenses against *P. aeruginosa*. Therefore, the ability of strain PA14 and the *cbrA* mutant to resist killing by neutrophils was also examined. As difficulties were experienced in obtaining neutrophils from mice, the neutrophil killing assays were performed using only freshly isolated human neutrophils incubated with strain PA14 and the *cbrA* mutant opsonized with fresh serum. Due to the shorter life span, enhanced killing propensity, and greater sensitivity of neutrophils, compared to macrophages, to bacterially induced damage, the incubation time of neutrophils with *P. aeruginosa* was reduced to 30 min and the survival of bacteria in gentamicin-treated neutrophils was followed for only 1 h. To compensate for these reduced incubation periods, both the concentration of neutrophils used and the MOI of *P. aeruginosa* were increased. As shown in Fig. 6B, assessment of the average bacterial cell counts, after the 30 min of incubation with neutrophils and gentamicin killing of extracellular bacteria (T0), revealed that uptake of the *cbrA* mutant by neutrophils was 13-fold greater than the uptake of strain PA14. Already at 30 min of incubation in fresh medium, we observed a decrease in bacterial cell counts for both PA14 and the *cbrA* mutant. After 1 h, the bacterial counts for both strains started to increase (data not shown). Similarly to the macrophage-killing results, the significantly enhanced uptake by neutrophils of the *cbrA* mutant compared to strain PA14 was followed by a greater percentage of killing of the *cbrA* mutant by neutrophils than that of the strain PA14.

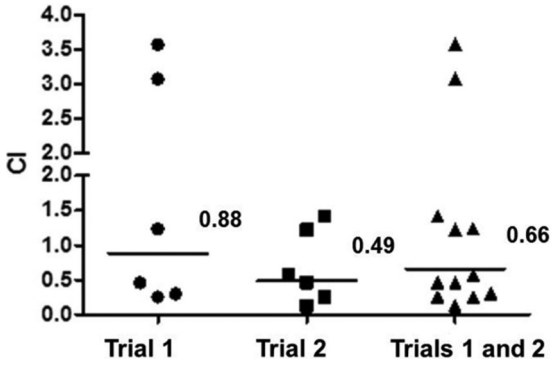

FIG 4 *In vivo* competitive growth of the *cbrA* mutant in competition with the PA14 strain in a rat model of chronic lung infection. Equal ratios of the WT and mutant were embedded in agarose beads and delivered to the rat lungs via intubation. Seven days postinfection, rats were sacrificed and lungs were recovered for CFU determinations. Each data point represents the competitive index (CI) for a single animal in each group. Symbols ●, ■, and ▲ represent the CI values determined for trial 1, trial 2, and trials 1 and 2 combined, respectively. The geometric means of the CIs from each trial are represented as a solid bar. The small difference in the mean CFU values between the mutant and the WT for each trial was determined to be statistically insignificant ($P > 0.05$) by the *F* test.

Increased susceptibility of the *cbrA* mutant toward killing by *Dictyostelium discoideum*. *D. discoideum*, an amoeba, can be used as a simple nonmammalian host system model to assess the pathogenicity of *P. aeruginosa* (33). Moreover, as *Dictyostelium* is a unicellular organism that feeds phagocytically upon bacteria, this system can also be used analyze phagocytosis. Here, we tested the relative ability of *D. discoideum* to kill the *cbrA* deletion mutant. Different numbers of amoebae were spotted onto bacterial lawns of strain PA14 or the *cbrA* mutant. The number of amoebae necessary to create a plaque on the bacterial lawn was determined as an assessment of virulence since a more virulent bacterium would kill the amoeba rather than vice versa. These analyses revealed a consistent, statistically significant 2- to 4-fold reduction in the numbers of amoebae required to form a plaque of identical size for the *cbrA* mutant compared to strain PA14 (Fig. 7). As a control, a similar 2- to 4-fold reduction in numbers of amoebae was determined for a mutant in the *pcrH* gene, which is essential for the virulence-related type III secretion system (Fig. 7) These findings were consistent with the above phagocytosis results with mammalian macrophages and neutrophils.

Transcriptional analysis of the *cbrA* mutant during *D. discoideum* infection. To investigate how CbrA contributed to the expression of *P. aeruginosa* virulence factors *in vivo*, microarray studies were performed comparing the global gene expression of the *cbrA* mutant to that of PA14 during *D. discoideum* infection (analogous studies with cells from mouse infections failed due to inadequate RNA amounts and quality). Two independent microarray experiments were performed, and 286 genes were identified to be differentially regulated in the *cbrA* mutant relative to PA14, by more than 2-fold, in both microarrays (see Table S1 in the supplemental material). A selection of these genes is presented in Table 1.

Analysis of the microarray data revealed dysregulation of a number of genes that might contribute to the reduced *in vivo* virulence observed for the *cbrA* mutant. Adaptive responses to oxidative stress are important for protecting against oxidative killing by phagocytes. We observed downregulation, in the *cbrA* mutant, of a number of genes, including *sodM*, *katE*, *rahU*, *pfpI*, and *osmC*, that are known or have been suggested to play important or putative roles in protecting *P. aeruginosa* against host oxidative killing and stress. Microarray analysis also revealed downregulation of genes involved in the biosynthesis of virulence factors. These include genes involved in rhamnolipid biosynthesis, such as *rhlA* and *rhlB*, those involved in pyoverdine (*pvd*), pyochelin (*pch*), and pyocyanin (*phz*) biosynthesis, the protease-encoding

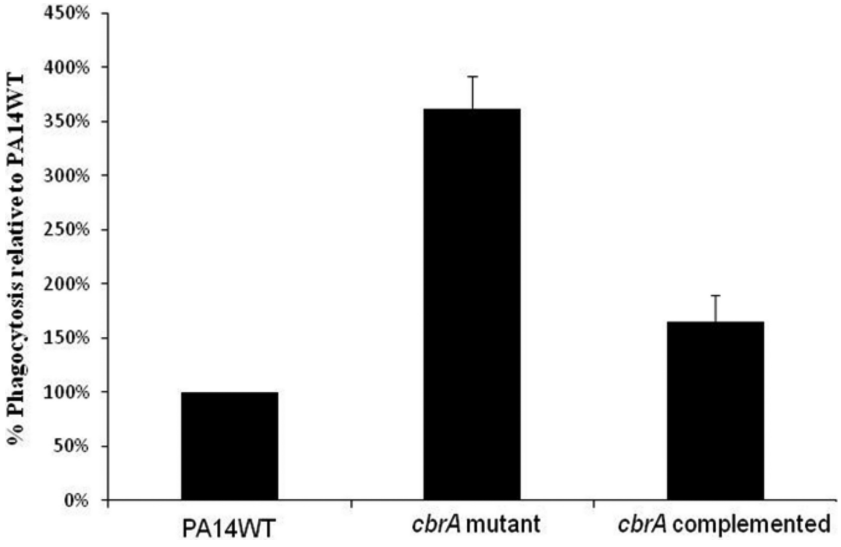

FIG 5 Phagocytosis of *P. aeruginosa* by human macrophages. PA14, *cbrA* mutant, and *cbrA* complemented strains were incubated with 1.5×10^5 cells/ml macrophages for 1 h at an MOI of 10. Subsequently, extracellular and attached bacteria were killed by treatment with gentamicin, macrophages were lysed with Triton X-100, and lysates were plated to enumerate viable intracellular bacteria. Results are expressed as the percentages of intracellular bacteria that were recovered relative to strain PA14. Each result represents the mean of 3 independent biological repeats, each assayed in triplicate. A statistically significant difference ($P < 0.05$) between the mutant and strain PA14 was determined by paired Student's *t* test.

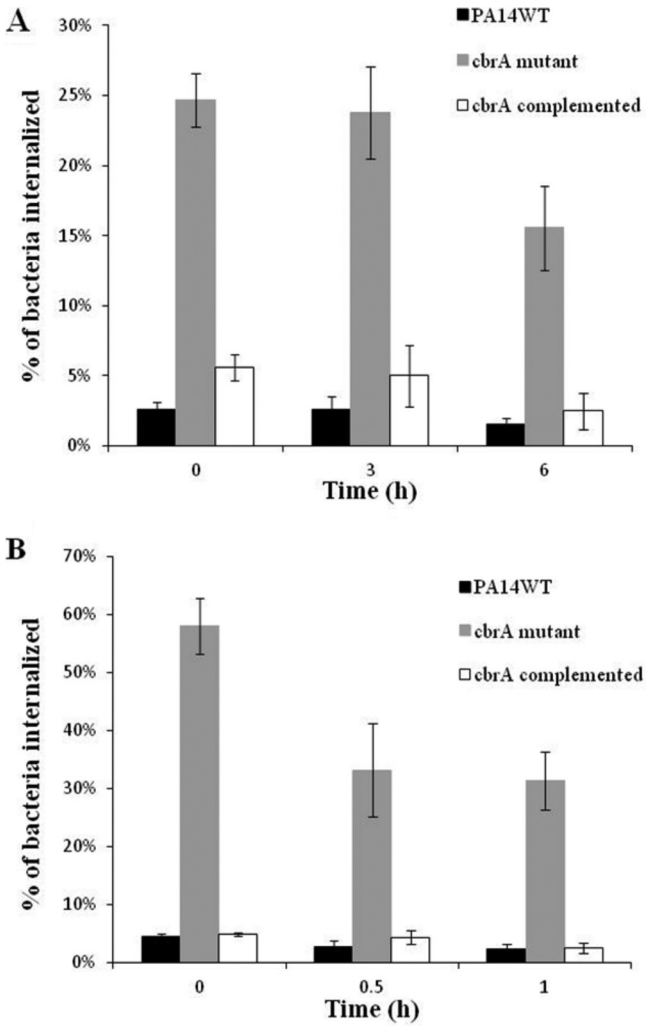

FIG 6 Killing of *P. aeruginosa* by phagocytes. Uptake and intracellular killing of PA14, the *cbrA* mutant, and the *cbrA* complemented strain by macrophages (A) and neutrophils (B) were determined. *P. aeruginosa* was incubated with 1.5×10^5 macrophages/ml or 2×10^6 neutrophils/ml for 1 h or 30 min, respectively. Following gentamicin treatment, either phagocytes were lysed and plated for viable intracellular bacteria (T0) or medium was replaced with fresh medium and the culture was incubated at 37°C for an additional 3 h (T3) and 6 h (T6) for macrophages or 0.5 h (T0.5) and 1 h (T1) for neutrophils. Results are expressed as the percentages of bacteria recovered from the phagocytes at each time point relative to the initial inoculation amount. Each result represents the mean of 3 independent biological repeats, each assayed in triplicate. Statistically significant differences were determined by paired Student's *t* test ($P < 0.05$, comparing the *cbrA* mutant and parent strain PA14 at T0; $P < 0.01$, comparing T0 and the different time points for the same strain).

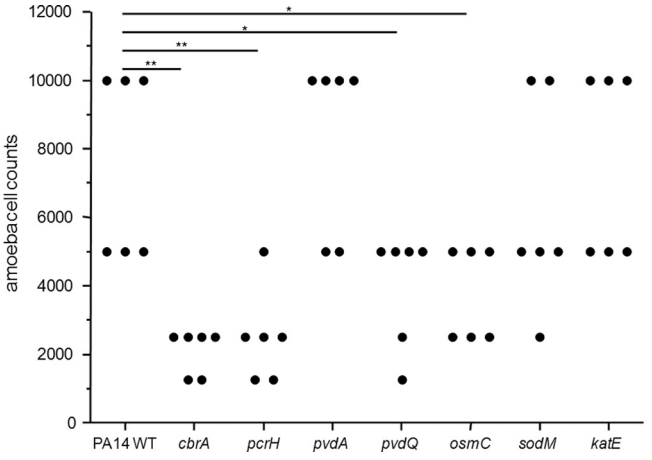

FIG 7 *D. discoideum* plate killing assay. Each point represents the number of amoebae required to form a plaque on the bacterial lawn of *P. aeruginosa* PA14 strains after 5 days of incubation. Experiments were performed with 6 independent biological repeats. Statistical significance was measured using the Mann-Whitney test (*, $P < 0.05$; **, $P < 0.01$, $n = 6$). Critically, growth of WT and the *cbrA* mutant on M9 plates was similar with or without amoebae, showing no observable differences.

genes *aprA*, *lasB*, and *piv*, *popB*, and *pvdQ*, which modulates the balance of quorum-sensing signaling molecules.

To further investigate the involvement of downregulated genes in the *cbrA*-mediated reduced virulence phenotype, we performed additional *D. discoideum* plate killing experiments using respective mutants of the Harvard PA14 mutant library (13). Knockout of the osmotically induced bacterial protein C (OsmC), as well as PvdQ, an enzyme that exhibits acyl-homoserine acylase activity in addition to its involvement in pyoverdine biosynthesis, resulted in a modest but significant 2-fold reduction in virulence in the respective amoeba plate killing experiments. In contrast, mutants with defects in pyoverdine production (*pvdA* mutants), rhamnolipid biosynthesis (*rhlA* and *rhlB* mutants), phenazine biosynthesis (*phzA* and *phzB* mutants), exoenzymes elastase (*lasB* mutants) and alkaline protease (*aprA* mutants), superoxide dismutase (*sodM* mutants), and catalase (*katE* mutants) exhibited no statistically significant differences in the numbers of amoeba required to form a plaque compared to the number obtained with wild-type strain PA14 (see Fig. 7 for examples).

DISCUSSION

In this study, the role of the sensor kinase CbrA in virulence *in vivo* was examined. Deletion of the *cbrA* gene resulted in a *P. aeruginosa* mutant strain that was significantly attenuated in virulence compared to the WT in mouse models of both acute lung infection and peritoneal infection. As the CbrAB TCS plays an important role in regulating the expression of several catabolic pathways and consequently in the utilization of a variety of organic compounds as the sole carbon source in *P. aeruginosa*, we investigated whether the reduced virulence of the *cbrA* mutant was simply due to a reduced ability to grow *in vivo*. The *in vivo* competitive growth analysis in the chronic rat lung model revealed that the *cbrA* mutant grew equivalently to the WT with a mean CI value of 0.66, which is within the range of error of this analysis. When combined with the normal virulence of a *cbrB* mutant, these data imply that altered growth is insufficient to explain the large (10- to 10^4-fold) reduction in virulence of the *cbrA* mutant compared to the WT in the acute lung and peritoneal infection models. Indeed, the normal virulence of the *cbrB* mutant, which has the same growth defects as the *cbrA* mutant, seems to argue against cross feeding of the *cbrA* mutant by WT in the competitive growth study in the rat lung, although this cannot be completely excluded due to the unique reduction in pyoverdine production of the *cbrA* mutant compared to the WT (12) and the consequent possibility of cross feeding. It was previously demonstrated that CbrA mediates the regulation of swarming, biofilm formation, and *in vitro* cytotoxicity toward HBE cells via CbrB and downstream small RNA *crcZ*

TABLE 1 Selected genes significantly dysregulated in the *cbrA* mutant during amoeba infection as determined by microarray analysis of global gene expression

Gene category and identity	Name[a]	Fold change[b]	Function
Virulence factors			
PA1249	*aprA*	−5.5	Alkaline protease
PA1708	*popB**	−5.3	T3SS[c] effector protein translocator
PA1707	*pcrH*	−2.4	Regulatory protein
PA1901	*phzC2**	−9.2	Phenazine biosynthesis protein
PA1902	*phzD2*	−9.3	Phenazine biosynthesis protein
PA1903	*phzE2*	−11.9	Phenazine biosynthesis protein
PA1904	*phzF2*	−11.2	Phenazine biosynthesis protein
PA1905	*phzG2*	−10.9	Phenazine biosynthesis protein
PA4210	*phzA1*	−11.0	Phenazine biosynthesis protein
PA4211	*phzB1*	−13.9	Phenazine biosynthesis protein
PA3478	*rhlB*	−4.6	Rhamnolipid biosynthesis protein
PA3479	*rhlA*	−8.5	Rhamnolipid biosynthesis protein
PA3724	*lasB*	−5.1	Elastase
PA4175	*piv*	−4.0	Protease IV
Iron acquisition			
PA2385	*pvdQ*	−10.7	Pyoverdine biosynthesis protein; 3-oxo-C12-homoserine lactone acylase
PA2386	*pvdA*	−22.5	Pyoverdine biosynthesis protein
PA2392	*pvdP*	−4.6	Pyoverdine biosynthesis protein
PA2393		−13.9	Pyoverdine biosynthesis protein
PA2394	*pvdN*	−15.3	Pyoverdine biosynthesis protein
PA2395	*pvdO*	−10.3	Pyoverdine biosynthesis protein
PA2396	*pvdF*	−7.6	Pyoverdine biosynthesis protein
PA2397	*pvdE*	−11.0	Pyoverdine biosynthesis protein
PA2398	*fpvA*	−15.0	Pyoverdine biosynthesis protein
PA2399	*pvdD**	−11.8	Pyoverdine biosynthesis protein
PA2400	*pvdJ*	−6.3	Pyoverdine biosynthesis protein
PA2413	*pvdH*	−13.5	Pyoverdine biosynthesis protein
PA2425	*pvdG*	−3.8	Pyoverdine biosynthesis protein
PA2426	*pvdS*	−17.5	Sigma factor; controls expression of pyoverdine
PA4221	*fptA*	−12.2	Fe(III)-pyochelin outer membrane receptor precursor
PA4224	*pchG*	−20.1	Pyochelin biosynthesis protein
PA4225	*pchF**	−7.7	Pyochelin biosynthesis protein
PA4226	*pchE*	−14.9	Pyochelin biosynthesis protein
PA4228	*pchD*	−15.2	Pyochelin biosynthesis protein
PA4229	*pchC*	−21.6	Pyochelin biosynthesis protein
PA4230	*pchB*	−25.0	Pyochelin biosynthesis protein
PA4231	*pchA*	−16.7	Pyochelin biosynthesis protein
Oxidative stress response			
PA0059	*osmC*	−3.7	Osmotically inducible protein
PA0122	*rahU*	−5.8	Modulator of innate immunity and inflammation in host cells
PA0355	*pfpI*	−16.2	Antimutator; general stress protection
PA2147	*katE*	−3.9	Catalase
PA4468	*sodM**	−18.2	Superoxide dismutase

[a] Expression of genes followed by an asterisk (*) was confirmed by qRT-PCR.
[b] Fold regulation of genes differentially expressed in the *cbrA* mutant relative to the WT. Average of two experiments. The negative numbers indicate transcript downregulation in the *cbrA* mutant.
[c] T3SS, type 3 secretion system.

and/or regulator Crc (12). In this study, we showed that the *cbrB* mutant exhibited WT phenotypes in the murine model of peritoneal infection and in uptake by macrophages. These results suggest that CbrA regulated *in vivo* virulence of *P. aeruginosa* independently of CbrB and also imply that alterations in the above *in vitro* virulence-related properties did not explain the attenuated virulence in animal models. The observation of independent regulation of virulence was consistent with observations regarding pyoverdine biosynthesis and antibiotic resistance, in that CbrA was able to regulate resistance to polymyxin B, ciprofloxacin, and tobramycin independently of CbrB involvement (12). We suggested previously (12) that this reflected the cross talk between CbrA and another regulatory system.

Despite the common use of cell lines to predict the effects of cells and mutants *in vivo*, they do not adequately represent the more complex situation in animals. For example, cell lines are usually immortalized (e.g., HBE), and this can lead to modifications of function, regulation, and growth characteristics. While

primary cells, such as PBMC, are more representative of the normal physiology and thus a better representative model of the *in vivo* state, these still lack the cellular complexity of intact organs. Moreover, *in vitro* results often correlate poorly with *in vivo* results due to the complex physiological environment *in vivo* that cannot be replicated precisely in *in vitro* models. One important and substantial difference is the lack of most host defenses in *in vitro* models. These considerations might explain why, despite the observation that the *cbrA* mutant exhibited enhanced *in vitro* cytotoxicity toward HBE cells, it was found to be significantly attenuated *in vivo*. In addition, there was an insignificant, ~1.5-fold increase in *in vitro* cytotoxicity toward human PBMC of the *cbrA* mutant compared to the WT, indicating that the effect of *cbrA* mutation on cytotoxicity was cell specific.

Since the metabolic deficiency of the *cbrA* mutant was unable to provide an explanation for the significant reduction in virulence of the mutant, we investigated whether CbrA plays a role in protecting *P. aeruginosa* from host defenses, particularly clearance by phagocytes. Intracellular killing following engulfment of microbes by phagocytic cells, such as macrophages and neutrophils, plays a major role in host innate defenses against *P. aeruginosa* infections (34). In this study, we showed that the deletion of the *cbrA* gene resulted in significantly increased uptake by phagocytes compared to the WT strain (Fig. 5). As a result of the enhanced uptake of the *cbrA* mutant, a greater number of cells of this mutant than of the WT were also cleared by phagocytes (Fig. 6). Thus, CbrA appears to play an important role in the *in vivo* virulence of *P. aeruginosa* by protecting the bacteria from phagocytosis. Based on the gene expression data for the mutant and wild type in conjunction with amoeba infection, we hypothesize that the downregulation, in the *cbrA* mutant, of multiple virulence factors that are known to impact on phagocytes and/or phagocytosis led to enhanced phagocytosis of the mutant. However, this cannot completely explain decreased virulence, since decreased virulence was strongly evident in leukopenic mice.

Moreover, our amoeba studies provide other potential explanations. For example, expression studies (Table 1) in the context of *D. discoideum* infections revealed the downregulation of several genes encoding known virulence factors that impact on virulence in mouse and *in vitro* models (7), including the protease-encoding genes *aprA*, *lasB*, and *piv*. Alkaline protease AprA can potently block phagocytosis and killing of *Pseudomonas* by human neutrophils and also interferes with complement activation (35, 36). The elastase LasB facilitates *P. aeruginosa* escapes from phagocytosis by degrading the pulmonary surfactant protein A (SP-A) (37) and antagonizes phagocytosis and killing by neutrophils (35). Protease IV, encoded by *piv*, is able to degrade several different surfactant proteins, including SP-A (21), and mediate corneal damage (38).

In the *D. discoideum* infection model, we also observed the downregulation of biosynthesis of rhamnolipids, which are virulence-associated hemolysins that are known to be able to lyse polymorphonuclear leukocytes and macrophages (39). Genes involved in pyoverdine (*pvd*), pyochelin (*pch*), and pyocyanin (*phz*) biosynthesis were also downregulated. Consistent with this observation, we previously demonstrated that pyoverdine biosynthesis genes and pyoverdine itself were substantially downregulated in a *cbrA* mutant, but the expression of most pyoverdine genes was unaffected by a *cbrB* mutation (12). Pyoverdin is in fact a required virulence factor in several animal models (40). Intriguingly, we observed similar, but somewhat milder, defects in growth in *Dictyostelium* for the *pvdQ* mutant, compared to the *cbrA* mutant and the WT. However, the *pvdA* mutant showed no defect relative to the WT and the *pvdQ* mutant effect might be related to quorum sensing, which is altered in this mutant. We also observed reduced *D. discoideum* virulence for the *osmC* mutant, in the OsmC osmosensor, although to our knowledge this mutant has not been tested in mouse models.

Interestingly, the majority of the genes downregulated in the *D. discoideum* infection model (Table 1), with the exception of those genes involved in pyoverdine, pyochelin, and pyocyanin biosynthesis, were not previously observed to be significantly dysregulated in a microarray of the *cbrA* mutant under swarming conditions (12), suggesting that these genes might be specifically activated by CbrA during amoeba infection. We also note that the expression of a number of these virulence-related genes, including *lasB*, *rhlAB*, *aprA*, and genes involved in pyoverdine (*pvd*), and pyochelin (*pch*) biosynthesis, are regulated by quorum sensing, consistent with the possibility that CbrA might promote resistance against amoeba-mediated killing in part by modulating quorum sensing. This is consistent with the observed 8.4-fold dysregulation of *pvdQ* (Table 1), encoding a 3-oxo-C12-homoserine lactone acylase that modulates the balance of quorum sensing signaling molecules.

We also observed (Table 1) downregulation of *popB*, a type III secretion gene involved in translocating virulence factors, such as ExoS, across the eukaryotic cell membrane, as well as *exsA*, involved in the expression of type III secretion genes (41, 42), and again a mutant in a type III secretion regulator, *pcrH*, was deficient in the *D. discoideum* infection model (Fig. 7). Thus, we conclude that the decreased virulence of the *cbrA* mutant does not owe itself to any single factor but rather to a combination of factors including dysregulated quorum sensing, virulence factors, iron-regulated systems, and osmosensing.

During bacterial infections in murine infection models, large numbers of leukocytes are recruited to the sites of infection. The impaired ability of the *cbrA* mutant to escape engulfment and killing by the phagocytes is likely to play a significant role in the inability of the *cbrA* mutant to sustain an infection at the site of initial inoculation and to protect against *D. discoideum*, as well as spread to disseminated sites (in the peritoneal infection model). Indeed, this is consistent with the normal competitive growth within the rat lung (Fig. 5), in which phagocytes are excluded by encasing *Pseudomonas* within agar beads. Previously, we showed that the *cbrA* mutant was completely abolished for swarming motility. In another species, *Serratia liquefaciens*, swarmer cells exhibited enhanced resistance to engulfment by *Tetrahymena* sp. (43); so, one possibility considered was that swarming might play a role in protecting *P. aeruginosa* from phagocytes. However, the unaltered virulence of the swarming-deficient *cbrB* mutant indicated that altered swarming could not *per se* explain the reduced virulence of the *cbrA* mutant. Since the *in vitro* conditions that promote swarming of *P. aeruginosa* mimic those of the mucous layer that overlays epithelial surfaces, it is possible that the bacteria use this form of motility to rapidly spread and colonize the mucosal surfaces of the body. We suggest that the *cbrA* mutant swarming defect might contribute to its inability to spread to disseminate to other sites (i.e., the blood and other organs) in the murine peritoneal infection model and work synergistically with the phagocytic defect.

Pathogenic bacteria, including *P. aeruginosa*, are exposed to a

variety of reactive oxygen and nitrogen species during oxidative killing by phagocytes, including superoxide, hydroxyl radical, hydrogen peroxide, peroxynitrite, and organic hydroperoxides (44). Phagocytes utilize the cytotoxic effects of these highly reactive oxygen and nitrogen species to damage the nucleic acids, proteins, and cell membrane of the infectious pathogens (45), and for *P. aeruginosa* to survive and proliferate in the host, it must overcome these oxidative stresses. *P. aeruginosa* is equipped with various strategies to defend against these toxic species. Interestingly, the microarray of the *cbrA* mutant during infection of *D. discoideum* revealed downregulation of a number of genes associated with the oxidative stress response in *P. aeruginosa* (Table 1). For example, we observed the downregulation of superoxide dismutase SodM, which plays a role in defending *P. aeruginosa* against the toxic effects of superoxides, a major component of oxidative killing by phagocytes (45). The catalase KatE has also been suggested to protect *P. aeruginosa* against hydrogen peroxide and osmotic stresses (46), and the PfpI protein protects the bacteria against DNA damage caused by the oxidative stress (47). The downregulated RahU protein inhibits antimicrobial nitric oxide production by macrophages (48). Also, the downregulated osmotically inducible protein OsmC has been implicated in *Escherichia coli* to play a role in defense against oxidative stress caused by exposure to organic hydroperoxides (49). Overall, these data are consistent with the possibility that CbrA might control oxidative stress responses and thus influence susceptibility to oxidative killing by phagocytes. Similarly, the known effect of CbrA on susceptibility to the cationic antimicrobial peptide polymyxin B (12) indicates that CbrA might also influence susceptibility to nonoxidative phagocytic killing by cationic antimicrobial peptides.

In conclusion, the data presented here indicate that CbrA has a major and multifactorial role in controlling virulence, but surprisingly this appeared to be independent of the participation of its cognate response regulator and *in vitro* virulence-related defects.

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