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In Vitro Analysis of Tobramycin-Treated *Pseudomonas aeruginosa* Biofilms on Cystic Fibrosis-Derived Airway Epithelial Cells[▽][†]

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P. aeruginosa forms biofilms in the lungs of individuals with cystic fibrosis (CF); however, there have been no effective model systems for studying biofilm formation in the CF lung. We have developed a tissue culture system for growth of *P. aeruginosa* biofilms on CF-derived human airway cells that promotes the formation of highly antibiotic-resistant microcolonies, which produce an extracellular polysaccharide matrix and require the known abiotic biofilm formation genes *flgK* and *pilB*. Treatment of *P. aeruginosa* biofilms with tobramycin reduced the virulence of the biofilms both by reducing bacterial numbers and by altering virulence gene expression. We performed microarray analysis of these biofilms on epithelial cells after treatment with tobramycin, and we compared these results with gene expression of (i) tobramycin-treated planktonic *P. aeruginosa* and (ii) tobramycin-treated *P. aeruginosa* biofilms on an abiotic surface. Despite the conservation in functions required to form a biofilm, our results show that the responses to tobramycin treatment of biofilms grown on biotic versus abiotic surfaces are different, as exemplified by downregulation of genes involved in *Pseudomonas* quinolone signal biosynthesis specifically in epithelial cell-grown biofilms versus plastic-grown biofilms. We also identified the gene PA0913, which is upregulated by tobramycin specifically in biofilms grown on CF airway cells and codes for a probable magnesium transporter, MgtE. Mutation of the PA0913 gene increased the bacterial virulence of biofilms on the epithelial cells, consistent with a role for the gene in the suppression of bacterial virulence. Taken together, our data show that analysis of biofilms on airway cells provides new insights into the interaction of these microbial communities with the host.

Pseudomonas aeruginosa is adept at establishing chronic infections in the lungs of individuals with cystic fibrosis (CF). CF is a common inherited genetic disorder, and pathology in the disease is brought about by improper chloride secretion due to mutations in the CF transmembrane conductance regulator (CFTR) (4, 14, 23). In the lung, this chloride transport defect, and the resulting altered airway physiology, lead to impairment of mucociliary clearance and production of thick mucus plugs in the airways (14, 16, 38, 51). These reactions predispose the CF patient to chronic microbial infection, and *P. aeruginosa* eventually becomes the dominant infecting microorganism from childhood through adulthood (14, 30). Despite the availability and aggressive use of antibiotics, *P. aeruginosa* colonization often becomes lifelong and is a major factor contributing to CF patient complications, including respiratory failure and death (14, 30, 38).

Current evidence suggests that one significant reason for the persistence of *P. aeruginosa* is its ability to form antibiotic-resistant biofilms in the lungs of CF patients. Indeed, antibiotic resistance profiles, quorum-sensing studies, and microscopic examination of microcolonies in CF patient lungs support this hypothesis (16, 22, 25, 38, 43). Biofilm formation is the result of global regulatory changes within the bacterium as a result of the harsh lung environment, and the appearance of *P. aeruginosa* biofilms correlates with chronic infection (18). Investiga-

tion into the genetic regulatory networks governing this transition between acute and chronic infection might assist in our understanding of *P. aeruginosa* persistence in the CF lung.

P. aeruginosa biofilm formation has been extensively studied on abiotic surfaces using a variety of static and flow cell assays (35). These investigations have revealed a multistep process leading to formation of bacterial communities attached to the abiotic substratum. However, a more relevant model system in which to study *P. aeruginosa* biofilm formation and virulence in the context of CF airway cells would be useful in order to understand airway infection in CF.

To better understand *P. aeruginosa* infection in the context of the CF airway epithelium, we developed a tissue culture-based system in which to study *P. aeruginosa* biofilm formation on human CF-derived lung epithelial cells in vitro. We report that tobramycin decreases the virulence of *P. aeruginosa* growing on human CF airway epithelial cells. Microarray analysis showed that the genes required for biogenesis of *Pseudomonas* quinolone signal (PQS), an important signaling molecule regulating virulence gene expression, are downregulated upon tobramycin treatment, specifically within the context of a biofilm grown on airway cells. We also identified a putative magnesium transporter that is involved in bacterial virulence toward the epithelial cells. Furthermore, our results show that the responses of *P. aeruginosa* to tobramycin treatment are markedly different depending upon whether the microbe is growing planktonically, in a biofilm on an abiotic surface, or in a biofilm on epithelial cells.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table S1 in the supplemental material. For all experiments, except for pyoverdine assays, bacteria were grown in LB overnight with appropriate anti-

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biotics. Pyoverdine assays were performed using cultures grown overnight in King's B medium (20 g/liter proteose peptone, 10 g/liter glycerol, 1.5 g/liter KH_2PO_4, 1.5 g/liter $MgSO_4$), and overnight cultures were adjusted to similar optical densities at 600 nm (OD_{600}) prior to assays. Wild-type *P. aeruginosa* PA14 and ΔPA0913 strains exhibited similar growth kinetics (data not shown).

Static coculture biofilm assay. CFBE41o− cells (CFBE cells) are human bronchial epithelial cells that are homozygous for the ΔF508 mutation of CFTR (5, 8). *P. aeruginosa* biofilms were grown on CFBE cells using a coculture model system. In this model, epithelial cells were seeded at a concentration of 10^6 cells/well in 6-well tissue culture plates or 2×10^5 cells/well in 24-well tissue culture plates and maintained in minimal essential medium (MEM) (Mediatech, Herndon, VA) with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. The cells were grown at 37°C and 5% CO_2 for 7 to 10 days before inoculation with bacteria. These conditions have been shown to lead to confluent cell monolayers and to allow the formation of tight junctions (5, 8). *P. aeruginosa* was inoculated at a concentration of approximately 2×10^7 CFU/ml into 1.5 ml MEM/well (without fetal bovine serum, penicillin, or streptomycin) for 6-well plates or 1.2×10^7 CFU/ml in 0.5 ml MEM/well for 24-well plates. These concentrations were equal to a multiplicity of infection of approximately 30:1 for both plate sizes relative to the number of epithelial cells originally seeded. The plates were incubated for 1 h at 37°C and 5% CO_2. After 1 h, the supernatant was replaced with fresh MEM supplemented with 0.4% arginine, and the system was incubated at 37°C and 5% CO_2 for the times indicated for each experiment. Epithelial-monolayer integrity was assessed by phase-contrast microscopy using a Leica DM IRB inverted microscope (Leica Microsystems, Wetzlar, Germany), and PA14 biofilm formation on CFBE cells was visualized by fluorescence microscopy examination of cocultures inoculated with *P. aeruginosa* PA14 carrying plasmid pSMC21, which constitutively expresses green fluorescent protein (GFP) (2). Images were analyzed with OpenLab 4.0.4 software (Improvision, Lexington, MA). To determine the numbers of biofilm CFU, we washed the cells two or three times with phosphate-buffered saline (PBS) to remove planktonic bacteria and treated the cells with 0.1% Triton X-100 for 10 to 15 min to lyse the epithelial cells. The lysate was vortexed for 3 min, serially diluted, and plated on LB. Calcofluor staining was accomplished by diluting a 1% solution of calcofluor (Fluorescent Brightener 28; Sigma, St. Louis, MO) 1:1,000 in the static coculture supernatant (52). Staining was assessed by microscopic examination after incubation with calcofluor for 1 h at 37°C.

Microarray analysis. For microarray analysis of CFBE/*P. aeruginosa* biofilms, coculture static biofilms were grown for 9 h in six-well tissue culture plates, whereupon the cells were washed two or three times with 2 ml PBS, and then fresh MEM was added. Replicate samples were then incubated in the presence or absence of 500 μg/ml tobramycin, a concentration approximately one-half that observed in the lungs of CF patients treated with tobramycin (13), for 30 min. The cells were then washed two or three times with 2 ml PBS, and RNA was harvested using a modification of the protocol from the RNeasy RNA isolation kit (Qiagen, Valencia, CA). Specifically, the cells were incubated with 600 μl of 1 μg/ml lysozyme in Tris-EDTA buffer for 10 min at room temperature. Then, the cells were homogenized with 600 μl RLT lysis buffer from the Qiagen RNeasy kit, and the homogenate was vortexed for several seconds and drawn through a 20-gauge needle 10 times. Six hundred microliters of 100% ethanol was then added, and the solution was applied to an RNeasy column. From this point, we followed the manufacturer's protocol for isolation of bacterial RNA. Mammalian RNA was removed using the Microb*Enrich* kit (Ambion, Austin, TX) according to the manufacturer's protocol. cDNA synthesis and microarray preparation, scanning, and analysis were carried out as previously described (24). Importantly, the multiwell format of the static model system allowed us to combine the RNAs harvested from several identical wells into one sample in order to increase the yield. In this manner, we were able to obtain sufficient high-quality RNA from the bacterial biofilm to carry out microarray analysis.

For microarray analysis of planktonic bacteria, we used mid-exponential phase cultures of *P. aeruginosa* PA14. This was achieved by growing *P. aeruginosa* in 10 ml MEM/2% LB until mid- to late exponential phase, diluting this bacterial culture 1:4 in fresh MEM/2% LB, and growing it for an additional 3 to 4 h to reach mid-exponential phase. Replicate cultures were incubated in the presence or absence of 5 μg/ml tobramycin for 30 min at 37°C. Bacterial RNA was then isolated and prepared for microarray analysis as previously described (24).

Raw microarray data were processed using a Bayesian analysis, as previously described (24). We chose a ±2.0-fold cutoff, as well as a natural log (ln) *P* value of <0.05, to identify significantly upregulated and downregulated genes. Data sets were compared using VennMaster (http://www.informatik.uni-ulm.de/ni /staff/HKestler/vennm/doc.html).

RT-PCR analysis. For transcriptional analysis of abiotic biofilms, we grew PA14 in six-well tissue culture plates using MEM supplemented with 0.4%

arginine, as described above for biofilms on CFBE cells, except without epithelial cells. The abiotic biofilms were grown for 24 h at 37°C and 5% CO_2 to reach approximately 10^7 to 10^8 CFU/well, similar to conditions for CFBE biofilm and planktonic microarray experiments as described above. These abiotic biofilms were washed twice with PBS and treated with 1 μg/ml tobramycin for 30 min at 37°C and 5% CO_2. RNA was harvested, and cDNA was synthesized as described above. Notably, we found that the Microb*Enrich* procedure was not needed to obtain bacterial RNA of sufficient concentration and quality for reverse transcriptase (RT) PCR (data not shown). Quantitative RT-PCR (qRT-PCR) was carried out as previously described (24) using primer pairs 913forRT/913revRT, 3330for/3330rev, 4326forRT/4326revRT, 4635for/4635rev, 4761forRT/4671revRT, PA5110for/PA5110rev, 5470for/5470rev, fptA1/fptA2, pqsAF1/pqsAR1, PA0044f/PA0044r, PA1706f/PA1706r, and PA1707F/PA1707R (see Table S2 in the supplemental material). Primers for *fbp* (PA5110, encoding fructose bisphosphatase) were used as a normalization control, because analysis of several data sets in our laboratory indicated that the transcript levels of the gene remained constant, regardless of the growth conditions tested (data not shown).

Transcriptional analysis of PA0913 was accomplished by semiquantitative RT-PCR. RNA was harvested from 6- to 9-h CFBE/PA14 biofilms or mid-exponential-phase planktonic PA14 cultures treated with tobramycin for 30 min, as described above for microarray analysis. cDNA synthesis and semiquantitative RT-PCR were performed as previously described (24, 32), using primer pair 913forRT/913revRT (see Table S2 in the supplemental material) at an annealing temperature of 56°C. We assayed *fbp* as a constitutive control.

Genetic constructs. (i) Gene deletions. Isogenic deletion mutants were created by allelic replacement, using suicide vectors that were assembled by single-step recombination of PCR fragments in *Saccharomyces cerevisiae* (41). Briefly, approximately 1,000 bp of 5′ and 3′ flanking regions for the target genes were PCR amplified using primer pairs 913Lfor/913Lrev, 913Rfor/913Rrev, 4635Rfor/4635Rrev, 5470Lfor/5470Lrev, 5470Rfor/5470Rrev, 3819Lfor/3819Lrev, 3819Rfor/3819Rrev, 4690igLfor/4690igLrev, 4690igRfor/4690igRrev, 4825Lfor/4825Lrev, 4825Rfor/4825Rrev, 3531Lfor/3531Lrev, 3531Rfor/3531Rrev, 4826Lfor/4826Lrev, and 4826Rfor/4826Rrev (see Table S2 in the supplemental material). Allelic-replacement vector pMQ30 (41) was digested with BamHI and EcoRI, and the digested vector, as well as 5′ and 3′ PCR fragments, was transformed into *S. cerevisiae* strain InvSc1 (Invitrogen, Carlsbad, CA) using an improved "lazybones" protocol, as previously described (41). This technique resulted in in vivo homologous recombination of vector and PCR fragments, thus creating deletion plasmids for the target genes (see Table S1 in the supplemental material). The isolated plasmids were transformed by electroporation into *Escherichia coli* strain S17-1. S17-1 transformants were conjugated with *P. aeruginosa* PA14 using standard techniques (24), and PA14 exconjugants were selected on LB plates containing gentamicin and nalidixic acid. At this point, the deletion constructs were integrated onto the bacterial chromosome at the gene locus via a single-crossover recombination event. These strains were grown overnight in liquid culture, and excision of the insert was accomplished by plating them on LB plates containing 10% sucrose to select for strains that had lost the *sacB* gene located on the suicide vector. This indicated that an allele replacement had occurred. The gene deletion was confirmed by PCR using primer pairs 913for/913rev, 4635For/4635Rev, 5470For/5470Rev, 3819for/3819rev, 4690for/4690rev, 4825for/4825rev, 3531for/3531rev, and 4826for/4826rev (see Table S2 in the supplemental material).

(ii) Complementation vectors. To complement the isogenic PA0913 deletion strain, the full-length PA0913 gene was PCR amplified using primer pair 913Cfor/913Crev (see Table S2 in the supplemental material). This fragment included approximately 500 bp upstream of the start codon, so that the natural promoters might be incorporated into the complementing construct. We digested this PCR fragment with EcoRI and ligated it into the EcoRI-restricted expression vector pMQ72 (41). The orientation of the fragment was determined by PCR and sequencing with primers F-t1t2 and R-Pbad (41). The insert oriented in the same direction as the P_{BAD} promoter was considered an inducible complementing clone (pSMC235), and insertion in the opposite orientation was used as a natural promoter expression clone (pSMC237). Cloning was carried out in *E. coli* strain DH5α. The PA0913 (*mgtE*) isogenic deletion mutant was transformed with these constructs by electroporation.

Cytotoxicity assays. The cytotoxicity of PA14 and the isogenic mutants was assessed by measuring lactate dehydrogenase (LDH) released from epithelial cells in coculture with the bacteria. Static biofilms were developed on CFBE cells for the times indicated in the text, and 400 μl of the culture medium was harvested. This medium was centrifuged to pellet bacteria and cell debris, and 300 μl of the supernatant was saved. LDH levels within this cell-free supernatant were assayed using the CytoTox 96 nonradioactive cytotoxicity assay (Promega, Madison, WI) according to the manufacturer's instructions. Assays were per-

formed in triplicate or quadruplicate, and the data represent at least three identical assays.

Exofactor assays. Pyoverdine was assayed from density-matched overnight cultures grown in King's B medium. Culture supernatants were passed through 0.22-μm filters, and the relative pyoverdine concentration was determined by measuring the OD_{404} (12). Pyocyanin production was assayed similarly to published methods (9). Briefly, density-matched overnight cultures grown in LB were filtered through 0.22-μm filters. Pyocyanin was chloroform extracted using equal volumes of culture filtrate and chloroform. After being vortexed for 30 seconds, the solutions were centrifuged for 2 min, and 200 μl of the bottom, organic layer was collected. The solutions were vortexed a second time for 30 seconds and centrifuged for 2 min; 300 μl of the organic phase was recovered and added to the previously collected 200 μl, 500 μl of 0.1 N HCl was added to this organic phase, and pyocyanin was extracted into the aqueous phase by 30 seconds of vortexing. Four hundred microliters of the upper, aqueous phase was combined with 600 μl 200 mM Tris-HCl, pH 8.0, and the relative pyocyanin concentration was determined by measuring the OD_{376}. Protease and lipase activities were assessed by measuring the zone of clearance after overnight incubation of 5 μl of density-matched overnight cultures in 3% skim milk/LB agar plates or 0.5% tributyrin emulsion agarose plates, respectively (12, 20). Tributyrin agarose plates were made by sonicating 1% tributyrin in 20 mM HEPES-500 mM NaCl, pH 7.4, for 3 min. The resultant emulsion was mixed with an equal volume of 20 mM HEPES-500 mM NaCl, pH 7.4, and 2% melted agarose, and the solution was poured into petri plates to solidify. Each of these assays was performed a minimum of three times using triplicate cultures.

Biofilm formation. Bacterial viable counts in biofilms in coculture with CFBE cells were determined as described above (see "Static coculture biofilm assay"). Formation and assessment of bacterial biofilms on polyvinyl chloride (PVC) plastic microtiter plates were performed using the crystal violet assay as previously described (34).

Microarray data accession number. Full microarray data for both the CFBE biofilm and planktonic experiments were deposited in the NCBI (GEO accession no. GSE10030).

RESULTS

Biofilm formation on CFBE cells. To investigate the interactions between *P. aeruginosa* and CF airway cells, we developed an in vitro model system of biofilm formation on epithelial cells. We grew CFBE cells, a human bronchial epithelial cell line with a CFTR ΔF508/ΔF508 genotype (5, 8), in standard multiwell (6-well and 24-well) tissue culture plates, in which they formed monolayers. These monolayers were inoculated with *P. aeruginosa* strain PA14 (referred to throughout as "PA14"), and biofilm formation was monitored in this static system over time by phase-contrast microscopy.

In initial studies, uninfected epithelial cells formed confluent monolayers (Fig. 1A), but after 4 to 6 h of exposure to PA14, most epithelial cells had lifted off the plastic, leaving only small patches of cells separated by large sections of empty space (Fig. 1B). This cell death presumably arose from bacterial killing of the airway cells and precluded the formation of biofilms. The type III secretion system (T3SS) of *P. aeruginosa* greatly affects bacterial virulence, and it is known to exert cytotoxic effects on epithelial cells (37, 39, 49). Based on data presented below, it seems likely that the T3SS might have been involved in the cell death we observed in our assays.

In an effort to identify conditions that delay bacterial killing of epithelial cells and thus support biofilm formation, we looked to models of biofilm formation on abiotic surfaces. The addition of arginine to minimal growth medium enhances the formation of *P. aeruginosa* biofilms on PVC plastic surfaces (6). We predicted by analogy that arginine might exert a similar biofilm-stimulatory effect in our CFBE biofilm cell culture model. Indeed, addition of arginine to the tissue culture medium resulted in the preservation of monolayer integrity after
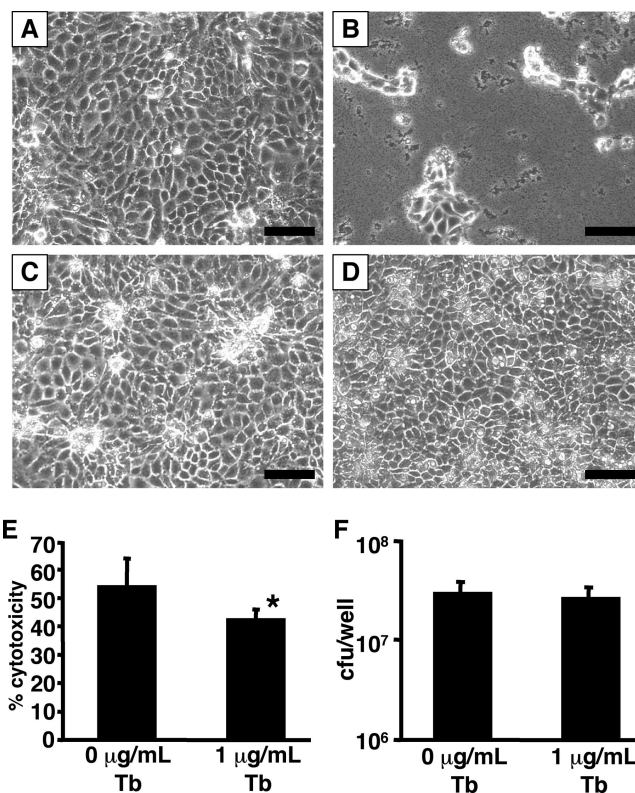

FIG. 1. Arginine and tobramycin prevent bacterial killing of CFBE epithelial cells. (A) An uninfected monolayer. (B and C) CFBE cell monolayers 6 h after inoculation with PA14 in the absence (B) or presence (C) of 0.4% arginine. (D) Intact CFBE cell monolayer after 8 h of biofilm development with arginine followed by 24 h of incubation with 1,000 μg/ml tobramycin. The tobramycin treatment did not clear the bacteria, which typically persisted at approximately 10^3 to 10^4 CFU/well. The images were taken by phase-contrast microscopy and are representative fields of view from several wells studied. Scale bars = 120 μm. (E) Treatment of 5-h coculture biofilms with 1 μg/ml tobramycin (Tb) for 2 h resulted in a small but significant reduction in bacterial virulence toward the CFBE cells compared to untreated samples. ∗, $P < 0.05$. (F) CFU determination of the biofilm cultures in panel E revealed equal bacterial titers in tobramycin-treated and untreated biofilms. The difference between the 0-μg/ml tobramycin and 1-μg/ml tobramycin samples was not statistically significant. The error bars indicate 1 standard deviation.

incubation with PA14 for up to ~9 h (Fig. 1C). Incubation for longer than 9 h resulted in monolayer destruction, even in the presence of arginine (data not shown). Enumeration of bacterial CFU bound to the epithelial cells after 1 h of incubation in the presence or absence of arginine revealed the same number of *P. aeruginosa* cells bound under both conditions, suggesting that arginine might affect downstream events in biofilm formation or might otherwise impact epithelial cell cytotoxicity (data not shown). We have termed this model of *P. aeruginosa* growth on epithelial cells the "static coculture biofilm system."

We next sought to determine whether the extended CFBE cell viability afforded by arginine was sufficient to support biofilm formation. We inoculated the static coculture biofilm system with PA14/pSMC21 (plasmid pSMC21 expresses GFP constitutively) (2). Fluorescence microscopy revealed clusters of *P. aeruginosa* cells scattered across the epithelial cell mono-
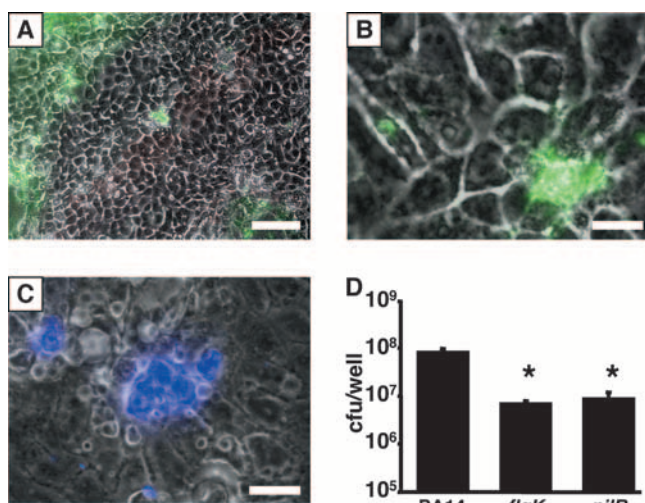
FIG. 2. PA14 forms biofilm microcolonies on a CFBE epithelial cell monolayer. (A and B) Incubation of CFBE cells with PA14/pSMC21 (GFP) resulted in clusters of GFP-PA14 scattered across the monolayer (A) that appeared as microcolonies bound to the surfaces of the cells (B). Panels A and B are phase-contrast images overlaid with the GFP channel. (C) Staining with calcofluor resulted in blue fluorescence of the microcolonies, as seen in a phase-contrast image overlaid with the blue channel. Scale bars = 120 μm (A) or 35 μm (B and C). (D) CFBE cells were inoculated with wild-type PA14 or strains carrying mutations in the *flgK* or *pilB* gene. Bacterial CFU determined at 7 h postinfection revealed >10-fold reduction in bacterial populations on cells infected with the *flgK* or *pilB* mutant. ∗, $P < 0.05$ compared to PA14. The error bars indicate 1 standard deviation.

layer within 6 to 8 h after inoculation (Fig. 2A). These clusters appeared to be attached to the epithelial cells, and they exhibited a mushroom-like morphology reminiscent of large *P. aeruginosa* macrocolonies developed on abiotic surfaces (7, 40). This structured architecture differentiated these macrocolonies from the "flat" biofilm phenotypes that *P. aeruginosa* can also form (21). We also observed PA14 as planktonic bacteria and in a variety of other biofilm stages, including reversibly and irreversibly bound individual bacteria and microcolonies of various sizes (Fig. 2B and data not shown).

These morphological observations suggested that PA14 forms biofilms on the CFBE cells. We next conducted additional experiments to determine whether the clusters of *P. aeruginosa* displayed properties typical of biofilms. One of the key features of bacterial biofilms is the production and secretion of an exopolysaccharide matrix that surrounds the biofilm bacteria (7). To demonstrate the presence of a polysaccharide matrix around the microcolony-like structures attached to the epithelial cells, we stained the bacteria with calcofluor, a fluorescent dye that binds to β(1-3) and β(1-4) polysaccharide linkages found in the biofilm matrices produced by many microorganisms (52). As expected, calcofluor stained these microcolonies (Fig. 2C).

To provide additional support for the view that the microcolonies on CFBE cells were biofilms, we inoculated CFBE cell monolayers with PA14 strains carrying mutations in the *flgK* or *pilB* gene, which encode components of flagella and the type IV pilus, respectively (34). Mutations that disrupt the biogenesis of these organelles negatively impact biofilm formation on abiotic surfaces (34). The type IV pilus is also an important

attachment factor for epithelial cell binding (48). After inoculation in the static CFBE coculture system with the *flgK* and *pilB* mutant strains, we assessed biofilm formation at 7 h by determination of the numbers of bacterial CFU attached to the epithelial cells. Similar to abiotic biofilm systems, the *flgK* and *pilB* mutants exhibited a marked decrease in bacterial CFU bound to the CFBE cells compared to wild-type PA14 (Fig. 2D). These findings underscore the similar requirements for biofilm formation on biotic and abiotic surfaces.

Bacterial colonies on the CFBE cells were extremely resistant to antibiotic treatment, another hallmark of biofilms. We treated static CFBE/PA14 biofilms for 24 h with up to 1,000 μg/ml tobramycin, a concentration measured in the lungs of CF patients (13). In this experiment, we measured >10³ CFU/well remaining on the cells after antibiotic treatment (data not shown), suggesting that the minimum bactericidal concentration was greater than 1,000 μg/ml tobramycin. CFU counts before the antibiotic treatment were comparable to those in Fig. 2D (approximately 10⁸ CFU/well). The antibiotic concentrations used during this treatment surpassed the minimum bactericidal concentration of tobramycin for planktonic PA14 and for abiotically grown PA14 biofilms (8 μg/ml and 400 μg/ml, respectively), as measured in a previous study from our group (32). We have also tested the antibiotic resistance of CFBE-grown biofilms by adding tobramycin at early time points in the presence and absence of arginine with no apparent difference in tobramycin resistance. PA14 grown on CFBE cells in the presence and absence of arginine displayed similar high-level tobramycin resistance (data not shown). Visualization of the epithelial monolayer after treatment of static CFBE/PA14 biofilms for 24 h with up to 1,000 μg/ml tobramycin showed that the CFBE cell monolayer was intact, and the CFBE cells appeared similar to uninoculated epithelial cells (Fig. 1D). Thus, even though ~10³ CFU/well of viable PA14 remained in the biofilm on the CBFE cells after tobramycin treatment, the bacteria did not kill the epithelial cells for at least 24 h.

The data above suggested that tobramycin treatment of *P. aeruginosa* biofilms on CFBE cells results in a decrease in the virulence of the microorganism toward the epithelial cells. Alternatively, decreased killing of the epithelial cells might have resulted from a lower number of bacteria. To distinguish between these possibilities, we tested a range of tobramycin concentrations for their effects on CFBE/PA14 biofilm CFU and cytotoxicity. We found that incubation of CFBE/PA14 biofilms with 1 μg/ml tobramycin elicited a small but significant decrease in bacterial cytotoxicity toward CFBE cells as measured by release of LDH from the epithelial cells (Fig. 1E) but had no effect on the biofilm CFU (Fig. 1F). Tobramycin concentrations above 1 μg/ml resulted in a decrease in both cytotoxicity and biofilm CFU (data not shown). These results suggest that tobramycin may exert two separable effects upon *P. aeruginosa* biofilms on CFBE cells: a decrease in viable bacteria and a decrease in bacterial virulence.

Microarray analysis of tobramycin-treated airway cells. The preceding results suggested that tobramycin might reduce the virulence of *P. aeruginosa* biofilms growing on CF-derived airway cells. Accordingly, microarray studies were conducted to identify genes that regulate virulence in response to tobramycin treatment. To accomplish this goal, we performed a mi-

croarray analysis of PA14 biofilms grown on CFBE cells treated with tobramycin. While treatment with 1 μg/ml tobramycin resulted in a significant decrease in bacterial cytotoxicity (Fig. 1E), this change was small, and we suspected that many transcriptional responses to tobramycin might be obscured by this low antibiotic dose. We therefore chose to treat CFBE/PA14 biofilms with 500 μg/ml tobramycin, and 30 min was chosen for the treatment time to be able to capture the early responses of PA14 biofilms to tobramycin. This concentration of tobramycin was 50% of the minimum bactericidal concentration for CFBE-grown biofilms (see above). Furthermore, CFBE/PA14 biofilms displayed antibiotic resistance phenotypes and epithelial cell viabilities when treated with 500 μg/ml tobramycin at 24 h similar to those in the experiments with 1,000 μg/ml tobramycin treatment described above (Fig. 1D).

To generate RNA for microarray studies, PA14 biofilms were grown on CFBE monolayers using the static system for 9 h. At that time, replicate samples were incubated in the presence or absence of 500 μg/ml tobramycin for 30 min, whereupon bacterial RNA was harvested and prepared for microarray analysis as described in Materials and Methods.

The microarray study was performed with triplicate samples, and a Bayesian analysis was used to provide a statistical assessment of significance (19, 24, 28). We chose cutoffs of ≥2.0-fold and an ln P value of <0.05. Overall, we found 338 bacterial transcripts that were upregulated upon tobramycin treatment and 500 transcripts that were downregulated (see Tables S3 and S4 in the supplemental material). Because tobramycin reduced PA14 virulence toward CFBE cells (Fig. 1D), we predicted that virulence-related genes would be downregulated by tobramycin. Most notably, we found that PQS biosynthesis was highly downregulated (Table 1). We also observed a decrease in transcript levels for phenazine synthesis (*phnA*, −15.3; *phnB*, −7.2), hydrogen cyanide synthesis (*hcnA*, −2.6; *hcnB*, −3.2; *hcnC*, −3.9), pyoverdine activity (*pvdA*, −4.2; *pvdD*, −3.1; *pvdE*, −4.8; *pvdS*, −4.3; *fpvA*, −26.3), and a probable phospholipase (PA3487, −3.0) (see Tables S3 and S4 in the supplemental material). However, there were no changes in the transcript levels of other known lipases or proteases. Three transcripts related to type III secretion were downregulated (*pscE*, −3.2; *pscI*, −2.6; and PA1692, −3.0), but no other type III gene was significantly changed (see Tables S3 and S4 in the supplemental material).

Intriguingly, we found very little change in known antibiotic resistance-related genes. Transcript levels for a probable drug efflux transporter gene (PA1541) were increased 2.9-fold (see Table S3 in the supplemental material), but the efflux transporter gene *mexB* was downregulated 2.4-fold and the *mexR* repressor was upregulated 2.5-fold. Furthermore, we saw no change in transcript levels for any other known or probable antibiotic-related genes, including *aph*, *bacA*, *cat*, *str*, and other *mex* genes (see Tables S3 and S4 in the supplemental material). These results suggest that the antibiotic resistance of *P. aeruginosa* biofilms on epithelial cells may involve the expression of other, unknown factors.

One theory put forth to explain increased antibiotic resistance of biofilms is the presence of specialized "persister" cells (26, 44). While few genes associated with this persister phenotype have been identified, none of these genes, including *glpD*,

TABLE 1. Selected genes stimulated or repressed by tobramycin in CFBE/PA14 biofilms[a]

Gene	Fold change	Description
PQS		
PA0996	−2.5	*pqsA* (biosynthesis of PQS)
PA0997	−19.5	*pqsB* (biosynthesis of PQS)
PA0998	−15.0	*pqsC* (biosynthesis of PQS)
PA0999	−10.1	*pqsD* (biosynthesis of PQS)
PA1000	−13.8	*pqsE* (biosynthesis of PQS)
Alginate		
PA0762	+8.6	*algU* (sigma factor)
PA0763	+6.5	*mucA* (anti-sigma factor)
PA5262	+3.6	*algZ* (alginate biosynthesis protein)
PA5255	+2.8	*algQ* (alginate biosynthesis regulator)
PA5261	+2.8	*algR* (alginate biosynthesis regulator)
PA5253	+2.1	*algP* (alginate biosynthesis regulator)
PA0764	+2.0	*mucB* (alginate biosynthesis negative regulator)
Iron		
PA3531	+16.5	*bfrB* (bacterioferritin)
PA4764	+4.2	*fur* (ferric uptake regulation)
PA0362	+2.1	*fdx1* (ferridoxin)
PA4131	−2.0	Probable iron-sulfur protein
PA3491	−2.0	Probable ferredoxin
PA5217	−2.3	Probable binding protein component of ABC iron transporter
PA5216	−2.4	Probable permease of ABC iron transporter
PA3812	−2.5	*iscA* (probable iron-binding protein)
PA3813	−2.8	*iscU* (probable iron-binding protein)
PA2399	−3.1	*pvdD* (pyoverdine synthetase)
PA3809	−3.4	*fdx2* (ferridoxin)
PA2386	−4.2	*pvdA* (pyoverdine synthesis)
PA2426	−4.3	*pvdS* (sigma factor, iron responsive)
PA4231	−4.4	*pchA* (salicylate biosynthesis, pyochelin biosynthesis)
PA2397	−4.8	*pvdE* (pyoverdine synthesis)
PA4225	−6.1	*pchF* (pyochelin synthetase)
PA4226	−7.7	*pchE* (pyochelin synthesis)
PA4228	−13.9	*pchD* (pyochelin synthesis)
PA4221	−25.3	*fptA* (pyochelin receptor precursor)
PA2398	−26.3	*fpvA* (ferripyoverdine receptor)
PA4229	−28.9	*pchC* (pyochelin synthesis)
PA4230	−29.9	*pchB* (salicylate biosynthesis, pyochelin synthesis)
Magnesium		
PA4635	+8.2	hypothetical MgtC protein
PA0913	+3.9	*mgtE* (magnesium transport)
PA4825	+2.8	*mgtA* (magnesium transport)

[a] The table shows the overall trends of several metabolic activities. Full microarray data can be found in the supplemental information.

gpsA, and *plsB* (45), displayed any change in transcript level in response to tobramycin in our study.

Despite modest changes in antibiotic and virulence gene expression, several interesting trends arose from our microarray analysis. For instance, several genes in the alginate regulatory cascade were highly upregulated by tobramycin (Table 1), including *algU*, *mucA*, and *algZ* (33, 36). However, there was no significant change in the expression of the core *alg* biosynthetic genes (e.g., *algD*), indicating that the increased expression of alginate-regulatory genes we observed in our

microarray might not result in a corresponding increase in alginate production.

Finally, numerous genes related to the synthesis and export of iron chelators were highly downregulated by tobramycin treatment, including *pchB*, *pchC*, *pchA*, *fptA*, *fpvA*, and *pvdD* (Table 1). Iron siderophores have been associated with the virulence of a number of different bacterial species, and thus, a reduction in the expression of these genes could account, at least in part, for the observed decrease in virulence (27).

The impacts of tobramycin on gene expression differ in planktonic cells, biofilms grown on abiotic surfaces, and biofilms grown on airway cells. To determine the impact of tobramycin treatment on planktonic bacteria, we performed further microarray analysis of planktonic *P. aeruginosa* treated with a subinhibitory concentration of tobramycin. Exponential-phase bacteria were treated with 5 μg/ml tobramycin for 30 min, and RNA was prepared for microarray analysis as described in Materials and Methods. This tobramycin concentration was chosen because it is approximately 50% of the planktonic minimum bactericidal concentration for the microorganism (32), analogous to the tobramycin dose used in the microarray studies of biofilm-grown bacteria described above. This planktonic subinhibitory concentration did not affect bacterial CFU during the time course of the experiment (data not shown). RNA from untreated planktonic cultures was used as a control.

We observed that far fewer genes were regulated under these conditions, with only 23 transcripts upregulated and 21 transcripts downregulated (see Tables S5 and S6 in the supplemental material). Additionally, the magnitude of these changes was much lower. The most highly upregulated gene from the planktonic microarray data set (PA4762) was increased only 3.6-fold, compared to the 37.5-fold increase observed for PA5471 in the CFBE/PA14 biofilm microarray (see Tables S3 and S5 in the supplemental material). Similarly, the maximally downregulated gene in the planktonic microarray (PA0284) was decreased 5.8-fold, compared to the maximum decrease of 29.9-fold (PA4230) found in the biofilm microarray (see Tables S4 snd S6 in the supplemental material). The decreased number of genes regulated and the lower magnitude of transcriptional changes seen in this planktonic data set may be due to the relatively low level of antibiotic treatment (5 μg/ml tobramycin) compared with treatment of CFBE-grown biofilms (500 μg/ml tobramycin). Many of the genes upregulated in the planktonic microarray encode heat shock-related proteins, such as *grpE*, *hslU*, and *dnaK* (see Table S5 in the supplemental material). The downregulated genes included a locus encoding sulfate binding and transport (PA0280 to PA0283), as well as a gene cluster encoding a probable ABC transport system (PA3441 to PA3446) (see Table S6 in the supplemental material).

When we compared the response of CFBE/PA14 biofilms treated with tobramycin to that of planktonic bacteria treated with tobramycin, we found little similarity between the two groups of data (Table 2; see Fig. S1 in the supplemental material). There were only two genes upregulated and only five genes downregulated under both conditions. Eight genes showed opposite effects between planktonic and biofilm cultures (upregulated in planktonic culture but downregulated in biofilms). These results suggest that the bacteria in biofilms on

TABLE 2. Comparison of biofilm and planktonic microarrays

Overlap	Gene	Description
CFBE up/planktonic up		
	PA0320	Conserved hypothetical protein
	PA4205	Hypothetical protein
CFBE down/planktonic down		
	PA0283	*sbp* (sulfate-binding protein)
	PA0284	Hypothetical protein
	PA2204	Probable component of ABC transporter
	PA3330	Probable short-chain dehydrogenase
	PA3931	Probable protease
CFBE down/planktonic up		
	PA0779	Probable ATP-dependent protease
	PA1596	*htpG* (heat shock protein)
	PA4385	*groEL* (chaperone-heat shock)
	PA4386	*groES* (chaperone-heat shock)
	PA4761	*dnaK* (chaperone-heat shock)
	PA4762	*grpE* (heat shock protein)
	PA5052	Hypothetical protein
	PA5054	*hslU* (heat shock protein)

epithelial cells react very differently to tobramycin than their planktonic *P. aeruginosa* counterparts.

Several years ago, Whiteley et al. published a microarray study of *P. aeruginosa* biofilms on granite surfaces treated with tobramycin (47). While this study reported transcriptional changes in only 20 genes, a comparison of their results on an abiotic surface with our CFBE/*P. aeruginosa* microarray results revealed several differences in transcriptional profiles between tobramycin-treated biofilms grown on abiotic surfaces and CFBE cells (see Table S7 and Fig. S1 in the supplemental material). Only four genes were upregulated in both data sets, and we found no overlap among downregulated genes. On the other hand, two of the tobramycin-upregulated genes on abiotic surfaces were downregulated in our study (the stress response factor genes *groES* and *dnaK*). Similar differences were seen when we compared the Whiteley et al. data with our planktonic microarray data (see Table S7 and Fig. S1 in the supplemental material). When we compared all three microarray studies, we discovered that no genes were upregulated under all conditions or downregulated under all conditions, although two genes that were downregulated in CFBE/PA14 biofilms during tobramycin treatment were upregulated in both abiotic biofilms and planktonic cultures during tobramycin treatment (see Table S7 and Fig. S1 in the supplemental material).

The differences we observed between our microarray data set of tobramycin-regulated genes in CFBE/PA14 biofilms and the microarray data of Whiteley et al. could be due to the very different experimental conditions in these studies (such as different abiotic surfaces, different media, and different tobramycin concentrations used). To address this issue, we assessed the expression of a select set of genes from bacteria grown in a
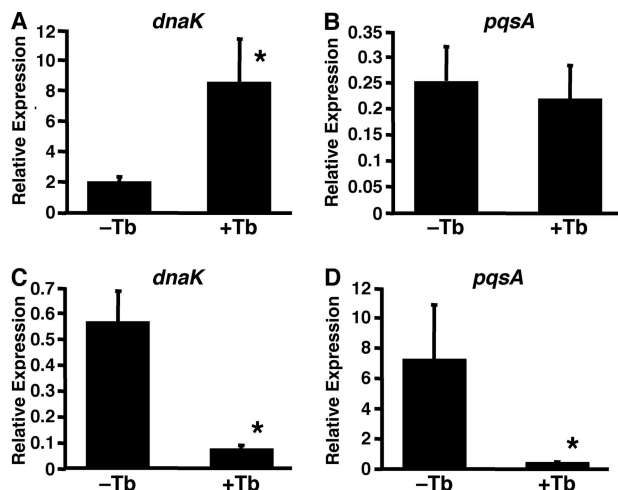
FIG. 3. Abiotic biofilms respond differently to tobramycin than biofilms on CFBE cells. Quantitative RT-PCR revealed that PA14 biofilms on PVC plastic plates demonstrated an increase in transcriptional expression of *dnaK* (A) but no change in *pqsA* expression (B) in response to tobramycin (Tb) treatment. On the other hand, tobramycin treatment of CFBE/PA14 biofilms resulted in a decrease in both *dnaK* (C) and *pqsA* (D) expression, as determined by qRT-PCR. ∗, $P <$ 0.05. The error bars indicate 1 standard deviation.

biofilm on an abiotic surface under conditions very similar to those used for the CFBE biofilm microarray and planktonic microarray experiments. PA14 biofilms were grown on a PVC plastic surface (six-well tissue culture plates without CFBE cells) for 24 h in MEM tissue culture medium with arginine supplementation, resulting in a bacterial CFU similar to that of CFBE/PA14 biofilms and planktonic PA14 populations used in the microarray experiments described above. The abiotic biofilms were treated for 30 min with 200 μg/ml tobramycin. This concentration of tobramycin was analogous to that used for the planktonic and CFBE-grown biofilms, that is, 50% of the minimum bactericidal concentration for abiotic PA14 biofilms (32). Thirty minutes after treatment with the antibiotic, bacterial RNA was harvested as described in Materials and Methods.

Using qRT-PCR, we showed that PA0913 (*mgtE*), PA4635 (*mgtC*), PA4326, and PA5470 were upregulated in abiotic-grown biofilms in response to tobramycin treatment (see Fig. S2 in the supplemental material). We also observed a downregulation in PA3330 and PA4221 (*fptA*) expression. These tobramycin-induced changes are similar to those observed in the microarray analysis of CFBE/PA14 biofilms (see Tables S3 and S4 in the supplemental material).

qRT-PCR analysis of abiotic biofilms also revealed an upregulation of PA4761 (*dnaK*) and no change in PA0996 (*pqsA*) expression in abiotic biofilms in response to tobramycin (Fig. 3A and B), as was observed in the study by Whiteley et al. (47), in contrast to the downregulation seen in the microarray analysis with both of these genes in CFBE/PA14 biofilms (see Table S4 in the supplemental material). We confirmed the downregulation of *dnaK* and *pqsA* expression in CFBE/PA14 biofilms by qRT-PCR analysis of tobramycin-treated CFBE/PA14 biofilms (Fig. 3C and D). Thus, while it is evident that similarities exist between the responses of biotic and abiotic *P.*

aeruginosa biofilms to tobramycin, clearly there are responses unique to tobramycin-treated CFBE/PA14 biofilms, such as *pqsA* transcriptional downregulation.

Mutational analysis. We next created isogenic deletion mutants in a number of genes in PA14 that were upregulated in the CFBE/PA14 biofilms upon tobramycin treatment. We tested these mutants for biofilm formation on CFBE cells (by CFU count), antibiotic sensitivity of the CFBE-grown biofilms, and virulence toward CFBE cells. While most of these deletion mutants displayed wild-type phenotypes, we discovered that a mutant in the gene PA0913 (*mgtE*) showed increased virulence on epithelial cells (see Table S8 in the supplemental material). When inoculated into culture with CFBE cells for 8 h, the PA0913 mutant damaged the CFBE monolayer, whereas PA14-infected monolayers remained intact at this time point (Fig. 4A).

We quantified this virulence effect by measuring the release of LDH from the epithelial cells to obtain a measure of the cytotoxicity of the bacteria. Over time, *P. aeruginosa* elicited a steady increase in the amount of LDH released from CFBE cells (see Fig. S3A in the supplemental material), and this experiment allowed us to choose the optimal time points at which to assess the cytotoxicity of wild-type and mutant strains in our system. When we compared LDH release from CFBE cells incubated with wild-type PA14 or with the PA0913 mutant, we discovered much greater cytotoxicity of the PA0913 mutant compared to wild-type PA14 (Fig. 4B). Complementation with the full-length gene on a multicopy plasmid attenuated the cytotoxicity of this mutant strain to a level indistinguishable from that of the wild type, and these effects were seen when PA0913 expression was driven from its natural promoter or a constitutive promoter (Fig. 4B and data not shown).

As mentioned above, PA0913 is a probable homolog of *mgtE*, encoding the magnesium transporter MgtE. Therefore, we tested isogenic deletion mutants in other predicted magnesium transporters in PA14, but we did not observe any virulence phenotypes, except with double and triple mutants involving mutation of PA0913 in combination with mutation of other putative magnesium transporter genes (see Table S8 in the supplemental material).

Varying biofilm biomass on epithelial cells may alter cytotoxicity due to changing numbers of bacteria. For instance, we noticed that decreased biofilm formation exhibited by *flgK* and *pilB* mutants (Fig. 2D) also resulted in significantly diminished cytotoxicity as determined by LDH release from CFBE cell monolayers (see Fig. S3B in the supplemental material). To investigate biofilm formation by the PA0913 mutant, we measured bacterial CFU of the biofilm 5 h after inoculation, the same time at which LDH measurements were made. We found no difference in numbers of bacteria between the wild type and the PA0913 mutant biofilms (Fig. 4C), indicating that the cytotoxicity of the PA0913 mutant was increased independently of the bacterial numbers. We also observed little to no difference in the formation of biofilms between the wild type and the PA0913 mutant over time using a 96-well PVC plastic plate as an abiotic surface for biofilm formation (Fig. 4D).

The PA0913 (*mgtE*) mutant requires the T3SS for increased virulence. As mentioned above, expression of the T3SS of *P. aeruginosa* contributes to the cytotoxicity of epithelial cells.
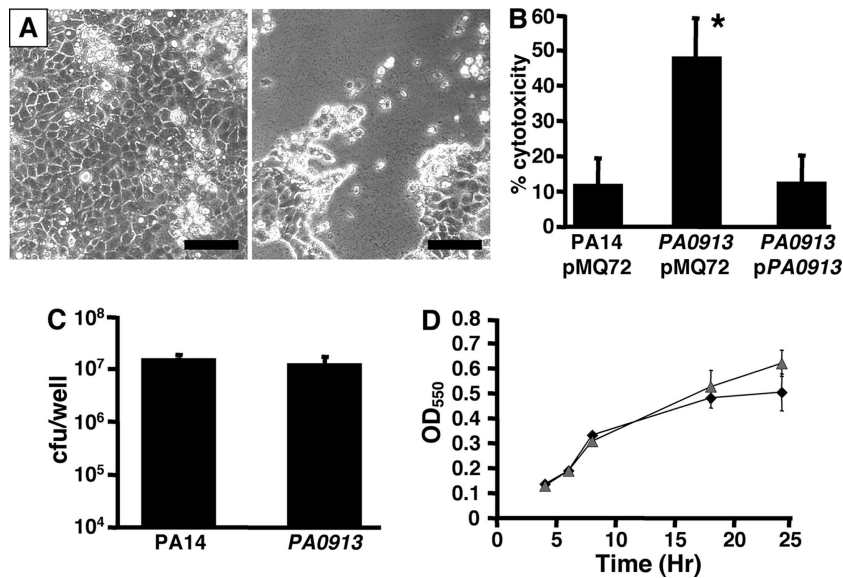
FIG. 4. The PA0913 (*mgtE*) gene affects virulence on CFBE epithelial cells. (A) Microscopic analysis revealed that monolayers infected with an isogenic deletion mutant in the PA0913 gene (right) were destroyed more quickly than monolayers infected with wild-type PA14 (left) at 8 h postinoculation. Scale bar = 120 μm. (B) The cytotoxicity of the ΔPA0913 mutant on the CFBE cells was analyzed at 5 h, as described in Materials and Methods. The ΔPA0913 mutant was significantly more cytotoxic than PA14 (*, $P < 0.05$), and this effect could be rescued by complementation with the full-length gene in *trans* (pPA0913). pMQ72 is the empty vector control. The error bars indicate 1 standard deviation. (C) Determination of the number of CFU in the biofilms on the CFBE cells demonstrated that a PA0913 isogenic deletion mutant developed bacterial CFU at 5 h postinoculation similar to that for wild-type PA14. (D) Wild-type PA14 and the ΔPA0913 mutant displayed similar biofilm formation kinetics on PVC plastic. Black diamonds, PA14; gray triangles, ΔPA0913.

The T3SS is also an important virulence factor in lung pathogenesis (31, 42), and considering the contact between *P. aeruginosa* and CFBE cells in our assays, it seemed likely that T3SS might be involved in biofilm-dependent cytotoxicity. To investigate the possible involvement of the T3SS in cytotoxicity, we inoculated CFBE cells with a ΔpscC deletion mutant, which is defective for the T3SS (17, 47a). As expected, this ΔpscC strain exhibited significantly attenuated cytotoxicity compared to the wild type in terms of LDH released from the CFBE epithelial cell monolayer (Fig. 5A). Intriguingly, a double deletion mutant in both *pscC* and PA0913 demonstrated low-level cytotoxicity, similar to the ΔpscC strain. Furthermore, release of LDH from cells incubated with either the ΔpscC strain or the double mutant was significantly abrogated compared to the single ΔPA0913 mutant (Fig. 5A), suggesting that the increased virulence phenotype of the PA0913 mutant requires a functional T3SS. In support of this hypothesis, we measured PA0913 gene expression in wild-type and ΔpscC biofilms by qRT-PCR, and we found a small but significant decrease in PA0913 transcript levels in the ΔpscC mutant (the relative expression level for ΔpscC was 0.104 ± 0.008 compared to 0.126 ± 0.015 for PA14; $P < 0.05$). On the other hand, qRT-PCR analysis of wild-type and ΔPA0913 biofilms revealed no difference in transcript levels of *pcrV* (PA1706, encoding a component of T3SS) or of *exoT* (PA0044, encoding a T3SS-secreted effector protein) (data not shown). However, we did observe a slight but significant increase in the transcript levels of *pcrH* (PA1707, encoding a component of T3SS; relative expression in ΔPA0913, 14.7 ± 2.9 compared to 11.8 ± 2.3 for the wild type; $P < 0.05$).

As a control experiment, we inoculated wild-type or mutant bacteria into MEM tissue culture medium in a Transwell cup
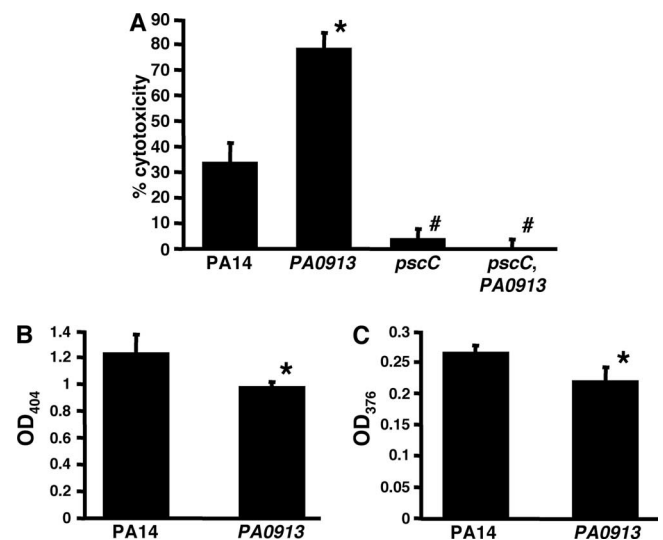

FIG. 5. Roles of T3SS and secreted virulence factors in the PA0913 (*mgtE*) mutant phenotype. (A) Mutation of PA0913 in the type III secretion-defective ΔpscC mutant background eliminates the cytotoxicity observed for the single ΔPA0913 strain. This cytotoxicity of the ΔPA0913 ΔpscC double mutant is similar to that of the single ΔpscC mutant. *, $P < 0.05$ compared to wild-type PA14; #, $P < 0.05$ compared to PA14 and to ΔPA0913. The error bars indicate 1 standard deviation. (B and C) We measured pyoverdine (B) and pyocyanin (C) secreted by PA14 and the ΔPA0913 mutant in overnight cultures using colorimetric assays, as described in Materials and Methods. Slight but significant decreases were seen in pyocyanin and pyoverdine production in the ΔPA0913 (*mgtE*) mutant. *, $P < 0.05$.
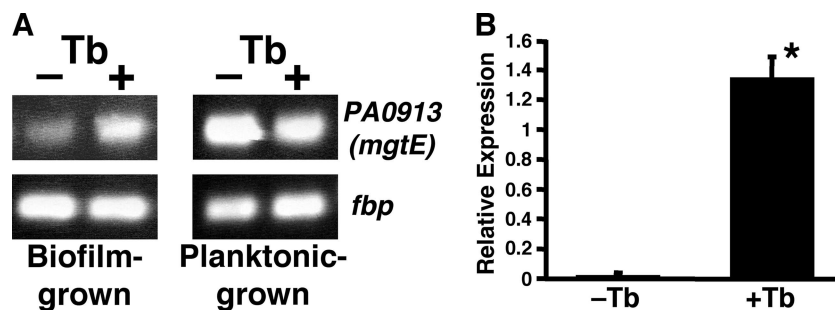
FIG. 6. Tobramycin stimulates expression of the PA0913 (*mgtE*) gene in biofilms on CFBE cells. (A) Semiquantitative RT-PCR demonstrated that tobramycin exposure led to an increase in PA0913 transcript levels in CFBE/PA14 biofilms (left) but not in planktonic cultures (right). *fbp* is a constitutively expressed control gene. Tb, tobramycin; − and + indicate that cDNA was prepared from untreated samples or tobramycin-treated samples, respectively. (B) qRT-PCR revealed a 38.3-fold enhancement of PA0913 gene transcription in PA14 biofilms on CFBE cells treated with 500 μg/ml tobramycin (+Tb). *, $P < 0.05$. The error bars indicate 1 standard deviation.

suspended above the epithelial cells. In this assay, the Transwell membrane prevented direct contact between the bacteria and the epithelial cells, but the bacterial supernatants (and hence, any secreted factors) could freely diffuse across the Transwell membrane and interact with the monolayer. Similarly, low-molecular-weight epithelial cell products could diffuse across the Transwell and stimulate bacterial gene expression. We measured the LDH released from Transwell-inoculated coculture and found essentially no cytotoxicity with the wild type or the mutants within the time frame of the experiment (data not shown). Thus, virulence effects observed with the PA0913 mutant appear to largely require contact-dependent mechanisms, such as the T3SS.

Mutating the PA0913 (*mgtE*) gene alters pyoverdine and pyocyanin production. As further controls, we also tested the levels of known secreted factors in wild-type and PA0913 mutant strains. Using spectrophotometric assays (see Materials and Methods), we measured pyoverdine and pyocyanin levels secreted in overnight cultures. Mutation of PA0913 resulted in a slight but significant decrease in the production of pyoverdine (Fig. 5B) and pyocyanin (Fig. 5C). We found no differences in protease and lipase expression between wild-type and mutant strains (data not shown), indicating that mutation of PA0913 did not have broad effects on the production of secreted virulence factors.

Regulation of the PA0913 gene by tobramycin. Our microarray analysis of CFBE/PA14 biofilms treated with tobramycin indicated that the antibiotic stimulates increased expression of the PA0913 gene (see Table S3 in the supplemental material). However, measurement of the cytotoxicity of the PA0913 mutant, as discussed above, was carried out in the absence of tobramycin, suggesting that regulation of the gene is complex. To begin to investigate the regulation of the PA0913 gene, we performed semiquantitative RT-PCR on RNA isolated from CFBE/PA14 biofilms treated with tobramycin for 30 min. We found that tobramycin treatment led to a dramatic increase in the levels of the PA0913 transcript compared to untreated biofilms (Fig. 6A, left), increasing transcript levels to approximately those observed in planktonic-culture-grown cells (see below). This result was confirmed by qRT-PCR analysis, revealing a 38-fold (±4.0 standard deviation) increase in the transcriptional levels of PA0913 in CFBE/PA14 biofilm treated with tobramycin (Fig. 6B). Importantly, low-level expression of

the PA0913 gene was observed in the absence of antibiotic treatment. This basal expression of PA0913 is likely to account, at least in part, for the virulence effects seen with biofilms of wild-type *P. aeruginosa* versus the PA0913 mutant in coculture biofilms (Fig. 4).

We next tested whether a similar tobramycin effect on PA0913 gene expression could be produced in planktonic cultures of PA14. Exponential-phase cultures were treated with a subinhibitory tobramycin concentration for 30 min, and we measured RNA levels of the PA0913 transcript by RT-PCR, as described above. Contrary to the biofilm phenotype observed above, high-level expression of the gene was observed in untreated planktonic cultures (Fig. 6A, right), and tobramycin had minimal effects upon the transcript levels of the gene. The *fbp* gene was used as a constitutive control in these reactions, and its transcript level remained unchanged regardless of experimental conditions.

DISCUSSION

We developed a static coculture tissue culture system in which *P. aeruginosa* biofilms form directly on human CF-derived airway cells in vitro. Some studies have suggested that *P. aeruginosa* initiates biofilm formation within the thick mucus layer of the CF airways (14). While *P. aeruginosa* biofilm formation may indeed occur in this mucus layer, it is also known that *P. aeruginosa* can interact with and bind to the epithelial cell surface of the lung, especially in the early stages of lung colonization (15, 31). Our study demonstrates that bacterial binding to the epithelial surface can lead to growth of biofilm microcolonies on the epithelial cells in culture (Fig. 2). The biofilms that formed on CFBE cells appeared similar to structured, mushroom-like biofilms formed under certain conditions on abiotic surfaces, including glass and mucin (21). The bacteria were bound to a surface (CFBE cells), were encased in a polysaccharide matrix (Fig. 2C), required factors necessary for abiotic-grown biofilms (Fig. 2D), and were extremely resistant to the antibiotic tobramycin. Our coculture biofilm model, therefore, may be useful for dissecting the early stages of the intricate relationship between *P. aeruginosa* and the CF airway epithelium.

Using our coculture biofilm model, we have discovered two conditions that influence bacterial virulence: arginine supple-

mentation and tobramycin treatment (Fig. 1). In regard to arginine, it is presently unclear why this amino acid inhibits bacterial virulence (Fig. 1) and promotes biofilm formation on both biotic and abiotic surfaces. Also unknown is whether arginine reduces virulence gene expression or upregulates biofilm formation pathways, although several recent studies suggest that these events may be linked (11). It is known that *P. aeruginosa* can metabolize arginine via several different pathways (29). Downstream signaling through these metabolic networks might influence the expression of virulence factors. On the other hand, arginine may act independently of known mechanisms. Furthermore, it is possible that arginine might affect the ability of the host cells to resist bacterial toxins and virulence factors. In any case, it is clear that arginine inhibits epithelial cell death sufficiently to allow biofilms to form, at least in the short term (Fig. 2). The role of arginine in biofilm formation and virulence is the subject of ongoing studies.

Our data indicate that tobramycin treatment of the biofilms likely impacts virulence in two ways: (i) by decreasing bacterial viability and (ii) by decreasing virulence. Although this decrease in virulence might be a result of a decreased number of bacteria after tobramycin treatment, we identified a tobramycin concentration (1 μg/ml) that inhibits virulence without affecting the number of biofilm CFU (Fig. 1E and F). Thus, we were able to separate these two phenotypes to demonstrate that tobramycin can specifically inhibit the virulence of *P. aeruginosa* biofilms on CF-derived airway cells in culture.

To identify tobramycin-regulated virulence factors, we exposed biofilms grown on airway cells to the antibiotic and monitored the response via microarray. To our knowledge, this is the first example of examining the gene expression of *P. aeruginosa* grown in biofilms on airway cells. In the wild-type strain, treatment of biofilms grown on CFBE epithelial cells with tobramycin induced the expression of a number of genes, including those that code for the AlgU/MucA/AlgZ regulators and PA0913, a predicted homolog of the magnesium transporter encoded by *mgtE* (Fig. 6). While there was no evidence that tobramycin positively regulated the genes required for alginate biosynthesis, it is intriguing to speculate that tobramycin treatment of *P. aeruginosa* infections in the CF lung might increase production of non-alginate-related genes regulated by the AlgU/MucA/AlgZ regulatory system.

In regard to the predicted *mgtE* homolog PA0913, because mutating the PA0913 gene results in increased virulence but does not impact the biofilms formed on airway cells (Fig. 4), we infer that the role of this gene and its product might be the down-modulation of virulence when bacteria are growing on airway cells in a biofilm. Consistent with this idea, treatment of the biofilm with tobramycin both increased PA0913 expression and decreased virulence. Given that the PA0913 gene codes for a putative magnesium transporter, we postulate that magnesium homeostasis might be important in the interaction between *P. aeruginosa* and the host. MEM contains 0.812 mM Mg^{2+}, and it would be interesting to investigate the effects of altering the magnesium concentration on biofilm formation and virulence. Interestingly, our genetic studies indicate that increased virulence of PA0913 requires the T3SS, although the relationship between PA0913 and the secretion system is unclear. It is important to note that we performed functional studies on just a small number of the genes that were differ-

entially expressed upon tobramycin treatment of the biofilms. Therefore, other genes besides PA0913 may participate in virulence, biofilm formation, or antibiotic resistance in the context of biofilms on airway cells.

Tissue culture systems are often used to investigate host/pathogen interactions in place of suitable animal models. Intriguingly, there have been a few studies demonstrating bacterial biofilm formation on cultured epithelial cells, including studies of *Haemophilus influenzae* and *Mycobacterium avium* biofilm formation on cultured human airway cells and *Salmonella enterica* biofilm formation on HEp-2 liver cells (3, 46, 50). While many researchers have looked at acute interactions of planktonic *P. aeruginosa* with cultured cells in vitro, to our knowledge, *P. aeruginosa* biofilm formation on epithelial cells has not been demonstrated in in vitro systems. Frisk et al. have reported interactions between *P. aeruginosa* and primary normal human airway epithelial cells grown as polarized monolayers on transwell inserts in vitro, and they observed these interactions up to 16 h after inoculation (10). However, no mention was made of biofilm formation, and the images presented depict individually binding bacteria. Considering our results, it is unclear why microcolony structures were not observed in the Frisk et al. study, although they used a different bacterial strain and grew epithelial cells on Transwell supports instead of on the well bottom, as in our study. Intriguingly, these researchers used normal airway cells as opposed to the ΔF508 homozygous cell line that we used here, pointing to a possible difference in the ability of *P. aeruginosa* to form biofilms on airway cells depending upon CFTR status. Current studies in our laboratories are investigating the formation of *P. aeruginosa* biofilms on other epithelial cell lines.

The different responses we observed between planktonic bacteria, CFBE/PA14 biofilms, and abiotic biofilms suggest physiological differences in *P. aeruginosa* depending upon environmental conditions (Tables 1 and 2; see Table S3 to S7 in the supplemental material). Similar to previous findings (1, 7, 47, 50), our results indicate that planktonic *P. aeruginosa* and biofilm *P. aeruginosa* populations are quite distinct from each other. Most intriguingly, the surface upon which a biofilm forms appears to influence responses to antibiotics. Taken together, our analysis of these three microarray experiments suggests that biofilm-grown *P. aeruginosa* reacts very differently to tobramycin than planktonic *P. aeruginosa*. While there are several experimental differences between our CFBE biofilm microarray study and the abiotic biofilm microarray analysis published by Whiteley et al., our analysis also indicates that *P. aeruginosa* biofilms behave differently when formed on CFBE epithelial cells versus an abiotic surface (see Table S7 in the supplemental material). Furthermore, our results suggest that the use of our coculture biofilm system can reveal novel virulence factors that studies of abiotic biofilms may have missed.

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