Pseudomonas aeruginosa AlgR Controls Cyanide Production in an AlgZ-Dependent Manner[▽]

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Pseudomonas aeruginosa is an opportunistic pathogen that causes chronic infections in individuals suffering from the genetic disorder cystic fibrosis. In *P. aeruginosa*, the transcriptional regulator AlgR controls a variety of virulence factors, including alginate production, twitching motility, biofilm formation, quorum sensing, and hydrogen cyanide (HCN) production. In this study, the regulation of HCN production was examined. Strains lacking AlgR or the putative AlgR sensor AlgZ produced significantly less HCN than did a nonmucoid isogenic parent. In contrast, *algR* and *algZ* mutants showed increased HCN production in an alginate-producing (mucoid) background. HCN production was optimal in a 5% O_2 environment. In addition, cyanide production was elevated in bacteria grown on an agar surface compared to bacteria grown in planktonic culture. A conserved AlgR phosphorylation site (aspartate at amino acid position 54), which is required for surface-dependent twitching motility but not alginate production, was found to be critical for cyanide production. Nuclease protection mapping of the *hcnA* promoter identified a new transcriptional start site required for HCN production. A subset of clinical isolates that lack this start site produced small amounts of cyanide. Taken together, these data show that the *P. aeruginosa hcnA* promoter contains three transcriptional start sites and that HCN production is regulated by AlgZ and AlgR and is maximal under microaerobic conditions when the organism is surface attached.

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen that can cause acute infections in patients suffering from cancer (6), human immunodeficiency virus/AIDS (27, 51), and severe burns (9). *P. aeruginosa* can also cause chronic infections in patients affected with the genetic disorder cystic fibrosis (CF) (8, 31). Mutation of the gene that encodes the CF transmembrane conductance regulator, a chloride ion channel found in epithelial cells, results in a dehydrated layer of mucus in the airways of the lungs. The impaired mucociliary clearance entraps bacteria and allows for the establishment of a chronic infection (65, 85). Pulmonary colonization by *P. aeruginosa* is the leading cause of morbidity and mortality of CF patients (8).

During chronic CF infection, *P. aeruginosa* strains acquire mutations that cause them to undergo a phenotypic switch from a nonmucoid to a mucoid phenotype. The mucoid phenotype results from the production of copious amounts of the capsular exopolysaccharide alginate (23). The presence of a mucoid strain in the CF lung is associated with a worsening clinical prognosis (22, 24). Production of alginate aids the organism in the evasion of phagocytosis and in resistance to killing by neutrophils and macrophages (11, 64, 74), quenching of oxygen intermediates (46, 76), and antibiotic resistance (1).

Alginate biosynthesis is regulated by multiple factors (34, 69) including the transcriptional regulator AlgR, a response regulator belonging to the bacterial two-component signaling system superfamily (21, 35). AlgR is required for virulence in an acute septicemia model (48) and regulates a variety of other virulence factors, including twitching motility (80, 81), hydrogen cyanide (HCN) production (13), and biofilm formation and quorum sensing (58).

The effects of cyanide on various cell types have been extensively tested, and depending on cell type and level of oxidative stress, cyanide causes either apoptosis or necrosis (66, 67). *P. aeruginosa* was one of the first bacteria shown to undergo cyanogenesis (18). Growing evidence suggests that HCN production may be an important virulence factor. The presence of cyanide in burn wounds was directly associated with *P. aeruginosa* infections (33). More recently, the ability of *P. aeruginosa* to paralyze and kill *Caenorhabditis elegans* in an in vitro model was shown to be dependent on *hcnC* (30). HCN production has also proven to be clinically relevant in the CF lung as (i) *P. aeruginosa* CF isolates produced higher levels of HCN than did laboratory strains (13), (ii) cyanide has been detected in the sputum of *P. aeruginosa*-infected CF and bronchiectasis patients (12, 26, 40, 71), and (iii) detection of HCN in sputum and bronchoalveolar lavage fluid directly correlated with a worsening prognosis (12). The reported HCN concentrations in the sputum of infected patients ranged from 1.6 to 3.25 times the lethal blood concentration (71).

HCN is a secondary metabolite of *P. aeruginosa* produced

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from glycine in a poorly understood oxidative reaction catalyzed by HCN synthase (15, 82). HCN is not produced by *P. aeruginosa* under anaerobic conditions (15) but is expressed under low oxygen tensions (14). Optimal production requires the anaerobic regulator ANR and the quorum sensing regulators LasR and RhlR, which bind the *hcnA* promoter at high cell densities during late exponential growth (62). There are two reported transcriptional start sites for *hcnA* in *P. aeruginosa* (62) and one in *Pseudomonas fluorescens* (45). *P. aeruginosa* T1 is regulated by the *rhl* and *las* quorum sensing systems, while T2 relies on a synergistic effect of LasR, RhlR, and ANR. GacA and RsmA function as posttranscriptional regulators of HCN production through juxtaposition of the *hcnA* ribosomal binding site and indirectly by positively and negatively regulating amounts of *N*-acylhomoserine, respectively (43, 61, 70). Additionally, AlgR directly regulates HCN production in *P. aeruginosa* through control of T1 and T2 of the *hcnA* promoter. Moreover, AlgR has recently been shown to repress HCN production in nonmucoid strains and to activate production in mucoid strains, demonstrating AlgR's ability to switch from a repressor to an activator (13).

The purpose of this study was to examine the role of AlgZ, the putative sensor for AlgR in *P. aeruginosa* HCN production. Two independent research groups identified the open reading frame (PA5262) that encodes AlgZ almost simultaneously (80, 89). The first group identified this gene while screening a transposon library for mutants that lost twitching motility and termed the gene *fimS* (80). The second group identified this same open reading frame while examining alginate production and called it AlgZ (89). The *algZ* or *fimS* gene is located directly upstream of *algR* and shares homology with other two-component sensors. PA3385 has also been referred to as *algZ* in the literature (2–4, 86) but has since been renamed *amrZ* (3). In addition to identifying a previously unreported *hcnA* transcriptional start site, the present study expands the role of AlgZ and AlgR by providing evidence that this putative sensor/regulator pair control HCN production through a surface attachment-specific mechanism that requires the conserved AlgR aspartate at position 54. These data suggest that AlgZ functions as a kinase in regulating HCN production in *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown on Luria-Bertani (LB) agar (Difco). *P. aeruginosa* was grown on *Pseudomonas* isolation agar (PIA; Difco). Both were incubated at 37°C overnight. LB agar was supplemented with kanamycin (25 μg/ml) or ampicillin (50 μg/ml), and PIA was supplemented with tetracycline (300 μg/ml), streptomycin (10 μg/ml), or gentamicin (10 μg/ml) for selection, plasmid maintenance, or genetic manipulation.

DNA manipulations. The oligonucleotides used in this study are listed in Table 2. The plasmid pZKO442, a derivative of pCVD442 (25), was used to create a 907-bp in-frame deletion of the *algZ* gene in multiple strains. The deletion plasmid was constructed by a two-step crossover PCR (37) with oligonucleotide primers algZkoXbaF, algZkoR′2, algZkoF′2, and algZkoSstR using the PAO1 chromosome as the template DNA. Oligonucleotides algZkoXbaF and algZkoSstR were used to engineer XbaI and SstI restriction enzyme sites to the 5′ and 3′ ends of the final PCR product, respectively. The resulting 1,743-bp fragment was cloned into pCVD442 using the restriction enzymes XbaI and SstI. The plasmid pUCP18Z was used to complement the *algZ* deletion strains. To construct this plasmid, a 2,326-bp fragment was amplified by PCR with oligonucleotides algZko1 and algZko4 to obtain the *algZ* open reading frame and promoter

TABLE 1. Strains used in this study

Strain or plasmid	Description	Mucoid phenotype[a]	Reference or source
P. aeruginosa strains			
PAO1	Wild type	NM	38
PAZ-1	Δ*algZ*	NM	This study
PAO6886	*algZ*::Tc^r	NM	89
PSL317	Δ*algR*	NM	48
PSL317Z	*algZ*::Tc^r Δ*algR*	NM	This study
PAO568	*mucA2*	M	29
PDO300	*mucA22*	M	54
PAZ568	*mucA2* Δ*algZ*	M	This study
PAO6884	*mucA2 algZ*::Tc^r	M	89
PAR568	*mucA2* Δ*algR*	NM	13
PAO6885	*mucA2 algZ*::Gm^r *algR*::Tc^r	NM	89
TUMC92	Clinical isolate	NM	13
TUMC92Z	Δ*algZ*	NM	This study
TUMC92R	Δ*algR*	NM	13
FRD-1	*mucA*	M	32
FRD-1Z	*mucA* Δ*algZ*	M	This study
FRD-1R	*mucA algR*::Sm^r	NM	50
WFPAPAO1	Wild type	NM	50
WFPA8	*algR*(D54N)	NM	50
WFPA13	*algR*(D85N)	NM	50
WFPA16	*algR*(D54N D85N)	NM	50
WFPA8A	*mucA algR*(D54N)	NM	This study
WFPA13A	*mucA algR*(D85N)	M	This study
WFPA16A	*mucA algR*(D54N D85N)	M	This study
WFPA8Z	*mucA algR*(D54N)	NM	This study
WFPA13Z	*mucA algR*(D85N)	M	This study
WFPA16Z	*mucA algR*(D54N D85N)	M	This study
WFPA8AZ	*mucA* Δ*algZ algR*(D54N)	M	This study
WFPA13AZ	*mucA* Δ*algZ algR*(D85N)	M	This study
WFPA16AZ	*mucA* Δ*algZ algR*(D54N D85N)	M	This study
PAO1 *vfr*	Δ*vfr*	NM	This study
PAK	Hyperpiliated	N	39
PAK *mucA*	*mucA22*	M	87
PAK *algR*	*algR*::Gm^r	NM	87
PAK *mucA algR*	*mucA22 algR*::Gm^r	NM	87
PA14	Wild type	NM	68
PA103	Wild type	NM	47
19660	Clinical isolate	NM	83
X13273	Clinical isolate	NM	83
S54485	Clinical isolate	NM	83
CF27	Clinical isolate	M	83
PAK *pscC*	Δ*pscC*	NM	84
PAK HCN KI	Δ*pscC*; PAO1 *hcnA* promoter	NM	This study
Plasmids			
pCR2.1	TA cloning vector; Ap^r Kan^r		Invitrogen
pCVD442	Gene replacement vector; Tc^r *sacB*^+		26
pZKO442	*algZ* in-frame deletion vector; Ap^r		This study
pUCP18	Expression vector; Tc^r		78
pUCP18Z	*algZ* expression vector for complementation		This study
pRK2013	Helper plasmid for conjugation, *tra* functions		28
pEXGmGW	Gateway converted pEX18Gm		83
phcnAKI	pEXGmGW with *hcnA* PAO1 promoter		This study

[a] M, mucoid; NM, nonmucoid.

region from the PAO1 chromosome. This fragment was cloned into pCR2.1 (Invitrogen) and subcloned into the XbaI and KpnI restriction sites of pUCP18.

DNA manipulations. In-frame deletions of *algZ* were created in laboratory and clinical *P. aeruginosa* strains by introducing the *algZ* deletion plasmid (pZKO442 [Δ*algZ*]) through triparental conjugation (42) and selection of carbenicillin-sensitive and sucrose-resistant double recombinants using the helper plasmid pRK2013 (28). Deletion of *algZ* was confirmed by PCR and Southern blotting with a digoxigenin-labeled probe (Roche) using oligonucleotides algZko1 and algZko4 (data not shown). Western blot analysis with a monoclonal anti-AlgR antibody, 3H9, confirmed that there were no polar effects on AlgR expression in *algZ* deletion strains. In-frame deletion of the *algR* gene was confirmed by PCR with oligonucleotides ArgHF and HemCR and Western blot analysis with anti-AlgR antibody (20). The PAO1 Δ*algR algZ*::Tc^r mutant was created by electroporating fragmented chromosomal DNA from PAO6886 (*algZ*::Tc^r) into PSL317

TABLE 2. Oligonucleotides used in this study

Name	Oligonucleotide sequence	Gene	Location[a]
algZkoXbaF′	CCCTCTAGACCCAGCAACTGGCAGGTGATCTCG	*algZ*	−889 to −866 (+)
algZkoR′2	ACGCCTGTCGCGTGCCTTTCGACGCTTGAATCGGATAGGCATCGACAGAG	*algZ*	−8 to +17 (−)
algZkoF′2	CGTCGAAAGGCACGCGACAGGCGT	*algZ*	+925 to +948 (+)
algZkoSstR′	CCCGAGCTCGAGGCCTTTCAGGTAGAGCTGGA	*algZ*	+1736 to +1758 (−)
algZKO1F	TGTCTTCCTGGTTGTCCTTGTTGTA	*algZ*	−1212 to −1188 (+)
S1algRhindIII	CCCAAGCTTCCAGAGGTTCGTCATCGACAATCAG	*algZ*	+1090 to +1114 (−)
algZKO4R	CGAGTGGCGATGCGGATT	*algZ*	+1950 to +1967 (−)
hcnAprimetext	GTGTTGACGACGTTCAAGAAGGTGCAT	*hcnA*	−495 to +24 (−)
hcnAS1BglI	GCAAAGCCGGCTCACCCGTGGAGG	*hcnA*	−3166 to −3137
PhcnA KI 5′	TACAAAAAAGCAGGCTCCGCTTCCGACCGCCCCAGGCGGCTGA	*hcnA*	−1622 to −1600 (+)
PhcnA KI 3′	TACAAGAAAGCTGGGTCCGCAGTTTTTCCAGCATCCGGGTCTA	*hcnA*	+533 to +508 (−)
ArgHF	ATATATGAGCTCGGACCTGTCCGACCTGTTCC	*argH*	−482 to −426 (+)
HemCR	ATATATGAGCTCGGCTGGCGTAGGTGTTCGAG	*hemC*	+1197 to +1177 (−)

[a] Location numbering is relative to the translational start site of gene. (+), coding strand; (−), antisense strand.

and selection of tetracycline-resistant recombinants, as described by Choi et al. (17). Deletion of *algR* and insertional inactivation of *algZ* was confirmed by Southern blot analysis using digoxigenin-labeled probes (Roche) created with primers ArgHF and HemCR or algZko1 and algZko4, respectively (data not shown).

The entire *hcnA* promoter region from PAO1 was amplified from genomic DNA with primers PhcnA KI 5′ and PhcnA KI 3′ (Table 2). The resulting PCR fragment (700 bp) was engineered with AttB1 and AttB2 sequences at the 5′ and 3′ ends, respectively, and cloned into pEXGmGW (84) via cloning to create the *hcnA* knock-in (KI) vector phcnAKI. This 700-bp PAO1 *hcnA* promoter fragment was introduced into an attenuated PAK strain (PAKΔpscC), which lacks a functioning type III secretion system, as previously described (84). Positive *hcnA* KI mutants were identified by PCR and confirmed by sequencing.

S1 nuclease protection assays. RNA for S1 nuclease protection assays was isolated from PAO1, PSL317 (Δ*algR*), and PAZ (Δ*algZ*) grown in LB to stationary phase using CsCl as previously described (52). An S1 nuclease protection assay was performed as previously described using 100 μg of RNA with the following modifications (51). The 519-bp region of the *hcnA* promoter ranging from −495 to +24 (numbering relative to the translational start site) was cloned into M13mp18. Single-stranded phage DNA was isolated and used as a template for the uniformly labeled ([α-^{32}P]dCTP; NEN DuPont) single-stranded DNA probe. Two probes were constructed using either of the oligonucleotides hcnAprimetext and hcnAS1BglI. Probes constructed with hcnAprimetext were digested with BglI, and probes constructed with hcnAS1BglI were cut with BglI. Digested probes were purified on a 5% polyacrylamide gel. Total RNA (100 μg) was hybridized to each probe at 67°C for 1 h. Reactions were then digested with S1 nuclease for 30 min and then precipitated. S1 reaction mixtures were electrophoresed through an 8% acrylamide-urea gel adjacent to a sequencing ladder generated using the same oligonucleotide as the probe.

HCN production assay of *P. aeruginosa* strains. HCN was measured using a modification of the protocols described by Zlosnik and Williams (91) and Gallagher and Manoil (30). Laboratory and clinical *P. aeruginosa* isolates were grown on PIA plates aerobically for 16 h at 37°C. The plates were then inverted and individually sealed with lids containing 1 ml of 4 M NaOH. The inverted plates were then placed inside a trigas incubator under microaerobic conditions (5% O_2, 5% CO_2, and 90% N_2) at 30°C for 4 h. After a 4-hour incubation at 30°C and after 20 h total, the NaOH/NaCN was collected and measured directly using a cyanide micro-ion-selective electrode (Lazar Research Laboratories) connected to an Accument Basic AB15 pH meter (Fisher Scientific) set to read mVs. Values recorded from known concentrations of a KCN standard ranging from 1 × 10^{-5} to 1 M were used to generate a line of regression on a semilog graph. Unknown values were calculated using the slope and *y* intercept of this line. For the time course, cells were incubated with the NaOH for 4 hours prior to the collection time indicated. Bacterial cells were collected from the plates, total protein was determined by the Bradford assay (Bio-Rad) as previously described (13), and HCN production was reported as μmol HCN/mg protein.

Pseudomonas isolation liquid medium (2% peptone, 0.14% $MgCl_2$, 1% K_2SO_4, 0.0025% Irgasan) broth cultures were grown in a sidearm flask with an attached enclosed chamber with shaking at 150 rpm under temperature and oxygen conditions identical to those of the plate-grown cultures. Four hours prior to the collection time point 1 ml of 4 M NaOH was added to the sidearm collection chambers to act as a cyanide trap. The micro-ion-selective electrode was used to measure the cyanide concentration in the NaOH/NaCN collected in the attached chamber and broth directly, and the concentrations were added together to obtain the total amount of cyanide produced in the broth culture. The cells were harvested by centrifugation, and total protein was determined as described above. Six biological replicates were performed for each strain. One-way analysis of variance (ANOVA) and the Tukey-Kramer multiple test were used for statistical analysis.

Optimization of oxygen concentration for *P. aeruginosa* HCN production. PAO1 was grown on PIA plates aerobically for 16 h at 37°C. The plates were then inverted, individually sealed with lids containing 1 ml of 4 M NaOH, and incubated for 4 additional hours at 30°C in a trigas incubator under various oxygen conditions (1%, 3%, 5%, 7%, 10%, and 15% O_2). The NaOH/NaCN and total protein were collected and measured as described above.

RESULTS

***P. aeruginosa* HCN production is surface attachment specific.** HCN is produced by *P. aeruginosa* during the transition from logarithmic to stationary growth phase at low oxygen tensions and high cell densities due to regulation of the *hcnA* promoter by the synergistic effects of the anaerobic regulator ANR and the quorum sensing regulators LasR and RhlR (14, 16, 62). Cyanide production is also under the control of the transcriptional regulator AlgR (13). RhlA and RhlI have recently been shown to be controlled by AlgR through an attachment-specific mechanism (58). A recent report showed increased cyanide production from *P. aeruginosa* grown in a static glass bead biofilm system (72). In order to determine if HCN production is increased during surface growth, HCN production by PAO1 was measured following growth of cells in broth and on agar media. Planktonic cultures of *P. aeruginosa* PAO1 produced a maximal amount of 0.94 μmol HCN/mg protein during the transition from mid-log to stationary phase (8 h) consistent with previous findings (14, 16) (Fig. 1A). Cultures grown on agar plates produced various amounts of HCN over the time course with an increase at 20 h to 1.5 μmol HCN/mg protein (Fig. 1B). These results show that surface-grown *P. aeruginosa* in stationary phase produces more HCN than do planktonically grown cells.

Optimal oxygen tension for *P. aeruginosa* HCN production. HCN is produced at low oxygen tensions during planktonic growth (14, 16), but the optimal oxygen concentration for surface-attached cells is unknown. To determine the range of oxygen concentrations that permit HCN production, *P. aeruginosa* was grown at various oxygen levels and HCN production
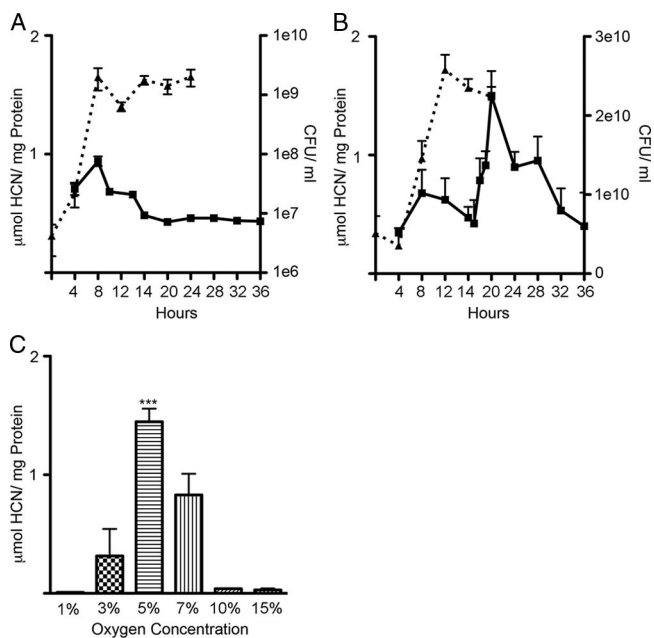
FIG. 1. PAO1 HCN production in liquid and plate media and under different oxygen tensions. (A) HCN production (solid squares and solid line) by PAO1 in liquid *Pseudomonas* isolation broth and bacterial CFU/ml (solid triangles and dotted line) over time. (B) HCN production (solid squares and solid line) by PAO1 grown on PIA plates and bacterial CFU/ml (solid triangles and dotted line) over time. (C) HCN production under various oxygen tensions. Statistical significance was determined by one-way ANOVA and the Tukey-Kramer test. ***, $P < 0.001$.

was measured. PAO1 produced the maximum amount of HCN in a 5% oxygen environment (Fig. 1C). Consistent with previous studies, HCN production was greatly reduced as oxygen tensions neared anaerobic or aerobic conditions (14, 16).

Identification of previously unidentified *hcnA* transcriptional start site. AlgR directly regulates HCN production in *P. aeruginosa* by controlling expression of *hcnA* from the T1 and T2 transcriptional start sites (13). The effects of *algZ* deletion on the transcription of the *hcnA* promoter were examined under microaerobic growth conditions by an S1 nuclease protection assay. This assay showed that AlgZ, like AlgR, is required for repression of *hcnA* transcription in a nonmucoid background (Fig. 2A). Interestingly, the assay also revealed an additional transcriptional start site (designated T3) upstream of the two previously identified sites (13, 62). The intensity of this band of protection indicates that the amount of transcript produced from the T3 site is equal to or greater than that of the T1 and T2 transcripts. The T3 start site is a G residue located −221 bp from the translational initiation codon.

A naturally occurring deletion upstream of *hcnA* results in low cyanide production. Previously, Wolfgang et al. identified a region upstream of *hcnA* in PAO1 (termed the *exoY* deletion region) that is absent in several other laboratory and clinical strains (83). The *hcnA* T3 transcriptional start site and previously identified AlgR and Lux β binding sites are all within this deleted region (Fig. 2B) (13, 62). Measurement of HCN production on the laboratory strain PAK, which features this chro-

mosomal deletion, showed low levels of cyanide production (~0.3 μmol/mg protein) in comparison to PAO1 (~1.4 μmol/mg protein) (Fig. 3A) when measured under microaerobic conditions. Mutations in PAK *algR*, *mucA2*, or both did not affect the amount of cyanide produced by these isogenic strains (Fig. 3A). Sequence analysis of the *hcnA* promoter region in strains PA14 (HCN production = 0.4 μmol/mg protein) and PA103 (HCN production = 0.1 μmol/μg protein) revealed that they did not contain the *hcnA* T3 promoter either (data not shown). Insertion of the PAO1 *hcnA* promoter onto the PAK chromosome by homologous recombination restored HCN production to PAO1 wild-type levels in the PAK background (Fig. 3A). Moreover, cyanide measurements of blood isolate X13273, urinary tract infection isolate S54485, eye isolate 19660, and clinical isolate CF27, all previously shown to contain the same deletion region (83), revealed that these strains also produce very little HCN (Fig. 3B).

AlgZ activates and represses HCN production in *P. aeruginosa*. Previous analysis of HCN production in *P. aeruginosa* strains indicated that mucoid strains produce approximately sevenfold more HCN than do their nonmucoid parental strains. In-frame *algR* deletions in nonmucoid strains produced ~3-fold more HCN and ~1.5-fold less HCN in a mucoid background, showing that AlgR switches from a repressor to an activator of the *hcnA* promoter depending upon mucoid status (13). Both AlgR and AlgZ are involved in alginate production (19, 89) and required for twitching motility (5, 80, 81). An S1 nuclease protection assay indicated that AlgZ also plays a role in HCN regulation (Fig. 2A). Cyanide production levels from in-frame *algZ* deletion and insertional *algZ* mutants in both nonmucoid and mucoid backgrounds were measured under microaerobic conditions on agar plates. Mutations in *algR*, *algZ*, or both resulted in a ~3-fold increase in HCN production in nonmucoid laboratory strains (Fig. 4A) compared to the wild type. In mucoid laboratory strains, mutations in *algR*, *algZ*, or both resulted in an ~3.5-fold decrease in HCN production (Fig. 4B). In order to determine if the results observed with laboratory strains and their isogenic mutants are consistent with those for clinical isolates, HCN production was measured in mucoid and nonmucoid CF isolates and their isogenic *algR* and *algZ* mutants. Consistent with the results for the laboratory strains, mutations in *algR* or *algZ* resulted in an ~1.5-fold increase in HCN production in nonmucoid clinical isolates (Table 3). In mucoid clinical isolates, mutations in *algR* or *algZ* resulted in a decrease in HCN production (Table 3). Additionally, complementation of the *algZ* mutant with the wild-type allele restored HCN production to wild-type levels in all strains (Fig. 4A and B). These results provide evidence that AlgZ works through AlgR to regulate HCN production.

AlgZ and AlgR regulation of HCN production is surface attachment specific. AlgZ and AlgR are both required for fimbrial biogenesis (80, 81) and affect alginate production (55, 56, 88). Both of these phenotypes are associated with biofilm formation and surface colonization (60, 78). Moreover, AlgR control of the *rhlI* and *rhlA* promoters has recently been shown to be surface attachment specific (58). In order to determine if AlgZ and AlgR regulation of HCN production was dependent on surface growth, cyanide assays were performed on nonmucoid and mucoid strains both grown in broth or on agar plates in stationary phase, 8 h for
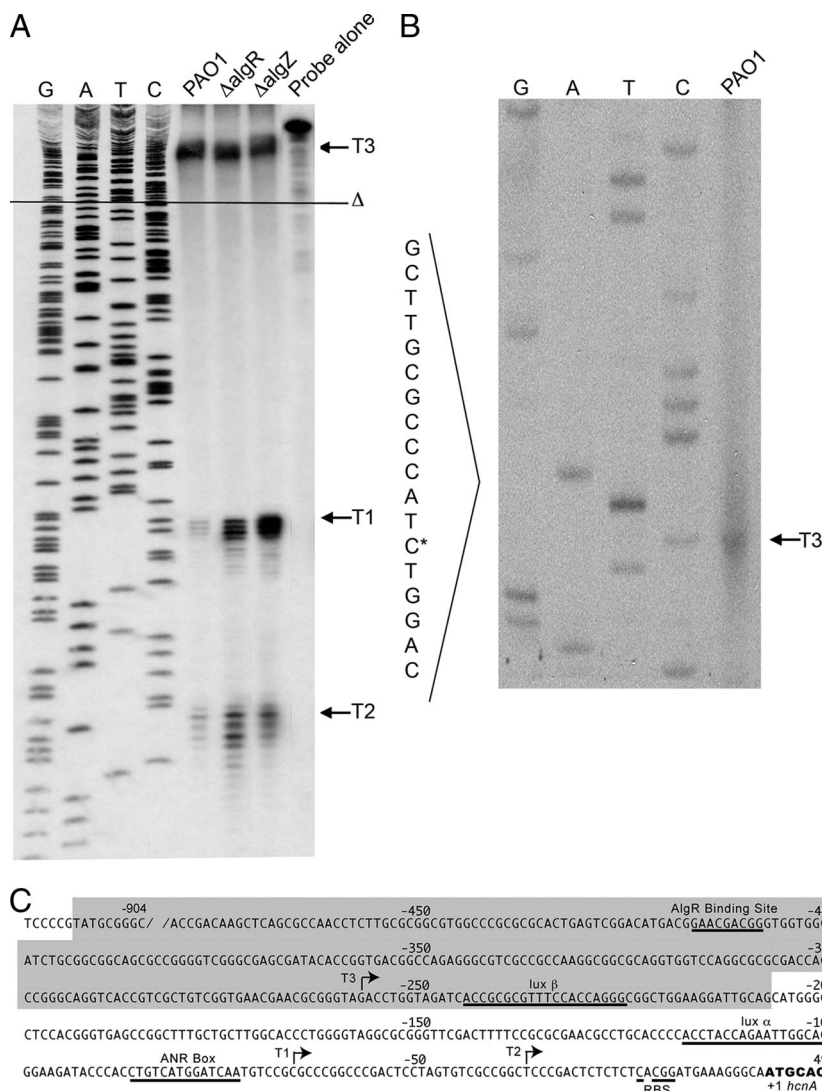
FIG. 2. The *hcnA* promoter is controlled by AlgZ and AlgR. (A) S1 nuclease protection assay of the *hcnA* promoter. Previously mapped T1 and T2 transcriptional start sites (indicated by respective labels and arrows) of the nonmucoid strain PAO1 and Δ*algR* and Δ*algZ* derivatives. The open triangle with the adjacent black line denotes the beginning of the common deletion region found upstream of the *hcnA* promoter. (B) S1 nuclease protection assay identifying T3 transcriptional start site. The arrow indicates the band of protection indicating the +1 start site. An asterisk denotes the first nucleotide in the antisense probe. (C) Sequence of the *hcnA* promoter region. The shaded sequence denotes the *exoY* deletion region. Binding sites are underlined for AlgR (AlgR Binding Site), ANR (ANR Box), RhlR and LasR (*lux* α and *lux* β), and ribosome (RBS). +1, HcnA translational start site; boldface nucleotides, *hcnA* open reading frame.

broth-grown strains and 20 h for plate-grown strains under microaerobic conditions (see Materials and Methods) (Fig. 1A and B). In agreement with our previous data, strains grown in broth produced approximately half (Table 4) the amount of HCN observed when the same strains were grown on plates (Fig. 4). In broth, neither *algR* nor *algZ* mutation had an effect on HCN production in either background (Table 4) whereas the same strains grown on agar plates resulted in threefold changes in HCN production (Fig. 4). Taken together, these results indicate that AlgZ/AlgR-dependent cyanide production was regulated through a surface-attachment-specific mechanism.

AlgR D54 is critical for HCN production. The aspartate at position 54 of AlgR is highly conserved among two-compo-

nent regulators and is able to accept phosphate from *E. coli* CheA in vitro (20, 50). An additional aspartate at position 85 is conserved in the AlgR homologues *Staphylococcus aureus* LytR and *E. coli* YehT but is not capable of accepting phosphorylation. AlgR D85 has been implicated in controlling the amount of pilin subunits exported (81). To determine the role of these conserved aspartates in HCN production, site-directed AlgR mutants containing D54N, D85N, or D54N D85N chromosomal alleles (81) were tested for their ability to produce HCN under microaerophilic conditions on PIA. These strains were made mucoid by mutating the *mucA* gene, and HCN production was measured from all isolates. Though the parental strain of these mutants, a PAO1 strain obtained from the Wozniak labora-
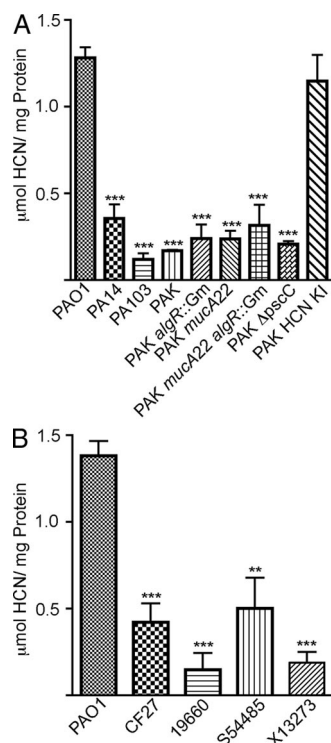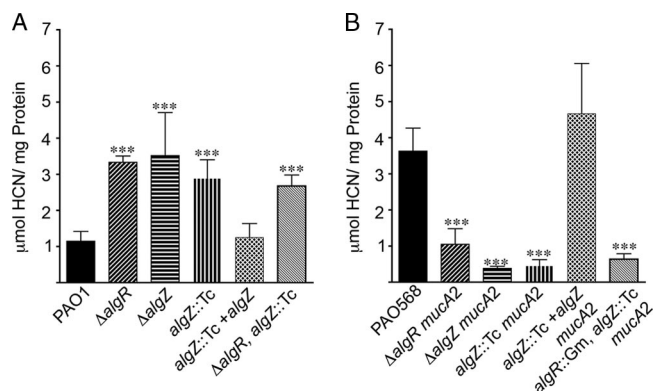
FIG. 4. AlgZ and AlgR control HCN production. HCN production for nonmucoid (A) and mucoid (B) *algZ* and *algR* mutants was measured as for Fig. 3. (A) PAO1, PSL317 (Δ*algR*), PAZ (Δ*algZ*), PAO6886 (*algZ*::Tc), PAO6886 harboring pUCP18Z (*algZ*::Tc + *algZ*), and PSL317Z (Δ*algR algZ*::Tc). (B) PAO568, PAR568 (Δ*algR mucA2*), PAZ568 (Δ*algZ mucA2*), PAO6884 (*algZ*::Tc *mucA2*), PAO6884 harboring plasmid pUCP18Z (*algZ*::Tc *mucA2* + *algZ*), and PAO6885 (*algR*::Gm *algZ*::Tc *mucA2*). Statistical significance was determined as for Fig. 3.

FIG. 3. Strains with the *exoY* chromosomal deletion region upstream of *hcnA* produced decreased amounts of cyanide. (A) HCN production from nonmucoid wild-type PAO1 and laboratory strains with the *exoY* deletion region (PA14, PA103, and PAK), the PAK *algR* deletion (PAK *algR*::Gm), the PAK *mucA22* mutation, the PAK *mucA22 algR* deletion, the PAK *pscC* deletion (PAK Δ*pscC*), and the PAK *pscC* deletion with the PAO1 *hcnA* promoter (PAK HCN KI). (B) HCN produced from PAO1, CF isolate CF27, eye isolate 19660, urinary tract infection isolate S54485, and blood isolate X13273. Statistical significance was determined by one-way ANOVA and the Tukey-Kramer test. $**$, $P < 0.01$, and $***$, $P < 0.001$, compared to the isogenic parent.

DISCUSSION

This study shows that mutations in *algZ* or *algR* affect HCN production in *P. aeruginosa* and that these effects are partially due to the phosphorylation state of AlgR. In addition, results shown here demonstrate that the *hcnA* promoter contains three transcriptional start sites with the most upstream T3 promoter being required for maximal activity. The *hcnA* promoter is controlled at transcriptional and posttranscriptional levels by many different regulators including ANR (62, 90), LasR and RhlR (62), GacA (7, 44, 61, 70), RsmA (10, 36, 44), and AlgR (13, 49). Two *hcnA* promoters have been previously reported for *P. aeruginosa*, termed T1 and T2 (62). T1 is regulated by quorum sensing alone, while T2 relies on a synergistic effect of LasR, RhlR, and ANR (62). In *P. fluorescens*, there are three RsmA/RsmE recognition elements that function as posttranscriptional regulators of the *hcnA* promoter by directly binding to the 5′ leader region (44). The chromosome of *P. aeruginosa* also contains *rsmA*, and deletion of this gene increased HCN production, suggesting that RsmA may function similarly in *P. aeruginosa* (63, 70). GacA also indirectly controls HCN production by positively regulating the *rhlI* promoter and hence amounts of *N*-butanoyl-homoserine-lactone (63, 70). The effects of *algZ* and *algR* deletion on *hcnA* transcription could be partially indirect, as AlgR represses *rhlI* transcription

tory, produced less cyanide than did our PAO1 strain, the same trends for HCN production were observed for an *algR* mutant and an *algZ* mutant as with our PAO1 strain (data not shown). The AlgR D54N and AlgR D54N D85N mutants produced increased HCN levels in nonmucoid strains (~2-fold [Fig. 5A]) while producing lower levels in mucoid (*mucA*::Tc[r]) strains (~3-fold [Fig. 5B]). Similar to the results obtained from assays of twitching motility (81) and alginate production (50), the D85N mutant had no effect on HCN production. Deletion of *algZ* resulted in the same effects on HCN production as those found with the AlgR D54N mutant (Fig. 5). These data show that repression of HCN production in nonmucoid strains and activation in mucoid strains are both dependent on AlgZ and AlgR D54. Taken together, these data strongly suggest that phosphorylation of AlgR D54 is required for HCN regulation and that AlgZ is required for phosphorylation of AlgR. However, these data also indicate that phosphorylation of AlgR is not the mechanism responsible for its ability to switch from a repressor in nonmucoid *P. aeruginosa* to an activator in mucoid isolates for HCN production.

TABLE 3. Effects of *algR* and *algZ* mutations in clinical isolates on HCN production

Strain	Description or genotype	HCN production (mean μmol/μg protein ± SD)
TUMC92	Clinical	5.64 ± 0.64
TUMC92R	Δ*algR*	9.30 ± 0.32
TUMC92Z	Δ*algZ*	8.02 ± 0.69
FRD-1	Clinical	7.60 ± 0.62
FRD-1R	Δ*algR*	2.38 ± 0.47
FRD-1Z	Δ*algZ*	2.89 ± 0.08

TABLE 4. Effects of *algR* and *algZ* mutations in broth-grown strains on HCN production

Strain	Genotype	HCN production (mean μmol/μg protein ± SD)
PAO1	Wild type	0.63 ± 0.05
PSL317	PAO1 Δ*algR*	0.55 ± 0.01
PAZ	PAO1 Δ*algZ*	0.55 ± 0.01
PAO568	*mucA2*	0.55 ± 0.01
PAR568	PAO568 Δ*algR*	0.57 ± 0.01
PAZ568	PAO568 Δ*algZ*	0.63 ± 0.01

when *P. aeruginosa* is attached (58). However, AlgR has been shown to bind directly to the *hcnA* promoter in vitro, indicating that it controls HCN production directly (13). The most plausible explanation is that AlgR is interacting with other regulators (RhlR, ANR, or others) to control *hcnA*. Further studies are under way to determine the effects of AlgR, ANR, RsmA, LasR, and RhlR interactions on the *hcnA* promoter.

S1 nuclease protection assays (Fig. 2) established that there is one additional transcriptional start site upstream of the two previously reported. The *hcnA'-'lacZ* translational fusion used in a previous study that contained the T3 transcriptional start site showed higher β-galactosidase activity than did those fusions that lacked this promoter (62). The new start site is located in the *exoY* deletion region and is found in several laboratory and clinical strains (83). Results from Fig. 3 indicate that T3 may be at least as important as T1 and T2, as the absence of this 5' region of the *hcnA* promoter resulted in extremely low levels of HCN production. These data suggest that the T3 promoter is responsible for a considerable amount of *hcnA* expression in *P. aeruginosa*. Interestingly, with the exception of PAK and CF27, most of the strains examined with this deletion region are *exoU* positive and exhibit increased type III secretion activity compared to PAO1, suggesting that only one of these virulence factors may be featured in a given strain (83).

It has been proposed that AlgZ acts as the sensor to AlgR in a two-component regulatory system (80, 89). The gene encoding AlgZ is directly upstream of *algR*, and both proteins share homology to two other sensor/regulator pairs, *S. aureus lytS* and *lytR* and *E. coli yehU* and *yehT*. In fact, AlgR, LytR, and AgrA compose a unique branch of the two-component system family, called the LytTR family (59). In this study we provide additional evidence that AlgZ acts as the AlgR sensor. Deletion of *algZ* resulted in the same phenotype as did an *algR* deletion and the site-directed *algR*(D54N) strain with respect to HCN production, strongly indicating that AlgZ acts through AlgR to control this promoter.

The AlgR response regulator responds to different environmental conditions depending upon the promoter examined. For instance, AlgR responds to nitrate concentrations on the *algD* promoter (57) and to surface contact or perhaps lowered oxygen tensions in control of the *rhlI* and *rhlA* promoters (58). HCN is not produced by *P. aeruginosa* under aerobic or anaerobic conditions (15) but is expressed under low oxygen tensions that range from 0.1 μM to 180 μM dissolved oxygen in liquid medium (14). This broad range of HCN production was not observed here (Fig. 1); however, the conditions used in this study were differing concentrations of atmospheric ox-

ygen and not dissolved oxygen. The results presented here show that HCN production was maximal at 5% (1.5 μM O₂) and is within the range previously reported. Part of the oxygen dependence can be explained by the requirement for the anaerobic regulator ANR for *hcnA* transcription (62, 90). However, it is not clear how or if AlgR and ANR interact directly. The current data support a model where AlgR is repressing *hcnA* and ANR is activating *hcnA*, in a nonmucoid background. In the mucoid background, the data indicate that there may be some synergy between these two transcriptional regulators to activate *hcnA*, as both are required for maximal *hcnA* expression.

As with AlgZ and AlgR control of the *rhlI* and *rhlA* promoters (58), changes in HCN production were not observed in liquid cultures of *P. aeruginosa* in strains containing mutations in *algR* or *algZ*. These results together suggest that AlgR may respond to either a microaerobic environment or surface attachment. Recently, Ryall et al. have shown that bacteria of the *Burkholderia cepacia* complex are cyanogenic under biofilm conditions (a glass bead system and agar plate) but not in
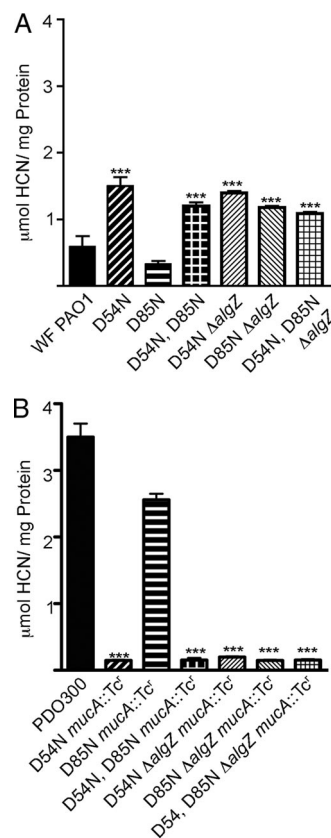

FIG. 5. AlgR D54 is critical for HCN. (A) HCN production was measured from WFPAO1, WFPAO1 with chromosomal *algR* mutation D54N (AlgR D54N), WFPAO1 with chromosomal *algR* mutation D85N (AlgR D85N), WFPAO1 with double chromosomal *algR* mutations D54N and D85N (AlgR D54N D85N), WFPAO1 containing chromosomal *algR* mutation D54N and an *algZ* deletion (D54N Δ*algZ*), and WFPAO1 containing chromosomal mutations in *algR* D54N and D85N and an *algZ* deletion (D54N D85N Δ*algZ*). (B) HCN production from mucoid derivatives (*mucA*::Tc) of the same site-directed *algR* and *algZ* mutants as those in panel A. Statistical significance was determined as described for Fig. 3.

liquid cultures (73). The results shown in Fig. 1A are in agreement with those studies. Cultures grown on agar have been described as open-air biofilms (41) and share many characteristics, including surface attachment, high cell densities, and coordinated cellular behavioral patterns (41, 75). Type IV pili could directly mediate either interaction and simultaneously act as a sensor for attachment and have been shown to affect gene transcription of *lipC* (53). Alternatively, surface growth could have a nonspecific effect. For example, surface growth could result in high local concentrations of signaling molecules (*N*-acylhomoserine lactones) or alterations in the local available O_2 concentration, all things known to affect HCN production (14, 62, 63). In order to address this possibility, HCN was measured in a *pilA* strain that resulted in a slightly lower but not statistically significant amount of HCN compared to the amount produced by PAO1 (data not shown). Further studies are being performed to elucidate this potentially important mechanism.

P. aeruginosa has been proposed to grow in a biofilm in the CF lung, and this environment also contains the low oxygen tension and high cell density required for HCN production (77, 85). A steep oxygen gradient is present within secondary bronchi, reported as more than 70 times lower than the partial pressure of oxygen (pO_2) found in the primary bronchi (85). Recently, reports have shown that HCN is detectable in the sputum and bronchoalveolar lavage fluid (12, 40, 71) of CF patients and is associated with impaired lung function. These reports are consistent with our previous data showing that 86% of CF isolates produced HCN (13) and with the data of others who have measured volatile compounds from CF sputum samples and detected HCN in vitro (12). Altogether, these data indicate that HCN may be an important virulence determinant for *P. aeruginosa* in CF.

Previous studies show that AlgZ and AlgR are both activators of twitching motility, with AlgZ acting in an AlgR-dependent manner. It has also been shown that AlgZ is a repressor of alginate production in the mucoid strain PAO568 (88), while AlgR acts as an activator (19, 55, 56). The conserved AlgR aspartate at position 54 does not play a role in alginate production in *P. aeruginosa* (50); however, it is required for twitching motility (81). The different phenotypes observed in alginate and twitching motility with respect to AlgR D54N suggest both a phosphorylation-dependent and a phosphorylation-independent mechanism for AlgR regulation. Analysis of HCN production by *P. aeruginosa* strains demonstrated that AlgR switches from a repressor to an activator of the *hcnA* promoter depending upon mucoid status (13). This study demonstrates that AlgR control of HCN production requires AlgR D54, indicating that some regulation occurs through the phosphorylation-dependent mechanism. In contrast, the AlgR switch from repressor to activator is still observed in the AlgR D54N site-directed mutants in comparisons of the nonmucoid to the mucoid background. These data provide evidence that phosphorylation is not the mechanism by which AlgR switches from a repressor to an activator in the mucoid background. Furthermore, these data suggest that AlgZ acts as a kinase in the microaerobic environment necessary for HCN production. Further investigation into the protein-protein interactions and posttranslational modification of AlgR is required to explain the repressor/activator switch.

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