

Expression Analysis of the *Pseudomonas aeruginosa* AlgZR Two-Component Regulatory System

Christopher L. Pritchett,^{a,b} Alexander S. Little,^a Yuta Okkotsu,^a Anders Frisk,^{c,*} William L. Cody,^{a,*} Christopher R. Covey,^a Michael J. Schurr^a

Department of Immunology and Microbiology, University of Colorado School of Medicine, Aurora, Colorado, USA^a; East Tennessee State University, Johnson City, Tennessee, USA^b; Tulane University Health Science Center, New Orleans, Louisiana, USA^c

Pseudomonas aeruginosa virulence components are subject to complex regulatory control primarily through two-component regulatory systems that allow for sensing and responding to environmental stimuli. In this study, the expression and regulation of the *P. aeruginosa* AlgZR two-component regulatory system were examined. Primer extension and S1 nuclease protection assays were used to identify two transcriptional initiation sites for *algR* within the *algZ* coding region, and two additional start sites were identified upstream of the *algZ* coding region. The two *algR* transcriptional start sites, RT1 and RT2, are directly regulated by AlgU, consistent with previous reports of increased *algR* expression in mucoid backgrounds, and RpoS additionally plays a role in *algR* transcription. The expression of the first *algZ* promoter, ZT1, is entirely dependent upon Vfr for expression, whereas Vfr, RpoS, or AlgU does not regulate the second *algZ* promoter, ZT2. Western blot, real-time quantitative PCR (RT-qPCR), and transcriptional fusion analyses show that *algZR* expression is Vfr dependent. The *algZ* and *algR* genes also are cotranscribed in both nonmucoid and mucoid backgrounds. Furthermore, *algZR* was found to be cotranscribed with *hemCD* by RT-PCR. RT-qPCR confirmed that *hemC* transcription in the PAO1 Δ *algZ* mutant was 40% of the level of the wild-type strain. Taken together, these results indicate that *algZR* transcription involves multiple factors at multiple start sites that control individual gene expression as well as coexpression of this two-component system with heme biosynthetic genes.

Pseudomonas aeruginosa is a human opportunistic pathogen capable of causing fatal infections in individuals with compromised innate immunity, such as those undergoing cancer treatment or those with severe burn wounds or cystic fibrosis (CF) (1–3). *P. aeruginosa* is one of the most clinically relevant organisms in CF patients due to its ability to establish chronic infections, characterized by the overproduction of an exopolysaccharide called alginate. Alginate overproduction (a phenotype called mucoidy) increases *P. aeruginosa* resistance against antimicrobials and phagocytosis and is a hallmark of clinical decline and worsening prognosis in patients with CF.

Alginate production is regulated in a complex manner by several transcriptional regulators, including the extracytoplasmic sigma factor AlgU (AlgT) (4–6). The activity of AlgU is increased in mucoid *P. aeruginosa* due to mutations in the *muca* anti-sigma factor gene that releases AlgU from the inner membrane (6–8). The enzymes encoded by the *algC* gene and the *algD* operon are responsible for alginate biosynthesis, modification, and export. The AlgR transcriptional regulator is an essential activator for the *algD* operon and the *algC* gene; deletion of *algR* in mucoid *P. aeruginosa* backgrounds results in a nonmucoid phenotype (9–11). As part of its regulon, AlgU also activates the transcription of the *algR* gene to increase alginate production. An AlgU-dependent *algR* transcriptional start site was determined in *muca22* strains to be located 73 bp upstream of the translational start site (4, 12). A constitutively active promoter had previously been proposed to be located in the *algR* promoter region, although it has not been mapped (4, 10, 12–15).

Although transcriptional regulation of *algR* has been examined briefly in previous work, the regulation of *algZ* has remained virtually unexplored. There are several pieces of data demonstrating that Vfr (for virulence factor regulator), a member of the 3',5'-cyclic AMP (cAMP) receptor protein (CRP) family of transcrip-

tional regulators (16), is tied into the regulation of the *algZR* system. A Vfr binding site has been identified upstream of the *algZ* coding region and was shown by gel shifts to be bound by Vfr (17). Additionally, it has been shown that the cAMP/Vfr-dependent (CVS) signaling pathway is suppressed in a *muca22* background, and that this suppression can be relieved by deletion of either *algZR* or *algU* (48).

Besides alginate production, AlgR also regulates a number of virulence factors in *P. aeruginosa*, including type IV pili, hydrogen cyanide and rhamnolipid production, the type III secretion system, the Rhl quorum-sensing system, and biofilm formation (19–26, 48, 67, 68). The response regulator encoded by *algR* (PA5261) and the putative cognate histidine kinase encoded by *algZ-fimS* (PA5262) are proposed to form a two-component regulatory system (TCS) (25, 27). TCSs transduce an environmental signal to the intracellular environment through a phosphotransfer reaction

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Address correspondence to Michael J. Schurr, michael.schurr@ucdenver.edu.

* Present address: Anders Frisk, Pelham, New York, USA; William L. Cody, University of Dallas, Irving, Texas, USA.

C.L.P., A.S.L., and Y.O. contributed equally to the manuscript.

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between the sensor kinase and response regulator. AlgR phosphorylation functions to modulate AlgR activity either as an activator or repressor of its target genes (14, 19, 20, 23, 24, 28, 29).

Because AlgZ and AlgR regulate a number of virulence factors, this TCS plays a key role in overall *P. aeruginosa* virulence. Therefore, understanding how these genes are transcriptionally controlled is important in dissecting their physiological role in the organism. Currently, one transcriptional start site has been identified upstream of *algR* that is AlgU dependent (4, 12). However, an additional start site also was detected but not characterized (12). In this study, transcriptional start site mapping was performed to provide a better understanding of the regulation of this two-component system. We present evidence that (i) two promoters control *algR* transcription, (ii) two promoters control *algZ* transcription, (iii) *algZ* and *algR* are cotranscribed, (iv) the *algZ* genes are cotranscribed with *hemCD*, (v) both promoters regulating *algR* transcription are directly AlgU dependent, (vi) RpoS plays a role in *algR* expression but not *algZ* expression, and (vii) Vfr regulates *algZR* expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* was grown at 37°C in lysogeny broth-Miller (LB-Miller) or *Pseudomonas* isolation agar (PIA; Difco). The LB-Miller medium used for *P. aeruginosa* was supplemented with tetracycline (300 µg/ml), gentamicin (150 µg/ml), or carbenicillin (300 µg/ml) as needed. *Escherichia coli* was cultivated at 37°C in LB-Miller and supplemented when necessary with ampicillin (100 µg/ml), gentamicin (15 µg/ml), kanamycin (50 µg/ml), or chloramphenicol (34 µg/ml).

The PAO1 *ΔalgU*, PAO1 *ΔmucA*, and PAO1 *rpoS::Gm ΔalgU* strains were constructed by cloning an in-frame deletion of *algU* or *mucA* into pEXG18Gm or pEX18Tc (35). The in-frame *algU* deletion was created by overlap extension (OE) PCR (37) using the oligonucleotides algUKO1, algUSOEF, algUSOER, and algUKO4 (see Table S1 in the supplemental material). The in-frame *mucA* deletion was created by OE PCR (37) using the oligonucleotides mucA-SOE-YO-1, mucA-SOE-YO-2, mucA-SOE-YO-3, and mucA-SOE-YO-4 (see Table S1 in the supplemental material). The pEX18Gm *ΔalgU* plasmid was conjugated into PAO1, and pEX18Tc *ΔalgU* plasmid was conjugated into the PAO1 *rpoS::Gm* strain (SS24 [31]). The pEX18Gm *ΔmucA* plasmid was conjugated into PAO1. Single recombinants were selected on Vogel-Bonner minimal medium (VBMM) supplemented with the appropriate antibiotic to create merodiploid strains. The merodiploids were resolved by an overnight outgrowth in LB followed by plating on VBMM (38), supplemented with 7.5% sucrose for counterselection. The *algU* or *mucA* mutation was confirmed by PCR using oligonucleotides algUKO1 and algU KO4 or mucA-SOE-YO-1 and mucA-SOE-YO-4, respectively, and the nucleotide sequences of the amplicons were determined.

A single *algZ-S-Tag* gene was integrated at the *attB* site on the *P. aeruginosa* chromosomes of the PAO1, PAO1 *rpoS::Gm*, PAO1 *ΔalgU*, PAO1 *rpoS::Gm ΔalgU*, and PAO1 *Δvfr* strains to create PAO1-Z, PAO1 *rpoS::Gm-Z*, PAO1 *ΔalgU-Z*, PAO1 *rpoS::Gm ΔalgU-Z*, and PAO1 *Δvfr-Z* strains, respectively. We could not insert this *algZ-S-Tag* allele into its correct location on the chromosome, because the S-Tag gene would disrupt *algR* transcription. The *algZ* gene was cloned by PCR using oligonucleotides algZ-1212FXbaI and S-Tag-HindIIIIR and cloned into PCR2.1. The *algZ-S-Tag* gene was excised by BamHI and EcoRV and subcloned into the mini-CTX vector. The mini-CTX *algZ-S-Tag* construct includes 1,212 bp of *algZ*'s upstream promoter region and encodes a carboxy-terminal S-Tag (Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-Asp-Ser). The mini-CTX *algZ-S-Tag* vector was moved into each strain using the helper conjugative *E. coli* strain harboring plasmid pRK2013, and single recombinants were selected as tetracycline resistant

on PIA plates supplemented with tetracycline (200 µg/ml). The inserted mini-CTX vector was removed from each construct's chromosome by conjugation with pFLP2 and plated on PIA supplemented with carbenicillin. After an overnight outgrowth without antibiotics, individual colonies were counterselected on 10% sucrose. The sucrose-resistant colonies were replica plated to PIA supplemented with tetracycline to confirm tetracycline sensitivity.

S1 nuclease protection assay analysis. The *P. aeruginosa* strain PAO1 total cellular RNA for the S1 nuclease protection assay was isolated from cells of cultures grown to stationary phase (optical density at 600 nm [OD₆₀₀] of 1.0 to 2.0) through a cushion of 5.7 M CsCl as previously described (12) or using the Qiagen RNeasy kit (Qiagen). The S1 nuclease protection assays were performed using 50 to 100 µg of RNA with the following modifications (12). A 1,910-bp PCR amplicon containing upstream sequence of *algR* using the primers algrS1R and algZko1 (see Table S1 in the supplemental material) was cloned in plasmid pCR2.1 (Invitrogen) and subsequently transferred into M13mp18. This fragment ranges from bp +34 to -1877 upstream of the *algR* translational start site. Single-stranded phage DNA was isolated and used as the template for the uniformly labeled [α -³²P]dCTP DNA probes generated using Klenow. The probes were digested using the appropriate restriction enzymes (New England BioLabs, Ipswich, MA) and purified on a 5% denaturing polyacrylamide-urea gel. The probes were hybridized to the RNA at 67°C for 12 to 24 h in 40 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) (pH 6.4), 0.1 mM EDTA (pH 8.0), and 0.4 M NaCl. Hybridizations were treated with 3,000 U of S1 nuclease (Promega) for 60 min at 37°C. A control containing the single-stranded probe alone and S1 nuclease also was performed. Reactions were precipitated and separated on a 6% polyacrylamide-8 M urea gel along with a sequencing ladder generated using the same oligonucleotides as the probes.

Primer extension. *P. aeruginosa* total RNA (20 to 30 µg) was isolated as described for the S1 nuclease protection assays. Radiolabeled oligonucleotides algrB5R' and ZT2-875R (see Table S1 in the supplemental material) were used in reverse transcription reactions using ThermoScript (Invitrogen). A temperature of 55°C was used for the extension. The same oligonucleotides used for primer extension also were used in sequencing reactions with the *algZ* upstream sequence in pCR2.1 (Invitrogen) using the Sequenase 7-deaza-dGTP kit (USB). Both primer extensions and sequencing reactions were run on a 6% acrylamide-8 M urea gel. The gel was dried and extension products detected using a phosphorimager (Bio-Rad).

Reverse transcriptase PCR (RT-PCR). Total RNA was isolated from cells grown in 100-ml LB cultures at 37°C and 250 rpm to an OD₆₀₀ of 2.0 and 2.5 (early and late stationary phases). RNA (250 ng) was reverse transcribed at 50°C using random primers and Superscript II as outlined by the manufacturer (Invitrogen). For PCR amplification of the cDNA, five different primer sets were used to generate 4 amplicons. To test for genomic contamination, oligonucleotides algZ-1212FXbaI and algrGSR6 were used and generated a 2,165-bp fragment. The *algR*-specific oligonucleotides algrTOPOF and algr-Y99F2-YO-R generated a 304-bp fragment. The *algZ*-specific oligonucleotides used were algrRSF2 and algrRSR6 and generated a 611-bp fragment. The *algZR*-specific oligonucleotides algrRSF2 and algr-Y99F2-YO-R were used to detect cDNA that spanned *algR* and *algZ* and generated a 1,045-bp fragment. The PCR cycle used was 30 s of denaturation at 94°C, 30 s of annealing at 60°C, and 2 min of elongation at 68°C and was repeated for 30 cycles using the AccuPrime *Taq* DNA polymerase (Invitrogen) and an optimized buffer of 2.5 mM MgCl₂ at pH 8.5.

Transcriptional fusion analysis. Promoter fragments were generated by PCR using both algrTRHindIII and algZNotIF2 for *algR* fusions or algZTRHindIII and algZ3forward for *algZ* (see Table S1 in the supplemental material). The *algR* RT2 promoter fusion was created by PCR using algZNotIF2 and algrP2HindIII to generate a 395-bp product (PAO1 genomic positions 5923976 to 5924370). The -35 region of *algR* RT2 was changed from 5'-GAACGTG-3' to 5'-TGGAGT-3' using the mu-

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or other characteristics	Reference or source
<i>P. aeruginosa</i>		
PAO1	Wild type	30
PSL317	PAO1 $\Delta algR$	20
PAZ	PAO1 $\Delta algZ$	22
PAO1 Δvfr	PAO1 Δvfr	Matthew Wolfgang
PAO1 <i>mucB</i>	PAO1 <i>mucB</i> ::Tc ^r	7
SS24	PAO1 <i>rpoS101::aacCI</i>	31
PDO300	PAO1 <i>mucA22</i>	32
PAO1 $\Delta algU$		This study
PAO1 <i>rpoS</i> ::Gm $\Delta algU$	SS24 (<i>rpoS101::aacCI</i>) and deletion of <i>algU</i>	This study
PAO1 $\Delta mucA$	PAO1 with <i>mucA</i> deleted	This study
PAO1-Z	PAO1 containing an <i>attB</i> site single copy of <i>algZ</i> -S-Tag gene with 1,212 bp of upstream promoter	This study
PAO1-Z $\Delta algU$	PAO1 $\Delta algU$ containing an <i>attB</i> site single copy of <i>algZ</i> -S-Tag gene with 1,212 bp of upstream promoter	This study
PAO1-Z <i>rpoS</i> ::Gm	PAO1 <i>rpoS</i> ::Gm containing an <i>attB</i> site single copy of <i>algZ</i> -S-Tag gene with 1,212 bp of upstream promoter	This study
PAO1-Z <i>rpoS</i> ::Gm $\Delta algU$	PAO1 $\Delta algU$ <i>rpoS</i> ::Gm containing an <i>attB</i> site single copy of <i>algZ</i> -S-Tag gene with 1,212 bp of upstream promoter	This study
PAO1-Z Δvfr	PAO1 Δvfr containing an <i>attB</i> site single copy of <i>algZ</i> -S-Tag gene with 1,212 bp of upstream promoter	This study
PAO1-RTF	PAO1 containing 1,077-bp <i>algR</i> :: <i>lacZ</i> promoter fusion inserted at chromosomal <i>attB</i> site	This study
PAO1 <i>vfr</i> RTF	PAO1 Δvfr containing 1,077-bp <i>algR</i> :: <i>lacZ</i> promoter fusion inserted at chromosomal <i>attB</i> site	This study
PAO1 ZTF	PAO1 containing 796-bp <i>algZ</i> :: <i>lacZ</i> promoter fusion inserted at chromosomal <i>attB</i> site	This study
PAO1 <i>vfr</i> ZTF	PAO1 Δvfr containing 796-bp <i>algZ</i> :: <i>lacZ</i> promoter fusion inserted at chromosomal <i>attB</i> site	This study
PAO1-RT2	PAO1 containing 422 bp of the <i>algR</i> RT2:: <i>lacZ</i> promoter fusion inserted at chromosomal <i>attB</i> site	This study
<i>mucA</i> -RT2	PAO1 $\Delta mucA$ containing 422 bp of the <i>algR</i> RT2:: <i>lacZ</i> promoter fusion inserted at chromosomal <i>attB</i> site	This study
<i>algU</i> -RT2	PAO1 $\Delta algU$ containing 422 bp of the <i>algR</i> RT2:: <i>lacZ</i> promoter fusion inserted at chromosomal <i>attB</i> site	This study
PAO1-SDM	PAO1 with 422 bp of the <i>algR</i> RT2:: <i>lacZ</i> promoter fusion inserted at chromosomal <i>attB</i> site; RT2 -35 <i>AlgU</i> binding region mutated from 5'-GAACTG-3' to 5'-TGGAGT-3'	This study
<i>mucA</i> -SDM	PAO1 $\Delta mucA$ with 422 bp of <i>algR</i> RT2:: <i>lacZ</i> promoter fusion inserted at chromosomal <i>attB</i> site; RT2 -35 <i>AlgU</i> binding region mutated from 5'-GAACTG-3' to 5'-TGGAGT-3'	This study
<i>E. coli</i>		
DH5 α	F ⁻ ϕ 80 <i>dlacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>phoA supE44</i> λ ⁻ <i>thi-1 gyrA96 relA1</i>	Invitrogen
Plasmids		
pRK2013	<i>tra</i> ⁺ <i>mob</i> ⁺ functions, Km ^r	33
Mini-CTX <i>lacZ</i>	Self-proficient integration vector with <i>tet</i> , Ω -FRT- <i>attP</i> -MCS, <i>ori</i> , <i>int</i> , and <i>oriT</i>	34
pFLP2	Flp recombinase	35
pVDZ'2R	<i>algR</i> complementation plasmid, tetracycline resistant	
Mini-CTX <i>algZ</i> -S-Tag	Mini-CTX containing 2.2-kb BamHI/EcoRV fragment of <i>algZ</i> and its promoter fused to carboxy-terminal S-Tag	
pCTX- <i>lacZ</i> RTF	Mini-CTX <i>lacZ</i> , containing 1,077 bp of the <i>algR</i> promoter, including RT1 and RT2	This study
pCTX- <i>lacZ</i> ZTF	Mini-CTX <i>lacZ</i> , containing 796 bp of the <i>algZ</i> promoter, including ZT1	This study
pCTX- <i>lacZ</i> RT1	Mini-CTX <i>lacZ</i> , containing 395 bp upstream of the <i>algR</i> promoter RT1	This study
pCTX- <i>lacZ</i> RT2	Mini-CTX <i>lacZ</i> , containing 422 bp upstream of the <i>algR</i> RT2 promoter	This study
-136	pVDX18 <i>algR</i> :: <i>xylE</i> , 110-bp fragment, -27 to -136 (RT1)	This study
-423	pVDX18 <i>algR</i> :: <i>xylE</i> , 397-bp fragment, -27 to -423 (RT1-RT2)	This study
-802	pVDX18 <i>algR</i> :: <i>xylE</i> , 776-bp fragment, -27 to -802 (RT1-RT2)	This study
-1428	pVDX18 <i>algR</i> :: <i>xylE</i> , 1,402-bp fragment, -27 to -1428 (RT1-ZT1)	This study
-1877	pVDX18 <i>algR</i> :: <i>xylE</i> , 1,851-bp fragment, -27 to -1877 (RT1-ZT1)	This study
pVDX18	IncQ, Amp ^r (CbR), <i>mob</i> ⁺ <i>xylE</i> ⁺	36
pRK2013	<i>tra</i> ⁺ <i>mob</i> ⁺ functions, Km ^r	33

tagenic oligonucleotides AlgUSDM2F and AlgUSDM2R (see Table S1). PCR products were cloned into pCR2.1 and then subcloned into miniCTX*lacZ* using the restriction enzymes NotI/HindIII (NEB). Fusion constructs were introduced into *P. aeruginosa* strains by triparental filter conjugation (39). Strains were selected for tetracycline resistance and then conjugated with pFLP2 (35). Strains were selected for carbenicillin resis-

tance, grown overnight without selection, and plated on PIA supplemented with 10% sucrose. To confirm the presence of the fusion constructs, PCR was performed using the forward oligonucleotide used to construct the fusion and the reverse oligonucleotide, lacZR-for-TF (see Table S1). Sonic cell extracts were prepared from strains grown on PIA or LB plates. Total protein was determined by the method of Bradford (Bio-

Rad). The kinetic readings at OD₄₂₀ were measured using a spectrophotometer in cuvettes with a 1-cm light path. The values were calculated based on the following formula: units/mg = (Abs/min)/(ε · [scr1] · P), where Abs/min is the change in OD₄₂₀ units per minute, ε is the extinction coefficient taken as 4,500 M⁻¹cm⁻¹ at OD₄₂₀, [scr1] is the light path, and P is the protein amount (0.01 mg) (40). The activity was expressed as mU/mg, where 1 U was defined as the enzymatic activity of β-galactosidase to convert 1 μmol *o*-nitrophenyl- β -D-galactopyranoside (ONPG) to *o*-nitrophenol per minute. Each strain was tested with at least three biological replicates.

Five *algR::xylE* promoter deletion fragments were generated by PCR using *algRecoR* as the 3' primer. The following are the 5' oligonucleotides used to construct the five fusions (see Table S1 in the supplemental material for sequences): *algRXbaF1*, *algRXbaF2*, *algRXbaF4*, *algZHindF5*, and *algZko1*. PCR products were cloned into pCR2.1 and then subcloned into pVDX18. Fusion constructs were introduced into *P. aeruginosa* by triparental filter conjugation (39). Sonic cell extracts were prepared from strains grown on PIA. Total protein was determined by the method of Bradford (Bio-Rad). Catechol 2,3-dioxygenase (CDO) activity was determined as previously described (15). The appropriate strain (PAO1 or PDO300) harboring the empty vector, pVDX18, was assayed and subtracted from the *algR::xylE* constructs.

Western blot analysis of AlgR and S-tagged AlgZ. *P. aeruginosa* strains were grown in liquid LB at 37°C for the times indicated in the figure legends. The bacteria were collected by centrifugation, resuspended in sterile phosphate-buffered saline (PBS), and lysed by sonication. Total protein concentrations were quantified by the Bradford protein assay (Bio-Rad). Ten micrograms of protein from cell extracts was separated by SDS-PAGE on 12% polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Bio-Rad), and the membranes were blocked overnight in TBST containing 5% skim milk and 1% bovine serum albumin (BSA).

For AlgR Western blotting, the membranes were probed using a 1:2,500 dilution of preabsorbed anti-AlgR rabbit polyclonal antibody followed by a 1:30,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit antibody (41) and detected using the ECL plus kit (GE Healthcare) and a Chemi-doc XRS system (Bio-Rad).

For the AlgZ-S-tagged Western blotting, the membranes were probed using a 1:5,000 dilution of anti-S-Tag mouse monoclonal antibody (Novagen, EMD Chemicals) followed by a 1:20,000 dilution of horseradish peroxidase-conjugated goat anti-mouse antibody (41) and detected using the Clarity Western ECL substrate kit (Bio-Rad) and a Chemi-doc XRS system (Bio-Rad).

RT-qPCR. For relative real-time quantitative PCR (RT-qPCR) analysis of *algR* and *algZ* expression, total RNA was extracted from cultures using RNeasy spin columns (Qiagen) and treated with DNase I (New England BioLabs). cDNA was generated using Superscript II reverse transcriptase and random primers (Invitrogen Life Science Technologies) according to the manufacturer's instructions under the following conditions: 25°C for 10 min, 37°C for 60 min, 42°C for 60 min, and 70°C for 10 min. Real-time PCRs were conducted in a LightCycler 480 instrument with LightCycler 480 probe master mix (Roche Applied Science). Fragments were amplified using primer/probe sets for *algR* (*algR* RTq-F, *algR* RTq-R, and *algR* probe), *algZ* (*algZ* RTq-F, *algZ* RTq-R, and *algZ* probe), *algZ* upstream (*algZ* Up RTq-F, *algZ* Up RTq-R, and *AlgZ* Up probe) and compared to the amplification of *rpoD* (*rpoD* RTq-F, *rpoD* RTq-R, and *rpoD* probe). Data were analyzed using LightCycler 480 software, release 1.5.0 (Roche Applied Science). Relative expression was normalized to *rpoD* cDNA levels as the reference gene in each sample.

Relative real-time quantitative PCR analysis also was performed using SYBR green incorporation for quantification of *fimU*, *hemC*, and *hemY* expression in PAO1 and the PAO1 Δ *algZ* mutant. RNA isolation was performed from stationary-phase cells grown to an OD₆₀₀ of 2.5 by using RNeasy plus (Qiagen), and then cDNA was synthesized with Superscript III (Invitrogen) using the recommended protocols. Real-time PCR was

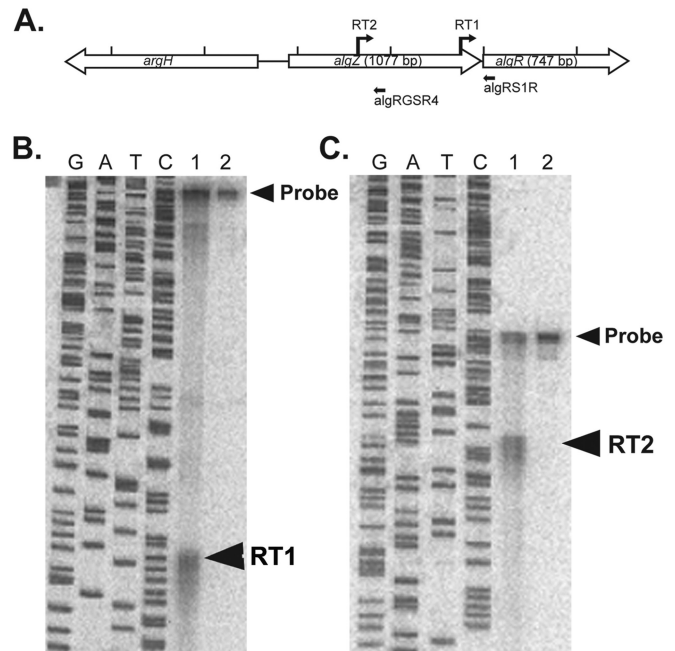


FIG 1 Two transcriptional start sites are located directly upstream of *algR*. (A) Diagram showing the relative location and orientation of *argH* (PA5263), *algZ* (PA5262), and *algR* (PA5261). The transcriptional start sites for *algR* identified previously (RT1) and in this paper (RT2) are indicated. Black arrows indicate the location of probes utilized for S1 nuclease protection assays and sequencing reactions. Each tick represents 500 bp. (B) S1 nuclease protection assay of *algR* transcriptional start site RT1 from nonmucoid PAO1. The four lanes on the left show sequencing reactions with corresponding nucleotides. Lane 1, hybridized probe from PAO1 cDNA; lane 2, S1 probe alone. The upper arrowhead indicates undigested probe, and the lower arrowhead indicates the transcriptional start site. (C) Identification of *algR* transcriptional start site RT2. Lanes: 1, protected probe; 2, probe alone.

performed using a Bio-Rad CFXConnect real-time system with primer pairs shown in Table S1 in the supplemental material and Power SYBR green PCR master mix (Life Technologies). Data were analyzed using CFX Manager 3.1 (Bio-Rad). Results are from three independent experiments performed in triplicate. All samples were normalized to the expression of the reference gene *rpoD* via the Pfaffl method (40).

RESULTS

Two transcriptional start sites control *algR* gene expression.

Previous studies demonstrated that *algR* transcription was activated by the AlgU sigma factor in phenotypically mucoid *P. aeruginosa*. An AlgU-dependent transcriptional start site for the *algR* gene was identified 73 bp upstream of the *algR* open reading frame in mucoid strain PAO568 and a *muca22* cystic fibrosis clinical isolate, FRD1 (4, 10, 12–15). However, *algR* transcription has not been examined in a wild-type nonmucoid strain, such as PAO1. Therefore, S1 nuclease protection assays were performed using two different oligonucleotides (Fig. 1A), which confirmed the previously identified proximal start site, RT1 (4, 12) (Fig. 1B). In addition, there was evidence that *algR* is transcribed from at least one other transcriptional start (10, 12, 14). S1 nuclease protection assays were performed to identify these other transcriptional start sites. A band of protection was identified at 698 bp upstream of the *algR* open reading frame (RT2), located within the *algZ* gene from cells grown to stationary phase (OD₆₀₀ of 1.5) in LB-Miller medium (Fig. 1C, lane 1). S1 nuclease protection

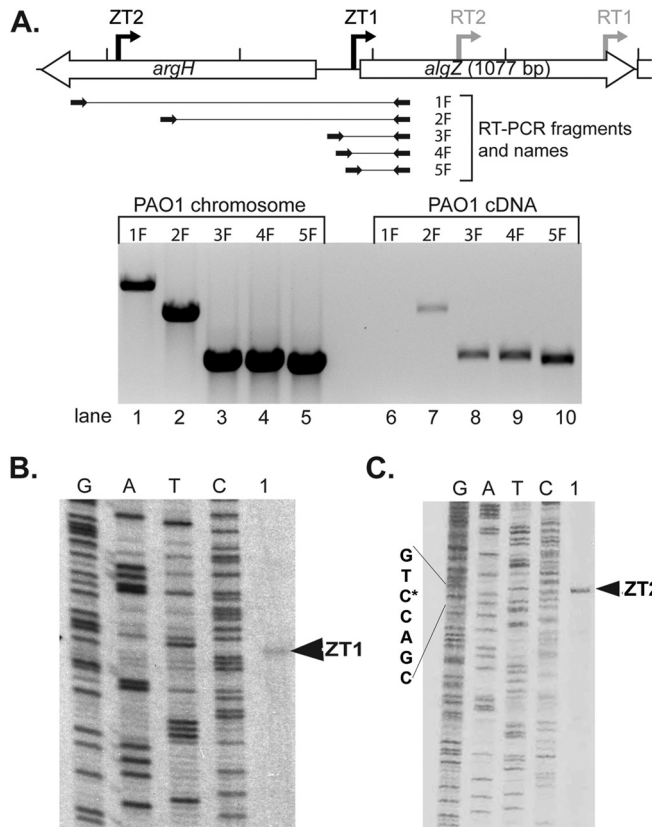


FIG 2 Two transcriptional start sites are located upstream of *algZ*. (A) Reverse transcription-PCR to elucidate the transcriptional start site(s) for *algZ*. (Top) Diagram showing the relative locations of *algZ* transcriptional start sites identified in this paper (ZT1 and ZT2), as well as the primers (black arrow) and amplicons (solid line) utilized for reverse transcription reactions. (Bottom) Representative agarose gel with amplification of promoter regions from PAO1 genomic template control (lanes 1 to 5) or cDNA generated from PAO1 (lanes 6 to 10). Amplified products 1F (lanes 1 and 6), 2F (lanes 2 and 7), 3F (lanes 3 and 8), 4F (lanes 4 and 9), and 5F (lanes 5 and 10) correspond to those in the top diagram, utilizing the algRGS6 oligonucleotide in every reaction and the following oligonucleotides for specific reactions: 1F, algZ-1212FXbaI; 2F, algZpromoter-766-F; 3F, algZpromoter-184-F; 4F, algZpromoter-167-F; and 5F, algZpromoter-126-F. (B) Primer extension using primer algZ Up RTq-R to identify the first *algZ* transcriptional start site, ZT1. The four lanes on the left show sequencing reactions with corresponding nucleotides. Lane 1, hybridized probe from PAO1 cDNA. (C) Primer extension using primer ZT2-875R to identify the second *algZ* transcriptional start site, ZT2.

assays indicated that the *algR* gene has two transcriptional start sites located 73 and 698 bp upstream from its translational start site (see Fig. S1 in the supplemental material).

The *algZ* gene has two transcriptional start sites. The location of the *algZ* transcriptional start sites were identified by RT-PCR analysis. An anchoring 3' oligonucleotide (algRGS6) within the *algZ* coding region was used with various 5' oligonucleotides upstream of *algZ* (Fig. 2A). PAO1 chromosomal DNA (Fig. 2A, lanes 1 to 5) and no-RT (data not shown) control templates were utilized as positive and negative controls, respectively. RT-PCR results showed that an *algZ* transcript is present at least 766 bp (Fig. 2A, lane 7) but less than 1.2 kb (Fig. 2A, lane 6) upstream of the *algZ* open reading frame.

Primer extension analyses were performed to define the location of the *algZ* start site using cDNA from 10-h PAO1 broth

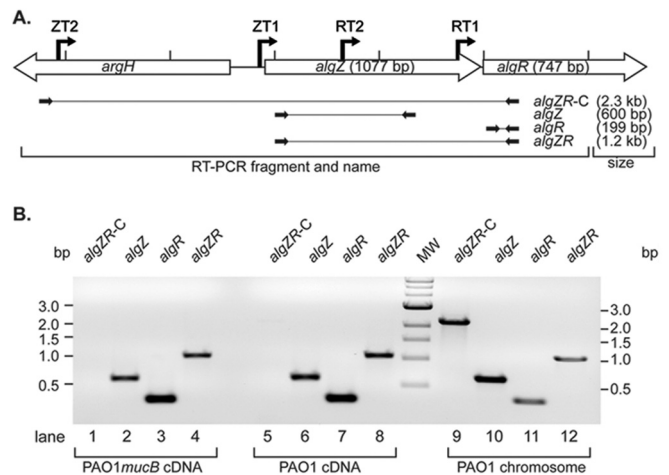


FIG 3 *algR* and *algZ* genes are cotranscribed in both the nonmucoid and mucoid backgrounds. (A) Diagram showing the relative location and orientation of *argH* (PA5263), *algZ* (PA5262), and *algR* (PA5261) with the locations of the transcriptional start sites (bent arrows) ZT2, ZT1, RT2, and RT1. Primers (black arrows) and amplicons (solid lines) utilized for reverse transcriptase reactions are indicated. (B) Representative agarose gel with amplification of cDNA generated from the PAO1 *mucB* strain (lanes 1 to 4), PAO1 (lanes 5 to 8), or PAO1 genomic template control (lanes 9 to 12). The *algZ* and *algR* products correspond to the expression of the respective genes; *algZR* amplifies both in a single transcript, and *algZR-C* controls for chromosomal contamination.

cultures. A start site was identified 23 bp upstream of the *algZ* open reading frame (Fig. 2B) and named ZT1. Because the RT-PCR results suggested a larger transcript existed that contained at least 766 bp upstream of *algZ* (Fig. 2A; also see Fig. S2 in the supplemental material), primer extension also was performed using a primer at bp -875 . The second transcriptional start site was identified 1,044 bp upstream of the *algZ* open reading frame (Fig. 2C; also see Fig. S2) and named ZT2.

The *algR* gene is cotranscribed with *algZ* in the nonmucoid and mucoid backgrounds. The band of full protection that runs with the probe alone in the S1 nuclease protection assay shown in Fig. 1C, and the close proximity of the two genes (separated by 4 bp), led to the hypothesis that *algZ* and *algR* are cotranscribed. Deletion of *algZ* in a *mucA22* background increased alginate production (27); therefore, RT-PCR analysis was performed to determine if *algR* expression is similar in a different genotype (*mucB*) that is also phenotypically mucoid and in wild-type strains. Oligonucleotides were designed for RT-PCR to amplify *algZ*, *algR*, *algZR*, and a region beyond the *algZ* ZT2 promoter (Fig. 3A). RNA isolated from both PAO1 *mucB* (Fig. 3B, lanes 1 to 4) and wild-type PAO1 (Fig. 3B, lanes 5 to 8) strains were converted to cDNA and compared to a PAO1 chromosomal control (Fig. 3B, lanes 9 to 12). Primer pairs were designed to amplify either the *algZ* (amplicon termed *algZ*) (Fig. 3B, lanes 2, 6, and 10) or *algR* (amplicon termed *algR*) (Fig. 3B, lanes 3, 7, and 11) gene alone, the 5' end of *algZ* into the coding region of *algR* (amplicon termed *algZR*) (Fig. 3B, lanes 4, 8, and 12), and a position well upstream of *algZ* ZT2 into the coding region of *algR* as a control for genomic DNA contamination (amplicon termed *algZR-C*) (Fig. 3B, lanes 1, 5, and 9). Amplification indicated that both *algZ* and *algR* are expressed and that they also are expressed as a cotranscript in both the *mucB* and wild-type backgrounds.

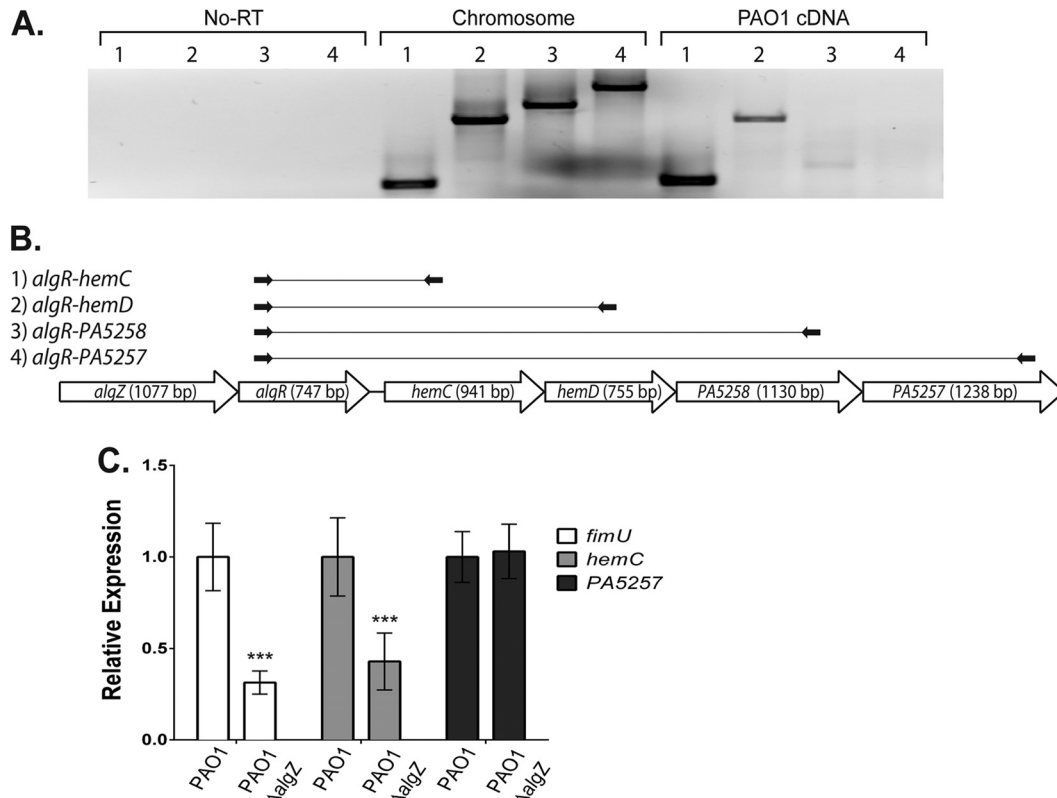


FIG 4 *algZR* and *hemCD* genes are cotranscribed in PAO1. (A) Amplification of *algR* and progressively further downstream genes *hemC*, *hemD*, *PA5258*, and *PA5257* from the no-RT control, PAO1 chromosomal DNA, and cDNA resolved in an agarose gel. (B) Diagram showing the locations of oligonucleotide sets used in panels A (oligonucleotide sets 1 to 4) and C (oligonucleotide sets 5 to 9), which use the following oligonucleotides: set 1, *algR*GSF7 and *hemC*NR; set 2, *algR*GSF7 and *hemD*-R2; set 3, *algR*GSF7 and *PA5258*-R2; set 4, *algR*GSF7 and *PA5257*-R2. Arrows indicate primers, and solid lines indicate amplicons. (C) RT-qPCR of *fimU*, *hemC*, and *PA5257* in the PAO1 and PAO1 Δ *algZ* mutant backgrounds normalized to *rpoD* expression.

The *algZR* genes are cotranscribed with *hemCD*. Previous characterization of an *algR* insertion mutant (CDM5/2) identified the location of the *hemC* and *hemD* genes directly downstream of *algR* (42). Additionally, this study showed that *algR* transcription was linked to these downstream genes in mucoid strains (the PAO568 *mucA22* mutant and a CF clinical isolate) but not a nonmucoid clinical derivative (42). These results indicated that *algR* is cotranscribed with heme biosynthetic genes in a mucoid but not a nonmucoid background. This discrepancy between mucoid and nonmucoid strains led to further investigation of whether the *hemCD* genes are cotranscribed with *algZR*. To test this possibility in PAO1, RNA from stationary-phase cells was converted to cDNA and examined by RT-PCR (Fig. 4). Initially, the transcription from *algR* to each downstream gene was examined (Fig. 4A and B, amplicon schematic 1 to 4). Figure 4A, lanes 1 and 2, show PCR products generated from PAO1 cDNA, indicating that an mRNA transcript from *algR* through *hemD* is expressed at this stage of growth (OD_{600} of 2.0; early stationary phase). However, Fig. 4A, lanes 3 and 4, do not show clear products generated from PAO1 cDNA, suggesting that this cotranscription does not extend into the downstream genes *PA5258* (*hemX*) and *PA5257* (*hemY*). The separation of expression between *hemCD* and *PA5258* (*hemX*)-*PA5257* (*hemY*) has been shown previously in a PA14 RNA sequencing (RNA-seq) experiment (43).

From our *algR* transcriptional mapping results (Fig. 1), we now know that the *algR* RT2 promoter was deleted when the PAO1

Δ *algZ* strain (PAZ) was created. In a comparison of PAO1 to the PAO1 Δ *algZ* mutant using RT-qPCR, a clear reduction in *fimU* expression was observed, which was expected, because *AlgZR* is known to regulate the expression of the *fimU* operon (Fig. 4C) (20, 44). These experiments also demonstrated that the PAO1 Δ *algZ* strain retained 40% of the *hemC* transcript found in PAO1, whereas the *PA5257* (*hemY*) transcript was unaltered (Fig. 4C). Taken together, *algR* and *hemC* transcription are linked according to both qualitative and quantitative assessment measures.

Expression of *algR* increases throughout growth. A previous report comparing the transcriptomes of PAO1 and a PAO1 *rpoS*::*aacC1* mutant (via microarray) showed a 2.5-fold reduction in *algR* expression in the mutant strain, suggesting that *algR* gene expression is regulated by *RpoS* in a nonmucoid background (45). This is consistent with a previous study that showed *rpoS* deletion abrogated mucoidy in FRD1 (31). To investigate the possible involvement of *RpoS* in *AlgR* levels, the expression of *algR* was monitored through the growth curve (Fig. 5). Transcriptional fusion data, utilizing a single-copy chromosomal *algR*::*lacZ* fusion, indicated a large increase in *algR* transcriptional activity from 6 to 16 h of growth and then a subsequently slight increase in expression from 16 to 24 h (Fig. 5A). A more rigorous examination of *AlgR* protein levels over 16 h showed a corresponding increase in *AlgR* levels and the CFU/ml of the culture (in Fig. 5B, bars indicate *AlgR* and the line depicts CFU/ml). When this was evaluated by Western blotting using anti-*AlgR* antibody and com-

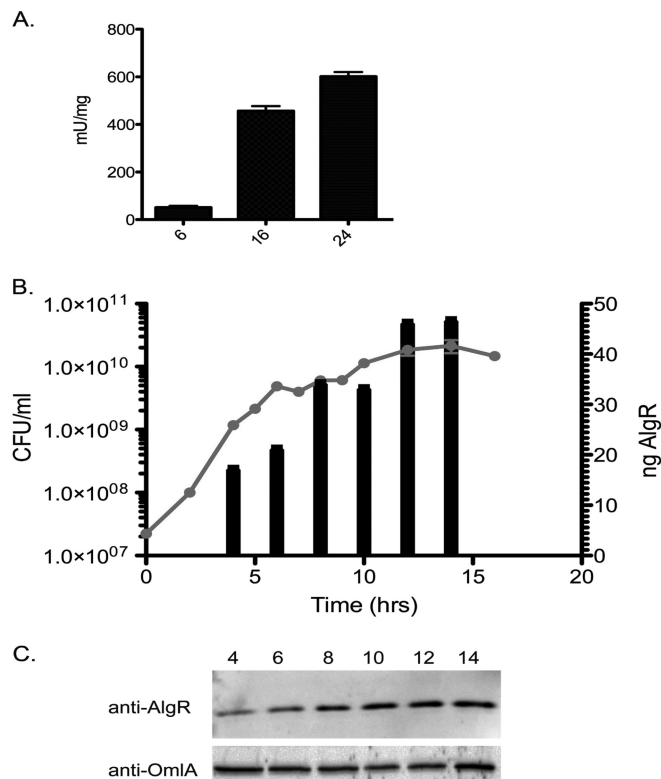


FIG 5 AlgR expression increased throughout the growth phase. (A) β -Galactosidase activity of an *algR::lacZ* fusion chromosomal transcription in PAO1 was measured over time. The fusion contains *algR* promoters RT1 and RT2 and was assayed in triplicate. (B) AlgR concentrations in PAO1 were quantified by comparison to purified AlgR over time by Western blotting. The left axis depicts CFU per milliliter (line) of culture. The right axis depicts concentration of AlgR (bars; values are ng AlgR). (C) Western blot of LB-grown PAO1 extracts to detect AlgR and OmlA (loading control) at 4, 6, 8, 10, 12, and 14 h. Anti-AlgR, rabbit polyclonal antibody used to detect AlgR; anti-OmlA, rabbit polyclonal antibody used to detect OmlA.

pared to OmlA levels as a loading control, there is a clear increase in the amount of AlgR over time into stationary phase (Fig. 5C).

The RT1 and RT2 *algR* promoters are AlgU dependent. Previous studies identified RT1 as the main AlgU-dependent *algR* promoter in a mucoid background (12). In light of the newly identified transcriptional start site, RT2, and the detection of an *algZ*-*algR* cotranscript, *algR* transcription was examined by transcriptional reporters with various fragments of the *algZR* promoter analyzed in PAO1 or mucoid PAO1 *mucA22* (strain PDO300) backgrounds. As shown in Fig. 6A, various 5' primers and a common 3' anchor were utilized to construct a set of *xylE* reporter fusions containing the promoter regions for RT1 (−136 and −423), RT1 and RT2 (−802), or RT1, RT2, and ZT1 (−1428 and −1877) (Fig. 6A). In a nonmucoid background, a fusion containing both *algR* promoters (RT2 and RT1) resulted in a higher level of *xylE* reporter activity than that of a fusion containing only RT1 (Fig. 6B, −802, −423, and −136). Additionally, a 2-fold increase in transcriptional activity was observed when an *algZ* promoter (ZT1) was included (Fig. 6B, −1428 versus −802). These results demonstrate that ZT1 may contribute to an *algZ*-*algR* cotranscript and enhance *algR* expression in the nonmucoid background.

In the mucoid PDO300 strain (PAO1 containing a *mucA22* allele; thus, increased AlgU activity), *algR* expression from the RT1 promoter was higher than that of wild-type PAO1 (Fig. 6B, −136 and −423). There was also a 2-fold increase in transcriptional activity when RT2 was included in the reporter (Fig. 6B, −423 versus −802), indicating that the RT1 and RT2 promoters are required for maximal transcriptional activity. In addition, the alignment of RT1 and RT2 promoters with the AlgU-binding consensus sequence (13) shows an identifiable AlgU-binding site in both RT1 and RT2 (Fig. 6C; also see Fig. S1 in the supplemental material), an appropriate distance from the transcriptional start site. These results suggest that AlgU activates both RT1 and RT2 in *mucA* strains and are in agreement with the presently accepted model that AlgU activates *algR* expression (4, 46). Unlike in PAO1, the addition of ZT1 in the reporter (Fig. 6B, −802 versus −1428) in a *mucA22* background failed to significantly increase transcriptional activity. Although a cotranscription product was detected in the *mucB* background (Fig. 3B, lanes 1 to 4), these transcriptional fusion data suggest that cotranscription is less relevant in a *mucA22* background.

To confirm that AlgU activates RT1 and RT2 in a *mucA* background, RT-qPCR experiments were performed using three primer sets. The following primer set labels were used: (i) *algZ* upstream measured the contribution of ZT2 and ZT1, (ii) *algZ* measured the contribution of ZT2, ZT1, and RT2, and (iii) *algR* measured the contribution of all four promoters (Fig. 6D). The relative abundances of transcripts of the PAO1 Δ *mucA* strain was compared to those of PAO1. The primer sets termed *algZ* and *algR* showed an approximately 2-fold increase in transcript levels in the Δ *mucA* strain compared to those of the wild type, which would be expected if the *algR* promoters are regulated by AlgU. The relative amount of *algZ* upstream transcript was lower in the Δ *mucA* strain than in the wild type, but this decrease was not statistically significant. Because the *algZ* upstream primer set only measured the contributions of ZT2 and ZT1 and both the *algZ* and *algR* primer sets include *algR* promoters, the increased *algR* transcript in the Δ *mucA* background was due primarily to the contributions of both RT2 and RT1.

Transcription of *algR* in the nonmucoid background is directly AlgU dependent and additionally regulated by RpoS. To further investigate the possibility of RpoS involvement and confirm a role for AlgU in *algR* expression, RT-qPCR was performed using three primer sets (as described for Fig. 6D) to evaluate *algZR* expression in (i) the PAO1 wild type and the (ii) PAO1 Δ *algR*, (iii) PAO1 *rpoS::Gm*, (iv) PAO1 Δ *algU*, and (v) PAO1 *rpoS::Gm* Δ *algU* mutants (Fig. 7). Comparison of the transcript levels in the PAO1 *rpoS::Gm* strain to those in wild-type PAO1 revealed that transcript levels downstream of RT2 (primer set *algZ*) were reduced to 52%, while transcript levels downstream of RT1 (primer set *algR*) were reduced to 64% relative to the wild type (Fig. 7A). On the other hand, there were no differences in transcript levels downstream of ZT1 and ZT2 (primer set *algZ* upstream) for all strains tested. Together, these results suggest that RpoS affects RT1 and RT2 transcription either through direct regulation or indirectly through AlgU but does not affect the transcription from the ZT1 and ZT2 promoters.

In the Δ *algU* background, transcript levels from RT2 also were reduced to 28% of the wild type (primer set *algZ*), supporting the findings shown in Fig. 6D. Interestingly, *algR* transcript levels downstream of both RT2 and RT1 (primer set *algZ* and *algR*) were

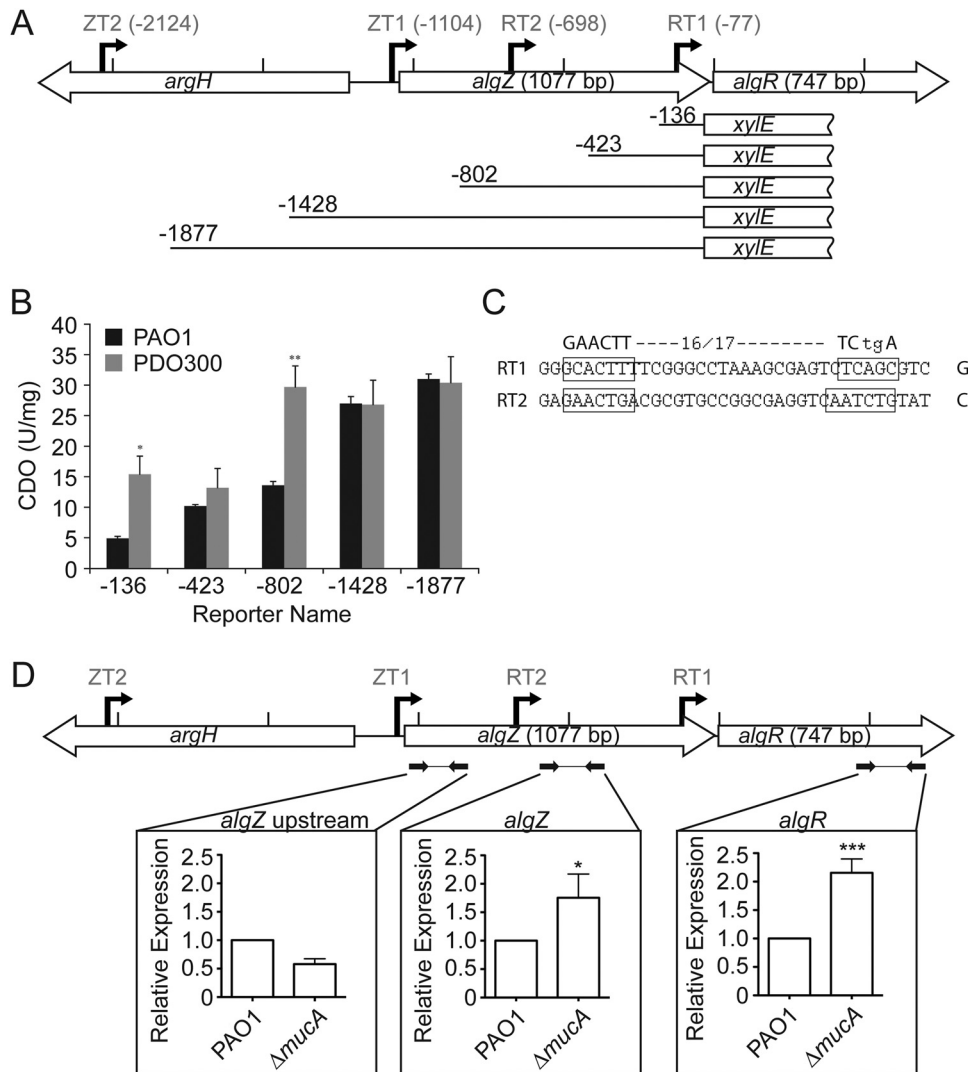


FIG 6 *algR* promoters RT1 and RT2 are AlgU dependent. (A) Diagram showing the relative location and orientation of *argH* (PA5263), *algZ* (PA5262), and *algR* (PA5261) with the locations of promoter fragments fused to the *xylE* gene. (B) Expression of XylE from the promoter fragments measured in CDO (U/mg) in both PAO1 and the mucoid strain PDO300. (C) Proposed AlgU binding sites upstream of RT1 and RT2. (D) RT-qPCR performed with cDNA generated from both PAO1 and the PAO1 Δ *mucA* mutant. Three regions were tested in each background, which included different promoters, including *algZ* upstream (ZT2 and ZT1), *algZ* (ZT2, ZT1, and RT2), and *algR* (ZT2, ZT1, RT2, and RT1). Data were analyzed by analysis of variance (ANOVA). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

further reduced in the *rpoS*::Gm Δ *algU* double mutant compared to the wild type (Fig. 7A). AlgR protein levels then were examined in (i) PAO1 and the mutant (ii) PAO1 Δ *algR*, (iii) PAO1 *rpoS*::Gm, (iv) PAO1 Δ *algU*, and (v) PAO1 *rpoS*::Gm Δ *algU* strains (Fig. 7B) to support the RT-qPCR data. In agreement with such data, AlgR protein levels were noticeably reduced in the *rpoS*::Gm Δ *algU* background compared to those in wild-type PAO1 at an OD₆₀₀ of 2.1 (Fig. 7B) and OD₆₀₀ of 2.5 (data not shown). These results indicate that both AlgU and RpoS are required for maximal expression of AlgR in the nonmucoid background. As transcript levels from the ZT1 and ZT2 promoters (primer set *algZ* upstream) were not reduced in the backgrounds examined by RT-qPCR, Western blotting additionally was performed using a chromosomal single-copy *AlgZ* expressing a C-terminal S-Tag and a monoclonal anti-S-Tag antibody to confirm expression levels. The AlgZ protein levels were unaffected in these backgrounds,

confirming the RT-qPCR results that transcription of *algZ* is neither RpoS nor AlgU dependent (Fig. 7B). To determine if AlgU controlled transcription from the RT2 promoter, a 413-bp transcriptional fusion containing only RT2 was assayed in PAO1 and the PAO1 *mucA22* strain. As indicated in Fig. 7C, there was a 2.6-fold increase in β -galactosidase activity in the *mucA22* background compared to PAO1 for this construct. When this fusion was tested in the PAO1 Δ *algU* strain, there was an 8-fold decrease in β -galactosidase activity compared to that of wild-type PAO1. Site-directed mutagenesis was performed on the -35 region of the RT2 transcriptional fusion to mutate the AlgU -35 binding site (from 5'-GAAGT-3' to 5'-ACTCCA-3'; see Fig. S1 in the supplemental material) and assayed in various strains. This site-directed AlgU -35 binding site mutant exhibited significantly decrease β -galactosidase activity compared to both the PAO1 (8-fold) and the *mucA22* mutant (15-fold) backgrounds. Together

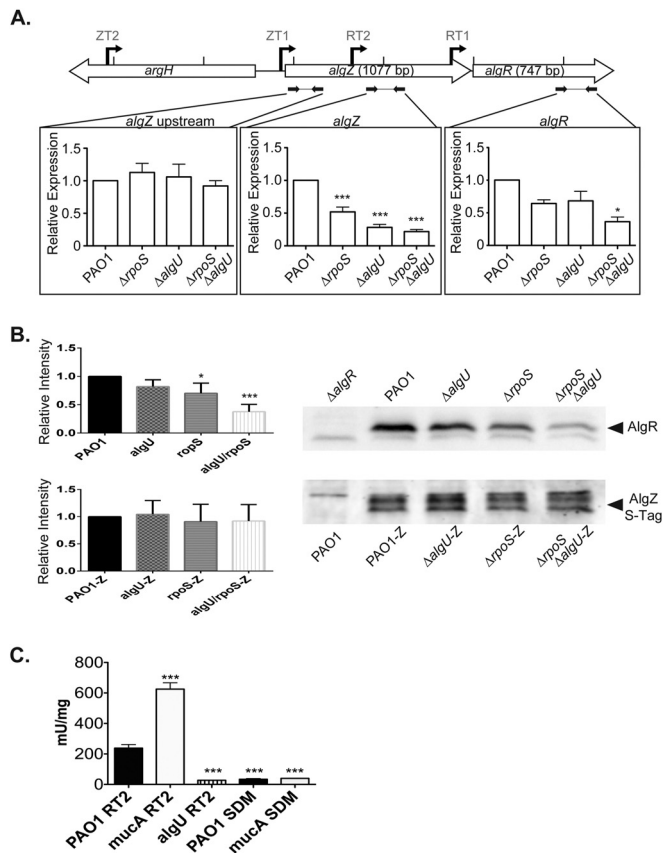


FIG 7 Transcription dependence of *algR* on both *rpoS* and *algU* in the non-mucoid background. (A) RT-qPCR performed with cDNA generated from PAO1 and the mutant strains *rpoS::Gm*, $\Delta algU$, and *rpoS::Gm \Delta algU* in the PAO1 background. Three regions were tested in each background, which included different start sites for different genes, *algZ* upstream (ZT2 and ZT1), *algZ* (ZT2, ZT1, and RT2), and *algR* (ZT2, ZT1, RT2, and RT1). ns, not statistically significant compared to PAO1. (B) Densitometry and representative Western blotting for expression of AlgR in the tested backgrounds, as well as the PAO1 $\Delta algR$ mutant, and for the expression of AlgZ in the tested backgrounds containing a single copy of *algZ* with a fused S-Tag and a PAO1 negative control. Densitometry was measured as fluorescent intensity relative to PAO1 and was averaged from four blots. (C) β -Galactosidase activity of an *algR::lacZ* fusion chromosomal transcription in PAO1 was measured over time. The RT2 fusion contains the *algR* RT2 promoter on the chromosomes of the PAO1 (PAO1 RT2), PAO1 $\Delta mucA$ (*mucA* RT2), and PAO1 $\Delta algU$ (*algU* RT2) strains. The -35 region of RT2 was mutated from 5'-GAAGCTG-3' to 5'-ACTCCA-3' in the RT2 fusion, termed SDM, and conjugated into PAO1 (PAO1 SDM, and the PAO1 $\Delta mucA$ mutant (*mucA* SDM). All of the constructs were assayed in triplicate. Data were analyzed by ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

with the data from Fig. 6, these data indicate that AlgU controls the expression from the RT2 *algR* promoter located within the *algZ* coding region.

Expression of *algZ* is *vfr* dependent. While the expression of *algZ* was unaffected in the tested sigma factor backgrounds shown in Fig. 7, previous reports suggested that there is a transcriptional link between Vfr and *algZR*, where Kanack et al. identified a putative Vfr binding site upstream of *algZ* ZT1 (see Fig. S2, boxed sequence, in the supplemental material). It also has been demonstrated that elevated levels of AlgR repress *vfr* transcription, resulting in the downregulation of the Vfr-dependent virulence factor regulon (17, 47, 48). To determine whether Vfr regulates *algZ*

transcription, transcriptional reporters utilizing either the first *algZ* promoter (ZT1) or both *algR* promoters (RT1 and RT2) were constructed and integrated in single copy into wild-type PAO1 or Δvfr mutant backgrounds (Table 2). Reporter activity from the *algR* promoter was unaffected in the Δvfr mutant, indicating that Vfr is not required for its expression alone. On the other hand, reporter activity from the *algZ* ZT1 promoter was significantly reduced in the Δvfr background to undetectable levels compared to the activity of wild-type PAO1. This indicates that while Vfr has no direct regulatory role on RT1 and RT2, the *algZ* ZT1 promoter is substantially, if not completely, dependent upon Vfr for expression, which is consistent with the previously identified Vfr binding site upstream of *algZ* ZT1 (17).

Western blotting and RT-qPCR were performed to examine AlgR, AlgZ, and AlgZR expression levels in PAO1 and PAO1 Δvfr strains. Western blot analysis using anti-S-Tag antibody directed to AlgZ expressing a C-terminal S-Tag and anti-AlgR polyclonal sera against cell extracts from PAO1 and PAO1 Δvfr strains showed decreased AlgZ and AlgR levels in the Δvfr mutant background (Fig. 8A and B). Confirmatory RT-qPCR performed on both PAO1 and PAO1 Δvfr strains indicated that transcript levels from ZT1 and ZT2 (*algZ* upstream) were reduced 62%, and transcript levels from ZT1, ZT2, and RT2 (*algZ*) were reduced by 49% in the PAO1 Δvfr mutant (Fig. 8C). Collectively, these data indicate that Vfr heavily influences the expression of *algZ*. Nevertheless, *algZ* expression is not totally abolished by the absence of Vfr, suggesting that AlgZ expression from the ZT2 promoter is controlled by a different transcription factor and accounts for approximately 40% of *algZ* transcription at late stationary phase (OD₆₀₀ of 2.5). Diminished *algR* expression likely is due to reduced cotranscription, because the PAO1 Δvfr mutant showed no detectable decrease in *algR* promoter activity (Table 2). Moreover, expression levels measured by RT-qPCR increased when the fragment tested moved from only including ZT1 and ZT2 (*algZ* upstream) to including RT2 (*algZ*) (Fig. 8C).

DISCUSSION

As a pathogen, *P. aeruginosa* is extremely versatile and survives in a wide variety of niches. *P. aeruginosa* is capable of rapidly responding to changes in its environment by the expression of the most appropriate genes and virulence factors for a particular environment. To maintain this responsiveness, layers of regulation are employed, including two-component regulatory systems (18), alternative sigma factors (49), quorum-sensing systems (50, 51), regulatory RNAs (52), and a variety of more complex and specialized regulatory elements (53). The expression of virulence deter-

TABLE 2 Vfr controls *algZ* expression

Strain	Chromosomal fusion ^a	β -Galactosidase activity (mU/mg)
PAO1 RTF	<i>algR::lacZ</i> ^b	499 \pm 51
PAO1 <i>vfr</i> RTF	<i>algR::lacZ</i>	494 \pm 32
PAO1 ZTF	<i>algZ::lacZ</i> ^c	164 \pm 24
PAO1 <i>vfr</i> ZTF	<i>algZ::lacZ</i>	ND ^d

^a Transcriptional fusions. β -Galactosidase activity was recorded as mU/mg of protein and was averaged for three separate experiments. Data are represented as mU/mg \pm standard error.

^b *algR::lacZ* promoter fusion of 1,077 bp inserted at the chromosomal *attB* site.

^c *algZ::lacZ* promoter fusion of 796 bp inserted at the chromosomal *attB* site.

^d ND, not detected.

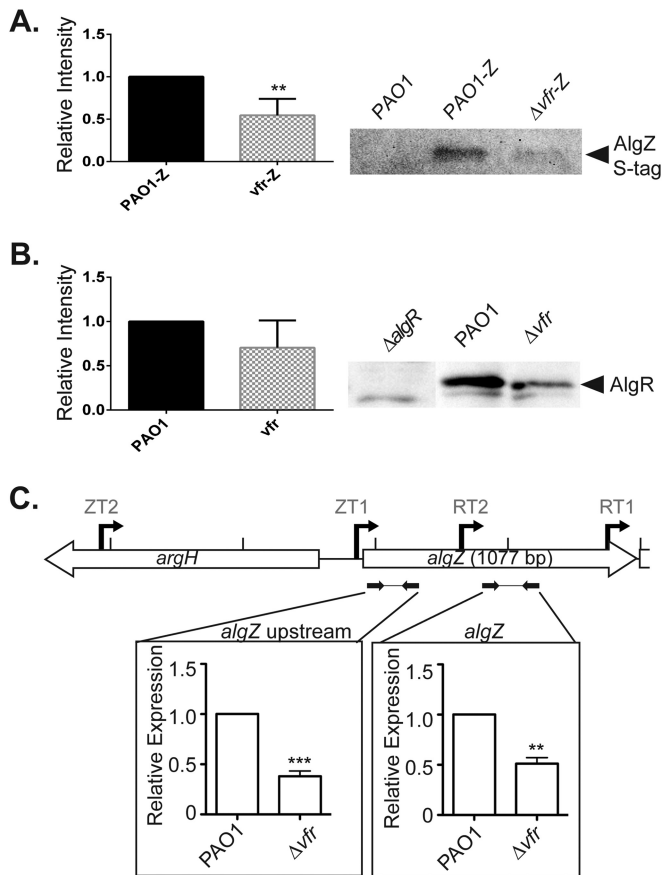


FIG 8 Vfr dependence of *algZR* transcription. (A) Densitometry and representative Western blotting for expression of AlgZ and AlgR in the PAO1 and mutant PAO1 Δ *algR* and PAO1 Δ *vfr* backgrounds and of AlGZ in the PAO1-Z and mutant PAO1 Δ *vfr*-Z backgrounds containing a single copy of *algZ* with a fused S-Tag and a PAO1 negative control. Densitometry was measured as fluorescent intensity relative to that of PAO1 averaged from four blots. (B) RT-qPCR performed with cDNA generated from PAO1 and PAO1 Δ *vfr* strains. Two regions were tested in each background and included different promoters, *algZ* upstream (ZT2 and ZT1) and *algZ* (ZT2, ZT1, and RT2). Data were analyzed by ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

minants is especially tightly controlled to prevent unnecessary expression of metabolically burdening systems, such as the exopolysaccharide alginate (54–57). One of the transcriptional regulators that controls alginate overproduction as well as other virulence factors, such as type IV pili, the type III secretion system, and rhamnolipid and cyanide production, is the response regulator AlgR (58). The proposed histidine kinase for AlgR, AlgZ (FimS), is required for type IV pili (25) function but also modulates alginate and rhamnolipid production by two- to threefold (24, 27). Consequently, determining the transcriptional regulators that control the AlgZR two-component regulatory system provides insights into the functions of many different *P. aeruginosa* virulence determinants.

The *algZR* transcriptional analysis revealed that multiple regulatory layers control this two-component regulatory system (Fig. 9). This study identified four transcriptional start sites, three of which were previously unknown. While an *algR* transcriptional start site dependent upon AlgU was identified previously in the mucoid strain PAO568 and the clinical isolate FRD1 (4, 12), it had

not been examined previously in a wild-type background. There also have been prior indications that *algR* is transcribed from at least one additional transcriptional start site (12, 14). Using primer extensions and S1 nuclease protection assays with the wild-type strain PAO1, the previously identified transcriptional start site (RT1), located at 73 bp upstream from the translational start site, was confirmed, and a newly identified start site was found 698 bp upstream of the *algR* open reading frame within the *algZ* gene (RT2). Previous data suggested a role for RpoS in *algR* expression based on microarray analysis (31). Our data are consistent with this study and showed increased AlgR expression in the stationary phase of growth (Fig. 5). However, when RT-qPCR was performed to examine expression from ZT1 and ZT2 (*algZ* upstream); ZT1, ZT2, and RT2 (*algZ*); and all four promoters (*algR*) in various sigma factor deletion strains, it appears that RpoS affects *algR* expression, either directly by activating RT1 and/or RT2 or indirectly, potentially through AlgU (Fig. 7). Interestingly, it appears that RpoS has no effect on *algZ* expression (Fig. 7A and B). Evidence that expression from RT2 is directly AlgU dependent includes (i) decreased *algR* expression in the PAO1 Δ *algU* mutant (Fig. 7A); (ii) decreased AlgR levels in the PAO1 Δ *algU* mutant (Fig. 7B); (iii) significantly decreased RT2 expression when the promoter was isolated and examined in the PAO1 Δ *algU* mutant (Fig. 7C); (iv) increased RT2 expression in the PAO1 *mutA22* mutant (Fig. 7C); and (v) abrogation of *algR* expression when the AlgU –35 region was mutated in strains tested (Fig. 7C). While control of RT1 by AlgU in a *mutA* background was known previously, the control of both RT1 and RT2 by AlgU in the wild-type PAO1 background is a bit more surprising and shows that this sigma factor may play a larger role in gene regulation in wild-type *P. aeruginosa* than previously appreciated. Interestingly, the *algR* promoter is the second promoter region identified where AlgU controls two different start sites within the same gene, the other promoter being *algU* itself (59). The reason for two independent start sites within the same gene promoters that are controlled by the same sigma factor is unknown. Perhaps there are differences in promoter affinity that respond to increased levels of the sigma factor. However, both of the AlgU-dependent start sites in the *algU* and *algR* promoters were discovered in PAO1, indicating that increased AlgU levels were not required to initiate transcription from both promoters. While the role of AlgU in wild-type *P. aeruginosa* has not been examined as thoroughly as in the mucoid backgrounds, it is known to play a regulatory role, since about 33% of AlgU present in nonmucoid cells is free in the cytoplasm (60, 61). Interestingly, the PAO1 Δ *algU* strain does not display the twitching motility phenotype, which has been explained by the reduced expression of both lectin-encoding genes *lecA* and *lecB*, since LecB is required for type IV pilus biogenesis (62). Our data suggest that the deletion of *algU* would decrease AlgR expression at least 2-fold, which likely would abrogate twitching motility through decreased *fimU* expression. The *algR* deletion strain also is impaired in twitching motility (20, 25).

Little has been reported on *algZ* transcriptional regulation. After identifying the furthest upstream transcriptional end, primer extensions were performed and identified two transcriptional start sites at bp –23 (ZT1) and –1044 (ZT2; numbering relative to the translational start site). Unlike the *algR* expression that was found to be AlgU and RpoS dependent, levels of expression from ZT1, ZT2, and AlgZ were unaffected when these sigma factors were not present. Although *algZ* expression was unaffected in the

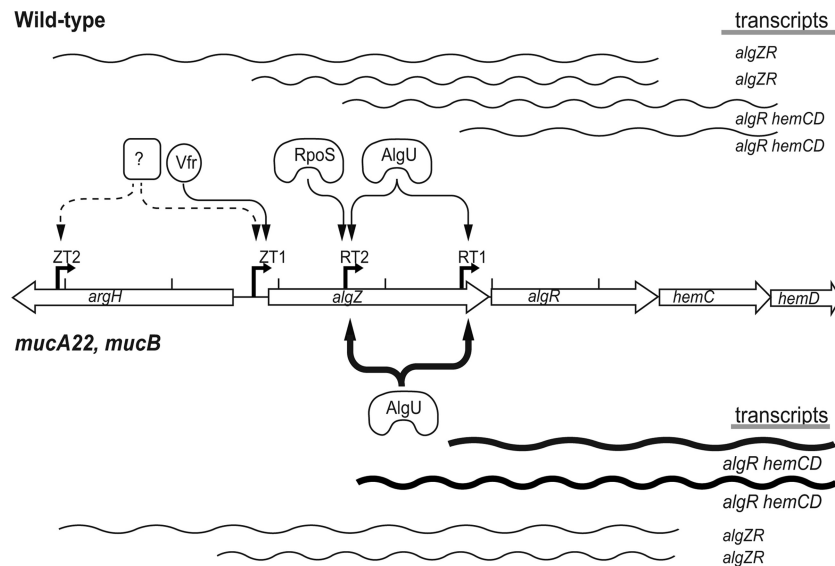


FIG 9 Model of the regulation and expression of the *algZR* system. The diagram shows the relative location and orientation of *argH* (PA5263), *algZ* (PA5262), *algR* (PA5261), *hemC* (PA5260), and *hemD* (PA5259), with the transcriptional start site locations of ZT2, ZT1, RT2, and RT1 indicated by bent arrows. Transcripts and regulatory elements are split horizontally between regulation in wild-type cells and mucoid (*mucA22, mucB*) cells. In wild-type cells, the regulation of RT1 and RT2 by AlgU (Fig. 7A and C), RT2 by RpoS (Fig. 7A), and ZT1 by Vfr (Fig. 8) are indicated by solid lines, with dashed lines indicating additional unknown elements regulating ZT2 and possibly ZT1. Above the regulatory elements are several transcripts produced by cotranscription from several promoters extending into *hemCD* (Fig. 4). In mucoid cells, the regulation of RT1 and RT2 is heavily dependent upon AlgU, as indicated by line thickness, and the regulation of ZT1 and ZT2 is unknown in this background, although *algZ* transcripts are detectable (Fig. 3B). The transcripts of *algR* are shown with thicker lines, as the *mucA* background was shown to have higher levels of *algR* transcripts by RT-qPCR (Fig. 6D).

tested sigma factor deletion backgrounds, further experimentation identified stationary-growth-phase dependence on the transcriptional factor Vfr for AlgZ through ZT1 (Fig. 8). Evidence that ZT1 is Vfr dependent includes (i) decreased *algZ* transcription levels from ZT1 and ZT2 by 62% in the Δvfr mutant background relative to the wild-type PAO1 (Fig. 8C); (ii) reduced AlgZ and AlgR levels according to Western blot analysis (Fig. 8A and B); and (iii) abrogation of transcriptional activity from an isolated ZT1 *algZ* promoter fusion in the PAO1 Δvfr mutant (Table 2). Another indication that Vfr controls AlgZ is the presence of a Vfr binding site (see Fig. S2 in the supplemental material) upstream of ZT1 identified by Kanack et al. (17).

Vfr is a member of the 3',5'-cAMP receptor protein (CRP) family of transcriptional regulators involved in regulating a variety of virulence phenotypes in *P. aeruginosa* (16). These virulence determinants include exotoxin A production, type IV pili, type III secretion, the *las* quorum-sensing system, and flagellar expression (47, 63–66). Interestingly, AlgR controls two of these same phenotypes, specifically type IV pili (20, 25, 44) and the type III secretion system (48, 67), but it does so through different genes than those controlled by Vfr. Interplay between AlgR and Vfr was shown previously when AlgR indirectly suppressed cAMP/Vfr-dependent (CVS) signaling in a *mucA22* background (48). Deletion of *algZR* or *algU* in a *mucA22* mutant restored CVS activity to wild-type levels, as measured through a synthetic *E. coli lac* P1 strain fused to *lacZ*. In support of these data, Vfr levels returned to normal in the same mutants, and plasmid-borne overexpression of *algZR* alone suppressed Vfr levels. Here, we show that AlgR-Vfr regulation is reciprocal in that Vfr directly controls expression from the *algZ* ZT1 promoter and *algZR* expression in a wild-type background. While ZT1 contains an adjacent Vfr binding site, the

ZT2 start site further upstream contains no such consensus sequence. Our RT-qPCR and Western analyses showed reduction but not total loss of *algZ* and AlgZ expression in the Δvfr mutant background. These results indicate that Vfr does not influence expression from ZT2, but it is likely controlled by another transcriptional regulator. Further experiments are necessary to identify such a regulator(s). We have observed *algZ* expression in multiple growth phases and have not observed any conditions that result in a total loss of expression (data not shown). These data suggest that the expression from ZT2 is constitutive, like that through RpoD.

The analysis of the *algZR* transcript indicated that these genes also are expressed as a cotranscript even in the mucoid background, where the AlgU-dependent *algR* promoters seem to dominate. The cotranscription of *algZR* has been reported in a PA14 RNA-seq experiment (43). This same study showed that *hemCD* and PA5258 (*hemX*)-PA5257 (*hemY*) are two separate transcriptional units. The differences between our data and that reported previously may be strain dependent, as our studies were conducted in PAO1 and the RNA-seq data are from PA14. However, the RNA-seq data may be a reflection of the most prevalent transcript identified. The cotranscription of *algR-hemC* previously has been demonstrated in a *mucA22* background, and another transcriptional start site has been mapped between *algR* and *hemC* by others and us, but nothing else was known (21, 42, 43). Our data suggest that *algZR* is at least transcriptionally connected to heme biosynthesis and also plays a role in controlling heme levels in *P. aeruginosa*. Data that support this suggestion include alterations in the regulation of catalase and cytochrome oxidase genes observed in our microarray studies where the PAO1 *algR::Tc^r* mutant was compared to PAO1 (20, 23).

The AlgZR system is known to be involved in regulating a considerable number of virulence factors, so fully elucidating the regulation of this system is vital for fully understanding their physiological role in both virulence and the biology of the organism as a whole. Transcriptional control by at least three distinct regulatory elements, Vfr, RpoS, and AlgU, allows for highly regulated expression based on several environmental conditions. However, several questions remain. (i) How is ZT2 regulated? (ii) What is the function of the large 1-kb noncoding intergenic region between ZT2 and the start of *algZ* translation? (iii) What is the biological significance of tying *algZ* expression to cAMP levels through Vfr? (iv) What is the biological significance of *algZR* and *hemCD* cotranscription?

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