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Novel Mouse Model of Chronic *Pseudomonas aeruginosa* Lung Infection Mimicking Cystic Fibrosis

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Pseudomonas aeruginosa **causes a chronic infection in the lungs of cystic fibrosis (CF) patients by establishing an alginate-containing biofilm. The infection has been studied in several animal models; however, most of the models required artificial embedding of the bacteria. We present here a new pulmonary mouse model without artificial embedding. The model is based on a stable mucoid CF sputum isolate (NH57388A) with hyperproduction of alginate due to a deletion in *mucA* and functional *N*-acylhomoserine lactone (AHL)-based quorum-sensing systems. Chronic lung infection could be established in both CF mice (*Cftr*$^{tmlUnc-/-}$) and BALB/c mice, as reflected by the detection of a high number of *P. aeruginosa* organisms in the lung homogenates at 7 days postinfection and alginate biofilms, surrounded by polymorphonuclear leukocytes in the alveoli. In comparison, both an AHL-producing nonmucoid revertant (NH57388C) from the mucoid isolate (NH57388A) and a nonmucoid isolate (NH57388B) deficient in AHL were almost cleared from the lungs of the mice. This model, in which *P. aeruginosa* is protected against the defense system of the lung by alginate, is similar to the clinical situation. Therefore, the mouse model provides an improved method for evaluating the interaction between mucoid *P. aeruginosa*, the host, and antibacterial therapy.**

The ability of the opportunistic pathogen *P. aeruginosa* to grow as a biofilm is an important aspect of the pathogenesis in the lung disease of patients with cystic fibrosis (CF). The transition from nonmucoid to a mucoid, alginate-overproducing phenotype initiates the fatal, chronic stage of the infection and correlates with a pronounced antibody response and immune complex-mediated chronic inflammation in the CF lungs (23). There is no clear interval between the initial colonization by *P. aeruginosa* and conversion to mucoidy (25), which indicates that it is due to spontaneous mutations, followed by selection of mucoid strains (8, 14). We have previously shown that biofilms of nonmucoid *P. aeruginosa* exposed to oxygen radicals from hydrogen peroxide or activated polymorphonuclear leukocytes (PMN) induce mutations in the *mucA* gene (35). MucA encodes an anti-sigma factor of AlgU required for expression of the alginate biosynthetic operon, leading to a mucoid phenotype (8, 34, 55). The mucoid *P. aeruginosa* biofilms withstand opsonization and phagocytosis by cells of the immune system (37, 48, 58), as well as an increased tolerance to toxic oxygen radicals (32, 35) and antibiotics (17, 19).

P. aeruginosa regulates the production of several virulence factors by cell-to-cell communication (quorum sensing [QS]) (30). Apparently, QS influences the biofilm formation (7, 18) and may therefore be important for the pathogenesis of *P. aeruginosa* lung infection in CF (9, 10, 38, 59).

Since the establishment of the first animal model of chronic *P. aeruginosa* lung infection in rats by Cash et al. in 1979 (4), several animal models of acute and chronic *P. aeruginosa* lung infection have been described (24, 50, 68). These models required that *P. aeruginosa* was embedded in an artificial biofilm (e.g., agar, agarose, or seaweed alginate) to prevent mechanical clearing (24). Furthermore, these models led to chronic infections of the conducting airways in mice, due to the size of the beads mechanically blocking the bronchi (72). Animal models addressing the role of *P. aeruginosa* alginate in pulmonary lung infection without the use of artificial embedding have not been used widely (2, 3, 60, 74).

We describe here a novel mouse model of chronic *P. aeruginosa* infection in CF. We use a stable mucoid clinical *P. aeruginosa* isolate (NH57388A) expressing QS, which can establish a chronic lung infection, both in the respiratory and conducting zones of the airways without artificial embedding. Furthermore, the role of alginate and QS in the pathogenesis of lung infection and inflammation is analyzed.

MATERIALS AND METHODS

P. aeruginosa **isolates.** The bacterial strains and plasmids used in the present study and their properties are shown in Table 1. The mucoid *P. aeruginosa* isolate NH57388A was initially screened for stability of the mucoid phenotype by serial passages every second or third day during 1 month on Blue agar plates (BAP [a modified Conradi Drigalski medium selective for gram-negative rods]; Statens Serum Institute, Copenhagen, Denmark) (20).

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TABLE 1. Strains and plasmids used in the present study

Strain or plasmid	Description	Source or reference
Strains		
E. coli		
HB101	*proA2 leuB6 thi-1 lacY1 hsdR hsdM recA13 supE44 rpsL20*	34
JM109	F′ *traD36 lac1*^{q} Δ(*lacZ*) *M15 proA*$^+$ *B*$^+$/*el4*$^-$ (McrA$^-$) Δ(*lac-proAB*) *thi gyraA96*(Na1^r) *endA1 hsdR17*(r_k^- m_k^+) *relA1 supE44 recA1*	34
P. aeruginosa		
NH57388A	Stable mucoid CF mouse sputum isolate, hyperproducing alginate, functional AHL-based QS, mutation in *mucA*	This study
NH57388B	Nonmucoid isolate from the same CF mouse sputum sample, nonfunctional AHL-based QS	This study
NH57388C	Nonmucoid isolate, derived in vitro from mucoid NH57388A, functional AHL-based QS, mutation in *algT*	This study
PAO1	Prototypic nonmucoid wild-type, functional AHL-based QS	B. H. Iglewski
Plasmids		
pRK600	Cm^r; *ori,* ColE1 RK2-Mob$^+$ RK2-Tra$^+$	28
pCD100	*F′traD36 lacI*^{q} (*lacZ*)M15 *proA*$^+$ *B*$^+$/*el4*$^-$ (*McrA*$^-$)(*lacproAB*)*thi gyr; Alg*TmucA(−)BCD operon	M. Kalai

PCR and sequencing. Alterations in *mucA* are the most frequent mutations found in mucoid CF isolates (3) and conversion to a nonmucoid phenotype has been associated with mutations in *algT*, which is a sigma factor, regulated by an anti-sigma factor, MucA (8). We sequenced *mucA*, *algT*, *mucB*, and *mucD* in the stable mucoid isolate NH57388A and in its nonmucoid revertant NH57388C. Amplification and sequencing of *mucA* was done by using the primers mucA1 (5′-CTCTGCAGCCTTTGTTGCGAGAAGC-3′) and mucA1 rev (5′-CTGCCAAGCAAAAGCAAAGGGAGG-3′). Amplification of *algT* was done by using the primers algT-F (5′-CCAAAGCAGGATGCCTGAAGACCT-3′) and algT-R (5′-GCAACTCGAGTTCATCCGCTTCGT-3′), and sequencing was done by using the primers algT-sek1 (5′-CCTGAGCCCGATGCAATCCATTTTCG-3′), algT-sek2 (5′-GGTATGCCTTGATGAAGGCTTCCTGC-3′), algT-sek3 (5′-GCTGTATCGGATCGCCATCAACACC-3′), and algT-sek4 (5′-GGACAGAGTTTCCTGGCAGGGCTTCAC-3′). Amplification of *mucB* was done by using the primers mucB1 (5′-ATCCGCCGTCAGTGGTACAG-3′) and mucB1-rev (5′-CGAGCAGGACGAGCAGGTAC-3′). The sequencing primers were mucB1, mucB1-rev, mucB-int1 (5′-CAGTGGTCCTTGCGGTGACT-3′), and mucB-int2 (5′-TTCAGCAGCAGCGACTTCAA-3′). Amplification of *mucD* was done by using the primers mucD1 (5′-GTCCGATTCGGCCTGAGTCT-3′) and mucD1-rev (5′-ACGCAGGTAACGGATTGACG-3′). The sequencing primers were mucD1, mucD1-rev, mucD-int1 (5′-GATCAACCCGGGTAACTCCG-3′), mucD-int2 (5′-AGATCTGCGAGTTGATGCCG-3′), mucD-int3 (5′-CATCCTCACCAACAATCACGTCGTGG-3′), mucD-int4 (5′-GCTCGCTACGGTCGGACAGG-3′), mucD-int5 (5′-CAGCGCAAGTCCCTGAGCATGG-3′), and mucD-int6 (5′-GCTTCATGTTGCCCACCAGGTGC-3′). The PCR conditions were 1.5 min at 94°C, 36 cycles of 2 min at 61°C, and 2.5 min at 72°C. Sequencing was performed on an ABI 3700 automatic DNA sequencer.

Complementation analysis. Triparental mating was used to mobilize recombinant plasmid pCD100 from *Escherichia coli* JM109 to *P. aeruginosa* NH57388C with the conjugation helper plasmid pRK600 (28) in *E. coli* HB101. Transconjugants of *P. aeruginosa* were selected on *Pseudomonas* isolation agar (PIA; Difco) containing 80 μg of tetracycline/ml, and complementation of alginate mutations in *P. aeruginosa* NH57388C was scored by the mucoid phenotype observed on the PIA plates after incubation at 37°C.

Batch culture conditions. *P. aeruginosa* were cultured on BAP from frozen stocks. Liquid inocula for the growth experiment were prepared by inoculating *P. aeruginosa* from a plate in filtered ox broth (Statens Serum Institut, Copenhagen, Denmark) overnight at 37°C and at 170 rpm. The bacteria were recovered by centrifugation, resuspended, and diluted to an optical density at 600 nm (OD_{600}) of 0.1 in 80 ml of fresh ox broth or ox broth supplemented with 1% glycerol. The batch cultures were incubated at 37°C (170 rpm) for 2 days. Samples were taken at different time intervals and centrifuged (23,000 × *g*, 30 min, 4°C), and the supernatant was analyzed for virulence factors (see below). The number of bacterial cells was determined as the OD_{600} or the number of CFU with BAP. All experiments were performed in triplicate.

Stability of the mucoid and nonmucoid phenotypes. The mucoid strain NH57388A, the nonmucoid strain NH57388B, and PAO1 were cultured aerobically for 4 weeks at 37°C in ox broth in both tubes and flasks under both static growth conditions, which led to spatial heterogeneity and multiple niches, and in shaking cultures (150 rpm) to maintain spatially homogeneity according to the methods of Rainey and Travisano (54) and Wyckoff et al. (73). Subcultures (100 μl) were taken from the surface, middle, and bottom from each tube and flask after 24 h and weekly thereafter and grown aerobically on BAP at 37°C for 3 days. The colony morphology (mucoid, nonmucoid, and small colony variants [SCV]) was determined. The static growth experiment was also carried out anaerobically in ox broth supplemented with 0.02% KNO_3 as the electron acceptor (which is similar to the concentration found in CF sputum [16]) and subcultured anaerobically (7% H_2, 7% CO_2, 86% N_2) and aerobically on 5% horse blood agar plates (State Serum Institute, Copenhagen, Denmark) and on BAP (supplemented with nitrate for anaerobic growth conditions). All subcultured phenotypes of the three strains, respectively, were checked for identity by pulsed-field gel electrophoresis as described previously (47).

LPS profile. Lipopolysaccharide (LPS) from *P. aeruginosa* was prepared as described previously (11), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and detected by silver staining.

Extraction of AHLs from culture supernatant. *P. aeruginosa* organisms were grown in 80 ml of LB broth or filtered ox broth supplemented with 1/10 A10 [20 g of $(NH_4)_2SO_4$, 60 g of $Na_2HPO_4 \cdot 2H_2O$, 30 g of KH_2PO_4, and 30 g of NaCl per liter (pH 6.4)] (5), followed by incubation at 37°C with shaking (170 rpm). Extraction was performed according to the method of Gram et al. (15). *N*-acylhomoserine lactones (AHLs) were extracted from early-stationary-phase cultures (30 ml) with acidified ethyl acetate (45 ml) overnight. Values were corrected for differences in cell densities. The organic phase was collected, which was subsequently evaporated under a gentle N_2 flow to dryness. The extract was dissolved in 500 μl of ethyl acetate.

Identification of AHLs by TLC. Analytical thin-layer chromatography (TLC) was done as described by Shaw et al. (56). Duplicate samples of AHL extracts (20 μl) were applied to C_{18} reversed-phase TLC plates (aluminum sheets RP-18 F254s [20 by 20 cm]; Merck Chrom Line), and chromatograms were developed with methanol-water (60:40 [vol/vol]). After separation, the dried plates were overlaid with 250 ml of LB medium (42°C) containing 2% agar and 2.5 ml of culture of the *Chromobacterium violaceum* CV026 indicator strain (36) monitoring short-chain AHLs. For the detection of medium- and long-chain AHLs, plates were overlaid with 250 ml of LB medium (42°C) containing 2% agar, 500 μl of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [40 mg/ml]; Sigma Chemical Co., St. Louis, Mo.), and 10 ml of culture of *Agrobacterium tumefaciens* indicator strain. Plates were incubated overnight at 30°C. The spots were compared visually with known amounts of AHL standards (Sigma).

Testing for QS-regulated virulence factors. As a guide to enzyme production, a qualitative test for screening for endochitinase (hydrolytic splitting of chitin polymer) and total protease (casein degradation on skim milk agar, detecting, i.e., alkaline protease and elastase [*lasA* and *lasB*]) activity was performed by the protocol of Nielsen and Sørensen (43) except with L-agar instead of 1/10 tryptic soy agar.

Production of exoproducts. (i) Endochitinase. Endochitinase assay was performed as described by Nielsen and Sørensen (42). Briefly, culture supernatant (200 μl) was mixed with carboxymethyl-chitin-remazol brilliant violet (100 μl; 2 mg ml^{-1}; Loewe Biochemica, Sauerlach, Germany) and sodium acetate buffer (100 μl; 0.05 M [pH 5.0]) and incubated for 20 min at 50°C in water bath. The reaction was terminated by addition of HCl (100 μl: 2 M), kept for 10 min on ice, and centrifuged at 10,000 × *g* at 4°C for 5 min. The absorbance (OD_{540}) of the supernatant was measured. Boiled supernatant with buffer and substrate was used as a blank. Endochitinase activity was expressed in units of measured A_{540} × 1,000 × min^{-1} × ml^{-1} as a mean of three independent replicates. The specific activity (enzyme units per unit of cell mass) was calculated as enzyme units OD_{540}/OD_{600} normalized to 1 ml of supernatant.

(ii) Protease. LasB protease (elastase) activity was measured by the method of Ohman et al. (46) by using the elastin Congo red (Sigma) as the substrate. Elastin Congo red (5 mg ml^{-1}) in 0.1 M Tris-maleate buffer (pH 7.0) and culture supernatant (500 μl) were incubated for 2 h at 37°C. Sodium phosphate buffer (1 ml; 0.7 M [pH 6.0]) was added to the samples, and the mixtures were placed on ice for 10 min to stop the reaction. After centrifugation (10,000 × *g* at 4°C for 15 min), the absorbance (OD_{495}) of the supernatant was measured. Supernatant plus assay buffer was used as a blank. The specific elastase (LasB) activity was measured as the absorbance of the supernatant (OD_{495}) divided by the OD_{600} of the culture normalized to 1 ml of supernatant. The production of endochitinase and protease activity from PAO1 was measured as a positive control. All values are the means of three independent replicates.

(iii) Alginate. The extraction of exopolysaccharide (alginate) was performed according to the method of Pedersen et al. (49) with slight modifications. Sample cultures (1 ml) were centrifuged for 30 min at 23,000 × *g* at 4°C. The pellet was discarded, and the supernatant containing the alginate was precipitated with ice-cold 99% ethanol (3 ml). The precipitate was centrifuged, (5,000 × *g*, 5 min) and then dissolved in 1 ml of sterile 0.9% saline. The content of uronic acid polymers (the component of alginate) in the samples was then analyzed by the carbazole-borate assay (31) with D-mannuronic acid lactone (Sigma) as a standard. All values are the means of three independent replicates.

Preparation of inoculum for infection of mice (experiments 1 and 2). Mucoid NH57388A or nonmucoid *P. aeruginosa* NH57388B strains were separately cultured in 80 ml of ox broth for 28 h at 37°C with shaking (170 rpm). Cells were harvested by centrifugation (23,000 × *g*, 30 min, 4°C) and resuspended in 2 ml of fresh ox broth, and the CFU was were counted. The cells were adjusted to the appropriate challenge inoculum by resuspension in their respective supernatant (crude alginate). Controls were cell-free supernatants.

Isolation of pseudomonal alginate (purified alginate). Mucoid *P. aeruginosa* NH57388A was cultured in 80 ml of ox broth supplemented with 1% glycerol, which promoted alginate production, for 28 h at 37°C with shaking (170 rpm). The cells were harvested (23,000 × *g*, 30 min, 4°C), and the culture supernatant (80 ml) was heated for 30 min at 80°C to inactivate enzymes. The alginate in the supernatant was precipitated by the addition of 3 volumes of ice-cold 99% ethanol and a withish cotton-like clod of alginate was harvested with a sterile bent-glass rod and repeatedly washed (three 5-min washes) in sterile 0.9% saline with gentle shaking. Afterward, the alginate was suspended in ca. 20 ml of sterile 0.9% saline and stirred vigorously on a magnetic stirrer overnight or until a homogeneous suspension was achieved (partly purified alginate). The alginate concentration of this suspension was determined by the carbazole-borate assay (31) and adjusted to a concentration of ca. 11 mg/ml.

Preparation of inoculum for infection of mice (experiments 3 and 4). Mucoid *P. aeruginosa* NH57388A, nonmucoid *P. aeruginosa* NH57388B, or nonmucoid *P. aeruginosa* NH57388C were separately cultured in 80 ml of ox broth supplemented with 1% glycerol for 28 h at 37°C with shaking (170 rpm). The cells were harvested and resuspended in 2 ml of fresh ox broth, and the CFU were counted. Afterward, *P. aeruginosa* cells were adjusted to appropriate challenge inoculum by dilution (1:10) in the pseudomonal alginate solution (purified alginate) (see above). Controls were sterile 0.9% saline mixed with purified alginate.

The challenge inoculum of *P. aeruginosa* was established by pilot experiment to be 10^8 CFU/ml for mice with CF and 10^9 CFU/ml for BALB/c mice.

Experimental animals. Female and male homozygotic ($CFTR^{-/-}$), transgenic *Cftr*^{tmlUnc}-TgN(FABPCFTR) mice (Jackson Laboratories, Bar Harbor, Maine), referred to here as "CF mice," bred with mixed a genetic background (63) were used. For comparison, we included normal female BALB/c mice (Bomholtg(angst)ard, Bomholtgaard, Ry, Denmark), which is the standard mouse used for the seaweed alginate bead model in our laboratory (41). The CF mice were 16 to 19 weeks old, and the BALB/c mice were 12 to 14 weeks old. The CF mice and the BALB/c mice were housed under specific-pathogen-free conditions at the Royal Veterinary and Agricultural University, Copenhagen, Denmark, and at the Panum Institute, University of Copenhagen, Copenhagen, Denmark, respectively.

Mouse infection model. Before challenge, mice were anesthetized subcutaneously with 0.2 ml of a 1:1 mixture of 25% Hypnorm (Janssen-Cilag), 25% Dormicum (Roche), and 50% sterile water and then tracheotomized (26). The mice were intratracheally challenged with 40 μl of planktonic mucoid (NH57388A), nonmucoid (NH57388B), or nonmucoid (NH57388C) *P. aeruginosa* strains, resulting in ca. 4 × 10^6 CFU/lung for CF mice and ca. 4 × 10^7 CFU/lung for BALB/c mice. The challenge was performed with a bead curved needle as previously described by Johansen et al. (26). The mice were sacrificed by injection of 2 ml of 20% pentobarbital (DAK) kg^{-1}.

Macroscopic description of the lungs. The lungs were scored in situ and after removal from the thoracic cavities according to the method of Johansen et al. (26) as follows: 1, normal; 2, swollen lungs, hyperemia, small atelectasis; 3, pleural adhesion, atelectasis, multiple small abscesses; and 4, large abscesses, large atelectasis, and hemorrhages.

Lung bacteriology. Randomly selected mice were prepared for quantitative bacteriology. The whole lung of each mouse was excised aseptically and homogenized in 5 ml of sterile 0.9% saline, and 100 μl of appropriately serial diluted lung homogenates samples were plated on BAP, incubated at 37°C, and inspected for *P. aeruginosa* colonies (i.e, CFU) after 35 to 40 h.

Histopathological studies. Randomly selected mice were used for lung histopathology. The lungs were fixed in formalin buffer (4% formaldehyde [pH 7.0]; Bie & Berntsen, Copenhagen, Denmark) for at least 1 week, followed by embedding in paraffin wax, and then cut into 5-μm sections (26). Mounted sections were stained with hematoxylin and eosin (HE) combined with Alcian blue–periodic acid-Schiff stain for exopolysaccharides (69). The cellular changes were assigned to acute or chronic inflammation groups by a scoring system (27) based on the proportion of PMN and mononuclear leukocytes (MN) in the inflammatory foci. Acute inflammation affected predominantly PMN (PMN, ≥90%; MN, ≤10%), and chronic inflammation affected predominantly MN (MN, ≥90%; PMN, ≤10%). The slides were blinded for mouse genotype.

Determination of alginate content in mouse lung homogenate. Mouse lung homogenate (500 μl) was extracted with ice-cold 99% ethanol (2 ml) and resuspended in sterile 0.9% saline (500 μl). The content of uronic acid (alginate) was quantified by the carbazole-borate assay (31). Lung homogenate from mice challenged with 0.9% saline in purified alginate was used as a blank.

Histopathologic autopsy of lungs from CF patients. The histopathology of the lungs of mouse model of chronic *P. aeruginosa* infection was compared to HE-stained 5-μm sections of lungs of six CF patients. These patients (two males and four females, ranging in age from 3 to 20 years old) died from chronic *P. aeruginosa* infection in 1974 to 1975 before the intensive maintenance antibiotic therapy was introduced in the Danish CF center (64). The duration of chronic *P. aeruginosa* infection was 6 weeks to 5 years, all patients harbored mucoid strains, the number of precipitating antibodies in serum to *P. aeruginosa* was 10 to 48 (normal, 0 to 1) (22) 2 days to 2 months before death, and the immunoglobulin G concentration in serum was 14.9 to 26.1 g/liter.

Statistical analysis. The Mann-Whitney U test was used to compare the data between two groups. The categorical data were analyzed by the chi-square test.

RESULTS

Stability of mucoid and nonmucoid phenotypes. Mucoid *P. aeruginosa* NH57388A streaked on BAP maintained stable mucoidy for weeks when subcultured daily (Fig. 1a). The mucoid strain (NH57388A), the nonmucoid strain (NH57388B), and PAO1 all maintained their colony phenotypes for 1 week in liquid batch cultures. After 2 weeks of static growth, subcultures on BAP of the nonmucoid NH57388B and PAO1 showed rough phenotypes and SCV, but the nonmucoid smooth phenotype continued to dominate. Subcultures on BAP showed essentially the same results from shaken growth in tubes and flasks; however, shaken growth in flasks resulted in the death of both nonmucoid NH57388B and PAO1 strains in contrast to the mucoid NH75388A strain, which survived for 4 weeks. Neither the nonmucoid NH57388B (Fig. 1b) nor the PAO1 strain showed any mucoid colonies at any of the growth experiments.

After 2 weeks of growth in liquid medium, subcultures on BAP of the mucoid NH57388A had the appearance of mucoid,

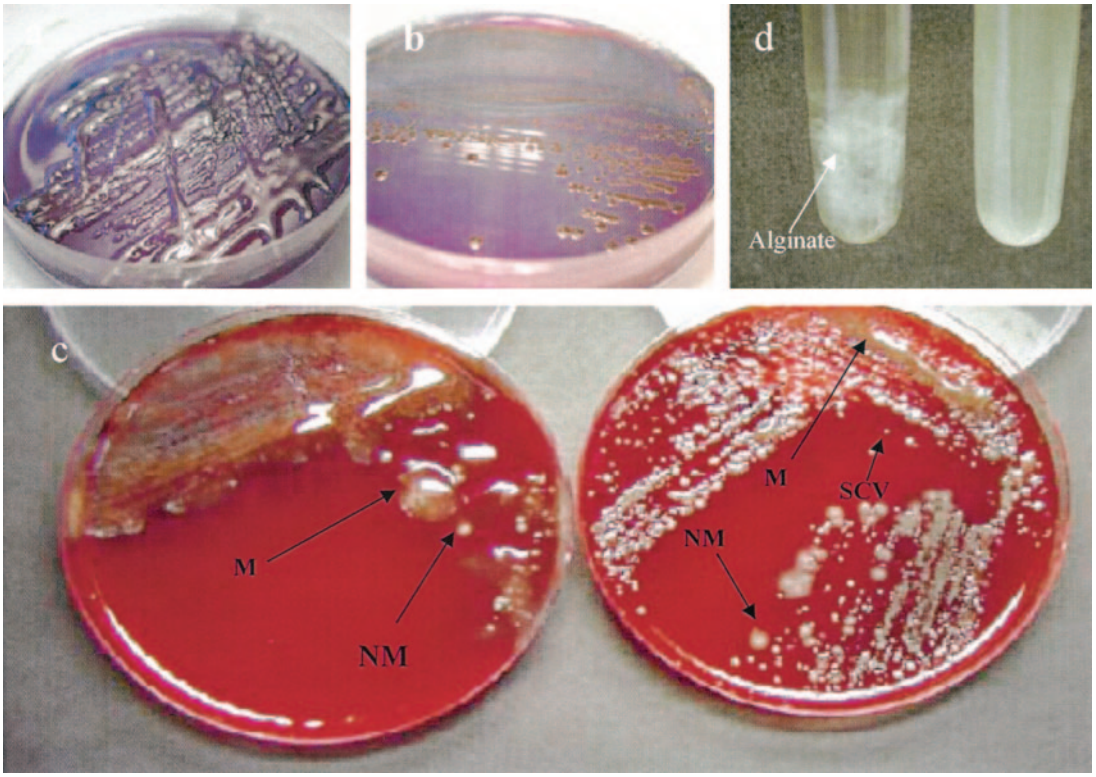

FIG. 1. Photos of a stable, mucoid *P. aeruginosa* NH57388A colonies after serial subculture on BAP (a), a stationary-shaken ox broth flask culture of nonmucoid *P. aeruginosa* NH57388B after 2 weeks (b), alcohol precipitation of alginate (white arrow) from stationary-shaken ox broth flask cultures (30 h) of mucoid *P. aeruginosa* NH57388A (left) and nonmucoid NH57388B (right) (d), and mucoid *P. aeruginosa* NH57388A after 14 days stationary-shaken (left) or static (right) aerobic culture in a tube (c). Three phenotypes are indicated by the black arrows in panel c: M, mucoid; NM, nonmucoid; and SCV.

nonmucoid (NH57388C), and SCV phenotypes. Shaking cultures showed a predominance of mucoid phenotypes (Fig. 1c, left) throughout the 4 weeks, whereas static cultures showed an increasing predominance of nonmucoid and SCV phenotypes, but some mucoid phenotypes continued to be present (Fig. 1c, right) There were no major differences between the surface, middle, and bottom cultures. Similar results were obtained at anaerobic growth.

The pulsed-field gel electrophoresis results of the different morphotypes from the three strains, respectively, showed that the mucoid, nonmucoid smooth, rough, and SCV types were identical (data not shown).

***mucA*, *algT*, *mucB*, and *mucD* sequence.** Sequencing of *mucA* in the stable mucoid NH57388A isolate and in the nonmucoid revertant NH57388C showed the deletion of a C residue at position 170 and a deletion of 105 bp starting at position 293 bp. The deletion of the C residue at position 170 generates a frameshift and a stop codon (TGA) at position 283 of the coding sequence. Sequencing of *algT* showed an AGCCC AGGC insertion at position 147 of the coding sequence leading to a frameshift in the nonmucoid revertant NH57388C. No changes in *mucB* and *mucD* were found in the nonmucoid revertant NH57388C compared to the mucoid NH57388A.

Complementation analysis. The nonmucoid *algT* mutant NH57388C spontaneously derived in vitro from ox broth cultures of the mucoid *P. aeruginosa* NH57388A could be complemented by the plasmid pCD100 containing the *algT* mucA(−)BCD operon, and thus conversion to mucoid phenotype was observed identical to that see in Fig. 1a.

LPS analysis. The nonmucoid isolates NH57388B/C had the typical ladder of smooth O polysaccharide, whereas the mucoid isolate NH57388A lacked the repeating O polysaccharide corresponding to semirough LPS (21) (data not shown).

AHL analysis. The mucoid clinical *P. aeruginosa* NH57388A isolate and its nonmucoid spontaneous segregant NH57388C produced several signal molecules. These were putatively identified as BHL (C4), HHL (C6), OOHL (oxo C8), ODHL (oxo C10), and OdDHL (oxo C12). Interestingly, no signal molecules were produced by the nonmucoid NH57388B isolate (data not shown).

Time course of alginate production. Ox broth cultures of *P. aeruginosa* were sampled and assayed for alginate production. All *P. aeruginosa* isolates had equal specific growth rates. However, a lower final maximum cell yield was found in mucoid *P. aeruginosa* NH57388A cultures (maximum OD_{600} of 2.2) relative to the nonmucoid isolates NH57388B, NH5733C, and PAO1 (maximum OD_{600}s of 3.52, 3.82, and 3.75, respectively). The production of alginate from the mucoid clinical isolate NH57388A paralleled growth from the early logarithmic phase to the stationary phase with a specific production of 2.12 mg of alginate ml^{-1} $cell^{-1}$ after 52 h (Fig. 1d, left). Virtually, no alginate could be determined from nonmucoid NH57388B (Fig. 1d, right), nonmucoid NH57388C, and PAO1cultures throughout growth cycle. Since glycerol is known to be alginate

TABLE 2. Mortality recorded after intratracheal *P. aeruginosa* challenge of CF mice and BALB/c mice with the mucoid strain +QS (NH57388A), the nonmucoid strain −QS (NH57388B), and the nonmucoid strain +QS (NH57388C)[a]

Parameter	Expt 1		Expt 2		Expt 3	Expt 4		
	CF mice (mucoid +QS NH57388A)	BALB/c mice (mucoid +QS NH57388A)	BALB/c mice (mucoid +QS NH57388A)	BALB/c mice (nonmucoid −QS NH57388B)	BALB/c mice (mucoid +QS NH57388A)	CF mice (mucoid +QS NH57388A)	CF mice (nonmucoid +QS NH57388C)	CF mice (nonmucoid −QS NH57388B)
Mortality (no. of animals dying/total no. tested) at day:								
1	1/26	0/15	1/22	1/15	1/20	0/13	2/15	1/13
2	3/26	0/15	1/22	1/15	1/20	2/13	3/15	0/13
3	4/26	0/15	4/22	1/15	1/20	3/13	2/15	1/13
4	4/26	0/15	3/22	0/15	1/20	0/13	1/15	0/13
5	5/26	0/15	2/22	0/15	0/20	1/13	1/15	0/13
6	0/26	0/15	1/22	0/15	0/20	0/13	0/15	1/13
7	0/26	0/15	0/22	0/15	0/20	0/13	0/15	0/13
Total	17/26	0/15	12/22	3/15	4/20	6/13	9/15	3/13
Mortality (%)	65***	0	55*	20	20	46	60**	23
95% Confidence interval	4–83	0–22	32–76	4–48	6–44	19–75	32–84	5–54

[a] Expt 1, inoculum of 5×10^6 CFU/lung (bacteria were instilled with crude alginate); Expt 2, inoculum of 5×10^7 CFU/lung (bacteria were instilled with crude alginate); Expt 3, inoculum of 5×10^7 CFU/lung (bacteria were instilled with purified alginate); Expt 4, inoculum of 5×10^6 CFU/ml (bacteria were instilled with purified alginate).

[b] * and **, $P < 0.05$ compared to strain NH57388B resuspended in culture supernatant (crude alginate [*]) or in purified alginate (**); ***, $P < 0.001$ compared to BALB/c mice.

promoting (70), the mucoid *P. aeruginosa* isolate NH57388A was cultured in ox broth plus 1% glycerol. Induction of additional alginate production was observed during late log phase after 15 h and early stationary phase after 25 h, resulting in a high final alginate production per cell (3.23 mg of alginate ml^{-1} $cell^{-1}$ after 52 h).

Production of QS-regulated exoproducts. When examined qualitatively, both protease and chitinase activity was detected in the mucoid NH57388A isolate, nonmucoid NH57388C, and PAO1. However, no activity was observed in the nonmucoid NH57388B isolate. Alginate production has been inversely correlated with protease activity (35). The specific elastase activity determined quantitatively in the culture supernatant of the mucoid NH57388A isolate was reduced approximately seven- and threefold compared to its isogenic nonmucoid isolate NH57388C and PAO1, respectively. The nonmucoid NH57388C isolate produced 2.3-fold more elastase than PAO1. The total specific chitinase activity of the mucoid NH57388A isolate was increased 1.2-fold compared to the PAO1 level. Elastase and chitinase activity could not be determined from the nonmucoid isolate NH57388B by the quantitative assays (data not shown).

Establishment of chronic lung infection with mucoid *P. aeruginosa* isolate NH57388A in different strains of mice. (i) Mortality and persistence of *P. aeruginosa* in the lungs of mice. We found that CF mice were more susceptible to infection with mucoid *P. aeruginosa* NH57388A (+QS) at an infectious dose of 5×10^6 CFU/lung than our standard BALB/c mice (Table 2, experiment 1). A total of 65% of the CF mice died within 7 days of infection, whereas no BALB/c mice died ($P < 0.001$). The peak mortality in CF mice occurred at between 3 and 5 days postchallenge. Of the surviving CF mice, high numbers of bacteria (6×10^8 CFU/lung; median, $P = 0.004$) were cultured from the lung 7 days postinfection demonstrating that bacterial proliferation had occurred. In contrast, nearly all BALB/c mice had cleared the mucoid bacteria (Fig. 2a). However, when the infectious dose was increased 10-fold, BALB/c mice were severely afflicted by mucoid *P. aeruginosa* lung infection (Fig. 2b).

As shown in Table 2 [experiment 2] and Fig. 2b, BALB/c mice challenged with the mucoid NH5833A isolate had a significantly higher mortality rate (55%) ($P < 0.05$) and bacterial load (3.12×10^6 CFU/lung, median) ($P = 0.003$) compared to a mortality of 20% and almost clearance of the bacteria in mice challenged with the nonmucoid NH57388B isolate. In experiments 1 and 2, *P. aeruginosa* cells resuspended in the supernatant, crude alginate, were used in the challenge inoculum. Since the culture supernatant contains virulence factors, which may influence the infection and mortality, the challenge inoculum was prepared by resuspending pelleted cells in purified alginate. The results in experiments 3 and 4 demonstrated a reduction in mortality in both CF mice and BALB/c mice ($P < 0.05$) compared to mice challenged with an inoculum made of cells resuspended in their supernatant (Table 2). The control study showed that no mice died after inoculation with cell-free culture supernatant ($n = 4$) or purified alginate diluted in 0.9% saline ($n = 4$). BALB/c mice challenged with the mucoid NH57388A isolate when the inoculum was made by cells resuspended in purified alginate showed high bacterial numbers (median, 2.5×10^6 CFU/lung) 7 days postinfection, and after 13 days bacteria could still be cultured from the lungs of the mice (Fig. 2c). Collectively, mucoidy was not lost by in vivo passage in the lungs of neither CF mice nor BALB/c mice and remained stable when plated on BAP (Fig. 3a). Interestingly, however, a few nonmucoid variants appeared among the mucoid colonies in two of six BALB/c mouse lung homogenates 13

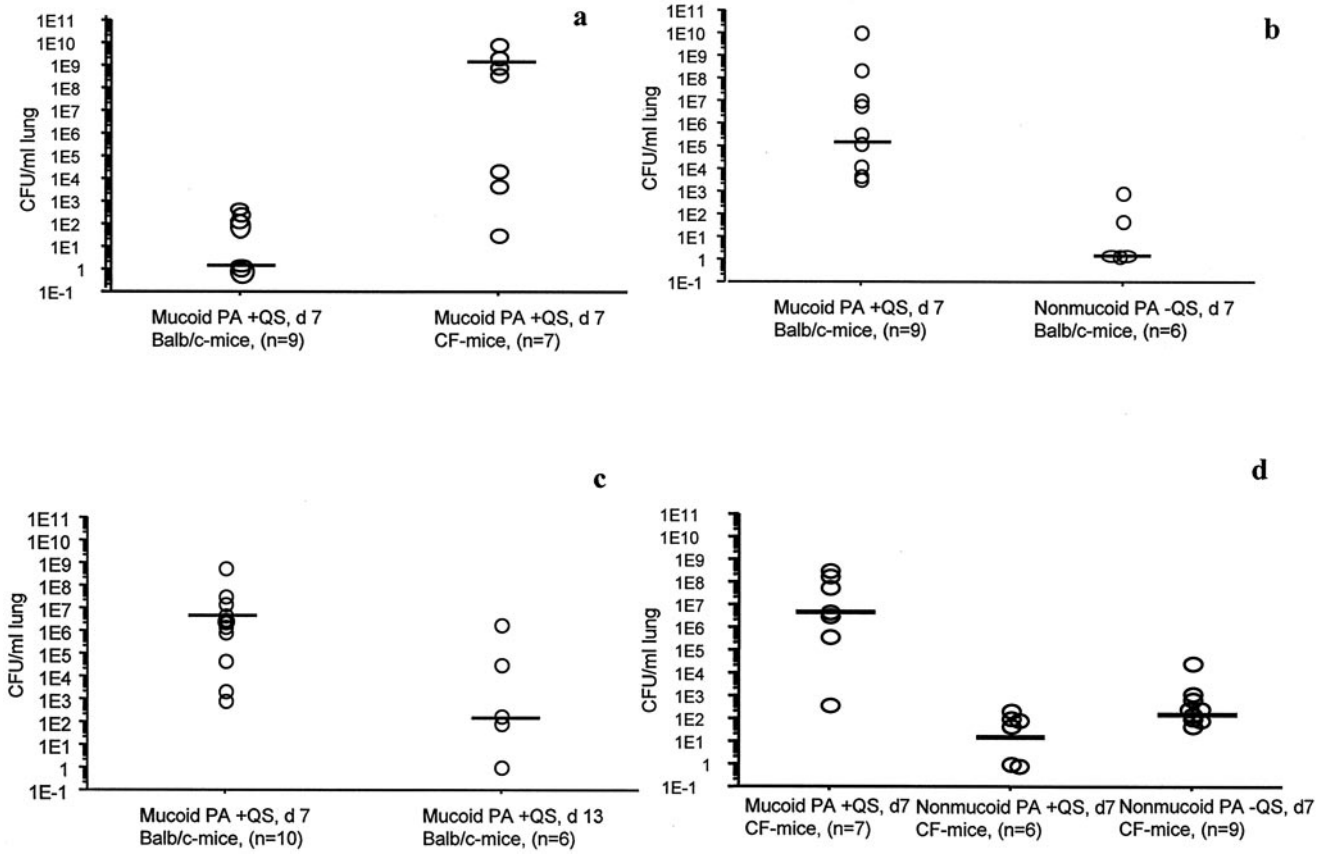

FIG. 2. (a and b) Bacterial CFU in the lung of mice after intratracheal challenge of *P. aeruginosa* resuspended in culture supernatant (crude alginate) with mucoid strain NH57388A at 5×10^6 CFU/lung in CF mice or 5×10^7 CFU/lung in BALB/c mice (a) or mucoid strain NH57388A or nonmucoid strain NH57388B at 5×10^7 CFU/lung in BALB/c mice (b). (c and d) Intratracheal challenge of *P. aeruginosa* resuspended in purified alginate with mucoid strain NH57388A at 5×10^7 CFU/lung in BALB/c mice (c) or mucoid strain NH57388A, nonmucoid strain NH57388C, or nonmucoid strain NH57388B at 5×10^6 CFU/lung in CF mice (d). Abbreviations: d, day; PA, *P. aeruginosa*; +QS, functional QS system; −QS, nonfunctional QS system.

days postinfection. These were never seen from lung homogenates plated 7 days postinfection. Infection with washed bacteria (NH57388A), 5×10^7 CFU/lung, tested in BALB/c mice ($n = 11$), led to bacterial clearance in five mice and a low concentration (median, 3.2×10^3 CFU/lung) in six mice by 7 days postinfection.

To elucidate the role of alginate and QS in the outcome of *P. aeruginosa* lung infection, CF mice were challenged with mucoid *P. aeruginosa* NH57388A (+QS), nonmucoid *P. aeruginosa* NH57388C (+QS), and nonmucoid *P. aeruginosa* NH57388B (−QS) strains.

As seen in Fig. 2d, the number of bacteria in the lung at 7 days postinfection was significantly higher ($P < 0.01$) in CF mice challenged with the mucoid isolate +QS compared to nonmucoid isolates +QS and −QS. The mortality (Table 2, experiment 4) was significantly higher ($P < 0.05$) among mice infected with the nonmucoid NH57388C +QS strain compared to mice infected with the nonmucoid strain NH57388B −QS strain, whereas the mucoid NH57388A +QS strain did not differ significantly from the two other groups ($P = 0.46$ and $P = 0.22$, respectively). Taken together, these data show that the mucoid phenotype was more resistant to host defenses than the nonmucoid phenotypes and that functional QS may contribute to the severity of the infection.

(ii) Determination of alginate content in mouse lung homogenate. Alginate could be precipitated with alcohol from the lung homogenate of CF mice challenged with mucoid *P. aeruginosa* NH57388A and quantitated to a median of 333 μg/ml (range, 195 to 946 μg/ml). Lung homogenates from mice challenged with cell-free culture supernatant or nonmucoid *P. aeruginosa* NH57388B or NH57388C (control) strains did not contain detectable alginate.

(iii) Macroscopic lung pathology. The lungs of both CF mice and BALB/c mice challenged with the mucoid *P. aeruginosa* NH57388A 5×10^6 or 5×10^7 CFU/lung, respectively, showed severe inflammation, including multiple abscesses, edema, hemorrhage, atelectasis, consolidated areas, and fibrinous adhesion to the thoracic wall. As judged by lung scoring, significantly more severe changes in lung pathology were found in CF mice challenged with the mucoid NH57388A isolate +QS compared to the nonmucoid isolates NH57388C +QS and NH57388B −QS ($P < 0.01$ and $P < 0.001$) (Table 3, experiment 4). Furthermore, BALB/c mice challenged with the mucoid NH57388A isolate +QS had significantly higher lung

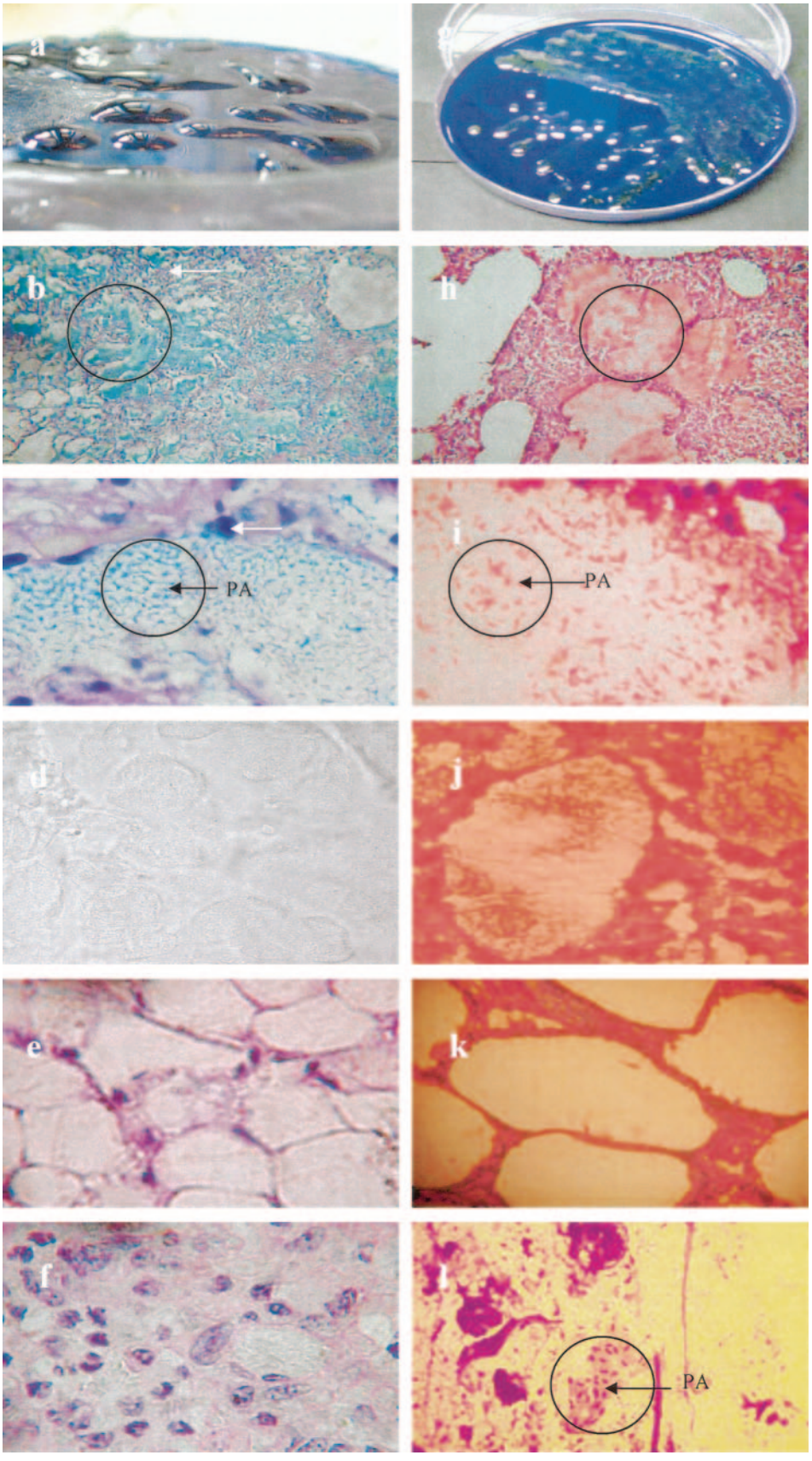
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TABLE 3. Macroscopic lung pathology after intratracheal *P. aeruginosa* challenge of CF mice and BALB/c mice with the mucoid strain +QS (NH57388A), the nonmucoid strain −QS (NH57388B), and the nonmucoid strain +QS (NH57388C)[a]

Score[c]	Pathology (no. of mice in scoring groups/total no. of mice challenged [%]) at day 7								
	Expt 1		Expt 2		Expt 3		Expt 4		
	CF mice (mucoid +QS*** NH57388A)	BALB/c mice (mucoid +QS NH57388A)	BALB/c mice (mucoid +QS**** NH57388A)	BALB/c mice (nonmucoid −QS NH57388B)	BALB/c mice (mucoid +QS* NH57388A)	BALB/c mice (mucoid +QS NH57388A)[b]	CF mice (mucoid +QS NH57388A)	CF mice (nonmucoid +QS** NH57388C)	CF mice (nonmucoid −QS***** NH57388B)
1		1/15 (7)		5/12 (42)					1/9 (11)
2		10/15 (67)		7/12 (58)		2/6 (33)	1/7 (14)	6/6 (100)	8/9 (89)
3	2/9 (22)	3/15 (20)	4/10 (40)		8/10 (80)	2/6 (33)	2/7 (29)		
4	7/9 (78)	1/15 (7)	6/10 (60)		2/10 (20)	2/6 (33)	4/7 (57)		

[a] See Table 2, footnote *a.* Asterisks indicate probabilities as follows: *, $P < 0.01$ compared to day 13; **, $P < 0.01$ compared to the mucoid strain NH57388A +QS; ***, $P < 0.001$ compared to BALB/c mice; ****, $P < 0.001$ compared to the nonmucoid strain NH57388B −QS; *****, $P < 0.001$ compared to the mucoid strain NH57388A +QS.

[b] Day 13, in this case.

[c] Score 1, normal; 2, swollen lungs, hyperemia, small atelectasis; 3, pleural adhesion, atelectasis, multiple small abscesses; 4, large abscesses, large atelectasis, and hemorrhages.

scores than mice infected with nonmucoid NH57388B isolate −QS ($P < 0.001$) and more severe lung pathology at day 7 compared to day 13 postinfection ($P < 0.01$) (Table 3, experiments 2 and 3). Mice infected with cell-free culture supernatant (crude alginate) or purified alginate showed areas of consolidation possibly due to extracellular virulence factors present in the supernatant.

(iv) Histopathology. Histological examination of lungs from CF mice and BALB/c mice 7 days postinfection challenged with the mucoid *P. aeruginosa* NH57388A strain at 5×10^6 or 5×10^7 CFU/lung, respectively, had an extensive focal endobronchial and alveolar *P. aeruginosa* infection (Fig. 3b to d) with a pronounced PMN infiltration. *P. aeruginosa* was presented in the alveoli as microcolonies of bacteria encapsulated in a matrix stained blue for polysaccharides (alginate) with Alcian blue (Fig. 3b and c). In contrast, BALB/c mice (Fig. 3e) and CF mice challenged with saline in purified alginate or the nonmucoid isolates NH57388B (Fig. 3f) or NH57388C showed signs of restoration of the lung tissue dominated by weak cellular infiltration on day 7 postinfection. Furthermore, staining of lung tissue from these mice with Alcian blue, resulted in no blue color, indicating absence of detectable alginate (Fig. 3e and f).

(v) Histopathologic autopsy of lungs from a CF patient. The characteristic macroscopic appearance of mucoid *P. aeruginosa* from bronchoalveolar lavage from a CF patient is shown in Fig. 3g. The histopathology showed that the conducting zone was filled with exudate and the respiratory zone was scattered by foci of extensive infiltration of PMN surrounded by few *P. aeruginosa* biofilms within the alveoli and alveolar ducts(Fig. 3 h to j). An alveolus without infection is shown in Fig. 3k. The microscopic appearance of the bacteria in these biofilms was similar to the biofilm seen in sputum from a CF patient with microcolonies of mucoid *P. aeruginosa* (Fig. 3l).

DISCUSSION

In this study we have established a new mouse model of chronic *P. aeruginosa* lung infection based on a stable mucoid clinical *P. aeruginosa* isolate NH57388A with hyperproduction of alginate without the use of artificial embedding agents. We have also shown that *P. aeruginosa* expressing QS may contribute to the severity of the lung inflammation and higher mortality (Table 2). Our model is superior to previously developed pulmonary models since the number of bacteria cultured from the lungs of the mice remained high throughout the infection period and could be established in both CF mice and normal BALB/c mice without embedding of the bacteria. Furthermore, the histopathologic features were similar to those of chronic *P. aeruginosa* lung infections in humans. Similar to our study, other studies have shown a proliferation of *P. aeruginosa* in the lungs of CF mice (66) and normal mice (39, 62) and an increase in mortality. However, these studies used agar or agarose-entrapped *P. aeruginosa* for the chronic infection. Recently, Coleman et al. (6) were able to produce a chronic *P. aeruginosa* colonization in the oropharynxes of wild-type, heterozygous, and homozygous CF mice infected with *P. aeruginosa* added to the drinking water, although lung infection was

FIG. 3. Photographs showing mucoid *P. aeruginosa* NH57388A isolated from a CF mouse lung 7 days postinfection. (a) Sections of mouse lung tissue at 7 days postinfection stained with HE and Alcian blue for detection of alginate. Mucoid *P. aeruginosa* NH57388A-infected CF mouse lung showing large *P. aeruginosa* biofilms (in black circles) encapsulated in alginate (blue color) in the alveolar space surrounded by PMN (white arrow) at ×40 (b) and ×100 (c) magnifications; scanning confocal photomicrographs of freeze microtomy sections of CF mouse lung tissue (40 μm) showing alveolus sacs filled with mucoid *P. aeruginosa* NH57388A biofilms (d) and lung tissues from a CF mouse challenged with saline (control) (e) and from a BALB/c mouse challenged with nonmucoid *P. aeruginosa* NH57388B (f). No alginate is seen. HE and Alcian blue stain were used in panel f. Mucoid *P. aeruginosa* microcolonies from bronchoalveolar lavage from a CF patient with chronic *P. aeruginosa* lung infection (g) and autopsy of a female CF patient who died from chronic *P. aeruginosa* lung infection showing alveoli sacs filled with *P. aeruginosa* biofilms (black arrow) at magnifications of ×100 (h), ×1,000 (i), and ×250 (j) with HE and Gram stain. Alveoli without infection (magnification, ×250; HE stain) (k) and sputum with a mucoid biofilm of *P. aeruginosa* (in black circle) from a CF patient (magnification, ×1,000; Gram stain) (l) are also shown.

established only in few homozygous CF mice and with a low bacterial load.

The liquid batch culture experiments showed that the mucoid *P. aeruginosa* NH57388A was unstable, especially under static growth conditions in which a nonmucoid revertant (NH57388C) appeared together with SCV. This is in accordance with the experiments of Wyckoff et al. (73) and similar to experiments by Rainey and Travisano (54) and Spiers et al. (61). Our nonmucoid revertant (NH57388C) had mutations in *algT* and could be complemented by plasmid pCD100 containing the *algT*mucA(−)BCD operon in agreement with a previous study (A. Heydorn, unpublished results) in which an *algT* mutant derived from the mucoid *P. aeruginosa* strain PDO300 (35) resulted in a mucoid phenotype by complementation with pCD100, thus supporting the idea that *algT* genes regulate the process of alginate conversion (8). The instability of mucoid *P. aeruginosa* is characteristic for CF strains and occurs especially under anaerobic conditions (8), which are found in sputum in the conductive airways (71). The in vitro results are in accordance with the results of the presented mouse model in which the mucoid phenotype splits off nonmucoid variants after 2 weeks in the lungs.

The mechanism of alginate hyperproduction in the clinical mucoid isolate NH57388A of *P. aeruginosa* was due to alteration of the *mucA* gene. It has previously been shown that *mucA* is the preferential site for conversion to mucoidy in vivo (3). The mucoid isolate +QS (NH57388A) could establish a persistent lung infection in mice, most likely due to hyperproduction of alginate (Fig. 2 and 3b to d) since mice infected with the nonmucoid isolates +QS and −QS (NH5733C and NH57388B) almost cleared the lung infection (Fig. 2b and d and 3f). The possibility that the differences in lung bacteriology and pathology between mucoid NH57388A and nonmucoid NH57388C infections is due to a mutation at a second site in NH57388C seems unlikely since complementation of the *algT* mutation in isolate NH57388C restored mucoidy. Taken together, this indicates that the production of alginate may confer a selective survival advantage for the mucoid isolate. Boucher et al. (3), Yu et al. (74), and Song et al. (60) also reported improved persistence in mice lungs of mucoid *P. aeruginosa* strains relative to nonmucoid strains. However, their mice were already sacrificed 4, 18, and 48 h, postinfection, respectively. In contrast, another animal study (2) comparing mucoid and nonmucoid *P. aeruginosa* strains failed to confer mucoidy as a selective advantage. This discrepancy may reflect differences in the virulence of the *P. aeruginosa* strains used and/or the genetic background of the animals (39, 67).

We found that CF mice were more sensitive to *P. aeruginosa* lung infection than BALB/c mice; however, since the genetic background of the mice and the biofilm model may influence the sensitivity of *P. aeruginosa* (39, 41), we cannot conclude that the difference is only due to the CFTR mutation in the CF mice. Hence, the BALB/c mice were concluded to be resistant compared to C57BL/6 mice in the agar-bead model of *P. aeruginosa* lung infection (13, 39). Previously work from our group has shown that the BALB/c mice were found to be relatively susceptible compared to C3H/HeN mouse in the seaweed alginate-bead model (40).

P. aeruginosa secretes several proteases that play a major role in pathogenesis, especially during acute infections (29). These proteases, e.g., elastase, have been inversely correlated with alginate production (35, 45). The higher mortality seen in mice challenged with the nonmucoid NH57388C isolate +QS compared to the isogenic mucoid NH57388A isolate +QS may be due to the ability of this isolate (NH57388C) to produce higher amounts of elastase (sevenfold) and probably other QS-regulated virulence factors such as exotoxin A.

Alginate from *P. aeruginosa* is a linear polymer of D-mannuronate and L-guluronate, which are highly soluble in water and chemically very similar to seaweed alginate (33); however, important differences exist. Pseudomonal alginate produces a more elastic biofilm (57), which may adapt more efficiently in the lungs of the mice. In addition, the alginate is O acetylated (12), which plays an important role in the ability of the polymer to act as a virulence factor (17, 32, 44, 52, 53). In our study, we could quantitate alginate from the mice lung with a median value of 333 μg/ml. In comparison, Pedersen et al. (51) found that the median concentration of alginate in sputum samples from CF patients was 40 μg/ml (range, 1 to 100 μg/ml).

Histological examination of our mouse lung sections showed alveolar sacs invaded with *P. aeruginosa* biofilm anchored in a protective alginate matrix surrounded by numerous PMN. Despite this significant PMN infiltration, it did not appear to influence bacterial clearance (Fig. 3b to d). This finding supports in vitro studies showing that alginate protects bacteria from the host immune system (32, 35, 51, 58). The lungs of the CF mice were characterized by foci of *P. aeruginosa* biofilms in the alveoli and alveolar ducts of the respiratory zone (Fig. 3b to d). Our findings are similar to those seen with CF patients (Fig. 3 h to j), as previously described by Baltimore et al. (1) and Tiddens (65). The bacteria in our mouse model were capable of spreading to the alveoli, which is more difficult to achieve when agar, agarose, and seaweed alginate beads are used. Hence, a mouse experiment with *P. aeruginosa* immobilized in seaweed alginate beads showed that beads can cause mechanical blocking and damage the bronchi (72). In addition, since the alveolar space is associated with volume and shape changes during breathing, the adaptation of a viscoelastic biofilm structure provided by *P. aeruginosa* native alginate might be advantageous compared to a more rigid seaweed alginate gel.

We thus present here a new animal model of chronic *P. aeruginosa* lung infection that may be more useful for reliable studies of the interactions between mucoid *P. aeruginosa* and the host defense mechanisms, as well as for studies of new therapeutic interventions for this disease.

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