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Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients

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ABSTRACT **Chronic respiratory infections with mucoid *Pseudomonas aeruginosa* are the leading cause of high mortality and morbidity in cystic fibrosis (CF). The initially colonizing strains are nonmucoid, but in the CF lung they invariably convert into the mucoid, exopolysaccharide alginate-overproducing form causing further deterioration and poor prognosis. Here we report the molecular basis of conversion to mucoidy. The *algU* gene is required for expression of the key alginate biosynthetic gene *algD* and encodes a protein homologous to σ^H, an alternative σ factor regulating sporulation and other post-exponential-phase processes in *Bacillus*. The *algU* gene and the negative regulators *mucA* and *mucB* constitute the gene cluster controlling conversion to mucoidy. We demonstrate a critical role of *mucA* in this process based on (*i*) the presence of frameshift mutations disrupting the *mucA* coding region in mucoid cells that were absent in nonmucoid parental strains, (*ii*) genetic complementation of *mucA* mutations with the *mucA*$^+$ gene, (*iii*) allelic replacements with specific mutant *mucA* genes causing conversion to mucoidy in previously nonmucoid cells, and (*iv*) detection of identical and additional *mucA* mutations in clinical mucoid strains isolated from the lungs of CF patients. These results suggest that the switch from the nonmucoid to mucoid state can be caused by inactivation of *mucA*, resulting in constitutive expression of alginate biosynthetic genes dependent on *algU* for transcription and that such mutants may be selected *in vivo* during chronic infections in CF.**

Cystic fibrosis (CF) is an autosomal recessive disorder characterized by abnormal chloride transport across the apical membranes of epithelial cells and altered mucus secretions (1, 2). The most significant complications of CF are respiratory sequelae responsible for approximately 90% of deaths in this disease (1, 3). The lungs of CF patients characteristically become colonized with *Pseudomonas aeruginosa* that cannot be eradicated (3, 4). The principal factor in the establishment of chronic *P. aeruginosa* colonization in CF is the conversion of the initially invading nonmucoid strains into the mucoid, exopolysaccharide alginate-overproducing form (5–7). The microcolony mode of growth of *P. aeruginosa*, embedded in exopolysaccharide biofilms in the lungs of CF patients (8), among other functions, plays a critical role in preventing effective opsonization and phagocytosis of *P. aeruginosa* cells (9). Under such circumstances, the conversion of *P. aeruginosa* to mucoidy becomes a critical persistence factor in CF.

Despite the critical role of mucoid *P. aeruginosa* in CF, the genetic basis for conversion to mucoidy has thus far eluded satisfactory definition. Previous genetic studies have mapped mutations causing mucoidy (*muc*) at 67.5 min on the *P. aeruginosa* strain PAO chromosome (10–12). More recently, some *muc* mutations (e.g., *muc-2* and *muc-22*) have been associated (13) with transcriptional activation of a distant, key alginate-biosynthesis gene *algD* (14). The activation of the *algD* promoter in mucoid cells has become the focus of efforts aimed at understanding molecular processes governing conversion to mucoidy. Several signal transduction and histone-like elements (15), which map in chromosomal regions different from *muc*, have been implicated in the regulation of the *algD* promoter. However, such studies have not explained the transition to constitutively mucoid forms seen in CF isolates. It has become increasingly clear that *muc* genes must be isolated and that mutations in these loci must be characterized to understand more completely the process of conversion to mucoidy.

We have sought to determine the nature of *muc* mutations. The isolation of a cosmid clone from nonmucoid *P. aeruginosa* capable of complementing *muc* mutations has recently been reported (16). The region critical for complementation to nonmucoidy contains three tightly linked genes, *algU*, *mucA*, and *mucB* (16, 17). Based on genetic and physical analyses, these genes map in the same region as the majority of *muc* mutations (16). The nucleotide sequence of all three genes from the standard genetic (nonmucoid) strain PAO1 and detection of their respective polypeptide products, with apparent M_r values of 27 kDa (AlgU), 20 kDa (MucA), and 33 kDa (MucB), have been reported (16, 17).

The first gene of the cluster, *algU*, plays a positive regulatory role (16). AlgU is homologous to σ^H, an alternative σ factor controlling developmental process in *Bacillus* (16, 18). When *algU* is insertionally inactivated in mucoid strains, *algD* transcription ceases, resulting in the nonmucoid phenotype (16). The precise role of *mucA* and *mucB* is not known. Here we examined whether the *algU–mucAB* region is a site of genetic alterations¶ causing conversion to mucoidy.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Mucoid and nonmucoid derivatives of *P. aeruginosa* PAO1 have been described (10). Clinical strains of *P. aeruginosa* were isolates from different CF patients and had different pyocin typing patterns. PAO381a2-3 and PAO381a22-1 were isolates generated by replacement of the chromosomal *mucA*$^+$ in PAO381 with plasmid-borne *mucA2* and *mucA22*, respectively. The plasmid ptac-mucA$^+$ was generated by cloning a 852-base-pair (bp) *Bst*YI fragment containing (*i*) the *mucA*$^+$ gene from PAO; (*ii*) 58 bp of the 3′ end of *algU*; and (*iii*) 167 bp of the 5′ end of *mucB* into the *Bam*HI site of pVDtac24 (19).

Genetic Manipulations, Allelic Replacements, and Complementation Analysis. Plasmids were transferred into *P. aeruginosa* by triparental matings (20). Nonreplicative plasmids

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Abbreviations: ASO, allele-specific oligonucleotide; CF, cystic fibrosis; IPTG, isopropyl β-D-thiogalactopyranoside.

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¶The sequences reported in this paper have been deposited in the GenBank data base [accession numbers L14760–L14766, respectively, for PAO381 (2222 bp), PAO568 (2200 bp), PAO578 (247 bp), CF1 (296 bp), CF8 (200 bp), CF14 (27 bp), and CF23 (30 bp)].

(in *Pseudomonas*) were based on pUC12 carrying a fragment with *mob* functions, permitting conjugal mobilization as described (16). For gene replacements with *mucA* alleles, PCR products containing sequenced *mucA* alleles from mucoid strains [PAO568 (*mucA2*) and PAO578 (*mucA22*)] were cloned as 1.5-kilobase (kb) *Eco*RI fragments (encompassing regions beginning 28 bp downstream of the *mucA* initiation codon and ending 16 bp past the stop codon of *mucB*) into pVDZ'2 (19) and were transferred into the nonmucoid strain PAO381. The exconjugants (all nonmucoid) were passaged multiple times on *Pseudomonas* isolation agar (Difco), supplemented with tetracycline (300 μg/ml). Strains with putative allelic exchanges were scored as mucoid colonies emerging on the lawn of nonmucoid cells at a low but detectable frequency. Multiple independent mucoid clones were isolated and cured of their respective plasmids. Purified mucoid strains were grown in cycles by alternating between Luria-Bertani (LB) medium and LB supplemented with antibiotics (50 μg of tetracycline and 1000 μg of carbenicillin per ml). Clones that lost the pVDZ'2 derivatives were identified as tetracycline-sensitive colonies.

For *mucA* complementation studies, the expression of *mucA*$^+$ was induced, when stated, by growth in the presence of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). The phenotype was scored as mucoid or nonmucoid after 48 hr of growth at 37°C.

DNA Amplification, Cloning, and Sequencing. Oligonucleotides UL3 (5'-CTGTCCGCTGTGATGG-3') and UR12 (5'-CGCCCCTGCTCCTCGA-3') were used for PCR amplification of the sequences beginning 24 bp downstream of the *mucA* initiation codon and ending 16 bp past the stop codon of *mucB*, resulting in a 1.5-kb PCR product. Where indicated, cycle sequencing of PCR products without their prior cloning was performed with BRL's double-stranded DNA cycle sequencing kit. In most cases, PCR products were cloned, and for each strain the nucleotide sequence of at least three independent PCR clones was determined by using Sequenase (version 2.0, United States Biochemical), double-stranded templates, and custom-made oligonucleotides as sequencing primers, spanning in regular intervals the *algU–mucAB* region. Sequences illustrating *muc* mutations were generated with the sequencing primer UR18 (5'-CGGGTATCGCTGGACG-3').

Differential Hybridization with ASO. The presence of *mucA2* on the chromosome was detected by differential hybridization of PCR-amplified *mucA* sequences with allele-specific oligonucleotide (ASO) 568 (5'-CAGGGGGCCAGGGGGC-3') and ASO 381 (5'-GAGCAGGGGGCGCCG-3'). DNA fragments generated by PCR amplification, containing *mucA* from PAO568 (*mucA2*) and PAO381 (*mucA*$^+$), were separated on an agarose gel; blotted onto a nitrocellulose membrane; hybridized with ASO 381 or ASO 568 in 5× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7) containing 0.10% polyvinylpyrrolidone, 0.10% bovine serum albumin, 0.10% Ficoll, and 0.1% SDS at 42°C; washed at 42°C in 2× SSC containing 0.1% SDS; and subjected to autoradiography.

Alginate Assay and Transcriptional Fusion Analysis. Growth conditions for activity measurements have been described (16). For transcriptional fusion measurements, all strains harbored pPAOM3 (20) with an *algD*::*xylE* transcriptional fusion. The activity of the reporter gene was determined as described (16). Alginate was measured in duplicates by the method of Knutson and Jeanes (21).

RESULTS

Detection of *muc* Mutations in Spontaneous Mucoid Derivatives of the Standard Genetic Strain, PAO. A collection of spontaneous mucoid derivatives of the *P. aeruginosa* strain PAO has been described (10). These derivative strains have permitted the mapping of several *muc* alleles (e.g., *muc-2* and *muc-22*) that confer mucoidy in conjugation and transduction crosses and are tightly linked to the *pruAB* marker (4, 10, 22). Recently, a similar linkage of *pruAB* and the *algU–mucAB* cluster, tagged with a cassette encoding tetracycline resistance on the PAO chromosome, has been demonstrated (16). This suggests that *algU–mucAB* may be the site of *muc* mutations.

In the course of performing gene replacements with *algU* in the mucoid strain PAO568 carrying the *muc-2* mutation (10), we noticed an informative class of recombinants regarding the location of the *muc-2* mutation. The gene replacements on the chromosome of PAO568 were carried out via homologous recombination with *algU*::tetracycline-resistance gene on a plasmid that cannot replicate in *Pseudomonas* (16). A set of experiments was performed using *algU–mucAB* cloned from nonmucoid *P. aeruginosa* (PAO1) modified by an internal deletion that simultaneously removed the 3' end of *algU* and the 5' end of the downstream gene *mucA* (Fig. 1). Two types of recombinants were anticipated: (*i*) nonmucoid strains that contained true gene replacements with inactivated *algU* (resulting from double crossovers) and (*ii*) mucoid strains that were results of single crossovers. As expected, all double crossover strains were nonmucoid because they lost a functional *algU*. The majority of single crossover strains were mucoid because they retained a functional copy of *algU*. However, a third class of recombinants was also observed that consisted of nonmucoid single crossovers (3–7% of >10,000 colonies from three independent crosses). Since the plasmid-borne genes originated from the nonmucoid strain PAO1, parental to the PAO568 lineage, a plausible explanation for the existence of nonmucoid single crossovers was that the recombination took place between the deletion in *mucA* on the plasmid and a putative mutation (*muc-2*) in *mucA* on the chromosome of the mucoid strain PAO568. Only such a crossover could restore a wild-type copy of *mucA* (dashed line, Fig. 1) resulting in the nonmucoid phenotype at the observed frequency. The mutation had to be located between the *Eco*RV site of *mucA*, where the 5' deletion in the plasmid construct ended (Fig. 1), and the 3' end of *mucA*.

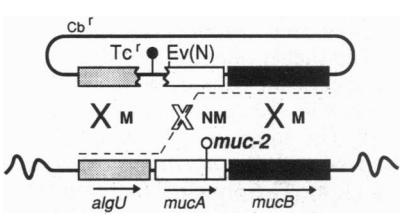

FIG. 1. Genetic organization of the locus controlling conversion to mucoidy in *P. aeruginosa* and recombinational events between the nonreplicative plasmid (above the dashed line) and chromosome (below the dashed line) that indicated the location of the *muc-2* mutation (⚲). A chromosomal segment of the mucoid strain PAO568 (*muc-2*) is shown. The plasmid insert with sequences from a nonmucoid strain [PAO1 (*mucA*$^+$)] was modified by (*i*) a 3' deletion of *algU*, (*ii*) a 5' deletion of *mucA*, and (*iii*) insertion of a cassette-encoding tetracycline resistance (Tc^r) (⚲) between the two genes. Since the plasmid vector used cannot replicate in *Pseudomonas* (16), all exconjugants obtained upon its introduction into PAO568 were recombinants with the PAO568 chromosome. The majority of single crossovers [Tc^{r}Cb^r (Cb^r = carbenicillin-resistance gene); indicated by X) resulted in retention of mucoid phenotype (M; filled **X** symbols) with the exception of the crossovers (open **X** symbol) between the 5' deletion in *mucA* on the plasmid and the *muc-2* mutation on the PAO568 chromosome. The latter events restored a wild-type copy of *mucA* (traced by a dashed line), resulting in the nonmucoid (NM) phenotype. All double crossovers (Tc^{r}Cb^s) were nonmucoid because of the inactivation of *algU* (not shown). Ev(N), *Eco*RV site converted into a *Not* I site.

To test this hypothesis, we cloned the corresponding region from the strain PAO568 by PCR and determined its complete nucleotide sequence in multiple independent clones. A duplication of eight nucleotides (positions 434–441; Fig. 2*A*) was observed within *mucA* in all PCR clones from PAO568 (Fig. 2*B*). The existence of this mutation was further confirmed by hybridization with ASOs 568 and 381 to PCR-amplified chromosomal sequences from PAO568 (*muc-2*) and its direct nonmucoid parental strain PAO381 (10) (Fig. 2*C*). Next, the entire algU–mucAB region was cloned by PCR from PAO568 and its parental strain PAO381, and the complete nucleotide sequence of this 2.2-kb region from both strains was determined in at least three independent clones each. The only difference between PAO381 (*muc*$^+$) and PAO568 (*muc-2*) was the octanucleotide duplication in *mucA*. We concluded that this was the *muc-2* mutation, and the corresponding *mucA* allele was designated *mucA2*. The *muc-2* mutation results in a frameshift causing premature termination of *mucA* (TGA at the position 450).

We next examined whether we could use ASO 568 and ASO 381 to screen other mapped *muc* mutations in PAO. For this purpose, PAO578, a mucoid derivative of PAO381 with the mutation *muc-22* mapping close to *muc-2* was used. The initial results were equivocal. Although ASO 568 (specific for the *mucA2* allele) did not hybridize with the PCR-amplified sequences from PAO578, the control oligonucleotide (ASO 381) did hybridize but with a reduced intensity relative to PAO381. This suggested that, although PAO578 did not have the octanucleotide duplication observed in *mucA2*, there were other alterations within the region complementary to the oligonucleotide probe. The corresponding region from PAO578 hybridizing weakly with the oligonucleotide 381 was cloned and sequenced. Instead of the duplication of the octanucleotide sequence in PAO568, there was a deletion of a G residue within a string of 5 G residues within the same general region (Fig. 2 *A* and *B*). Since this was a deletion of one nucleotide, the net result was a similar frameshift as in PAO568, placing the same TGA termination codon in frame with the *mucA* sequence. The mutant allele in PAO578 was designated *mucA22*.

Gene Replacements with *muc-2* and *muc-22* Alleles Result in Conversion to Mucoidy and Activation of the *algD* Promoter. To prove that the specific changes in the *mucA* gene observed in PAO568 (*mucA2*) and PAO578 (*mucA22*) are responsible for conversion to mucoidy, we performed gene replacements with these alleles in nonmucoid cells and determined whether conversion to mucoidy correlated with inheritance of a specific mutant *muc* allele. To this aim, the *mucA2* allele (on a fragment lacking the 5′ end of the gene) was placed on a broad host-range vector pVDZ′2 and introduced into the nonmucoid strain PAO381. Mucoid colonies arose at a very low but detectable frequency on the lawn of nonmucoid cells harboring the plasmid, a phenomenon which has never been observed with other DNA fragments cloned on the same vector. This indicated that there had been exchange of the plasmid-borne *mucA2* with the wild-type allele on the PAO381 chromosome. This was confirmed by curing the plasmid followed by hybridization of the chromosomal sequences with ASO 568. All mucoid colonies showed positive hybridization with ASO 568. The chromosomal *mucA* sequences from two such mucoid isolates were amplified by PCR and sequenced. This analysis (Fig. 3) confirmed the presence of an eight-nucleotide duplication characteristic of the *mucA2* allele used as input in this allelic replacement experiment.

The same type of experiment was performed using a different *mucA* allele. A DNA fragment carrying the *mucA22* allele from PAO578 was cloned on pVDZ′2 and introduced into PAO381. Mucoid strains were obtained in the fashion described for *mucA2* replacements. Randomly picked mu-

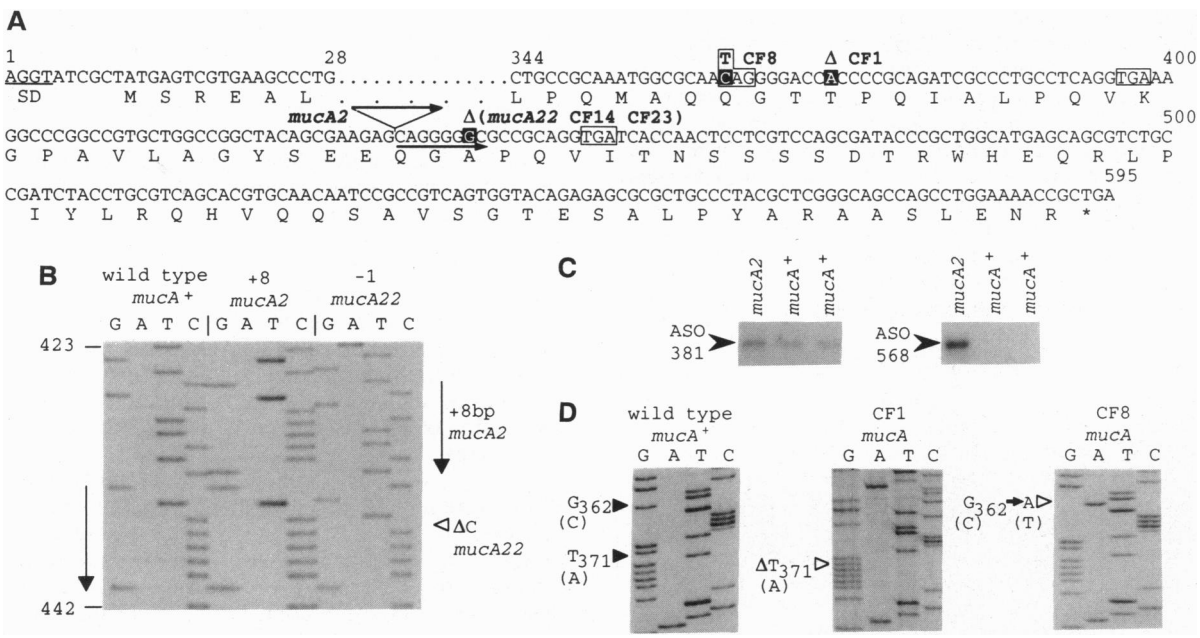

FIG. 2. Mutations causing mucoidy in *P. aeruginosa*. (*A*) The region of *mucA* containing mutations causing conversion to mucoidy is shown [dots indicate omitted sequence; the complete sequence of the gene has been communicated elsewhere (17)]. Highlighted are nucleotides missing or substituted in mucoid strains (CF1, CF8, CF14, CF23, and PAO578). Arrows indicate duplicated sequence (eight nucleotides) in PAO568 (*mucA2*). Boxed are termination codons (TGA or TAG) placed in frame with the *mucA* sequence as a result of *muc* mutations. (*B*) DNA sequence (from position 423 to 442) of the wild-type mucA$^+$, *mucA2*, and *mucA22* alleles. The location of the duplicated sequence in *mucA2* (+ 8 bp) is indicated by arrows; triangle and ΔC denote a missing nucleotide (−1 bp) in *mucA22*. (*C*) Detection of *muc* mutations by hybridization with ASOs. PCR-amplified sequences containing *mucA* from PAO568 (*mucA2*) and PAO381 (*mucA*$^+$) were blotted onto nitrocellulose membrane and hybridized with ASO 381 or ASO 568. Under the conditions used, ASO 568 hybridizes to *mucA2* only while ASO 381 hybridizes to most *mucA* alleles. (*D*) The sequence of *mucA* mutations in CF1 (deletion of a nucleotide at 371) (*Center*) and CF8 (C-362 → T-362 transition) (*Right*) are compared to the wild-type sequence of the nonmucoid strain PAO1 (*Left*). The antisense strand is shown (nucleotides in parentheses correspond to the residues in *A*).

coid isolates were subjected to plasmid curing, PCR amplification of the *mucA* region, and DNA sequence analysis. PCR products from independently obtained mucoid derivatives were sequenced, and all displayed four G residues at positions 436–440, where the parental nonmucoid strain PAO381 (*mucA*$^+$) contained 5 G residues. This sequence was identical to the *mucA22* allele used as input in this particular gene replacement experiment. These analyses showed that replacements of the wild-type *mucA* gene on the chromosome with two different *mucA* alleles carrying frameshift mutations resulted in conversion to mucoidy.

We next examined whether the exchange of *mucA*$^+$ with *mucA2* caused activation of *algD*. Mucoidy is dependent on a strong transcriptional activation of the *algD* gene (14) encoding a key biosynthetic enzyme for the mucoid exopolysaccharide alginate. The plasmid pPAOM3 (20) containing an *algD*::*xylE* transcriptional fusion was introduced into one such mucoid strain, and the reporter gene activity was determined (Table 1). The results of such measurements indicated that the gene replacement with the *mucA2* allele resulted in a 320-fold activation of the *algD* promoter, which correlated with the levels of alginate production.

Complementation of a *mucA* Mutation by the Wild-Type *mucA* Gene Results in Suppression of Alginate Production and Nonmucoid Phenotype. Frameshift mutations in *mucA*, which result in conversion to mucoidy and activation of the *algD* promoter, suggest a negative regulatory role for the *mucA* gene product. If this is correct, then it should be possible to complement a mutation in *mucA* to nonmucoidy by the plasmid-borne wild-type *mucA* gene. To test this hypothesis, a 852-bp *Bst* YI fragment containing the *mucA* gene from the nonmucoid strain PAO1 was cloned behind the *tac* promoter on the broad-host-range plasmid pVDtac24, resulting in the construct termed "ptac-mucA$^+$." This plasmid also carries the *lacI*^{q} gene, which renders *tac* transcription dependent on the presence of the inducer IPTG. The plasmid was transferred into the mucoid strain PAO568 (*mucA2*) by triparental conjugation, and exconjugants were examined for colony morphology and alginate production on plates supplemented with 1 mM IPTG or in the absence of the inducer. The colonies grown in the absence of IPTG showed a mucoid morphology identical to that of the parental strain PAO568. When the same strain harboring ptac-mucA$^+$ was grown in the presence of 1 mM IPTG, it displayed a nonmucoid phenotype. This was accompanied by a decrease in detectable alginate production by a factor of 15 (Table 2). These experiments showed that mucoid phenotype can be complemented by a functional *mucA* gene alone, thus additionally confirming that the observed *muc* mutations are responsible for mucoid phenotype and that *mucA* plays a negative regulatory role.

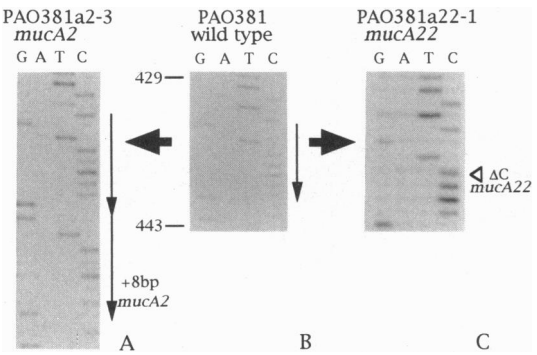

FIG. 3. DNA sequence analysis of gene replacements with the *mucA2* and *mucA22* alleles resulting in conversion to mucoidy. Allelic replacements are explained in the text. Horizontal arrows indicate the direction of gene replacements: from the nonmucoid parental strain PAO381 (*Center*) to mucoid derivatives (*Left* and *Right*). PAO381a2-3 and PAO381a22-1 are representative mucoid isolates generated by replacements of *mucA*$^+$ in PAO381 with *mucA2* and *mucA22*, respectively. Numbering and markings are as in Fig. 2.

Table 1. Conversion to mucoidy and activation of *algD* transcription as a result of gene replacement with the *mucA2* allele

Strain*	Phenotype†	Alginate production‡	*algD*::*xylE* activity, units/mg of CDO§
PAO381 (*mucA*$^+$)	NM	<0.1	0.14 ± 0.03
PAO381a2-3 (*mucA2*)	M	64.9	44.9 ± 1.0

*For transcriptional fusion measurements, all strains harbored pPAOM3 with an *algD*::*xylE* transcriptional fusion. PAO381a2-3 was one of several mucoid isolates generated by exchange of the chromosomal *mucA*$^+$ in PAO381 with plasmid borne *mucA2*.
†Phenotype was scored as mucoid (M) or nonmucoid (NM).
‡Alginate production was expressed in μg of alginate per mg of wet cell weight.
§Transcriptional activity was expressed as units of catechol 2,3-deoxygenase (CDO; the *xylE* gene product) per mg of crude protein extracts ± SEM.

Detection of *mucA* Mutations in Mucoid *P. aeruginosa* Isolates from CF Patients. To determine whether similar mutations in *mucA* occur in mucoid CF isolates, we tested several strains from different patients. Strains previously shown (16) to be complemented with plasmids carrying the *algU–mucAB* region from PAO1 were examined. Two randomly chosen strains that showed a reduced hybridization with ASO 381 were subjected to PCR and sequence analysis. These strains, CF14 and CF23, displayed a mutation identical to the *mucA22* allele (a loss of one G within the string of five G residues in the wild-type *mucA*), resulting in a premature termination of *mucA* at the same position as in PAO578 (Fig. 2*A*).

Next, the strains that did not show reduced hybridization with ASO 381 were examined. One such cystic fibrosis isolate (CF1) was subjected to the same cloning and sequencing procedure as outlined above. No changes were detected within the location of the *mucA2* and *mucA22* mutations. Instead, a deletion of a single nucleotide at the position 371 was detected (Fig. 2 *A* and *D*). This deletion was confirmed by sequencing multiple clones. This frameshift mutation also results in a premature termination of *mucA*, although at an upstream termination codon (position 396). Another CF strain from this category, CF8, was examined, and a different mutation was observed. There was a transition of C into a T (Fig. 2 *A* and *D*) at position 362 of the *mucA* sequence. Interestingly, this mutation, although not causing a frameshift, generates a stop codon (TAG) at this position (Fig. 2*A*). These results showed that mutations inactivating *mucA*, identical or similar to the *mucA* mutations responsible for conversion of PAO to mucoidy, can be observed in clinical CF isolates.

Strains that can be complemented with *algU–mucAB* from PAO1 were also found in which no mutation in *mucA* could be detected in the two general areas where *mucA2* (and *mucA22*) or mutations in CF1 (and CF8) were observed. Similar to these strains, PAO581, a mucoid PAO derivative that carries a *muc* mutation (*muc-25*) which has not been mapped by transduction (10, 22), did not have sequence

Table 2. Complementation of the *mucA2* mutation to nonmucoidy in PAO568 harboring ptac-mucA$^+$

IPTG	Phenotype	Alginate production, μg/mg of wet cell weight
—	M	59.8
1 mM	NM	3.6

IPTG was used as inducer of tac transcription. M, mucoid; NM, nonmucoid.

differences relative to its nonmucoid parent PAO381 within the regions examined here. These results are consistent with the existence of additional types of mutations within the *algU–mucAB* region or participation of additional sites or processes affecting conversion to mucoidy (10, 16).

DISCUSSION

This report identifies *mucA* as a major site for mutations causing mucoidy in *P. aeruginosa*. The *mucA* gene and a tightly linked downstream gene, *mucB*, are both required for suppression of mucoidy (16, 17). A strong activation of *algD* transcription and conversion to mucoidy are observed when *mucA* (frameshift mutations reported here) or *mucB* (17) are inactivated on the chromosome of previously nonmucoid strains provided that the first gene of the cluster (*algU*) is intact.

The gene replacements presented in this work have been performed by using sequenced mutant *mucA* alleles found in mucoid PAO derivatives and CF isolates, thus confirming that the observed *muc* mutations cause conversion to mucoidy. Complementation data further support this notion, although the ability of plasmid-borne *mucAB* to suppress mucoidy (16, 17), even in strains in which mutations may be outside *mucA*, permits additional explanations. Insertional inactivation of *mucA* (e.g., with a cassette encoding tetracycline resistance) has not been carried out, since such mutations would cause interpretation problems (e.g., polar effects) because of the presence of another negative regulator (17), *mucB*, immediately downstream of *mucA*. Upon completion of experiments presented here, a study was published (23) presenting the sequence of the *algN* gene, a negative regulator downstream of the *algST* locus, that also has been shown to play a role in the conversion to mucoidy (12). Our comparison of the two sequences indicates that *mucB* (17) and *algN* are the same genes. Incidentally, a partial nucleotide sequence of the region upstream of *algN/mucB* that overlaps with the *mucA* gene but lacks its 5′ end, was also shown, although a coding sequence corresponding to *mucA* was apparently not recognized. This displayed region in ref. 23 encompasses the sequence in which we report here five G residues in nonmucoid strains (*mucA*$^+$) but four G residues in several PAO (*mucA22*) and CF mucoid derivatives. By inspection of the presented sequence originating from the mucoid CF strain FRD (23), we have noticed that it contains four G residues and may represent the mutation causing mucoidy in that particular clinical isolate.

Mucoidy in *P. aeruginosa* has received attention mainly because of its association with CF. However, almost all *P. aeruginosa* strains have the genetic capacity to synthesize alginate, suggesting that this system must play a role in other ecological niches. The vast majority of *P. aeruginosa* biomass in nature exists as the form embedded in the exopolysaccharide biofilm attached to surfaces (24, 25). It has been shown that *P. aeruginosa* undergoes interconversions between the free-floating planktonic form and the sessile form in biofilms, a process that has been viewed as a developmental or cell differentiation phenomenon (22, 24). Regulation of alginate production by a factor (AlgU) homologous to an alternative σ factor Spo0H (σ^H), controlling the initial stages of development in *Bacillus* spp. (e.g., sporulation and competence) (18), may reflect the nature of regulatory processes controlling development of biofilms. Although the precise activities of *mucA* and *mucB* remain to be biochemically defined, the genetic evidence suggests that they suppress the function or expression of *algU*. There are now ample examples of accessory factors associated with or linked to alternative σ factors in *Bacillus* and other organisms that posttranslationally modify (e.g., inhibit) their function (26–31). By analogy, MucA and MucB may play a similar role. This system, along with signal transduction regulators and histone-like elements (15), is likely designed to control development of biofilms in response to appropriate environmental cues. Superimposed on this regulatory network, mutations in *mucA* that lock the system in its constitutive state, which is favorable because of the antiphagocytic properties of the mucoid coating (4, 9), are being selected in the course of chronic respiratory infection in CF.

In addition to the improved understanding of the molecular mechanisms controlling an important bacterial virulence factor, several aspects of the regulation of mucoidy presented here may shed some light on developmental processes in Gram-negative organisms. The finding that AlgU shows similarities with a σ factor specializing in developmental processes of a Gram-positive sporulating organism suggests that bacterial cell differentiation phenomena may share common regulatory mechanisms.

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