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Alginate Synthesis in *Pseudomonas aeruginosa*: Environmental Regulation of the *algC* Promoter

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The exopolysaccharide alginate is a major virulence factor of *Pseudomonas aeruginosa* strains that infect the lungs of cystic fibrosis patients. The synthesis of alginate is almost uniquely associated with the pathogenicity of *P. aeruginosa* within the environment of the cystic fibrosis lung. The gene *algC* is one of the essential alginate biosynthetic genes and codes for the enzyme phosphomannomutase. In this report, we present data on the transcriptional regulation of *algC* expression. The activity of the *algC* promoter is modulated by the response regulator, AlgR1, a member of the two-component signal transduction protein family, which also regulates other alginate-specific promoters. In both mucoid (alginate-positive) and nonmucoid (alginate-negative) *P. aeruginosa* strains, transcriptional activation of *algC* increased with the osmolarity of the culture medium. This osmolarity-induced activation was found to be dependent on AlgR1. AlgR1 was found to interact directly with the *algC* promoter. Deletion mapping, in conjunction with mobility shift assays, showed that AlgR1 specifically bound with two regions of *algC* upstream DNA. A fragment spanning nucleotide positions −378 to −73 showed strong specific binding, while a fragment located between positions −73 and +187 interacted relatively weakly with AlgR1. Phosphorylation of the AlgR1 protein resulted in the stimulation of its in vitro ability to bind to the *algC* promoter region (a fragment spanning nucleotides −378 to −73). Transcription from the *algC* promoter, which has significant homology with the RNA polymerase σ-54 (RpoN) recognition sequence, decreased in an *rpoN* mutant of *P. aeruginosa*.

Chronic and recurrent bacterial infections play a critical role in the progression of pulmonary disease in patients with cystic fibrosis (CF). One clinical manifestation of CF is the production of large amounts of hyperviscous bronchial secretions, which allow the proliferation of mucoid, alginate-producing *Pseudomonas aeruginosa* in the CF respiratory tract (20). Alginate is an exopolysaccharide composed of β-1,4-linked D-mannuronic acid and L-gluronic acid (13). Alginate production is thought to be important for the survival of *P. aeruginosa* in the lungs of CF patients (36). The presence of alginate exacerbates the respiratory difficulties resulting from the abnormally viscous CF lung environment. Since the alginate layer surrounding *P. aeruginosa* in the CF respiratory tract provides a protective barrier against antibiotics and the host immune defenses (18, 20, 43), the prevention of alginate synthesis by *P. aeruginosa* in the CF lung may enhance existing treatment strategies (20, 43). Thus, compounds that inhibit enzymes or regulatory proteins required for alginate synthesis in *P. aeruginosa* have potential use as therapeutic agents.

Alginate synthesis by *P. aeruginosa* is a rare phenomenon and is associated with the CF lung. It is therefore important to study the regulation of alginate genes to understand how their expression is influenced by the CF lung environment. Several alginate biosynthetic genes are clustered around the 34-min region of the *P. aeruginosa* chromosome, with the *algD* gene at the far 5′ end. The *algD* promoter is activated by environmental conditions, such as high osmolarity, nitrogen or phosphate starvation, and ethanol-induced membrane perturbation (1, 8, 9). Some of these environmental conditions are similar to those encountered in the CF lung. Several proteins are known to regulate the transcription of the *algD* promoter (20). Among these proteins are AlgR1 and AlgR2, which are members of the two-component bacterial signal transduction protein family. AlgR1 (7) is the response regulator that binds DNA far upstream of the *algD* promoter and activates transcription (14). AlgR1 functions as a transcriptional regulator of genes such as *algD* (7), *algR1* (16), and the neuraminidase gene *nanA* (4). AlgR2 is the kinase that undergoes autophosphorylation in the presence of ATP or GTP and transfers the acquired phosphate to AlgR1 (30, 31). Functional AlgR2 is important for AlgR1-mediated transcriptional activation of the *algD* promoter (15).

We previously described the cloning, sequencing, and regulation of the *algC* gene (42). It is interesting to note that *algC* maps at the 10-min region of the *P. aeruginosa* chromosome in a 60-kb *Dra*I (35) fragment along with a cluster of alginate regulatory genes, *algR1*, *algR2*, and *algR3*, while all the other known alginate biosynthetic genes map at the 34-min region (Fig. 1). The activities of all the alginate biosynthetic enzymes assayed so far, including the *algC* product, phosphomannomutase, are higher in mucoid than in nonmucoid cells (25, 32). To understand how *algC*, despite being unlinked to the alginate biosynthetic gene cluster, is regulated in a manner similar to *algD*, we carried out a detailed analysis of *algC* regulation at the level of transcription. In particular, we studied the effect of environmental conditions on *algC* transcription and the role of AlgR1 as a global regulator of alginate gene expression.

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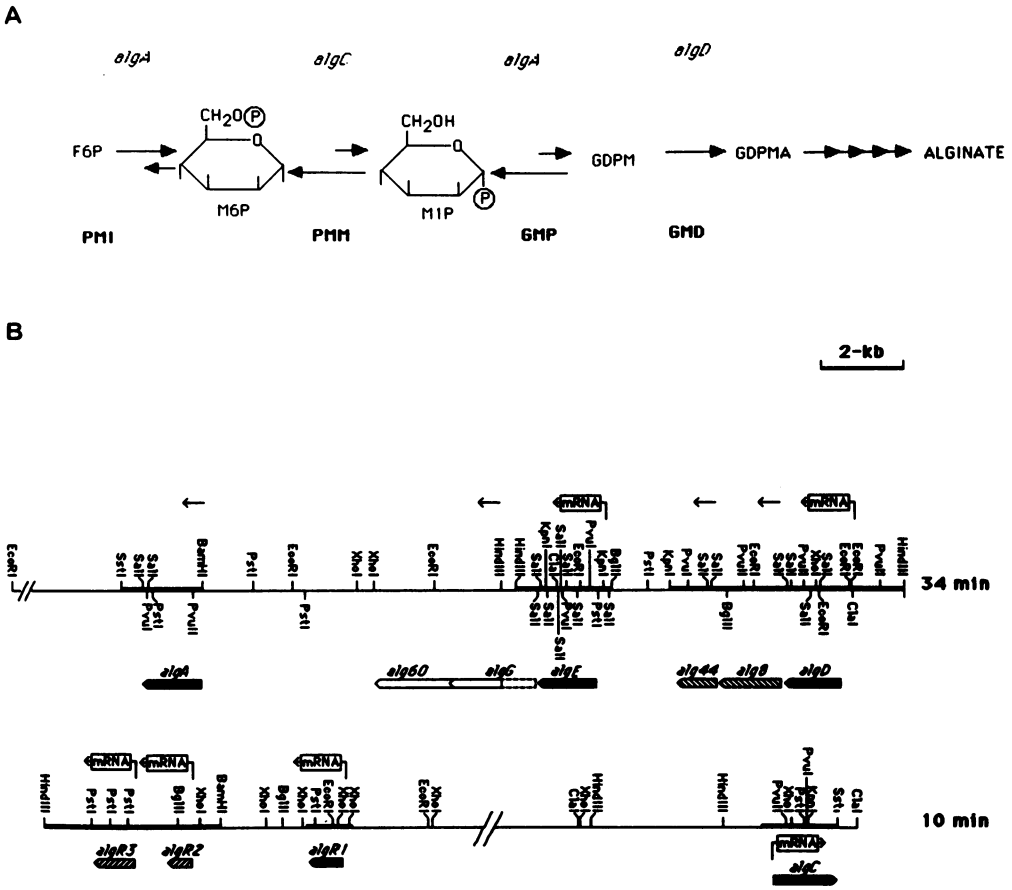

FIG. 1. Alginate biosynthetic pathway in *P. aeruginosa* and two gene clusters involved in alginate synthesis. (A) The enzymes catalyzing the known reactions in the pathway are as follows: PMI, phosphomannose isomerase; PMM, phosphomannomutase, GMP, GDP-mannose pyrophosphorylase; and GMD, GDP-mannose dehydrogenase. The genes encoding these enzymes are indicated above the respective enzymes. The known reaction intermediates in the pathway are as follows: F6P, fructose 6-phosphate; M6P, mannose 6-phosphate; M1P, mannose 1-phosphate; GDPM, GDP-mannose; and GDPMA, GDP-mannuronic acid. The steps between the formation of GDPMA and that of alginate include polymerization, acetylation, export, and epimerization (shown by arrows) and are not yet well characterized. (B) Organization of genes involved in alginate synthesis. The locations of the two gene clusters on the *P. aeruginosa* chromosome are indicated in minutes. The *algC* gene maps on a 60-kb *Dra*I fragment along with the *algR1*, *algR2*, and *algR3* genes (35). Restriction enzyme sites are indicated by vertical lines. Sequenced regions of DNA are indicated by thick horizontal lines. Thin horizontal lines denote unsequenced regions of DNA. Arrows indicate the direction of transcription with the transcriptional start site (mRNA), when known. Filled bars indicate proteins encoded by the indicated gene and confirmed by N-terminal amino acid sequencing. Hatched areas denote protein coding regions predicted from DNA sequence analysis of the indicated gene. Open areas indicate proteins encoded by the indicated gene for which the exact coding region has not been determined.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* CF613 and CF613-NIG were kindly provided by S. Lory. The promoter probe constructs were initially transformed into *Escherichia coli* CSH50 and then mated into *P. aeruginosa* strains. The exonuclease III deletion constructs were initially transformed into *E. coli* MV1184.

Media and culture conditions. Liquid cultures were grown in Luria-Bertani broth (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter of distilled deionized water) or YTG (10 g of tryptone, 5 g of yeast extract, and 2 g of glucose per liter of distilled deionized water). The solid media used were Luria broth solidified with 1.5% agar (for *E. coli*) and *Pseudomonas* isolation agar (PIA) (for *P. aeruginosa*). All cultures were grown at 37°C, and liquid cultures were aerated by being shaken at 250 rpm, unless otherwise specified. Antibiotic concentrations used for the plasmid-containing strains were as follows: carbenicillin (*P. aeruginosa*), 350 μg/ml; ampicillin (*E. coli*), 75 μg/ml; tetracycline, 50 and 300 μg/ml for *E. coli* and *P. aeruginosa*, respectively; and kanamycin, 75 μg/ml and 1.2 mg/ml for *E. coli* and *P. aeruginosa*, respectively. *P. aeruginosa* and *E. coli* strains harboring *algC-lacZ* transcriptional fusion plasmids were used for the determination of *algC* activation in media of different osmotic strengths. The strains were grown in YTG containing no NaCl, 0.3 M NaCl, or 0.44 M sucrose. Cultures were incubated for 18 h (mid-stationary phase for *P. aeruginosa*). At the mid-exponential phase, the specific cell growth rates of the strains were comparable with the doubling times, varying between 2.17 and 1.92 h. For the hyperproduction of AlgR1, *P. aeruginosa* (8822) and *E. coli* (MV1184) strains harboring plasmid pJK66R1 (containing the *algR1* gene under the control of the inducible *tac*

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant characteristics[a]	Source or reference
Strains		
E. coli		
CSH50	Str^r ProF *ara* Δ(*pro-lac*) *strA thi*	21
HB101	F$^-$ *hsdS20* (r$^-$ m$^-$) *recA13 ara-14 proA2 lacY1 galK2 rpoL20 xyl-5 mtl-1 supE44* (Str^r)	2
MV1184	*ara* Δ(*lac-proAB*) *rpsL thi* (ϕ80*lacZ*ΔM15) Δ(*srl-rec306*::Tn*10*) (Tet^r) F'[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	39
P. aeruginosa		
8821	*his-1 alg*$^+$ (CF isolate, unstable mucoidy)	5
8822	*his-1 alg-1* (spontaneous nonmucoid revertant of 8821)	5
8830	*his-1 alg*$^+$ (ethyl methanesulfonate mutant of 8822, stable mucoidy)	5
8852	*his-1 alg-22 algR1*	5
8858	*his-1 alg-28 algC*	42
CF613	Mucoid isolate from a CF patient	B. Ramsey and S. Lory
CF613-NIG	CF613 *rpoN*::*aph*	38
Plasmids		
pIJ1363	Tc^r; pSA *ori*	28
pJRD215	Km^r Sm^r IncQ *mob*$^+$	6
pKRZ-1	Ap^r promoter probe vector containing *lacZ* as a reporter gene in vector pUCD615	29
pRK2013	ColE1 *mob tra*$^+$ (RK2) Km^r	10
pMMB66EH	Ap^r *tac* promoter	11
pUC118	Ap^r *lacZ*"M13 intergenic region	39
pUC119	Polylinker of pUC118 inverted	
pJK66R1	Ap^r *algR1* gene cloned under *tac* promoter control	14
pNZ63	Ap^r *algC-lacZ* transcriptional fusion cloned into pKRZ-1	42

[a] Ap^r, Km^r, and Tc^r, resistance to ampicillin, kanamycin, and tetracycline, respectively.

promoter) or control vector pMMB66EH were grown as previously described (14).

DNA manipulations. Plasmid DNA isolation, ligation, and transformation and other DNA manipulations were carried out as described earlier (14) or by established procedures (33). Restriction enzymes, T4 DNA ligase, T4 DNA polymerase, and the large fragment of DNA polymerase I (the Klenow fragment) were used as specified by the manufacturers. Vector DNA was treated with calf intestinal alkaline phosphatase. For deletion analysis, we used plasmid pRM55, which harbors a 2.6-kb fragment containing the *algC* coding region (42) and approximately 1 kb of DNA upstream of the transcriptional initiation site. This fragment was cloned into the *Hin*cII site of pUC119 (39) in the *Eco*RI-*Hin*dIII orientation. Exonuclease III derivatives of pRM55 with deletions from the 5' end were constructed by the method of Henikoff (12) and are indicated in Fig. 2 (bent arrows show the deletion endpoints). The deletion constructs described in Fig. 3 were cloned from the respective pRM55 deletion derivatives into broad-host-range plasmid pIJ1363 (28) for subsequent complementation studies. Plasmid pNZ55 was constructed by cloning the entire 2.6-kb *algC* fragment into pIJ1363.

DNA sequence determination and analysis. The dideoxynucleotide chain termination method of Sanger et al. (34) was modified and used for the DNA sequence determination at 37°C with Sequenase TM DNA polymerase as recommended by the manufacturer (United States Biochemicals, Cleveland, Ohio). To reduce electrophoretic band compression, we routinely incorporated 7-deaza-GTP in the reactions instead of dGTP (22). In some experiments, 20% formamide was added as a component of the polyacrylamide gel mixture. DNA fragments to be sequenced were cloned into pUC118 and pUC119 and propagated in MV1184 to be used for generating single-stranded DNA (39–41). DNA obtained from pRM55 and exonuclease III deletion derivatives was used for sequencing of the coding strand. In addition to the primers used previously, custom-made primers were obtained for sequencing of the reverse strand (14). The host strain used was *E. coli* MV1184. Preparation of sequencing gels, electrophoresis conditions, and autoradiography were as described previously (42), except that prior to autoradiography, gels were fixed in a 10% acetic acid–10% methanol solution for 10 min, transferred to Whatman 3 MM paper, and dried. DNA sequence analysis was done and the results were analyzed with FASTA software, which includes the GenBank and EMBL data bases (27).

Conjugation of recombinant plasmids into *P. aeruginosa*. DNA fragments that were cloned into broad-host-range vector pIJ1363 were mobilized from *E. coli* to various *P. aeruginosa* strains by triparental mating with *E. coli* HB101(pRK2013) as the helper strain (10).

Genetic complementation analysis. Merodiploid strains were constructed by conjugating constructs into *P. aeruginosa* 8852 or 8858. To isolate clones that complemented the *algC* mutation in *trans*, we plated mating mixtures on PIA, with appropriate antibiotic selection. Colonies exhibiting a mucoid appearance were purified and reexamined for the Alg$^+$ phenotype after incubation at 37°C on PIA plates. Plasmid DNA molecules were isolated from transconjugants that possessed an Alg$^+$ phenotype after single-colony isolation on PIA plates containing the proper antibiotic. This DNA was used to transform calcium chloride-treated *E. coli* MV1184, with appropriate antibiotic selection. Single colo-

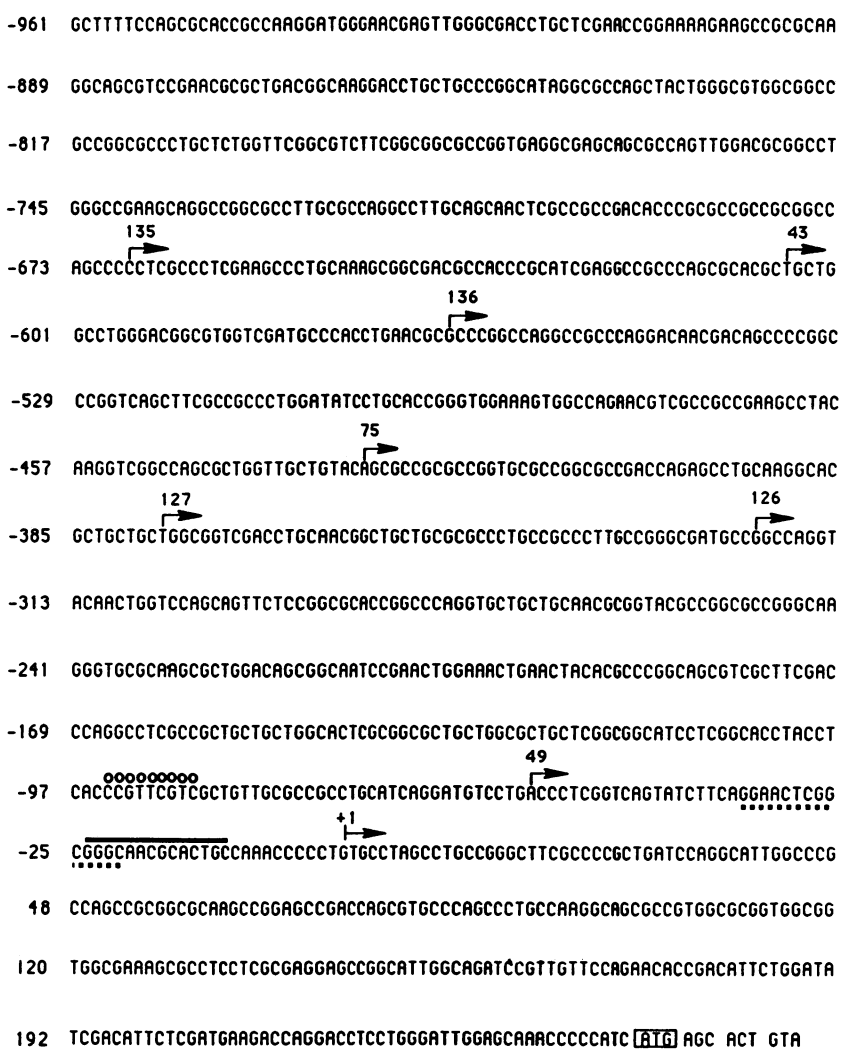

FIG. 2. Nucleotide sequence of the DNA region upstream of the *algC* translational start site. A ca. 1.2-kb sequence with the translational initiation codon of *algC* at the 3′ end is shown. Nucleotides are numbered with the transcriptional initiation site as +1 (42). Only the coding strand is shown. The straight horizontal arrow indicates the orientation of transcription. Bent arrows indicate the 5′ endpoints of the deletion products used in the complementation studies, with the designations of the deletions given above the arrows. Sequences that are homologous to the consensus *rpoN* recognition sequence, GG-N_{10}-GC (from positions −24 to −11), designated promoter 1, are marked by a heavy solid line. The sequence GG-N_{10}-GC (from positions −34 to −21), designated promoter 2, which also is present in the region upstream of the *algD*, *algE*, and *algR1* genes, is underscored by a heavy broken line. The consensus AlgR1 binding sequence (centered at position −87) is shown with open circles over the specific bases. The translational start codon, ATG, is boxed.

nies exhibiting the proper antibiotic resistances were used in matings to reintroduce the plasmid constructs into the recipient strains.

Enzyme assays. Crude extracts of *P. aeruginosa* or *E. coli* to be used for β-galactosidase assays were prepared by thawing cell pellets in 7 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 5 mM β-mercaptoethanol and 1 mM $MgSO_4$. Cells were disrupted by sonication three times for 20 s at 120 W each time on ice. The sonicated suspensions were centrifuged at 40,000 × *g* for 30 min at 4°C. The resultant supernatants were used directly for β-galactosidase assays by the procedure of Miller (21). The formation of *o*-nitrophenol was spectrophotometrically determined at an A_{420}. β-Galactosidase specific activities were defined as nanomoles of *o*-nitrophenol produced per minute per milligram of crude extract protein at 28°C and pH 7.0. Glucose 6-phosphate dehydrogenase activity in crude extracts was determined as described by Lessie and Jones (19).

Protein determinations. Protein concentrations were determined by the method of Bradford (3), as outlined in the Bio-Rad protein assay instruction manual, with bovine serum albumin as the reference protein for the construction of standard curves.

Mobility shift DNA binding assay. Lysates of *P. aeruginosa* harboring pJK66R1 (to allow the hyperproduction of AlgR1) were tested for DNA binding activity by an electrophoretic mobility shift assay. Crude cell extracts were prepared as described by Kato and Chakrabarty (14). A typical DNA binding assay was performed with a total volume of 20 μl containing 25 mM Tris-HCl (pH 8.0), 0.05 mM dithiothreitol, 20 mM KCl, 0.2 mM EDTA, 0.05 μg of salmon sperm DNA, and 11.25% (vol/vol) glycerol. Typically between 20

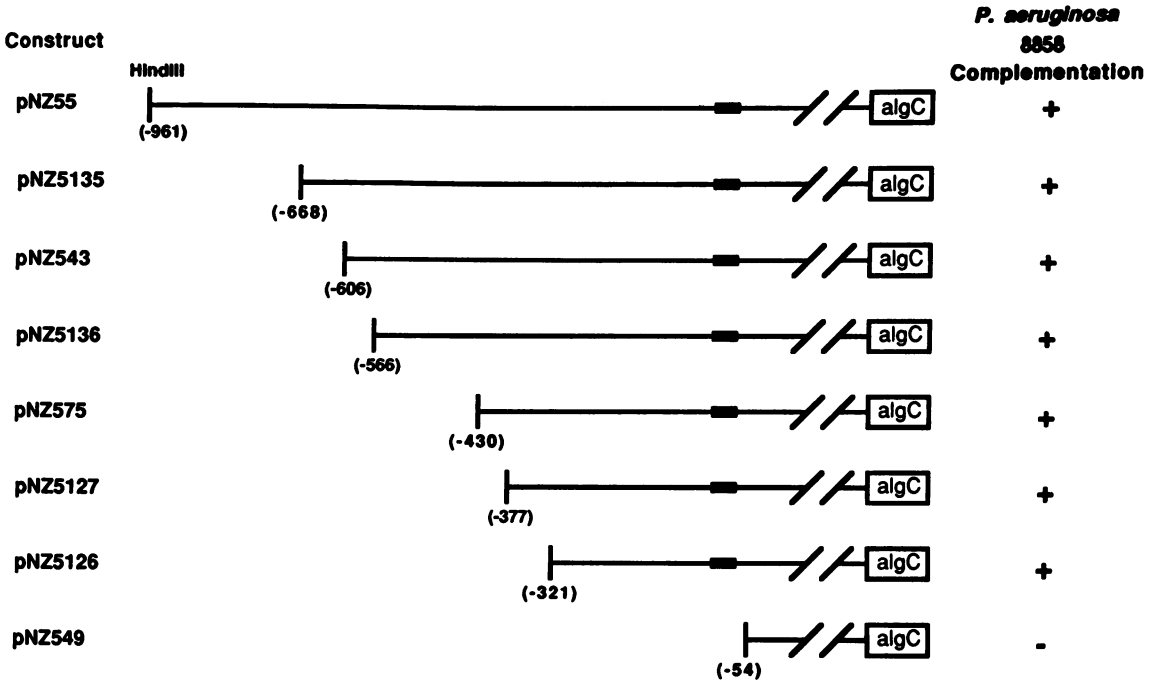

FIG. 3. Complementation of *P. aeruginosa algC* mutant strain 8858 with exonuclease III-generated deletion constructs. Unidirectional deletions from the 5′ end of the 2.6-kb fragment containing the *algC* gene were generated by limited digestion of linearized plasmids with exonuclease III. Residual fragments of desired lengths were subcloned into broad-host-range vector pIJ1363 and introduced into strain 8858. The resulting merodiploids were observed for complementation: +, complementation (restoration of the mucoid, alginate-producing phenotype); −, no complementation.

and 200 ng of protein was used in the assay. DNA fragments (probe DNA) tested for binding were labeled with [α-^{32}P]dATP (3,000 Ci/mmol) by filling in of 3′ recessive ends with the Klenow fragment (the large fragment of DNA polymerase I) (33). Binding reactions were carried out for 10 min at room temperature. After incubation, 1 μl of neutralized 0.1% bromophenol blue was added to the reaction mixture. Samples were loaded onto a horizontal 5% (wt/vol) native polyacrylamide gel after the gel was preelectrophoresed for 0.5 h at 140 V with continuous buffer (6.7 mM Tris-HCl [pH 8.0]), 3.3 mM sodium acetate, 1.0 mM EDTA) recirculation at 4°C. The samples were electrophoresed for 1 h at 140 V. Subsequently, the gel was dried and exposed to Kodak XAR-5 film overnight in the presence of an amplifying screen.

Competitive DNA binding assay. In this experiment, specific and nonspecific competitor DNAs were used (see the legend to Fig. 4) to establish the specificity of the observed DNA complexes formed. Only one modification in the mobility shift assay protocol was made for these experiments. The labeled probe and the cold specific or nonspecific DNAs were added simultaneously to the binding mixture prior to the addition of the AlgR1 preparations. The specific *algC* competitor DNA fragment (containing DNA from positions −378 to −73, harboring the AlgR1 binding site) was cloned into pUC119 to generate plasmid pNZCR1. The specific *algD* competitor DNA fragment (containing DNA from positions −563 to −399, harboring the AlgR1 binding site) was cloned into pUC119 to generate plasmid pNZDR1.

Phosphorylated AlgR1 binding assay. To determine whether the phosphorylation of AlgR1 would lead to more efficient binding of the protein to the target DNA, we preincubated the AlgR1 preparation (20 ng) with various amounts of ATP or the nonhydrolyzable analog ATPγS (1 nM, 10 nM, 100 nM, or 1 μM) for 1 min. The labeled *algC* probe containing the consensus AlgR1 binding site (nucleotides −378 to −73) was immediately added to the binding mixture, and the mixture was incubated for 10 min at room temperature before being loaded onto a 5% native polyacrylamide gel. The gel was treated in the same manner as that described above.

Nucleotide sequence accession number. The DNA sequence of the region upstream of the *algC* transcriptional initiation site from positions −961 to −180 has been assigned GenBank accession number L00980.

RESULTS

Effect of osmolarity on *algC* gene expression. One environmental factor relevant to CF is osmotic stress. The increased osmolarity of bronchial secretions in CF patients is caused by, among other factors, elevated levels of sodium and chloride ions. As shown in Table 2, in both mucoid and nonmucoid *P. aeruginosa* strains (8821 and 8822, respectively), the level of *algC* expression increased as the medium osmolarity (i.e., the NaCl or sucrose concentration) was increased. When the effect of NaCl was examined, maximum activation was achieved at 0.3 M NaCl (Table 2). A higher concentration of NaCl resulted in decreased activation (data not shown). A similar activation of *algC* transcription was observed with sucrose at 0.44 M, which is isotonic to NaCl at 0.3 M (Table 2). An increased level of gene expression resulting from growth in an osmotic environment is not a general phenomenon, since the levels of enzymes such as glucose 6-phosphate dehydrogenase did not change in response to increased medium osmolarity (data not

TABLE 2. Activation of the *algC* promoter in *P. aeruginosa* strains grown under osmotic conditions

P. aeruginosa strain/plasmid[a]	Relevant characteristics	β-Galactosidase sp act[b] (U/mg) in the presence of:		
		0 M NaCl or sucrose	0.3 M NaCl	0.44 M sucrose
8821/pNZ63	Wild type; *algC-lacZ*; mucoid CF isolate	82	444	554
8822/pNZ63	Alg⁻ *algC-lacZ*	52	324	260

[a] Cells were grown for 18 h in YTG medium or YTG medium supplemented with 0.3M NaCl or 0.44 M sucrose as indicated.

[b] One unit of β-galactosidase activity was defined as 1 nmol of *o*-nitrophenol formed per min. Specific activities are expressed as units per milligram of crude extract protein and were measured in at least three independent experiments, with a standard error of <10%.

shown). Interestingly, the osmotic regulation of *algC* expression is dependent on AlgR1, since in the *algR1* mutant strain 8852, no activation was observed under hyperosmotic conditions. In *E. coli*, a 3.6-fold increase in *algC* expression was observed in response to high osmolarity (0.3 M NaCl). *algC* promoter activation was not appreciably modulated by environmental conditions, such as oxygen tension, pH, nutrient limitation, or ethanol-induced membrane perturbation. Some of these conditions were shown to allow activation of the *algD* promoter (8, 9).

Role of σ-54 in *algC* regulation. The sequence of the *algC* promoter indicated the presence of two putative sequences resembling σ-54 recognition motifs: promoter 1 (P1), with the sequence GG-N_{10}-GC, from positions −24 to −11, and promoter 2 (P2), with the sequence GG-N_{10}-GC, from positions −34 to −21 (Fig. 2). We therefore wanted to determine whether *algC* transcription was dependent on the *rpoN* gene product σ-54 in *P. aeruginosa*. *algC-lacZ* transcriptional fusion plasmid pNZ63 was introduced into *rpoN*$^+$ wild-type strain CF613 and *rpoN* mutant strain CF613-NIG. When the level of β-galactosidase activity was measured, *algC* expression was found to be reduced in both hypo- and hyperosmotic environments (0 M and 0.3 M NaCl, respectively) as a result of the *rpoN* mutation. β-Galactosidase activities were 118 and 40 U/mg for the wild-type and mutant strains with 0 M NaCl and 529 and 178 U/mg for those strains with 0.3 M NaCl, respectively, under the same conditions as those used for the experiments shown in Table 2. These results suggest that the *rpoN* gene product is necessary for the maximal expression of *algC*. It should be noted, however, that substantial promoter activity was observed in the *rpoN* deletion mutant.

Analysis of the nucleotide sequence upstream of the *algC* translational initiation site. The complete nucleotide sequence of the *P. aeruginosa algC* open reading frame along with approximately 200 bp of upstream DNA, the direction of transcription, and the transcriptional initiation site were previously determined (42). To study the molecular details of *algC* regulation, we determined the complete nucleotide sequence of the upstream DNA region up to nucleotide position −961 (Fig. 2).

Minimum sequences necessary for *algC* expression. It was previously shown that an *algC* DNA fragment containing the whole *algC* gene and up to 67 bp upstream of the transcriptional start site was not sufficient for complementation of *algC* mutant strain 8858 (42). To determine the minimum sequence necessary for *algC* expression, we constructed a series of deletions from the 5′ (*Hin*dIII) end of the *algC* upstream DNA. By use of exonuclease III, various portions of the 5′ noncoding sequences were removed, and the resulting fragments were cloned into broad-host-range vector pIJ1363. Representative deletions were selected for further analysis. The corresponding plasmid designations and the deletion endpoints, which were determined by DNA sequence analysis, are indicated in Fig. 2 and 3. To analyze the extent of *algC* expression in vivo, we introduced each plasmid construct into *P. aeruginosa algC* mutant strain 8858. The expression of *algC* was then studied by complementation analysis. The colonies were scored as +, for complementation (restoration of the mucoid phenotype of 8858), or −, for no complementation (Fig. 3). When the upstream DNA was deleted up to nucleotide −54 (in pNZ549), the complementation ability was lost. However, the complementation ability of the construct harboring up to nucleotide −321 of the upstream DNA suggests that a sequence(s) between nucleotide positions −54 and −321 is necessary for efficient complementation of *algC* mutant strain 8858.

AlgR1 binds to two regions of the *algC* promoter, upstream and downstream of the mRNA start site. Interestingly, *algC* upstream DNA was found to contain the consensus AlgR1 binding sequence CCGTTCGTCN$_5$ at positions −94 to −81 (Fig. 2). This consensus site was previously found by gel mobility shift assays and footprinting experiments to be involved in the regulation of *algD* transcription via the direct binding of AlgR1 (14). To determine whether the dependence of *algC* gene expression on AlgR1 was also due to a direct interaction of the *algR1* gene product with the *algC* upstream DNA, we performed a series of DNA binding experiments. A set of deletions (with some overlapping endpoints) within the *algC* far-upstream region was produced by use of exonuclease III, and their endpoints were determined by DNA sequence analysis. Five consecutive fragments spanning nucleotides −961 to +187 were used in mobility shift assays to determine whether the AlgR1 preparation would bind any of these regions under our experimental conditions (Fig. 4). Efficient mobility shift was observed with fragment IV, indicating its affinity for AlgR1. Fragment IV is the only fragment that contains a DNA sequence that is perfectly homologous to the consensus AlgR1 binding sequence. Relatively low AlgR1 binding affinity was observed with fragment V (nucleotides −73 to +187), which contains a DNA sequence, CCGTTGTTC (+161 to +169), that is partially homologous to the consensus AlgR1 binding sequence, CCGTTCGTC. The presence of this weakly binding sequence, however, does not allow efficient expression, as determined by complementation analysis (Fig. 3). No observable retardation of the *algC* fragment was detected when *P. aeruginosa* 8821 or 8822 cell extracts containing vector pMMB66EH (instead of pJK66R1) were added to the reaction mixture in place of the extract containing hyperproduced AlgR1. These results suggest that AlgR1 has at least two binding sites within the *algC* DNA sequence upstream of the translational initiation site. The highest-affinity binding site is centered approximately 87 bp upstream of the transcriptional start site and is critical for downstream expression of the *algC* gene.

To determine the specificity of AlgR1 for the fragment containing the consensus AlgR1 binding sequence, we performed competitive binding reactions (Fig. 5). The complex formed between the *algC* probe (containing DNA from nucleotide positions −378 to −73) and AlgR1 was resistant to the addition of low concentrations of cold nonspecific (whole plasmid pUC119) competitor DNA (lanes 7 to 10),

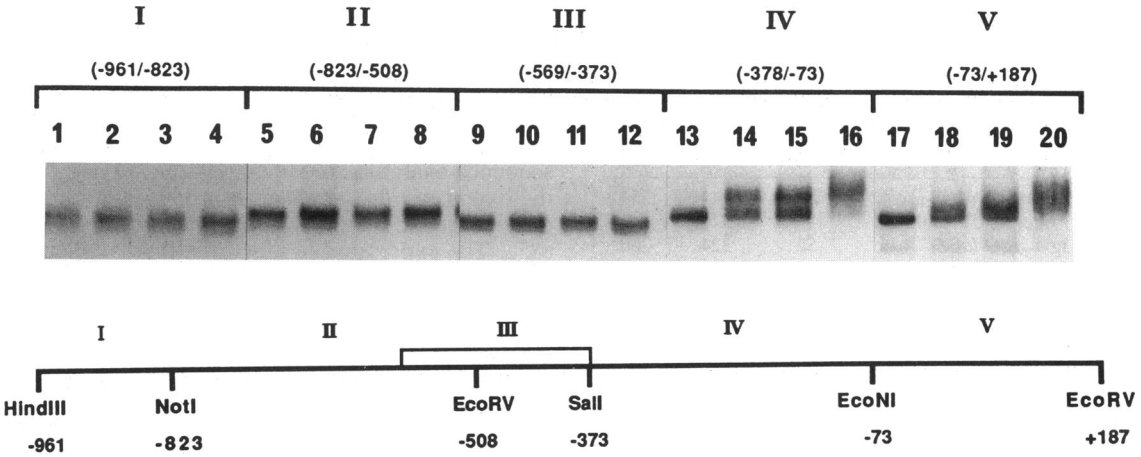

FIG. 4. Binding of AlgR1 to the *algC* regions upstream of the translational initiation site. A mobility shift assay was performed with cell extracts of *P. aeruginosa* harboring plasmid pJK66R1 (with *algR1* under the control of the *tac* promoter) and grown in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG). Five different fragments spanning sequences from −961 to +187 are shown. Each labeled fragment (500 cpm) was incubated with the following amounts of AlgR1 and analyzed on a 5% polyacrylamide gel: lanes 1, 5, 9, 13, and 17, radiolabeled fragments alone; lanes 2, 6, 10, 14, and 18, 60 ng of AlgR1 protein; lanes 3, 7, 11, 15, and 19, 100 ng of AlgR1 protein; and lanes 4, 8, 12, 16, and 20, 185 ng of AlgR1 protein.

compared with the results obtained with the same amounts of cold specific (*algC*) competitor DNA (lanes 3 to 6). When the same amounts of another specific competitor DNA, the *algD* upstream region containing the consensus AlgR1 binding sequence, were added to the binding reaction mixture, the amounts of bound complex were significantly reduced (lanes 11 to 14), suggesting that the consensus binding sequence in both of these fragments was responsible for AlgR1 binding.

Binding of phosphorylated AlgR1. Two-component-system proteins are involved in numerous adaptive responses in bacteria, such as virulence, nitrogen regulation, osmoregulation, chemotaxis, sporulation, and phosphate regulation (26). In each of these cases, the sensor protein responds to a signal by undergoing autophosphorylation, and the phosphate is subsequently transferred to the cognate response regulator protein. The phosphorylated form of the response regulator is most often a transcriptional activator. As mentioned earlier, in the case of the regulation of alginate synthesis by *P. aeruginosa*, AlgR2 is the protein kinase that is autophosphorylated and that transfers its phosphate to the response regulator, AlgR1 (30, 31). To determine what role the phosphorylation of AlgR1 may play in regulating *algC* transcription, we incubated the AlgR1 preparation with various concentrations of ATP and its analog ATPγS. When low concentrations of the AlgR1 preparation (which also contains AlgR2) were incubated with increasing amounts of ATP, the ability of AlgR1 to bind to the *algC* probe increased (Fig. 6, lanes 7 to 10). When the nonhydrolyzable analog ATPγS was substituted for ATP, no enhancement of binding was observed (Fig. 6, lanes 3 to 6). This result suggests that ATP hydrolysis, resulting in the phosphorylation of AlgR1, as shown previously (31), is responsible for the enhanced binding of AlgR1.

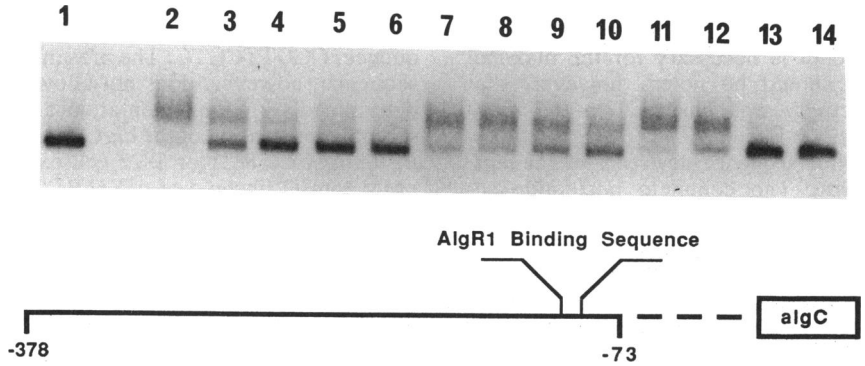

FIG. 5. Competitive binding of AlgR1 to the *algC* promoter region. Shown is a mobility shift DNA binding assay (top panel) with a 300-bp fragment containing the AlgR1 binding sequence upstream of the *algC* transcriptional initiation site (bottom panel). Lane 1 contained the free probe only. Lane 2 was the gel mobility shift control with 100 ng of AlgR1. Lanes 3 to 14 contained equal amounts of AlgR1 (100 ng) and different amounts and types of competitor DNAs. Lanes 3 through 6 contained nonradioactive specific competitor DNA (plasmid pNZCR1; 1, 3, 5, and 10 μg, respectively) with sequences spanning the *algC* promoter region from positions −378 to −73. Lanes 7 through 10 contained nonspecific competitor DNA (plasmid pUC119; 1, 3, 5, and 10 μg, respectively). Lanes 11 through 14 contained specific competitor DNA (plasmid pNZDR1; 1, 3, 5, and 10 μg, respectively) with sequences spanning the *algD* promoter region from positions −563 to −399. Note that this region of the *algD* promoter contains a consensus AlgR1 binding sequence (14).

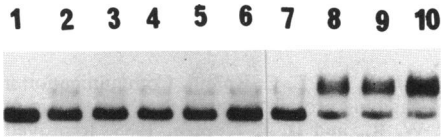

FIG. 6. Binding of the phosphorylated form of the AlgR1 protein to the *algC* promoter region. A 300-bp fragment containing the AlgR1 binding site from the *algC* promoter region was used as target DNA for the mobility shift assay. AlgR1 (20 ng) was incubated for 1 min with various amounts (final concentrations, 1 nM, 10 nM, 50 nM, and 1 μM) of ATPγS or ATP at room temperature. After incubation, the *algC* DNA probe (500 cpm) was added to the reaction mixture to complete the DNA binding assay. Lanes: 1, free probe; 2, DNA protein binding assay control containing 20 ng of AlgR1; 3 to 6, gel mobility shifts with increasing amounts of the ATP analog, ATPγS; 7 to 10, gel mobility shifts with increasing amounts of ATP.

DISCUSSION

This paper describes studies on the regulation of *algC*, the gene that is located outside the alginate biosynthetic gene cluster in the *P. aeruginosa* chromosome. Our studies revealed some interesting properties of the regulation of this distantly located biosynthetic gene. The most important aspect of the regulation of the *algC* promoter appears to be the coordination between its regulation and that of the *algD* promoter. On the basis of our observations, it can be suggested that a global regulatory network may be involved in regulating and modulating the expression of alginate genes in response to environmental signals present in the CF-affected lung.

Our studies on the effect of medium osmolarity indicated that the *algC* promoter, like the *algD* promoter (1), is activated in a hyperosmotic environment. This activation appears to involve the response regulator, AlgR1. However, the role of RpoN (σ-54) in *algC* transcription may represent a deviation from the pattern of *algD* promoter activity. It is unclear whether the *algD* promoter requires RpoN for its maximal transcriptional activity, since the data reported from two different laboratories are contradictory (16, 24). Furthermore, unlike the *algD* promoter, the *algC* promoter contains two sequence elements resembling the RpoN recognition motif. One of these GG-N_{10}-GC elements is located from positions −24 to −11, a location normally found in RpoN-dependent promoters. Thus, the partial dependence of *algC* promoter activation on RpoN may be due to the normal position of its recognition site. While the *rpoN* gene was originally identified by its involvement in the transcription of genes that encode enzymes of nitrogen assimilation and nitrogen fixation, it is recognized to be involved in the transcriptional activation of a broader set of genes that are functionally unrelated (17, 38).

Both the *algD* and *algR1* genes of *P. aeruginosa* are activated by the *algR1* gene product, AlgR1 (7, 16). In addition, AlgR1 also activates the neuraminidase gene in *P. aeruginosa* in a hyperosmotic medium (4). Neuraminidase is believed to be involved in the adherence of *P. aeruginosa* to host epithelial cells (4). Therefore, AlgR1 appears to regulate other pathogenesis-related genes besides those involved in alginate biosynthesis. It has recently been demonstrated by DNA footprinting experiments that AlgR1 binds at 14-mer sites having the sequence CCGTTCGTCN$_5$ and located upstream of the *algD* transcriptional initiation site (14). The first two sites are located far upstream of the *algD* transcriptional initiation site, at nucleotide positions −465 to −452 and −389 to −376, and have been shown to be critical for high-level expression of *algD* (14). AlgR1 has also been shown to bind weakly with the *algD* promoter region between positions −144 and +11 (23). In the case of *algC* promoter regulation, the similar weak binding of AlgR1 to fragment V (Fig. 4) may be due to the presence of a sequence having appreciable homology with the consensus AlgR1 binding sequence. The importance of all the bases within the AlgR1 consensus binding site is not known. Mutational alterations of specific bases within this site followed by AlgR1 binding studies will shed light on the role of such a binding site in alginate gene expression.

The AlgR2-AlgR1 phosphotransfer reaction seems to play a critical role in the sensory signal transduction system regulating alginate gene expression. Many bacterial signal transduction systems rely on a phosphorelay involving at least one kinase and one response regulator (37). The autophosphorylation of AlgR2 and the subsequent transfer of phosphate from AlgR2 to AlgR1 have been demonstrated (30, 31). Using a crude cell lysate with hyperproduced AlgR1 in the presence of AlgR2, we demonstrated enhanced binding of AlgR1 in the presence of ATP (Fig. 6), suggesting that the phosphorylation of AlgR1 enhances its affinity for *algC* upstream DNA. Phosphorylation of the response regulators of bacterial two-component signal transduction systems is known to enhance their DNA binding activity (26). Thus, the ability of AlgR1 phosphate to bind its cognate binding site at a higher affinity than AlgR1 is not surprising. Further studies on different aspects of the DNA binding properties of AlgR1 and AlgR1 phosphate may shed light on the details of the AlgR1-DNA interaction process, which may influence the expression not only of *algC* but also of several other genes that are regulated by AlgR1 in *P. aeruginosa*.

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