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Control of *Pseudomonas aeruginosa algZ* Expression by the Alternative Sigma Factor AlgT

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AlgZ controls *Pseudomonas aeruginosa* alginate synthesis by activating *algD*, yet *algZ* expression is not detectable in nonmucoid strains. Mobility shift and Western blot assays revealed that *algZ* expression requires the sigma factor AlgT. The mapped *algZ* transcription start site revealed a consensus AlgT-dependent promoter that, when mutated, substantially reduced *algZ* transcription.

Individuals with cystic fibrosis (CF) are predisposed to pulmonary infections with a number of bacteria, including *Pseudomonas aeruginosa* (8). Whereas initial colonizing *P. aeruginosa* strains are nonmucoid, over the course of chronic infection, alginate-producing (mucoid) strains emerge. There is a distinct correlation between the appearance of mucoid *P. aeruginosa* and a worsening clinical prognosis for CF patients (8, 14). Control of alginate biosynthesis is complex and involves multiple genes. The *algD* gene, which is the first gene in the alginate biosynthetic operon and encodes GDP-mannose dehydrogenase, undergoes strong transcriptional activation in mucoid cells (6, 8). In a previous study, we discovered AlgZ (PA3385; reference 18), a protein that bound with high affinity and specificity to sequences upstream of the *algD* promoter and was essential for *algD* activation (3). Sequence analysis revealed that *algZ* encodes a protein of the ribbon-helix-helix family of DNA binding proteins (2). Members of this group include the Arc and Mnt repressors of bacteriophage P22, the methionine repressor protein MetJ, and NikR, a repressor of the high-affinity nickel uptake operon in *Escherichia coli* (2, 5, 15).

In earlier work, we observed that the expression or activity of *algZ* was correlated with the mucoid phenotype and dependent on the alternative sigma factor AlgT, which is also designated and annotated AlgU (8, 18). However, the mechanism by which AlgT controlled *algZ* was not investigated (3). An electrophoretic mobility shift assay that validates these data is depicted in Fig. 1A. In this experiment, extracts from mucoid strain FRD1 (13), as well as three isogenic mutants harboring either an *algT*::Tn*501* allele (FRD440; reference 7), an *algZ*::*xylE aacC1* allele (FRD1200), or the *algT*::Tn*501* *algZ*::*xylE aacC1* double mutation (FRD1202), were prepared and examined for binding to DNA upstream of the *algD* promoter. The *algZ*::*xylE aacC1* mutation was constructed by previously described methods (16, 19), with pDJW588, a pEX18Ap-derived plasmid (10) with a 2.2-kb *Sma*I fragment containing *xylE-aacC1* from pX1918G (10) inserted at the *Xho*I site within the *algZ* coding sequence (2). The *algD* DNA fragment was identical to that used in earlier AlgZ-*algD* DNA binding studies (2, 3). In addition, an extract from *E. coli* cells expressing recombinant AlgZ derived from BL21(DE3)/pPJ145 was examined for *algD* DNA binding activity. Plasmid pPJ145, which expresses wild-type AlgZ, was generated by PCR amplification of pDJW585 (2) with primers *algZ*9 (5′-CCCCCATATGCGCCCACTGAAACAGG-3′) and *algZ*23 (5′-GCGCTACGCGTGGGCGGCCGCGCTCAGGCCTGGG-3′) and subsequent cloning into pET29a (Novagen). All plasmids containing *algZ* originated from pDJW585, which is pUCP21T (17) harboring *algZ* derived from FRD1 on a 1.8-kb *Bam*HI fragment. AlgZ present in the parental strain, FRD1, formed several protein-DNA complexes (Fig. 1A, lane 1). The migration of these complexes was identical to that observed with extracts of *E. coli* cells expressing recombinant AlgZ (Fig. 1A, lane 6). Previous competition studies indicated that this binding was highly specific (3). This is also evidenced by the fact that no binding was observed when an extract from an *algZ* mutant was tested (Fig. 1A, lane 3). Significantly, no binding was detected when an *algT* mutant was examined (Fig. 1A, lane 2). This suggested that either the activity or the expression of *algZ* was AlgT dependent. To distinguish these, a His-tagged AlgZ protein was expressed from BL21(DE3)/pPJ138 cells, purified, and used to make AlgZ antibodies. Plasmid pPJ138 was constructed by cloning an *algZ* PCR fragment derived by amplification of pDJW585 with primers *algZ*9 (above) and *algZ*10 (5′-CCCCTCGAGGGCCTGGGCCAGCTCCGCATCG-3′) into pET29a. For antibody production, approximately 1 mg of His-tagged AlgZ derived from BL21(DE3)/pPJ138 was resolved by preparative sodium dodecyl sulfate (SDS)–15% polyacrylamide gel electrophoresis (PAGE), followed by brief staining with Coomassie blue, exhaustive destaining, and excision of the band representing AlgZ. This material was used as an immunogen by a commercial vendor (Covance, Denver, Pa.) to generate polyclonal antiserum in New Zealand White rabbits. Western blot analysis was performed essentially as previously described (11), with AlgZ antiserum at a 1:50,000 dilution and chemiluminescent reagents and in accordance with the procedures outlined by Amersham. This antiserum recognized the purified 14-kDa recombinant His-tagged AlgZ protein (Fig. 1B, lane 6), as well as a faster-migrating 12-kDa wild-type AlgZ protein in an extract of *E. coli* expressing native

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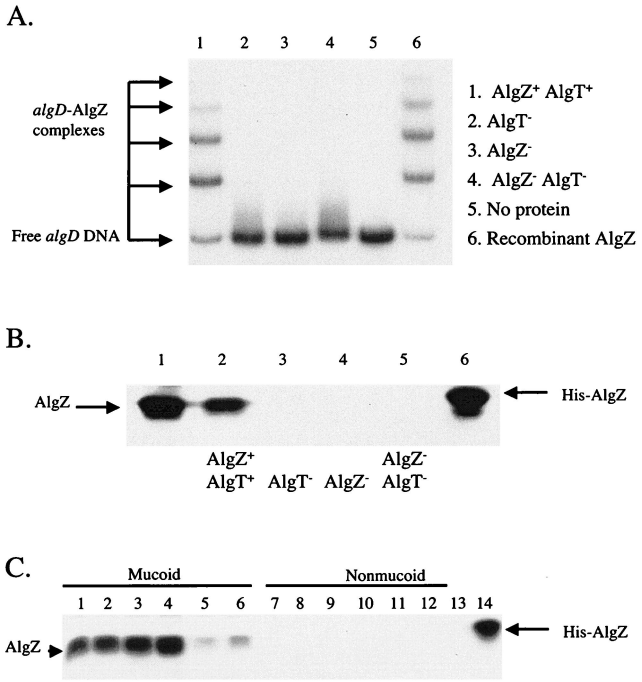

FIG. 1. (A) Analysis of AlgZ binding to *algD* by electrophoretic mobility shift assay. Approximately 2 pmol of a labeled *algD* fragment was left untreated (lane 5) or incubated with cell extracts derived from the following strains: lane 6, 100 ng of BL21(DE3)/pPJ145; lanes 1 to 4, 5 μg derived from FRD1 (Alg$^+$), FRD440 (*algT*::Tn*501*), FRD1200 (*algZ*::*xylE aacC1*), and FRD1202 (*algZ*::*xylE aacC1 algT*::Tn*501*), respectively. (B) Western blot analysis of AlgZ. Extracts or purified AlgZ were resolved by SDS–15% PAGE and probed with antibodies to AlgZ. Lane 1, 1.0 μg of extract of *E. coli* BL21(DE3)/pPJ145; lanes 2 to 5, 35 μg of extract prepared from FRD1 (lane 2), FRD440 (lane 3), FRD1200 (lane 4), and FRD1202 (lane 5). Lane 6 contains 280 ng of purified His-tagged AlgZ from BL21(DE3)/pPJ138. (C) Western blot analysis of AlgZ in representative CF isolates. Extracts or purified AlgZ was resolved by SDS–15% PAGE and probed with antibodies to AlgZ. Lane 1 contains 25 μg of FRD1 extract. Lanes 2 to 6, contain 25 μg each of extracts prepared from mucoid CF isolates. Lane 7 contains 25 μg of FRD440 extract. Lanes 8 to 12 contain 30 μg of extracts prepared from nonmucoid CF isolates. Lane 13 contains 30 μg of FRD1200. Lane 14 contains 200 ng of purified, His-tagged AlgZ from BL21(DE3)/pPJ138.

AlgZ (Fig. 1B, lane 1). AlgZ was detected in extracts derived from mucoid strain FRD1 but not in those from the *algZ* mutants (Fig. 1B, compare lanes 2 and 4). Additionally, there was no detectable AlgZ when an extract from the isogenic *algT* mutant was examined (Fig. 1B, lane 3). Taken together, the data in Fig. 1A and B strongly suggest that the absence of AlgZ DNA binding in the *algT* mutant is due to a lack of *algZ* expression and not modulation of the DNA binding activity of AlgZ.

Our earlier work showed that AlgZ binding activity was present in all of the mucoid strains examined yet absent in nonmucoid strains derived from CF patients (3). We used AlgZ antiserum to screen extracts from additional mucoid and nonmucoid *P. aeruginosa* strains derived from CF patients. These results confirm that AlgZ is highly expressed in mucoid strains derived from CF patients but absent in nonmucoid strains (Fig. 1C). This also indicates that the expression of

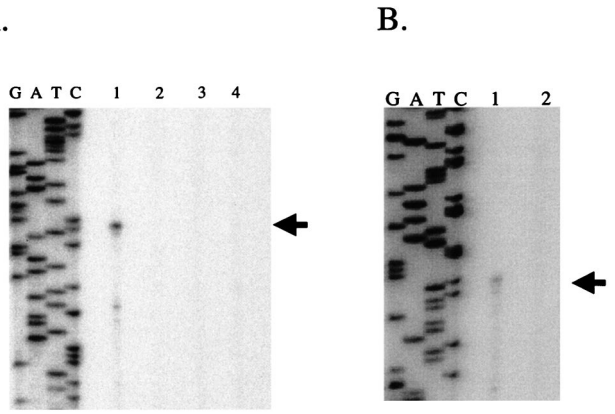

FIG. 2. (A) Primer extension analysis of *algD*. Oligonucleotide *algD*1 was end labeled and used in a primer extension experiment with 50 μg of total cellular RNA from the following strains: lane 1, FRD1 (Alg$^+$); lane 2, FRD440 (*algT*::Tn*501*); lane 3, FRD1200 (*algZ*::*xylE aacC1*); lane 4, FRD1202 (*algT*::Tn*501 algZ*::*xylE aacC1*). The arrow represents the start site of *algD* transcription (20). The *algD* sequencing ladder (GATC) was produced from pDJW220 (20) with the same oligonucleotide (*algD*1) used for synthesis of the probe in the primer extension experiment. (B) Primer extension analysis of *algZ*. Oligonucleotide *algZ*15 (see Fig. 3A) was end labeled and used in a primer extension experiment with 50 μg of total cellular RNA from the following: lane 1, FRD1 (Alg$^+$); lane 2, FRD440 (*algT*::Tn*501*). The arrow represents the start site of *algZ* transcription. The *algZ* sequencing ladder (GATC) was produced from pDJW585 with the same oligonucleotide (*algZ*15) used for synthesis of the probe in the primer extension experiment.

AlgZ parallels the mucoid status of the cell and suggests that AlgZ-mediated activation of *algD* is conserved among mucoid CF isolates.

The above data suggest that AlgT controls *algD* expression and alginate synthesis in part through control of *algZ*. If this is the case, transcription of both *algD* and *algZ* should be reduced or eliminated in *algT* mutants. Data previously obtained from our laboratory and others have demonstrated that AlgT is essential for *algD* transcription (6, 9, 20). We performed primer extension experiments as outlined previously (20) to determine if the control exerted by AlgZ at *algD* was at the previously studied AlgT-dependent promoter (6, 20). RNA was harvested from parental mucoid strain FRD1, as well as isogenic *algT*, *algZ*, or *algT algZ* mutants, and analyzed for *algD* expression by primer extension. RNA from *P. aeruginosa* cells cultured in LBNS (10 g of tryptone and 5 g of yeast extract per liter) (A_{600} = 0.5) was purified by standard techniques (1). The oligonucleotide used for primer extension was *algD*1 (5′-AAC AGGTTGAGTTTGTCCCT-3′, position +86 to position +66 relative to the start of *algD* transcription) (20) and was end labeled with polynucleotide kinase with [γ-^{32}P]ATP as previously described (1). All detectable *algD* transcription originated from the previously mapped promoter (Fig. 2A, lane 1). Expression at this promoter was absolutely dependent on sigma factor AlgT (lane 2), as well as *algZ* (lanes 3 and 4). These data support the conclusions that AlgZ is an essential positive transcriptional activator of *algD* and that AlgZ controls *algD* through the previously characterized AlgT-dependent promoter.

To test whether AlgT was responsible for *algZ* transcription

A.

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   1 TGACAATCTCTTGAACGACCGGTGGTCAGAAGGCCATCATCCAACCCCCAGTAAAAAACTAGGCTTTTGCTGAATCGCTC 80

  81 GCATTTCAGATAGCTGGTACTGGCAAAACGCCGGCACGCAACGCACAATTGACGTCAACTAATTGTTGTTTCCGAAATAA 160

            -35                    -10
           GAACtt                  TCtNA
 161 CAGACTGAACGGCTAGCGGAAATCGTGGTCATAGGCCCGAGAACAATGAACGCTTCCTCGGCACCGCTTTTCCCTGACTC 240

algZ11                           GATCC
algZ12          CTCGAG

 241 TACAGGTTCAATGT ATG CGC CCA CTG AAA CAG GCA ACT CCT ACC TAC TCC AGC CGT ACC GCT
                     M   R   P   L   K   Q   A   T   P   T   Y   S   S   R   T   A
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algZ15

B.

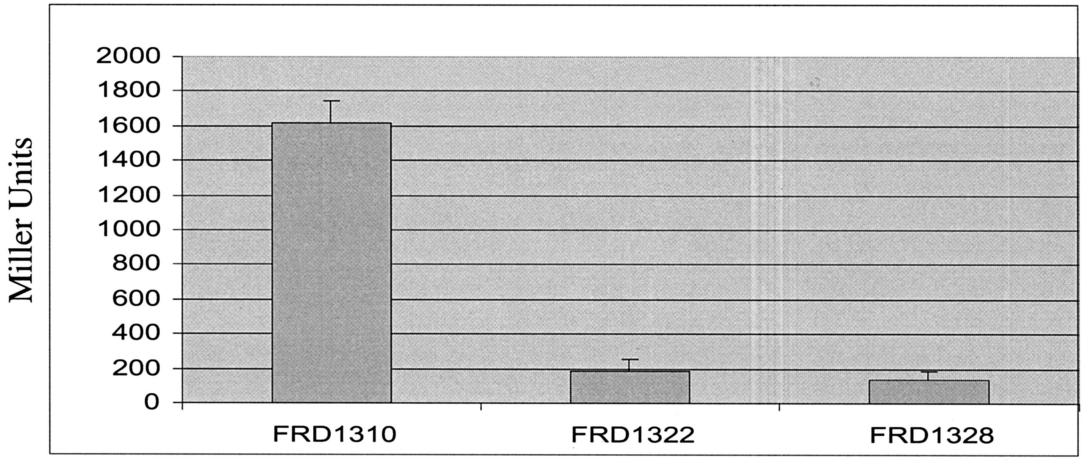

FIG. 3. (A) Upstream regulatory sequences at *algZ*. The sequences immediately upstream of the *algZ* coding region are depicted. The small arrow represents the position of the mapped *algZ* transcription start site, and the proposed AlgT promoter is underlined. Sequences on top of this represent the consensus AlgT promoter (8). Sequences under this represent mutant alleles *algZ11* and *algZ12*. (B) *algZ-lacZ* transcription studies. Strains FRD1310 (FRD1 *algZ-lacZ*), FRD1322 (FRD1 *algZ12-lacZ*), and FRD1328 (FRD1 *algZ11-lacZ*) were cultured on LBNS plates and assayed for *algZ-lacZ* transcriptional activity.

and to map a promoter(s) required for *algZ* expression, primer extension analysis was performed on RNA derived from *P. aeruginosa* strains FRD1 and FRD440 (Fig. 2B). A primer positioned just downstream of the *algZ* translation initiation site (*algZ*15 [5′-GTTGCCTGTTTCAGTGGGC-3′]) (Fig. 3A) was end labeled, hybridized to RNA, and reverse transcribed. A primer extension product of 78 nucleotides was observed when RNA from parental ($AlgT^+$) strain FRD1 was examined (Fig. 2B, lane 1). However, no primer extension signal was observed when RNA from the *algT* mutant FRD440 was examined (Fig. 2B, lane 2, arrow in Fig. 3A). This is consistent with data in Fig. 1B, which indicate no AlgZ protein present in *algT* mutants. Inspection of the sequences immediately upstream of the *algZ* transcription start site revealed a partial match with the consensus AlgT promoter recognition sequence (8), GAACTT 16/17 bp TCTNA (Fig. 3A). This suggested that the alternative sigma factor AlgT was required directly for expression from this promoter.

To determine if the consensus AlgT promoter functioned in vivo, site-directed mutagenesis was used to disrupt the putative promoter. Two allelic variants were created that harbored mutations (Promega Altered Sites) in the putative −10 (*algZ11*) and −35 (*algZ12*) sequences (Fig. 3A). Promoter sequences from wild-type *algZ*, as well as *algZ11* and *algZ12*, were amplified by PCR with primers *algZ*15 (5′-GTTGCCTGTTTCA GTGGGCG-3′) and *algZ*24 (5′-GGTGTAGACCAAGCTTG AAGGAGACTG-3′) and cloned into mini-CTX-*lacZ* (4) to form *algZ-lacZ* operon fusions. Plasmids harboring the *algZ-lacZ* fragments were integrated in single copy into the FRD1 chromosome at the neutral *attB* site as described elsewhere (4, 21), resulting in strains FRD1310 (FRD1 *algZ-lacZ*), FRD1322 (FRD1 *algZ12-lacZ*), and FRD1328 (FRD1 *algZ11-lacZ*). β-Galactosidase levels (Miller units [12]) were determined from FRD1310, FRD1322, and FRD1328 cells recovered from LBNS plates. Compared with the wild-type *algZ-lacZ* levels expressed in FRD1310, mutations in the −35 or −10 sequence resulted in a substantial reduction in *algZ* transcription (88.5 or 91.5%, respectively; Fig. 3B).

Taken together, these data provide evidence that expression of *algZ* requires the alternative sigma factor AlgT. Most likely, AlgT is directly involved in *algZ* promoter recognition and transcription initiation since a promoter that is similar to a consensus AlgT promoter was identified upstream of the mapped transcription start site and mutations in the −35 or −10 element significantly reduced but did not completely eliminate *algZ* transcription.

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