Gene *algD* Coding for GDPmannose Dehydrogenase Is Transcriptionally Activated in Mucoid *Pseudomonas aeruginosa*

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Transcriptional regulation of alginate biosynthesis by *Pseudomonas aeruginosa* was studied. A DNA region complementing the *alg-5* mutation within the alginate gene cluster was found by RNA-DNA dot blot and Northern hybridization to be transcriptionally activated in mucoid *P. aeruginosa*. This region was subcloned as a 3.2-kilobase *Bgl*III-*Cla*I DNA fragment on the broad-host-range controlled transcription vector pMMB24, and gene products were analyzed by expression from the *tac* promoter. A 48-kilodalton polypeptide was detected in extracts of *P. aeruginosa* and ³⁵S-labeled *Escherichia coli* maxicells. By using the same expression system, GDPmannose dehydrogenase activity was detected in both *P. aeruginosa* and *E. coli*. Thus, gene *algD* coding for this enzyme was found to be present in the transcriptionally active DNA area. Insertion of the *xylE* gene within the *Bgl*III-*Cla*I fragment disrupted the induction of the 48-kilodalton polypeptide, GDPmannose dehydrogenase activity, and *alg-5* complementing ability. With the *algD*-*xylE* transcription fusion, activation of *algD* gene expression was shown to occur in mucoid *P. aeruginosa* of different origins. In addition, regulation of the *algD* promoter activity was demonstrated to be mediated by a diffusible factor.

Cystic fibrosis (CF) is the most common lethal genetic disorder in the Caucasian population (11). Although the genetic defect of CF produces clinical manifestations of a generalized exocrinopathy, the most serious complication of this disease is chronic pulmonary infection (45). In the course of prolonged antibiotic treatment the initial bacterial pathogens *Staphylococcus aureus* and *Haemophilus influenzae* are replaced in CF lungs by *Pseudomonas aeruginosa* (5). The *P. aeruginosa* strains that initially invade CF lungs have a typical nonmucoid phenotype, but as the infection persists and antibiotic therapy continues, these strains characteristically shift to the mucoid, alginate-producing form (13). Slimy exopolysaccharide alginate is a polymer of O-acetylated, β (1-4)-linked D-mannuronic acid and its epimer L-guluronic acid (15, 29) which, when combined with the abnormally viscous CF lung mucus, causes respiratory duct obstruction. During the terminal stages of the disease, the predominant mucoid *P. aeruginosa* organisms are associated with clinical deterioration, resulting in a high patient fatality rate (11).

In contrast to their prevalence in CF lungs, mucoid *P. aeruginosa* strains revert to the nonmucoid form in vitro at a high rate (23, 24). This phenomenon has previously hampered genetic studies of alginate biosynthesis. Recently, however, the availability of *alg* mutants derived from a stable mucoid strain (8) and *alg* temperature-sensitive mutants (22) has allowed cloning of alginate-specific DNA fragments by genetic complementation (8, 9, 22). Our laboratory has demonstrated that, with the exception of one mutation (*alg-22*) mapping near the *argH* gene at 19 min of the *P. aeruginosa* chromosome (8), most of the mutations affecting alginate production are located near the *argF* gene at 45 min, forming a cluster that can be subdivided into at least six complementation groups (10). One gene, *algA*, was functionally identified in this cluster by intergeneric complementation (9) and direct assaying of the overproduced gene product (20) as coding for phosphomannose isomerase

(PMI). However, very little is known about the other alginate-specific genes, or the environmental and genetic factors causing the emergence of alginate-producing strains, exclusive to the CF lungs. Here we report a transcriptional activation in mucoid *P. aeruginosa* of a chromosomal region within the alginate gene cluster and identification of its gene product and function as GDPmannose dehydrogenase (GMD).

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this work are listed in Table 1. Exposure of *P. aeruginosa* to ethyl methanesulfonate (Sigma Chemical Co.) was performed as described previously (12).

Media and bacterial growth. *Escherichia coli* strains were grown in LB medium (GIBCO Laboratories) supplemented with ampicillin (40 μ g/ml), tetracycline (25 μ g/ml), kanamycin (50 μ g/ml), and 1 mM isopropyl- β -thiogalactopyranoside (IPTG) when required. *P. aeruginosa* strains were grown in LB liquid medium supplemented with carbenicillin (300 μ g/ml) or tetracycline (50 μ g/ml), when required, and 1 or 5 mM IPTG as indicated. *P. aeruginosa* was grown on *Pseudomonas* isolation agar (Difco Laboratories) supplemented with carbenicillin (1,000 μ g/ml) or tetracycline (300 μ g/ml) and 5 mM IPTG when required. All incubations were at 37°C.

Conjugal plasmid transfer and genetic complementation. Plasmid transfers from *E. coli* to *P. aeruginosa* were performed by triparental filter matings as described before (12), using helper strain *E. coli* HB101(pRK2013). Genetic complementation was scored by observing the mucoid phenotype of transconjugants and by assaying alginate as described previously (26).

DNA biochemistry. Plasmid DNA isolation, restriction endonuclease digestion, and other DNA manipulations were performed as described previously (12, 30).

RNA isolation. Total cellular RNA was isolated by an adaptation of the CsCl purification procedure (21) for bacterial samples. Overnight cultures of *P. aeruginosa* were

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TABLE 1. Bacterial strains and plasmids

Species and strain or plasmid	Pertinent properties	Reference
<i>E. coli</i>		
HB101	<i>hsdS20 (hsdR hsdM) recA pro rpsL20</i> (Str ^r)	30
JM83	<i>ara Δ(lac-pro) rpsL (Str^r) thi φ80 dlacZΔM15</i>	43
CSR603	<i>phr-1 recA1 uvrA6</i>	39
<i>P. aeruginosa</i>		
8821	<i>his-1 Alg⁺</i> (unstable)	8
8822	<i>his-1 alg-1</i> (spontaneous)	8
8830	<i>his-1 Alg⁺</i> (stable)	8
8835	<i>his-1 alg-5</i>	8
SR6339-1	<i>Alg⁺</i> (fresh CF isolate)	This study
SR6339-2	<i>alg</i> (spontaneous)	This study
PAO2003	<i>argH rec-2 alg</i>	7
PAOM18	<i>argH rec-2 Alg⁺</i> (unstable, ethyl methanesulfonate induced)	This study
Plasmid		
pMMB24	12.7 kb, IncQ/P4 <i>mob⁺ tra Ap^r tac lacI^a</i>	2
pRK2013	48 kb, ColE1 <i>mob⁺ tra⁺</i> (RK2) Km ^r	19
pVDZ'2	20.5 kb, IncP1 <i>mob⁺ tra lacZ'</i>	This study
pAD2	32.5 kb, IncP1 <i>mob⁺ tra Tc^r alg-44⁺ alg-8⁺ alg-5⁺</i>	8
pVD211	15.9 kb, Ap ^r <i>alg-5⁺ tac lacI^a</i> (sub-clone of <i>algD</i> from pAD2 in pMMB24)	This study
pTS71	14.2 kb, ColE1 Ap ^r <i>xylDEGF⁺</i> (sub-clone of TOL plasmid in pBR322)	25
pVD2X	32.2 kb, Tc ^r <i>alg-44⁺ alg-8⁺ alg-5</i> (<i>algD-xylE</i>) (<i>algD-xylE</i> transcription fusion)	This study

diluted 100 times into the fresh medium. After the initial growth for 2 h, IPTG was added to a final concentration of 5 mM when needed. The total period of growth was 4 h. Cultures were rapidly cooled in dry ice-ethanol and centrifuged in a Sorvall GSA rotor for 10 min at 6,000 rpm and 4°C. Pellets were washed with 5 ml of ice-cold lysis buffer (50 mM Tris hydrochloride, pH 7.0) and suspended in another 5 ml of lysis buffer at room temperature. Subsequently, 1 ml of 20% sodium dodecyl sulfate (SDS) was added and mixtures were incubated at 56°C for 5 min. Solid CsCl (4 g) was dissolved and an additional 5 ml of lysis buffer was added to each sample. After mixing, cell debris was removed by centrifugation for 10 min at 11,000 rpm in a Sorvall SM-24 rotor at room temperature. Clear supernatant liquids were layered over a 2-ml cushion of 5.7 M CsCl. RNA was pelleted by centrifugation at 35,000 rpm in an SW50.1 rotor for 12 to 20 h at 15°C. RNA pellets were suspended in 300 µl of water, extracted with chloroform-isoamyl alcohol (24:1), precipitated by adding 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol, and finally stored at -20°C.

RNA-DNA dot blot and Northern hybridization analyses. The concentration of RNA was determined spectrophotometrically at 260 nm. Glyoxylation of RNA, dot blot, and Northern hybridization were performed as described previously (42). Gene Screen Plus hybridization transfer membranes (E. I. du Pont de Nemours & Co., Inc.; New England Nuclear products) were used for RNA immobilization. DNA fragments were extracted from agarose gels as described before (14) and labeled to high specific activity by the mixed oligodeoxynucleotide primer technique (17), using

[³²P]dCTP (3,000 Ci/mmol; Amersham Corp.). RNA size and hybridization standards were 23S, 16S, and 5S rRNAs from Boehringer Mannheim Biochemicals.

Maxicell analysis and protein gel electrophoresis. Plasmid-encoded proteins were labeled with [³⁵S]methionine (800 Ci/mmol; Amersham), using *E. coli* maxicell strain CSR603 as described previously (39). Induction of transcription from the *tac* promoter was performed by incubation of the culture in the presence of 1 mM IPTG. Protein gel electrophoresis of products from maxicell analysis was performed on 10% SDS-polyacrylamide gels as described before (27). Prior to autoradiography, the gels were fixed in a solution of 10% acetic acid and 10% methanol for 30 min, washed for 30 min in water, impregnated with 1 M sodium salicylate for 1 h, and dried under vacuum. ¹⁴C-methylated protein standards were from Amersham.

Total cellular proteins were solubilized in the electrophoresis loading buffer (27) and separated on 5 to 20% gradient SDS-polyacrylamide gels in the presence of 4 M urea. Gels were stained with Coomassie brilliant blue R-250 (Bio-Rad Laboratories) as described previously (16). Protein standards were from Bio-Rad Laboratories.

Enzyme assays. All enzyme assays were performed at 25°C. In all samples protein concentration was determined by the method of Bradford (3). Assay of GMD in cell-free *P. aeruginosa* and *E. coli* extracts prepared as described before (20) was performed according to published procedures (38). GDPmannose-dependent reduction of NAD⁺ was monitored by following A₃₄₀. Catechol 2,3-dioxygenase (CDO; EC 1.13.11.2) was assayed as described previously (35).

RESULTS

Transcriptional activation of a region within the alginate gene cluster in mucoid *P. aeruginosa*. To elucidate the molecular mechanisms by which factors in CF lungs promote the emergence of mucoid *P. aeruginosa* strains, we studied the in vivo transcript formation from various alginate-specific chromosomal regions that had been previously isolated in our laboratory (8, 9). To this end we used RNA-DNA dot blot hybridization analysis, probing total cellular RNA isolated from mucoid strain 8821 and its spontaneous nonmucoid revertant, strain 8822, with different cloned segments of alginate gene cluster at 45 min (Fig. 1B). Earlier characterization of *algA*, which codes for PMI, showed that the transcription of this gene was very low in both mucoid and nonmucoid strains, below the detection limits of RNA-DNA dot blot hybridization (20). However, using the insert of recombinant plasmid pAD2 as a probe (Fig. 1C), we observed a strong hybridization signal with RNA from the mucoid *P. aeruginosa* but not from the nonmucoid strain (data not shown). The insert of plasmid pAD2 spans most of the alginate gene cluster at 45 min of the *P. aeruginosa* chromosomal map (Fig. 1B) of the *P. aeruginosa* transcriptionally active region, we used various segments of the pAD2 insert as hybridization probes. Comparison of the total cellular RNA from strains 8821 and 8822 revealed a significant increase in the hybridization signals with the RNA from mucoid strain 8821 only when *EcoRI* fragments C and D of the pAD2 insert were used as the hybridization probes (Fig. 2). Further characterization of overlapping fragments (data not shown) localized the transcriptionally active area in mucoid cells to the 3.2-kilobase (kb) *BglII*-*ClaI* fragment (*BglII*-*ClaI* fragment A) that included *EcoRI* fragments C and D.

The transcriptionally activated region complements the *alg-5* mutation and encodes a 48 kilodalton (kDa) polypeptide.

FIG. 1. Alginate biosynthetic pathway; genes and recombinant plasmids used in this study. (A) Alginate biosynthetic pathway. Intermediates: F6P, fructose 6-phosphate; M6P, mannose 6-phosphate; M1P, mannose 1-phosphate; GDPM, GDPmannose; GMA, GDPmannuronic acid. Enzymes: A, PMI; B, phosphomannomutase; C, GDPmannose pyrophosphorylase; D, GMD. Steps E, F, G, and I are putative enzymatic activities involved in the steps of polymerization, epimerization, and export through the cell membrane. Closed triangles denote enzymatic steps conclusively determined here or elsewhere (9, 20). (B) Chromosomal regions with *alg* mutations and genes. Genes coding for PMI, *algA* (20), and GMD, *algD*, are located at the extreme ends of the alginate gene cluster (10) mapped at 45 min of the *P. aeruginosa* PAO chromosomal map. The position of these two genes corresponds to the locations of *alg-43* and *alg-5* mutations, respectively (10). Mutation *alg-22* was mapped at 19 min of the chromosome (8) and is part of the DNA region which, when chromosomally amplified, induces alginate production (12). (C) Plasmids used in this study. Plasmid pAD2 is a cosmid clone complementing *alg-5*, *alg-8*, and *alg-44* mutations (8). Plasmids pVD210 and pVD211 are clones of *Bgl*III-*Cla*I fragment A, with the ends converted into the *Bam*HI sequence by the use of linkers, into the *Bam*HI site of broad-host-range expression vector pMMB24 (2). Also shown is the insert portion of plasmid pVD2X used in the transcription fusion expression studies. The 2.2-kb DNA fragment from pTS71 (25) containing *xylE* was inserted into a single *Xho*I site of the *algD* gene. Restriction endonuclease sites: B, *Bam*HI; Bg, *Bgl*III; C, *Cla*I; E, *Eco*RI; H, *Hind*III; P, *Pst*I; X, *Xho*I.

To determine the function(s) encoded by the transcriptionally active DNA region, we constructed fusions of the *Bgl*III-*Cla*I fragment A with *tac* promoter on the broad-host-range expression vector pMMB24. This vector also carries the *lacI^q* gene. It has been shown previously that *tac* promoter is functional in *Pseudomonas* spp. and that it can be sufficiently repressed if *lacI^q* is present on the same plasmid (8). As a result, plasmids pVD210 and pVD211 were obtained with both orientations of *Bgl*III-*Cla*I fragment A in the *Bam*HI site downstream of the *tac* promoter (Fig. 1C). When these plasmids were introduced into *P. aeruginosa* strain 8835 carrying mutation *alg-5* (8) (mapping in the region designated as *algD*; Fig. 1B), only plasmid pVD211 restored the mucoid phenotype in the presence of IPTG. This result suggested that (i) the *Bgl*III-*Cla*I fragment A lacked a functional promoter and (ii) the transcriptional direction of the gene complementing the *alg-5* mutation was from *Cla*I towards the *Bgl*III site. However, a certain level of complementation of the *alg-5* mutation with pVD211 was observed even without induction with IPTG. This may reflect an

incomplete repression of the *tac* promoter by the *lacI^q* gene product in *P. aeruginosa*. This result also indicated the importance of a high-level expression of the pVD211 insert for alginate production.

To determine how many detectable proteins were coded by *Bgl*III-*Cla*I fragment A, we introduced plasmids pVD210 and pVD211 into the *E. coli* maxicell strain CSR603 (39). Maxicell analysis (Fig. 3) showed the IPTG-dependent appearance of a new 48-kDa polypeptide (P48) only with pVD211. IPTG-dependent expression of the pVD211 insert in *P. aeruginosa* strain 8822 also produced a polypeptide with electrophoretic mobility identical to that of P48, as seen on SDS-polyacrylamide gels (Fig. 4).

Gene *algD* coding for GMD is present on *Bgl*III-*Cla*I fragment A. We recently demonstrated the functional identity of the *algA* gene product (Fig. 1) (20). This confirmed the role of PMI in the alginate biosynthetic pathway. However, expression of *algA* was below the detection limits of RNA-DNA dot blot hybridization, suggesting that it was not subject to strong transcriptional activation. Another pro-

FIG. 2. Dot blot hybridization analysis of RNA from mucoid and nonmucoid *P. aeruginosa*. Filters shown in panel A, with 10 μ g of total cellular RNA per dot from (1) mucoid strain 8821 and (2) nonmucoid strain 8822 and (3) rRNA from *E. coli* were hybridized with different 32 P-labeled *Eco*RI fragments (A to E) of a 9.5-kb *Hind*III-*Hind*III insert of plasmid pAD2 depicted in panel B. See legend to Fig. 1 for abbreviations of restriction endonucleases.

posed alginate biosynthetic enzyme (Fig. 1), GMD seemed a good candidate for the strong transcriptional regulation observed with *Bgl*III-*Cla*I fragment A. We reasoned that the oxidation of GDPmannose to GDPmannuronic acid by GMD might serve to commit sugar intermediates to exopolysaccharide synthesis in mucoid cells. To test whether such an enzyme was coded by the DNA region that is under transcriptional control, we applied the same approach that led to the identification of PMI activity associated with *algA* (20). In that case, the *algA* gene was overexpressed with the *tac* promoter, and the PMI activity was demonstrated to be dependent on the induction with IPTG. In a similar type of experiment, GDPmannose-dependent reduction of NAD^+ was observed in extracts of *P. aeruginosa* strain 8822 harboring plasmid pVD211 when induced with IPTG (Table 2). Although this strongly suggested that GMD is coded by a pVD211 insert, the possibility existed that the product of the gene residing on *Bgl*III-*Cla*I fragment A induced GMD synthesis by acting on some other site of the *P. aeruginosa* chromosome. To rule out this possibility, we expressed pVD211 in *E. coli* strain JM83. Again, GMD activity was observed only upon induction with IPTG (Table 2). Thus, by expressing the cloned gene on plasmid pVD211 in two different species, we have identified GMD activity as coded by *Bgl*III-*Cla*I fragment A that is transcriptionally activated in mucoid *P. aeruginosa*. Since this enzyme catalyzes the fourth reaction (step D, Fig. 1A) specific for alginate biosynthesis, we assigned the corresponding gene as *algD*.

Insertional inactivation of the *algD* gene abolishes GMD activity, synthesis of P48 protein, and complementation of the *alg-5* mutation. Insertion of a 2.2-kb *Xho*I-*Xho*I DNA fragment containing the *xylE* gene from the TOL plasmid of *P. putida* (subcloned in pBR322) (25) into the *Xho*I site of pVD211 (plasmid pVD211X) resulted in the loss of GMD activity (Table 2). Since the *xylE* gene was inserted in the

direction of transcription of the *algD* gene, CDO activity, coded by the *xylE* gene, was induced by IPTG in the same extracts (Table 2). In addition, the IPTG-dependent induction of P48 was abolished in extracts of *Pseudomonas* cells harboring plasmid pVD211X. Instead, a new polypeptide was produced with the expected size (35 kDa) of the *xylE* gene product (data not shown). These results strongly suggest that P48 represents the GMD enzyme or, more likely, its subunit. Furthermore, inactivation of the dehydrogenase activity and loss of P48 were also accompanied by loss of the *alg-5* complementing ability. This suggested that *alg-5* might be a mutation within the *algD* structural gene.

Control of *algD* transcription. To test whether the apparent absence of *algD* transcription in strain 8822 reflected a high instability of the *algD* RNA sequences, we attempted to detect mRNA from *Bgl*III-*Cla*I fragment A cloned behind the *tac* promoter in nonmucoid *P. aeruginosa*. Plasmid pVD211 (Fig. 1C) was introduced into nonmucoid strain 8822, and transcription from the *tac* promoter was induced with IPTG. As a result, formation of the transcript specific for *Bgl*III-*Cla*I fragment A was readily observable in the nonmucoid *P. aeruginosa* strain 8822. The level of transcription from pVD211 was comparable to that from its natural promoter in the mucoid background (Fig. 5, lanes 5 and 6). This result suggests that the difference in the amounts of *Bgl*III-*Cla*I fragment A mRNA observed between mucoid and nonmu-

FIG. 3. Maxicell analysis of the gene residing on *Bgl*III-*Cla*I fragment A. Plasmids pVD210 and pVD211 were introduced into *E. coli* CSR603, and 35 S-labeled proteins were analyzed by electrophoresis through an SDS-polyacrylamide gel. Lanes: 1, 14 C-methylated protein standards lysozyme (14.3 kDa), carbonic anhydrase (30 kDa), ovalbumin (46 kDa), bovine serum albumin (69 kDa), and phosphorylase b (92.5 kDa); 2 and 3, *E. coli* CSR603 harboring pVD211, uninduced and induced with IPTG, respectively; 4 and 5, *E. coli* CSR603 harboring a pVD210, uninduced and induced with IPTG. Arrow indicates the position of P48 protein.

coid strains is most likely due to the inactivity of the *algD* promoter in nonmucoid cells. We further studied whether the transcriptional activation of *algD* promoter was transmitted by a diffusible factor. First we examined the relative level of transcript formation when *algD* was present on a plasmid. Plasmid pAD2 was introduced into strains 8821 and 8822 by conjugal plasmid transfer. This plasmid replicates with an estimated copy number of five to eight (19). Using *Bgl*III-*Cla*I fragment A as a hybridization probe, we observed an approximate eightfold increase in the hybridization signal with the RNA from mucoid strain 8821 harboring pAD2 as compared with the signal from the same mucoid strain alone (Fig. 5, lanes 2 and 4). Transcript formation was not detectable over background levels in the nonmucoid strain 8822 even in the presence of plasmid pAD2 (Fig. 5, lane 3). This was further confirmed by Northern hybridization analysis of RNA isolated from the mucoid and nonmucoid *P. aeruginosa* strains harboring pAD2. Results shown in Fig. 6 reveal the presence of two distinct mRNA species (2.2 ± 0.6 and 1.7 ± 0.5 kb) only in mucoid cells when *Bgl*III-*Cla*I fragment A was used as a hybridization probe. These results suggested that transcriptional control of *algD* might be mediated in *trans* via a putative diffusible factor. However, if the activation of transcription was due to some DNA rearrangement, then it should be dependent on the phenotype of the strain from which the plasmid was transferred. Nevertheless, when pAD2 isolated from mucoid strain 8821 was introduced by conjugal plasmid transfer into the nonmucoid strain 8822, there was no increase in the level of transcript formation from *Bgl*III-*Cla*I fragment A (Fig. 5, lane 10). There was also no effect on the nonmucoid phenotype. Conversely, when pAD2, isolated from either mucoid strains

TABLE 2. GMD activities in crude extracts of bacteria harboring plasmids pVD211 and pVD211X^a

Species, strain, and plasmid	IPTG (mM)	Sp act (per mg of protein)	
		GMD (mU) ^b	CDO (mU) ^c
<i>P. aeruginosa</i> 8822			
pVD211	0	<0.1	ND ^d
	1	24.0	ND
<i>E. coli</i> JM83			
pVD211	0	<0.1	ND
	1	1.6	ND
pVD211X	0	<0.1	114
	1	<0.1	6,190

^a Plasmid pVD211X is described in the text.

^b One unit is defined as the amount of enzyme reducing 1 mol of NAD⁺ per min.

^c One unit is defined as the amount of enzyme oxidizing 1 μ mol of catechol to the 2-hydroxymuconic semialdehyde, a product with a molar extinction coefficient of 4.4×10^4 at 375 nm.

^d ND, Not determined.

8821 and 8830 or nonmucoid strain 8822, was introduced into mucoid strain 8821, we observed in all cases comparably high levels of transcriptional activity (Fig. 5, lanes 7, 8, and 9).

Transcription fusion studies of *algD* and *xylE* genes. The observed increase in mRNA levels in mucoid cells harboring plasmid pAD2 suggested that activation of DNA transcription occurred in *trans* by a putative diffusible factor. To further test this hypothesis, we constructed a transcription (operon) fusion of *algD* with the *xylE* gene (25) coding for the easily assayable CDO enzyme normally absent in *P. aeruginosa*. The transcriptional fusion, when present on a plasmid, would permit us to determine whether plasmid-coded *algD* sequences were activated in mucoid cells. Transcriptional fusion plasmid pVD2X was constructed by cloning the *Hind*III-*Hind*III insert of pAD2 into the pRK290-derived, broad-host-range, low-copy vector pVDZ'2 (V. Deretic, D. K. Chatterjee, S. Chandrasekharappa, and A. M. Chakrabarty, manuscript in preparation) to obtain a plasmid with a single *Xho*I site. Into that *Xho*I site located within *Bgl*III-*Cla*I fragment A and *Eco*RI fragment C (Fig. 1C), a 2.2-kb *Xho*I-*Xho*I fragment containing the *xylE* structural

FIG. 4. Expression of the *algD* gene in *P. aeruginosa* from the *tac* promoter. Cells of nonmucoid strain 8822 harboring plasmid pVD211 were grown with (lane 1) or without (lane 2) IPTG. Total cellular extracts (100 μ g of protein per lane) were prepared and electrophoresed as described in Materials and Methods. Arrow indicates P48 protein. Bars indicate protein M_r standards.

FIG. 5. Control of transcription of *algD* gene present on the plasmid. Filters contained 10 μ g of total cellular RNA per dot from: 1, nonmucoid strain 8822; 2, mucoid strain 8821; 3, strain 8822 harboring plasmid pAD2; 4, strain 8821 harboring plasmid pAD2; 5 and 6, strain 8822 harboring plasmid pVD211 with no IPTG and 5 mM IPTG in the culture, respectively; 7, 8, and 9, strain 8821 harboring plasmid pAD2 transferred from mucoid strains 8821 and 8830 and nonmucoid strain 8822, respectively; and 10, strain 8822 harboring plasmid pAD2 transferred from strain 8821. Hybridization probe was ³²P-labeled *Bgl*III-*Cla*I fragment A.

FIG. 6. Northern blot hybridization analysis of transcripts from *BglIII-ClaI* fragment A. Total cellular RNA from (1) nonmucoid strain 8822 and (2) mucoid strain 8821, both harboring plasmid pAD2, was electrophoresed through a 1.2% agarose gel, blotted to the filter membrane, and hybridized with ^{32}P -labeled *BglIII-ClaI* fragment A, as described in Materials and Methods.

gene (921 base pairs) and a part of the upstream genes of the *xylDEGF* operon, but not the operator-promoter region (25, 33), was cloned. We obtained constructs with both orientations of *xylE* fragment relative to the *HindIII-HindIII* insert as well as the total combined insert relative to the vector. When plasmid pVD2X, having *xylE* in the same direction of transcription as *algD* (Fig. 1C), was introduced into *P. aeruginosa*, a 10-fold increase of CDO activity was observed in mucoid versus nonmucoid cells (Table 3). The same effect was observed even when the total insert of pVD2X was in the opposite orientation relative to the vector. The low basal level of CDO activity in nonmucoid cells harboring pVD2X was also observed in cells harboring constructs with *xylE* in orientation opposite to *algD* gene transcription (data not shown). This was probably due to some transcription initiation intrinsic to the 2.2-kb *XhoI-XhoI* fragment containing the *xylE* gene. Thus, experiments with the *algD-xylE* transcription fusion confirmed that transcriptional activation of *algD* was occurring in *trans* in mucoid cells.

We further used the *xylE-algD* transcription fusion to study whether the phenomenon of the *algD* gene activation could be observed in other mucoid *P. aeruginosa* strains. Mucoid *P. aeruginosa* strains SR6339-1 and PAOM18 were isolated from the CF patient sputum and by ethyl methanesulfonate treatment of the nonmucoid PAO2003 strain, respectively. Plasmid pVD2X was introduced into these strains and into strain SR6339-2, a spontaneous nonmucoid revertant of strain SR6339-1, as well as into strain PAO2003. Similar to the mucoid-nonmucoid strain

pair 8821-8822, the level of *xylE* gene product was increased in the mucoid strains compared with the nonmucoid revertant or parental strains (Table 3). This result suggested the general significance of the activation of *algD* transcription for the occurrence of the mucoid phenotype.

DISCUSSION

Determining the mechanisms by which alginate production is controlled in *P. aeruginosa* is of central importance to understanding the underlying reasons for *Pseudomonas* infection in CF lungs. We have demonstrated here that the transcriptional activation of the *algD* gene, coding for GMD, is an important component for alginate production and maintenance of the mucoid phenotype. Based on the studies of enzyme activities present in extracts of *Azotobacter vinelandii* (37) and *P. aeruginosa* (36), it was suggested that the synthesis of alginate might be similar to the biosynthesis of alginic acid in the brown alga *Fucus gardneri* (28). In our laboratory, recent genetic (10) and biochemical (20) studies confirmed that PMI catalyzes the first step of alginate biosynthesis branching from the glycolytic intermediate fructose 6-phosphate. In the latter study, we have shown that PMI activity was only detected in *P. aeruginosa* extracts when the corresponding gene *algA* was overexpressed under the *tac* promoter (20). Using the same approach, we were able to identify the *algD* gene as coding for GMD. The identification of a gene coding for GMD and its enzyme product confirms the fourth step of the proposed pathway. Thus, the proposed second and third steps (Fig. 1A) appear to be catalyzed by phosphomannosemutase and GDPmannose pyrophosphorylase, respectively, although their genes and gene products have not been characterized yet. The controlled overexpression of other alginate genes cloned downstream of the *tac* promoter may prove useful in their identification.

All classes of nucleotide-linked four-electron transfer dehydrogenases, including UDPglucose dehydrogenase and histidinol dehydrogenase, are known to consist of an even number of identical subunits having M_r in the range of 50,000 (18). Several lines of evidence support the notion that the subunit M_r of GMD is 48,000. (i) Inactivation of *algD* by insertion of *xylE* into the single *XhoI* site of pVD211 caused the disappearance of both the P48 polypeptide and GMD activity from the cell extracts. (ii) We have recently observed that GMD activity copurifies with P48 during ion-exchange high-pressure liquid chromatography (unpublished observation). (iii) The complete nucleotide sequence of the DNA region from 180 base pairs upstream to 1,840 base pairs downstream of the *ClaI* site has been determined (V. Deretic, J. F. Gill, and A. M. Chakrabarty, manuscript in

TABLE 3. Specific activities of CDO in crude extracts of mucoid and nonmucoid *P. aeruginosa* harboring *algD-xylE* transcription fusion plasmid pVD2X

Strain	CDO activity (mU/mg) ^a	Alginate production (μg/mg) ^b
8821	10,290	126
8822	960	<1.5
SR6339-1	7,690	82
SR6339-2	630	<1.5
PAOM18	3,400	42
PAO2003	850	<1.5

^a CDO activity as defined in footnote c, Table 2.

^b Alginate production expressed in micrograms of alginate per milligram of cell wet weight.

preparation). A single open reading frame was found in the same orientation as the direction of transcription determined in this work. This open reading frame has a coding capacity for a 47.6-kDa protein, which is in a good agreement with the 48-kDa polypeptide observed on SDS-polyacrylamide gels. Also, the *XhoI* site was found 560 base pairs downstream of the ATG codon. Thus, insertion of the *xylE* gene was made within the structural gene coding for the GMD enzyme.

Expression of the *algD* gene was achieved by transcription from the *tac* promoter in both *E. coli* and *P. aeruginosa*. In both organisms the protein having an M_r of 48,000 was obtained but yields differed by at least an order of magnitude, possibly reflecting the differences in translation or transcription signals between *P. aeruginosa* and *E. coli*. The high yield of *algD* gene product obtained in *P. aeruginosa* harboring pVD211 (estimated as 2% of the total cellular protein) represents a significant purification step and should facilitate isolation of the GMD enzyme. Since GMD might represent a critical enzyme for the emergence of mucoid *P. aeruginosa*, purification of this enzyme and study of its biochemical properties are of considerable importance.

The relatively high expression level of *algD* in mucoid strains and the apparent absence of transcription in nonmucoid strains imply a very stringent control of the *algD* promoter. Transcriptional control of *algD* appears to be mediated by a diffusible factor, possibly an activator. Activator proteins have been shown to control the transcription of a number of the biodegradative operons in *Pseudomonas* spp. (34, 40). Although a repression-like mechanism cannot be disregarded at present, the absence of any effect similar to the repressor titration, when extra copies of the promoter sequences were present in *trans*, argues in favor of *algD* activation. In addition, when plasmid pVD2X, having insertionally inactivated the *algD* gene, was present in mucoid strain 8821, we observed a significantly reduced level of alginate production (data not shown). In this case, the plasmid-borne *algD* promoter might compete for the activator with the functional copy present on the chromosome.

The clustering of most of the alginate genes (10) suggested a possible polycistronic nature in their transcription. However, the existence of a highly transcribed region of the chromosome confined to the DNA fragment complementing only a single mutation (*alg-5*) strongly suggested that at least this area might form an independent transcription unit. Another line of evidence supported this possibility. Insertion of the 2.2-kb *XhoI* fragment containing the *xylE* gene into the *XhoI* site of the pAD2 insert (plasmid pVD2X, Fig. 1C) abolished *alg-5* complementing ability. However, two other mutations, *alg-8* and *alg-44*, mapping downstream from *algD*, were fully complemented by pVD2X (unpublished observation). The absence of any observable polar effect on these mutations suggests that at least one part of the alginate gene cluster belongs to a separate transcription unit. The sizes of mRNAs observed by Northern hybridization analysis argue in favor of such a hypothesis. In addition, Northern hybridization revealed the presence of two RNA transcripts produced from *BglII*-*ClaI* fragment A in mucoid cells. This may indicate that another transcription unit, also undergoing activation in mucoid cells, begins within *BglII*-*ClaI* fragment A, immediately downstream of the *algD* gene. It is also possible that two separate sites exist for either transcription initiation or termination of the *algD* gene. Such a phenomenon has been seen in other systems. For example, transcription of the β -lactamase gene of pBR322 is known to terminate at three different sites (44), while in *Vibrio harveyi*

four different mRNA species can be transcribed from genes encoding luciferase subunits (32). However, two RNA molecules could be produced by sequential fragmentation processing of the primary transcript (41). S1 nuclease protection studies are under way to explore these possibilities.

Several systems have been developed to assay promoter activity in *E. coli*, based on transcriptional fusions with the structural genes for β -galactosidase (6), galactokinase (31), tetracycline resistance (1), and chloramphenicol acetyltransferase (4). No comparable equivalent has been developed for use in *P. aeruginosa*. However, the *xylE* gene was recently used in *P. putida* as a promoter probe (25). In this report we have used *xylE* to construct a transcription fusion with the *algD* promoter. Studies with this fusion confirmed our findings of increased *algD* transcription in mucoid cells, using RNA-DNA dot and Northern blot hybridization analyses, providing strong evidence for the existence of a *trans*-acting regulatory factor. Construction of a transcriptional fusion system with the regulated *algD* promoter should facilitate studies of the conditions affecting expression of the *algD* gene. In addition, screening of CDO levels in available *alg* mutants (8, 10) harboring transcription fusion plasmid pVD2X indicates that the *alg-22* mutation abolishes transcription of the *algD* gene (Deretic et al., manuscript in preparation). This suggests a positive regulation of *algD* by a DNA region previously shown to induce alginate production in nonmucoid *P. aeruginosa* when chromosomally amplified (12).

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