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Genetic Mapping of Chromosomal Determinants for the Production of the Exopolysaccharide Alginate in a *Pseudomonas aeruginosa* Cystic Fibrosis Isolate

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Mucoid *Pseudomonas aeruginosa* strain FRD, a sputum isolate from a cystic fibrosis patient, was used to develop a genetic system. The mucoid appearance is due to the biosynthesis of the exopolysaccharide alginate and is a potential virulence factor of the organism. The sex factor plasmid FP2 was used for uninterrupted genetic exchange to investigate the nature of spontaneous mutations which produce frequent alginate-negative (Alg^-) derivatives. Crosses between Alg^+ donors and Alg^- recipients demonstrated linkage between alginate genes and chromosomal markers. Crosses between an Alg^- donor and Alg^- recipients produced Alg^+ recombinants at frequencies that varied, depending on the recipient strains used. This indicated that more than one genetic locus was associated with spontaneous mutation leading to loss of the mucoid character. Three classes of Alg^- mutants were identified. Genetic exchange experiments showed that the loci of the alginate (*alg*) mutations of the three mutant classes are in the same region of the chromosome. The sex factor plasmid R68.45 was used for nonpolarized chromosome transfer and demonstrated close linkage between chromosomal markers (*his-1*, *met-1*) and *alg* markers. This was consistent with the data obtained in FP2-mediated crosses. Thus, the evidence obtained indicated that the *alg* genes which undergo frequent mutation are chromosomal, that several loci are involved, and that these *alg* loci are apparently clustered on the chromosome.

Chronic pulmonary infections by *Pseudomonas aeruginosa* are responsible for much of the morbidity and mortality associated with cystic fibrosis (2). Doggett et al. (4) have shown that patients with cystic fibrosis are colonized first with typical nonmucoid *P. aeruginosa*, but mucoid forms of these strains later emerge. The occurrence of the mucoid forms is rare in *P. aeruginosa* infections other than cystic fibrosis (3). In contrast, *P. aeruginosa* in the mucoid form is isolated from the majority of cystic fibrosis patients (4, 13). Thus, isolation of such strains from the respiratory tract is almost a diagnostic test for cystic fibrosis (20). Mucoid strain colonization also correlates with a deteriorating clinical course, suggesting that the mucoid coating possessed by the organisms plays a significant role in the pathogenesis of *P. aeruginosa* in cystic fibrosis (2, 4). Respiratory infections with heavily mucoid strains are seldom eradicated despite intensive treatment with antibiotics (17).

The mucoid appearance of *P. aeruginosa* is due to the production of alginate, an acetylated exopolysaccharide composed of 1,4-linked D-mannuronic acid and L-guluronic acid (5, 15). *P. aeruginosa* alginate is similar to the exopolysaccharide produced by *Azotobacter vinelandii* (7) and to alginates produced by certain species of brown seaweed (15), differing from the latter by containing *O*-acetyl groups. The maximum rate of exopolysaccharide synthesis by *P. aeruginosa* has been shown to occur during exponential growth (19).

Alginate-producing (Alg^+) strains of *P. aeruginosa* are unstable with respect to this characteristic and revert to nonmucoid forms at a high rate when cultured in vitro (9, 19, 23). The mechanisms underlying the instability of the Alg^+ phenotype are not completely understood. The pathway of alginate biosynthesis by *P. aeruginosa* and its genetic control are unknown. This study was undertaken to examine the genetics of alginate biosynthesis by *P. aeruginosa* and the loci associated with its in-

stability. To this end, a genetic system was established in an Alg^+ strain, *P. aeruginosa* FRD, isolated from a cystic fibrosis patient.

MATERIALS AND METHODS

Organisms and plasmids. *P. aeruginosa* strain FRD1 was a mucoid (Alg^+) isolate from the sputum of a cystic fibrosis patient. Table 1 lists derivatives of parental strain FRD1. The genetic symbol *alg* was used to designate alginate biosynthesis. *P. aeruginosa* strains carrying sex factor plasmids PAO381 *leu-38* (FP2) and PAO25 *leu-10 argF-10* (R68.45) were the kind gifts of D. Haas, Eidgenosische Technische Hochschule, Zurich, Switzerland. Strains were stored in sterile skim milk at −35°C.

Culture media. L-broth was 1.0% tryptone (Difco Laboratories), 0.5% yeast extract (Difco), 0.5% NaCl, and 0.1% D-glucose. Minimal medium was that described by Vogel and Bonner (21). A defined, alginate-promoting (AP) medium was a modification of that described by Mian et al. (19) and contained 100 mM D-gluconate, 100 mM monosodium glutamate, 7.5 mM NaH_2PO_4, 16.8 mM K_2HPO_4, and 10 mM $MgSO_4$. Amino acid supplements were at a final concentration of 1 mM. For solid media, agar (Difco) at a concentration of 1.5% was added.

Mutant isolation. To obtain alginate-deficient (Alg^-) mutants, L-broth (2 ml) in a sterile tube was inoculated by loop with a single mucoid colony and incubated at 40°C without aeration for 24 to 48 h. The culture was streaked with a loop on L-agar and incubated at 37°C overnight. An Alg^- (nonmucoid) colony was selected and purified on L-agar.

To obtain auxotrophs of *P. aeruginosa* FRD, cells were grown in L-broth at 37°C with aeration to a density of about 5×10^8 cells/ml, collected by centrifugation, and suspended in a buffer consisting of 0.2 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.0) (Sigma Chemical Co.), 70 mM K_2HPO_4, 28 mM KH_2PO_4, 7.5 mM $(NH_4)_2SO_4$, and 0.4 mM $MgSO_4$. Cells were incubated with 10 μl of ethyl methane sulfonate (Sigma) per ml at 37°C for 60 min, washed in L-broth, resuspended in L-broth, and incubated with aeration at 37°C overnight. Enrichment of auxotrophs was performed according to Watson and Holloway (22) by contraselection with 2 mg of carbenicillin per ml in minimal medium containing the supplements required for the parental strains.

TABLE 1. *Strains of P. aeruginosa FRD used*

Strain	Genotype		Origin
FRD1	Prototroph, Alg^+		This paper
FRD107	*pro-3* (FP2)		This paper
FRD113	*pro-3* (R68.45)		This paper
FRD114	*met-1 arg-1 ilv-2*		This paper
FRD120	*pro-3 alg-15* (FP2)		FRD107
FRD122	*his-1 ilv-1 arg-5*		This paper
FRD123	*his-1 ilv-1 arg-5*	*alg-16*	FRD122
FRD125	*met-1 arg-1 ilv-2*	*alg-17*	FRD114
FRD141 to FRD160	*his-1 ilv-1 arg-5*	*alg-23* to *alg-42*	FRD122

Alginate assay. Overnight cultures in AP medium (0.05 ml) were used to inoculate 10 ml of AP in 125-ml Erlenmeyer flasks. The flasks were incubated at 37°C in a rotary shaker (Lab-Line Instruments) at 180 rpm for 22 h. Culture supernatants were obtained by centrifugation at 27,000 × *g* for 30 min and extensively dialyzed against water at 5°C. The uronic acid content was measured according to the procedure of Knutson and Jeanes (14), and the amount of alginate present in a given sample was calculated from a standard curve run side by side with purified alginate (Aldrich Chemical Co.).

Construction of *P. aeruginosa* FRD strains with chromosome donor ability. Sex factor plasmids FP2 and R68.45, described previously (12), were transferred by conjugal mating from *P. aeruginosa* PAO to *P. aeruginosa* FRD. Donors and recipients were grown overnight in L-broth, and then equal volumes were mixed. The mixture (0.1 ml) was spread over the surface of an L-agar plate and incubated at 37°C for 5 h. The growth was spread with a loop onto appropriately supplemented minimal medium or AP agar containing 3 μg of $HgCl_2$ per ml for FP2 selection or 250 μg of kanamycin sulfate (Sigma) per ml for R68.45 selection. After incubation at 37°C for 3 days, isolated colonies were purified on the same medium and then purified on L-agar. Chromosome donor ability was tested in uninterrupted matings.

Uninterrupted matings. Recipient and donor cells for all matings were grown in L-broth at 37°C on a rotary shaker (180 rpm) to midexponential phase (5×10^8 cells/ml). For FP2-mediated matings, recipient cells were collected by centrifugation and suspended in one-fourth volume of L-broth. Conjugal matings were initiated by mixing 5 ml of recipient cells (2×10^9 cells/ml) with 5 ml of donor cells (5×10^8 cells/ml) in a 250-ml Erlenmeyer flask. After 90 min of incubation at 37°C without agitation, cells were collected by centrifugation at 10,000 × *g* for 15 min and suspended in 5.0 ml of saline. The mixture (0.1 ml) was spread over appropriately supplemented AP agar plates, which were incubated at 37°C for 2 to 3 days. Unmixed control cultures were treated in a similar fashion.

In R68.45-mediated matings, donor and recipient cells were collected by centrifugation and gently suspended in saline at cell densities of 1×10^9 and 4×10^9 cells/ml, respectively. Equal volumes of the cell suspensions were mixed and spread (0.1 ml) over appropriately supplemented AP agar plates. When high recombination frequency was expected, a 10^{-1} dilution of the donor was used. Plates were incubated at 37°C for 2 to 3 days. Unmixed control cultures were treated in a similar fashion.

Analysis of recombinants. Frequency of recombination was the number of recombinants for a given selected marker per 10^8 donor cells. Coinheritance of an alg^+ marker with a given selected marker was the percentage of Alg^+ recombinants among the total number of recombinants obtained. Recombinants in-

heriting the Alg$^+$ phenotype were scored on the basis of their obvious mucoid growth on AP medium. The percentage of coinheritance of unselected markers was determined by purifying recombinants on selective AP medium and then replica plating onto media selective for other markers.

RESULTS

Isolation of Alg$^-$ mutants. Alg$^-$ derivatives of *P. aeruginosa* FRD were obtained without mutagenic agents. Incubation in L-broth at 40°C without aeration apparently shifted mucoid cultures rapidly to late stationary phase, with conversion of the population to predominantly Alg$^-$ cells (approximately 50%). It was not determined whether the Alg$^-$ phenotype had a growth advantage over the Alg$^+$ type under these conditions. *P. aeruginosa* FRD culture supernatants (AP medium) contained approximately 1 mg of alginate per ml, whereas supernatants of Alg$^-$ derivatives contained no detectable alginate (<5 μg/ml) (data not shown). The spontaneous reversion of Alg$^-$ derivatives back to the Alg$^+$ phenotype was never observed among several thousand colonies examined.

Linkage of Alg markers with other chromosomal markers in FP2-mediated matings. In the development of a genetic system in *P. aeruginosa* FRD, auxotrophic markers were induced and sex factor plasmids were introduced. Recipient strain FRD123 had the *alg-16* marker in addition to the three chromosomal markers (*his-1*, *ilv-1*, and *arg-5*) of its parental strain, FRD122. Donor strain FRD107 Alg$^+$ carried the sex factor plasmid FP2, which has been shown to promote polarized chromosome transfer from one origin (12). Early markers usually show a higher frequency of recombination than later markers in polarized chromosome transfer. Thus, in the cross between FRD107 (FP2) and FRD123, the gradient in frequencies of recombination indicated that the recipient auxotrophic markers were in the gene order: *ilv-1 arg-5 his-1* (Table 2).

Recombinants were then tested for the coinheritance of unselected markers, including *alg-16*$^+$, to establish linkage relationships. A selected proximal marker coinherits unselected markers in a gradient fashion such that nearby markers are inherited more frequently than distal markers. Selected *ilv-1*$^+$ recombinants were 100% *arg-5*$^+$, and *arg-5*$^+$ recombinants were 100% *ilv-1*$^+$, thus, the two markers must be closely linked (Table 2). Of the *ilv-1*$^+$ recombinants, 48.0% were *alg-16*$^+$ and fewer were *his-1*$^+$ (45.6%). Similarly, *arg-5*$^+$ recombinants were more often *alg-16*$^+$ (55.2%) than *his-1*$^+$ (52.8%). These data indicated that *alg-16* was a chromosomal marker and part of a gradient of transmission in the gene order: *ilv-1 arg-5 alg-16 his-1*. Selection for *his-1*$^+$ resulted in coinheritance of all unselected markers, which is consistent with its terminal position in the gene order given above.

Recipient strain FRD125, the *alg-17* mutant of multiple auxotroph FRD114, was used in FP2-mediated matings with FRD107 (Table 3). The relative recombination frequencies arranged in descending order suggested the gene order: *ilv-2 met-1 arg-1*. When scored for unselected markers of the recipient, *ilv-2*$^+$ recombinants rarely inherited *met-1*$^+$ (8.3%), *alg-17*$^+$ (8.3%), or *arg-1*$^+$ (7.2%). In contrast, *met-1*$^+$ recombinants frequently inherited *alg-17*$^+$ (89.3%) and less frequently inherited *arg-1*$^+$ (83.4%). As in the previous cross, an *alg*$^+$ marker acted as a chromosomal marker with segregation to the recipient in a gradient fashion. Unlike the previous cross, *met-1*$^+$ and *arg-1*$^+$ recombinants coinherited proximal markers at frequencies of less than 100%. This presumably was because the FRD125 auxotrophic markers were more spread out on the chromosome than those of FRD123 and, thus, less likely to be included in crossovers of later markers. The above data indicated that the chromosomal markers of FRD125 were in the gene order: *ilv-2 met-1 alg-17 arg-1*.

Linkage of *alg* markers to the chromosome in R68.45-mediated matings. The possibility of chromosomal locations for *alg* markers in *P. aeruginosa* strain FRD was also analyzed with the sex factor plasmid R68.45. Unlike FP2, R68.45 promotes nonpolarized

TABLE 2. *Analysis of ilv-1*$^+$, *arg-5*$^+$, *and his-1*$^+$ *recombinants in the conjugation cross between FRD107 (FP2) and FRD123 Alg*$^-$ [a]

Selected marker	No. of recombinants/10^8 donor cells	No. of recombinants tested	% Coinheritance of unselected marker			
			ilv-1$^+$	*arg-5*$^+$	*alg-16*$^+$	*his-1*$^+$
ilv-1$^+$	80.1	250	100	100	48.0	45.6
arg-5$^+$	59.8	250	100	100	55.2	52.8
his-1$^+$	35.8	200	100	100	100	100

[a] Matings were performed in L-broth without interruption before plating on selective AP medium. Values shown represent one of three comparable experiments.

TABLE 3. *Analysis of ilv-2⁺, met-1⁺, and arg-1⁺ recombinants in the conjugation cross between FRD107 (FP2) and FRD125 Alg⁻*[a]

Selected marker	No. of recombinants/10^8 donor cells	No. of recombinants tested	% Coinheritance of unselected marker			
			ilv-2⁺	*met-1⁺*	*alg-17⁺*	*arg-1⁺*
ilv-2⁺	127	635	100	8.3	8.3	7.2
met-1⁺	41.4	290	63.4	100	89.3	83.4
arg-1⁺	7.6	53	62.3	81.1	86.8	100

[a] Matings were performed in L-broth without interruption before plating on selective AP medium. Values shown represent one of three comparable experiments.

transfer of the chromosome from multiple origins (10). The recombinants of R68.45 matings have been shown to inherit short segments of the donor chromosome, usually not exceeding 15 to 20 min (10).

In the cross between FRD113 (R68.45) and FRD123, *his-1⁺* recombinants were frequently *alg-16⁺* (48.1%), but *ilv-1⁺* recombinants were rarely *alg-16⁺* (0.7%) (Table 4). This indicated that *his-1⁺* was much closer to the *alg-16* marker than was *ilv-1*. However, *his-1⁺* recombinants coinherited *ilv-1⁺* at a frequency (17.7%), high enough to suggest that these two markers may be on opposite ends of a 15- to 20-min segment of the chromosome.

In the conjugal mating between FRD113 (R68.45) and FRD125, *met-1⁺* recombinants coinherited *alg-17⁺* at a frequency of 41.2%, which indicated close linkage between these two markers (Table 5). The *ilv-2⁺* and *arg-1⁺* recombinants were only 1.3 and 0% *alg-17⁺*, respectively, indicating no significant linkage to this *alg* marker. All recombinants of this cross had a low coinheritance frequency of unselected auxotrophic markers (0 to 4%), indicating that these markers were probably widely separated on the chromosome.

The data obtained in the above-mentioned R68.45-mediated crosses were consistent with those obtained in FP2-mediated crosses. However, in R68.45-mediated recombination, a decline in the overall recombination frequency appeared to be associated with selection for markers closely linked to *alg* markers. These local changes in recombination frequency were not apparent in the FP2-mediated crosses.

Alginate mutant classes. The Alg⁻ phenotype, which readily occurred spontaneously, could be due to mutations in one or several loci on the *P. aeruginosa* FRD chromosome. To evaluate this, 20 independent and spontaneous Alg⁻ derivatives of FRD122 were isolated. The resulting strains, FRD141-160 with markers *alg-23* to *-42*, respectively, were the recipient strains in genetic crosses. The donor was FRD120 (FP2), an Alg⁻ derivative of FRD107. Selection was made for *his-1⁺*.

TABLE 4. *Linkage analysis of the alg-16 locus in the cross between FRD113 (R68.45) and FRD123 Alg⁻*[a]

Selected marker	No. of recombinants/10^8 donor cells	No. of recombinants tested	% Coinheritance of unselected marker		
			ilv-1⁺	*alg-16⁺*	*his-1⁺*
ilv-1⁺	428	257	100	0.7	0.7
his-1⁺	53	158	17.7	48.1	100

[a] Matings were performed on AP plates. Values shown represent one of two comparable experiments.

Among the 20 Alg⁻ × Alg⁻ crosses, three recipients produced *his-1⁺* recombinants that were also Alg⁺ at a frequency of approximately 0.7% (Table 6). These three recipients were designated as class A Alg⁻ mutants. Eleven recipient strains produced recombinants that were Alg⁺ at very low or undetectable frequencies (<0.005 to 0.02%) and were designated class B Alg⁻ mutants. A group of six recipient strains had a rough colony type on L-agar that was clearly distinguishable from the smooth morphology of strains in classes A and B. In crosses with FRD120, these rough strains produced recombinants that were never observed to be Alg⁺ (<0.005%) and were designated class C Alg⁻ mutants. The donor in these crosses, FRD120, also had this rough colony appearance and was probably a class C Alg⁻ mutant.

Relative positions of *alg* genetic loci of Alg⁻ mutants. To determine the relative positions of the *alg* loci on the chromosome of Alg⁻ mutants, matings were performed between FRD107 (FP2) and representatives of the Alg⁻ classes: class A, FRD151; class B, FRD153 and FRD142; and class C, FRD146 (Table 7). When selection was made for *his-1⁺*, all recipient strains produced 100% Alg⁺ recombinants, suggesting that all *alg* markers were before *his-1* relative to the FP2 origin.

Selection was also made for the proximal marker *ilv-1⁺*. The percentage of recombinants which are Alg⁺ should be inversely related to the distance an *alg* marker is from *ilv-1*. The recipient strains of the three Alg⁻ classes pro-

TABLE 5. *Linkage analysis of the alg-17 locus in the cross between FRD113 (R68.45) and FRD125 Alg⁻*[a]

Selected marker	No. of recombinants/10^8 donor cells	No. of recombinants tested	% Coinheritance of unselected marker			
			ilv-2⁺	*met-1*⁺	*alg-17*⁺	*arg-1*⁺
ilv-2⁺	300	150	100	0	1.3	0
met-1⁺	73	294	4.1	100	41.2	0
arg-1⁺	1,070	321	1.2	1.2	0	100

[a] Matings were performed on AP plates. Values shown represent one of two comparable experiments.

TABLE 6. *Characteristics of alg marker classes in strains of P. aeruginosa FRD*

Alg⁻ mutant class[a]	FRD strains[b] (Alg⁻)	Colonial morphology on L agar	% Alg⁺ recombinants[c]
A	141, 151, 158	Smooth	0.7
B	142, 143, 144, 145, 148, 150, 153, 155, 156, 159, 160	Smooth	BD[d]–0.02
C	146, 147, 149, 152, 154, 157	Rough	BD

[a] Class of Alg⁻ mutants based on percentage of Alg⁺ recombinants.

[b] All strains shown were derived from FRD122 *his-1 ilv-1 arg-5* Alg⁺.

[c] Determined in recombination crosses with FRD120 (FP2) Alg⁻ with selection for *his-1*⁺. Values shown represent examination of approximately 20,000 recombinants per strain in two experiments.

[d] BD, Below detection (<0.005%).

TABLE 7. *Linkage relationships between alg mutations of classes A, B, and C[a] and chromosomal markers*

Recipient[b]	Alg⁻ mutant class	Marker selected	No. of recombinants/10^8 donor cells	No. of recombinants tested	% Alg⁺ recombinants
FRD151	A	*ilv-1*⁺	90.2	541	47.9
		his-1⁺	26.7	213	100
FRD153	B	*ilv-1*⁺	119.2	715	50.5
		his-1⁺	40.4	323	100
FRD142	B	*ilv-1*⁺	98.0	686	47.5
		his-1⁺	38.3	306	100
FRD146	C	*ilv-1*⁺	90.6	634	52.9
		his-1⁺	40.6	325	100

[a] Matings were performed between FRD107 (FP2) Alg⁺ and the recipients shown in L-broth without interruption before plating on selective AP medium. Values shown represent one of two comparable experiments.

[b] All recipients were derived from FRD122 *his-1 ilv-1 arg-5* Alg⁺ (see Table 6).

duced *ilv-1*⁺ recombinants that were Alg⁺ at approximately the same frequency (50%) (Table 7). These data indicated that the *alg* markers of classes A, B, and C were close to one another on the *P. aeruginosa* FRD chromosome.

DISCUSSION

The genetic basis underlying the Alg⁺ (mucoid) phenotype of *P. aeruginosa* has recently drawn considerable interest. Markowitz et al. (16) evaluated the possibility of plasmid-borne genes associated with alginate production, but could find no evidence for this. Martin (18) demonstrated induction of the mucoid phenotype with bacteriophage, but concluded that the colonial change did not result from lysogenic conversion. Fyfe and Govan (6) have recently reported the location of a chromosomal determinant of exopolysaccharide production by using a mucoid variant of *P. aeruginosa* PAO, a normally nonmucoid but genetically well-characterized strain (12).

We have undertaken the development of a genetic system in a mucoid strain, *P. aeruginosa* FRD, isolated from the sputum of a cystic fibrosis patient. It is not known whether the genetic mechanisms of alginate biosynthesis in strains pathogenic for cystic fibrosis patients have unique characteristics. Thus, it was considered preferable to use a cystic fibrosis isolate of *P. aeruginosa* for such genetic studies of alginate biosynthesis.

The results of this study showed that spontaneous *alg* mutations associated with mucoid instability are located on the chromosome of *P. aeruginosa* FRD. In FP2-mediated crosses, *alg*⁺ markers were transferred in a gradient fashion in polarized chromosome transfer, as were auxotrophic markers. Coinheritance of the Alg⁺ phenotype by the recombinants showed linkage relationships that placed *alg* markers near and between *met-1* and *his-1*. The conjugative plasmid R68.45 has been shown to mobilize the chromosome of *P. aeruginosa* in a nonpolarized fashion from multiple sites (10). The analysis of recombinants from R68.45-mediated matings between *P. aeruginosa* FRD strains confirmed that *alg*⁺ genes are chromosomal. As above, linkages to *met-1* and *his-1* were demonstrated.

Genetic methods were used to determine whether the instability of the Alg⁺ phenotype was due to one or more genetic loci. Crosses between an Alg⁻ donor and Alg⁻ recipients produced Alg⁺ recombinants at various frequencies

which depended upon the recipient strains used. Three classes of Alg^- mutants were distinguishable by these experiments. Strains designated class A Alg^- mutants produced frequent Alg^+ recombinants (0.7%), indicating that the *alg* mutations of the donor (FRD120) and recipient were far enough apart to allow genetic crossovers between them. In crosses with Alg^- donor FRD120, class B Alg^- mutants produced Alg^+ recombinants at very low or undetectable frequencies, indicating that class B *alg* mutations are too close to the donor *alg* mutation to permit frequent recombination between them. Group C Alg^- mutants were never observed to produce Alg^+ recombinants in crosses with the Alg^- donor. Group C Alg^- mutants also had a rough colonial morphology, unlike the smooth appearance of the strains in classes A and B. The association between colony type and alginate biosynthesis is uncertain at this time. However, these data suggest that a mutation which blocks the synthesis of the exopolysaccharide, alginate, may also affect synthesis of lipopolysaccharide in the cell wall.

In crosses with an Alg^+ donor strain (FRD107), representative strains of Alg^- classes A, B, and C produced Alg^+ recombinants at approximately the same frequency. This indicated that the *alg* loci of the three Alg^- mutant classes are in the same region of the chromosome. Considering the above-mentioned crosses with the Alg^- donor, these data indicate the *alg* loci of the three Alg^- classes are very closely linked and may be in a single operon or even in a single cistron. Investigations to determine whether one or more genes are affected in spontaneous Alg^- mutants are in progress. A search for general transducing phage to study the fine structure of this region of the chromosome is underway. Known transducing phages, F116 and G101 typically used for strain PAO, do not form plaques on strain FRD, and extending their host range to strain FRD by mutation has not thus far been possible (D. E. Ohman and A. M. Chakrabarty, unpublished data).

An interrupted mating system in *P. aeruginosa* FRD has not yet been developed. Nalidixic acid has successfully been used with strain PAO to counterselect against the donor for time of entry matings (11). Repeated attempts to obtain nalidixic acid-resistant mutants of strain FRD that will produce recombinant colonies in the presence of this agent have been unsuccessful. Other methods for counterselection are under investigation. The interrupted mating technique is advantageous in that it would provide a more precise location of *alg* markers with respect to other chromosomal markers and would permit comparisons of strain FRD with strain PAO with respect to the general organization of the chromosome.

A question left unanswered concerns the unstable nature of the Alg^+ phenotype. The alg^+ markers exhibit an apparently high mutability for conventional chromosomal genes. The in vitro instability of this characteristic has been well documented (8, 9, 20, 23). We have been unable to demonstrate that a bacteriophage plays a necessary role in alginate biosynthesis and its instability. Induction with mitomycin C of temperate phages carried by Alg^+ strain FRD produced a lysate which would not form plaques on Alg^- strain FRD, and conversion to the mucoid form was not observed (Ohman and Chakrabarty, unpublished data). Although no plasmids have been detected in strain FRD which might interact with chromosomal alg^+ determinants, we cannot rule out the possibility of an extrachromosomal element controlling the expression of chromosomal *alg* genes. Govan (8) provided evidence indicating that mucoid instability was due to spontaneous mutation to the nonmucoid form followed by a growth rate advantage of the nonmucoid revertants in unshaken broth cultures.

The mechanism of the spontaneous mutations leading to the nonmucoid form has not been determined. Cultures started from single Alg^+ colonies were routinely converted to predominantly Alg^- cultures in only 24 h (see Materials and Methods), suggesting that something other than simple mutation was involved. The mechanism by which mucoid strains arise in vivo is also unknown. A tenable explanation for these observations would be the involvement of transposable genetic elements in the control of alginate biosynthesis. A greater knowledge of the genes responsible for alginate biosynthesis by *P. aeruginosa* may first be required to understand the mechanisms of their control.

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