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THE EFFECT OF ALGINATE ON THE INVASION OF CYSTIC FIBROSIS RESPIRATORY EPITHELIAL CELLS BY CLINICAL ISOLATES OF *PSEUDOMONAS AERUGINOSA*

A. Rene D. Massengale, F. J. Quinn, A. Williams, S. Gallagher, and S. C. Aronoff □ *Departments of Microbiology and Immunology and Pediatrics, Robert C. Byrd Health Sciences Center, West Virginia University, Morgantown, West Virginia, USA*

□ *Chronic infection in the cystic fibrosis (CF) lung is characterized by* Pseudomonas aeruginosa *strains that overproduce the mucoid exopolysaccharide, alginate. Previous experiments have shown that long-term survival of* P. aeruginosa *in the CF lung may be facilitated by increased adherence and decreased invasion of respiratory epithelial cells. Therefore, mucoid and nonmucoid clinical isolates of* P. aeruginosa *were assayed for their ability to associate with and invade the CF respiratory epithelial cell line, CF/T43. Association assays and gentamicin exclusion assays demonstrated that mucoid* P. aeruginosa *associates with and invades CF/T43 cell monolayers significantly less than nonmucoid* P. aeruginosa *strains* ($P = .004$, $.02$). *Fluorescence microscopy invasion assays confirmed these results. The differences in association and invasion by the* P. aeruginosa *strains were not due to differences in lipopolysaccharide phenotype or cytotoxicity for CF/T43 respiratory epithelial cells. Exogenous bacterial alginate had no effect on the invasion of CF respiratory epithelia by a nonmucoid strain. Invasion assays with the wild-type* P. aeruginosa *strain PAO1 and isogenic* algU *and* mucA *mutant strains failed to show differences in invasion* ($P = .25$). *We conclude that (i) mucoid* P. aeruginosa *isolates associate with and invade CF/T43 respiratory epithelial cells with less efficiency than nonmucoid* P. aeruginosa, *(ii) these differences are not due to variations in lipopolysaccharide phenotype between strains, (iii) neither exogenous nor endogenous alginate affects the ability of* P. aeruginosa *to invade CF/T43 respiratory epithelial cells, and (iv) invasion of CF/T43 respiratory epithelial cells by a laboratory reference strain of* P. aeruginosa *does not appear to be regulated by AlgU.*

Keywords *alginate, cystic fibrosis, invasion,* Pseudomonas aeruginosa

The establishment of chronic *Pseudomonas aeruginosa* infection in the cystic fibrosis (CF) lung marks the beginning of a severe inflammatory response

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Address correspondence to A. Rene D. Massengale, at her current address: National Institute for Occupational Safety and Health, Health Effects Laboratory Division, 1095 Willowdale Road, MS4050, Morgantown, WV 26505, USA. E-mail: zhg3@cdc.gov

that leads to a decline in lung function and the subsequent death of the patient [1–4]. Most *P. aeruginosa* strains recovered from CF sputa are of a mucoid phenotype and overproduce the mucoid exopolysaccharide, alginate [5, 6]. In addition, large amounts of free alginate are found in expectorated sputum [20]. The majority of genes encoding enzymes that are responsible for alginate synthesis are located in a gene cluster at 34 minutes on the *P. aeruginosa* chromosome [5, 8]. This gene cluster exhibits an operonic structure and appears to be cotranscribed with *algD*, the initial gene in the cluster [9].

AlgU is an extreme stress sigma factor that regulates alginate synthesis as well as the expression of other genes [10–12]. Expression of this sigma factor is required for transcription from the *algD* promoter [12, 13]. Yu and colleagues [11], demonstrated that *P. aeruginosa algU* mutants were more sensitive to killing by reactive oxygen intermediates, macrophages, and neutrophils. However, systemic virulence was increased in a mouse model of acute infection as indicated by decreased time-to-death after infection with the *algU* mutants as compared to infection with wild-type strains [11]. These findings suggest that AlgU is responsible for the regulation of multiple bacterial defense and virulence mechanisms.

Bacterial adherence to host cells usually precedes bacterial invasion [14]. Both lipopolysaccharide and pilin have been implicated as possible ligands for *P. aeruginosa* adherence to host tissue [15–18]. Alginate has also been shown to enhance adherence of *P. aeruginosa* to host tissue [19]. Invasion of *P. aeruginosa* into corneal epithelia cells has also been well documented [20, 21]. However, pathological studies of the CF lung indicate that most *P. aeruginosa* remain localized in the airway and invade host cells very little [22]. Furthermore, these strains actually multiply the surface of CF respiratory epithelia in contrast to normal respiratory epithelia [11, 23, 24]. Because entry of bacteria into host cells and subsequent exfoliation is viewed as a possible host defense mechanism [25], the lack of bacterial internalization by CF respiratory epithelia may actually contribute to the ongoing *Pseudomonas* colonization that occurs in these patients.

In this study we compared the invasion of CF/T43 respiratory epithelial cells by mucoid and nonmucoid clinical isolates of *P. aeruginosa* and examined the role of alginate and the sigma factor, AlgU, in the invasion of CF/T43 respiratory epithelial cells by a laboratory reference strain. Our results indicate that (i) mucoid *P. aeruginosa* isolates associate with and invade CF/T43 respiratory epithelial cells with less efficiency than nonmucoid *P. aeruginosa*, (ii) these differences are not due to variations in lipopolysaccharide phenotype between strains, (iii) neither exogenous nor endogenous alginate affects the ability of *P. aeruginosa* to invade CF/T43 respiratory epithelial cells, and (iv) invasion of CF/T43 respiratory epithelial cells by a laboratory reference strain of *P. aeruginosa* does not appear to be regulated by AlgU.

MATERIALS AND METHODS

Bacteria

Clinical isolates of *P. aeruginosa* were provided by the Microbiology Laboratory at West Virginia University Hospital (Morgantown, WV) and were recovered from the sputa of CF patients and the blood of nonaffected individuals. Isolates were classified as mucoid or nonmucoid based on the phenotype at the initial isolation from the patient. All bacteria were stored at -70°C. *P. aeruginosa* strains used in this study are listed in Table 1. Strains PAO381a2-3 and PAO6852 are isogenic mutants of the reference strain PAO1. PA381a2-3 (*mucA*) was created by replacing the PAO1 chromosomal *mucA* with a mutant *mucA* gene from a mucoid strain [13]. PAO6852 (*algU*) was created by replacing the PAO1 chromosomal *algU* gene with an inactivated *algU* gene disrupted by a tetracycline resistance cassette (*algU::Tc*^{r}) [11].

Growth curve assays were performed by inoculating multiple tubes containing 1 mL keratinocyte growth medium with 1×10^{8} colony forming units per milliliter (cfu/mL) bacteria. A separate tube for each time point was inoculated and aliquots from each tube were diluted and plated at either 0.5, 1, 2, 4, or 8 hours to determine concentration. Three tubes per strain were inoculated and averaged at each time point.

Cell Cultures

The CF/T43 cell line is a CF human respiratory epithelial cell line trans-

TABLE 1 Strains Used in This Study

Strain	Description	Source or reference
P. aeruginosa 27853	Muc^{-} reference strain	American Type Culture Collection
P. aeruginosa PAO1	Muc^{-} parent strain	33
P. aeruginosa PAO381a2-3	Muc^{+} strain (*mucA*)	33
P. aeruginosa PAO6852	Muc^{-} strain (*algU::Tc*^{r})	27
P. aeruginosa CFM1	Muc^{+} clinical isolate	WVU Hospital
P. aeruginosa CFM2	Muc^{+} clinical isolate	WVU Hospital
P. aeruginosa CFM3	Muc^{+} clinical isolate	WVU Hospital
P. aeruginosa CFM4	Muc^{+} clinical isolate	WVU Hospital
P. aeruginosa CFM5	Muc^{+} clinical isolate	WVU Hospital
P. aeruginosa CFM6	Muc^{+} clinical isolate	WVU Hospital
P. aeruginosa CFNM1	Muc^{-} clinical isolate	WVU Hospital
P. aeruginosa CFNM2	Muc^{-} clinical isolate	WVU Hospital
P. aeruginosa CFNM3	Muc^{-} clinical isolate	WVU Hospital
P. aeruginosa NCFNM1	Muc^{-} clinical isolate	WVU Hospital
P. aeruginosa NCFNM2	Muc^{-} clinical isolate	WVU Hospital
P. aeruginosa NCFNM3	Muc^{-} clinical isolate	WVU Hospital
P. aeruginosa NCFNM4	Muc^{-} clinical isolate	WVU Hospital

Muc^{+}, mucoid phenotype; Muc^{-}, nonmucoid phenotype.

formed with the pZIPneoSV(X)-1/XV40T retrovirus [3, 26]. CF/T43 respiratory epithelial cells were grown to confluence in 24-well tissue culture plates with keratinocyte growth medium supplemented with bovine pituitary extract (30 μg/mL), epidermal growth factor (0.1 μg/mL), insulin (5 μg/mL), hydrocortisone (0.5 μg/mL), and gentamicin sulfate (50 μg/mL; Clonetics, San Diego, CA).

Preparation of Alginate

The alginate stock solution (10 mg/mL) was made by dissolving either alginic acid from *Macrocystis pyrifera* (Sigma, St Louis, MO) or purified bacterial alginate isolate from *P. aeruginosa* in sterile phosphate-buffered saline (BioWhittaker, Walkersville, MD) [27]. The stock alginate solutions (10 mg/mL) were serially diluted in phosphate-buffered saline to a total volume of 100 μL prior to use. Due to the viscosity of the alginate stock solution, the dilutions were premixed with the culture medium and the bacterial inocula in microcentrifuge tubes before addition to all cultures.

Gentamicin Survival Assay

Gentamicin survival assays as described by Miller and Falkow were modified as follows [28]. Strains of *P. aeruginosa* were grown overnight in Luria Bertaini broth and standardized by optical density. Aliquots were diluted and plated on Luria Bertaini agar plates to determine the actual stock concentration (stock count). Confluent CF/T43 monolayers in 24-well tissue culture plates (Costar, Cleveland, OH) were washed 3 times with antibiotic-free supplemented keratinocyte growth medium to remove residual antibiotic and nonviable cells. Approximately 1×10^8 bacteria were added to each well along with antibiotic-free supplemented keratinocyte growth medium to a total volume of 1000 μL/well. This inoculum was determined in preliminary control experiments described below and correponds to approximately 50 to 100 bacteria per epithelial cell in each well. The plates were then centrifuged at 1250 revolutions per minute (rpm) and incubated at 37°C in 5% carbon dioxide.

After 4 hours of incubation, some infected CF/T43 monolayers were washed 3 times, lysed with trypsin (0.025%)-Tween 20 (0.1%) (BioWhittaker) serially diluted, and plated to determine the number of bacteria associated with the monolayer for each strain (association count). The association index was calculated by dividing the association count by the stock count. Other infected CF/T43 monolayers were treated with 1000 μg/mL gentamicin sulfate (Sigma) for 2 hours to remove extracellular bacteria then lysed with trypsin-Tween 20, serially diluted, and plated to determine the number of bacteria that invaded the respiratory epithelial cells

(invasion count). The invasion index was calculated by dividing the invasion count by the stock count. This experiment was done in triplicate and the mean indices used for statistical analysis. Gentamicin assays in the presence of exogenous bacterial alginate and alginic acid were performed similarly with the addition of alginate dilutions to separate wells.

Control invasion assays were initially performed to determine the optimum average stock inoculum. Parallel invasion assays with varying inoculum concentrations (10^5–10^9) *P. aeruginosa* 27853 and *Escherichia coli* JM109 were performed as described above.

Fluorescence Microscopy Assays

Bacteria were fluorescently labeled as previously described by Drevets and Elliott [29]. Preliminary control experiments were performed to determine the following: (1) optimum fluorescein concentration that allows maximum bacterial staining with minimum nonspecific background fluorescence (2 mg/mL), and (2) optimum ethidium bromide concentration that effectively counterstains (quenched fluorescein fluorescence) of bacteria with minimum nonspecific background fluorescence. Test strains were labeled by overnight growth in broth containing 2 mg/mL of the fluorochrome 5-(((2(carbohydrazino)methyl)thio)-acetyl)-amino-fluorescein (fluorescein, Bio-Probe, Eugene, OR). Aliquots were diluted and plated on Luria Bertaini agar plates to determine the stock count. Confluent CF/T43 monolayers were washed 3 times with antibiotic-free supplemented keratinocyte growth medium to remove residual antibiotic and nonviable cells. Approximately 1×10^8 bacteria in antibiotic-free supplemented keratinocyte growth medium were added to each well. The plates were centrifuged at 1250 rpm and incubated for 4 hours at 37°C in 5% carbon dioxide. The monolayers were washed 3 times and the coverslips transferred to glass slides. The CF/T43 respiratory epithelial cells were counterstained with 25 μg/mL ethidium bromide (Sigma) to differentiate the extracellular bacteria. The monolayers were then viewed by fluorescence microscopy. The number of intracellular bacteria in approximately 2000 CF/T43 respiratory epithelial cells were counted and expressed as the number of intracellular bacteria per 1000 CF/T43 respiratory epithelial cells per 1×10^8 bacterial inoculum.

Cytotoxicity Assays

Cytotoxicity assays were performed as described by Fleiszig and colleagues [20]. Test strains were grown overnight in Luria Bertaini broth and standardized by optical density. Confluent CF/T43 monolayers were washed 3 times with antibiotic-free supplemented keratinocyte growth medium and

approximately 1×10^8 bacteria were added to each well along with antibiotic-free supplemented keratinocyte growth medium to a total volume of 1000 μL/well. Control wells contained culture media without bacteria. The plates were centrifuged at 1250 rpm and incubated at 37°C in 5% carbon dioxide. For time zero values, infected CF/T43 monolayers were immediately washed 3 times with antibiotic-free supplemented keratinocyte growth medium, and treated with trypsin-EDTA (BioWhittaker) to lift the epithelial cells. Trypan blue dye (Sigma) was added to the treated wells and aliquots of the cells were counted on a hemocytometer. The number of nonviable cells in 250 total cells was recorded. The count was repeated after 4 hours of incubation, then the percent nonviable cells was determined.

For alginate cytotoxicity assays, CF/T43 monolayers were washed 3 times and a final alginate concentration of 1000 μg/mL was added to each monolayer. The plate was incubated at 37°C in 5% carbon dioxide. Some CF monolayers were immediately washed 3 times with antibiotic-free supplemented keratinocyte growth medium and treated with trypsin-EDTA to life the epithelial cells. Trypan blue dye was added to the treated wells and aliquots of the cells were counted on a hemocytometer. Two hundred fifty epithelial cells were counted per aliquot and the frequency of stained, nonviable cells recorded. The count was repeated after 4 hours of incubation and the increase in the number of nonviable cells was determined.

Preparation of Lipopolysaccharide

Lipopolysaccharide (LPS) was obtained from each clinical isolate and characterized for the presence of *O*-side chains and the presence of LPS core oligosaccharide. LPS from each strain was prepared according to the method of Zaidi and colleagues [18]. Each strain was grown in 100 mL Luria Bertaini broth overnight at 37°C in a shaker incubator, then pelleted and resuspended in 10% sodium lauryl sarcosine to lyse the cells. The cells were then centrifuged at 20,000 × *g* for 15 minutes to remove cellular debris, and the supernatant was removed. Four volumes of 95% ethyl alcohol were added to the supernatant and incubated for 18 hours at 4°C. The precipitate was collected by centrifugation, resuspended in phosphate-buffered saline, and ultracentrifuged for 3 hours at 100,000 × *g*. The pellet containing LPS was first treated with a mixture containing DNase (0.1 mg/mL), RNase (0.1 mg/mL), calcium chloride (4 μM), and magnesium chloride (4 μM) overnight at 37°C and then treated with pronase (0.1 mg/mL) for 2 hours at 56°C. The LPS was then ultracentrifuged again for three hours at 100,000 × *g*, the pellet redissolved in distilled water and lyophilized. LPS from each strain was redissolved in distilled water and analyzed via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The gels were silver stained by the method of Tsai and Frasch [30].

Statistics

Differences among groups were determined by analysis of variance and least significant difference tests. Correlation of indices with alginate concentration was determined by Spearman's correlation test.

RESULTS

Association and Invasion of CF Respiratory Epithelial Cells by Mucoid and Nonmucoid Clinical Isolates of *P. aeruginosa*

Control invasion assays were initially performed with the laboratory reference strain *P. aeruginosa* ATCC 27853 and a negative control strain, *Escherichia coli* JM109, to determine the optimum stock inoculum for association assays and gentamicin exclusion assays. These experiments indicated that *E. coli* JM109 exhibits very low invasion of epithelial cells at all inoculum concentrations tested (10^5–10^9) (data not shown). The number of *P. aeruginosa* recovered intracellularly increased linearly with inoculum concentration; therefore, a mid-range inoculum concentration of 1×10^8 was selected corresponding to a multiplicity of infection of 50 to 100 bacteria per epithelial cell. This value is similar to *P. aeruginosa* concentrations routinely found in sputum samples from infected CF lungs [31, 32]. The mean invasion index for *E. coli* JM109 at this inoculum concentration is less than 3% of *P. aeruginosa* 27853 (data not shown). Similar results were obtained with *P. aeruginosa* PAO1.

To determine the ability of mucoid and nonmucoid clinical isolates of *P. aeruginosa* to associate with and invade CF respiratory epithelial cells, association assays and gentamicin survival assays of CF/T43 monolayers were performed with thirteen clinical isolates (Table 1). Table 2 contains the mean association and invasion indices for these strains. The mean association index

TABLE 2 Association With and Invasion of CF Respiratory Epithelial Cells by Mucoid and Nonmucoid Clinical Isolates of *P. aeruginosa* as Determined by the Gentamicin Survival Assay

Phenotype	Association index[a] (mean ± *SE*)	Invasion index[b] (mean ± *SE*)
Mucoid (n = 6)	0.7 ± 0.2	$(3.5 \pm 1.7) \times 10^{-4}$
Nonmucoid (n = 7)	16.5 ± 5.1	$(3.8 \pm 2.0) \times 10^{-3}$
P value[c]	.004	.02

[a] Defined as the number of bacteria associated with the monolayer after 4 hours of infection (association count) divided by the initial inoculum. Values represent the mean ± standard error of 3 separate experiments.

[b] Defined as the number of bacteria recovered after gentamicin treatment divided by the initial inoculum. Values represent the mean ± standard error of 3 separate experiments.

[c] Determined by analysis of variance.

of the mucoid strains was significantly less (20-fold) than that of the nonmucoid strains. The mean invasion index of the mucoid strains was also significantly less (10-fold) than the nonmucoid strains tested. To determine if differences in association were due to differences in either growth rate or lag phase duration between strains, the growth curves of *P. aeruginosa* strains 27853 (nonmucoid), CFM2 (mucoid), and NCFNM1 (nonmucoid) were determined (Figure 1). There was no significant difference in bacterial concentration at any time point tested, suggesting that differences in association were not due to differences in growth rate of either mucoid or nonmucoid strains. These data suggest that mucoid strains of *P. aeruginosa* associate with

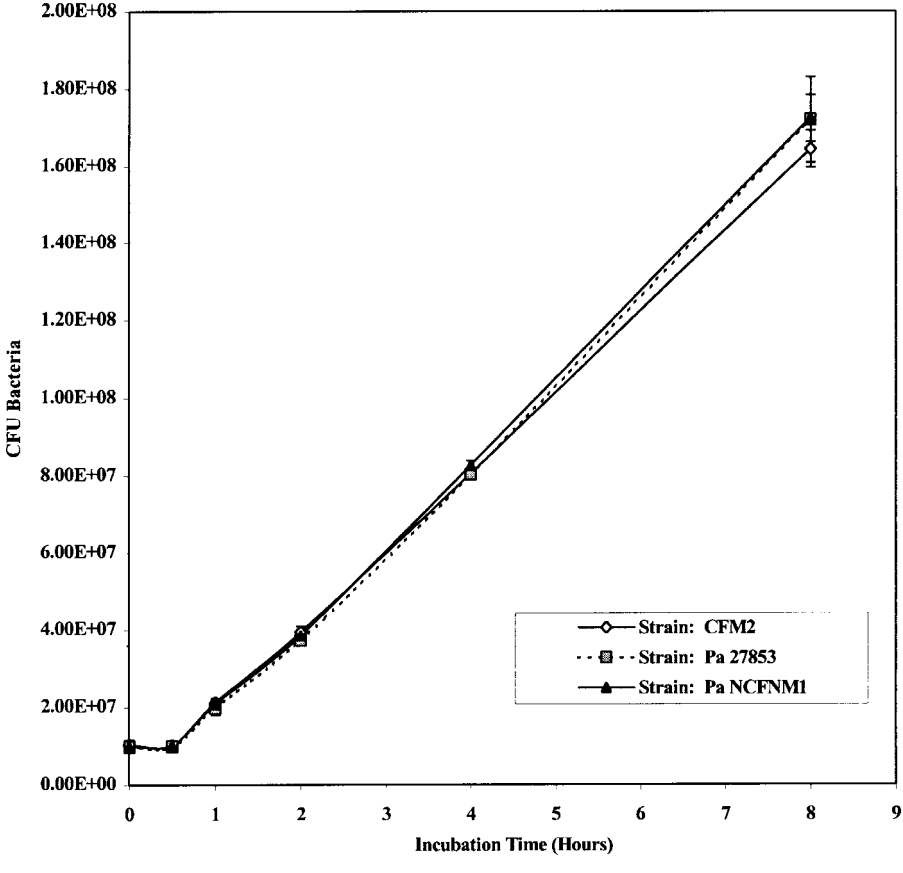

FIGURE 1 Change in bacterial concentration over time. To assess the change in bacterial concentration over time, separate 1-mL aliquots of keratinocyte growth medium were inoculated with 3 representative strains of *P. aeruginosa* (1×10^8 bacteria). Three separate aliquots were sampled for each time point, diluted, and plated to determine concentration (colony-forming units [CFU] per mL). Bacterial concentrations were determined at 0, 0.5, 1, 2, 4, and 8 hours. There was no significant difference in bacterial concentration between strains within any single time point. The mean bacterial concentrations after 4 hours incubation were as follows: *P. aeruginosa* CFM2, $(8.06 \pm 0.061) \times 10^7$; *P. aeruginosa* 27853, $(8.04 \pm 0.148) \times 10^7$; and *P. aeruginosa* NCFNM1, $(8.27 \pm 0.129) \times 10^7$.

and invade CF respiratory epithelia less efficiently than nonmucoid *P. aeruginosa.*

To confirm that differences in invasion detected in the gentamicin survival assay accurately represented invasion differences between strains, invasion assays using fluorescence microscopy were performed with representative mucoid and nonmucoid clinical isolates. The mean number of intracellular bacteria per 1000 CF/T43 respiratory epithelial cells per 10^8 inoculum of each strain from three separate experiments were as follows: CFM1 (3.7 ± 0.6), CFM4 (2.8 ± 0.0), and NCFNM1 (14.8 ± 1.0). Least significant difference analysis indicated that there were significantly more nonmucoid NCFNM1 intracellular bacteria than mucoid CFM1 or CFM4 (F ratio = 186.3, $P = .0007$). Therefore, differences in invasion as measured by gentamicin exclusion assays represented true differences in number of intracellular bacteria.

Lipopolysaccharide has been identified as a possible ligand for adherence of *P. aeruginosa* to respiratory epithelial cells [4]. To determine if differences in invasion were due to differences in LPS phenotype, LPS from *P. aeruginosa* PAO1 and each *P. aeruginosa* clinical isolate was purified and analyzed by SDS-PAGE and silver staining. The gels revealed that only *P. aeruginosa* NCFNM1 and *P. aeruginosa* CFM4 had smooth LPS with core oligosaccharide, but no *O*-antigen side chain. *O*-antigen side chains and core oligosaccharide were present in all other strains examined. *P. aeruginosa* NCFNM1 is a non-CF, nonmucoid clinical isolate exhibiting significantly higher invasion that the mucoid CF clinical isolate *P. aeruginosa* CFM4 (mean invasion indices: NCFNM1 = $[3.3 \pm 1.4] \times 10^{-3}$; CFM4 = $[2.1 \pm 0.7] \times 10^{-6}$). The presence of similar LPS phenotypes in both mucoid and nonmucoid strains suggests that no correlation between adherence or invasion and LPS phenotype or presence of LPS oligosaccharide core exists.

Cytotoxic Effect of *P. aeruginosa* Clinical Isolates and Alginate on CF Respiratory Epithelial Cells

Gentamicin exclusion assays are based on the concept that bacteria which invade host cells are protected from subsequent gentamicin treatment by the intact host cell membrane. Because bacterial cytotoxicity for epithelial cells can compromise epithelial cell integrity and affect the results of gentamicin exclusion assays, the cytotoxic effects of the clinical *P. aeruginosa* isolates on CF/T43 cell monolayers were determined. Trypan blue dye exclusion assays revealed no significant differences in the number of nonviable CF/T43 respiratory epithelial cells after 4 hours of infection with mucoid or nonmucoid strains ($P = .49$; Table 3). The percent of nonviable CF/T43 respiratory epithelial cells adjusted for normal epithelial cell attrition was less than 5%.

TABLE 3 The Cytotoxic Effect of Representative Clinical Isolates of *P. aeruginosa* on CF Respiratory Epithelial Cells

Strain	Percent Nonviable CF/T43 cells (mean ± *SE*)[a]
CFM1	4.1 ± 1.1
CFM2	5.7 ± 0.9
CFNM1	5.2 ± 1.4
NCFNM1	3.2 ± 0.2
27853	3.0 ± 1.8
P value	.488

[a] The percent nonviable CF/T43 respiratory epithelial cells for each strain was calculated by subtracting the percent nonviable cells at 0 hours from the percent nonviable cells at 4 hours. The values represent the mean ± standard error of 3 separate experiments.

High concentrations of exogenous alginate are produced by mucoid *P. aeruginosa* colonizing the CF lung [5, 6]. Therefore, the reduction in CF/T43 cell invasion by mucoid *P. aeruginosa* could also potentially be due to cytotoxic effects of exogenous alginate on the respiratory epithelial cells. To determine if the presence of exogenous alginate is cytotoxic to CF/T43 respiratory epithelial cells, trypan blue dye exclusion assays were performed following incubation of CF/T43 monolayers in the presence of 0, 10, 100, and 1000 μg/mL alginate. These assays revealed no differences in the number of nonviable CF/T43 respiratory epithelial cells after exposure to any concentration of alginate (data not shown). Thus, exogenous alginate was not cytotoxic to CF/T43 respiratory epithelial cells, and this potential cytotoxcity was not responsible for the apparent differences in invasion between mucoid and nonmucoid clinical isolates.

Effect of Exogenous Alginate on the Invasion of CF Respiratory Epithelial Cells by *P. aeruginosa*

Because exogenous bacterial alginate has been shown to affect the adherence of *P. aeruginosa* to host tissue [19], it is possible that exogenous alginate also affects the invasion of respiratory epithelial cells in some way. Therefore, the effect of exogenous alginate on the association and invasion of CF/T43 respiratory epithelial cells by the highly invasive, nonmucoid strain, *P. aeruginosa* NCFNM1, was determined in the presence of 0, 10, 100, and 1000 μg/mL alginic acid (data not shown) and bacterial alginate (Table 4). There was no significant difference in invasion in the presence or absence of exogenous alginic acid or bacterial alginate ($P = .6, .9$, respectively). These indices also were independent of the alginate concentration in the wells (alginic acid:

TABLE 4 The Effect of Exogenous Alginate on the Invasion of CF Respiratory Epithelial Cells by *P. aeruginosa* NCFNM1

Alginate concentration (μg/mL)	Mean invasion index (mean ± *SE*)[a]
1000	$(2.95 \pm 1.3) \times 10^{-3}$
100	$(2.9 \pm 0.9) \times 10^{-3}$
10	$(3.12 \pm 1.1) \times 10^{-3}$
1	$(2.74 \pm 0.8) \times 10^{-3}$
0	$(1.98 \pm 0.7) \times 10^{-3}$

[a] The values represent the mean ± standard error for 3 experiments. There was no significant difference between the invasion indices for each group ($P = .6$). The invasion index did not correlate with the alginate concentration as calculated by Spearman's correlation test ($P = .6$, .8, respectively).

invasion correlation coefficient = −.7, $P = .3$; bacterial alginate correlation coefficient = .3, $P = .6$).

Effect of *mucA* and *algU* Mutations on the Invasion of CF Respiratory Epithelial Cells by *P. aeruginosa*

Alginate produced by *P. aeruginosa* is not only given off by the cell, but also forms a thick protective layer around the cell surface. The alginate biosynthetic gene cluster is activated by the extreme stress sigma factor, AlgU [12, 13]. This sigma factor is thought to be negatively regulated by the anti–sigma factor, MucA, that prevents the binding of AlgU to its promoter sequences thus inhibiting alginate synthesis [33]. To determine if the production of this endogenous alginate by the bacterium affects invasion, gentamicin exclusion assays were done with the wild-type, nonmucoid *P. aeruginosa* PAO1 and the isogenic mutants PAO381a2-3 (*algU*) and PAO6852 (*mucA*) (Table 5). There was no significant difference in the invasion indices for the nonmucoid parent PAO1, the mucoid strain PAO381a2-3 (*mucA*), or the nonmucoid strain PAO6852 (*algU*, $P = .3$). This indicates that neither mutations in *algaU* and *mucA* nor the endogenous production of alginate affect the invasion of CF/T43 respiratory epithelial cells by the laboratory reference strain *P. aeruginosa* PAO1.

DISCUSSION

We tested the association and invasion of a CF respiratory epithelial cell line by 13 clinical isolates of *P. aeruginosa*. Our studies showed that nonmucoid *P. aeruginosa* invade CF/T43 cells more efficiently than mucoid

P. aeruginosa. Fleiszig and colleagues recently demonstrated an inverse relationship between bacterial cytotoxicity for epithelial cells and bacterial invasion of epithelial cells via gentamicin survival assays [20]. In the present report, trypan blue exclusion assays demonstrated that differences in invasion were not due to variations in cytotoxicity of the clinical isolates for the CF/T43 respiratory epithelial cells. Furthermore, LPS analysis indicated that differences in invasion were not due to differences in LPS phenotype. The addition of exogenous alginate had not effect on the invasion of CF/T43 respiratory epithelial cells by a nonmucoid *P. aeruginosa* strain. The production of alginate as defined by mutations in *algU* or *mucA* had no effect on the invasion of CF/T43 respiratory epithelial cells by the laboratory reference strain *P. aeruginosa* PAO1.

Uptake of bacteria by host epithelial cells may serve as a host defense mechanism [25]. Following phagocytosis, bacteria can be removed from the airway by cell desquamation or some other means. Prevention of this mechanism would potentially allow the persistence of the organism within the host as seen in the colonization of the CF lung by *P. aeruginosa*. Recently, Goldman and colleagues [23] demonstrated that *P. aeruginosa* persist in the CF airway due to a defect in a host defensin activity at high chloride concentration characteristic of the CF lung. Also, Pier and colleagues demonstrated that purified complete core LPS inhibited *Pseudomonas* uptake by respiratory epithelial cells more effectively than incomplete core LPS [25]. In a mouse pneumonia model, the addition of complete core LPS combined with the *Pseudomonas* inoculum prior to instillation into the mouse lung resulted in the survival of more bacteria than the addition of incomplete core LPS [25]. These observations suggest that reduced *Pseudomonas* invasion of respiratory epithelia results in increased bacterial survival in the airway [25]. Furthermore, because mucoid *P. aeruginosa* strains are most commonly isolated from older, chronically infected CF patients, it is reasonable to hypothesize that mucoid *P. aeruginosa* invades CF respiratory epithelial cells less than nonmucoid *P. aeruginosa*. Our finding that mucoid *P. aeruginosa* strains invade CF/T43 respiratory epithelial cells less efficiently than nonmucoid strains supports this hypothesis. However, we cannot exclude the possibility that the mean difference in invasion was due to an even greater increase in association of nonmucoid strains over mucoid strains. Future experiments will determine whether these differences in invasion are due to differences in adherence or actual differences in the ability of the mucoid and nonmucoid strains to invade epithelial cells.

The ligands involved in the adherence of *P. aeruginosa* have not been completely defined. Both pilin and LPS have been identified as potential ligands for the adherence of *P. aeruginosa* to the surface of epithelial cells [3, 16–18]. Ramphal and colleagues demonstrated, through competitive inhibition assays, that exogenous pili inhibited the adherence of a nonmucoid

TABLE 5 The Invasion of Defined Isogenic Mutants of *P. aeruginosa* into CF Respiratory Epithelial Cells as Determined by Gentamicin Survival Assays

Strain	Phenotype	Invasion index[a] (mean ± *SE*)
PAO1	Nonmucoid	$(1.7 \pm 0.6) \times 10^{-3}$
PAO381a2-3	Mucoid	$(2.7 \pm 1.0) \times 10^{-3}$
PAO6852	Nonmucoid	$(1.1 \pm 0.6) \times 10^{-3}$
P value[b]		.3005

[a] Defined as the number of bacteria recovered after gentamicin treatment divided by the initial inoculum. The values represent the mean ± standard error of 3 experiments.

[b] Determined by analysis of variance. There is no significant difference between the invasion indices.

P. aeruginosa strain to injured tracheal cells [16]. Saiman and Prince demonstrated that *P. aeruginosa* bound to asialoGM1 residues on the surface of respiratory epithelial cells [17]. The role of *P. aeruginosa* LPS outer core as a ligand for corneal cell adherence was subsequently demonstrated by Zaidi and colleagues [18]. Furthermore, Gupta and colleagues have demonstrated that both pilin and LPS from *P. aeruginosa* bind asialoGM1 residues, supporting the idea that both pilin and LPS may serve as *P. aeruginosa* ligands to epithelial cells [15]. These experiments indicate that more than one ligand may be involved in *Pseudomonas* adherence to host tissue. It is currently unknown which the predominant ligand is, the selective conditions for either ligand, or if other ligands may also play a role in *P. aeruginosa* adherence to host tissue. Characterization of the LPS from each clinical isolate revealed no correlation between invasion and type of LPS present. This suggests that differences in invasion were not due to differences in LPS ligand. However, these results do not rule out differences in adherence efficiency between strains due to variations in other ligands. Further experiments will explore the role of other ligands and their effect on the adherence and invasion of individual clinical isolates. This also highlights the need for further experiments that more clearly define the ligands involved in *P. aeruginosa* adherence to respiratory epithelia.

The conversion to mucoidy of *P. aeruginosa* is most common in the CF environment and appears to confer a selective advantage to the colonizing strain. The proposed roles of alginate in pathogenesis are extensive [5]. Alginate serves as a physical barrier to opsonization and subsequent phagocytosis [5], scavenges hypochlorite and reactive oxygen intermediates [32, 34], and facilitates the formation of microcolonies within the airways [5, 6]. It also has been implicated in the adherence of mucoid strains to the respiratory epithelia [16, 19]. In the present report, the addition of exogenous alginate had no effect on the invasion of CF/T43 respiratory epithelial cells

by a nonmucoid *P. aeruginosa* strain. If bacterial production of alginate itself inhibits invasion of CF respiratory epithelial cells, then CF/T43 cell invasion by a mucoid strain of *P. aeruginosa* should be reduced as compared to a nonmucoid, isogenic strain. Preliminary assays of isogenic *P. aeruginosa* PAO1 mutants either producing or not producing alginate in this study revealed that bacterial production of alginate had no effect on the invasion of CF/T43 respiratory epithelia cells.

The sigma factor AlgU lies in a gene cluster that also contains the downstream genes *mucA*, *mucB*, *mucC*, and *mucD*. The downstream genes are thought to be negative regulators of *algU* expression [5, 10]. MucA is thought to be an anti–sigma factor involved in posttranslational modification of AlgU [33]. This is supported by the finding that disruption of *mucA* results in conversion to the mucoid phenotype [13]. If bacterial production of alginate inhibits *Pseudomonas* invasion of CF respiratory epithelial cells, then a mutant strain with an inactivated *mucA* gene should be less invasive than its isogenic, wild-type parent. Similar results should be obtained in the comparison of an *algU* mutant strain with its isogenic parent. However, the results obtained with invasion of CF/T43 respiratory epithelial cells by the isogenic mutants failed to support the role of AlgU and MucA in regulation of invasion. Due to significant differences that may exist between the laboratory reference strain *P. aeruginosa* PAO1 and the uncharacterized clinical isolates, further experiments are underway to determine how these mutations affect a representative clinical isolate.

The results of recent studies suggest that reduced CF respiratory epithelial cell invasion by *Pseudomonas* is a potentially important process in persistence of the organism in the CF airway. Although in the current study we found that mucoid *P. aeruginosa* invaded CF respiratory epithelial cells significantly less than nonmucoid *P. aeruginosa*, we also demonstrated that this difference was not due to differences in LPS, cytotoxicity, or the mucoid phenotype itself. Because the sigma factor, AlgU, has been shown to regulate other functions within the cell, the possibility remains that some other factor under the control of AlgU may be responsible for this observed difference in invasion. Future experiments should continue to explore the role of AlgU and its possible relationship to bacterial invasion. The specific mechanisms involved in regulation of adherence and invasion of CF respiratory epithelia by *P. aeruginosa* remain to be determined. A better understanding of these mechanisms and the host-pathogen interaction will facilitate the development of potential therapies for chronic *Pseudomonas* infections in the CF patient.

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