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Characterization of a Locus Determining the Mucoid Status of *Pseudomonas aeruginosa*: AlgU Shows Sequence Similarities with a *Bacillus* Sigma Factor

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Overproduction of the exopolysaccharide alginate by *Pseudomonas aeruginosa* results in mucoid colony morphology and is an important virulence determinant expressed by this organism in cystic fibrosis. Mucoidy is transcriptionally regulated by signal transduction systems and histone-like elements. One point of convergence of regulatory elements controlling mucoidy is the *algD* promoter. A newly described genetic locus required for *algD* transcription was characterized in this study. This DNA region, cloned from a nonmucoid PAO strain, was initially isolated on the basis of its ability to suppress mucoidy when present on a plasmid. The suppressing activity was observed in several mucoid PAO derivatives, including strain PAO568, in which the mapped *muc-2* mutation is responsible for its mucoid phenotype, and in close to 40% of cystic fibrosis strains tested. Protein expression studies detected two polypeptides with apparent molecular masses of 27.5 and 20 kDa encoded by the region required for the suppression activity. The gene encoding the polypeptide with an apparent molecular mass of 27.5 kDa, termed *algU*, was further characterized. A functional chromosomal copy of *algU* was found to be necessary for the expression of mucoidy. Insertional inactivation of *algU* on the chromosome of the mucoid strain PAO568 abrogated alginate production and *algD* transcription. DNA sequence analysis revealed sequence similarity of the predicted *algU* gene product with σ^H (Spo0H), a sigma factor involved in the control of sporulation and competence in *Bacillus* spp. Physical mapping revealed that *algU* resided on the same *Spe*I fragment (F) as did the *pruAB* locus, known to be tightly linked with genetic determinants (*muc*) which can confer mucoidy in genetic crosses. When the chromosomal *algU* copy was tagged with a Tc^r cassette (*algU*::Tc^r), a tight genetic linkage of *algU* with *pruAB* was demonstrated by F116L-mediated generalized transduction. Moreover, *algU*::Tc^r derivatives of PAO568 (originally carrying the *muc-2* marker) lost the ability to transfer mucoidy in genetic crosses. These results suggest that *algU*, a regulator of *algD* transcription showing sequence similarity to an alternative sigma factor, and the genes immediately downstream of *algU* may be associated with a locus participating in the differentiation into the mucoid phenotype.

Overproduction of the exopolysaccharide alginate results in mucoid colony morphology, a well-recognized virulence determinant expressed by *Pseudomonas aeruginosa* infecting individuals with cystic fibrosis (CF) (18, 27, 51). The altered lung environment of CF patients renders their respiratory tract prone to colonization by a characteristic succession of bacterial pathogens and their morphological forms (27). *P. aeruginosa* is of particular importance since it causes an intractable chronic infection and is responsible for much of the morbidity and mortality currently seen in CF patients (27). The initially colonizing strains of *P. aeruginosa* are nonmucoid, but there is an almost inevitable change into the mucoid phenotype (27, 51). The emergence of mucoid forms of *P. aeruginosa* is an important indicator associated with a worsened clinical outlook (27, 28).

Considerable information is now available concerning alginate biosynthesis and its regulation (18, 51). A key event leading to the expression of mucoidy is the transcriptional activation of *algD* (11). The *algD* gene heads the cluster of alginate biosynthetic genes located at 34 min of the *P. aeruginosa* chromosome (Fig. 1). *algD* encodes GDPmannose dehydrogenase, an enzyme which catalyzes double oxidation of GDPmannose into its uronic acid, a reaction that channels sugar intermediates into alginate production (11). The amounts of secreted alginate and colony morphology correlate directly with the level of *algD* transcription in all strains and under all conditions tested (11, 12, 50).

The *algD* promoter is under control by bacterial signal transduction systems (*algR* and *algB*, located at 9 and 11 min, respectively) (10, 65) and histone-like elements (*algP*, linked to *algR*) (13, 15, 34, 38). When *algR* is insertionally inactivated, *algD* expression is blocked (50). AlgR directly interacts with the *algD* promoter (33, 48, 49). This response regulator binds with differing affinities to three sites (RB1, RB2, and RB3) defined by a decanucleotide core sequence (ACCGTTCGTC or its variants) within the *algD* promoter (49, 50). AlgR can undergo phosphorylation typical of the two-component environmentally responsive systems (17) and may also interact with small-molecular-weight phosphorylated metabolites (17).

Similar systems and phosphorylation reactions control many complex physiological and developmental processes in other bacteria (60), including regulation of virulence (46), in response to environmental signals. Various growth conditions can affect mucoidy and *algD* expression in a strain-dependent manner (12). Even in a single strain, multiple factors can modulate expression of *algD* and, correspond-

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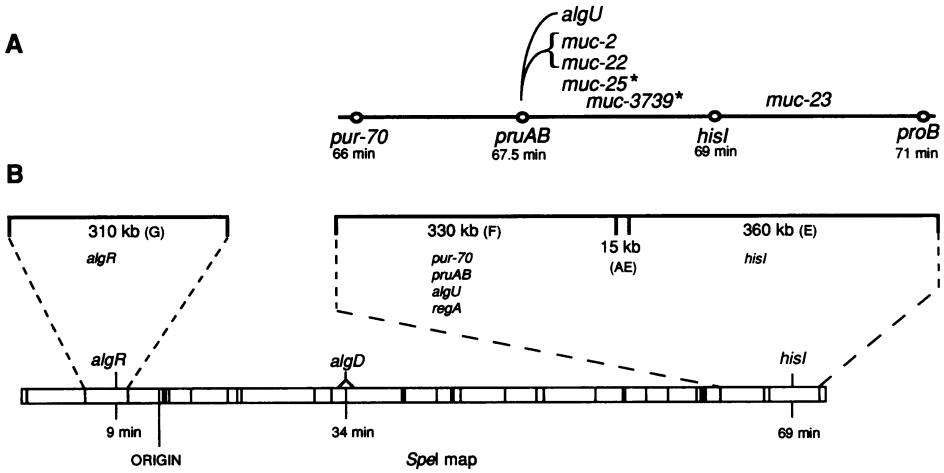

FIG. 1. Locations of *muc* loci and *algU* on genetic and physical maps of *P. aeruginosa* PAO. (A) Genetic map of the late region of the *P. aeruginosa* chromosome. Genetic markers *pur-70*, *pruAB*, *hisI*, and *proB* are linked with the *muc* loci. *muc-2*, *muc-22*, and *algU* are cotransducible with *pruAB* (indicated by arcs). *muc-25* and *muc-3739* map between *hisI* and *pur-70*; it is not known whether they are cotransducible with *pruAB* (indicated by asterisks). The *muc-23* marker maps between *hisI* and *proB*. (B) Positions of several genetic markers, *alg* genes, and probes used in this study on a physical map (*Spe*I) of *P. aeruginosa* PAO. The *algD* gene hybridizes to two *Spe*I fragments. Fragments E (360 kb), F (330 kb), and G (310 kb) are enlarged. The genetic map of the late region and the corresponding *Spe*I fragments are aligned to permit overlaps of markers known to hybridize to a given fragment, but precise relative positions are not known. Probes known to hybridize, or that are shown here to hybridize, to a given *Spe*I fragment are displayed below corresponding fragments.

ingly, the level of alginate production (12, 50). Such studies have been facilitated by the use of mucoid derivatives of the standard genetic strain PAO, e.g., PAO568 and PAO578, which display induction of *algD* transcription in response to growth on nitrate instead of ammonia as the nitrogen source and to the presence of high salt concentrations in the medium (12, 50). Induction by growth on nitrate is absolutely *algR* dependent (47, 50). Although several of the proposed environmental factors may be linked to the specificities of the CF lung (27), the complexity of the environmental regulation of mucoidy, the emergence of mucoid strains, and the maintenance of their phenotypes are difficult to explain on the basis of only the currently available information. Additional regulatory elements that modulate alginate synthesis probably exist. These elements may include the putative histidine protein kinases/phosphatases interacting with AlgR and AlgB, as well as the sigma factor involved in *algD* transcription. It has been suggested that σ^{54} plays a role in *algD* transcription (14, 35), but when the *rpoN* gene encoding this alternative sigma factor was inactivated in *P. aeruginosa*, the alteration had no effect on mucoidy, mRNA start site, and levels of *algD* transcription (50).

The existence of additional regulatory elements is supported by the early genetic studies performed prior to the more recent elucidation of the alginate biosynthetic pathway and transcriptional regulation at *algD* (24, 25, 43). These reports strongly suggest that several loci, termed *muc*, mapping in the late region of the *P. aeruginosa* chromosome participate in the emergence of mucoid strains (24, 25, 43). By means of chromosomal genetic exchange in PAO, the known *muc* loci have been mapped to the late region of the PAO chromosome between the *pur-70* (66 min) and *proB* (71 min) loci (Fig. 1). The existence of multiple *muc* linkage groups was indicated on the basis of the position of *muc* loci relative to those of additional genetic markers in this region, such as *hisI* (69 min) and *pruAB* (67.5 min) (Fig. 1) (23, 24, 27, 43). More recently, another locus termed *algST*, linked to *hisI* (22), has been implicated in the control of mucoidy (22, 51). None of these loci have been characterized at the molecular level, and their nature and function are currently not known.

In an effort to identify putative additional factors controlling *algD*, we cloned several new genes affecting mucoidy, one of which, *algU*, was studied in detail in this work.

MATERIALS AND METHODS

Media and bacterial growth. *Escherichia coli* was grown on LB supplemented with tetracycline (10 μg/ml), ampicillin (40 μg/ml), and kanamycin (25 μg/ml) when required. *P. aeruginosa* was grown on LB and minimal media (12, 44) and on *Pseudomonas* isolation agar (PIA) (Difco). The nitrogen-free medium (P), used to test the ability to utilize proline (supplemented at a concentration of 20 mM) as the sole carbon and nitrogen source, has been previously described (44). Other amino acids were supplied at 1 mM when necessary. Media for environmental modulation by different nitrogen sources (nitrate or ammonia) have been described previously (12, 50). NaCl at 300 mM was added to LB when required (12). Antibiotic supplements for *P. aeruginosa* were 300 μg of tetracycline per ml for PIA, 50 μg of tetracycline per ml for LB and minimal media, and 300 μg of carbenicillin per ml for all media.

Plasmids and bacterial strains. Strains of *P. aeruginosa* and plasmids used in this study are shown in Table 1. Strains PAO669 and PAO670 were derivatives of *P. aeruginosa* PAO568 (*muc-2*). Strain PAO669 was generated by integration of a nonreplicative plasmid carrying an *algD*::*xylE* fusion on the chromosome of PAO568. An 11.5-kb *Hin*dIII fragment carrying *algD* with *xylE* inserted in the *Xho*I site of *algD* was cloned in the *Hin*dIII site of pCMobB (47), and the resulting plasmid pDMDX was conjugated into PAO568. pCMobB and its derivative pDMDX cannot replicate in *P. aeruginosa* but can be effectively mobilized into this bacterium (47). Cb^r exconjugants were obtained and tested for the presence of other plasmid markers (development of a yellow

TABLE 1. Bacterial strains, plasmids, and bacteriophage

Strain, plasmid, or phage	Relevant properties[a]	Reference
P. aeruginosa		
PAO1	Prototroph Alg$^-$	31
PAO1293	Prototroph Alg$^-$	55
PAO568	FP2$^+$ *muc-2* (Alg^{+i}) *leu-38*	24
PAO578	FP2$^+$ *muc-22* (Alg$^+$) *leu-38*	24
PAO579	FP2$^+$ *muc-23* (Alg$^+$) *leu-38*	24
PAO581	FP2$^+$ *muc-25* (Alg$^+$) *leu-38*	24
PAO540	*cys-5605 his-5075 argA171* Alg$^-$	24
PAO669	FP2$^+$ *muc-2* (Alg^{+i}) *leu-38* Cb^r *algD*$^+$ *algD*::*xylE* (derived from PAO568)	This work
PAO670	FP2$^+$ *algU*::Tc^r (Alg$^-$) (derived from PAO568)	This work
PAO964	*pru-354 ami-151 hut C107* Alg$^-$	44
PAM425	*muc-3739* (Alg$^+$) *lys-13*	43
Plasmids		
pLA2917	IncP1 *mob*$^+$ *tra cos*$^+$ Tc^r Km^r	1
pCMob	ColE1 *mob*$^+$ (RK2) *tra cos*$^+$ Ap^r (Cb^r) Tc^r	47
pSF4	Ori (p15A) *mob*$^+$ (RK2) *cos*$^+$ Tc^r	57
pRK2013	ColE1 *mob*$^+$ *tra*$^+$ (RK2) Km^r	21
pT7-5	ColE1 Ap^r ϕ10 promoter-*Eco*RI-polylinker-*Hin*dIII	61
pT7-6	ColE1 Ap^r ϕ10 promoter-*Hin*dIII-polylinker-*Eco*RI	61
pGP1-2	Ori (p15A) p_L T7 gene 1 (T7 RNA polymerase) P_{lac}-*c*1857 Km^r	61
pVDZ'2	IncP1 *mob*$^+$ *tra lacZ'* (*lacZ*a) Tc^r	9
pCMR7	*algR* as 827-bp *Hin*dIII-*Bam*HI in pT7-6	48
pPAOM3	pVDX18 IncQ/P4 *algD*::*xylE* Ap^r (Cb^r)	37
pMO011809	*hisI*$^+$ (cosmid clone in pLA2917)	55
pMO012046	*algU*$^+$ (cosmid clone in pLA2917)	This work
pDMU1	*algU*$^+$ (6-kb *Hin*dIII-*Eco*RI fragment from pMO012046 subcloned on pVDZ'2)	This work
pDMU4/76	*algU*$^+$ as ΔU4/76 subcloned on pVDZ'2	This work
pRCW1	6-kb *Hin*dIII-*Nsi*I subclone from cosmid pMO011809	This work
pDMU100	pUC12 *mob*$^+$ *algU*::Tc^r Ap^r (Cb^r)	This work
pDMDX	pCMobB *algD*::*xylE mob*$^+$ *Ap*^{r} *(Cb*^{r}*)*	This work
Phage		
F116L	Generalized transduction phage	40

[a] Alg^{+i}, inducible production of alginate resulting in mucoid phenotype (12); Alg$^+$, mucoid phenotype; Alg$^-$, nonmucoid phenotype.

color when sprayed with a solution of catechol [37]), and insertions on the chromosome were verified by Southern blot analysis. Strain PAO669 was mucoid and produced alginate on inducing media. PAO670, a strain used to determine effects of the inactivation of *algU* on the chromosome, was constructed by gene replacement of the chromosomal *algU* with an insertionally inactivated *algU* (*algU*::Tc^r). This was accomplished as follows. A 2.4-kb *Hin*dIII-*Eco*RI fragment from ΔU4/76 was inserted into pUC12. The resulting construct was digested with *Eco*RV, and *Not*I linkers were added. A *Not*I-modified Tc^r cassette (32) was inserted, and the resulting plasmid was digested with *Eco*RI. A 1.4-kb *Eco*RI fragment with *mob* from pCMobA (originating from pSF4) (47, 57) was inserted into this site to produce pDMU100. This plasmid was transferred into *P. aeruginosa* PAO568 by conjugation, and exconjugants were selected on PIA supplemented with tetracycline. Since pUC12 and its derivative pDMU100 cannot replicate in *P. aeruginosa*, Tc^r strains had this plasmid integrated on the chromosome via homologous recombination. Double-crossover events were identified as Tc^r Cb^s strains; chromosomal DNA was extracted and digested with appropriate enzymes, and gene replacements were verified by Southern blot analysis. CF strains were from a combined collection of mucoid isolates from CF patients in Edinburgh, Scotland, and San Antonio, Tex. Cosmid clones not shown in Table 1 are described in Results. The source of *regA* was a 1.9-kb *Pst*I-*Xho*I subclone in mp18 (30). The use of *E. coli* strains for subcloning in pVDZ2 (JM83), triparental conjugations (HB101 harboring pRK2013), and deletion subcloning (WB373) has been described elsewhere (14, 38).

Nucleic acid manipulations and recombinant DNA methods. All DNA manipulations and Southern blot analyses were carried out according to previously published methods (14, 38, 50, 55) or standard recombinant DNA procedures (3). Radiolabeled probes (3) were generated by using the random-priming labeling method and [α-^{32}P]dCTP (3,000 Ci/mmol; DuPont NEN). Procedures for RNA extraction and S1 nuclease analysis have been previously published (14, 38). Construction of the cosmid clone library has been described elsewhere (55). Overlapping deletions of the clones in M13 were generated as previously described (14). DNA was sequenced by a modification of the chain termination method (substitution of dGTP by its analog 7-deaza-dGTP to avoid compressions) as previously described (38) and using 17-bp or custom-made primers when needed. Similarity searches were performed by using the FASTA program (52) and GenBank data bases as well as through the NBRF-PIR protein identification resource network server.

Genetic methods. Clones made in broad-host-range plasmids (pVDX18 and pVDZ'2) were transferred into *P. aeruginosa* by triparental filter matings as described previously (37), using *E. coli* harboring pRK2013 as the helper. Cosmid clones were mobilized into *P. aeruginosa* from *E. coli* S17-1 (59) as previously reported (55). Generalized transduction using F116L (40) was performed as follows. Serially diluted (to achieve near confluency) single-plaque preparations of F116L were grown mixed with the donor strain in top agar

for 17 h at 37°C. The top agar was scraped, phage was eluted in an equal volume of TNM (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 10 mM $MgSO_4$) and centrifuged at 9,000 rpm in an SM24 rotor, and the supernatant was filtered through a 0.45-μm-pore-size membrane to generate the transducing phage stock (used within 1 month). Freshly grown overnight recipient cells (500 μl) were incubated with 500 μl of transducing phage stock (diluted to 5×10^9; multiplicity of infection, 5:1) for 20 min at 37°C. Cells were centrifuged for 1 min in a microcentrifuge and resuspended in 1 ml of TNM. Aliquots were plated on selective medium and incubated for 1 to 2 days; strains were purified on selective medium and then spot tested for coinheritance of unselected markers.

Enzyme and alginate assays and scoring of suppression of mucoidy. Catechol 2,3-dioxygenase (CDO), the gene product of *xylE*, was assayed in cell sonic extracts as previously described (37). The activity was monitored in 50 mM phosphate buffer (pH 7.5)–0.33 mM catechol by following the increase of A_{375} in a Shimadzu UV160 spectrophotometer. The molar extinction coefficient of the reaction product, 2-hydroxymuconic semialdehyde, is 4.4×10^4 at 375 nm. Suppression of mucoidy by plasmid-borne genes was monitored on PIA plates unless specified otherwise, and the phenotypic appearance of the colonies was scored as mucoid or nonmucoid. A control strain harboring the vector without an insert was always used for comparison. Alginate was assayed by a colorimetric method (36).

Visualization of gene products by using the T7 RNA polymerase/promoter system. Polypeptides encoded by cloned genes were visualized by expression in *E. coli*, using a temperature-inducible T7 expression system (plasmid vectors pT7-5 and pT7-6 and T7 RNA polymerase encoded by pGP1-2) (61) and protein labeling with [^{35}S]methionine and [^{35}S]cysteine (Expre^{35}S^{35}S protein labeling mix; 1,000 Ci/mmol; DuPont NEN) with previously described modifications (38, 47). Proteins were separated on sodium dodecyl sulfate (SDS)–12% polyacrylamide gels. ^{14}C-labeled methylated proteins (Amersham) were used as molecular weight standards. The gels were fixed in 10% acetic acid, washed with H_2O, and impregnated with 1 M salicylic acid, and bands representing radiolabeled polypeptides were detected by autofluorography at −70°C.

Pulsed-field gel electrophoresis and Southern blot analysis. Localization of genes on the *Spe*I map of *P. aeruginosa* PAO was performed by previously published methods (55, 58). *Spe*I fragments were identified by comparison with the lambda phage concatemeric ladder ranging in size from 48.5 to 582 kb (55) as well as on the basis of hybridization to the previously mapped genes (55, 58).

Nucleotide sequence accession number. The sequence reported here has been deposited in GenBank (accession number LO2119).

RESULTS

Isolation of cosmid clones affecting mucoidy in *trans*. Several genetic studies have indicated that *muc* loci affect mucoidy when present in *trans*. For example, it has been observed that R′ derivatives of R68.45, which carry *pruAB*$^+$ and an adjacent *muc* locus from a nonmucoid PAO strain, are capable of switching off (suppressing) alginate production in mucoid strains PAO568, PAO578, and PAO581 (23). This effect appeared to be specific since another mucoid PAO derivative, strain PAO579, was not affected (23). This finding suggested to us that changes in mucoidy could be used as a screening tool to clone and isolate additional regulatory genes. Generation of a comprehensive genomic library from *P. aeruginosa* has been reported previously (55). Several cosmids from this library have been successfully used for construction of a combined physical and genetic map of *P. aeruginosa* PAO (55). This cosmid library was constructed in pLA2917 (which can replicate in *P. aeruginosa*) by using DNA from a derivative of the strain PAO1 (nonmucoid) (31, 55). The library was introduced into several mucoid strains by conjugation, and 10 independent and nonoverlapping clones capable of altering the mucoid character were isolated: pMO010533, pMO010921, pMO011021, pMO011537, pMO011644, pMO011744, pMO011801, pMO011809, pMO011920, and pMO012046. Two of the clones had previously been described as carrying other genetic markers (55). pMO011809 contains *hisI* and has been used to demonstrate that this locus resides on the *Spe*I fragment E (Fig. 1, 360 kb) in the late region of the chromosome (55). In the same study, pMO011644 was shown to carry the *oruI* gene, also mapping in the late region of the chromosome but hybridizing to a different *Spe*I fragment (Fig. 1, 330 kb; fragment F). One of the clones, pMO012046, rendered a significant number of strains completely nonmucoid and was chosen for further study. The locus affecting alginate production on this chromosomal fragment was designated *algU*.

Deletion mapping of the *algU* locus. To facilitate molecular characterization of *algU*, this locus was examined by deletion mapping. Subcloning of the ability of *algU* to suppress alginate production and mucoid phenotype was done by using the broad-host-range subcloning vector pVDZ′2 (9). Initially, a 6-kb *Hin*dIII-*Eco*RI fragment from pMO012046 was found to carry the suppressing activity and was subjected to further deletion mapping. Two series of consecutive overlapping deletions were produced from each end of the 6-kb fragment (Fig. 2), using the previously described deletion-subcloning strategy (14). Subclones of these deletion products in pVDZ′2 were transferred by conjugation into PAO568, a mucoid derivative of the standard genetic strain PAO (24). The exconjugants were screened for the loss of mucoid character. A summary of this analysis is shown in Fig. 2A. All deletion clones which retained the suppressing activity caused phenotypically indistinguishable effects; all negative deletions completely lost the ability to affect mucoidy. The activity was delimited to a region demarcated by the endpoints of deletions ΔU4/76 and ΔUM9.

***algU* has a strain-specific effect on suppression of mucoidy.** It has been shown that different mucoid PAO derivatives and clinical CF isolates display significant differences in *algD* promoter activity and alginate production in response to modulation by environmental stimuli, such as the salt concentration in the medium or growth on nitrate (12). For example, the *algD* promoter in strains PAO568 and PAO578 is induced by salt or growth on nitrate (12), although the effects differ in magnitude. PAO568 and PAO578 carry *muc* determinants designated *muc-2* and *muc-22* (24), respectively, which map close to each other and to *pruAB* (23, 25). PAO579 has a different *muc* locus (designated *muc-23*) which maps between *hisI* and *proB* (Fig. 1) and displays a completely opposite response to increased salt concentration in the medium compared with PAO568 and PAO568 (12). Another, possibly different *muc* locus is represented by *muc-3739* (strain PAM425) (43). When plasmid pDMU1, containing an active *algU* locus on the 6-kb *Hin*dIII-*Eco*RI insert in pVDZ′2, was introduced into a panel of strains representative of different mucoid PAO derivatives and CF

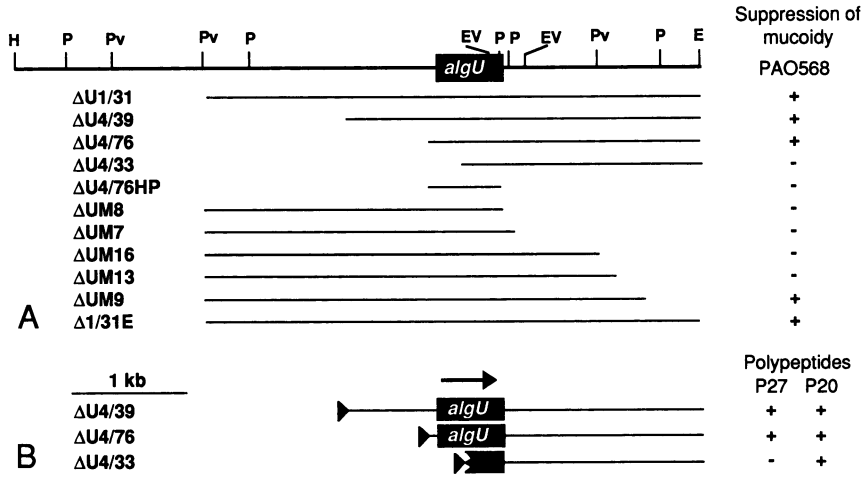

FIG. 2. Deletion mapping of the *algU* locus. Different deletion products of a 6-kb *Hin*dIII-*Eco*RI fragment from pMO012046, which suppresses mucoidy in *trans*, were subcloned on the broad-host-range plasmid pVDZ'2 and conjugated into PAO568 (*muc-2*), and exconjugants were scored for the loss of mucoid phenotype. +, loss of mucoidy; −, no effect (mucoid phenotype retained). Lines represent regions spanned by DNA fragments. Only the location of *algU* is shown; the boundaries of the other gene(s) (see text) are not known. Bar, 1 kb. Restriction sites: E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II. (B) Position of the coding region for P27 (the *algU* gene), as determined by its expression in a T7 system. Overhead arrow, direction of *algU* transcription; P27 and P20, two polypeptides of 27.5 and 20 kDa, respectively, detected in expression studies (see Results); filled triangle, T7 promoter; + and −, production and no production, respectively, of corresponding polypeptides by a given construct.

clinical isolates, a specific pattern of suppression of mucoidy was observed (Table 2). pDMU1 rendered *muc-2*, *muc-22*, and *muc-25* strains (PAO568, PAO578, and PAO581) nonmucoid. In contrast, it had no detectable effect on the *muc-23* strain PAO579 and a *muc-3739* strain (PAM425). It also affected a substantial number of mucoid clinical isolates (7 of 18 tested). Congruent with these results was the finding that the mucoid phenotype of some of the strains not affected by *algU* was affected by a different clone. For example, strain PAM425, which was not affected by pDMU1, lost its mucoid character when pRCW1, containing a 6-kb *Hin*dIII-*Nsi*I subclone from cosmid pMO011809 (55), was introduced (Table 2). pRCW1 affected three of eight CF isolates tested. Thus, the CF strains fell into three categories: (i) those affected by pDMU1, (ii) those affected by pRCW1, and (iii) those not affected by either plasmid.

TABLE 2. Strain-specific suppression of mucoidy by *algU*

Strain[a]	Suppression of mucoidy[b] with plasmid[c]:		
	pVDZ'2	pDMU1	pRCW1
PAO568 (*muc-2*)	−	+	−
PAO578 (*muc-22*)	−	+	−
PAO581 (*muc-25*)	−	+	−
PAO579 (*muc-23*)	−	−	−
PAM425 (*muc-3739*)	−	−	+
CF strains	− (18/18)[d]	+ (7/18)[e]	+ (3/8)[f]

[a] PAO strains are isogenic mucoid derivatives of *P. aeruginosa* PAO381 carrying different mapped *muc* markers (24) (Fig. 1). PAM425 is a cross between PAO and a mucoid clinical *P. aeruginosa* isolate, Ps3739 (43); the corresponding *muc-3739* locus has been mapped (43) (Fig. 1). CF strains were mucoid *P. aeruginosa* isolates from different CF patients.

[b] Scored on PIA supplemented with tetracycline as + (the strain underwent transition from mucoid to nonmucoid status when harboring the plasmid) or − (the strain remained mucoid when harboring the plasmid).

[c] pDMU1 is *algU* from PAO1 cloned as a 6-kb *Hin*dIII-*Eco*RI fragment on the broad-host-range vector pVDZ'2 (9). pRCW1 is a subclone of a 6-kb *Hin*dIII-*Nsi*I fragment (see Results) from pMO011809 in pVDZ'2.

[d] Of 18 strains tested, none were affected by the vector pVDZ'2.

[e] Of 18 strains tested, 7 lost mucoidy when harboring pDMU1. The strains affected by pDMU1 were different from those affected by pRCW1, except in one case with variable results. Not all strains tested with pRCW1 were tested with pDMU1 and vice versa.

[f] Of 8 strains in which pRCW1 was introduced, 3 lost mucoidy. See footnote *e*.

The results presented in this section indicated that (i) the suppression of mucoidy in *trans* was strain dependent, (ii) *algU* affected a significant number of CF isolates, and (iii) there was a correlation between different *muc* linkage groups and different clones exerting effects.

Two polypeptides, P27 and P20, are encoded by the region affecting mucoidy in *muc-2*, *muc-22*, and *muc-25* strains. Since deletion inactivation of the *algU* locus from either end had similar effects, suppression of mucoidy was unlikely to be due to the titration of a diffusible factor (e.g., AlgR) by its binding to DNA. Whether this locus had a coding capacity for a possible *trans*-acting factor was tested by analysis of [^{35}S]methionine- and [^{35}S]cysteine-labeled polypeptides encoded by the insert in a T7 expression system. The results of these studies are illustrated in Fig. 3. Two polypeptides, with apparent molecular masses of 27.5 kDa (P27) and 20 kDa (P20), were observed as encoded by the *algU*-containing DNA fragment. The consecutive deletions were then used to establish the order of genes and their importance for the suppressing activity (Fig. 3). Deletions extending from the *Hin*dIII end abolished P27 synthesis while not affecting P20, thus establishing the order of genes as P27 followed by P20. The gene encoding P27 was designated *algU*. Deletion ΔU4/33, which lost the ability to produce P27 but still directed the synthesis of P20, was no longer capable of suppressing mucoidy. Thus, *algU* was necessary for the activity of this region.

Suppression of mucoidy by *algU* is exerted at the level of *algD* transcription. Both *algD* and *algR* undergo transcriptional activation in mucoid cells (14). The difference in transcription is very profound at the *algD* promoter, which remains silent in nonmucoid cells and is highly active in mucoid strains (11, 12, 14). *algR* is transcribed from two

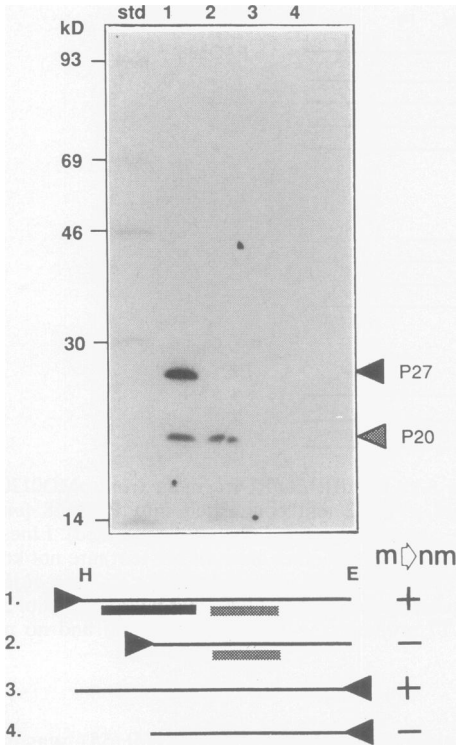

FIG. 3. T7 expression analysis of polypeptides encoded by the *algU* locus. [^{35}S]methionine- and [^{35}S]cysteine-labeled polypeptides encoded by different deletion derivatives of the *algU* region were separated by SDS-polyacrylamide gel electrophoresis and visualized by autofluorography. Lanes and DNA constructs: std, ^{14}C-labeled methylated protein standard (Amersham); 1, ΔU4/39 cloned in pT7-6; 2, ΔU4/33 cloned in pT7-6; 3, ΔU4/39 cloned in pT7-5; 4, ΔU4/33 cloned in pT7-5. Filled triangle, P27; stippled triangle, P20. Triangle at the beginning or end of a line designates the direction of transcription from the T7 promoter. Filled rectangle, the location of the gene encoding P27. The position of the gene encoding P20 (stippled rectangle) is shown arbitrarily. +, ability of the insert to suppress the mucoid phenotype in PAO568 (transition from mucoidy to nonmucoidy) when cloned in pVDZ'2; −, no suppression of mucoidy.

promoters, one distal and constitutive (47, 50) and the other proximal and induced in mucoid cells (14). We investigated whether the presence of *algU* affected transcription of *algD* and *algR*. To assay *algD* transcription under different conditions in the presence of *algU* on a plasmid, we first constructed a transcriptional fusion of *algD* and *xylE* (used as a reporter gene [37]) on the chromosome of PAO568. The strain was constructed as a merodiploid for *algD*, with one intact copy of *algD*, while the other was inactivated as a result of the fusion with *xylE* (strain PAO669; for construction details, see Materials and Methods).

The parental strain PAO568 (24) has a remarkable feature in that it displays a broad dynamic range of *algD* expression (12). Both *algD* transcription and colony morphology (changing from nonmucoid to mucoid) respond dramatically to inducing conditions (high salt concentration in the medium or growth on nitrate) (12). Strain PAO669 retained these properties (since PAO669 was merodiploid for *algD*, it could synthesize alginate). The induction of *algD* on the chromosome of PAO669 was analyzed to verify the previously established parameters of *algD* response to environmental conditions (12, 37, 50). The results of *xylE* fusion assays and phenotypic induction of mucoidy indicated that the chromosomal fusion reacted to environmental modulation in the same manner as previously reported for *algD-xylE* fusions on plasmids (Table 3). Introduction of plasmid pDMU4/76, carrying *algU* and capable of suppressing mucoidy, into PAO669 resulted in a complete loss of alginate synthesis and *algD* transcription. No induction was observed in response to environmental stimuli known to induce *algD* in PAO568 (12) (Table 3). When PAO669 harboring pDMU4/76, which displayed nonmucoid colony morphology, was transferred to a medium that no longer supplied selective pressure for plasmid maintenance, colonies segregated into outgrowing mucoid and nonmucoid sectors (data not shown). This was accompanied by a loss of the plasmid in mucoid segregants, as evidenced by the loss of Tc^r in such cells. The Tc^s bacteria (devoid of pDMU4/76) had *algD* activity restored, as indicated by activities of the chromosomal *algD-xylE* fusion in strains purified from the corresponding sectors. The mucoid segregants grown on PIA showed CDO (the *xylE* gene product) activities ranging from 1.76 to 2.01 U/mg, while the nonmucoid strains originating from the same colonies had CDO activities ranging from 0.401 to 0.445 U/mg of protein in crude cell extracts. The effect of *algU* on *algD* was confirmed by S1 nuclease protection analysis of *algD* mRNA levels (data not shown). The S1 nuclease protection experiments also indicated that neither of the *algR* promoters was affected in PAO568 harboring *algU* on a plasmid (not shown). These results strongly suggested that the effect of *algU* on mucoidy was at the level of *algD* transcription.

Insertional inactivation of the *algU* locus on the chromosome of PAO568 renders cells nonmucoid and abrogates *algD* transcription. The experiments presented in the previous

TABLE 3. Effects of plasmid-borne *algU* from PAO1 on *algD* transcription in the *muc-2* background

Strain[a]	Phenotype[b]	CDO (U/mg)[c] in given growth conditions[d]			
		LB	LB+NaCl	NH_4	NO_3
PAO669	M	0.43 (ND)	2.84 (ND)	0.22 (±0.02)	5.69 (±1.19)
PAO669(pVDZ'2)	M	0.76 (±0.14)	4.61 (±1.19)	0.59 (±0.10)	3.25 (±0.47)
PAO669(pDMU4/76)	NM	0.39 (±0.08)	0.40 (±0.08)	0.20 (±0.03)	0.20 (±0.02)

[a] PAO669 is a derivative of PAO568 (*muc-2*) in which an *algD-xylE* fusion has been placed on the chromosome. Plasmid pDMU4/76 was constructed by cloning the deletion product ΔU4/76 (Fig. 2) into pVDZ'2. This plasmid suppresses mucoidy in *muc-2*, *muc-22*, and *muc-25* PAO derivatives.

[b] Scored on inducing media (PIA, LB+NaCl, and NO_3). M, mucoid; NM, nonmucoid.

[c] Determined in cell extracts as previously described (37). One unit of CDO is defined as the amount of enzyme that oxidizes 1 μmol of catechol per min at 24°C. Standard error is given in parentheses. ND, not determined.

[d] Growth conditions and media were as previously reported (12). LB+NaCl, LB supplemented with 300 mM NaCl; NH_4 and NO_3, minimal media with ammonia and nitrate, respectively, as the nitrogen sources. The composition and use of these media for *algD* induction have been previously described (12, 50).

sections were not sufficient to conclude that *algU* participates in *algD* promoter regulation under normal circumstances. To investigate this possibility and to explore whether *algU* is a positive or a negative regulator of *algD* transcription, we insertionally inactivated this locus on the chromosome. Transposon mutagenesis of *algU* on a plasmid using Tn*5* proved to be uninterpretable, possibly because of the reported instability of Tn*5* in *P. aeruginosa* (26), and was not pursued further. Instead, a Tc^r cassette was inserted into a conveniently located restriction site within the *algU* region. These experiments were performed as follows. (i) The presence of two closely spaced *Eco*RV sites (Fig. 2) was noted in the region where the gene encoding P27 (*algU*) resided. This determination was based on the estimated size of the gene needed to encode a 27.5-kDa polypeptide and the detailed mapping of the coding region of *algU* by using T7 expression analysis (summarized in Fig. 2B) and was further confirmed by DNA sequence analysis (see below). Since the endpoint of the last positive deletion still producing P27 was located 540 bp upstream from the first *Eco*RV site, we concluded that this site must be within the *algU* coding region. (ii) A suicide plasmid (pDMU100) was constructed (see Materials and Methods) in which the 2.4-kb *Hin*dIII-*Eco*RI fragment from ΔU4/76 was placed on pUC12 which cannot replicate in *P. aeruginosa*. *Eco*RV sites within the *algU* insert were converted into *Not*I specificity, and a Tc^r cassette (32), modified as a *Not*I fragment, was inserted. After addition of a DNA fragment with the *mob* functions to facilitate plasmid mobilization into *P. aeruginosa* (57), the final construct (pDMU100) was conjugated into PAO568 and Tc^r exconjugants were selected. These strains were expected to have the plasmid with *algU*::Tc^r integrated on the chromosome via homologous recombination. Two possible types of recombinants were anticipated: (i) merodiploids for *algU*, retaining an active *algU* copy, which would have an insertion of the entire plasmid as the result of a single crossover event and (ii) true gene replacements, products of double crossovers, in which case the plasmid moiety and the associated markers would be lost. We have observed in other gene replacement studies using this procedure that double-crossover events on the *P. aeruginosa* chromosome are frequent and that they range from 10 to 70% for various genes studied (unpublished results), obviating in all cases examined the need for a positive selection against markers encoded by the plasmid moiety. In nine independent experiments with *algU*::Tc^r, 1,663 Tc^r exconjugants were examined. Of these, 29% lost Cb^r encoded by the plasmid moiety, indicative of double-crossover events. All such Tc^r Cb^s strains were nonmucoid and did not produce alginate under any of the conditions tested. Most of the colonies with Tc^r and Cb^r markers (results of single-crossover events and thus expected to have a functional copy of *algU*) were mucoid, while a portion of such strains showed a delayed mucoid phenotype (mucoidy was developing after 3 to 4 days, compared with 48 h needed for the parental strain PAO568). Further experiments with Tc^r Cb^s recombinants using Southern blotting analysis confirmed that these nonmucoid strains had a true gene replacement with the chromosomal copy of *algU* disrupted by the Tc^r cassette (Fig. 4). Moreover, when the mutation in such strains was purified by transduction (using the generalized transducing phage F116L) into the parental strain PAO568, all Tc^r transductants displayed a nonmucoid phenotype. One of the *algU*::Tc^r derivatives characterized in these experiments (strain PAO670) was used to investigate *algD* transcription. This time, the previously characterized *algD-xylE* fusion

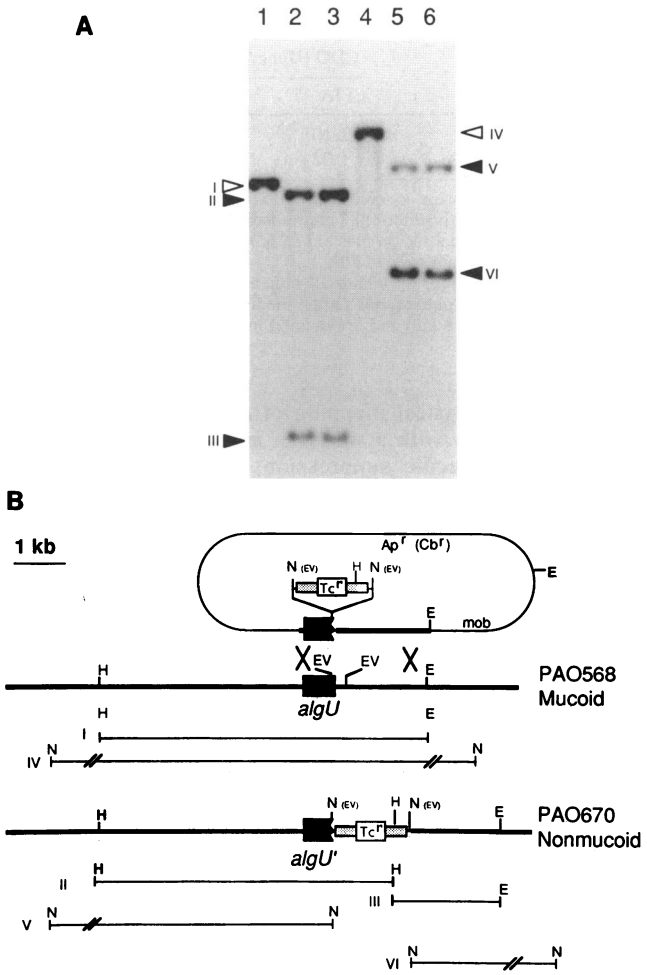

FIG. 4. Insertional inactivation of *algU* on the PAO568 chromosome. (A) Southern blot analysis of chromosomal DNA from PAO568 (lanes 1 and 4) and from PAO670 (lanes 2 and 5) digested with *Hin*dIII-*Eco*RI (lanes 1 and 2) and *Not*I (lanes 4 and 5). Lanes 3 and 6 show *Hin*dIII-*Eco*RI and *Not*I digests, respectively, of another nonmucoid derivative of PAO568 which, like PAO670, underwent a gene replacement of *algU* with *algU*::Tc^r. (B) Events leading to the gene replacement in PAO670. Plasmid pDMU100 (oval) was constructed as described in Materials and Methods and conjugated into PAO568, and double-crossover mutants were selected. Different *algU* variants and resulting restriction fragments in PAO568 and PAO670 are shown. I (*Hin*dIII-*Eco*RI) and II (*Not*I), chromosomal fragments of PAO568 hybridizing (open triangles) to the *algU* probe (ΔU4/76). Filled triangles, fragments in PAO670 hybridizing with the probe. II and III, fragments detected after digestion with *Hin*dIII and *Eco*RI. V and VI, fragments detected after digestion with *Not*I. Oval, plasmid pDMU100 (thin line, vector sequences; thick line, *algU* insert). Filled rectangle, *algU*. Jagged edge indicates incomplete *algU*. Stippled rectangle, Tc^r cassette. X, crossover points (chosen arbitrarily). Thick horizontal lines, chromosomal regions of PAO568 and PAO670. Thin lines, location of restriction fragments detected on the blot. // indicates that the fragment is longer than actually shown. Horizontal bar, 1 kb. Small vertical bars, restriction sites. N, *Not*I; N(EV), *Eco*RV site converted into *Not*I. Other sites are as in Fig. 2.

plasmid pPAOM3 (37) was introduced into PAO670, and *algD* promoter activity was assayed. These results (Table 4) indicated that inactivation of the *algU* locus on the chromosome resulted in a loss of *algD* transcription and strongly suggested a positive role for *algU* in *algD* expression.

TABLE 4. Analysis of *algD* transcription in PAO670 (*algU*::Tc^r)

Strain[a]	CDO (U/mg)[b] in given growth conditions[c]		
	PIA	LB+NaCl	NO_3
PAO568(pPAOM3)	12.10	11.54	10.95
PAO670(pPAOM3)	1.02	1.85	1.40

[a] PAO568 (*muc-2*) is the mucoid parental strain of PAO670. PAO670 has *algU* insertionally inactivated on the chromosome. Both strains harbored the *algD-xylE* transcriptional fusion plasmid pPAOM3.
[b] Relative error did not exceed 20%.
[c] PIA is a rich medium on which all mucoid strains, including PAO568, present their mucoid phenotype. Other media induce mucoidy and *algD* transcription in PAO568 (12) and are defined in Table 3, footnote *d*.

Genetic and physical mapping of *algU* indicates its close linkage or identity with a subset of *muc* loci. Plasmid-borne *algU* showed specific suppression of mucoidy in strains containing *muc-2* and *muc-22*. These and other *muc* loci have been suggested to participate in the emergence of mucoid strains (24, 43), although their nature and mechanism of action have not been studied. Extensive information is available on the linkage of *muc* to genetic markers within the late region of the PAO chromosome (23–25, 43) (Fig. 1). Of particular significance is the cotransducibility of *muc-2* and *muc-22* with the *pru-354* marker (a mutation in *pruAB*, genes required for the utilization of proline as the sole carbon and nitrogen source [44]) demonstrated by bacteriophage F116L-mediated genetic exchange (23, 25). This result indicates that these *muc* loci and the *pruAB* genes are very close, since F116L can transduce regions in the range of 1 min of the chromosome.

We took two approaches to localize *algU* on the chromosome. The first one was based on the recently determined physical map of *P. aeruginosa* PAO (55); in these experiments, *algU* was used as a probe for Southern hybridization analysis of *Spe*I fragments separated by pulsed-field gel electrophoresis. The second approach was to map *algU* via F116L transduction; in this case, we took the advantage of having a strain (PAO670) with the *algU* gene on the chromosome tagged with the Tc^r cassette and monitored the coinheritance of *pruAB* with Tc^r.

The results of Southern blot analyses with *Spe*I-digested PAO chromosome subjected to separation by pulsed-field gel electrophoresis are illustrated in Fig. 5. As explained in the figure legend, several consecutively applied probes were used to confirm identification of the *Spe*I fragments. The *algU* gene hybridized to the 330-kb *Spe*I fragment (F) known to carry two genetic markers linked to *muc-2* and *muc-22*: *pur-70* at 66 min and *pruAB* at 67.5 min (55). This finding indicated that *algU* may be close to the *muc-2* and *muc-22* markers. To explore this possibility, cotransducibility of *pruAB* with *algU*::Tc^r was tested. The results of transductional crosses between PAO670 (*algU*::Tc^r on the chromosome of PAO568 [*muc-2*]) and PAO964 (*pru-354*), a mutant in *pruAB* which cannot grow on proline as the sole carbon and nitrogen source, revealed a high degree of coinheritance of *pruAB* with *algU*::Tc^r (Table 5). The percent coinheritance of Tc^r with *pruAB* corresponded closely to the values previously reported for *muc-2* and *muc-22* (20 to 49%) (23, 25). In a control experiment, no coinheritance of *hisI* and Tc^r was observed with use of the same transducing phage lysates (Table 5). Significantly, no mucoid transductants (expected from the transfer of *muc-2*) among over 700 colonies examined were observed in these crosses regardless of whether the selection was for *pru*$^+$ or Tc^r. This observation was in sharp contrast with the results obtained with the recipient strain PAO964 and the donor strain PAO568 (*muc-2*; the strain parental to PAO670). Normally, 49% of the *pru*$^+$ colonies are mucoid in transductions involving PAO568 and PAO964 (23, 25). Although PAO568 in our hands had the capacity to transfer the *muc-2* marker conferring mucoidy upon the recipient cells, its *algU*::Tc^r derivative PAO670 completely lost this ability. This effect could be attributed to the insertional inactivation of *algU* in PAO670. These results strongly suggest that *algU* is located close to the *muc* loci represented by *muc-2* and *muc-22* and may even be allelic with these determinants.

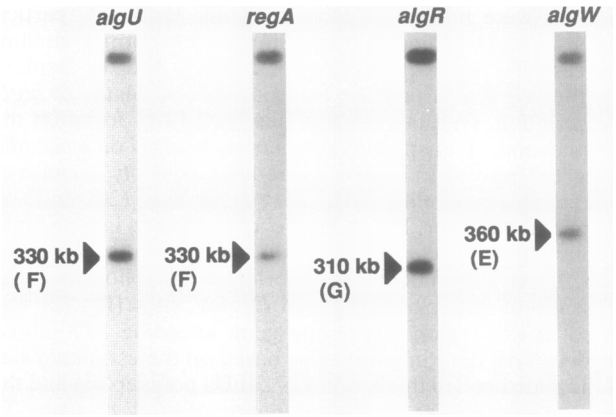

FIG. 5. Physical mapping of *algU* on the chromosome of *P. aeruginosa*. Shown is a Southern blot hybridization of various probes (indicated above each strip) with PAO1 DNA digested with *Spe*I; fragments were separated on agarose gels by pulsed-field gel electrophoresis and blotted onto a membrane. The radiolabeled probes were hybridized, autoradiograms were obtained, probes were stripped of the filter and checked for completeness of the process, and the blot was reprobed with a different gene. Probes: *algU*; *regA*, a gene that regulates toxin A synthesis (30); *algR*, a response regulator controlling *algD* transcription (10); *algW*, a 6-kb *Hin*dIII-*Nsi*I fragment from pMO011809 that also affects mucoidy (see Results) (55). Horizontal bar, chromosomal DNA retained within the well hybridizing with all probes. The *Spe*I fragments hybridizing to corresponding probes are indicated by triangles; their sizes and designations (letters in parentheses), based on the physical map (*Spe*I) of the *P. aeruginosa* chromosome, are indicated.

TABLE 5. Cotransduction of *algU* and *pruAB*[a]

Donor × recipient	Selected marker[b]	% Coinheritance of the unselected marker[c]	
		Tc^r	Mucoidy
PAO670 × PAO964	*pru-354*$^+$	20.3	0 (<0.3)
PAO670 × PAO540	*hisI*$^+$	0 (<0.25)	0 (<0.25)

[a] F116L transduction was performed by using an *algU*::Tc^r derivative of PAO568 (*muc-2*) (strain PAO670) as the donor and PAO964 (*pru-354*) or PAO540 (*cys-5605 his-5075 argA171*) as the recipient. PAO670 is nonmucoid as a result of the inactivation of *algU* by the insertion of a Tc^r cassette. PAO964 and PAO540 are nonmucoid.
[b] *pru-354* is a mutant allele of *pruAB* (44). PAO964 (*pru-354*) cannot grow on proline as the sole carbon and nitrogen source. The selection was performed for *pruAB*$^+$ or *hisI* as described in Materials and Methods.
[c] *pruAB*$^+$ transductants (300 colonies) and *hisI* transductants (400 colonies) were tested for coinheritance of Tc^r. Tc^r in transduction crosses originates from *algU*::Tc^r on the PAO670 chromosome. No strain displayed mucoid character in at least two independent transduction experiments. In a reciprocal experiment, in which Tc^r was the selected marker, a 50% coinheritance of *pruAB*$^+$ with Tc^r was observed (not shown).

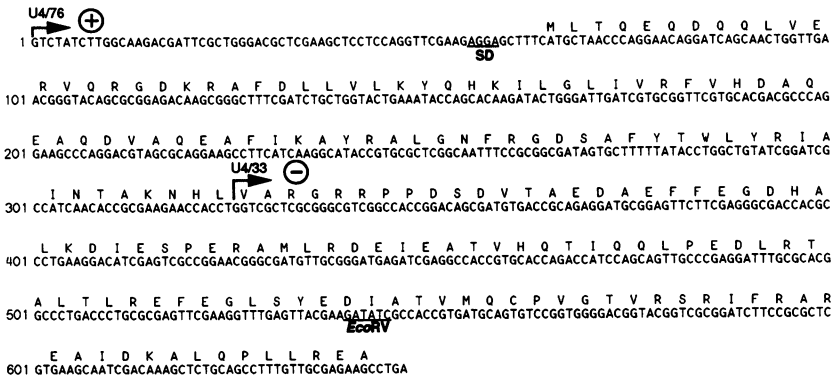

FIG. 6. DNA sequence of *algU*. Bent arrows denote the endpoints of deletions. U4/76 suppresses mucoidy and produces P27 (+); U4/33 has no effect on mucoidy and is not capable of producing P27 (−). *Eco*RV, a site used for insertional inactivation of *algU* on the chromosome, is shown.

AlgU shows sequence similarity with σ^H (Spo0H), a sigma factor required for developmental processes in *Bacillus subtilis*. To gain information about the nature and possible function of genetic elements within the *algU* region, the nucleotide sequence of the DNA region from the endpoint of deletion ΔU4/76 (the last 5′ deletion positive for suppression of mucoidy and synthesis of P27) and extending through one of the *Eco*RV sites used for insertional inactivation of *algU* was determined (Fig. 6). An open reading frame was identified within the region defined as *algU* by deletion and functional mapping. This sequence contained translational initiation signals, conformed with *Pseudomonas* codon usage (63), and was in the direction of transcription determined in T7 expression studies. When a global homology search was performed by using the translated sequence of *algU* with GenBank and NBRF data bases, two known proteins showed statistically significant similarity with AlgU: σ^H (Spo0H) from *Bacillus licheniformis* and *B. subtilis* (Fig. 7). σ^H is dispensable for growth and is primarily required for initiation of sporulation and other developmental processes (competence) in *B. subtilis* (20, 62). The sequence similarity observed (24.9% identity over the entire length of both sequences with an optimized score of 155), although limited, was equivalent to the extent of similarity of σ^H to other known sigma factors (ranging between 22 and 31% identity with optimized scores of between 113 and 145) (20). All regions noted in several sequence compilations and alignments of sigma factors (29, 41) were represented in the regions of homology between Spo0H and AlgU. The predicted pI of AlgU was 5.315, similar to the pI of Spo0H (5.052 to 5.146). A relatively low pI is characteristic of sigma factors (45) and is known to cause anomalous mobility of several members of this class of proteins during SDS-polyacrylamide gel electrophoresis (45). This may help explain a discrepancy in the observed electrophoretic mobility corresponding to 27.5 kDa and the predicted molecular mass of AlgU from the sequence (22,194 Da) which is in the range of discrepancies reported for several sigma factors (45). *B. subtilis* σ^H shows electrophoretic mobility corresponding to 30 kDa, while its predicted M_r is 25,331 (5).

DISCUSSION

In this work, we have presented the cloning and molecular characterization of *algU*, a newly described factor participating in the control of mucoidy in *P. aeruginosa*. AlgU affects mucoid phenotype and *algD* transcription and shows sequence similarity with the sigma factor σ^H (Spo0H) from

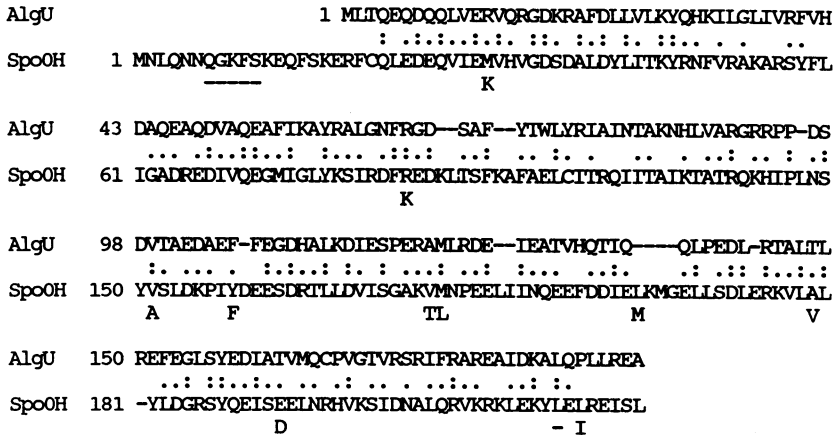

FIG. 7. Sequence similarities of AlgU and Spo0H. Double dots indicate identities; single dots indicate conserved amino acid substitutions. The Spo0H sequence from *B. licheniformis* (20) is shown. Letters and dashes below the line with the Spo0H from *B. licheniformis* indicate amino acid substitutions and absence of the corresponding amino acids, respectively, in Spo0H from *B. subtilis* (20).

B. subtilis and *B. licheniformis*. *algU* has been mapped in this study by physical and genetic means and is located in the late region of the *P. aeruginosa* chromosome. This is the same area where several linkage groups of the previously genetically identified *muc* loci are known to map (24, 43). The *muc* markers confer mucoidy during chromosomal exchange between mucoid donors and nonmucoid recipients (24, 43). The *algU* and adjacent downstream genes are tightly linked and possibly allelic with one such *muc* linkage group, *muc-2* and *muc-22*, defined as the cluster of *muc* loci cotransducible with *pruAB*.

The genomic library from which *algU* originated was generated by using DNA from a nonmucoid PAO strain. It has been postulated that *muc* markers are mutations conducive to mucoidy and that *muc-2*, *muc-23*, and *muc-3739* represent mutant alleles of the respective wild-type *muc* genes (24, 43). This hypothesis is based on the findings that the genetic transfer of *muc* markers confers mucoidy (24). It will be of interest to compare functional properties of the *algU* regions cloned from different mucoid and nonmucoid strains. Work is under way to compare the sequence reported here and that of the downstream region with the corresponding sequences from the *muc-2* and *muc-22* PAO derivatives PAO568 and PAO578 (24). Our preliminary results suggest the presence of mutations affecting and possibly inactivating the genes downstream of *algU*. For example, an alteration within the gene encoding P20 (*mucA*) that may represent the *muc-2* allele has been found in strain PAO568 (43a). Whether and how mutations in the downstream genes affect the expression or function of the *algU* gene product, or whether they act independently of *algU*, is currently being investigated.

Experiments described here indicate that the *algU* region is different from *muc-23* and *muc-3739* (24, 43). This observation is in agreement with results of previously published genetic studies suggesting that several groups of genes affecting mucoidy exist in the late region (24, 25, 43, 51). The relationship of *algU* to *algST*, another more recently reported locus (22, 51), is not known since these genes have been mapped in a different strain of *P. aeruginosa* (FRD) (22). Although *algST* appears to be in the late region of the chromosome, unlike *algU*, it has been reported as not cotransducible with the *pruAB* genes (22) and to encode a 34-kDa polypeptide (64). However, molecular characterization of additional *muc* loci and *algST*, as well as determination of their DNA sequence, is needed to make more conclusive comparisons.

In this study, we focused our attention on the *algU* gene. Another locus (from pMO011809) preliminarily characterized here also maps in the late region of the chromosome but hybridizes to a different *Spe*I fragment. We have previously suggested that some of the *muc* loci may carry mutations which alter the function of putative protein kinases/phosphatases interacting with AlgR, a response regulator directly controlling *algD* transcription (12). However, the first characterized gene from this region, *algU*, shows no similarity with this class of proteins. The work on genes from pMO011809 and other cloned regions will continue in that direction.

The similarity of the predicted *algU* gene product with a known sigma factor combined with the requirement for *algU* in *algD* transcription suggests a possible function for AlgU. Although the percent identity between AlgU and σ^H is relatively low, many important residues (29, 41) are conserved. Several clusters of similar residues are recognizable, and they follow the pattern of conserved regions and subregions postulated to play distinct roles in sigma factor function (29, 41). Limited regions of homologies with other sigma factors that did not appear in global homology searches were also observed (data not shown). These additional similarities are not random; the regions of similarity between AlgU and RpoD from different organisms display 25% identity and encompass conserved subregions such as 2.1, 2.2, 2.3, and 2.4, including the *rpoD* box, and a part of the 14-mer region (41). These blocks of sequence conservation have been implicated in binding to core (2.1), strand opening (2.3), and −10 recognition (2.4) in several sigma factors (41). It should be pointed out that σ^H itself shows limited similarity with other sigma factors (20). It belongs, according to a recent classification, to group 3 of alternative sigma factors, which display the highest divergence from primary sigma factors (41). σ^H shows 22% identity with *B. subtilis* σ^A (RpoD) (20). AlgU has 20.5% identity with *B. subtilis* σ^A in a 151-amino-acid overlap and 19.2% identity with *E. coli* σ^{70} in a 156-amino-acid overlap (not shown).

It may also be of interest that the *algD* promoter sequence lacks a recognizable similarity with canonical −10/−35 regions transcribed by major sigma factors. The *algD* promoter does not depend on σ^{54} (50). A consensus sequence for σ^H promoters has been proposed (53, 62). It has been noted that subgroups of homologous alternative sigma factors from group 3 (41) recognize promoters that share some similarity even when their biological functions are dissimilar (6, 41). Experiments are in progress to determine which of the residues in the *algD* promoter may be important for *algD* transcription. It will also be of interest to examine whether *algU* is needed for *algD* expression in *muc* mutants other than those clustered in the *algU* region (e.g., *muc-23* and *muc-3739*), as might be expected if *algU* was the sigma factor acting at *algD*. Preliminary experiments with a chromosomal *algD*::*lacZ* fusion in a λ lysogen of *E. coli*, which is completely inactive unless the *algU* gene is provided in *trans*, support such a function for this factor.

The general direction of this research was to clone additional regulatory genes controlling mucoidy. A cloning strategy has been applied on the basis of the rationale that mucoidy may be affected when genes involved in the control of *algD* transcription are present in several copies on a plasmid. Ten different DNA fragments that can reduce or totally suppress mucoidy have been obtained in this way. Most of these clones hybridize to different *Spe*I fragments corresponding to various positions on the genetic map (e.g., around 40, 50, 66.5, and 67.5 min), suggesting that the regulation of alginate may be affected by many different loci on the chromosome. Direct or indirect involvement of a multitude of genes is frequently encountered in the regulation of very complex processes such as bacterial development (19, 42).

It has been proposed that the overproduction of alginate by *P. aeruginosa* in CF represents a modified differentiation or developmental process (25, 28). Chronic respiratory infections with *P. aeruginosa* in CF are characterized by the growth of this organism in biofilms, frequently referred to as the microcolony mode of growth (8), which affords adherence to the substrate and protection against host defense mechanisms, in particular phagocytosis (27, 39). Exopolysaccharide synthesis by *P. aeruginosa* outside the CF lung plays a role in the formation of biofilms (7), a process which represents differentiation from a planktonic (mobile) to a sessile (exopolysaccharide-embedded) cell type (2, 7). Alternation between two metabolically and morphologically different forms, the free-swimming planktonic cell and the

immobilized cell within the biofilm adherent to a surface, is the preferred mechanism used by *P. aeruginosa* for survival in natural environments (8, 25). One possibility is that the emergence of mucoid strains in CF represents a variation of these processes. It may be worth noting that the exopolysaccharide alginate participates in the developmental process of encystment (into dormant cells resistant to dehydration) in another known alginate-producing bacterium, *Azotobacter vinelandii* (56).

Other pathogenic bacteria undergo complex adaptation processes. It has been recently suggested that *Bordetella pertussis*, the causative agent of whooping cough, undergoes sequential differentiation stages (dependent on signal transduction) as it progresses through the infectious cycle (54). *Chlamydia trachomatis* alternates during its infectious cycle (4) between two morphologically distinguishable developmental stages (reticulate and elementary body), involving a histone-like element homologous to AlgP (H_p1; participating in the control of mucoidy [13, 15]). The complexity of the regulation of mucoidy (18) and the involvement of several signal transduction systems (10, 18, 65), histone-like proteins (13, 34), and, as reported in this work, an element (AlgU) displaying sequence similarity to σ^H, a sigma factor involved in developmental processes (sporulation and competence) in *Bacillus* spp., suggest that the control of alginate production may be another example of such regulatory phenomena in bacterial pathogenesis.

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