JOURNAL OF BACTERIOLOGY, June 1984, p. 1115–1121
0021-9193/84/061115-07$02.00/0

Vol. 158, No. 3

Cloning and Expression in *Pseudomonas aeruginosa* of a Gene Involved in the Production of Alginate

JOANNA B. GOLDBERG AND DENNIS E. OHMAN*

Department of Microbiology and Immunology, University of California, Berkeley, California 94720

Received 23 November 1983/Accepted 21 March 1984

Pseudomonas aeruginosa strains isolated from patients with cystic fibrosis commonly produce a capsule-like exopolysaccharide called alginate. The alginate-producing (Alg^+) phenotype results in a mucoid colony morphology and is an unstable trait. A mutant of *P. aeruginosa* FRD (a cystic fibrosis isolate) was obtained which was temperature sensitive for alginate production (Alg^{ts}). At elevated growth temperatures (41°C), no alginate was detected in culture supernatants of the Alg^{ts} mutant, but yields of alginate increased as the temperature of incubation was reduced. The mutation responsible for the Alg^{ts} phenotype, *alg-50*(Ts), has been mapped to a region of the FRD chromosome closely linked to *trp-2*. The *alg-50*(Ts) marker did not map near the *met-1*-linked chromosomal mutations responsible for the instability of the Alg^+ phenotype. A broad host range cosmid cloning system based upon derivatives of plasmid RK2 was used to construct a *P. aeruginosa* clone bank. After transfer of the clone bank to the Alg^{ts} mutant, hybrid plasmids were obtained which complemented the Alg^{ts} defect. Deletion mapping of the original 20.3 kilobases of *P. aeruginosa* DNA cloned showed that a 4.7-kilobase fragment would complement the *alg-50*(Ts) mutation.

Alginate produced by *Pseudomonas aeruginosa* is an acetylated exopolysaccharide composed of 1,4-linked D-mannuronic acid and L-guluronic acid (14, 21). Alginate-producing (Alg^+) strains have a distinctive mucoid colony morphology. Alg^+ strains of *P. aeruginosa* are commonly found only in chronic respiratory infections of cystic fibrosis patients; these mucoid strains are rare in the general population of clinical *P. aeruginosa* isolates (8–10, 12). The secretion of the capsule-like material, alginate, is believed to contribute to the predilection and persistence of *P. aeruginosa* in respiratory tract infections of cystic fibrosis patients. Alg^+ organisms appear to have an increased resistance to phagocytosis (1). Alg^+ strains also exhibit reduced yields of proteases compared with their respective nonmucoid (Alg^-) derivatives, an effect which may contribute to the chronic nature of these pulmonary infections (26).

Patients with cystic fibrosis are first colonized with typical nonmucoid *P. aeruginosa*, but mucoid forms of these strains later emerge (13). Mucoid mutants of *P. aeruginosa* have also been isolated in vitro from wild-type nonmucoid strains, suggesting that the genetic information necessary for alginate production is present, though silent, in nonmucoid *P. aeruginosa* (17, 22). Alg^+ strains of *P. aeruginosa* are unstable with respect to this trait and convert to Alg^- forms when cultured in vitro (18, 24, 25). Genetic mapping of spontaneous alginate (*alg*) mutations by Ohman and Chakrabarty (25) was done in *P. aeruginosa* FRD, a sputum isolate from a cystic fibrosis patient. This study identified a region of the FRD chromosome where the spontaneous mutations occur which lead to the Alg^- phenotype.

The biosynthetic pathway for alginate in *P. aeruginosa* has not been determined. Alginate is also produced as an exopolysaccharide by *Azotobacter vinelandii*, and a pathway for its biosynthesis in this organism has been proposed (27). Studies by Banerjee et al. (2) suggest that the biosynthetic pathway of alginate in *P. aeruginosa* may not be the same as that in *A. vinelandii*. Recent studies on *P. aeruginosa* by Pugashetti et al. (28), however, indicate that GDP-mannose may be an intermediate in alginate biosynthesis as it is in the pathway in *A. vinelandii*.

This report describes a genetic characterization of an *alg* mutation located in a region of the chromosome of *P. aeruginosa* FRD and distinct from mutations involved with the instability of the Alg^+ phenotype. To study the alginate biosynthetic pathway in *P. aeruginosa*, an alginate gene (*alg-50*$^+$) was cloned in *P. aeruginosa* FRD. A cosmid cloning system, originally constructed for *Rhizobium meliloti* (16), was used in *P. aeruginosa*. This system allowed for the expression of *P. aeruginosa* DNA in *P. aeruginosa* and for technical genetic manipulations to be performed more easily in *Escherichia coli*.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are shown in Table 1.

Growth media. L-medium was 1% tryptone (Difco Laboratories), 0.5% yeast extract (Difco), and 0.5% NaCl (pH. 7.2). T-medium was L-medium without yeast extract. NY-medium was 0.8% nutrient broth (Difco) and 0.5% yeast extract (Difco). Minimal medium (VB-agar) was that described by Vogel and Bonner (31). AP-medium was a defined medium previously described (25). A minimal salts base used here was previously described (26). Media were solidified with 1.5% agar (Difco). Antibiotics were used in selection media at the following concentrations: tetracycline (Tc), 10 μg/ml for *E. coli* and 100 μg/ml for *P. aeruginosa*; kanamycin (Km), 25 μg/ml for *E. coli* and 500 μg/ml for *P. aeruginosa*; carbenicillin (Cb), 50 μg/ml for *E. coli* and 300 μg/ml for *P. aeruginosa*.

Assay for alginate. The amount of alginate present in culture supernatants was determined as previously described (26).

Mutagenesis and screening for mutants. Bacterial cultures to be mutagenized were grown in L-broth at 37°C with aeration to the early stationary phase (~8×10^8 cells per ml). Samples (2 ml) of culture were treated with 50 μl of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (5 mg/ml in ethanol)

* Corresponding author.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype[a]	Source or reference
E. coli		
HB101	*proA2 leuB6 thi-1 lacY1 hsdR hsdM recA13 rpsL20*	G. Stetler
MM294	*endoI hsdR pro*	F. Ausubel
BHB2688	N205 *recA* (λ *imm*^{434} cIts *b2 red3 Eam4 Sam7*)(λ)	L. Enquist
BHB2690	N205 *recA* (λ *imm*^{434} cIts *b2 red3 Dam15 Sam7*)(λ)	L. Enquist
P. aeruginosa		
FRD1	Alg$^+$, prototrophic, cystic fibrosis isolate	(25)
FRD40	*pro-3*	(25)
FRD45	*pro-3 alg-45*	This study
FRD110	*met-1 trp-2*	This study
FRD130	*met-1 arg-1 trp-2* Nal^r Alg$^+$ stable	This study
FRD261	*met-1 arg-1 trp-2* Nal^r *alg-50*(Ts)	This study
FRD262	*met-1 arg-1* Nal^r *alg-50*(Ts)	This study
FRD320	*met-1*	This study
FRD321	*met-1* Alg$^-$	This study
FRD322	*met-1 alg-50*(Ts)	This study
Plasmid		
pLAFR1	IncP Tc^r λ *cos*$^+$	F. Ausubel
pRK2013	ColE1-Tra(RK2)$^+$ Km^r	J. Downard
R68.45	IncP Tc^r Cb^r Km^r Tra$^+$ Cma$^+$	D. Haas

[a] Abbreviations: Tc^r, tetracycline resistance; Cb^r, carbenicillin resistance; Km^r, kanamycin resistance; Nal^r, nalidixic acid resistance; Str^r, streptomycin resistance; IncP, incompatability group P; Tra$^+$, self-transmissible; Cma$^+$, chromosome mobilization ability.

and incubated at 37°C for 10 min. Cells were washed twice in L-broth and incubated overnight in 30 ml of L-broth at 30°C with aeration. To isolate a mutant of *P. aeruginosa* which was temperature sensitive for alginate production, the mutagenized overnight cultures were diluted (1:50) in fresh L-broth and incubated at 37°C with aeration to a cell density of 5×10^8 cells per ml. This culture was diluted, spread on L-agar plates, and incubated overnight at 41°C. Nonmucoid colonies were marked and reincubated overnight at room temperature. Colonies which developed a mucoid appearance were purified and retested for alginate production at 30 and 41°C.

Genetic mapping. Construction of strains with chromosome donor ability was previously described (25). Plasmid R68.45-mediated matings were performed as previously described (25) except that recombinants were selected and purified on VB-agar supplemented with the appropriate growth factors. Coinheritance of an *alg* marker with a given selected marker was scored by its mucoid phenotype on L-agar.

Isolation of *P. aeruginosa* genomic DNA. Total DNA from *P. aeruginosa* was obtained by a modification of the procedure of Meade et al. (23). *P. aeruginosa* was incubated in L-broth at 37°C to the mid-logarithmic phase (5×10^8 cells per ml). The cells from a 40-ml culture were pelleted by centrifugation, washed twice in saline, and suspended in 10 ml of cold ET buffer (10 mM EDTA, 10 mM Tris-hydrochloride [pH 8.0]). Lysozyme (1 ml; Sigma Chemical Co.; 1 mg/ml in ET buffer) was added, and the mixture was incubated at 37°C for 15 min. Then, 1.2 ml of a Sarkosyl-pronase solution (10% Sarkosyl, 5 mg of pronase per ml in ET buffer) was added, and the mixture was incubated at 37°C for 1 h. This mixture was extracted four times with 10 ml of phenol (Aldrich Chemical Co.) previously saturated with TES buffer (10 mM Tris-hydrochloride [pH 8.0], 1 mM EDTA, 50 mM NaCl) and three times with 10 ml of chloroform-isoamyl alcohol (mixed in the ratio of 24:1). To the aqueous phase was added 1.2 ml of 3.0 M ammonium acetate. A 20-ml volume of isopropanol was layered above the mixture at room temperature. The two phases were mixed with a glass rod, thus spooling the DNA. The rod with DNA was blotted of excess liquid and placed in 4.0 ml of TE buffer (10 mM Tris-hydrochloride [8.0], 1 mM EDTA) at 5°C overnight to dissolve the DNA.

Isolation of plasmid DNA. Plasmid DNA was isolated from *E. coli* by the method of Birnboim and Doly (3). Plasmid DNA from *P. aeruginosa* was isolated by a procedure based on methods developed by Casse et al. (5). *P. aeruginosa* was grown at 37°C for 18 h in 500 ml of L-broth with maximum aeration. Cells were pelleted by centrifugation, washed in saline, and suspended in 10 mM Tris-hydrochloride (pH 8.0) to a concentration of 0.1 g of wet pellet per ml. A 5-ml sample of cells was rapidly mixed with 100 ml of freshly prepared lysis buffer (1% sodium dodecyl sulfate, 1 mM EDTA, 10 mM Tris [pH 12.45]), stirred for 5 min at room temperature, and incubated for 10 min in a 37°C bath. Twenty milliliters of 2.0 Tris-hydrochloride (pH 7.0) was added to the lysate at room temperature, with stirring for 5 min. Solid NaCl was added to 3%, and the mixture was incubated for 20 min at room temperature. The mixture was extracted with 100 ml of phenol (previously saturated with TES buffer) by stirring for 15 min and centrifuging at 8,000 × *g* for 15 min. The clear aqueous upper phase was treated with 100 ml of chloroform-isoamyl alcohol (24:1) by stirring for 10 min and centrifuging at 8,000 × *g* for 10 min. The upper aqueous phase was removed and mixed with a 0.1-volume of 3.0 M sodium acetate. Two volumes of cold (−20°C) 95% ethanol were added and incubated at −20°C overnight. The DNA was pelleted by centrifugation at 8,000 × *g* for 30 min at 5°C and then suspended in TE buffer.

Enzymes. Restriction endonucleases *Eco*RI and *Hin*dIII, as well as T4 DNA ligase, were purchased from New England BioLabs. The conditions for enzyme reactions were as specified by the manufacturer.

Construction of a *P. aeruginosa* FRD1 clone bank. *P. aeruginosa* FRD1 genomic DNA was partially digested with *Eco*RI to yield fragments predominantly in the range of 15 to 30 kilobases (kb). Restriction fragments were separated by electrophoresis (4 h, 100 V) in a horizontal agarose gel apparatus, using 40 mM Tris-acetate (pH 8.0)–8 mM sodium acetate–1 mM EDTA electrophoresis buffer. DNA fragments were visualized by staining with ethidium bromide (1 μg/ml) and photographed in short-wavelength UV light. Partially digested genomic DNA (75 μg) was layered onto each of two 11-ml linear gradients of 10 to 40% sucrose (Schwartz/Mann) in 20 mM Tris-hydrochloride (pH 8.0)–10 mM EDTA–50 mM NaCl. Centrifugation was for 16 h at 23,000 rpm in a SW41 Ti rotor at 25°C. Fractions (0.5 ml) were collected from the bottom of the tube. Samples (50 μl) of each fraction were monitored for DNA size on a 0.5% agarose gel. Those containing molecules predominantly 15 to 20 kb in size were pooled for construction of the clone bank. This DNA was dialyzed against TE buffer and concentrated by ethanol precipitation as follows. A 0.1-volume amount of 3.0 M sodium acetate was added, followed by 2.5-volumes of cold (−20°C) ethanol. The mixture was incubated in a dry ice-ethanol bath for 30 min (or at −20°C overnight). DNA was pelleted by centrifugation at 12,000 × *g* for 15 min at 5°C and then suspended in TE buffer (100 μl).

Cosmid cloning vector pLAFR1 (16) was isolated from *E. coli*, extracted with phenol, and purified by ethidium bromide-cesium chloride centrifugation. *P. aeruginosa* FRD1 chromosomal *Eco*RI fragments (10 μg) were mixed with pLAFR1 (2 μg) previously digested to completion with *Eco*RI and were ligated in a total volume of 25 μl with DNA ligase at 16°C for 18 h. The DNA in this mixture was extracted with chloroform-isoamyl alcohol (24:1), precipitated with ethanol (as described above), and dissolved in 10 μl of 10 mM Tris-hydrochloride (pH 7.8). This DNA was packaged into phage λ particles in vitro. Lambda packaging extracts were prepared by the method of Hohn (20), using the λ lysogenic strains BHB2688 and BHB2690. Samples of packaging extracts were stored at −70°C and thawed on ice immediately before use. Three microliters (~0.5 μg) of the DNA to be packaged was added to the extracts, mixed gently, and incubated at room temperature for 1 h. The reaction was stopped by the addition of 300 μl of λ dil buffer (10 mM Tris-hydrochloride [pH 7.5], 10 mM $MgSO_4$, 50 mM NaCl, 0.01% gelatin) and 2 drops of chloroform and vortexed for 1 s. The tube was spun in a microfuge (12,000 × *g*) for 3 min, and the supernatant was stored at 4°C until use.

Clone bank recipient, *E. coli* HB101, was grown overnight in T-broth containing 0.2% maltose at 30°C. Cells were pelleted by centrifugation and suspended in 0.5 volume of L-broth containing 10 mM $MgSO_4$. The packaging mixture of lambda particles containing recombinant DNA molecules was added to 1 ml of strain HB101 and incubated for 10 min at room temperature to allow infection. The mixture was transferred to 3 ml of L-broth and incubated at 37°C with aeration for 45 min to allow expression of the vector-encoded Tc resistance gene. The culture (0.1 ml) was spread onto plates of NY-agar containing Tc and incubated overnight at 37°C. Colonies (~300 per plate) were scraped from 50 plates and suspended in T-broth (~2 × 10^9/ml), glycerol was added to 10%, and samples (2 ml) were stored at −70°C.

Transfer of recombinant DNA to *P. aeruginosa*. Matings were performed as follows, using the conjugative properties of pRK2013 (15) to mobilize pLAFR1-recombinant DNA molecules to *P. aeruginosa*. *E. coli* MM294(pRK2013) and the mixture of *E. coli* HB101 containing the pLAFR1-FRD1 clone bank were grown overnight at 30°C in L-broth containing Km and Tc, respectively. Samples (0.1 ml) of the two *E. coli* cultures were mixed in 2 ml of L-broth with a sample (0.1 ml) of a culture of *P. aeruginosa* grown to the stationary phase in L-broth. Suspensions were filtered onto 0.45-μm filters (Millipore Corp.) and incubated on the surface of an L-agar plate (cell-side up) at 30°C overnight. Cells were suspended in 5 ml of saline and spread on VB-agar or AP-agar; both media contained Tc and the appropriate growth factors for the selection of *P. aeruginosa*. Plates were incubated for 2 to 3 days at 37°C (or at 41°C where indicated below).

Genetic complementation analysis. Merodiploid *P. aeruginosa* strains were constructed by conjugating pLAFR1-derived plasmids into *P. aeruginosa* FRD261 carrying *alg-50*(Ts), a temperature-sensitive mutation in a gene for alginate production (described below). To isolate clones from the pLAFR1-FRD1 clone bank which complemented the *alg-50*(Ts) mutation in *trans*, matings were plated on AP-agar containing Tc and incubated at 41°C for 3 days. Those colonies exhibiting a mucoid appearance were purified and reexamined for the Alg^+ phenotype after incubation at 41°C on L-agar containing Tc. Plasmid DNA molecules were isolated from transconjugants which possessed an Alg^+ phenotype at the nonpermissive temperature (41°C) and were used to transform calcium-treated (7) *E. coli* HB101 to Tc^r. For routine complementation assays with purified clones of *Pseudomonas* DNA in *E. coli*, matings with strain FRD261 were plated on VB-agar containing Tc and incubated for 2 to 3 days at 37°C. Colonies were purified on the same medium. The transconjugants were streaked onto two plates of L-agar containing Tc and incubated overnight at 30 and 41°C. These strains were examined for Alg^+ at 30°C (permissive temperature) and scored for Alg^+ at 41°C (nonpermissive temperature).

Southern blot hybridization of cloned DNA with FRD1 genomic DNA. Recombinant plasmid DNA was nick translated, ^{32}P labeled (29), and hybridized to a Southern blot (30) of *Eco*RI-digested *P. aeruginosa* FRD1 DNA.

RESULTS

Isolation of a mutant temperature sensitive for alginate production. *P. aeruginosa* FRD, like most mucoid (Alg^+) strains isolated from cystic fibrosis patients, forms Alg^- derivatives upon subculture without mutagenic agents (25). To isolate Alg^- mutants which were the result of mutagenesis, it was advantageous to reduce the background of naturally occurring Alg^- mutants. *P. aeruginosa* FRD130 was a mutant identified as very stable with respect to the Alg^+ trait, in that it rarely formed spontaneous Alg^- derivatives (D. E. Ohman and A. M. Chakrabarty, unpublished data). Strain FRD130 was mutagenized, and surviving cells were screened for the formation of nonmucoid colonies at 41°C which became mucoid upon reincubation at lower temperatures. Strain FRD261 was detected (among ca. 5,000 colonies examined) as having a mutation [*alg-50*(Ts)] which conferred a temperature-sensitive phenotype for the production of alginate (Alg^{ts}); it had a typical Alg^+ colony morphology when grown at 30°C and a typical Alg^- colony morphology when grown at 41°C.

Genetic mapping of *alg-50*(Ts) on the *P. aeruginosa* FRD chromosome. The linkage of the *alg-50*(Ts) marker with selectable chromosomal markers was examined in R68.45-mediated matings. Plasmid R68.45 promotes nonpolarized transfer of the *P. aeruginosa* chromosome from multiple origins, and recombinants generally inherit only short segments (~10 to 20 min) of the donor chromosome (19). In the cross between strains FRD40(R68.45) and FRD261 (Table 2), *trp-2*$^+$ recombinants were frequently (75%) *alg-50*$^+$ (i.e., no longer Alg^{ts}). The *met-1*$^+$ and *arg-1*$^+$ recombinants rarely coinherited *alg-50*$^+$ from the donor (8 and 2%, respectively). Selection for repair of any one of the three auxotrophic markers rarely led to coinheritance of either of the other two donor alleles (Table 2), indicating that the three chromosomal markers were widely separated on the FRD chromosome. These results indicated that the *alg-50*(Ts) marker was closely linked to *trp-2* on the

TABLE 2. Linkage of *alg-50*(Ts) to *trp-2* in the cross between strains FRD40(R68.45) Alg^+ and FRD261 Alg^{ts}

Selected marker[a]	% Coinheritance of unselected marker[b]			
	met-1$^+$	*arg-1*$^+$	*trp-2*$^+$	*alg-50*$^+$
met-1$^+$	100	8	8	8
arg-1$^+$	0	100	2	2
trp-2$^+$	4	5	100	75

[a] Matings were performed on VB-agar.

[b] Values shown represent one of three comparable experiments in which 100 recombinants were tested. Marker *alg-50*(Ts) was scored as Alg^+ at 30°C and Alg^- at 41°C.

TABLE 3. Analysis of the recombinants in the cross between strains FRD45(R68.45) Alg$^-$ and FRD261 Alg^{ts}

Selected marker[a]	% Coinheritance of unselected marker[b]				
	met-1$^+$	*arg-1*$^+$	*trp-2*$^+$	*alg-45*	*alg-50*$^+$
met-1$^+$	100	0	1	54	0
arg-1$^+$	0	100	1	0	5
trp-2$^+$	1	0	100	0	70

[a] Matings were performed on VB-agar.

[b] Values shown represent one of three comparable experiments in which 100 recombinants were tested. Marker *alg-50*(Ts) was scored as Alg$^+$ at 30°C and Alg$^-$ at 41°C. Marker *alg-45* was scored as Alg$^-$ at all temperatures.

P. aeruginosa FRD chromosome. In contrast, previous studies showed that *alg* mutations associated with the spontaneous conversion to the Alg$^-$ phenotype were located near the *met-1* marker (25).

We further examined the two distinct chromosomal regions (i.e., *met-1* linked and *trp-2* linked) which contained *alg* markers in the following recombinant analysis. The cross above was repeated but with a spontaneous Alg$^-$ derivative of donor strain FRD40(R68.45). The resulting donor strain, FRD45(R68.45), had the spontaneous (i.e., *met-1*-linked) *alg-45* marker. In the conjugal mating between strains FRD45(R68.45) and FRD261, *trp-2*$^+$ recombinants again coinherited the wild-type allele (*alg-50*$^+$) at a high frequency (70%), even though the donor was Alg$^-$ (Table 3). The *met-1*$^+$ recombinants again did not coinherit *alg-50*$^+$ but did inherit the *alg-45* marker (i.e., Alg$^-$ at any temperature) at a high frequency (54%).

Strain construction with the *alg-50*(Ts) marker. Strain FRD261 Alg^{ts} was derived from strain FRD130, a mutant which was alginate stable. To determine whether the *alg-50*(Ts) marker had the same phenotype in a wild-type Alg$^+$ strain as in an alginate-stable mutant, the *alg-50*(Ts) marker was transferred in an R68.45-mediated mating to the wild-type background in the following manner. A donor strain, FRD262, was constructed by isolating a spontaneous *trp-2*$^+$ derivative of strain FRD261, and then plasmid R68.45 was transferred to it by conjugation. Recipient strain FRD110 was Alg$^+$ and had the *trp-2* marker. In the cross between strains FRD262(R68.45) and FRD110, *trp-2*$^+$ recombinants were found to coinherit the *alg-50*(Ts) marker at a high frequency (60%) and to express the Alg^{ts} phenotype (data not shown). One recombinant of this cross (strain FRD322) with the *alg-50*(Ts) marker was purified and used in the following experiments.

Effects of temperature on alginate production. The *alg-50*(Ts) marker was conveniently scored on L-agar plates as mucoid and nonmucoid growth at 30 and 41°C, respectively. The following experiment verified that the Alg^{ts} phenotype on agar correlated with quantitative changes in the production of alginate. Isogenic strains with the Alg$^+$, Alg^{ts}, and Alg$^-$ phenotypes were incubated in L-broth with maximum aeration at various temperatures to saturation (24 h), and yields of alginate in culture supernatants were measured. Alg$^+$ strain FRD320 produced alginate at all temperatures, with maximum yields of alginate (752 μg/ml) when incubated at 30°C (Table 4). Strain FRD322 Alg^{ts} (described above) produced no detectable alginate at 41°C, but yields of alginate increased as the temperature of incubation was lowered. At 25°C, strain FRD322 Alg^{ts} produced more alginate than did strain FRD320 Alg$^+$. As expected, strain FRD321 Alg$^-$ produced no detectable alginate at any temperature. The growth rates of the Alg$^+$, Alg^{ts}, and Alg$^-$ strains in L-broth (measured in units of turbidity) during the logarithmic phase were indistinguishable; final cell densities were the same in Alg$^+$ and Alg^{ts} cultures at each temperature of incubation, and the Alg$^-$ cultures generally grew to slightly higher cell densities at the respective incubation temperatures (data not shown). The possibility that the *alg-50*(Ts) mutation could be a defect in central metabolism was examined. Strains FRD320, FRD321, and FRD322 were examined for the ability to grow on agar media containing minimal salts and the following carbon sources at a final concentration of 30 mM: glycerol, D-fructose, D-mannitol, D-glucose, D-gluconate, L-glutamic acid, and succinate. All strains, including FRD322, used these compounds as sole carbon sources for growth at 41 as well as 30°C.

Molecular cloning of the *alg-50*$^+$ gene by genetic complementation in *P. aeruginosa*. A versatile cloning system originally developed for *Rhizobium* strains was used for *Pseudomonas* strains. This system consists of two plasmids derived from RK2, a plasmid of incompatability group P-1 that is very similar, if not identical, to plasmids RP1, RP4, and R68 (4). A derivative of RK2, pRK290, had been developed by Ditta et al. (11) as a broad host range cloning vector. Plasmid pRK290 is 20 kb in size, confers Tc resistance, and is mobilizable but not self-transmissible. Another derivative of RK2, pRK2013, had been developed by Figurski and Helinski (15) and consists of a ColE1 replicon containing the RK2 conjugal transfer functions. Plasmid pRK2013 mobilizes pRK290 in *trans* to many gram-negative recipients (11). Recently, Friedman et al. (16) converted pRK290 to a cosmid (pLAFR1) by ligating into it a DNA fragment containing the λ phage *cos* site. A pLAFR1 clone bank of *P. aeruginosa* FRD1 DNA was constructed for the isolation of the *alg-50*$^+$ gene (see above). An average insert size of ca. 23 kb was observed as described by Friedman et al. (16) for pLAFR1. Assuming that the *P. aeruginosa* FRD genome size is equivalent to that of *E. coli* (4,100 kb), this pLAFR1-FRD1 clone bank would only need to contain ca. 820 members to guarantee 99% completeness (6, 16).

The pLAFR1-FRD1 clone bank was mated (en masse) into FRD261 Alg^{ts}, and Tc^r transconjugants were selected. Mating mixtures were plated on AP-agar, a defined medium shown to enhance the Alg$^+$ phenotype (26). Plates were incubated at 41°C where FRD261 is normally nonmucoid. The complementation of the *alg-50*(Ts) mutation by 5 clones (among ca. 3,000 examined) was detected by their mucoid appearance at the elevated temperature. Plasmid DNA molecules (designated pJG1 to pJG5) were prepared from each clone-containing *P. aeruginosa* and used to transform *E. coli* HB101 to Tc^r. There was no apparent change in

TABLE 4. Effect of temperature on alginate production by Alg$^+$, Alg$^-$, and Alg^{ts} strains of *P. aeruginosa* FRD

Strain	Phenotype	Alginate concn (μg/ml) at[a]:			
		25°C	30°C	37°C	41°C
FRD320	Alg$^+$	388	752	470	132
FRD322	Alg^{ts}	644	212	68	BD
FRD321	Alg$^-$	BD	BD	BD	BD

[a] All strains were initially incubated at 30°C in L-broth to a cell density of 2 × 10^8 cells per ml. Cultures were diluted (1:100) in fresh L-broth and incubated at the temperature indicated for 24 h with maximum aeration. Cultures were diluted (1:2) in saline to reduce viscosity, and cells were removed by centrifugation. Supernatants were exhaustively dialyzed against distilled water and assayed for alginate concentration. BD, Below detection.

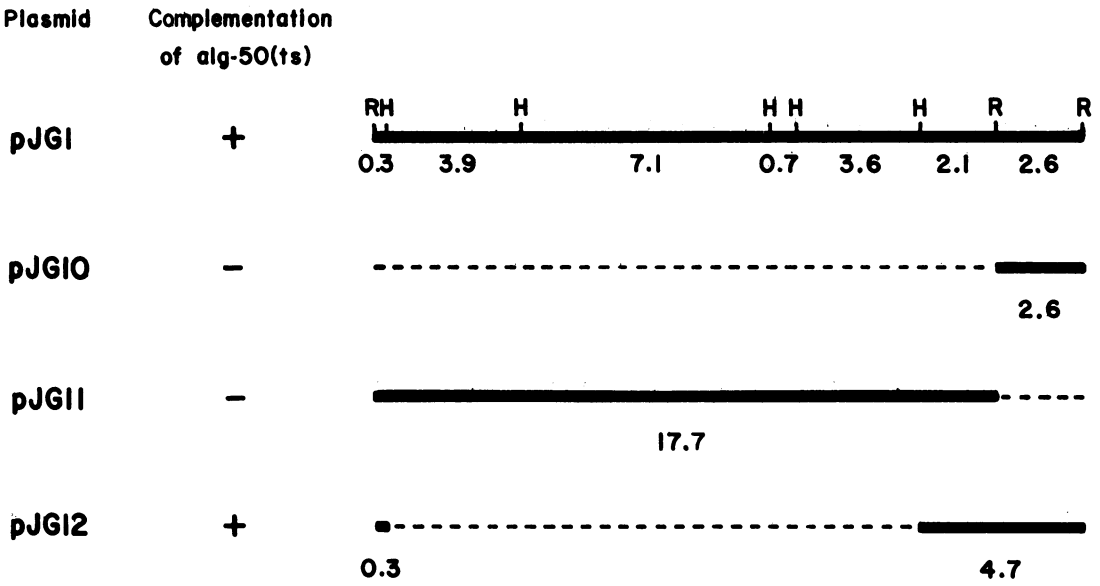

FIG. 1. Partial restriction endonuclease map of *alg-50*$^+$ DNA from *P. aeruginosa* FRD1. Restriction enzyme sites for *Eco*RI (R) and *Hin*dIII (H) are shown for the *P. aeruginosa* DNA in pJG1. DNA fragment sizes (kb) are indicated. Dashed lines in plasmids pJG10, pJG11, and pJG12 represent deletions of DNA fragments. The result (+ or −) of a complementation analysis of that plasmid with a genomic *alg-50*(Ts) mutation is shown.

the phenotype (e.g., conversion to mucoid) of *E. coli* due to these clones. Digestion with *Eco*RI of these plasmid DNA molecules from HB101 transformants showed that all five clones contained the same restriction fragments (2.6 and 17.7 kb), except that pJG5 contained an additional *Eco*RI fragment (7 kb). When the five purified clones in *E. coli* were mated back into strain FRD261, all Tc^r transconjugants were Alg$^+$ at 41°C, indicating that these clones provided genetic complementation of the *alg-50*(Ts) mutation. It was confirmed that the cloned 2.6- and 17.7-kb *Eco*RI fragments were of *P. aeruginosa* origin. ^{32}P-labeled pJG1 DNA was shown to hybridize with two restriction fragments (2.6 and 17.7 kb) on a Southern blot of *Eco*RI-digested *P. aeruginosa* FRD1 genomic DNA; no signal was detectable with *E. coli* HB101 DNA (data not shown).

Deletion mapping of *alg* DNA from *P. aeruginosa* by complementation analysis. Clone pJG1, which complements the *alg-50*(Ts) mutation, was chosen for further characterization. A preliminary restriction map of the 20.3-kb, wild-type, cloned DNA was constructed (Fig. 1). To determine the approximate location of the *alg-50*$^+$ gene in this fairly large DNA fragment, deletions of the DNA were obtained in vitro, and their effect on *alg-50*(Ts) complementation was examined. Plasmid pJG1 was partially digested with *Eco*RI; the DNA was religated and transformed into *E. coli* HB101. Tc^r transformants were screened for loss of one of the two *Eco*RI fragments by preparation of plasmid DNA and restriction analysis. Among several *Eco*RI fragment deletion derivatives isolated, pJG10 carried the 2.6-kb *Eco*RI fragment and pJG11 carried the 17.7-kb fragment (Fig. 1). When either *Eco*RI fragment deletion derivative was mated into strain FRD261, Tc^r transconjugants showed no change in the Alg^{ts} phenotype. This indicated that the expression of *alg-50*$^+$ on the cloned DNA was interrupted when the internal *Eco*RI site between the two *Eco*RI fragments of pJG1 was disrupted. The location of the *alg-50*$^+$ gene on the cloned DNA was further examined by testing *Hin*dIII fragment deletion derivatives in the complementation test. Plasmid pJG1 was digested to completion with *Hin*dIII, and the DNA was religated and then transformed into strain HB101. Plasmid pJG12 represents one of several deletion derivatives isolated in which all *Hin*dIII fragments were deleted from the cloned *P. aeruginosa* DNA. The *Hin*dIII sites in pJG1 are all in the larger (17.7-kb) *Eco*RI fragment. Thus, most (15.3 kb) of the 17.7-kb *Eco*RI fragment was deleted in the construction of pJG12. When pJG12 was mated into strain FRD261, all Tc^r transconjugants possessed the Alg$^+$ phenotype at the elevated temperature (41°C). Thus, the *alg-50*$^+$ gene and sufficient information for its expression appear to be located on a 4.7-kb (2.1 kb plus 2.6 kb) *Hin*dIII-*Eco*RI fragment (Fig. 1).

DISCUSSION

The isolation of Alg$^-$ mutants with defects in the pathway of alginate biosynthesis has been complicated by the fact that alginate production in *P. aeruginosa* is an unstable trait. Alg$^+$ strains convert to nonmucoid forms upon in vitro culture. The mutations responsible for spontaneous *alg* mutations map in the *met-1* region of the chromosome of *P. aeruginosa* FRD (25). A mutant strain which rarely formed spontaneous nonmucoid colonies was used in this study, and it was possible to obtain *alg* mutations other than those in the *met-1* region of the FRD chromosome. After mutagenesis of this alginate-stable strain, a mutant was isolated which was temperature sensitive for alginate production. The mutation conferring the Alg^{ts} phenotype, *alg-50*(Ts), was shown to map near *trp-2* and was not linked to *met-1* (Table 2). A cross was performed which involved both *alg-50*(Ts) and a spontaneous *alg* marker (Table 3), and both markers were shown to segregate to their two respective chromosomal locations. Although originally obtained in an alginate-stable strain, the Alg^{ts} phenotype was not dependent upon this genetic background. The *alg-50*(Ts) marker could be transferred to a typical Alg$^+$ strain (i.e., which produced sponta-

neous Alg$^-$ derivatives) by strain construction techniques, and the Alg^{ts} phenotype was retained.

Alginate yields in cultures of an *alg-50*(Ts)-containing strain (FDR322) were inversely related to the temperature of growth (Table 4). Alginate in culture supernatants was not detectable when strain FRD322 was grown at 41°C; yields of alginate increased as the temperature of incubation was reduced. The Alg$^+$ strain produced maximum yields of alginate when incubated at 30°C. It may be significant that the *alg-50*(Ts)-carrying strain actually produced more alginate than did the Alg$^+$ strain when the temperature of incubation was low (25°C). This suggests that the nature of the *alg-50*(Ts) mutation may involve a shift in the temperature optimum of an enzyme (or regulatory polypeptide) involved in alginate biosynthesis.

A pathway for the biosynthesis of alginate in *A. vinelandii* has been proposed (27), but the pathway which follows in *P. aeruginosa* has not been defined. A recent study on *P. aeruginosa* by Banerjee et al. (2) indicated that, unlike *A. vinelandii*, glyceraldehyde 3-phosphate was an intermediate in the biosynthesis of alginate from gluconeogenic precursors. This study examined alginate production by mutants of *P. aeruginosa* FRD with enzyme defects in central metabolism that affected the ability to utilize certain carbon sources. When *alg-50*(Ts)-containing strain FRD322 was examined in our studies for the ability to utilize these carbon sources (glycerol, D-fructose, D-mannitol, D-glucose, D-gluconate, L-glutamic acid, and succinate), growth comparable to that of the wild-type strain was observed (at 30 and 41°C). Thus, the defect in alginate biosynthesis due to the *alg-50*(Ts) mutation appeared not to be in central metabolism but, rather, was beyond the proposed glyceraldehyde 3-phosphate intermediate. Also, the growth rate and maximum cell mass of strain FRD322 at 30 and 41°C were indistinguishable from those of an isogenic strain with the *alg-50*$^+$ wild-type allele under similar conditions; this indicated that the reduced yields of alginate at elevated temperatures were not the result of a general growth defect. The role of the *alg-50*$^+$ gene in alginate biosynthesis is under investigation, using various genetic constructions, and will be the subject of a future communication.

In this report, we described the use of the cosmid pLAFR1 (16) as an *Eco*RI cloning vector in the study of alginate biosynthesis in *P. aeruginosa*. After ligation to *P. aeruginosa* DNA fragments of an appropriate size (15 to 30 kb), pLAFR1 derivatives were packaged in vitro into λ phage particles and transfected into *E. coli* at high efficiency. In the presence of the conjugative functions of helper plasmid pRK2013, this clone bank (pLAFR1-FRD1) was conjugated en masse into a recipient strain of *P. aeruginosa* FRD which contained the *alg-50*(Ts) mutation. Among the merodiploid strains thus constructed, clones carrying the wild-type allele (*alg-50*$^+$) were obtained by screening for complementation of the mutant phenotype. Complementation of auxotrophic mutations in strain FRD has also been possible with this clone bank (D. Ohman, unpublished data). Thus, this cloning system permits the expression of *P. aeruginosa* DNA in *P. aeruginosa* and is not dependent upon obtaining expression in *E. coli*.

The *P. aeruginosa* FRD1 clone bank described here contained a relatively large average fragment size of ca. 23 kb. Cloning large inserts had several advantages. Any gene of interest should be found after the examination of only about 820 random clones. Here, ca. 1 in 600 transconjugants examined contained DNA complementary to *alg-50*(Ts). A clone bank derived from a partial restriction digest made it possible to isolate genes which might otherwise have been disrupted by the restriction enzyme used. In this study, all clones which complemented *alg-50*(Ts) contained two *Eco*RI fragments. In vitro deletion of either *Eco*RI fragment of pJG1 abolished its complementation activity in *P. aeruginosa*. When most of the cloned DNA was deleted by the removal of *Hin*dIII fragments, the internal *Eco*RI site and complementation of *alg-50*(Ts) were maintained. Thus, the *alg-50*$^+$ gene was shown to reside on a 4.7-kb *Hin*dIII-*Eco*RI fragment and may be located at the internal *Eco*RI site. The pathway of alginate biosynthesis obviously requires several genes, and cloning large inserts may increase the probability of preserving potential alginate gene clusters intact. Genetic manipulations are in progress to determine whether other alginate genes are located on pJG1.

Little is currently known about the function, organization, and control of alginate (*alg*) biosynthetic genes in *P. aeruginosa*. We have found a new chromosomal locus involved in alginate production. By adapting a clone bank technology to *P. aeruginosa*, we have been able to perform a preliminary characterization of a fragment of the FRD chromosome containing at least one alginate gene, *alg-50*$^+$. Attempts are now being made to understand its regulation and to identify other genes which play a role in the biosynthesis of alginate by mucoid *P. aeruginosa*.

ACKNOWLEDGMENTS

We thank M. West and M. Lang for excellent technical assistance.

This work was supported in part by Public Health Service grant AI19146 from the National Institute of Allergy and Infectious Diseases, a grant from the American Lung Association of California, and a Biomedical Research Support Grant from the National Institutes of Health.

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