

Clustering of Mutations Affecting Alginic Acid Biosynthesis in Mucoid *Pseudomonas aeruginosa*

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A 10-kilobase DNA fragment previously shown to contain the phosphomannose isomerase gene (*pmi*) of *Pseudomonas aeruginosa* was used to construct a pBR325-based hybrid that can be propagated in *P. aeruginosa* only by the formation of a chromosomal-plasmid cointegrate. This plasmid, designated pAD4008, was inserted into the *P. aeruginosa* chromosome by recombination at a site of homology between the cloned *P. aeruginosa* DNA and the chromosome. Mobilization of pAD4008 into *P. aeruginosa* PAO and 8830 and selection for the stable acquisition of tetracycline resistance resulted in specific and predictable changes in the pattern of endonuclease restriction sites in the phosphomannose isomerase gene region of the chromosomes. Chromosomal DNA from the tetracycline-resistant transformants was used to clone the drug resistance determinant with *Bgl*II or *Xba*I, thereby allowing the "walking" of the *P. aeruginosa* chromosome in the vicinity of the *pmi* gene. Analysis of overlapping tetracycline-resistant clones indicated the presence of sequences homologous to the DNA insert of plasmid pAD2, a recombinant clone of *P. aeruginosa* origin previously shown to complement several alginate-negative mutants. Restriction mapping, subcloning, and complementation analysis of a 30-kilobase DNA region demonstrated the tight clustering of several genetic loci involved in alginate biosynthesis. Furthermore, the tetracycline resistance determinant in PAO strains transformed by pAD4008 was mapped on the chromosome by plasmid FP2-mediated conjugation and was found to be located near 45 min.

Pseudomonas aeruginosa has the ability to produce an acetylated exopolysaccharide known as alginic acid (27, 31). This polymer is composed of beta 1,4-linked D-mannuronic and L-guluronic acids and is similar to the polysaccharide produced by *Azotobacter vinelandii* (33) and the marine brown alga *Fucus gardneri* Silva (26). Alginate-producing (*Alg*⁺) strains of *P. aeruginosa* produce copious quantities of alginic acid and as a result have a distinctive mucoid colony morphology. These mucoid variants are found exclusively in association with the respiratory tract infections that accompany cystic fibrosis (12). It is this mucoid form that ultimately predominates in the cystic fibrosis-damaged lung and is the major pathogen isolated in the sputum cultures of patients in advanced stages of the disease. An interesting aspect of the mucoid phenotype is that it is unstable in vitro (18, 19), because mucoid strains revert quite readily to the more typical nonmucoid form at a high frequency.

The mapping of mutations involved in alginate biosynthesis by *P. aeruginosa* has recently been reported. Mutations responsible for the spontaneous conversion of *Alg*⁺ to *Alg*⁻ have been mapped near a *his* locus on the chromosome of *P. aeruginosa* PAO (16) and in the *met-1* region on the chromosome of *P. aeruginosa* FRD (32). Goldberg and Ohman (17) have mapped an *alg* mutation in the *trp-2* region on the chromosome of *P. aeruginosa* FRD and have shown that this mutation is distinct from those mutations involved in the instability of the *Alg*⁺ phenotype. Our laboratory has also mapped an *alg* mutation (*alg-22*) and the DNA segment that complements this mutant to about 19 min of the *P. aeruginosa* PAO chromosome (9). In addition, we have recently cloned the phosphomannose isomerase gene (*pmi*) and demonstrated the importance of this gene in alginate biosynthesis

(10). In this report, we continue our genetic characterization of alginate biosynthesis and offer evidence for the clustering of mutations affecting alginate biosynthesis on the *P. aeruginosa* chromosome in close proximity to the *pmi* gene. Furthermore, we provide information regarding the chromosomal location of the alginate gene cluster on the *P. aeruginosa* PAO chromosome.

MATERIALS AND METHODS

Bacterial strains, growth media, and genetic procedures. The bacteria and plasmids used in the manipulation of genes involved in alginate biosynthesis are listed in Table 1. *Alg*⁻ mutants used previously for complementation analysis and the procedures for the isolation of other *Alg*⁻ mutants of the stable alginate-producing strain 8830 that were new to this study have been described (9). Vogel-Bonner minimal medium (40) without citrate was used to select recombinants in all of the bacterial crosses. Stock solutions of glucose and acetamide were filter sterilized and added to the minimal medium at a final concentration of 50 mM. Required amino acids were added to the minimal medium at 50 µg/ml. All other media and the antibiotic concentrations used for selection of resistant transformants were as described previously (9, 10).

To construct FP2⁺ donor strains, strain PTO13 was used as the donor, and plasmid transconjugants were isolated and purified on selective minimal agar containing mercuric chloride (12 µg/ml). Donors for FP2-mediated conjugations were maintained on Pseudomonas Isolation Agar (Difco Laboratories, Detroit, Mich.) supplemented with tetracycline hydrochloride (Sigma Chemical Co., St. Louis, Mo.) and mercuric chloride at 400 and 30 µg/ml, respectively. Uninterrupted matings with FP2 were carried out by growing recipient and donor cells in L broth at 37°C to mid-exponential phase (5×10^8 cells per ml). Matings were

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TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Genotype or phenotype ^a	Reference
<i>P. aeruginosa</i>		
8830	<i>his-1 Alg</i> ⁺ (stable)	9
8835	<i>his-1 alg-5</i>	9
8837	<i>his-1 alg-7</i>	9
8838	<i>his-1 alg-8</i>	9
8846	<i>his-1 alg-16</i>	9
8849	<i>his-1 alg-19</i>	9
8853	<i>his-1 alg-23</i>	9
8857	<i>his-1 alg-27</i>	9
8860	<i>his-1 alg-30</i>	9
8872	<i>his-1 alg-42</i>	9
8873	<i>his-1 alg-43</i>	9
8874	<i>his-1 alg-44</i>	9
8886	<i>his-1 alg-59</i>	This study
8887	<i>his-1 alg-60</i>	This study
8888	<i>his-1 alg-61</i>	This study
8889	<i>his-1 alg-62</i>	This study
8890	<i>his-1 alg-63</i>	This study
8891	<i>his-1 alg-67</i>	This study
8892	<i>his-1 alg-69</i>	This study
8893	<i>his-1 alg-71</i>	This study
8894	<i>his-1 alg-72</i>	This study
8895	<i>his-1 alg-73</i>	This study
8896	<i>his-1 alg-75</i>	This study
8897	<i>his-1 alg-76</i>	This study
PTO13	<i>trp-6 FP2</i> ⁺	39
PAO25	<i>leu-10 argF10 FP</i> ⁻	21
PAO222	<i>ilv-226 his-4 lys-12 proA82 met-28 trp-6 FP</i> ⁻	21
PAO515	<i>met-9011 ami-151 nalA FP2</i> ⁻	R. V. Miller
PAO1042	<i>pur-67 cys-59 proB65 thr-9001 FP</i> ⁻	R. V. Miller
PAO2001	<i>argH32 str-39 cm1-2 FP</i> ⁻	7
PAO2003	<i>argH32 str-39 cm1-2 rec-2 FP</i> ⁻	7
PAO2198	<i>argG9036 leu-9014 lys-9015 met-9020 trp A,B, catA chu-9002 nar9011 FP2</i> ⁻	R. V. Miller
CH60	Tet ^r transconjugant of 8830 with pAD4008	This study
AL30	Tet ^r transconjugant of PAO25 with pAD4008	This study
AL40	Tet ^r transconjugant of PAO222 with pAD4008	This study
<i>E. coli</i>		
AC80	<i>thr leu met hsdR hsdM</i>	9
Plasmids		
FP2	Hg ^r Tra ⁺ Cma ⁺	23
pRK2013	ColE1 Tra ⁺ (RK2) Km ^r	14
pBR325	Am ^r Tc ^r Cm ^r	6
pCP13	IncP Tc ^r Km ^r	9
pMMB22	IncQ Am ^r Sm ^r <i>tac lacI</i> ^q	2
pAD2	See Fig. 3	9
pAD4001	<i>alg-43</i> ⁺	10

^a Abbreviations: Tet^r and Tc^r, tetracycline resistance; Am^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Sm^r, streptomycin resistance; Hg^r, mercury resistance; IncP, incompatibility group P; IncQ, incompatibility group Q; Tra⁺, self transmissible; Cma⁺, chromosome mobilization ability.

initiated by mixing 5 ml of recipient cells with 5 ml of donor cells in a 250-ml Erlenmeyer flask. After 90 min of incubation at 37°C without shaking, cells were collected by centrifugation and suspended in 5 ml of saline (0.85% NaCl). Portions (100 μ l) were plated on appropriately supplemented minimal agar plates and incubated for 2 to 3 days. Unmixed control

cultures were treated in a similar fashion. Triparental matings in which *Escherichia coli* HB101(pRK2013) (14, 15) was used as a source of the mobilizing plasmid pRK2013 were performed as described by Ruvkun and Ausubel (36).

Isolation and manipulation of DNA. The cloning, transformation, and DNA purification procedures used have been described previously (9, 10). Recombinant plasmids were detected by the miniscreening method of Holmes and Quigley (24). DNA fragments were isolated from 0.7% agarose gels with the aid of DEAE-cellulose membrane strips (NA-45; Schleicher & Schuell, Inc., Keene, N.H.) as described previously (10). Restriction endonucleases were purchased from New England BioLabs, Inc., Beverly, Mass. T4 DNA ligase, DNA polymerase, and calf alkaline phosphatase were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Inc. Agarose gel electrophoresis and DNA hybridization procedures have also been described previously (9).

Chemicals. Ethyl methanesulfonate, acetamide, and isopropyl- β -D-thiogalactopyranoside were purchased from Sigma.

RESULTS

Construction and insertion of pAD4008 into the *P. aeruginosa* chromosome. The purposes of this current study were twofold. First, we intended to "walk" the *P. aeruginosa* chromosome in the vicinity of the *pml* gene to isolate overlapping DNA subclones which could harbor other genes involved in alginate biosynthesis. Second, we intended to determine the genetic location of the region of DNA that contains the *P. aeruginosa pml* gene. To accomplish these goals, we took advantage of the fact that ColE1-derived plasmids such as pBR325 cannot replicate in *P. aeruginosa* (21). Similar logic was the basis for a procedure developed to replace bacterial chromosome segments with recombinant plasmid segments in *E. coli* strains defective in DNA polymerase I (*polA*) (20). With this strategy, we constructed plasmid pAD4008 by cloning a 10-kilobase (kb) DNA segment, which contains the *P. aeruginosa pml* gene, into pBR325. The 10-kb *EcoRI* fragment of plasmid pAD4001 (10) which harbors the *pml* gene toward one end of the fragment was removed from its broad-host-range replicon and cloned into the unique *EcoRI* site of pBR325. Plasmid pAD4008 is depicted at the top of Fig. 1. Because the 10-kb fragment was subcloned from a 20-kb *BamHI* cosmid clone (10), a region containing 100 base pairs of polylinker DNA from the vector pCP13 was present at one end of the DNA fragment.

Since plasmid pAD4008 will not replicate autonomously in *P. aeruginosa*, its maintenance within *P. aeruginosa* is solely dependent on its ability to integrate into the chromosome by recombination at the site of homology in the *pml* gene region. The homologous recombinational event envisioned between pAD4008 and the *P. aeruginosa* chromosome is depicted in Fig. 1. It can be seen that the integration event should have produced a direct nontandem duplication of bacterial sequences separated by pBR325 vector sequences. pAD4008 was mobilized from *E. coli*, with the help of pRK2013, into *P. aeruginosa* PAO25 and 8830. The matings were then plated on Pseudomonas Isolation Agar plates containing tetracycline (400 μ g/ml) to select for those colonies containing pAD4008 as a plasmid integrate. The transmission of pAD4008 to *P. aeruginosa* 8830 or PAO25 occurred at a relatively low frequency (approximately 10⁻⁹ transformant per recipient cell). When pBR325 was mobilized into these Rec⁺ *P. aeruginosa* strains, no tetracycline-resistant transformants could be isolated (frequency,

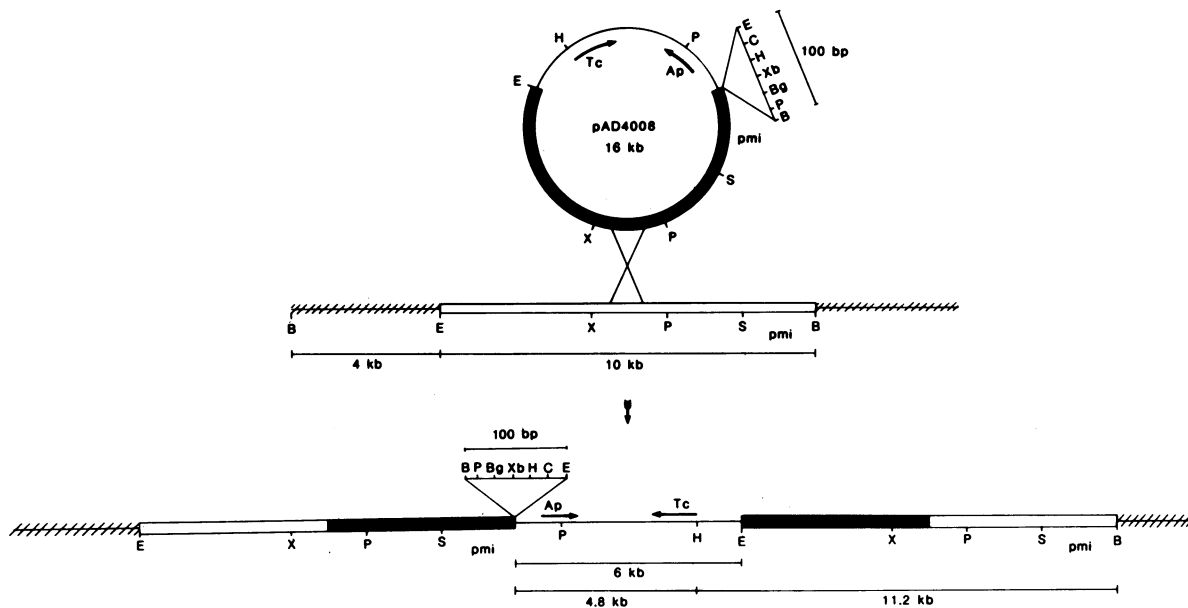


FIG. 1. Homologous recombination between plasmid pAD4008 and the *P. aeruginosa* chromosome. The circle at the top of the figure is pAD4008; —, pBR325 vector DNA; ■, 10-kb segment of cloned *P. aeruginosa* DNA; □, chromosomal homolog of the 10-kb cloned fragment; //, flanking chromosomal sequences. The triangle near the *pmi* gene is a 100-base pair (bp) polylinker with the indicated restriction sites. The point of recombination is given arbitrarily. The bottom line represents the predicted structure of the *P. aeruginosa* chromosome in the region of pAD4008 insertion. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; C, *Cl*aI; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sst*I; X, *Xho*I; Xb, *Xba*I; Tc, tetracycline resistance; Ap, ampicillin resistance.

$<10^{-10}$). Thus, transmission of pAD4008 appeared to be due to integration at a site of homology with the cloned DNA. Also, as expected, transmission of the drug resistance marker of plasmid pAD4008 was dependent on an intact recombinational system since no tetracycline-resistant colonies were obtained (frequency, $<10^{-10}$) when the recombination-deficient mutant strain PAO2003 served as the recipient.

To demonstrate directly the insertion of pAD4008 into the *P. aeruginosa* chromosome, we subjected total chromosomal DNA from the parental strains PAO25 and 8830 and from the respective plasmid integrate strains AL30 and CH60 to restriction and hybridization analysis. Chromosomal and pAD4008 fragments generated by cleavage with either *Eco*RI or *Hind*III were separated on a 0.7% agarose gel and transferred to a nitrocellulose filter. The filter was then hybridized with 32 P-labeled pBR325 DNA. The radioactive probe failed to hybridize with any of the *Eco*RI (Fig. 2, lanes 2 and 3) or *Hind*III (Fig. 2, lanes 7 and 8) fragments of parental DNA. However, the probe did recognize a 6.0-kb *Eco*RI fragment from each of the tetracycline-resistant plasmid integrate strains (lanes 4 and 5). The second, faintly hybridizing band in lane 1 represents a partial digest of plasmid pAD4008. Lane 6 demonstrates the hybridization profile of pAD4008 after it was digested with *Hind*III. DNA fragments of 4.8 and 11.2 kb were identified by the probe. An inspection of the insertion event in Fig. 1 indicates that in transformed DNA the pBR325 probe should hybridize to a 4.8-kb *Hind*III fragment and to a *Hind*III fragment greater than 11.2 kb in size. The hybridization profile in Fig. 2 (lanes 9 and 10) reveals bands consistent with the predicted sizes of 4.8 and >11.2 kb.

Walking the *P. aeruginosa* CH60 chromosome in the vicinity of the *pmi* gene. Our results suggest that use of the restriction endonuclease *Xba*I, *Bgl*II, or both would be helpful in isolating flanking sequences 5' of the *pmi* gene since single

site for *Xba*I and *Bgl*II appeared in the 100-base-pair polylinker adjacent to the pBR325 sequences and no sites for these enzymes existed either within pBR325 or within the adjacent 9.9-kb chromosomal *Eco*RI-*Bam*HI fragment (Fig. 1, bottom). Therefore, Tc^r clones generated by digestion of the plasmid integrate chromosome with *Xba*I or *Bgl*II should

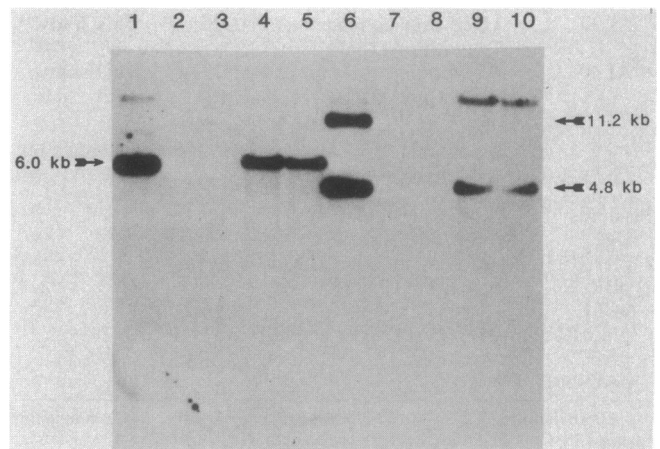


FIG. 2. Southern analysis of chromosomal DNA. Purified DNA from the *P. aeruginosa* parental strains PAO25 and 8830, the tetracycline-resistant transformant strains AL30 and CH60, and plasmid pAD4008 was digested with *Eco*RI or *Hind*III. DNA fragments were resolved through a 0.7% agarose slab gel, blotted to a nitrocellulose support, and probed with 32 P-labeled pBR325 DNA. In lanes 1 through 5, DNA was digested with *Eco*RI, and in lanes 6 through 10, DNA was digested with *Hind*III. Lanes: 1 and 6, pAD4008; 2 and 7, strain PAO25 parental DNA; 3 and 8, strain 8830 parental DNA; 4 and 9, strain AL30 DNA; 5 and 10, strain CH60 DNA.

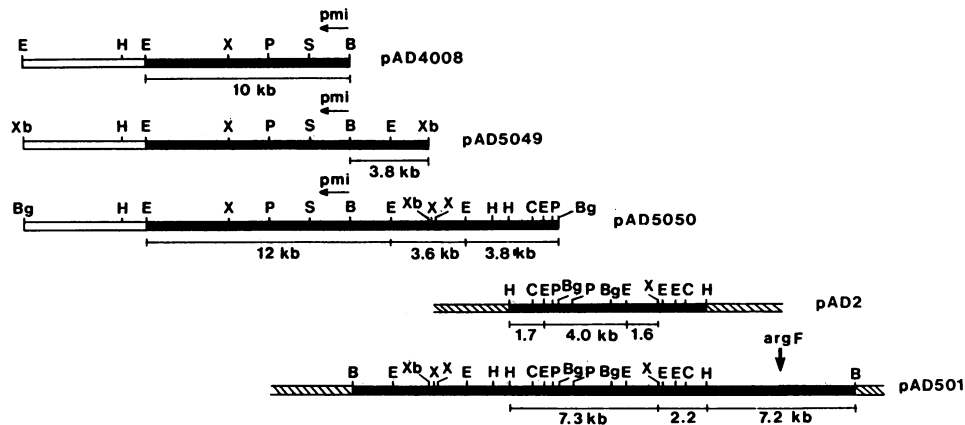


FIG. 3. Physical maps of the recombinant plasmids pAD4008, pAD5049, pAD5050, pAD2, and pAD501. ■, Cloned *P. aeruginosa* DNA; □, pBR325 vector DNA; ▨, broad-host-range plasmid pCP13. Abbreviations for restriction sites are as in the legend for Fig. 1.

have contained chromosomal DNA which spanned the *pmi* gene. Chromosomal DNA of *P. aeruginosa* CH60 was digested to completion with *Xba*I, ligated, and transformed into *E. coli* AC80. Selection for tetracycline-resistant colonies yielded many Tc^r clones which were analyzed by the plasmid miniscreening method of Holmes and Quigley (24). The *Xba*I-generated Tc^r colonies all contained the same cloned sequences as judged by their restriction profile. This *Xba*I-generated plasmid was designated pAD5049 (Fig. 3). From restriction endonuclease analysis, it appeared that pAD5049 represented an overlapping segment of DNA that contained sequences 3.8 kb 5' of the *Bam*HI site near the *pmi* gene. These newly cloned sequences, however, lacked *Bgl*II recognition sites, so *Bgl*II was next used to isolate Tc^r clones from the CH60 chromosome. Cloning and characterization of the resulting Tc^r plasmids indicated the presence of a single plasmid species. This plasmid, designated pAD5050, appeared to contain sequences cloned within pAD5049 but, in addition, now contained a segment of DNA which extended about 10 kb 5' from the *pmi* gene. A profile of the restriction maps of plasmids pAD5049 and pAD5050 has been summarized to allow a comparison with the physical maps of their inserts (Fig. 3).

In a previous paper (9), we reported on the isolation of three recombinant plasmids each of which complemented several different Alg^- mutants of *P. aeruginosa* 8830. Two of the recombinant clones, pAD1 and pAD3, have been previously characterized (9, 10). The remaining recombinant plasmid, which was designated pAD2, has not been fully characterized. However, when a partial restriction map of the 9.5-kb *Hind*III insert fragment of pAD2 was compared with the previously isolated overlapping DNA clone pAD5050, a DNA region of approximately 2.4 kb representing the right- and leftmost portions of pAD5050 and pAD2, respectively, appeared identical (Fig. 3). To find out whether any sequence homology existed between pAD2 and pAD5050, a Southern hybridization was done. Plasmid pAD5050 was labeled with [32 P]dCTP and used to probe various digests of plasmids pAD2 and pAD5050 and of *P. aeruginosa* chromosomal DNAs from strains PAO25 and 8830. Plasmid pAD2 was digested with *Hind*III alone (Fig. 4A, lane 1), with a combination of *Hind*III and *Eco*RI (lane 2), and with a combination of *Xho*I and *Hind*III (lane 3). The corresponding lanes of the autoradiogram (Fig. 4B) demonstrate that pAD5050 hybridized specifically with the 9.5-kb *Hind*III fragment (lane 1), the 4.0-kb *Eco*RI and 1.7-kb

*Hind*III-*Eco*RI fragments (lane 2), and the 7.3-kb *Hind*III-*Xho*I fragment (lane 3) of pAD2. The slowest migrating DNA bands in Fig. 4A, lanes 1 through 3, represent the cosmid cloning vehicle pCP13 which also hybridized to the pAD5050 probe because of homology with pBR325. The reason for the diminished hybridization in lane 2 is not clear. This hybridization profile was consistent with the suggestion that pAD5050-cloned sequences are contiguous with pAD2-cloned sequences on the *P. aeruginosa* chromosome. To confirm that this region of DNA has sequence homology with the chromosome of *P. aeruginosa*, pAD5050 was also hybridized to blots of total *P. aeruginosa* DNA. Strains PAO25 and 8830 had identical DNA hybridization profiles when probed with pAD5050 (Fig. 4, lanes 5, 6, 8, 9). Taken together, the restriction profile similarities and the hybridization data suggest that the chromosomally derived sequences present within pAD5050 were contiguous with the sequences present on the DNA insert of pAD2 and indicate for the first time that some of the loci involved in alginate biosynthesis may be clustered.

Clustering of mutations affecting alginate biosynthesis. By using the alginate-negative mutants already described (9) and those new to this study (Table 1), we subcloned the region containing pAD5050 and pAD2 to correlate specific regions with genetic complementation groups. Various restriction endonuclease-generated fragments were subcloned into the broad-host-range vectors pCP13 or pMMB22, and the ability of the derivative plasmids to complement the various *alg* mutants was determined. A compilation of the results is shown in Fig. 5. Plasmid pAD4028 (10), which was previously found to contain the *P. aeruginosa pmi* gene, was able to complement several of our *alg* mutants to the Alg^+ phenotype. The direction of transcription of the *pmi* gene (indicated by the arrow in Fig. 5) was determined by studies involving the sequencing of the *pmi* gene and the positioning of the gene next to the *E. coli tac* promoter (A. Darzins, B. Frantz, R. Vanags, and A. M. Chakrabarty, manuscript in preparation). Subcloning of the 7.2-kb *Bam*HI-*Hind*III fragment from pAD5050 into pCP13 resulted in the construction of plasmid pAD5056. When pAD5056 was mobilized into all of our *alg* mutants, only strain 8887 (*alg-60*) was complemented. The complementing ability of pAD5056 was further localized by subcloning the 6.1-kb *Xho*I-*Bgl*II fragment of pAD5050 into pCP13. This subclone is represented as plasmid pAD5053. It seems evident from the results illustrated in Fig. 5 that the 3.2-kb *Xho*I-*Hind*III fragment common to

both pAD5056 and pAD5053 was responsible for complementing mutant strain 8887. In addition to its ability to complement mutant 8887, pAD5053 was also capable of complementing mutant 8897. Therefore, plasmids pAD5056 and pAD5053 defined two separate genetic complementation groups very close to the *pmi* gene.

Plasmid pAD2, on the other hand, has the ability to complement several *alg* mutants (9). Subcloning of the 9.5-kb *Hind*III fragment of pAD2 resulted in the establishment of several other distinct genetic complementation groups. For instance, plasmid pSK2005, which contains the 4.6-kb *Hind*III-*Bgl*II fragment of pAD2 subcloned into pCP13, and plasmid pAD320, which contains the 7.3-kb *Cl*aI fragment of pAD2 subcloned into the same vector, both complemented mutant strain 8835 (*alg-5*) (9). However, plasmid pAD320, in addition to mutant strain 8835, was also able to complement several other *alg* mutations (Fig. 5). To further differentiate these *alg* mutants, we constructed plasmids pSK2001 and pAD5059. Plasmid pSK2001, which contained the 4.2-kb *Xho*I-*Pst*I fragment of pAD2 in pCP13, was found to complement only mutants 8838, 8846, 8857, and 8891 (Fig. 5). Still another group of *alg* mutants represented by 8874, 8889, and 8893 was specifically found to be complemented when the 4.0-kb *Eco*RI fragment of pAD2 was cloned next to the *E. coli tac* promoter in the broad-host-range expression vector pMMB22. Only when induced with 5 mM isopropyl- β -D-thiogalactopyranoside was plasmid pAD5059 able to restore the alginate biosynthetic capabilities to these three *alg* mutants. As expected of any gene under the transcriptional control of the *lacI^a-tac* promoter system in the absence of the inducer isopropyl- β -D-thiogalactopyranoside, no production of alginate by the three mutants harboring pAD5059 could be detected (data not shown). This suggests that a gene involved in alginate biosynthesis was present within plasmid pAD5059 but that, as cloned, it most likely lacked a functional promoter. Based on these subcloning and complementation data, we concluded that there were at least five distinct genetic complementation groups involved in alginate biosynthesis within close proximity of the *pmi* gene.

Conjugational mapping of the *alg* gene cluster. The genetic location of the clustered alginate region was next determined by localizing the chromosomal position of the tetracycline resistance determinant in two PAO strains harboring pAD4008. Derivatives of AL30 and AL40 containing chromosome-mobilizing plasmid FP2 were constructed as described above and used as donors in various crosses with recipient strains PAO25, PAO222, PAO515, PAO1042, and PAO2198. Exconjugants were selected for repair of the appropriate marker(s) and scored for coinheritance of tetracycline resistance (Tc^r). No linkage of Tc^r with the markers *pur-67* (89 min), *cys-59* (94 min), *proB65* (2 min), *ilv-226* (8 min), *his-4* (17 min), *lys-12* (20 min), or *met-28* (30 min) was observed among 100 recombinants examined from the crosses AL30(FP2) \times PAO222 and AL30(FP2) \times PAO1042; however, a low frequency of linkage (1%) with both *trp 6* (35 min) and *proA82* (38 min) was detected (data not shown). A higher conjugational linkage to Tc^r was detected with *thr-9001* recombinants (42 min) and with *argF10* or *argG9036* recombinants (45 min). In addition, selection for later mapping markers located distal to *argF*, such as *ami-151* (50 min) and *met-9011* (55 min), results in lower linkage values (Table 2; Fig. 6). The results of these single marker selection experiments suggest that the clustered alginate region mapped near the *argG* locus, which is situated at about 45 min.

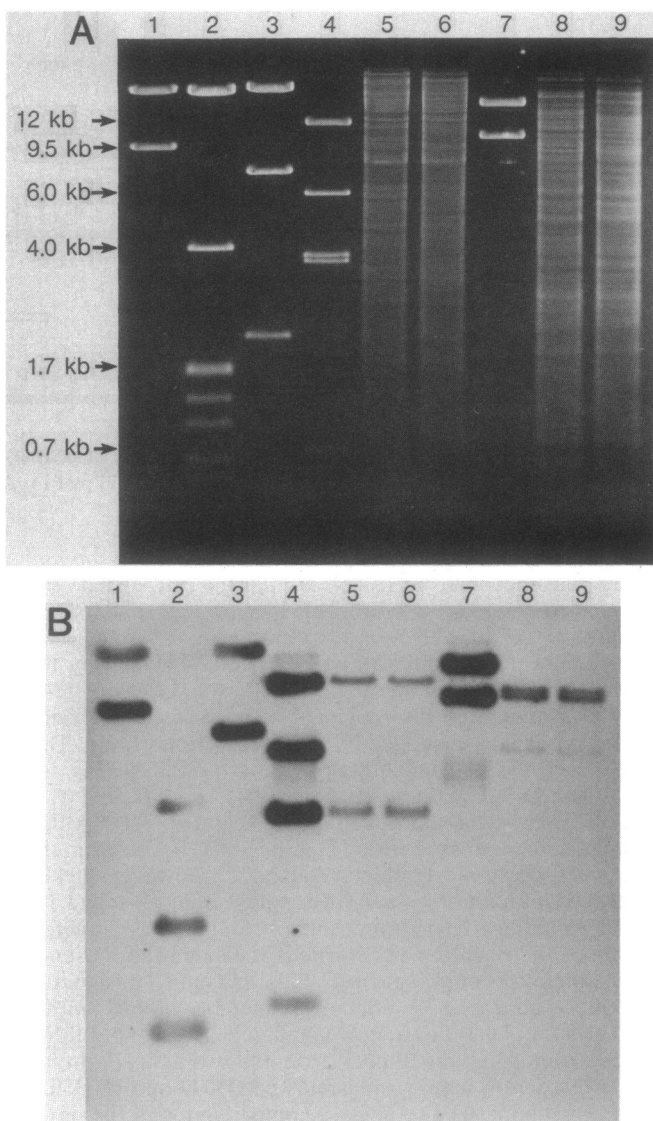


FIG. 4. Hybridization of plasmid pAD5050 to plasmid pAD2 and total DNA from parental strains PAO25 and 8830. Ethidium bromide-stained agarose gel (A) and autoradiogram of the corresponding Southern blot (B) after hybridization with ^{32}P -labeled pAD5050. Lanes: 1, *Hind*III-cut pAD2; 2, *Hind*III- and *Eco*RI-cut pAD2; 3, *Hind*III- and *Xho*I-cut pAD2; 4, *Eco*RI-cut pAD5050; 5, *Eco*RI-cut strain PAO25 total DNA; 6, *Eco*RI-cut strain 8830 total DNA; 7, *Xho*I-cut pAD5050; 8, *Xho*I-cut strain PAO25 total DNA; 9, *Xho*I-cut strain 8830 total DNA. Size standards (designated by arrows) were established from molecular weights of DNA fragments determined previously.

To verify this map location, double-selection experiments with the markers *argF10*, *leu-10*, *ami-151*, *met-9011*, *argG9036*, *lys-9015*, and *leu-9014* were carried out. Double selection for *ami-151* and *met-9011* recombinants showed only a 15% colinkage to the tetracycline resistance determinant of strain AL40. The *argF10 leu-10* double recombinants showed 85% linkage with Tc^r . However, selection for *argG lys-9015* and *argG leu-9014* double recombinants confirmed that the *alg* gene cluster mapped between *argG* and *leu-9014* but most likely resided closer to *argG* (Table 2; Fig. 6). Furthermore, we were not able to demonstrate that the

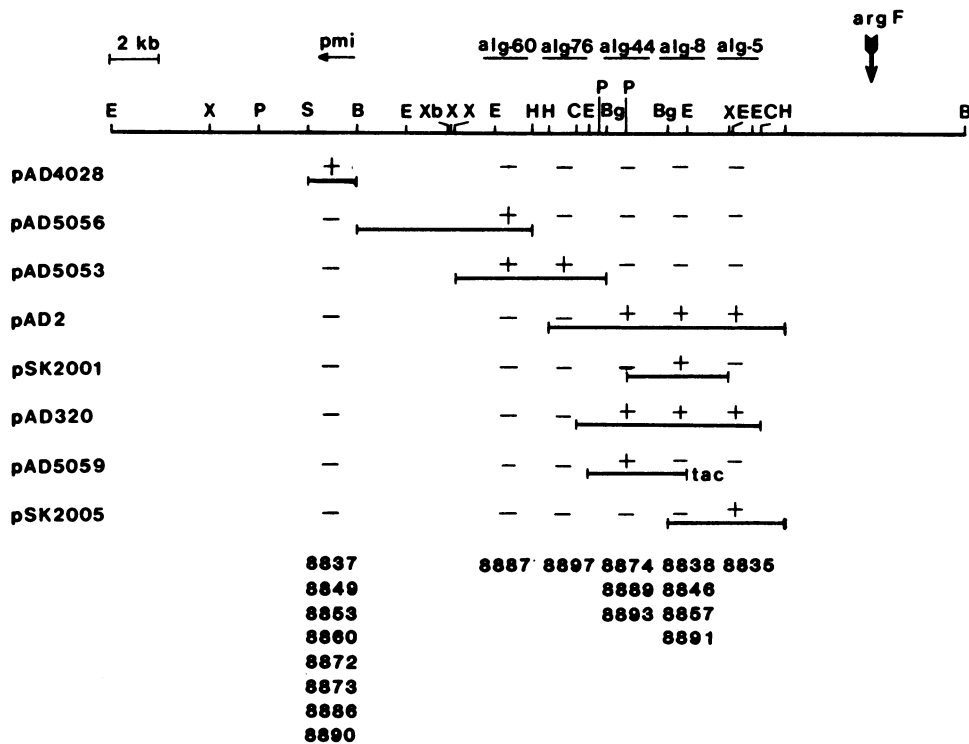


FIG. 5. Characterization of the clustered alginate region. Each subclone was introduced into each of the Alg mutants by triparental crosses (14, 15), and transconjugants were selected for by tetracycline resistance (pCP13) or carbenicillin resistance (pMMB22). Transconjugants were tested for restored alginate-synthesizing ability. The boundaries of the indicated *alg* loci located above the restriction map are approximate. Abbreviations: +, positive complementation to Alg⁺; -, lack of complementation. See legend to Fig. 1 for restriction site designations.

argG⁺, *argF*⁺, or *lys-9015* alleles were within the cloned 30-kb region containing the *alg* gene cluster.

Cloning of the *argF* gene. The genetic determinant for pyocin AP41 has recently been located on the *P. aeruginosa* chromosome near *argF* at about 45 min (38). A comparison of the restriction endonuclease maps of the cloned *alg* region and the R' plasmid pNM501 (38) revealed striking similarities with respect to their *Hind*III and *Bam*HI sites. These results prompted the cloning of the *argF* gene in the expectation that the *alg* genes might be picked up along with *argF*. A strain 8830 *Bam*HI-pCP13 cosmid library (10) was mobilized into *P. aeruginosa* PAO25, and *argF*⁺ tetracycline-

resistant colonies were selected. The physical map of one *argF*⁺ cosmid clone, designated pAD501, is presented in Fig. 3. We were able to demonstrate the presence of the *argF* gene on a 9.4-kb *Xho*I-*Hind*III fragment (Fig. 3). In addition to the *argF* gene, plasmid pAD501 also contained the cloned *alg* gene cluster 5' of the *pmi* gene. This was achieved by demonstrating complementation of the appropriate *alg* mutants with pAD501 (data not shown). Therefore, the gene arrangement, as deduced from our linkage and cloning analyses and from the results of Sano and Kageyama (38) is as follows: early *leu-9014 lys-9015 pyoAP41 pmi (alg-43) alg-60 alg-76 alg-44 alg-8 alg-5 argF argG met-9020* late.

TABLE 2. Conjugational mapping of the *P. aeruginosa* *alg* gene cluster

Donor	Recipient	Selection ^a	% Coinheritance to Tet ^b
AL30(FP2)	PAO1042 (<i>thr-9001</i>)	Thr ⁺	12 (36/300)
AL40(FP2)	PAO2198 (<i>argG9036</i>)	Arg ⁺	80 (240/300)
AL40(FP2)	PAO25 (<i>argF</i>)	Arg ⁺	64 (132/205)
AL40(FP2)	PAO515(<i>ami-151</i>)	Ami ⁺	10 (15/150)
AL40(FP2)	PAO515 (<i>met-9011</i>)	Met ⁺	4 (12/300)
AL40(FP2)	PAO515 (<i>ami-151 met-9011</i>)	Ami ⁺ , Met ⁺	15 (30/200)
AL40(FP2)	PAO25 (<i>argF leu-10</i>)	Arg ⁺ , Leu ⁺	85 (132/156)
AL40(FP2)	PAO2198 (<i>argG9036 lys-9015</i>)	Arg ⁺ , Lys ⁺	97 (290/300)
AL40(FP2)	PAO2198 (<i>argG9036 leu-9014</i>)	Arg ⁺ , Leu ⁺	97 (291/300)

^a Ami⁺, Acetamide utilization, Thr⁺, no requirement for threonine; Arg⁺, no requirement for arginine; Leu⁺, no requirement for leucine; Lys⁺, no requirement for lysine; Met⁺, no requirement for methionine.

^b Numbers in parentheses indicate the number of transconjugants containing the unselected marker per number of transconjugants scored.

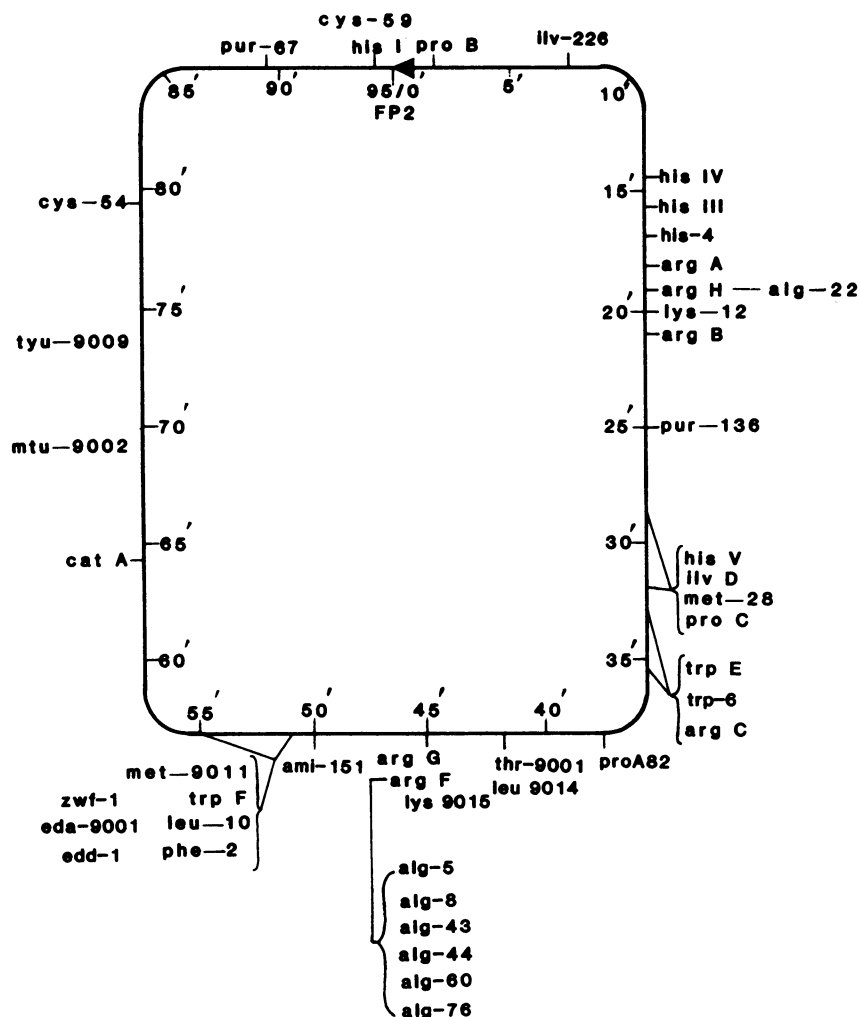


FIG. 6. Chromosome map of *P. aeruginosa* PAO with markers relevant to the present study. The map is based on a recent version by B. W. Holloway as depicted by Haas (21). Only a representative mutant of each *alg* complementation group is shown. Linked genetic markers are enclosed in brackets.

DISCUSSION

We demonstrated the integration of ColE1 hybrid plasmid pAD4008 into the *P. aeruginosa* chromosome by recombination between a segment of cloned *P. aeruginosa* DNA on the plasmid and its chromosomal analog. Tetracycline-resistant transformants analyzed by hybridization were found to have the plasmid integrated as predicted. This technique can be extended one step further by using this site-directed integration for gene replacement as described by Guttererson and Koshland (20). The site-directed integration of ColE1 plasmids into the *P. aeruginosa* chromosome should be of value in mapping other cloned *P. aeruginosa* DNAs for which precise genetic loci are not known.

We have already described the isolation of a *P. aeruginosa* DNA segment (plasmid pAD4) that harbors the *pml* gene at the extreme right end of that DNA segment (10). Plasmid pAD4 alone did not have the capability of complementing any other *alg* (non-*pml*) mutants within our collection (data not shown), so chromosomal walking experiments were conducted to find out whether other genes were involved in alginate biosynthesis 5' of the *pml* gene. By walking the chromosome of the plasmid integrate strain CH60, we

cloned sequences homologous to the DNA insert of plasmid pAD2. This result was our first hint that several genes involved in alginate biosynthesis may be clustered. The ensuing subcloning and complementation testing confirmed our assumption regarding the clustering of several alginate biosynthetic functions. Specifically, in addition to the *pml* gene, we identified at least five different and distinct alginate complementation groups in one region of the strain 8830 chromosome.

Although most of our *alg* mutants have been complemented by one of the various DNA subclones from either this clustered region or from the chromosomal region near the *argH* gene (9), a few of the *alg* mutants have not been complemented. These mutants could possibly represent still other alginate complementation groupings not yet identified by DNA cloning. Support for this possibility lies in the reported cloning of a DNA segment that complements an *alg* mutation (*alg-50*) in *P. aeruginosa* FRD (17). The restriction map of the DNA segment from plasmid pJG1 appears to be quite different from that seen within the clustered *alg* region. This finding suggests that other DNA segments harboring genes directly or indirectly involved with alginate biosynthesis may yet be present on the chromosome. Since exopoly-

saccharide synthesis and export represent multigenic processes, it is not surprising that mutations at many loci and in many different genes can affect these multigenic processes.

To confirm that the *P. aeruginosa* PAO chromosome contains an analogous region of DNA corresponding to the clustered *alg* region of strain 8830, we also performed chromosomal walking experiments in the PAO plasmid integrate strain AL30. Strain PAO DNA segments homologous to the DNA regions isolated from strain 8830 were recovered, suggesting a similar arrangement of DNA sequences in the region of the two chromosomes (A. Darzins, unpublished data). This conclusion is also consistent with the similar chromosomal hybridization profiles of strains PAO and 8830 that we obtained (Fig. 4). Consequently, the alginate gene cluster identified in strain 8830 was mapped in strain PAO and was shown to be located at 45 min of the PAO chromosome, very close to the *argF* gene.

A number of chromosomal catabolic genes that have been mapped in *Pseudomonas* species are known to exhibit extensive clustering (5, 11, 29, 34). It was recognized early, however, that the genes for biosynthetic pathways were nonclustered in *P. aeruginosa* (13) which is quite unlike the distribution in *E. coli* or *Salmonella typhimurium* (1, 37). For example, the histidine genes form a contiguous sequence of nine structural genes in enteric bacteria, whereas at least five unlinked loci are involved in *P. aeruginosa* (30). And unlike enteric bacteria, in which the *trp* genes form a single operon, there are three unlinked groups of genes in *P. aeruginosa* and *P. putida* (8). The data that we have presented here suggest a clustered arrangement of genes involved in a biosynthetic process. Coincidentally, many of the genes involved in carbohydrate catabolism reside in the 45- to 55-min region of the chromosome (25). For instance, Roehl et al. (34) previously demonstrated the tight clustering of genes corresponding to three inducible enzymes of the central pathways of carbohydrate metabolism. These genes were eventually mapped in the 50- to 55-min region of the chromosome. Furthermore, loci affecting expression of the phosphoenolpyruvate-fructose 1-phosphotransferase system, *pts* (35), and pyruvate carboxylase, *pyc* (34), have been mapped near the *argF* locus at 45 min (Fig. 6). The results of the present study strongly suggest that some, but not all, of the genes involved in alginate biosynthesis are located near several loci that are involved in carbohydrate metabolism. Since the catabolism of carbohydrate moieties such as glucose, gluconate, fructose, and mannitol is required for alginate production (3, 4, 28), it is interesting that some of the genes involved in these two processes are located in the same region of the chromosome. We expect that additional studies with the cloned *alg* cluster will be useful in understanding the molecular details of both the alginate biosynthetic pathway and the regulatory mechanism(s) that govern alginate gene expression.

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