The Transcriptional Regulator AlgR Controls Cyanide Production in 
*Pseudomonas aeruginosa*


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*Pseudomonas aeruginosa* is an opportunistic pathogen that causes chronic lung infections in cystic fibrosis (CF) patients. One characteristic of *P. aeruginosa* CF isolates is the overproduction of the exopolysaccharide alginate, controlled by AlgR. Transcriptional profiling analyses comparing mucoid *P. aeruginosa* strains to their isogenic algR deletion strains showed that the transcription of cyanide-synthesizing genes (*hcnAB*) was ~3-fold lower in the algR mutants. S1 nuclelease protection assays corroborated these findings, indicating that AlgR activates *hcnA* transcription in mucoid *P. aeruginosa*. Quantification of hydrogen cyanide (HCN) production from laboratory isolates revealed that mucoid laboratory strains made sevenfold more HCN than their nonmucoid parental strains. In addition, comparison of laboratory and clinically derived nonmucoid strains revealed that HCN was fivefold higher in the nonmucoid CF isolates. Moreover, the average amount of cyanide produced by mucoid clinical isolates was 4.7 ± 0.85 μmol of HCN/mg of protein versus 2.4 ± 0.40 μmol of HCN/mg of protein for nonmucoid strains from a survey conducted with 41 *P. aeruginosa* CF isolates from 24 patients. Our data indicate that (i) mucoid *P. aeruginosa* regardless of their origin (laboratory or clinically derived) produce more cyanide than their nonmucoid counterparts, (ii) AlgR regulates HCN production in *P. aeruginosa*, and (iii) *P. aeruginosa* CF isolates are more hypercyanogenic than nonmucoid laboratory strains. Taken together, cyanide production may be a relevant virulence factor in CF lung disease, the production of which is regulated, in part, by AlgR.

*Pseudomonas aeruginosa* is an opportunistic pulmonary pathogen of patients with cystic fibrosis (CF), where it is the major cause of morbidity and mortality. *P. aeruginosa* is able to persist and exacerbate damage in the lungs that ultimately results in respiratory failure. One unique feature of *P. aeruginosa* CF isolates is the overproduction of the exopolysaccharide alginate that phenotypically results in a mucoid colony morphology (23). The first committed step for alginate production is transcriptional activation of the *algD* gene (12), an event that requires the AlgR regulator and the alternative sigma factor AlgU (AlgT) (11, 14, 26, 34, 35, 38, 39, 46). Mucoidy is also associated with the chronic phase of CF airway disease where the bacteria acquire increased resistance to various antibiotics and phagocytic cells. Furthermore, increasing evidence suggests that *P. aeruginosa* may be in a microaerophilic or anaerobic microenvironment trapped within biofilms in the thick mucus lining the airways of CF patients (48, 55, 59). Under such conditions, the organisms are able to produce alginate and maintain mucoidy (25, 48, 56, 59).

It has long been recognized that *P. aeruginosa* generates poisonous cyanide as a secondary metabolite (7). Hydrogen cyanide (HCN) is produced from glycine (7, 54) in a poorly understood oxidative reaction catalyzed by HCN synthase (8, 9, 53) whose expression requires the lasIRhl tandem of the intercellular signaling process known as quorum sensing (43, 44). HCN is not produced when the organism is grown under strict anaerobic conditions when supplied nitrate as a terminal electron acceptor (7) but rather occurs optimally at low oxygen tensions (~5%) during the transition from exponential to stationary phase when bacteria are at high cell densities and fully capable of quorum sensing (6, 10, 43, 44). An early report (22) describing the detection of HCN in *P. aeruginosa*-infected wounds from burn patients gave cause to believe that the cyanogenic properties of the organism may be a contributing factor in its pathogenicity. Further support for this hypothesis stems from recent studies demonstrating that a mutant defective in *hcnC* had a strongly reduced ability to kill the nematode *Caenorhabditis elegans* in an experimental infection model (18). In addition, recent transcriptional profiling analyses revealed that mucoid *P. aeruginosa* actively transcribes *hcnA*, encoding HCN synthase (16), further suggesting that cyanide production may be an important virulence factor.

The present study extends the recent work (29) examining the molecular basis underlying the ability of AlgR to control virulence in *P. aeruginosa*. Through the use of Affymetrix Gene Chip technology, we previously identified many potential genes under AlgR control, one of which was *hcnA* (30). Here, we
show that (i) mucoid \textit{P. aeruginosa} strains produce copious amounts of HCN, (ii) AlgR regulates this process, and (iii) HCN production in clinical CF isolates is significantly elevated over that of laboratory strains.

**MATERIALS AND METHODS**

\textbf{Bacterial strains, plasmids, and growth conditions.} The \textit{P. aeruginosa} strains used in this study are listed in Table 1. \textit{P. aeruginosa} strains PAO6857 (mucB::Tc'), PAO568 (mucA2), PAR568 (mucA2 AlgR), and PAR6857 (mucB::Tc' AlgR) were grown in modified \textit{Pseudomonas} isolation media (20 g of peptone/liter, 7 mM MgCl$_2$, 5 mM K$_2$SO$_4$, 2% glycerol, pH 7.0) under microaerophilic conditions for \textit{S1} nuclease protection assays. Overnight cultures were diluted 1:100 in 500-mL aeration flasks capped with rubber stoppers and then placed at 37°C with slow shaking at 100 rpm for microaerophilic growth conditions. These cultures were grown to an optical density at 600 nm (OD$_{600}$) of 0.4. The plasmid used for \textit{algR} complementation was pCMR7 (algR') (38). Routine overnight cultures were grown in Luria-Bertani broth at 37°C in a rotary shaker incubator. Carbencillin (300 μg/mL) was supplemented as needed for plasmid maintenance and genetic manipulations.

\textbf{DNA manipulations.} The \textit{algR} gene was deleted from strains as previously described (30). Briefly, an \textit{algR} deletion plasmid (pRK0442 [algR]) was introduced into clinical and laboratory \textit{P. aeruginosa} strains by triparental conjugation (28), and double recombinants were obtained by selection of carbenicillin-sensitive and sucrose-resistant colonies (15). Deletion of \textit{algR} was confirmed by PCR with oligonucleotide primers ArgHf (5' - ATATATGAGCTCGACCGTGCTGACCTGTTCC-3') and HemCr (5' - ATATATGAGCTCGACCGTGCTGACCTGTTCC-3') and Southern blot (data not shown) and Western blot analysis with anti-AlgR (see Fig. 2A and 4B) (13).

\textbf{Western blot analysis of AlgR.} \textit{P. aeruginosa} strains were grown aerobically in Luria-Bertani broth at 37°C overnight. The bacteria were collected by centrifugation and resuspended in 50 mM Tris-HCl (pH 8.0)−150 mM NaCl and lysed by sonication. Total protein concentrations were quantified by the Bradford protein assay (Bio-Rad). Cell extract (30 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 5% to 20% polyacrylamide gradient gels and transferred to a polyvinylidene difluoride membrane (GE Osmonics). The membranes were probed using a 1:2,000 dilution of anti-AlgR mouse monoclonal antibody (13) followed by a 1:2,000 dilution of horseradish peroxidase-conjugated goat anti-mouse monoclonal antibody and the signal was detected using the Opti-4CN substrate kit (Bio-Rad).

\textbf{Qualitative gaseous HCN production assay of \textit{P. aeruginosa} strains.} An initial screen of \textit{P. aeruginosa} CF isolates was performed by testing qualitatively for gaseous HCN production using a modified method of Castric and Castric (5). Whatman filter paper strips were soaked with 5 mg each of copper(H) ethyl acetocetate and 4,4'-methylenebis(N,N-dimethylamine) dissolved in 2.5 mL of chloroform and allowed to air dry. The strips were placed in the lids of L agar plates inoculated with \textit{P. aeruginosa} CF clinical isolates and incubated inverted overnight at 37°C. Relative HCN production was indicated by a change in the color of the strips from white to blue. As a control, L agar plates with no bacteria and the qualitative assay strip were incubated in the same incubator with CF isolates on different plates at the same time.

\section*{RESULTS}

\textbf{Cyanide production is elevated in mucoid \textit{P. aeruginosa}.} Analysis of the transcriptional profile comparing mucA2 and mucB::Tc' mucoid \textit{P. aeruginosa} strains to their isogenic \textit{algR} deletion strains showed that \textit{hcnA} transcription was ~3-fold lower in the \textit{algR} mutant strains (unpublished data). These results are consistent with those of Fioredi and Deretic, who reported that \textit{hcnA} transcription is sixfold greater in the \textit{mucA2} mucoid strain PAO578I than in its isogenic \textit{algU} mutant (16). Since AlgR is required for alginate production (11) and controlled by AlgU (35), we postulated that AlgR may be...
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activating hcnA transcription in mucoid P. aeruginosa bacteria. Therefore, cyanide was measured directly in mucoid and nonmucoid laboratory strains of PAO1 and PAO381. The nonmucoid laboratory strains PAO1 and PAO381 produced an average of 0.49 μmol of cyanide/mg of protein. In contrast, the mucA mucoid derivatives of these strains, PDO300 and PAO568, respectively, each produced approximately seven times the amount of cyanide (Fig. 1). In addition, PAO6857 (mucB::Te') produced 3.3-fold more cyanide than PAO1 (Fig. 1). Taken together, these results indicate that mucoid derivatives of P. aeruginosa produce more cyanide than their nonmucoid parental strains.

MucP. aeruginosa requires AlgR for complete hcnA expression and cyanide production. To determine if AlgR plays a role in a mucA2 derivative of P. aeruginosa, algR was deleted from P. aeruginosa strains PAO568 and PDO300 (Fig. 2A). An S1 nuclease protection assay performed on the hcnA promoter from PAO568 and its algR deletion strain PAR568 revealed that transcription from hcnA promoter T1 was elevated in PAO568 compared to the same promoter in PAR568 (Fig. 2B). HCN production was then quantified in the mucoid mucA2 strains PDO300 and PAO568 and their respective algR deletion strains. This comparison revealed that the mucoid mucA parental strain produced an average of 1.5-fold more cyanide than the algR mutants (Fig. 1). Moreover, overexpression of algR in trans in the algR deletion strains restored cyanide production (Fig. 1).

To determine if AlgR affected hcnA transcription in a mucB mucoid P. aeruginosa strain, algR was deleted in PAO6857 (mucB::Te') to generate the strain PAR6857 (mucB::Te' ΔalgR) (Fig. 2A). An S1 nuclease protection analysis was performed on the hcnA promoter by using total RNA from these strains grown microaerophilically and revealed AlgR activated the T1 and T2 promoters of hcnA (Fig. 2B and C). Moreover HCN determinations revealed that PAR6857 produced 1.9-fold more HCN per milligram of protein than its algR deletion strain PAR6857 (Fig. 1). This result is consistent with the transcriptional profile analysis of the same strain and its algR deletion mutant that indicated hcnA is activated threefold by AlgR. Taken together, these results suggest that AlgR activates cyanide production by ~2-fold in mucoid laboratory strains of P. aeruginosa, irrespective of the nature of the mutation (mucA, mucB) conferring the mucoid phenotype.

AlgR binds to the hcnA promoter region. Since hcnA transcription is algR dependent and there is an AlgR binding site within the hcnA promoter (~410 to ~402; GAACgACGG, where the lowercase “g” represents a departure from the reverse and complement of the algDalgC consensus AlgR binding sequence [30]), we tested the ability of purified AlgR to bind to the hcnA promoter region in an in vitro gel mobility shift assay. AlgR caused a shift in mobility compared to the probe alone, indicating that AlgR is capable of binding to the hcnA promoter region (Fig. 3). The addition of nonradioactive hcnA (specific competitor) reduced the amount of probe shifted by AlgR in a dose-dependent manner, indicating specificity of AlgR for the hcnA promoter region. Additionally, nonradioactive pUC12 was added as a nonspecific competitor to ensure that competition was not due to nonspecific AlgR DNA binding. These results provide in vitro evidence that AlgR binds specifically to the hcnA promoter DNA.

Clinical P. aeruginosa CF isolates produce elevated cyanide levels. To determine the prevalence of cyanide production among P. aeruginosa CF isolates, we performed a qualitative HCN production assay on 167 clinical P. aeruginosa isolates from 103 CF patients. This assay showed that 74% of all isolates examined produced HCN (data not shown). Furthermore, 83% of CF patients from the Tulane University Medical Center were found to carry at least one positive P. aeruginosa HCN-producing strain. To confirm these results, the amount of cyanide produced by 41 separate clinical CF P. aeruginosa isolates from 24 CF patients was quantified. The average amount of cyanide produced by all 41 strains was 3.5 μmol of cyanide/mg of protein, indicating that the majority of CF patients surveyed harbored P. aeruginosa strains that constitutively produced HCN when tested in vitro. When HCN production by both mucoid and nonmucoid isolates from 12 patients was compared, the amount of cyanide detected averaged 4.4 ± 1.1 and 2.5 ± 0.5 μmol of HCN/mg of protein, respectively. Then examined eight mucoid strains from additional patients for whom there were no nonmucoid isolates and found an average amount of 5.2 ± 1.2 μmol of HCN/mg of protein produced. We also examined nine nonmucoid strains from patients that did not carry mucoid isolates and determined that these strains produced an average of 1.36 ± 0.6 μmol of HCN/mg of protein. Overall, the amount of cyanide produced by mucoid P. aeruginosa CF isolates was approximately twice as high as that of nonmucoid CF isolates (Fig.
4A). This is in agreement with the results we obtained with the mucoid laboratory strains of *P. aeruginosa*.

**AlgR activates cyanide production in mucoid CF clinical isolates and represses HCN production in nonmucoid CF isolates.** In order to assess the role of AlgR in clinical CF isolates on HCN production, *algR* was deleted from the well-characterized mucoid CF isolate FRD1 (Fig. 4B). The amount of cyanide produced by the mucoid CF isolate FRD1 was compared with its isogenic *algR* mutant which revealed that FRD1 *algR* produced only 1.05 μmol of HCN/mg of protein, an amount eightfold lower than that for FRD1 (Fig. 4C). Interestingly, among CF clinical isolates, there were nonmucoid isolates that produced copious amounts of HCN (for example, TUMC92, 5.5 μmol of HCN/mg of protein, and TUMC197, 6.25 μmol of HCN/mg of protein) despite their nonmucoid phenotype. Thus, to ascertain the relevance of AlgR in regulating HCN production in nonmucoid clinical CF isolates, isogenic *algR* mutants were constructed for the nonmucoid hypercyanogenic clinical isolates TUMC92 and TUMC197 (Fig. 4B) and cyanide production was measured. We observed a threefold increase in cyanide production in the clinical *algR* mutant TUMC-197R and a 1.8-fold increase in cyanide production from the *algR* clinical isolate TUMC-92R (Fig. 4C). These results are consistent with our observation that AlgR repressed HCN production in the wild-type nonmucoid *P. aeruginosa* strain PAO1 (30). These results provide strong evidence that AlgR activates cyanide production in mucoid CF clinical isolates and represses its production in nonmucoid isolates.

**DISCUSSION**

The effects of HCN have been examined on many cell types (2, 24, 41). In these studies, HCN exposure resulted in neuronal necrosis (41) and inhibition of metalloenzymes including cytochrome *c* oxidase (49). One report of KCN exposure to the immortalized epithelial lung A549 cells resulted in double-stranded DNA breaks below 0.5 Mbp, indicating that endog-
nous nuclease activity was induced in a dose-dependent manner after KCN exposures (50). These data suggest that A549 cells exposed to 1 mM KCN may undergo necrosis. Cell death via necrosis results in increased inflammation and infiltration of polymorphonuclear leukocytes to the site of infection. Excess inflammation due to migration of polymorphonuclear leukocytes and lung necrosis are pathological hallmarks of CF airway infection (1).

In this study, we quantified the amount of HCN produced by mucoid and nonmucoid laboratory and CF isolates of *P. aeruginosa* and showed that AlgR, a transcriptional regulator of alginate biosynthesis (11, 38–40) and twitching motility (51, 52), plays a central role in controlling HCN production. Although HCN-producing *P. aeruginosa* strains have been isolated from burn patients (22), the only evidence that HCN may be a virulence factor in *P. aeruginosa* stems from an experimental infection model utilizing *C. elegans* (18). Since at physiological pH and ambient temperatures and above, cyanide exists predominantly as volatile HCN gas, it is possible to envision that HCN generated by *P. aeruginosa* CF strains could diffuse into the environment through exhaled breath with no deleterious affects. However, a recent report (19) describing the detection of cyanide in CF patient sputum could be interpreted as indicating that cyanide, in the form of either gaseous HCN or soluble cyanide (CN−), remains in the thick mucus layer harboring *P. aeruginosa* biofilms, thus being potentially available for diffusion into surrounding lung epithelial cells.

The conditions in *P. aeruginosa*-infected lung tissue appear to be optimal for cyanide production given that the thick CF mucus (48, 55, 59) provides an ideal environment for the growth of cells to high cell densities as a biofilm under microaerophilic conditions, processes controlled in part by quorum sensing (43, 44). Interestingly, there are two reported promoters for the *hcnA* gene, T1, controlled by quorum-sensing regulators alone, and T2, which appears to rely on a synergistic action of LasR, RhlR, and ANR (Fig. 2C) (43). Five regulatory proteins have been identified for the *hcnA* promoter: GacA (45), ANR (45, 62), LasR, RhlR (43), and RsmA (42). The global regulator GacA positively controls HCN synthesis as well as other secondary metabolites and exoenzymes (45). *P. aeruginosa* gacA or *anr* mutants produce very little HCN (45, 62). One positive regulator of anaerobic respiration, ANR, is required for anaerobic growth of *P. aeruginosa* (58). LasR and RhlR are quorum-sensing regulators required for transcription of the *hcnA* promoter (43). RsmA (regulator of
secondary metabolites) functions as a pleiotropic posttranscriptional regulator of HCN synthesis directly and also indirectly by negatively regulating the amounts of quorum sensing N-acylhomoserine lactones (42). Our data provide strong evidence that AlgR also regulates hcnA transcription by affecting both T1 and T2 promoters, indicating that AlgR and LasR and/or RhlR and ANR coordinate to regulate these promoters. Moreover, we have discovered through transcriptional profiling experiments that there are other genes (algM, PA1557, and arcD) of the AlgR regulon that are coregulated by AlgR and ANR (30). Further studies are needed to elucidate the mechanism by which these transcriptional regulators may potentially interact.

Under anaerobic growth conditions, P. aeruginosa is able to produce alginate and maintain its production (25, 48, 56, 59). Our survey of both laboratory and CF isolates demonstrated that mucoid P. aeruginosa produced more HCN than nonmucoid strains. These findings are consistent with the fact that environmental conditions favoring cyanogenesis by P. aeruginosa also favor alginate production (25, 48, 55, 59). Alginate is considered to be a major virulence factor in the CF lung (23) with the first committed step of biosynthesis being the transcription of algD, which is activated in mucoid P. aeruginosa (12). Transcription of algD requires AlgR as well as the alternative sigma factor AlgU (AlgT) (11, 14, 26, 34, 35, 38, 39, 46). AlgR is currently known as a transcriptional activator of the algD (11, 38–40) and algC (61) promoters and is required for type IV pilus function (51, 52). The switch from the nonmucoid to the mucoid phenotype in P. aeruginosa is known to involve mutations in mucA or mucB, resulting in activation of AlgU (AlgT) (20, 33, 34, 36, 57) and subsequently AlgR (35).

Recently, it was shown that AlgR is capable of acting as a repressor of transcription on the hcnA promoter and HCN production in the nonmucoid strain PAO1 (30). Results from our present study indicate that AlgR is an activator of hcnA transcription and HCN production in both mucoid laboratory and CF clinical isolates. Taken together, these results strongly suggest that AlgR is capable of switching from a repressor in the nonmucoid phenotype to an activator in mucoid P. aeruginosa (Fig. 5). This interpretation is consistent with results demonstrating that the switch from nonmucoid to mucoid phenotypes involving mutations in mucA or mucB results in activation of AlgU (AlgT) (20, 33, 34, 36, 57) and also AlgR (35). The resultant activation of AlgR may be due to increased levels or posttranslational modifications; however, our data do not discern between these two possible mechanisms for AlgR activation of cyanide production. There is evidence that phosphorylation of AlgR is required for twitching motility (52), yet the kinase for AlgR has not been identified. The protein involved in the ability of AlgR to switch from a repressor to an activator of twitching motility may be AlgZ/FimS, as an insertion inactivation of algZ/fimS results in the loss of twitching motility (50). We have preliminary evidence indicating that AlgZ/FimS is also involved in activation of AlgR in mucoid P. aeruginosa, consistent with the findings on AlgZ/FimS in regards to the twitching motility phenotype.

Because cyanide could have extremely potent detrimental affects on respiratory epithelial cells, novel strategies for the future treatment of CF airway disease may involve inhibitors of both the quorum sensing and AlgR regulatory cascades, both of which are required for cyanide biosynthesis in P. aeruginosa.

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