Mucoid *Pseudomonas aeruginosa* in Cystic Fibrosis: Characterization of *muc* Mutations in Clinical Isolates and Analysis of Clearance in a Mouse Model of Respiratory Infection

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A distinguishing feature of *Pseudomonas aeruginosa* isolates from cystic fibrosis (CF) patients is their mucoid, exopolysaccharide alginate-overproducing phenotype. One mechanism of conversion to mucoidy is based on mutations in the *algU* *mucABCD* cluster, encoding the stress σ factor AlgU and its regulators. However, conversion to mucoidy in laboratory strains can be achieved via mutations in other chromosomal sites. Here, we investigated mechanisms of the emergence of mucoid *P. aeruginosa* in CF by analyzing the status of *mucA* in a collection of mucoid *P. aeruginosa* isolates from 53 CF patients. This negative regulator of *algU*, when inactivated under laboratory conditions, causes conversion to mucoidy. The overall frequency of *mucA* alterations in mucoid CF isolates was 84%. Nucleotide sequence analyses revealed that the majority of the alterations caused premature termination of the *mucA* coding sequence. Comparison of paired nonmucoid and mucoid *P. aeruginosa* isolates from three CF patients indicated the presence of *mucA* mutations only in the mucoid strains. Interestingly, mucoid *P. aeruginosa* isolates from urinary tract infections also had mutations in the *mucA* gene. Clearance of CF isolates from the murine lung was investigated in an aerosol infection model with C57BL/6J, BALB/c, and DBA/2NHsd mice. Two CF strains, selected for further study based on the dependence of their alginate production on the concentration of salt in the medium, were used to examine the effects of mucoidy on pulmonary clearance. Statistically significant improvement in recovery from the murine lung of viable mucoid *P. aeruginosa* cells relative to the nonmucoid bacteria was observed in the majority of mouse strains tested. Collectively, the results reported here suggest that *mucA* is most likely the preferential site for conversion to mucoidy in CF and that alginate overproduction in *mucA*-mutant *P. aeruginosa* improves its resistance to the innate clearance mechanisms in the lung.

Pseudomonas aeruginosa is an opportunistic pathogen causing chronic respiratory infections in cystic fibrosis (CF) patients (14). *P. aeruginosa* is also a common agent of urinary tract infections (UTIs) and can cause fatal infections in burned or neutropenic patients (2). The most prominent feature of *P. aeruginosa* infecting CF patients is its conversion into the mucoid, exopolysaccharide alginate-overproducing phenotype (14). This phenomenon has been associated with the establishment of the chronic phase of respiratory infections in CF (30). Based on in vitro analyses, overproduction of alginate by *P. aeruginosa* is believed to have several effects, including inhibition of phagocytosis (36, 39), protection from reactive oxygen intermediates (22, 37), and a number of biofilm-related phenomena (21). However, the comprehensive role of mucoidy in pathogenesis has not been defined. Earlier clearance studies with animal models have failed to confirm unequivocally the possibility that mucoid strains have a selective advantage in the respiratory tract (1, 41). Nevertheless, significant attention has been given to the elucidation of the genetic mechanism of conversion to mucoidy (44). In a series of recent studies (5, 24, 25), such processes have been delineated at the molecular level with laboratory strains and have been tested with only a limited number of CF isolates.

Based on classical genetic studies by Govan and colleagues (12), several mutations (termed *muc*) responsible for sponta-

neous conversion to mucoidy in laboratory strains have been mapped to at least two loci on the *P. aeruginosa* chromosome (Fig. 1A). One such site (represented by the *muc-23* mutation [Fig. 1A]) has been mapped between *hisI* at 69 min and *proB* at 71 min, while the other major locus (represented by the *muc-22* mutation) has been found to be cotransducible with the *pruAB* marker at 67.5 min (11). More recent analyses have permitted a detailed characterization of the latter site, which consists of a cluster of five genes, *algU* *mucABCD* (Fig. 1B) (5, 23, 25). The first gene in this cluster, *algU* (alternative name, *algT* [7]), encodes the *P. aeruginosa* equivalent of the extreme heat shock sigma factor σ^E in enteric bacteria (45) and appears to be the principal positive regulator of mucoidy. The downstream genes, *mucABCD*, have been shown to negatively regulate AlgU activity (35); individual inactivation of *mucA*, *mucB*, or *mucD* in laboratory strains causes overt conversion to mucoidy (5, 24, 25), while *mucC* has only mild effects (4).

Spontaneous mutations in the *mucA* gene have been found upon conversion to the mucoid phenotype under laboratory conditions. Thorough genetic investigations have demonstrated that *mucA* alterations were responsible for the mucoid status in a subset of laboratory derivatives (25). However, not all model strains of *P. aeruginosa* investigated thus far carry mutations in *mucA*, suggesting the existence of alternative pathways of conversion to mucoidy. For example, point mutations in *mucA* have been found in the few mucoid *P. aeruginosa* strains from CF patients examined to date (25), but only a fraction of spontaneous mucoid *P. aeruginosa* isolates obtained in the laboratory carry mutations in *mucA*. This has cast some doubt on how important *mucA* is in CF, since the frequency of

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FIG. 1. Genetic loci controlling conversion to mucoidy in *P. aeruginosa* and the role of *mucA*. (A) The *algU mucABCD* cluster (linked to *pruAB* at 67.5 min) encodes the extreme stress sigma factor AlgU (Pa σ^E [AlgT]) and its negative regulators. This cluster is the site of one class of *muc* mutations (5, 10, 12, 25). Another major site is represented by the *muc-23* mutation mapping between *hisI* at 69 min and *proB* at 71 min (11). (B) The open square and solid or shaded circles indicate positive and negative roles, respectively, of the genes within the *algU mucABCD* cluster in the expression of the mucoid phenotype. Inactivation of *algU* results in loss of mucoidy. Inactivation of *mucA*, *mucB*, or *mucD* causes conversion to a mucoid phenotype in laboratory strains. Spontaneous mutations in *mucA* are seen in a subset of laboratory-derived mucoid strains (5, 25) and in the majority of mucoid CF isolates (this work). Inactivation of *mucC* (shaded circle) has no overt phenotype, except in combination with other *muc* mutations when it augments their effects (4). Half arrows show oligonucleotides used to generate fragments corresponding to the regions of *mucA* used for SSCP analysis (I, II, III, and IV); numbers are relative to the previously established nomenclature (25). (C) Model depicting how mutations in *mucA*⁺ cause conversion to mucoidy in CF strains. In wild-type nonmucoid cells and under normal physiological conditions, AlgU is inhibited by MucA (35). Inactivation of *mucA* in CF isolates releases AlgU, leading to increased expression of the response regulator *algR*. AlgU and AlgR cooperate to activate the key alginate biosynthetic gene *algD*, causing the mucoid phenotype.

mucA mutations in mucoid strains including CF isolates remained unknown. In an effort to define the most common genetic alterations causing conversion to mucoidy in *P. aeruginosa* infecting CF patients, we initiated investigations with a collection of fresh CF isolates. Employing single-strand conformation polymorphism (SSCP) and automated DNA sequencing analyses, we determined here that the mutations in the *mucA* gene are found in the majority (84%) of mucoid isolates from CF patients. These findings suggest that *mucA* is the preferential site for conversion to mucoidy in vivo. We also initiated investigations of the role of conversion to mucoidy in an aerosol infection mouse model by using *P. aeruginosa* CF isolates and laboratory strains with characterized *mucA* mutations. We report here a differential clearance from murine lung of mucoid and nonmucoid strains, indicating improved survival of alginate-producing strains in the respiratory tract.

MATERIALS AND METHODS

Bacterial strains. Strains of *P. aeruginosa* originated from 53 CF patients from two geographic locations and represent independent isolates as determined by pyocin typing (unless indicated otherwise). Isolates CF52 to CF55 were obtained from one patient. All laboratory strains of *P. aeruginosa* used in this study were

derivatives of the nonmucoid strain PAO381 (11). PAO578I carries *mucA22* and constitutively produces alginate (25). PAO578II carries *mucA22* and displays a medium-dependent mucoid phenotype due to the second site suppressor mutation *sup-2* that occurred in the PAO578I background (5, 34). PAO6888 is an *algD::Gm^r* derivative of PAO578II constructed by chromosomal gene replacements.

Media and growth conditions. *Escherichia coli* was grown on Luria broth (LB) supplemented with kanamycin (25 μ g/ml) and gentamicin (10 μ g/ml) as required. *P. aeruginosa* was grown on *Pseudomonas* isolation agar (PIA; Difco) or in LB supplemented with NaCl (60, 100, or 300 mM), gentamicin (150 μ g/ml), carbenicillin (400 μ g/ml), or 1 mM isopropylthio- β -D-galactopyranoside (IPTG) as required. All incubations, including those for aerosolization, were carried out at 37°C. For *P. aeruginosa* aerosol studies, bacteria were grown for 12 h and centrifuged at 3,000 $\times$ g. The pellet was resuspended in 5 ml of cold 10 mM phosphate-buffered saline (pH 7.4) plus 1% Protease Peptone. The stock concentration was determined by plating, with the remaining 5 ml used for aerosolization. For SSCP assays, overnight cultures of CF isolates were centrifuged at 3,000 $\times$ g and washed twice in cold phosphate-buffered saline, and chromosomal DNA was isolated with cetyltrimethylammonium bromide.

SSCP and automated DNA sequence analysis. SSCP detects mutations based on conformational differences between wild-type and mutant sequences. This is accomplished by denaturing relatively short DNA fragments followed by refolding of single-stranded DNA and analysis of conformational isomers by electrophoresis on native gels. To perform the SSCP analysis, the *mucA* gene was subdivided into the four arbitrary segments, I to IV. PCR fragments for each region were generated with the following ³²P-radiolabeled oligonucleotides: I, UL22 (5'-AGCCTGACACAGCGGC3') and UR17 (5'-TCGTCGCCAGGGCAG3'); II, UL23 (5'-GCGCGGTCCGTCATGC3') and UR33 (5'-CAGGGCGTCGTTCTGG3'); III, UL8 (5'-CGGTGACCCCTGGCGT3') and UR18 (5'-CGGGTATCGCTGGACG3'); and IV, UL20 (5'-CGTGCTGGCCGGCTAC3') and UR34 (5'-CAGGGAGGTGGTGCGC3'). The PCR products were diluted 1:50 in 0.1% sodium dodecyl sulfate–10 mM EDTA (pH 8.0) and then 1:2 in denaturing dye (48% formamide, 25% [wt/vol] bromophenol blue, 25% [wt/vol] xylene cyanol, 10 mM EDTA [pH 8.0]). Samples were heated to 80°C for 5 min, with 2 μ l immediately loaded on a 0.5 $\times$ MDE gel (FMC). Gels were electrophoresed (6 W) at room temperature in 0.6 $\times$ TBE (100 mM Tris, 100 mM boric acid, 2 mM EDTA [pH 8.0]). Radiolabeled bands were detected by autoradiography at -70°C. Standard automated DNA sequence technology was used to determine the alterations in *mucA*.

Gene replacements and genetic complementation. pHYDGX, a suicide plasmid used for inactivation of *algD* on the chromosome, was constructed by placing the 11-kb *Hind*III fragment from pVD2X (6) into the *Hind*III site of pCMobB (27). The *Xho*I *xylE* gene fragment was replaced with the *Gm^r* *Xho*I fragment from pK111G (16). The homologous recombination between the insert of the plasmid pHYDGX and the chromosome was carried out as previously described (5). Triparental conjugation with pRK2013 as the helper plasmid was used to introduce pHYDGX into PAO578II, and *Gm^r* exconjugants were selected on PIA supplemented with gentamicin. *Gm^r* Cb^r recombinants were identified by replica plating. Gene replacements were confirmed by Southern blot analysis. The plasmids pVDtac24 and *ptac-mucA*⁺ and complementation of the chromosomal *mucA* alleles have been previously described (25).

Animal aerosol infection model. Three mouse strains, BALB/c (Jackson Laboratory, Bar Harbor, Maine), C57BL/6J (Jackson Laboratory), and DBA/2NHSd (Harlan-Sprague Dawley, Indianapolis, Ind.), were used. Mice (8 weeks old) were exposed to *P. aeruginosa* aerosols in groups of five to eight animals in an inhalation exposure system (Glas-Col) for 30 min as described elsewhere (43). Following nebulization of bacteria, cloud decay for 15 min, and a subsequent 15-min decontamination by UV irradiation, mice were removed from the inhalation chamber. The animals were euthanized with CO₂ either immediately for determination of the initial deposition of bacteria or 4 h postinfection. *P. aeruginosa* CFU were determined in lung homogenates by plating.

Alginate and TNF- α assays. Determination of uronic acid (alginate) was performed as previously described (19). Levels of tumor necrosis factor alpha (TNF- α) in lung tissue homogenate were determined 4 h postexposure by the Quantikine M mouse TNF- α immunoassay (R&D Systems).

Statistical analysis. SuperANOVA (analysis of variance) software (version 1.11; Abacus Concepts) was used to perform all statistical analyses (analysis of variance and Fisher's protected least-significant-difference post hoc test).

RESULTS

Mutations in the *mucA* gene in mucoid *P. aeruginosa* isolates from CF patients. To determine the frequency of mutations occurring in the *mucA* gene among mucoid *P. aeruginosa* isolates from CF patients, a collection of 55 CF strains was examined. The majority (53 strains) were isolated from different CF patients. All strains were characterized by pyocin typing, and only those isolates that differed by this criterion (unless specified otherwise) were included in further study. The *mucA*

FIG. 2. Representative sample of SSCP analyses of the *mucA* gene from clinical mucoid *P. aeruginosa* isolates. The strains were initially screened for SSCP differences in region III (C), in which the majority of the observed alterations were found. Strains not displaying changes in region III were further systematically analyzed for potential alterations in regions I, II, and IV (A, B, and D, respectively). The PAO1 pattern is shown as a standard for each region. Two bands were detected by SSCP in CF16 (carrying a 42-bp deletion); only the top band is shown in the section of the gel displayed here. With the exceptions indicated in the text and Table 1 (e.g., strain CF40), isolates carrying mutations in region III were not routinely investigated for additional changes in other regions of *mucA*.

sequence was subdivided into four arbitrary segments suitable for SSCP analysis (regions I, II, III, and IV) (Fig. 1B). Based on SSCP analysis (Fig. 2), the overall frequency of *mucA* mutations in CF isolates was 84%. The majority of altered SSCP patterns (77%) were found (Fig. 2C) within the third quarter of the *mucA* sequence (Fig. 2B, region III). Additional alterations (7%) were found in other segments (regions I, II, and IV) (Fig. 1B and 2A, B, and C). DNA sequencing analysis (Fig. 3) was carried out on a subset of 41 strains which showed representative *mucA* alterations by SSCP. The most frequently observed *mucA* mutations fell into two categories: frameshifts and C-to-T transitions which resulted in stop codons (Table 1). The most common mutation (observed in 25% of the strains carrying *mucA* mutations (Fig. 3 and Table 1) was a deletion of a G within the homopolymeric stretch of five G residues located between positions 429 and 433 of the *mucA* coding sequence (436 and 440 relative to the ribosomal binding site of *mucA* [Fig. 3]) and was designated *mucA*ΔG440 (residue 440 chosen arbitrarily) according to the previously established nomenclature (25, 44). Interestingly, this mutation has also been identified in mucoid laboratory strains and is identical to

*mucA*Δ22, one of the first mapped (12) and characterized (25) *muc* alleles at 67.5 min. Homopolymeric runs of nucleotides have been identified as the basis for frameshift mutations in some phase variation systems (17), and the relatively high frequency of *mucA*ΔG440 alleles among mucoid CF isolates may reflect similar processes. Another frequently encountered alteration was a C-to-T transition at position 362 (Fig. 3) generating a stop codon in place of a glutamine codon. In addition, several less-prominent clusterings of mutations were observed (Fig. 3). Since the vast majority of the characterized alleles in CF isolates represented truncated versions of *mucA*, it was concluded that these changes were most likely not polymorphisms but mutations associated with the mucoid phenotype of the CF strains. This notion is supported by previously published studies with allelic replacements (with the most typical allele, *mucA*ΔG440) in laboratory strains, which have demonstrated that a loss of *mucA* function causes conversion to mucoidy (25). To experimentally verify this conclusion, a selection of CF strains with *mucA* alleles characterized in this work, CF60 (the 5'-most mutation), CF40 (carrying an insertion of a T at position 295 and the *mucA*ΔG440 mutation), and CF53

FIG. 3. Mutations in the *mucA* gene from mucoid clinical isolates of *P. aeruginosa*. Shown is the *mucA* sequence of PAO1; differences observed in the clinical strains are indicated as follows: CF, mucoid isolates from CF patients; UT, mucoid urinary tract isolates; boldface letters, insertions, deletions, or substitutions of indicated nucleotides; inverted triangles, insertions; overscored nucleotides, positions of single nucleotide deletions (for *mucA*ΔG440 all five G residues are overscored because of the ambiguity); Δ, frameshifts; ΔStart and ΔStop, beginnings and ends, respectively, of large deletions; underlined triplets, stop codons brought into frame via upstream frameshift mutations (TGA₂₉₄, TGA₃₉₆, TGA₄₅₀, and TGA₄₈₇ [also see Table 1]) or resulting from C-to-T transitions; arrow underneath the sequence, 8-bp duplication in CF42 (identical to the duplication previously seen in the laboratory strain PAO568 [25]); and overhead arrows, long repeats demarcating the ends of the deletion in CF16.

TABLE 1. Mutations in the *mucA* gene of mucoid *P. aeruginosa* CF isolates^a

Strain and alteration	<i>mucA</i> mutation ^b	Stop ^c or change ^d
Frameshifts		
-1		
CF1	ΔA at 371	TGA at 396
CF4	ΔA at 253	TGA at 294
CF5	ΔG at 250	TGA at 294
CF14	ΔG at 440	TGA at 450
CF15	ΔG at 440	TGA at 450
CF22	ΔG at 440	TGA at 450
CF23	ΔG at 440	TGA at 450
CF26	ΔG at 440	TGA at 450
CF31	ΔG at 440	TGA at 450
CF41	ΔA at 253	TGA at 294
CF45	ΔG at 440	TGA at 450
CF51	ΔG at 440	TGA at 450
CF55	ΔG at 440	TGA at 450
+1		
CF9	Insertion of a T at 407	TGA at 487
CF19	Insertion of a C at 295	TGA at 487
CF33	Insertion of a T at 471	TGA at 487
CF37	Insertion of a T at 262	TGA at 487
CF59	Insertion of a G at 167	TGA at 487
+2		
CF21	Insertion of a GG at 265	TGA at 294
CF42	Insertion of 8 bp at 440	TGA at 450
C→T transitions		
CF2	C→T at 362	TAG at 362
CF3	C→T at 359	TAA at 359
CF6	C→T at 377	TAG at 377
CF8	C→T at 362	TAG at 362
CF13	C→T at 362	TAG at 362
CF20	C→T at 446	TAG at 446
CF25	C→T at 362	TAG at 362
CF28	C→T at 359	TAA at 359
CF29	C→T at 377	TAG at 377
CF34	C→T at 377	TAG at 377
CF43	C→T at 434	TAG at 434
CF46	C→T at 362	TAG at 362
CF52	G→T at 431	TAG at 431
Deletions		
CF16	Δ 42 bp at 352	In frame
UT49	Δ 13 bp at 182	TGA at 293
UT50	Δ 74 bp at 352	TGA at 487
Other		
CF40	Insertion of T at 295, ΔG at 440	V ₉₅ /A ₁₄₄ ^e
CF53	C→T at 560	P184S
CF57	C→T at 362	TAG at 362
	(G→T at 431)	TAG at 431
CF58	G→T at 379	Q123H
	Insertion of C at 249, ΔA at 253	K80N
	C→T at 279	A90V
	C→T at 357	A116V
CF60	G→T at 32	TAA at 32

^a CF1, CF8, CF14, and CF23 have been reported previously (23). Based on SSCP patterns, strains CF10, CF17, CF38, CF39, and CF44 appeared identical to PAO578; CF36 and CF37 appeared identical to CF25; and CF11 appeared identical to CF9. SSCP patterns of strains CF12, CF18, CF24, CF27, CF30, CF32, CF47, and CF54 gave no indications of a change in *mucA*. These strains were not subjected to sequence analysis.

^b ΔG at 440 is an arbitrary choice of a G residue within the homopolymeric stretch of five G's from 436 to 440. An entry in parentheses represents the second (downstream) nonsense mutation.

^c Termination codon generated at the site of the alteration or placed in frame because of a frameshift.

^d Change relative to the PAO1 sequence of *mucA*.

^e Replacement of coding sequence between V₉₅ and A₁₄₄ due to insertion of T₂₉₅ and ΔG440.

(the 3'-most mutation, which is also one of the rare missense changes observed in *mucA*), were subjected to genetic complementation by plasmid-borne *mucA*⁺. All strains tested displayed a nonmucoid phenotype when complemented by *mucA*⁺, although some variability in dependence of the complementation on induction of *ptac-mucA*⁺ by IPTG was observed, possibly reflecting differences between the mutant *mucA* alleles tested (Table 2). These findings are in keeping with the previously reported gene replacements on the chromosome of laboratory strains (5, 25) and strongly suggest that *mucA* is the site causing conversion to mucoidy in the CF isolates tested. Collectively, these observations strongly support the conclusion that *mucA* is the predominant site for *P. aeruginosa* conversion to mucoidy in CF.

Analysis of paired mucoid and nonmucoid *P. aeruginosa* strains from CF patients. Establishment of the chronic infection phase in patients with CF coincides with the conversion of *P. aeruginosa* to the mucoid phenotype (20, 30). Since coexisting mucoid and nonmucoid strains can be found in some patients, it was of interest to compare mucoid and nonmucoid strains isolated from the same lung. For this analysis, three pairs of CF isolates obtained from three different patients were characterized as having indistinguishable pyocin patterns when the paired mucoid and nonmucoid strains were compared. For all three paired strains, the mucoid *P. aeruginosa* CF isolate had an apparent region III SSCP pattern change whereas its nonmucoid counterpart did not have such a change (Fig. 4A). The *mucA* region III from the mucoid isolates CF45 and CF46 was sequenced, and the specific mutations were identified (*mucA*ΔG440 and *mucA*C362T, respectively) (Table 1). Based on the SSCP patterns, the alteration in CF44 appeared identical to that in CF45 (*mucA*ΔG440) and was not sequenced. These findings are consistent with the interpretation that mutations in the *mucA* gene occurred in the mucoid isolate, although a parent-progeny relationship between nonmucoid and mucoid strains could not be ascertained.

Multiple mucoid *P. aeruginosa* isolates from a single CF patient may carry different *mucA* mutations. It has been observed that individual CF patients can carry a heterogeneous population of mucoid isolates (32). To assess the status of the *mucA* gene in multiple mucoid strains colonizing the same patient, we examined *mucA* in a set of four mucoid strains (CF52, CF53, CF54, and CF55) isolated from a single CF patient. First, SSCP analyses of region III of *mucA* suggested that only one strain (CF55) had a mutation in this region (Fig. 4B). However, SSCP and sequence analyses of other regions of *mucA* indicated that three of the four strains analyzed (with the exception of CF54) contained alterations in the *mucA* gene (Table 1). All strains with detectable mutations in *mucA* had different alterations: CF52 had a C→T transition generating a termination codon at 431, CF53 had a C→T transition at 560, and CF55 had a frameshift mutation (ΔG at 440). This suggests that multiple mucoid isolates of *P. aeruginosa* present in one patient can emerge independently or have independent origins, as evidenced by the different *mucA* alleles observed.

Mucoid *P. aeruginosa* isolates from UTIs and mutations in *mucA*. The association of mucoid *P. aeruginosa* strains with the respiratory tracts of patients with CF has been well documented (14). However, mucoid strains of *P. aeruginosa* can also be isolated from other infection sites, albeit at a lower frequency. For example, mucoid *P. aeruginosa* strains have been noted in UTIs (26). To determine if mutations in the *mucA* gene can be selected in additional infection sites, we examined four mucoid urinary tract isolates. SSCP patterns in three of these strains indicated changes in *mucA* (Fig. 4C). The DNA sequences were determined for two of these strains (UT49 and

TABLE 2. Complementation of mucoidy to a nonmucoid phenotype by *mucA*⁺ in *P. aeruginosa* isolates from CF patients

Isolate	<i>mucA</i> mutation	Plasmid	IPTG (1 mM)	Phenotype ^a	Alginate production (μg/mg [wet wt] of cells) ^b
CF40	Insertion of T ₂₉₅ , ΔG440	pVDtac24	—	M	23.7 ± 1.9
		<i>ptac-mucA</i> ⁺	—	NM	3.7 ± 0.5
		<i>ptac-mucA</i> ⁺	+	NM	2.5 ± 0.2
CF53	C→T ₅₆₀	pVDtac24	—	M	88.7 ± 6.4
		<i>ptac-mucA</i> ⁺	—	NM	6.1 ± 1.0
		<i>ptac-mucA</i> ⁺	+	NM	5.2 ± 0.8
CF60	G→T ₃₂	pVDtac24	—	M	67.9 ± 8.3
		<i>ptac-mucA</i> ⁺	—	M	54.6 ± 1.8
		<i>ptac-mucA</i> ⁺	+	NM	10.1 ± 1.8

^a M, mucoid phenotype; NM, nonmucoid phenotype.^b Production of alginate is expressed as micrograms of uronic acid per milligram (wet weight) of cells ± standard error. All *P* values (M versus NM) were <0.003.

UT50) and showed the presence of a relatively large deletion in region III of *mucA* in UT50 (Fig. 2 and 4C and Table 1) and a smaller deletion in region II of *mucA* in UT49 (Fig. 2 and Table 1). These results suggest that *mucA* mutations in *P. aeruginosa* can cause mucoidy in infection sites other than the CF lung.

Differential clearance of mucoid and nonmucoid *P. aeruginosa* strains from the murine lung. A respiratory infection model in mice that uses *P. aeruginosa* aerosols has recently been developed to investigate inflammatory processes during extended exposure to this pathogen (43). Here, we employed the same aerosol delivery technology (see Materials and Methods) to investigate how the mucoid or nonmucoid status of *P. aeruginosa* affects its pulmonary clearance by using a select number of characterized CF isolates and laboratory *mucA* mutants. In preliminary analyses, several nonmucoid and mucoid CF isolates were compared, but the clearance was highly variable even among nonmucoid strains. To overcome the problem of the complex and unknown nature of the heterogeneity among clinical strains, we resorted to the use of a subset of CF isolates which contained *muc* mutations but whose mucoid or nonmucoid status could be manipulated, depending on the growth conditions. One such CF isolate, CF40 (*mucA*ΔG440), displayed a mucoid phenotype only in the presence of increased salt concentrations. Aerosolization of CF40 after growth in the presence of 100 mM NaCl (optimal for alginate production by this strain) resulted in 45.2% ± 4.2% survival in the lung at 4 h postinfection in C57BL/6J mice. In the absence of alginate induction (no salt added to the growth medium), CF40 displayed a statistically significant reduction in survival (14.8% ± 0.7%) (*P* = 2.1 × 10⁻² [Table 3]). Another CF isolate (CF45) with alginate production dependent upon the concentration of salt in the medium was also tested. This strain displayed optimal induction of alginate production when grown in the presence of 60 mM NaCl. Aerosol challenge of C57BL/6J mice with CF45 grown in the presence of 60 mM NaCl, conditions which rendered this strain mucoid, resulted in 25.1% ± 4.4% survival at 4 h (Table 3). Challenge with the same strain, grown in the absence of salt (which rendered CF45 nonmucoid), resulted in 7.5% ± 1.3% survival at 4 h in C57BL/6J mice, which was lower than that of the mucoid form of the same strain (*P* = 4.0 × 10⁻²) (Table 3). In both cases tested, the mucoid status (or growth on high salt) was associated with a higher rate of survival in the lung.

Alginate production in some *muc* derivatives of the standard genetic strain PAO is also dependent on the concentration of salt in the medium. One such strain, PAO578II, which remains

nonmucoid unless salt concentrations are adjusted to higher levels, displayed 21.0% ± 3.3% survival in C57BL/6J mice when grown under standard conditions (Table 3). Upon increasing the salt concentration to a level (300 mM) coinciding with the induction of alginate production in PAO578II, there was an increase in survival in the mouse lung (43.7% ± 4.5%; *P* = 1.6 × 10⁻²). In the presence of 60 mM NaCl in the growth medium, a concentration which was not sufficient to render PAO578II mucoid, there was no significant change in pulmonary clearance of this strain relative to the growth in the absence of added salt (*P* = 0.86).

To further determine if the observed improvement in survival of the strains tested was due to the induction of mucoidy, the key alginate biosynthetic gene *algD* (6) was inactivated in PAO578II. Growth of the resulting strain, PAO6888 (*mucA*Δ22

FIG. 4. SSCP analysis of *mucA* region III in paired nonmucoid (NM) and mucoid (M) *P. aeruginosa* isolates (A), multiple independent mucoid isolates from the same CF patient (B), and mucoid isolates from UTIs (C). (A) C1272 and CF44, C1287 and CF45, and C1319 and CF46 are paired nonmucoid and mucoid strains (C followed by a number, nonmucoid; CF44, CF45, and CF46, mucoid). Each pair was isolated from a different CF patient. (B) Region III from multiple mucoid *P. aeruginosa* strains (differing by pyocin typing) isolated from one patient. (C) SSCP analysis of the *mucA* gene from mucoid *P. aeruginosa* urinary tract isolates.

TABLE 3. Decreased clearance of mucoid *P. aeruginosa* from the murine lung

Strain ^a	Genotype ^b	Concn of NaCl (mM) ^c	Phenotype ^d	% <i>P. aeruginosa</i> survival in mice ^e		
				DBA/2NHsd (n = 20)	C57BL/6J (n = 60)	BALB/c (n = 20)
CF40	<i>mucA</i> insertion of T ₂₉₅ ΔG440	100	NM	ND	14.8 ± 0.7	ND
CF40	<i>mucA</i> insertion of T ₂₉₅ ΔG440		M	ND	45.2 ± 4.2	ND
CF45	<i>mucA</i> ΔG440		NM	35.4 ± 11.7	7.5 ± 1.3	6.4 ± 0.7
CF45	<i>mucA</i> ΔG440	60	M	72.4 ± 4.7	25.1 ± 4.4	5.5 ± 0.7
PAO381	<i>mucA</i> ⁺		NM	3.7 ± 0.8	20.5 ± 5.6	10.5 ± 1.3
PAO578I	PAO381 <i>mucA22</i>		M	17.2 ± 0.6	38.9 ± 8.9	41.6 ± 8.5
PAO381	<i>mucA</i> ⁺	60	NM	ND	24.8 ± 4.8	ND
PAO578II	PAO578I <i>sup-2</i>		NM	ND	21.0 ± 3.3	ND
PAO578II	PAO578I <i>sup-2</i>		NM	ND	20.6 ± 12.9	ND
PAO578II	PAO578I <i>sup-2</i>	300	M	ND	43.7 ± 4.5	ND
PAO6888	PAO578II <i>algD::Gm^r</i>		NM	ND	12.8 ± 2.2	ND

^a PAO578I is a constitutive mucoid derivative of PAO381. PAO578II is a derivative of PAO578I with a suppressor mutation (*sup-2* [5, 34]) which attenuates the mucoid phenotype and renders it dependent on the concentration of salt in the medium.

^b Known alterations in *mucA* are listed. *mucA22* and *sup-2* have been previously described (25, 34).

^c NaCl concentrations were optimized for maximal alginate production by individual CF isolates.

^d M and NM, mucoid phenotype and nonmucoid phenotype, respectively, on the medium used to grow *P. aeruginosa* for clearance studies (see Materials and Methods). Strain phenotypes on LB and PIA were as follows: CF45, NM (LB) and M (PIA); CF40, NM (LB) and M (PIA); PAO381, NM on both media; PAO578I, M on both media; PAO578II, NM (LB) and M (PIA).

^e Survival at 4 h is expressed as the percentage of the initial CFU deposited in the lung. Initial bacterial depositions were as follows: BALB/c, 5.3×10^5 to 3.7×10^6 ; C57BL/6J, 2.2×10^5 to 3.5×10^6 ; and DBA/2NHsd, 7.0×10^5 to 9.5×10^6 . The numbers of bacteria remaining were determined by plating of total lung homogenate after 4 h. *n*, number of animals per mouse strain used in determining initial deposition and 4-h clearance. *P* values (*t* test) were as follows: DBA/2NHsd, $P = 4.33 \times 10^{-2}$ for the two forms of CF45 and $P = 3.91 \times 10^{-2}$ for PAO381 versus PAO578I; C57BL/6J, $P = 2.10 \times 10^{-3}$ for the two forms of CF40, $P = 1.92 \times 10^{-2}$ for the two forms of CF45, $P = 1.64 \times 10^{-4}$ for PAO381 versus 578I, $P = 1.57 \times 10^{-2}$ for PAO578I and PAO578II at 0.3 M NaCl, and $P = 3.59 \times 10^{-3}$ for PAO578II at 0.3 M NaCl and PAO6888; BALB/c, $P = 2.20 \times 10^{-2}$ for PAO381 versus PAO578I. ND, not determined.

algD::Gm^r Alg⁻), in the presence of 300 mM NaCl (where it remained nonmucoid because of the *algD* mutation) resulted in a low rate of survival, somewhat below the levels observed with PAO578II (*algD⁺*) grown in the absence of salt (Table 3; $P = 5.0 \times 10^{-1}$). In addition, when C57BL/6J mice were challenged with the nonmucoid strain PAO381 (parental to PAO578II), grown in the presence of added salt, no significant change in pulmonary clearance was observed (Table 3). Collectively, these findings suggest that the observed reduction in lung clearance of PAO578II upon addition of salt can be attributed to mucoidy rather than to induction of alternative defense mechanisms. In further confirmation of the role of alginate production in improved survival of *P. aeruginosa* in the murine lung, the strain PAO578I was tested (PAO578I is isogenic with PAO381 and PAO578II but is constitutively mucoid under all conditions), and it displayed improved survival relative to its parental strain, PAO381 (38.9% ± 8.9% for PAO578I versus 20.5% ± 5.6% and 24.8% ± 4.8% for PAO381; $P = 1.0 \times 10^{-4}$ [ANOVA]). These data strongly suggest that production of alginate reduces pulmonary clearance of *P. aeruginosa* in the murine lung.

Analysis of *P. aeruginosa* clearance in different strains of mice. It has been suggested that the susceptibility to *P. aeruginosa* in mouse models of respiratory infections depends on the mouse strain used (29). For example, BALB/c mice have been found to be more resistant to *P. aeruginosa* than DBA/2NHsd mice (29), while C57BL/6J mice are considered to have an intermediate sensitivity to *P. aeruginosa* (28). To examine whether the advantage of mucoid versus nonmucoid *P. aeruginosa* organisms observed in C57BL/6J mice can be detected in mice with different innate susceptibilities to *P. aeruginosa*, BALB/c and DBA/2NHsd mice were challenged with CF45 cells grown under conditions conducive to the mucoid or nonmucoid phenotype. In the DBA/2NHsd mouse, challenge with the mucoid form of CF45 resulted in a detectable increase in survival relative to challenge with the nonmucoid CF45 strain (72.4% ± 4.7% and 35.4% ± 11.7%, respectively; $P = 1.0 \times 10^{-4}$).

When the isogenic strains PAO381 and PAO578I were compared in DBA/2NHsd, C57BL/6J, and BALB/c mice, a statistically significant decrease ($P = 4.0 \times 10^{-4}$ [ANOVA]) in pulmonary clearance, coinciding with the production of alginate, was seen in all three mouse strains (Table 3). The mucoid strain PAO578I displayed survival rates of 41.6% ± 8.5%, 38.9% ± 8.9%, and 17.2% ± 0.6%, while the nonmucoid parental strain PAO381 had survival rates of 10.5% ± 1.3%, 20.5% ± 5.6%, and 3.7% ± 0.8% at 4 h postinfection in BALB/c, C57BL/6J, and DBA/2NHsd mice, respectively (Table 3). In only one case examined (mucoid versus nonmucoid CF45 in BALB/c mice) were no statistically significant differences in clearance observed. These results indicate that with a single exception, various combinations of *P. aeruginosa* and mouse strains tested favored mucoid *P. aeruginosa* over the nonmucoid matching strain for survival in the murine lung.

***P. aeruginosa* clearance and TNF-α levels.** We next wanted to assess whether the reduced clearance of mucoid strains could be attributed to potential differences in levels of proinflammatory cytokines induced by mucoid versus nonmucoid bacteria. Levels of TNF-α, a cytokine implicated in promoting *P. aeruginosa* clearance from the murine lung (28), were measured in the lungs at the time of bacteriological evaluation. In all challenged animals, irrespective of the mucoid or nonmucoid status of the strain used, a significant increase in TNF-α was observed upon exposure to *P. aeruginosa* (2.4 to 13.9 ng/g of lung tissue 4 h postinfection) over the levels seen in unexposed animals (0.02 ± 0.005 ng/g of lung tissue). Although overall TNF-α levels varied between the mouse and *P. aeruginosa* strains used ($P = 5 \times 10^{-3}$ [ANOVA]), no statistically significant correlation between TNF-α levels and mucoid versus nonmucoid status could be observed in the majority of mice exposed to *P. aeruginosa* ($P = 0.46$ [ANOVA]). Overall, only in BALB/c mice exposed to mucoid and nonmucoid forms of CF45 was there a statistically significant decrease in TNF-α levels in animals infected with a mucoid *P. aeruginosa* strain (Table 4; $P = 2.5 \times 10^{-2}$ [Fisher's protected least-significant

TABLE 4. TNF- α levels in mice exposed to aerosols of mucoid and nonmucoid *P. aeruginosa*

Isolate	Concn of NaCl (mM)	Pheno-type	Concn of TNF- α (ng/g of lung tissue) in mice ^a		
			DBA/2NHsd	C57BL/6J	BALB/c
CF40	100	NM	ND	3.2 $\pm$ 1.0	ND
CF40		M	ND	2.4 $\pm$ 0.7	ND
CF45		NM	7.0 $\pm$ 0.8	6.4 $\pm$ 0.2	13.9 $\pm$ 0.5
CF45	60	M	5.7 $\pm$ 0.1	6.6 $\pm$ 1.1	8.0 $\pm$ 1.1
PAO381		NM	4.8 $\pm$ 0.5	ND	9.9 $\pm$ 3.7
PAO578I		M	6.7 $\pm$ 0.7	ND	11.2 $\pm$ 4.2
PAO578II	300	NM	ND	6.6 $\pm$ 0.9	ND
PAO578II		M	ND	2.8 $\pm$ 0.6	ND
PAO6888		NM	ND	4.4 $\pm$ 0.6	ND

^a TNF- α levels were determined at 4 h after the initial deposition of each *P. aeruginosa* CF isolate. *P* values (ANOVA) for TNF- α were 5.3×10^{-3} for TNF- α levels (overall), 7.6×10^{-1} for TNF- α versus *P. aeruginosa* survival, and 4.6×10^{-1} for survival (Table 3) by mucoid (M) and nonmucoid (NM) *P. aeruginosa* strains versus TNF- α . ND, not determined.

difference test)). However, this was also the only case in which a mucoid strain did not show a statistically significant reduction in clearance over the paired nonmucoid control (Table 3; *P* = 0.36). It is worth noting that C57BL/6J mice exposed to mucoid CF40 and PAO578II displayed the lowest levels of TNF- α (Table 4), coinciding with the highest rates of survival in this mouse strain (Table 3). However, statistically significant parallels between reduced TNF- α levels and survival were not observed for the whole set of data (*P* = 0.76 [ANOVA]). Moreover, the highest survival rate of *P. aeruginosa* recorded in this study (72% [Table 3]), which was observed in DBA/2NHsd mice, was not associated with any extreme values for TNF- α (Table 4). Thus, no apparent correlations could be made between TNF- α levels and survival rates of mucoid versus nonmucoid strains of *P. aeruginosa* in the murine lung at the time point examined. These findings suggest that the observed reduction in clearance of mucoid strains cannot be attributed to effects on TNF- α levels. In conclusion, the observed advantage of strains expressing the mucoid phenotype may be at least partially independent of the levels of inflammatory cytokines provoked by the infection.

DISCUSSION

In this study, we have identified the *mucA* gene as the major site of genetic changes causing conversion of *P. aeruginosa* to mucoidy in CF patients. Alterations in the *mucA* gene have been detected at a high frequency (84%) in CF strains. The majority of *mucA* alterations result in a truncation of the *mucA* gene via introduction of premature stop codons, frameshifts, or other open reading frame disruptions. The nature of the *mucA* lesions in CF strains argues against the possibility that the detected alterations are simple polymorphisms. The complementation to nonmucoidy of the representative *mucA* CF mutants by a *mucA*⁺ gene further supports such conclusions. The interpretation that *mucA* lesions cause conversion to mucoidy in CF strains is consistent with the previously reported gene replacement studies (25). In such studies, replacements of the chromosomal *mucA*⁺ gene with mutant *mucA* alleles (which included *mucA*22, the PAO equivalent of the most common CF allele, *mucA* Δ G440) resulted in conversion to mucoidy. It also appears that a strong selection for *mucA* mutants exists in the lungs of CF patients, explaining the higher frequency of *mucA* alterations among clinical mucoid strains than among spontaneous mucoid derivatives obtained in the laboratory. In

this work, we have also addressed the role of the overproduction of alginate in the survival of *P. aeruginosa* in the lung. The subtle but statistically significant reduction in pulmonary clearance of mucoid strains in a mouse model of respiratory infection observed with CF isolates and isogenic mucoid and nonmucoid derivatives of PAO suggests increased resistance of the alginate-producing forms of *P. aeruginosa* to the innate defenses in the respiratory tract.

Although the *mucA* gene has received attention as a possible site of mutations causing conversion to mucoidy in *P. aeruginosa* (5), it has been a general belief that other mechanisms, besides inactivation of *mucA*, may also cause conversion to the mucoid phenotype. For example, early analyses of a collection of laboratory strains and a limited number of CF isolates have indicated that *mucA* may not be the only candidate for the major site of mutations causing mucoidy (5). In keeping with this notion, other genes in the *mucABCD* cluster (5) and at least in one additional locus (11) on the *P. aeruginosa* chromosome, have been shown to represent potentially significant sites of mutations causing conversion to mucoidy. Based on the analyses reported here, *mucA* appears to be the most frequent site of mutations in strains infecting CF patients. Moreover, since the observed frequency of alterations in *mucA* (84%) is based on an SSCP assay, which does not necessarily permit detection of all mutations, this value should be taken as an underestimate of the incidence of *mucA* mutations in mucoid *P. aeruginosa* CF isolates. While the specific reasons for the apparent preponderance of mutations in *mucA* among clinical strains are not known at present, it is possible that mutations in other sites, albeit causing mucoidy (12), confer some other disadvantages in vivo. Such effects may offset the benefits of alginate overproduction during infection but may not be critical for growth under laboratory conditions.

Mucoid *P. aeruginosa* strains can also be found in infection sites other than CF, including the urinary tract. The analyses of the *mucA* gene from mucoid *P. aeruginosa* urinary tract isolates have uncovered alterations similar to those observed in CF strains. Intriguingly, earlier reports comparing UTI and CF strains have indicated that *P. aeruginosa* isolates from CF and UTIs may have considerable phenotypic similarities (8, 26, 32). It is not known whether and to what extent the selective pressures and clearance mechanisms are comparable in the two infection sites, but they seem to favor the emergence of mucoid strains via what appears to be a common conversion pathway. Furthermore, the presence of *mucA* mutations in UTI isolates confirms the notion that mucoid *P. aeruginosa* can be found under clinical conditions different from CF. It should also be mentioned that three different *mucA* SSCP band patterns were observed for region III in a preliminary analysis of *P. aeruginosa* immunotypes O1 to O20 (unpublished results). The reasons for these polymorphisms, which remain to be characterized, are currently not known, but they could reflect the history of these strains (which, to our knowledge, has not been described in the literature) in the context of the infections that served as their source.

It is worth noting that the process of *mucA* inactivation in mucoid CF isolates is random, with a number of possible outcomes leading to the loss of *mucA* function. The loss of lipopolysaccharide O side chains in clinical isolates of *P. aeruginosa* has also been reported to include random mutations (9). Thus, the variations in expression of the two major exopolysaccharides of *P. aeruginosa* appear not to be based on programmed genetic events. In keeping with this notion, nonmucoid variants derived in vitro from mucoid mutants do not seem to represent direct reversions of *mucA* mutations (34). However, the observed clustering of mutations at several locations within re-

gion III and other positions within *mucA* potentially reflects specific interactions with other participants in the control of AlgU activity (e.g., MucB and MucC). Work is in progress to define the functional domains of MucA and how MucA interacts with AlgU on one end and with MucB and MucC on the other. In this context, the collection of mutants described in this work is expected to assist the ongoing structure-function analyses of MucA.

In an earlier analysis comparing *P. aeruginosa* mucoid and nonmucoid strains (using a mucoid parent and its nonmucoid revertant), no differences in lung clearance of the strains tested were reported upon intratracheal instillation in guinea pigs (1). In contrast, we find that pulmonary clearance from the murine lung in the infection model used here is affected by the mucoid or nonmucoid status of *P. aeruginosa*. It has also been reported that the susceptibility to *P. aeruginosa* infections differs between mouse strains (28). In this study, we found that susceptibility to *P. aeruginosa* aerosol challenge in DBA/2NHsd, BALB/c, and C57BL/6J mice varies with the bacterial strain used (Table 3; $P = 3.6 \times 10^{-2}$ [ANOVA]). Despite the increased levels of the proinflammatory cytokine TNF- α in each mouse strain exposed to *P. aeruginosa*, comparison of the TNF- α levels induced in BALB/c, C57BL/6J, and DBA/2NHsd mice revealed no statistically significant differences. It is possible that our findings are at variance with those in the previous reports (1, 28) because of the differences in bacterial strains, bacterial delivery (aerosol versus intratracheal instillation), and clearance analysis. Independent of these considerations, our results obtained with three different strains of mice suggest that alginate production improves the survival of *P. aeruginosa* in mouse lungs. Such differences may easily be missed or masked by other variables if the strains are not carefully matched and the aerosol delivery is not controlled with the utmost care. Furthermore, the known effects of initial bacterial deposition levels on the course of clearance (40) must be taken into account and controlled carefully.

Studies presented here and elsewhere (5, 35, 42) support the following model (Fig. 1C) for the role of *mucA* mutations in *P. aeruginosa* infections in CF. The high frequency of *mucA* mutations found in mucoid CF isolates strengthens the notion that alterations in *mucA* represent the major pathway leading to the conversion to the mucoid phenotype in the lungs of CF patients. In the initially colonizing nonmucoid cell, as illustrated in Fig. 1C, MucA binds to AlgU (15, 35), preventing efficient transcription of the key alginate biosynthetic gene *algD*. Inactivation of *mucA* by spontaneous mutations, as observed in the CF isolates presented here, permits AlgU to transcribe the response regulator gene *algR*. AlgU and AlgR, in cooperation, activate *algD* (and possibly additional elements), leading to alginate overproduction and the mucoid phenotype. Based on the observed advantage of mucoid strains, as reflected in the improved survival rates relative to nonmucoid *P. aeruginosa* in the model of aerosol infection in mice employed in this work and further supported by the previously reported in vitro studies suggesting various protective attributes of alginate (21, 22, 30, 36, 37, 39), it is possible to conclude that the mucoid phenotype may promote persistence of *P. aeruginosa* in the respiratory tract. Whether the roles of *mucA* mutations and contributions of alginate production to the pathogenesis of CF end with the effect of impeding pulmonary clearance of *P. aeruginosa* is currently not known (33). Furthermore, it would be of interest to study the potential interplay between the mucoid phenotype in *P. aeruginosa* and the recently recognized host factors in CF, which fall into several categories: e.g., reduced sialylation of glycoconjugates and increased adhesion (46), altered bactericidal properties of epithelial secretions

ascribed to modified salt content (13, 38), uptake by epithelial cells (31), and endogenous or infection-dependent elevation of proinflammatory cytokines (3, 18). Investigations are under way to address the effects of mucoidy in *P. aeruginosa* on the histopathology of the lung and production of inflammatory cytokines during prolonged exposure to the pathogen. These and additional studies may provide new information potentially critical for better control of respiratory infections in CF.

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REFERENCES

- Blackwood, L. L., and J. E. Pennington. 1981. Influence of mucoid coating on clearance of *Pseudomonas aeruginosa* from lungs. *Infect. Immun.* **32**:443–448.
- Bodey, G. P., R. Bolivar, V. Fainstein, and L. Jadeja. 1983. Infections caused by *Pseudomonas aeruginosa*. *Rev. Infect. Dis.* **5**:279–313.
- Bonfield, T. L., J. R. Panuska, M. W. Konstan, K. A. Hillard, J. B. Hillard, H. Ghnaim, and M. Berger. 1995. Inflammatory cytokines in cystic fibrosis lungs. *Am. J. Respir. Crit. Care Med.* **152**:2111–2118.
- Boucher, J. C., M. J. Schurr, H. Yu, and V. Deretic. Role of *mucC* in the regulation of alginate production and stress sensitivity in *Pseudomonas aeruginosa*. Submitted for publication.
- Boucher, J. C., J. M. Martinez-Salazar, M. J. Schurr, M. H. Mudd, H. Yu, and V. Deretic. 1996. Two distinct loci affecting conversion to mucoidy in *Pseudomonas aeruginosa* in cystic fibrosis encode homologs of the serine protease HtrA. *J. Bacteriol.* **178**:511–523.
- Deretic, V., J. F. Gill, and A. M. Chakrabarty. 1987. Gene *algD* coding for GDPmannose dehydrogenase is transcriptionally activated in mucoid *Pseudomonas aeruginosa*. *J. Bacteriol.* **169**:351–358.
- DeVries, C. A., and D. E. Ohman. 1994. Mucoid-to-nonmucoid conversion in alginate-producing *Pseudomonas aeruginosa* often results from spontaneous mutations in *algT*, encoding a putative alternate sigma factor, and shows evidence for autoregulation. *J. Bacteriol.* **176**:6677–6687.
- Doggett, G., G. M. Harrison, and R. E. Carter. 1971. Mucoid *Pseudomonas aeruginosa* in patients with chronic illnesses. *Lancet* **i**:236–237.
- Evans, D. J., G. B. Pier, M. J. Coyne, Jr., and J. B. Goldberg. 1994. The *rbf* locus from *Pseudomonas aeruginosa* strain PA103 promotes the expression of O antigen by both LPS-rough and LPS-smooth isolates from cystic fibrosis patients. *Mol. Microbiol.* **13**:427–434.
- Flynn, J. L., and D. E. Ohman. 1988. Cloning of genes from mucoid *Pseudomonas aeruginosa* which control spontaneous conversion to the alginate production phenotype. *J. Bacteriol.* **170**:1452–1460.
- Fyfe, J. A. M., and J. R. W. Govan. 1983. Synthesis, regulation and biological function of bacterial alginate. *Prog. Ind. Microbiol.* **18**:45–83.
- Fyfe, J. A. M., and J. R. W. Govan. 1980. Alginate synthesis in mucoid *Pseudomonas aeruginosa*: a chromosomal locus involved in control. *J. Gen. Microbiol.* **119**:443–450.
- Goldman, M. J., G. M. Anderson, E. D. Stolzenberg, U. P. Kari, M. Zasloff, and J. M. Wilson. 1997. Human β -defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. *Cell* **88**:553–560.
- Govan, J. R. W., and V. Deretic. 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol. Rev.* **60**:539–574.
- Hershberger, C. D., R. W. Ye, M. R. Parsek, Z.-D. Xie, and A. M. Chakrabarty. 1995. The *algT* (*algU*) gene of *Pseudomonas aeruginosa*, a key regulator involved in alginate biosynthesis, encodes an alternative σ factor (σ^B). *Proc. Natl. Acad. Sci. USA* **92**:7941–7945.
- Ishimoto, K. S., and S. Lory. 1989. Formation of pilin in *Pseudomonas aeruginosa* requires the alternative sigma factor (*rpoN*) of RNA polymerase. *Proc. Natl. Acad. Sci. USA* **86**:1954–1957.
- Jonsson, A. B., G. Nyberg, and S. Normark. 1991. Phase variation of gonococcal pili by frameshift mutation in *pilC*, a novel gene for pilus assembly. *EMBO J.* **10**:477–488.
- Khan, T. Z., J. S. Wagner, T. Bost, J. Martinez, F. J. Accurso, and D. W. H. Riches. 1995. Early pulmonary inflammation in infants with cystic fibrosis. *Am. J. Respir. Crit. Care Med.* **151**:1075–1082.
- Knutson, C. A., and A. Jeanes. 1976. A new modification of the carbazole reaction: application to heteropolysaccharides. *Anal. Biochem.* **24**:470–481.
- Koch, C., and N. Hoiby. 1993. Pathogenesis of cystic fibrosis. *Lancet* **341**:1065–1069.

21. Lam, J., R. Chan, K. Lam, and J. W. Costerton. 1980. Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. *Infect. Immun.* **28**:546–556.
22. Learn, D. B., E. P. Brestel, and S. Seetharama. 1987. Hypochlorite scavenging by *Pseudomonas aeruginosa* alginate. *Infect. Immun.* **55**:1813–1818.
23. Martin, D. W., B. W. Holloway, and V. Deretic. 1993. Characterization of a locus determining the mucoid status of *Pseudomonas aeruginosa*: AlgU shows sequence similarities with a *Bacillus* sigma factor. *J. Bacteriol.* **175**:1153–1164.
24. Martin, D. W., M. J. Schurr, M. H. Mudd, and V. Deretic. 1993. Differentiation of *Pseudomonas aeruginosa* into the alginate-producing form: inactivation of *mucB* causes conversion to mucoidy. *Mol. Microbiol.* **9**:497–506.
25. Martin, D. W., M. J. Schurr, M. H. Mudd, J. R. W. Govan, B. W. Holloway, and V. Deretic. 1993. Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. *Proc. Natl. Acad. Sci. USA* **90**:8377–8381.
26. McAvoy, M. J., V. Newton, A. Paull, J. Morgan, P. Gacesa, and N. J. Russell. 1989. Isolation of mucoid strains of *Pseudomonas aeruginosa* from non-cystic fibrosis patients and characterization of the structure of their secreted alginate. *J. Med. Microbiol.* **28**:183–189.
27. Mohr, C. D., and V. Deretic. 1990. Gene-scrambling mutagenesis: generation and analysis of insertional mutations in the alginate regulatory region of *Pseudomonas aeruginosa*. *J. Bacteriol.* **172**:6252–6260.
28. Morissette, C., E. Skamene, and F. Gervais. 1995. Endobronchial inflammation following *Pseudomonas aeruginosa* infection in resistant and susceptible strains of mice. *Infect. Immun.* **63**:1718–1724.
29. Morissette, C., C. Francoeur, C. Darmond-Zwaig, and F. Gervais. 1996. Lung phagocyte bactericidal function in strains of mice resistant and susceptible to *Pseudomonas aeruginosa*. *Infect. Immun.* **64**:4984–4992.
30. Pedersen, S. S. 1992. Lung infection with alginate-producing, mucoid *Pseudomonas aeruginosa* in cystic fibrosis. *APMIS* **100**(Suppl. 28):1–79.
31. Pier, G. B., M. Grout, T. S. Zaidi, J. C. Olsen, L. G. Johnson, J. R. Yankaskas, and J. B. Goldberg. 1996. Role of mutant CFTR in hypersusceptibility of cystic fibrosis patients to lung infections. *Science* **271**:64–67.
32. Pugashetti, B. K., H. M. Metzger, Jr., L. Vadas, and D. S. Feingold. 1982. Phenotypic differences among clinically isolated mucoid *Pseudomonas aeruginosa* strains. *J. Clin. Microbiol.* **16**:686–691.
33. Schurr, M. J., and V. Deretic. 1997. Microbial pathogenesis in cystic fibrosis: coordinate regulation of heat shock response and conversion to mucoidy in *Pseudomonas aeruginosa*. *Mol. Microbiol.* **24**:411–420.
34. Schurr, M. J., D. W. Martin, M. H. Mudd, and V. Deretic. 1994. Gene cluster controlling conversion to alginate-overproducing phenotype in *Pseudomonas aeruginosa*: functional analysis in a heterologous host and role in the instability of mucoidy. *J. Bacteriol.* **176**:3375–3382.
35. Schurr, M. J., H. Yu, J. M. Martinez-Salazar, J. C. Boucher, and V. Deretic. 1996. Control of AlgU, a member of the σ^E -like family of stress sigma factors, by the negative regulators MucA and MucB and *Pseudomonas aeruginosa* conversion to mucoidy in cystic fibrosis. *J. Bacteriol.* **178**:4997–5004.
36. Schwarzmann, S., and J. R. Boring III. 1971. Antiphagocytic effect of slime from a mucoid strain of *Pseudomonas aeruginosa*. *Infect. Immun.* **3**:762–767.
37. Simpson, J. A., S. E. Smith, and R. T. Dean. 1989. Scavenging by alginate of free radicals released by macrophages. *Free Radical Biol. Med.* **6**:347–353.
38. Smith, J. J., S. M. Travis, E. P. Greenberg, and M. J. Welsh. 1996. Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. *Cell* **85**:1–20.
39. Speert, D. P. 1994. *Pseudomonas aeruginosa* infections in patients with cystic fibrosis, p. 183–236. In A. L. Baltch and R. P. Smith (ed.), *Pseudomonas aeruginosa* infections and treatment. Marcel Dekker, Inc., New York, N.Y.
40. Toews, G. B., G. N. Gross, and A. K. Pierce. 1979. The relationship of inoculum size to lung bacterial clearance and phagocytic cell response in mice. *Am. Rev. Respir. Dis.* **120**:559–566.
41. Woods, D. E., P. A. Sokol, L. E. Bryan, D. G. Storey, S. J. Mattingly, H. J. Vogel, and H. Ceri. 1991. *In vivo* regulation of virulence in *Pseudomonas aeruginosa* associated with genetic rearrangement. *J. Infect. Dis.* **163**:143–149.
42. Yu, H., M. Mudd, J. C. Boucher, M. J. Schurr, and V. Deretic. 1997. Identification of the *algZ* gene upstream of the response regulator *algR* and its participation in control of alginate production in *Pseudomonas aeruginosa*. *J. Bacteriol.* **179**:187–193.
43. Yu, H., M. Hanes, C. E. Crisp, J. C. Boucher, and V. Deretic. *Pseudomonas aeruginosa* pathogenesis in cystic fibrosis: pulmonary clearance and inflammation in a model of repeated inhalation exposure. Submitted for publication.
44. Yu, H., M. J. Schurr, J. C. Boucher, J. M. Martinez-Salazar, D. W. Martin, and V. Deretic. Molecular mechanism of conversion to mucoidy in *Pseudomonas aeruginosa*. In S. Silver, T. Nakazawa, and D. Haas (ed.), *Pseudomonas*, in press. ASM Press, Washington, D.C.
45. Yu, H., M. J. Schurr, and V. Deretic. 1995. Functional equivalence of *Escherichia coli* σ^E and *Pseudomonas aeruginosa* AlgU: *E. coli* *rpoE* restores mucoidy and reduces sensitivity to reactive oxygen intermediates in *algU* mutants of *P. aeruginosa*. *J. Bacteriol.* **177**:3259–3268.
46. Zar, H., L. Saiman, L. Quittell, and A. Prince. 1995. Binding of *Pseudomonas aeruginosa* to respiratory epithelial cells from patient with various mutations in the cystic fibrosis transmembrane regulator. *J. Pediatr.* **126**:230–233.