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Cloning of Genes Controlling Alginate Biosynthesis from a Mucoid Cystic Fibrosis Isolate of *Pseudomonas aeruginosa*

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Mucoid strains of *Pseudomonas aeruginosa* isolated from the sputum of cystic fibrosis patients produce copious quantities of an exopolysaccharide known as alginic acid. Since clinical isolates of the mucoid variants are unstable with respect to alginate synthesis and revert spontaneously to the more typical nonmucoid phenotype, it has been difficult to isolate individual structural gene mutants defective in alginate synthesis. The cloning of the genes controlling alginate synthesis has been facilitated by the isolation of a stable alginate-producing strain, 8830. The stable mucoid strain was mutagenized with ethyl methanesulfonate to obtain various mutants defective in alginate biosynthesis. Several nonmucoid (Alg^-) mutants were isolated. A mucoid *P. aeruginosa* gene library was then constructed, using a cosmid cloning vector. DNA isolated from the stable mucoid strain 8830 was partially digested with the restriction endonuclease *Hin*dIII and ligated to the *Hin*dIII site of the broad host range cosmid vector, pCP13. After packaging in lambda particles, the recombinant DNA was introduced via transfection into *Escherichia coli* AC80. The clone bank was mated (en masse) from *E. coli* into various *P. aeruginosa* 8830 nonmucoid mutants with the help of pRK2013, which provided donor functions in *trans*, and tetracycline-resistant exconjugants were screened for the ability to form mucoid colonies. Three recombinant plasmids, pAD1, pAD2, and pAD3, containing DNA inserts of 20, 9.5, and 6.2 kilobases, respectively, were isolated based on their ability to restore alginate synthesis in various strain 8830 nonmucoid (Alg^-) mutants. Mutants have been assigned to at least four complementation groups, based on complementation by pAD1, pAD2, or pAD3 or by none of them. Introduction of pAD1 into the spontaneous nonmucoid strain 8822, as well as into other nonmucoid laboratory strains of *P. aeruginosa* such as PAO and SB1, was found to slowly induce alginate synthesis. This alginate-inducing ability was found to reside on a 7.5-kilobase *Eco*RI fragment that complemented the *alg-22* mutation of strain 8852. The pAD1 chromosomal insert which complements the *alg-22* mutation was subsequently mapped at ca. 19 min of the *P. aeruginosa* PAO chromosome.

Pseudomonas aeruginosa is an important pathogen in patients with a variety of predisposing conditions such as extensive burns, immunosuppressive therapy, and leukemia. One of the highest incidences of *P. aeruginosa* infection, however, is seen in patients with cystic fibrosis (CF) (10, 26). An interesting aspect of the chronic respiratory infection seen in CF is the ability of *P. aeruginosa* to be isolated in various morphological forms. During the course of the disease, *P. aeruginosa* first appears with a nonmucoid colony morphology, that is, without alginate production capabilities. However, over time and with the initiation of antibiotic therapy, the nonmucoid form changes into an alginate-producing mucoid form (13). This mucoid form then predominates and is the major pathogen isolated in the sputum culture of the terminal patient. Mucoid strains of *P. aeruginosa* isolated from CF patients secrete large amounts of an exopolysaccharide known as alginic acid (14, 32). This exopolysaccharide is composed of 1,4-linked D-mannuronic acid and L-guluronic acids, contains *O*-acetyl groups, and is similar to the polymer produced by *Azotobacter vinelandii* (18) and a marine alga (28).

It is known that isolates of mucoid *P. aeruginosa* are unstable with respect to alginate production and revert to the more typical nonmucoid phenotype when cultured in vitro (19, 21, 39). The mutations conferring nonmucoidy are known to map on the chromosome near a *his* locus and presumably occur on a regulatory gene controlling the expression of alginate biosynthesis genes (17, 33). Because of the very high frequency of spontaneous mutations to nonmucoidy, it has not been possible to characterize and genetically map structural gene mutations in the alginate pathway. To gain a better understanding of the mode and control of alginate biosynthesis, we have undertaken studies to isolate mutants defective in alginate synthesis in a CF isolate of mucoid *P. aeruginosa*. In this paper, we report on the isolation of stable alginate-producing *P. aeruginosa* 8830 and the molecular cloning of three segments of chromosomal DNA which contain at least some of the genes that control alginic acid biosynthesis. This paper also describes the characterization of one of the recombinant clones which has been shown to induce alginate synthesis in spontaneous nonmucoid mutants and nonmucoid clinical and natural isolates of *P. aeruginosa*. A restriction map of the cloned insert DNA was constructed and the chromosomal segment was successfully mapped on the PAO chromosome with the help of an R68.45 R′ plasmid constructed in vivo.

MATERIALS AND METHODS

Strains and plasmids. *P. aeruginosa* 8821 is a His^- mutant derived from a mucoid isolate from the sputum of a CF patient. Strain 8822 is a spontaneous nonmucoid derivative of strain 8821. Strain 8830 is a stable alginate-producing organism derived from strain 8822. The alginate-negative (Alg^-) mutants were derived from strain 8830 after mutagenesis. The relevant genotypic characteristics and nature of the plasmids borne by strains used in this study are given in Table 1. Strain stocks were stored in sterile skim milk at −35°C.

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TABLE 1. Bacterial strains

Bacterial strain	Plasmid	Genotype	Reference
P. aeruginosa			
8821		*his-1* Alg^+	This paper
8822		*his-1 alg-1* (spontaneous)	This paper
8830		*his-1* Alg^+ (stable)	This paper
8832		*his-1 alg-2*	This paper
8835		*his-1 alg-5*	This paper
8837		*his-1 alg-7*	This paper
8838		*his-1 alg-8*	This paper
8840		*his-1 alg-10*	This paper
8846		*his-1 alg-16*	This paper
8849		*his-1 alg-19*	This paper
8852		*his-1 alg-22*	This paper
8853		*his-1 alg-23*	This paper
8855		*his-1 alg-25*	This paper
8857		*his-1 alg-27*	This paper
8858		*his-1 alg-28*	This paper
8860		*his-1 alg-30*	This paper
8861		*his-1 alg-31*	This paper
8862		*his-1 alg-32*	This paper
8863		*his-1 alg-33*	This paper
8872		*his-1 alg-42*	This paper
8873		*his-1 alg-43*	This paper
8874		*his-1 alg-44*	This paper
8882		*his-1 alg-52*	This paper
8885		*his-1 alg-55*	This paper
PAO2003		*argH32 str-39 cml-2 rec-2* FP^-	(5)
PAO25	R68.45	*leu-10 argF10* FP^-	(22)
PAO2003	pAD50	*argH32 str-39 cml-2 rec-2* FP^-	This paper
E. coli			
HB101	pLAFR1	*recA hsdR hsdM pro leu*	(16)
HB101	pCP13	*pro leu recA hsdR hsdM*	(16; personal communication)
HB101	pRK2013	*pro leu recA hsdR hsdM*	(15, 16)
AC80		*thr leu met hsdR hsdM*	(4)

Media and cultural conditions. L broth was 1% tryptone (Difco Laboratories)–0.5% yeast extract (Difco)–0.5% NaCl–0.1% D-glucose. When L broth plus maltose was required, maltose (0.4%) was substituted for glucose. Minimal medium was described by Brammer and Clarke (2), except that the trace element solution was omitted. For solid media, agar (Difco) was added at a concentration of 1.5%. Strains of *P. aeruginosa* were maintained on Pseudomonas Isolation Agar (Difco) and were supplemented with 300 μg of tetracycline per ml when necessary. Amino acids were added to a final concentration of 1 mM. Carbon sources were added to a final concentration of 50 mM.

Patch mating, which was carried out for the isolation of R′ plasmids, was performed essentially as described by Holloway (24).

Isolation of a stable alginate-producing *P. aeruginosa* strain. A single mucoid colony of strain 8821 was inoculated into 5 ml of L broth in a sterile tube and incubated at 42°C without aeration for 48 h. A sample of the culture was streaked with a loop onto an L agar plate and incubated at 37°C overnight. A single nonmucoid colony (8822) was selected and purified on Pseudomonas Isolation Agar plates. An isolated colony of strain 8822 was inoculated into 10 ml of L broth and grown at 37°C with aeration until mid-log-phase growth was obtained. The culture was centrifuged, washed in phosphate-buffered saline, and suspended in a buffer consisting of 0.2 M Tris-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 methanesulfonate (EMS; Sigma) per ml at 37°C for 1 h, washed with phosphate-buffered saline, suspended in minimal medium plus histidine, and incubated overnight at 37°C with shaking. After incubation, the culture was diluted and plated on L agar plates so that individual colonies could be screened for the presence of typically mucoid colonies.

Mutant (Alg^-) isolation. The stable alginate-producing strain 8830 was mutagenized with EMS as previously described (33). Colonies were screened on L agar plates for the appearance of nonmucoid colonies. Visibly nonmucoid colonies were picked, purified, and maintained on Pseudomonas Isolation Agar plates.

Alginate assay. Cells grown overnight in liquid media were used to inoculate 20 ml of fresh media. Incubation was carried out in 50-ml sterile Erlenmeyer flasks in a gyratory shaker (Lab-Line Instruments Inc.) at 37°C. Samples of 0.5 ml were taken at intervals and centrifuged, and the alginic acid content was estimated in the supernatants after extensive dialysis with distilled water for 18 h at 5°C. Alternatively, growth of mucoid *P. aeruginosa* from agar plates was scraped off with a glass rod and resuspended in 10 ml of water. After vortexing, the bacteria were removed by centrifugation, and the supernatant was dialyzed at 5°C with several changes of distilled water. The modified procedure of Knutson and Jeanes (27) was used to assay alginate. Purified alginic acid was purchased from Sigma.

DNA isolation. Plasmid vector DNA was prepared from 1-liter L broth cultures grown at 37°C. Preparative amounts of plasmid DNAs were isolated by using a modification (6) of the procedure of Casse et al. (3). The refractive index of the precipitated DNA solutions was adjusted to 1.3940 with CsCl (Bethesda Research Laboratories), and the DNA was centrifuged in a 50 Ti rotor at 40,000 rpm for 48 h. Plasmid bands were removed, extracted with isopropanol to remove the ethidium bromide, and dialyzed against three changes of TE buffer (10 mM Tris-hydrochloride [pH 7.5], 1 mM EDTA). Total *P. aeruginosa* DNA was isolated by the procedure of Marmur (30). DNA was finally dissolved in TE buffer, and the optical density at 260 nm was determined. Purified DNA was kept at −20°C until required.

Restriction enzymes, partial restriction digestion for cloning, and ligation. Enzymes used for digestions were *Eco*RI, *Hin*dIII, *Bgl*II, *Xho*I, *Pst*I, and *Bam*HI from New England Biolabs. Digestions were carried out according to the instructions of the supplier. The *Hin*dIII or *Hin*dIII-*Eco*RI digests of phage λ *c*I857 *S*am7 DNA were used as standards.

To determine the optimal conditions for the partial digestion of *P. aeruginosa* chromosomal DNA, reactions, each containing 4 μg of DNA in a final volume of 50 μl, were set up with a fixed amount of enzyme for variable amounts of time. Reactions were stopped with 5 μl of stop mix (7% sodium dodecyl sulfate, 1.07% bromophenol blue, 33% glycerol), and the entire volume was loaded onto a 0.7% agarose gel. Once the appropriate digestion times had been determined, partial digestions were carried out and stopped by the addition of 5 μl of 0.5 M EDTA. The DNA was extracted with 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium

citrate)–saturated phenol, extracted twice with chloroform-isoamyl alcohol (24:1), and precipitated with ethanol at −70°C. The DNA pellet was dried under vacuum, suspended in sterile water, and used for ligation with the cosmid vector pCP13 completely digested with the same enzyme.

The DNAs to be ligated were mixed at a final concentration of 300 μg/ml (vector DNA, 225 μg/ml; chromosomal DNA, 75 μg/ml). The final mix for ligation (50 μl) was adjusted to 10 mM $MgCl_2$–10 mM Tris-hydrochloride (pH 7.5), heated at 70°C for 5 min, cooled slowly at room temperature, and placed on ice for 30 min. Dithiothreitol (10 mM)–100 μM ATP–0.2 U of T4 DNA ligase was added per 5 μg of DNA. Incubation was overnight at 14°C. The ligation reaction was monitored by agarose gel electrophoresis.

Agarose gel electrophoresis and hybridization analysis. Agarose gel electrophoresis was done on 0.7% slab gels, using a horizontal electrophoresis apparatus. The gel was run, using 36 mM Tris-chloride–30 mM NaH_2PO_4–1 mM EDTA (pH 7.8) as the gel and an electrode buffer. The gel was electrophoresed for 16 h at 30 mA of constant current and stained with an electrode buffer containing 1 μg of ethidium bromide (Sigma) per ml for 20 min. The gel was visualized with the use of a UV transilluminator.

The DNA fragments were transferred onto a sheet of nitrocellulose filter (BA85; Schleicher & Schuell, Inc.) by the method of Southern (36) with an additional step: the gel was treated with 0.25 N HCl for 20 min at room temperature before the denaturation step. DNA was labeled in vitro by the nick translation procedure described by Maniatis et al. (29). The filter was prehybridized for 4 h in 5× SSPE (1× SSPE is 0.18 M NaCl plus 0.01 M NaH_2PO_4 plus 1.0 mM EDTA)–5× Denhardt reagent (8)–200 μg of denatured and sonicated salmon sperm DNA (Sigma) per ml at 65°C. Nick-translated DNA (4×10^7 cpm per filter) was added, and hybridization was carried out overnight at 65°C. The filter was washed twice in 2× SSPE–0.1% sodium dodecyl sulfate and then twice again in 0.1× SSPE–0.1% sodium dodecyl sulfate for 20 min per wash at room temperature before the filter was blotted dry and exposed to X-ray film (Kodak X-Omat AR). [α-^{32}P]dCTP was obtained from Amersham Corp.

Lambda in vitro packaging procedures. In vitro packaging of the ligated DNA was performed by using packaging extracts (Amersham) according to the recommendations of the manufacturer. Recombinant DNA molecules packaged in vitro in lambda heads were used to infect *Escherichia coli* AC80 as described by Hohn (23). Triparental matings in which *E. coli* HB101(pRK2013) (15, 16) was used as a source of the mobilizing plasmid pRK2013 were performed as described by Ruvkun and Ausubel (34).

RESULTS

Cloning strategy and isolation of a stable alginate-producing strain of *P. aeruginosa*. Since the nature of the spontaneous mutations leading to nonmucoidy is not presently known, our initial cloning strategy involved attempts to complement the spontaneous nonmucoid derivatives to the Alg^+ phenotype. Repeated attempts to complement various nonmucoid strains to Alg^+ met with failure. To overcome this problem, we sought to obtain from a mucoid strain mutagen-induced nonmucoid mutants to be used in complementation studies. The cloning of the genes controlling alginate biosynthesis, however, could still be hampered by the instability of the mucoid phenotype, so it would be advantageous to obtain a strain of *P. aeruginosa* that expresses mucoidy stably without spontaneously transforming to the nonmucoid phenotype. Such a stable strain would not only be vital in obtaining mutagen-induced nonmucoid (Alg^-) mutants for cloning purposes but might also yield some insight into the control mechanisms governing alginate synthesis.

Observations by Govan and Fyfe (20) have led to the development of a technique for the isolation of mucoid variants in vitro from nonmucoid cells based on enhanced resistance to carbenicillin. Since exposure to the alkylating agent EMS increased the frequency with which a mucoid colony could be isolated by this method, the strain 8821 nonmucoid derivative strain 8822 was mutagenized and screened for the presence of mucoid colonies. After screening ca. 10^5 colonies, several mucoid colonies were noted and isolated as pure cultures. Upon testing the stability of the mucoid phenotype in unshaken broth cultures, one strain, designated 8830, was chosen for further study because of its seemingly stable nature. When the stability of strain 8830 was compared with that of parent strain 8821, it was found that strain 8821 spontaneously produced many nonmucoid colonies (ca. 50%) after only 24 h of incubation at 42°C. However, when strain 8830 was incubated under identical conditions, it failed to give rise to nonmucoid colonies, even upon repeated and longer incubations (data not shown). The screening of 2×10^5 colonies has not revealed the presence of any spontaneously arising nonmucoid colony types. Figure 1 demonstrates the growth rates and alginate yields of strain 8830 and parent strain 8821. Whereas strain 8821 makes large quantities of alginate (1,600 μg/ml) in liquid synthetic media with glucose as the carbon source, strain 8830 surprisingly makes barely detectable levels of alginate (<20 μg/ml) in such media. The inability of strain 8830 to synthesize extracellular alginate in a liquid medium seems to be independent of the carbon source since the exopolysaccharide is not produced with any growth substrate thus far used. Strain 8830, however, is not defective in alginate synthesis on solid media and appears just as mucoid as parent strain 8821. The exopolysaccharides of both 8821 and 8830 were isolated from agar plates and subjected to the alginate assay of Knutson and Jeanes (27). The spectral scan of the exopolysaccharides in this assay from both 8821 and 8830 gave spectra typical of purified alginate, and in almost identical yield. It is clear, therefore, that although the stable mucoid strain is impaired in its ability to produce alginic acid in liquid media, it does retain the ability to synthesize this exopolysaccharide on solid media. Chemical analysis has demonstrated that the polysaccharide produced on the solid media is alginate; however the location and nature of the mutation conferring the stable phenotype has not been determined as yet.

Isolation and characterization of strain 8830 nonmucoid mutants. Nonmucoid mutants of stable strain 8830 were obtained by mutagenesis with EMS. Screening ca. 4×10^4 colonies yielded 21 nonmucoid mutants, which were recognized by their inability to produce a typically mucoid colony or alginate on solid media. Table 1 lists the mutants isolated and their numerical designation. To determine that the defect(s) affecting alginate synthesis was not a result of mutagenic lesions in the carbohydrate metabolism genes, each mutant was tested for its ability to grow on minimal medium supplemented with different carbon sources. No growth aberrations were noted when the mutants were plated on solid media supplemented with glucose, gluconate, glycerol, mannitol, fructose, glutamate, or succinate (data not shown). We therefore believe that the mutants that have been isolated are nonmucoid as a consequence of mutation(s) in the genes controlling alginate biosynthesis and not

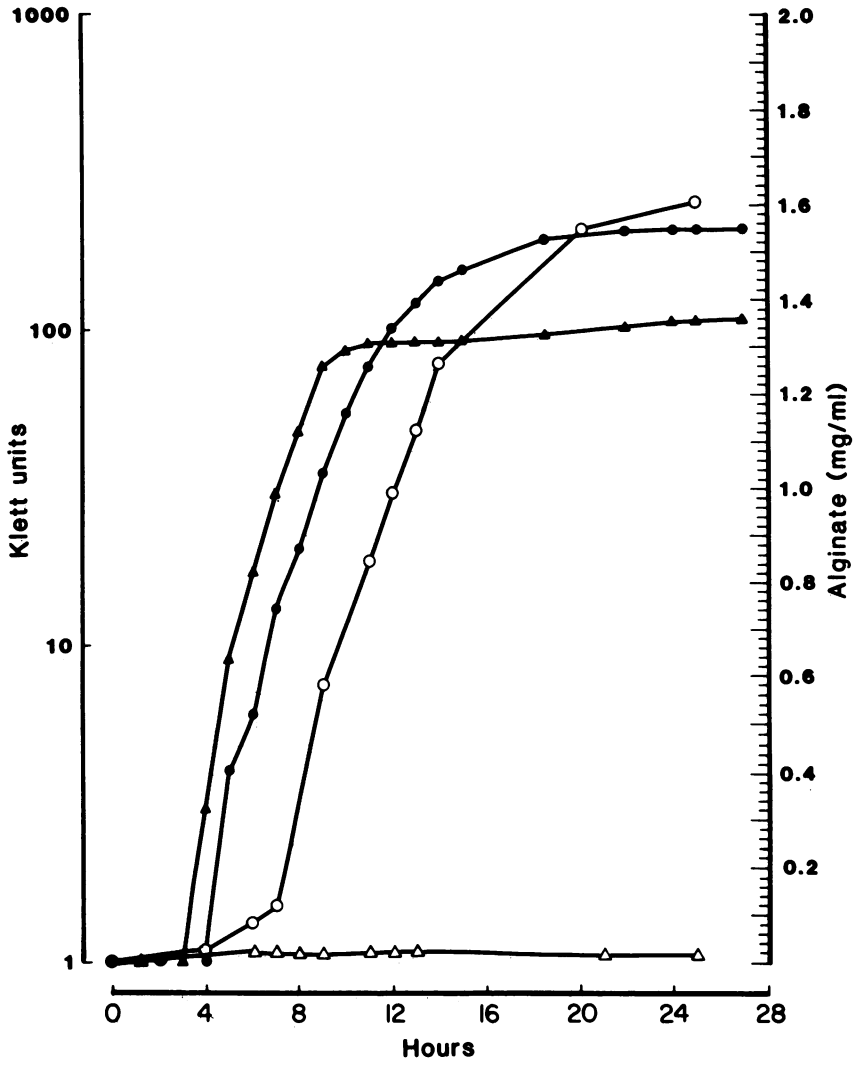

FIG. 1. Growth and alginate yields of *P. aeruginosa* strains in minimal medium plus glucose. Symbols: strain 8821 (Alg^+) growth (●) and alginate production (○); strain 8830 (Alg^+) growth (▲) and alginate production (△).

because of defects in the enzymes of carbohydrate metabolism.

Cosmid cloning of the genes controlling alginate biosynthesis. Cloning the genes controlling alginate biosynthesis was carried out with a *Hin*dIII-generated genomic library of strain 8830, using the broad host range cosmid vector, pCP13 (16; F. M. Ausubel, personal communication). This vector, derived from the cosmid vector pLAFR1 (Fig. 2), is 23 kilobases (kb) in size, confers tetracycline and kanamycin resistance, contains many unique restriction sites, and is mobilizable but not self-transmissible (Mob^+ Tra^-). The ligated DNA was packaged in vitro into lambda phage particles, and the recombinant DNA molecules were then transfected into *E. coli* AC80. The cosmid clone bank was mated with the help of plasmid pRK2013 into one of the nonmucoid mutants, strain 8852 (*alg-22*). pRK2013 (15) contains the RK2 *tra* functions and Km^r gene ligated to a ColE1 replicon and will mobilize the recombinant pCP13 derivatives into various gram-negative hosts. After mating, exconjugants were selected by tetracycline resistance and screened for the presence of mucoid colonies. A single mucoid colony was obtained after screening 2×10^4 colonies. The tetracycline resistance determinant (designated pAD1) of this mucoid colony was mobilized back into *E. coli* AC80 and then reintroduced into the nonmucoid mutant 8852. The cloned sequences contained on pAD1 are able to complement the mutant 8852 and restore the synthesis of alginate (Fig. 3). pAD1 is also capable of restoring alginate synthesis in the *alg-52* mutant 8882 (Table 2). Based on these results, mutant 8874 (*alg-44*) was chosen as the recipient for further complementation studies. Complementation of the mutant 8874 was accomplished with the isolation of the recombinant plasmid pAD2 from the strain 8830 clone bank. pAD2 restores the ability of mutant 8874 to synthesize alginate (Fig. 3), as well as restores the ability of several other nonmucoid mutants (Table 2). Based on this complementation profile, mutant 8873 (*alg-43*) was selected as a recipient for still further cloning attempts since it was not complemented with either pAD1 or pAD2. Screening of the tetracycline-resistant exconjugants after clone bank mating with mutant 8873 yielded a single mucoid colony containing a third recombinant plasmid, pAD3. The ability of pAD3 to

complement the *alg-43* mutation is shown in Fig. 3, and its ability to restore alginate synthesis in other mutants is shown in Table 2. Mutants generally fell into one of the three major complementation groups identified. Five Alg$^-$ mutants were not complemented with pAD1, pAD2, or pAD3 and may represent a fourth or other complementation groups not yet identified. In all cases, nonmucoid mutants on receiving the appropriate plasmids pAD1, pAD2, or pAD3 not only became mucoid but produced alginic acid on solid media but not in liquid media. No other polysaccharides besides alginate were made during complementation.

Restriction and homology studies with pAD1, pAD2, and pAD3. Plasmid DNA was isolated from *E. coli* AC80 containing pAD1, pAD2, or pAD3. Digestion of these plasmids with *Hin*dIII produced in all three cases a fragment that comigrates with the linearized vector and other fragments representing inserts (Fig. 4A, lanes 1 through 3). By using phage lambda *Hin*dIII fragments as size markers, it was established that ca. 20.0, 9.5, and 6.2 kb of inserted DNA were present in pAD1, pAD2, and pAD3, respectively. Each recombinant plasmid digested with *Hin*dIII liberates only a single insert fragment of a different size. Double digestion of the plasmid DNAs with first *Hin*dIII and then *Eco*RI gives the profile seen in Fig. 4A, lanes 4 through 6. pAD1 and pAD2 appear to contain a number of interior *Eco*RI sites within their cloned inserts, whereas pAD3 seems to contain only one interior *Eco*RI site. For instance, the 6.2-kb insert of pAD3 is cut into two smaller fragments of 3.5 and 2.7 kb.

The DNA fragments in Fig. 4A were transferred to a nitrocellulose filter and probed with nick-translated ^{32}P-labeled pAD1 DNA. Hybridization analysis of the three cosmid clones, using pAD1 as a probe, is shown in Fig. 4B; pAD1 is found to hybridize to the linearized vector (lanes 1 through 6) and to pAD1 sequences (lanes 1 and 4). However, no sequence homology seems to exist between the cloned fragment of pAD1 and the inserts of pAD2 or pAD3 (Fig. 4B, lanes 2, 3, 5, and 6). This experiment has been repeated, using pAD2 as a hybridization probe, and no sequence homology can be demonstrated between the cloned fragment of pAD2 and the inserts of pAD1 and pAD3 (data not shown). This suggests that genes involved in alginate synthesis are present on at least three different *Hin*dIII fragments of the strain 8830 chromosome.

Induction of alginate synthesis in a spontaneous nonmucoid strain of *P. aeruginosa*. Mobilization of pAD1 into the spontaneous nonmucoid strain 8822, followed by subsequent selection on Pseudomonas Isolation Agar-tetracycline plates yielded colonies that were visibly mucoid within 48 h at 37°C. Similar mobilizations of pAD2, pAD3, or the vector alone in such spontaneous nonmucoid cells, however, failed to generate mucoid colonies. Subcloning the pAD1 *Eco*RI fragments and introduction of the cloned fragments have revealed that this mucoid-inducing effect resides on the largest *Eco*RI fragment (7.5 kb) of pAD1 (Fig. 5). The largest *Eco*RI fragment of pAD1 was inserted into the single *Eco*RI site of pRK290 (15) to generate the recombinant plasmid pAD1000 which complements the nonmucoid mutation in strain 8852 (*alg-22*).

The cells of various nonmucoid strains such as 8822, PAO, and SB1 harboring the vector or each of the recombinant plasmids were grown for 48 h at 37°C. At the end of the growth period, the cells were washed off, resuspended, and centrifuged, and the supernatant was assayed for its alginate content by the carbazole assay (Table 3). The results demonstrate that only pAD1 and pAD1000 are able to induce alginate synthesis in spontaneous nonmucoid strains of *P. aeruginosa*. The polysaccharide isolated from these plasmid-harboring cultures has been subjected to acid hydrolysis and compared to commercial alginate treated similarly by using thin-layer chromatography. In all cases, the polysaccharide

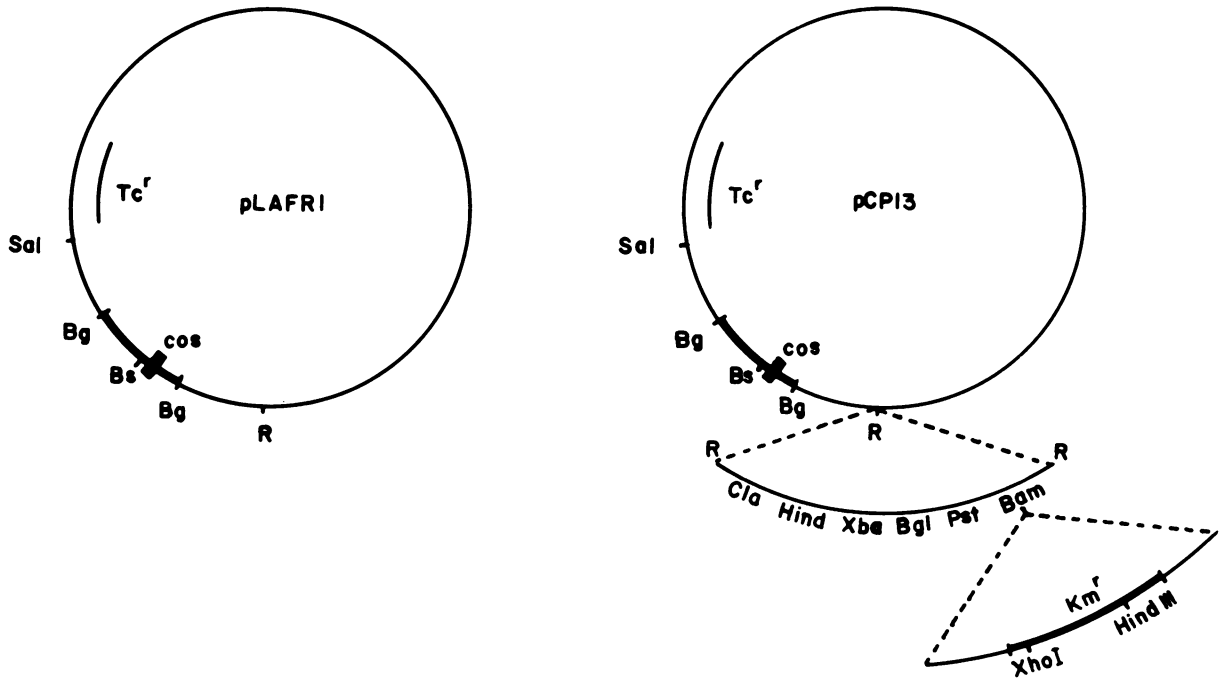

FIG. 2. Restriction maps of cosmid cloning vectors pLAFR1 and pCP13. Bam, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EII; Cla, *Cla*I; R, *Eco*RI; Hind, *Hin*dIII; Pst, *Pst*I; Sal, *Sal*I; Xba, *Xba*I; Tc^r, tetracycline resistance; Km^r, kanamycin resistance.

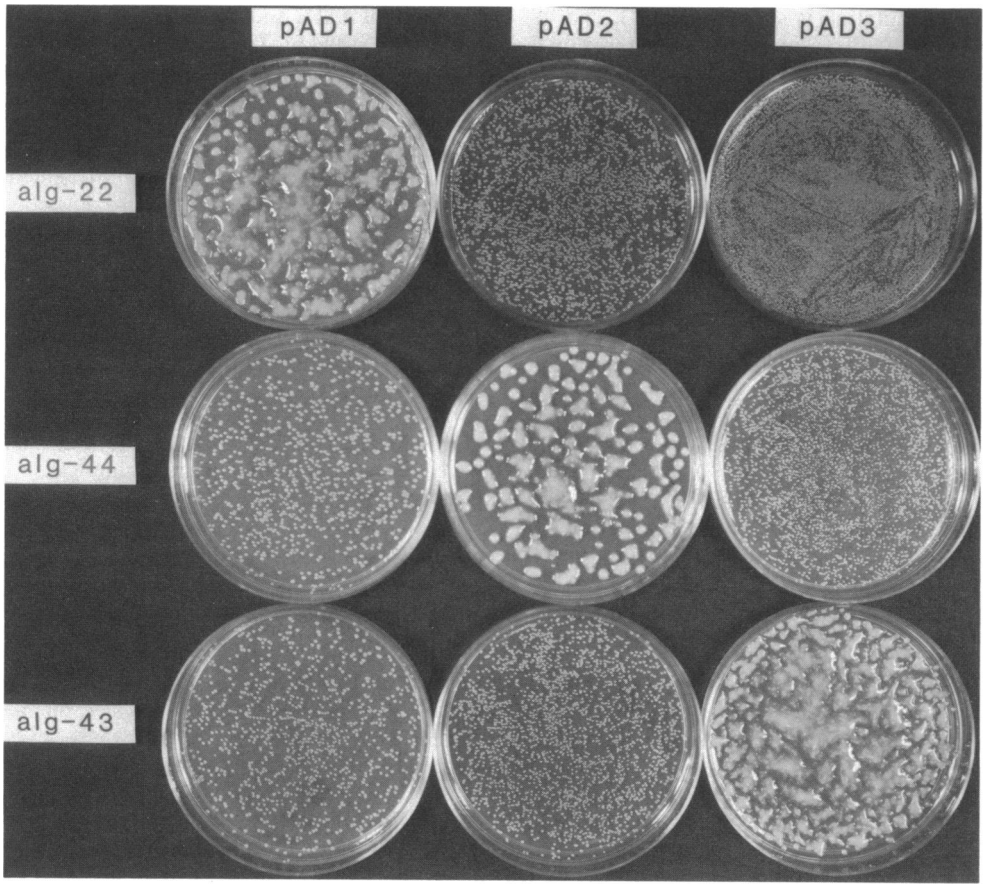

FIG. 3. Suppression of the nonmucoid (Alg^-) phenotype of *P. aeruginosa* mutants 8852 (*alg-22*), 8874 (*alg-44*), and 8873 (*alg-43*) by the introduction of the recombinant cosmids pAD1, pAD2, and pAD3, respectively.

was found to contain both mannuronic and guluronic acid residues, similar to authentic alginate (data not shown). The ability of pAD1 and pAD1000 to induce alginate synthesis is not restricted to the spontaneous nonmucoid strain 8822. Using spontaneous nonmucoid strains derived from other mucoid CF isolates and strain PAO, as well as hydrocarbon-utilizing strain SB1 isolated from oil-contaminated soil, we have been able to induce alginate synthesis in these strains, as well by using pAD1 or pAD1000. Thus various clinical or environmental isolates of *P. aeruginosa* can be complemented to the Alg^+ phenotype only by pAD1 or pAD1000 but not by pCP13, pAD2, or pAD3.

Genetic mapping of pAD1 chromosomal insert. *P. aeruginosa* PAO, although phenotypically nonmucoid, has the potential of producing alginate on selection for carbenicillin resistance (20). Therefore, PAO must have a full complement of alginate biosynthesis genes. These genes, however, are regulated in some unknown manner as they are not usually expressed. Since a fairly detailed linkage map exists for PAO, it has been possible to locate the chromosomal sequences present in pAD1 on the PAO chromosome. The pAD1 chromosomal segment was mapped by virtue of the fact that pAD1 contains the $argH^+$ allele which is located at ca. 19 min on the PAO linkage map. PAO2003, which contains the *argH* marker, was found to be phenotypically

TABLE 2. Complementation of strain 8830 Alg^- mutants

Mutation	Strain	Complementation[a] by:		
		pAD1	pAD2	pAD3
alg-2	8832	–	–	–
alg-5	8835	–	+	–
alg-7	8837	–	–	+
alg-8	8838	–	+	–
alg-10	8840	–	+	–
alg-16	8846	–	+	–
alg-19	8849	–	–	+
alg-22	8852	+	–	–
alg-23	8853	–	–	+
alg-25	8855	–	+	–
alg-27	8857	–	+	–
alg-28	8858	–	–	–
alg-30	8860	–	–	+
alg-31	8861	–	–	–
alg-32	8862	–	–	–
alg-33	8863	–	+	–
alg-42	8872	–	–	+
alg-43	8873	–	–	+
alg-44	8874	–	+	–
alg-52	8882	+	–	–
alg-55	8885	–	–	–

[a] +, Positive complementation (to Alg^+); –, lack of complementation.

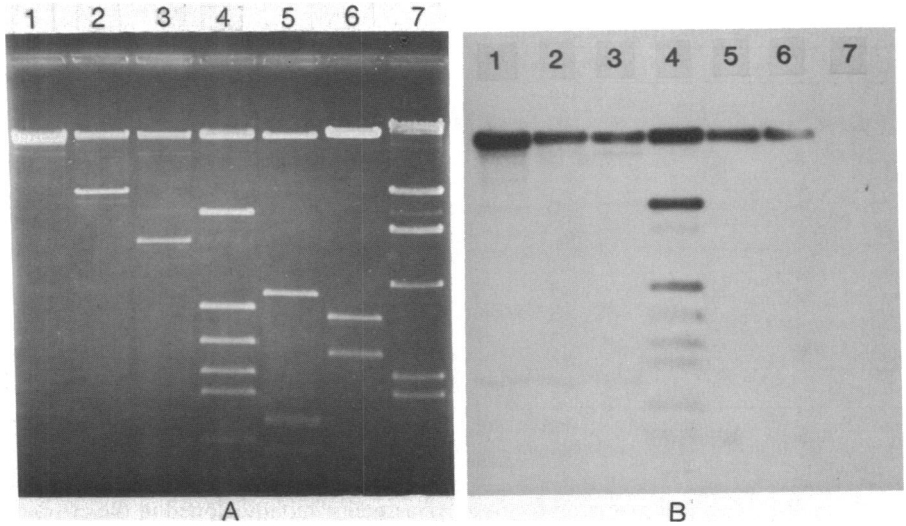

FIG. 4. Analysis of DNA from the recombinant cosmids pAD1, pAD2, and pAD3. (A) Ethidium bromide-stained gel and (B) autoradiogram of the corresponding Southern blot after hybridization with ^{32}P-labeled pAD1 as the probe. Lanes 1 through 3, single digestion of pAD1, pAD2, and pAD3, respectively, with *Hin*dIII; lanes 4 through 6, double digestion of pAD1, pAD2, and pAD3, respectively, with *Hin*dIII-*Eco*RI; lane 7, size markers of phage lambda DNA *Hin*dIII fragments.

Arg$^+$ when harboring pAD1, indicating the presence of the *argH*$^+$ allele. PAO2003 harboring the plasmid pAD1000 which contains the 7.5-kb *Eco*RI fragment of pAD1 was not complemented to Arg$^+$, suggesting that the complete *argH*$^+$ allele is not contained within the 7.5-kb *Eco*RI fragment.

To map the pAD1 chromosomal insert directly onto the PAO chromosome, it was necessary to isolate chromosomal segments from PAO homologous to pAD1. Recent advances in genetic techniques in *P. aeruginosa*, using R′ plasmids constructed in vivo from R68.45, have been very useful in characterizing long segments of DNA (24). With this in mind, R′ plasmids bearing the *argH*$^+$ gene were isolated from the Arg$^+$ transconjugants obtained by mating PAO25(R68.45) as a donor and the *argH recA* mutant PAO 2003 as a recipient. Arg$^+$ clones appeared at a frequency of ca. 10^{-8} per donor cell. A few of the colonies containing putative R′ plasmids were examined for the presence of large plasmids normally absent in PAO2003. One clone, designated PAO2003(pAD50), was shown to have similar properties as *P. aeruginosa* strains carrying R68.45, such as resistance to carbenicillin, kanamycin, and tetracycline. Plasmid DNA from the Arg$^+$ strain PAO2003(pAD50) was purified and subjected to agarose gel electrophoresis after digestion with *Eco*RI or *Eco*RI-*Hin*dIII (Fig. 6, lanes 1 and 3). The total length of the R′ plasmid pAD50 was estimated to be ca. 160 kb from the sum of the lengths of *Hin*dIII or *Bam*HI fragments (data not shown). The DNA fragments in Fig. 6A were transferred to a nitrocellulose filter and probed with nick-translated ^{32}P-labeled pAD1 DNA. The hybridization profile (Fig. 6B, lanes 3 and 4) demonstrates that the Arg$^+$ R′ plasmid pAD50 contains all of the sequences present on pAD1. Preliminary subcloning of the *Hin*dIII fragments of pAD50 has shown that the sequences homologous to pAD1 from PAO also reside on a 20-kb *Hin*dIII fragment and that this fragment also has the ability to complement PAO2003 to Arg$^+$ (data not shown).

DISCUSSION

P. aeruginosa is subject to a great deal of native genetic variation. Changes can occur in many recognizable properties of the organism, including colony morphology, pigment production, resistance to toxic agents, phage and bacteriocin susceptibility, and antigenic properties (25, 39). Another example of this unusual genetic variation is evident in mucoid, alginate-producing strains of normally nonmucoid *P. aeruginosa*. The unusual feature of the *P. aeruginosa* strains producing alginate is that they are almost exclusively found in association with respiratory tract infections accompanying CF (12, 13). It has been suggested that exopolysaccharide production by these mucoid variants provides the organism with a survival advantage in the CF lungs (9, 11). Although there is no generally accepted explanation for the emergence of the mucoid variant in vivo and dissociation to the nonmucoid form in vitro, recent studies involving genetic approaches to this medical problem have yielded interesting

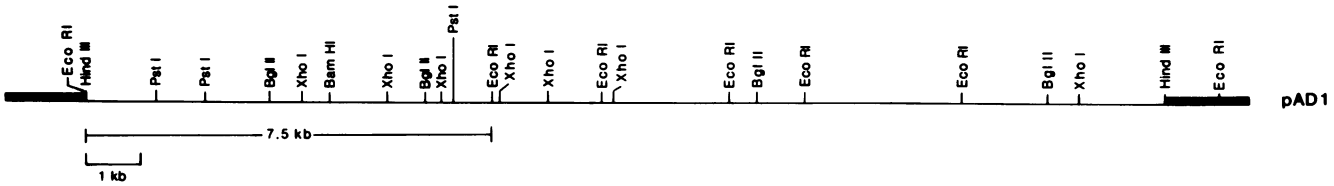

FIG. 5. Restriction map of the 20-kb *Hin*dIII fragment of pAD1. The heavy line represents the vector DNA, and the thin line represents the cloned *Hin*dIII fragment of pAD1. The length of the largest *Eco*RI fragment is marked (7.5 kb).

TABLE 3. Induction of alginate synthesis in spontaneous nonmucoid strains of *P. aeruginosa* harboring various plasmids

Strain (plasmid)[a]	Alginate (μg/ml)
8822(pCP13)	<5
8822(pAD1)	220
8822(pAD1000)	100
8822(pAD2)	<5
8822(pAD3)	<5
PAO1	<5
PAO1(pAD1)	200
PAO1(pAD2)	<5
PAO1(pAD3)	<5
SB1	<5
SB1(pAD1)	225
SB1(pAD2)	<5
SB1(pAD3)	<5

[a] Strain 8822 is a CF isolate, strain PAO is a clinical isolate that has been used widely in genetic studies (24), and strain SB1 has been isolated from oil-contaminated soil samples.

results. Fyfe and Govan (17) have demonstrated chromosomal locations for the mutations determining mucoidy in strain PAO. Similarly, Ohman and Chakrabarty (33), using a mucoid CF isolate of *P. aeruginosa*, obtained evidence that the gene(s) which undergoes frequent spontaneous mutations to nonmucoidy is chromosomal and that such mutations occur very close to one another, presumably in a single gene.

There is no direct evidence that these spontaneous mutations occur in a regulatory gene, rather than in one or more of the structural genes. The evidence presented in this paper appears to suggest that the sites are in a regulatory gene. This is because EMS mutagenesis of nonmucoid cells not only allows reversion to alginate synthesis, but the same mutation also affects the stability (subsequent mutability to nonmucoid) of the character and leads to a lack of ability of the cells to synthesize alginate in liquid media. The latter property is presumably due to altered sensitivity of one or more of the proteins controlling alginate biosynthesis to osmotic pressure, suggesting that such proteins might be membrane associated. Growth on the solid media, where the cells are not subject to osmotic pressure variations, leads to copious amounts of alginate production by such cells. An assay for cell-associated alginate during growth of the stably mucoid strain 8830 cells in liquid media demonstrates the presence of very little alginate, suggesting that lack of the polysaccharide production is not due to a defect in the secretion of the alginate but to a defect in its formation.

Recent studies in our laboratory in which sugar-negative mutants were used have demonstrated the primary role of the Entner-Doudoroff pathway enzymes in the synthesis of alginate from glucose, mannitol, or gluconate and the role of the glyceraldehyde 3-phosphate dehydrogenase reaction of the synthesis of alginate from gluconeogenic substrates such as glutamate (1). Such sugar-negative mutants affected in the various enzymes of carbohydrate metabolism can grow well on some carbon sources but fail to grow on others. The nonmucoid mutants which we have isolated from strain 8830 had no such characteristics and could grow well on all carbon sources normally used as growth substrates. We, therefore, believe that the nonmucoid phenotype demonstrated by these alginate-negative mutants is due to mutations in the genes controlling biosynthesis of alginate itself and not in any enzymes involved in carbohydrate metabolism.

The cosmid library used in this work was the product of ca. 3.2×10^5 recombinants, which is many times the number of recombinants needed to produce a clone bank with 99% probability of finding a single gene (7). The three recombinant plasmids, pAD1, pAD2, and pAD3, which seem to contain genes essential for alginate synthesis do not appear to have any sequences in common with each other based on three lines of evidence. First, each recombinant contains as an insert a single *Hin*dIII fragment of a different size. Subsequent digestion with *Eco*RI yields various numbers and sizes of fragments for each cloned insert. Second, no cross complementation of the nonmucoid mutants was seen with these plasmids, since those mutants complemented by one plasmid were not complemented by the other two. Finally, hybridization data reveals that no sequence homology exists between pAD1, pAD2, or pAD3 cloned DNA inserts. The studies reported in this paper point to the nonidentical nature of the fragments isolated from the *P. aeruginosa* clone bank. However, the rather small sizes of the pAD2 and pAD3 inserts may be due to deletions of larger fragments initially cloned in the cosmid vector, and the lack of overlapping homologous regions may be traced to such deletion formations.

In as much as pAD1, pAD2, and pAD3 do not represent overlapping sequences, it is not clear whether these fragments exist as adjacent sequences on the *P. aeruginosa* chromosome and provide for the clustering of at least some of the genes controlling alginate biosynthesis. Studies aimed at localizing the pAD1 chromosomal insert on the PAO chromosome succeeded in mapping it to ca. 19 min of the *P. aeruginosa* PAO chromosome. This was demonstrated, first, by the fact that pAD1 harbors the *argH*$^+$ allele and then by showing that an in vivo-constructed R′ plasmid (pAD50), which contains DNA from the region around the *argH* allele, contained sequences identical to those of the pAD1 chromosomal insert. The map locations of the pAD2 and pAD3 chromosomal inserts, however, have not yet been established.

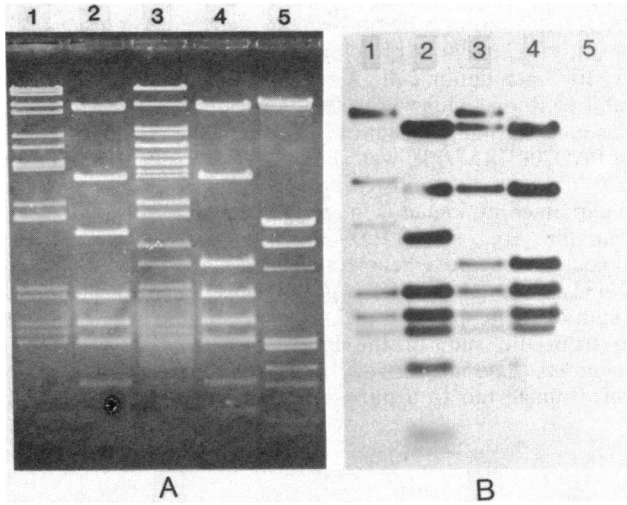

FIG. 6. Detection of pAD1 sequences on the R′ plasmid pAD50 by agarose gel electrophoresis and Southern hybridization. (A) Agarose gel electrophoresis of the recombinant plasmids pAD1 and pAD50. Lanes 1 and 2, pAD50 and pAD1, respectively, digested with *Eco*RI; lanes 3 and 4, pAD50 and pAD1, respectively, double digested with *Hin*dIII and *Eco*RI; lane 5, size markers of phage lambda DNA digested with *Eco*RI and *Hin*dIII. (B) Corresponding autoradiogram of the gel in (A) after hybridization with ^{32}P-labeled pAD1 as the probe.

Over the past few years, genetic manipulation has opened new avenues in which to examine gene control of pathogenicity and virulence factors. The native genetic variation evident in *P. aeruginosa* suggests that a genetic mechanism other than point mutation may be responsible for the frequency of the changes seen and the aspects of the phenotype involved. Recently, the importance of gene rearrangement in the process of pathogenesis has been recognized. In *Neisseria gonorrhoeae*, it has been shown that the conversion of the pilus gene from the expressed state to the nonexpressed state involves the rearrangement of segments of the chromosomal DNA of the gonococcus (31). An analogous regulation of gene expression through chromosome rearrangement is "phase variation" in *Salmonella typhimurium* (35). In this case the production of two antigenic types of flagella is controlled by the inversion of a particular segment of the bacterial chromosome. Similar genetic switches controlling inversion of the phage Mu G loop region are known (37). Finally, antigenic variation in trypanosomiasis is a sequential expression of a series of antigens on the surface of the parasite which depends on the movement of a basic copy of the surface antigen gene to a new location (38). The very high frequency of mucoid-to-nonmucoid conversion appears to suggest that a mechanism other than mutations, viz., a genetic switch similar to the inversion of Mu G region or *Salmonella* H2 antigenic phase variation region might be involved in this phenomenon.

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