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Identification of AlgR-Regulated Genes in *Pseudomonas aeruginosa* by Use of Microarray Analysis

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The *Pseudomonas aeruginosa* transcriptional regulator AlgR controls a variety of different processes, including alginate production, type IV pilus function, and virulence, indicating that AlgR plays a pivotal role in the regulation of gene expression. In order to characterize the AlgR regulon, *Pseudomonas* Affymetrix GeneChips were used to generate the transcriptional profiles of (i) *P. aeruginosa* PAO1 versus its *algR* mutant in mid-logarithmic phase, (ii) *P. aeruginosa* PAO1 versus its *algR* mutant in stationary growth phase, and (iii) PAO1 versus PAO1 harboring an *algR* overexpression plasmid. Expression analysis revealed that, during mid-logarithmic growth, AlgR activated the expression of 58 genes while it repressed the expression of 37 others, while during stationary phase, it activated expression of 45 genes and repression of 14 genes. Confirmatory experiments were performed on two genes found to be AlgR repressed (*hcnA* and *PA1557*) and one AlgR-activated operon (*fimU-pilVWXY1Y2*). An S1 nuclease protection assay demonstrated that AlgR repressed both known *hcnA* promoters in PAO1. Additionally, direct measurement of hydrogen cyanide (HCN) production showed that *P. aeruginosa* PAO1 produced threefold-less HCN than did its *algR* deletion strain. AlgR also repressed transcription of two promoters of the uncharacterized open reading frame *PA1557*. Further, the twitching motility defect of an *algR* mutant was complemented by the *fimTU-pilVWXY1Y2E* operon, thus identifying the AlgR-controlled genes responsible for this defect in an *algR* mutant. This study identified four new roles for AlgR: (i) AlgR can repress gene transcription, (ii) AlgR activates the *fimTU-pilVWXY1Y2E* operon, (iii) AlgR regulates HCN production, and (iv) AlgR controls transcription of the putative cbb_3-type cytochrome PA1557.

Pseudomonas aeruginosa is a gram-negative, opportunistic pathogen capable of causing acute septicemia in patients with severe burns or severe immunodeficiency and chronic pneumonia in individuals with the genetic disease cystic fibrosis (CF) (5). The chronic pneumonia caused by *P. aeruginosa* is the leading cause of morbidity and mortality of CF patients (24). The mucoid phenotype of *P. aeruginosa*, characterized by production of the exopolysaccharide alginate, is almost exclusively associated with chronic CF pneumonia (10, 15, 26, 43, 60). Alginate, composed of a linear copolymer of β-D-mannuronic and α-L-guluronic acids (17, 30, 31), confers a selective advantage on *P. aeruginosa* in the CF patient lung. Alginate insulates the bacterium from killing mechanisms of phagocytes such as hypochlorite (52–54) and prevents phagocytosis of *P. aeruginosa* by neutrophils and macrophages (43). Because of the selective advantage that mucoidy confers on *P. aeruginosa*, the mechanism of alginate production has been studied extensively (26).

Alginate production is tightly controlled by a number of transcriptional regulators (26). One alginate regulatory system involves the MucA, MucB, MucC, and MucD proteins (6, 36, 38, 51) and their regulation of the sigma factor AlgU (AlgT) (27, 37, 61). Two studies examining clinical CF isolates from different locations found that a large percentage of the mucoid strains had *mucA* mutations. The first study, using CF isolates from North America and Europe, reported that 84% of mucoid isolates tested contained a mutation in *mucA* (7), while the second study, using CF strains isolated in Australia, found that 22 of the 50 (44%) mucoid strains tested contained *mucA* mutations (4). Mutations in *mucA* prevent MucA, an anti-sigma factor, from binding to AlgU, thus allowing AlgU to initiate transcription of *algD* and subsequently the alginate biosynthetic operon (36, 51). Additionally, the periplasmic protein MucB (51) is required for alginate regulation since mutations in *mucB* also cause the conversion of *P. aeruginosa* from a nonmucoid to a mucoid phenotype (35).

The transcriptional regulator AlgR is also required for alginate production (13). AlgR is a member of the LytTR family of two-component transcriptional regulators (12, 41). AlgR regulates alginate production by binding to three sites within the *algD* promoter, thereby activating transcription (39, 40). It has been proposed that AlgR causes a looping of the *algD* promoter that is required for transcriptional activation (50). Additionally, AlgR regulates alginate production through *algC* by binding to its promoter (62, 63). AlgC is a bifunctional enzyme with both phosphomannomutase and phosphoglucomutase activity (9) that is required not only for alginate production (62) but also for rhamnolipid production (42) and lipopolysaccha-

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TABLE 1. *P. aeruginosa* strains and plasmids

Strain or plasmid	Genotype	Reference or source
P. aeruginosa strains		
PAO1	Wild type	B. Holloway
PSL317	Δ*algR*	This study
Plasmids		
pCMR7	pVD*tac*24 *algR*	39
pVDZ′2R	pVDZ′2R *algR*	32
pCVD442	ori RSK *mob sacB* Ap^r	16
pRKO442	pCVD442 Δ*algR*	This study
pVD*tac*39	IncQ/P4 mob^+ *tac* $lacI^q$	11
pVD*tac*PIL	pVD*tac*39 *fimT fimU pilV pilW pilX pilY1 pilY2 pilE*	This study

ride (LPS) expression (9). AlgR binds to three positions within the *algC* promoter region (21, 63), yet the orientation and positioning of the AlgR binding sites differ in the *algC* and *algD* promoters. The differences in orientation of the AlgR binding sites and of the AlgR binding affinities between the *algC* and *algD* promoters and their effects on the mechanism of AlgR regulation in vivo have not been clearly established.

More recent studies have indicated that AlgR regulates additional genes besides those required for alginate production. AlgR has been shown to be required for twitching motility (59), a type of surface motility utilizing type IV pili. Recently we have shown that AlgR is required for virulence in an acute septicemia mouse model. This study also demonstrated that the cellular concentrations of AlgR are critical for proper virulence since the overexpression of AlgR in PAO1 made the organism avirulent in a murine septicemia model (32). Taken together, these studies expanded AlgR's role in virulence beyond its known role as a regulator of alginate production and implied a possible role for AlgR in acute *P. aeruginosa* infections as well.

While *algR* mutations have been documented to impact the phenotypes of twitching motility and reduced virulence, the genes that AlgR regulates in these processes have not been identified. The determination of the genomic sequence of the laboratory wild-type strain *P. aeruginosa* PAO1 (55) facilitated the development of an Affymetrix GeneChip microarray for *P. aeruginosa* PAO1. In this study, we used the *P. aeruginosa* Affymetrix GeneChip array to examine the expression profiles of nonmucoid *P. aeruginosa* strains grown to mid-log and early stationary phases and used AlgR overexpression in PAO1 to identify genes regulated by AlgR.

MATERIALS AND METHODS

Genetic manipulations. *P. aeruginosa* strains and plasmids used in this study are listed in Table 1. The *algR* mutation in PSL317 was generated by crossover PCR deleting all but 30 bp of *algR*. Briefly, two initial PCRs were done, the first using the primers argHF (5′-ATATATGAGCTCGGACCTGTCCGACCTGTTCC-3′) and MR5 (5′-CGTCGTATGCATCAGCTCTGA-3′) and the second using the primers MR6 (5′-GAGCTGATGCATACGACGCAGGACATTCATAAGCTCAGC-3′) and hemCR (5′-ATATATGAGCTCGGCTGGCGTAGGTGTTCGAG-3′). The products of these reactions contain complementary sequence (the 5′ end of primer MR6 is complementary to primer MR5) that allow for their use as the template for a subsequent crossover PCR, using argHF and hemCR as the primers. This product was cloned into pCR2.1 (Invitrogen) and subsequently subcloned into the suicide plasmid pCVD442 (16), creating pRKO442. Plasmid pRKO442 was moved into PAO1 by triparental conjugation as previously described (28), and exconjugates were initially selected for plasmid insertion by carbenicillin resistance and then for plasmid recombination by sucrose resistance. Potential recombinants were tested by PCR using the primers argHF and hemCR, and *algR* deletion was confirmed by Southern blotting (data not shown).

For the complementation of the twitching motility defect, a PCR fragment containing the operon *fimTU-pilVWXY1Y2E* was generated using the oligonucleotides fimTF (5′-ATATATGAGCTCAAGTCCCGCGACCAGTGCG-3′) and pilER (5′-ATATATGAGCTCCTGGTTCGACGGTGTGCG-3′) and the proofreading polymerase Expand HiFi (Roche). This fragment was cloned into pCR2.1 (Invitrogen) and then subcloned into pVD*tac*39 (11), creating the plasmid pVD*tac*PIL. The orientation of the insert relative to the *tac* promoter was determined by HindIII digest.

RNA isolation and preparation for Affymetrix GeneChip analysis. For mid-logarithmic-phase growth experiments, five independent replicates of *P. aeruginosa* strains PAO1 and PSL317 (PAO1 Δ*algR*) were grown in 100 ml of Luria-Bertani (LB) broth in a 250-ml baffled flask vigorously shaken at 37°C to an optical density at 600 nm (OD_{600}) of 0.4. For the AlgR overexpression experiments, three independent replicates of PAO1 harboring the plasmid pCMR-7 were grown in the presence of 300 μg of carbenicillin/ml to an OD_{600} of 0.2, at which time IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 1 mM. The culture was then grown to an OD_{600} of 0.4. For the stationary-phase experiments, three independent replicates of PAO1 and PSL317 were grown in 100 ml of LB broth in a 250-ml baffled flask vigorously shaken at 37°C to an OD_{600} of 0.6. After the cultures were chilled in a dry ice-ethanol bath to stop RNA synthesis, approximately 10^9 bacteria were removed (1 ml for the mid-log cultures, 0.5 ml for the stationary-phase cultures), collected by centrifugation (8,000 × *g* for 5 min at 4°C), resuspended in Tris-EDTA with 1 mg of lysozyme/ml, and incubated for 5 min at room temperature. Total RNA was isolated using the RNeasy minikit (Qiagen) per the manufacturer's instructions.

The quality of the RNA was assessed on an Agilent Bioanalyzer 2100 electrophoretic system pre- and post-DNase treatment (Fig. 1). The RNA was treated with 2 U of DNase I (Ambion) for 15 min at 37°C to remove contaminating DNA. The reaction was stopped by the addition of 25 μl of DNase stop solution (50 mM EDTA, 1.5 M sodium acetate, and 1% sodium dodecyl sulfate). The DNase I was removed by phenol-chloroform extraction followed by ethanol precipitation. Ten micrograms of total RNA was used for cDNA synthesis, fragmentation, and labeling according to the Affymetrix GeneChip *P. aeruginosa* genome array expression analysis protocol. Briefly, random hexamers (Invitrogen) were added (final concentration, 25 ng/μl) to the 10 μg of total RNA along with in vitro-transcribed *Bacillus subtilis* control spikes (as described in the Affymetrix GeneChip *P. aeruginosa* genome array expression analysis protocol). cDNA was synthesized using Superscript II (Invitrogen) 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. RNA was removed by alkaline treatment and subsequent neutralization. The cDNA was purified with use of the QIAquick PCR purification kit (Qiagen) and eluted in 40 μl of buffer EB (10 mM Tris-HCl, pH 8.5). The cDNA was fragmented by DNase I (0.6 U/μg of cDNA; Amersham) at 37°C for 10 min and then end labeled with biotin-ddUTP with use of the Enzo BioArray Terminal Labeling kit (Affymetrix) at 37°C for 60 min. Proper cDNA fragmentation and biotin labeling were determined by gel mobility shift assay with NeutrAvadin (Pierce) followed by electrophoresis through a 5% polyacrylamide gel and subsequent DNA staining with SYBR Green I (Roche).

Microarray data analysis. Microarray data were generated using Affymetrix protocols. Absolute expression transcript levels were normalized for each chip by globally scaling all probe sets to a target signal intensity of 500. Three statistical algorithms (detection, change call, and signal log ratio) were then used to identify differential gene expression in experimental and control samples. The detection metric (presence, absence, or marginal) for a particular gene was determined using default parameters in MAS software (version 5.0; Affymetrix). Batch analysis was performed in MAS to make pairwise comparisons between individual experimental and control GeneChips in order to generate change calls and a signal log ratio for each transcript. These data were imported into Data Mining Tools (version 3.0; Affymetrix) via an Affymetrix Laboratory Information Management System. Transcripts that were absent under both control and experimental conditions were eliminated from further consideration. Statistical significance of signals between the control and experimental conditions ($P < 0.05$) for individual transcripts was determined using the *t* test. We defined a positive change call as one in which greater than 50% of the transcripts had a call of increased (I) or marginally increased (MI) for up-regulated genes and decreased (D) or marginally decreased (MD) for down-regulated genes. Finally,

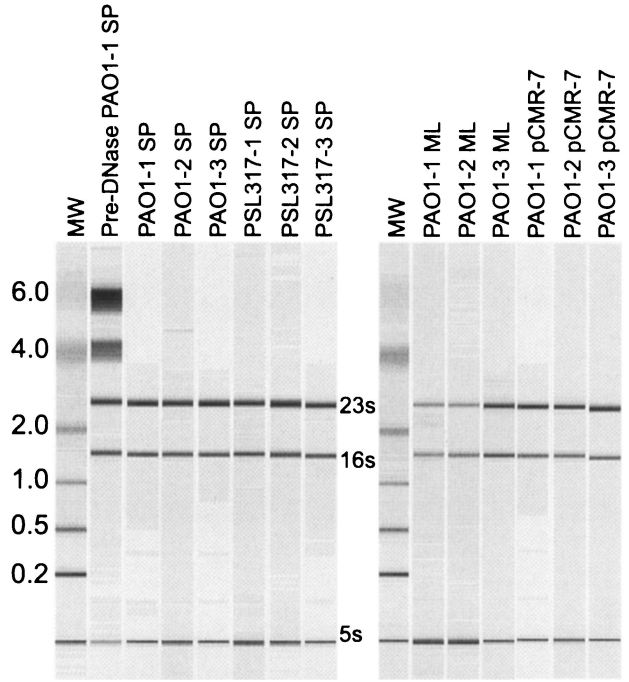

FIG. 1. Agilent electrophoretograms of RNA used to generate cDNA hybridized to the Affymetrix *Pseudomonas* GeneChips. Shown are the elctrophoretograms obtained from an Agilent Bioanalyzer 2100 on RNA samples for two of the three conditions examined in this study. MW, molecular size standards, in kilobases. Pre-DNase PAO1-1 SP, total RNA sample from *P. aeruginosa* PAO1 grown to stationary phase before treatment with DNase. PAO1-1 SP, total RNA sample from *P. aeruginosa* PAO1 grown to stationary phase after DNase treatment. PAO1-2 SP and PAO1-3 SP, independent replicates of total RNA from stationary-phase-grown *P. aeruginosa* PAO1 after DNase treatment. PSL317-1 SP, PSL317-2 SP, and PSL317-3 SP, three independent replicates of total RNA samples from *P. aeruginosa* PSL317 (Δ*algR*) grown to stationary phase after DNase treatment. PAO1-1 ML, PAO1-2 ML, and PAO1-3 ML, total RNA samples from three independent replicates of *P. aeruginosa* PAO1 grown to mid-logarithmic phase after DNase treatment. PAO1-1 pCMR-7, PAO1-2 pCMR-7, and PAO1-3 pCMR-7, total RNA samples from three independent replicates of *P. aeruginosa* PAO1 harboring the AlgR overexpression plasmid pCMR-7 grown to mid-logarithmic phase after DNase treatment.

the mean value of the signal log ratios from each comparison file was calculated. Only those genes that met the above criteria and had a mean signal log ratio of ≥ 1 for up-regulated transcripts and ≤ 1 for down-regulated transcripts were kept in the final list of genes. Signal log ratio values were converted from $\log_2$ and expressed as fold changes. The original raw data files have been deposited in the Cystic Fibrosis Foundation Therapeutics, Inc.-Genomax shared workspace. These files are not publicly available.

Genome searches. The PAO1 genome sequence was obtained from the *Pseudomonas* Genome Project (www.pseudomonas.com) (55). Sequence data were imported into MacVector (version 7.0; Eastman Kodak) for analysis. The subsequence search of the PAO1 genome was done using the known AlgR binding sequences (CCGTTCGTC, CCGTTTGTC, CCGTTGTTC, or CCGTG CGTC), allowing up to two mismatches. Gene function information was obtained from the PseudoCap annotation project (www.pseudomonas.com).

Twitching motility assay. Twitching motility was determined as previously described (1). Briefly, overnight cultures of *P. aeruginosa* strains PAO1, PSL317 in LB medium, and PSL317(pVD*tac*PIL) grown in LB medium with 300 μg of carbenicillin/ml were stabbed through a twitching motility plate (1% tryptone, 0.5% yeast extract, 1% NaCl, 1% agar) supplemented with 1 mM IPTG. The plates were incubated at 37°C for 48 h, at which time the agar was removed, the bacteria attached to the plate were stained with crystal violet, and the diameter of the zone of twitching was measured.

Western blot analysis. *P. aeruginosa* strains PAO1, PSL317, and PSL317(pVD*tac*PIL) were grown in LB medium with aeration to mid-log phase (OD_{600} of 0.4). The stains were collected by centrifugation (6,000 × *g* for 2 min), washed, resuspended in 50 mM Tris-HCl (pH 8.0)–150 mM NaCl, and then lysed by sonication. Protein extracts (25 μg) were separated on a 4 to 20% gradient polyacrylamide gel (Invitrogen) and then electroblotted onto a polyvinylidene difluoride membrane. The membrane was probed with an anti-AlgR monoclonal antibody (14), detected using a horseradish peroxidase-conjugated goat anti-mouse monoclonal antibody (Bio-Rad Laboratories), and developed using Opti-4CN (Bio-Rad Laboratories).

S1 nuclease protection assay and primer extension analysis. The RNA for the S1 nuclease protection assay was isolated from mid-log-phase-grown PAO1 and PSL317 with the use of CsCl as previously described (37). The S1 nuclease protection assay was performed as previously described (37) with the following modifications. The *hcnA* 354-bp promoter region ranging from −330 to +24 (numbering relative to translational start site) was cloned into M13mp18. Single-stranded phage were isolated and used as the template for the uniformly labeled ([α-^{32}P]dCTP) single-stranded DNA probe generated using the oligonucleotide hcnAprimext (5′-GTGTTGACGACGTTCAAGAAGGTGCAT-3′). The probe was digested using BglI and purified on a 5% polyacrylamide gel. The S1 nuclease reaction was performed as previously described (37) with the use of 50 μg of RNA from each strain. The sequencing ladder was generated using the same primer that was used to make the probe.

For the primer extension assay on the *PA1557* promoter, RNA was isolated from PAO1, PSL317, and PAO1(pCMR7) grown as described above with the RNeasy minikit (Qiagen). The primer extension was done as previously described (18) with slight modifications. Briefly, the PA1557R′ primer (5′-GCGG ACCACCTTGTAGTTATAGGCG-3′) was end labeled with [γ-^{32}P]ATP with the use of polynucleotide kinase and purified through a G-25 spin column (Amersham). The primer was hybridized to 10 μg of total RNA in hybridization buffer (0.5 M KCl, 0.24 M Tris-HCl [pH 8.3]), incubated at 95°C for 1 min and 55°C for 2 min, and then placed on ice for 15 min. Superscript II (Invitrogen) was added, and the primers were extended according to the manufacturer's protocol. The extension reaction mixtures were loaded next to a sequencing ladder generated using the same primer.

HCN quantification. Hydrogen cyanide (HCN) produced by *P. aeruginosa* strains was quantified using the protocol of Gallagher and Manoil (23) with slight modifications. In brief, strains PAO1, PSL317, PAO1(pCMR7), and PSL317(pVDZ′2R) were grown on *Pseudomonas* isolation agar (Difco) for 24 h at 37°C and then enclosed without the lid in individual sealed plastic bags that contained 1 ml of 4 M NaOH. After 4 h of incubation, the NaOH was diluted to 0.09 M to bring it within linear range of a KCN standard curve. Then 105-μl aliquots of the samples were mixed with 350 μl of a 1:1 mixture of 0.1 M *O*-dinitrobenzene (ACROS) in ethylene-glycol monoethyl ether (ACROS) and 0.2 M *p*-nitrobenzaldehyde (ACROS) in ethylene-glycol monoethyl ether. After 30 min of incubation at room temperature, the OD_{578} was measured as previously described (23) and the HCN produced by each strain was quantified by comparison with a KCN standard curve.

RESULTS

Transcriptional profile of the AlgR regulon in *P. aeruginosa* PAO1 during mid-log growth phase. We have recently shown that AlgR is essential for virulence of nonmucoid *P. aeruginosa* in an acute murine septicemia model (32). Since PAO1 does not produce significant amounts of alginate and since LPS was unaffected in the *algR* mutant strain PAO700 (*algR*::Gm^r), the known AlgR-regulated genes, *algD* and *algC*, probably were not the reason for the differences in virulence observed between PAO1 and PAO700 (*algR*::Gm^r). Furthermore, this study also suggested that AlgR regulates genes involved in *P. aeruginosa* response to oxidative stress (32). Therefore, in order to determine the extent of the AlgR regulon, we initiated a microarray study using the Affymetrix *P. aeruginosa* GeneChip Array. In the initial analysis, RNA was isolated from *P. aeruginosa* grown in LB medium to mid-logarithmic growth phase, mimicking the same conditions that were used in our

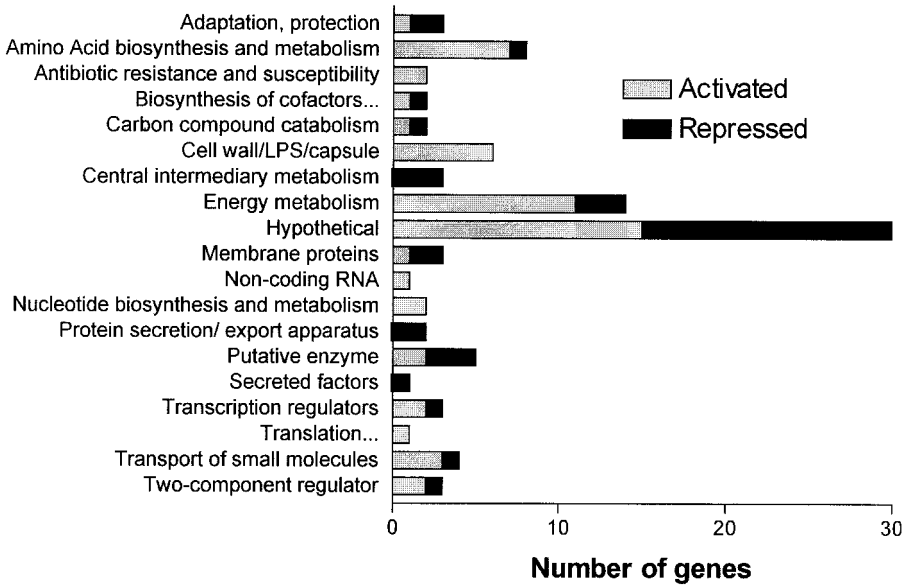

FIG. 2. Functional classes of AlgR-regulated genes from mid-logarithmic growth phase. The genes were identified as either activated or repressed by AlgR with the use of the comparison of gene expression in PAO1 compared to PSL317 (Δ*algR*) grown as described in Materials and Methods. All genes that had a significant difference in expression ($P < 0.05$ as determined by *t* test) are included. Functional classes were determined using the *Pseudomonas* Genome Project website (www.pseudomonas.com) on 14 January 2004.

previous virulence study (32). In order to identify the AlgR-regulated genes, transcripts from the wild-type strain PAO1 were compared to those of its isogenic *algR* in-frame deletion mutant PSL317 (Δ*algR*) (see Materials and Methods). Here, AlgR activated the expression of 58 genes (Fig. 2) that fall into 16 of the functional classes used by the *Pseudomonas* Genome Project. Most of the AlgR-activated genes in mid-log phase (15) are categorized as hypothetical, uncharacterized, or unknown, but AlgR also activates a number of genes involved in (i) energy metabolism (11 genes), (ii) amino acid biosynthesis and metabolism (7 genes), (iii) cell wall-LPS-capsule production (6 genes), and (iv) the transport of small molecules (3 genes). These data indicate that AlgR may be required for the global expression of genes in *P. aeruginosa* and not just as a regulator for the specific pathways of alginate production and twitching motility.

A previous examination of protein expression in PAO1 and PAO700 (*algR*::Gm^r) by two-dimensional gel electrophoresis found that 17 proteins were repressed by AlgR (32). Consistent with these results were those of the transcriptional analysis study comparing PAO1 and PSL317 (Δ*algR*), whereby AlgR was found to repress expression of 37 genes during mid-logarithmic growth (Fig. 2). Two functional classes were identified as having the largest number of both AlgR-activated and AlgR-repressed genes: energy metabolism (11 activated, 5 repressed) and amino acid biosynthesis and metabolism (7 activated, 4 repressed). Table 2 includes a list of these genes that showed the highest degree of AlgR regulation, defined as greater-than-threefold activation or repression. Overall, the analysis of the microarray data suggests that AlgR is capable of affecting diverse functions in *P. aeruginosa* through either activation or repression.

Transcriptional profile of the AlgR regulon in *P. aeruginosa* PAO1 during stationary growth phase. In *P. aeruginosa* the sigma factor RpoS has been shown to regulate a number of virulence factors in stationary phase, including two which are AlgR regulated: alginate production and type IV pili (56). With this in mind, we hypothesized that AlgR acts as a regulator not only in mid-logarithmic phase as shown in this study but also in stationary phase. We therefore compared the transcriptional profiles from PAO1 and PSL317 (Δ*algR*) grown to early stationary phase in LB broth. Our analysis identified 45 genes that are activated by AlgR when *P. aeruginosa* enters stationary phase (Fig. 3). These genes fell into 14 functional classes with the majority (12 genes) of the genes categorized as hypothetical, followed by putative enzymes (6 genes), genes encoding putative membrane proteins (6 genes), genes involved in motility and attachment (6 genes), and genes encoding proteins for the transport of small molecules (5 genes).

The analysis of PAO1 and PSL317 gene expression from stationary phase also identified 14 genes that are repressed by AlgR in early stationary phase (Fig. 3), once again indicating that AlgR activated and repressed gene expression. These genes were spread fairly evenly among eight functional classes. The genes from this study which had a greater-than-threefold activation or repression by AlgR are listed in Table 3. All but two of the most highly AlgR-regulated genes are activated by AlgR conditionally in early stationary phase. Other than *algR* itself, only one open reading frame (ORF), *PA3472*, encoding a hypothetical protein, was identified as highly AlgR regulated in both mid-logarithmic growth (Table 2) and early stationary phase (Table 3). The large number of AlgR-regulated genes that do not overlap between mid-log and stationary phases indicates that AlgR may regulate two independent sets of genes under these conditions.

Transcriptional profile of the AlgR regulon in *P. aeruginosa* PAO1 overexpressing AlgR. It is known that AlgR expression is increased during mucoid growth due to AlgU-initiated ex-

TABLE 2. Genes that had a greater-than-threefold AlgR activation or repression in PAO1 during mid-logarithmic growth

PA no.	Gene name	Fold activation[a]	*P* value[b]	Protein description[c]
PA0141		−4.2	0.049	Conserved hypothetical protein
PA0171		3.5	0.020	Hypothetical protein
PA1127		−4.1	0.001	Probable oxidoreductase
PA1414		−9.4	0.004	Hypothetical protein
PA1429		−3.4	0.020	Probable cation-transporting P-type ATPase
PA1546	*hemN*	−5.0	0.030	Oxygen-independent coproporphyrinogen III oxidase
PA1555		−7.4	0.025	Probable cytochrome *c*
PA1556		−8.3	0.041	Probable cytochrome *c* oxidase subunit
PA1557		−4.4	0.042	Probable cytochrome oxidase subunit (cbb_3 type)
PA2119		−7.5	0.019	Alcohol dehydrogenase (Zn dependent)
PA2194	*hcnB*	−3.0	0.047	HCN synthase HcnB
PA2491		14.3	<0.001	Probable oxidoreductase
PA2493	*mexE*	7.5	<0.001	RND[d] multidrug efflux membrane fusion protein MexE precursor
PA2494	*mexF*	17.3	<0.001	RND multidrug efflux transporter MexF
PA3278		−8.0	0.030	Hypothetical protein
PA3309		−6.8	0.041	Conserved hypothetical protein
PA3458		−3.0	0.022	Probable transcriptional regulator
PA3472		3.7	<0.001	Hypothetical protein
PA3582	*glpK*	−4.0	0.032	Glycerol kinase
PA4607	*oprG*	−8.0	0.028	Outer membrane protein OprG precursor
PA4348		−4.4	0.040	Conserved hypothetical protein
PA4352		−4.5	0.024	Conserved hypothetical protein
PA4354		6.1	0.026	Conserved hypothetical protein
PA4577		−4.6	0.023	Hypothetical protein
PA4613	*katB*	3.2	0.017	Catalase
PA4916		−3.2	0.048	Probable *c*-type cytochrome
PA5171	*arcA*	−6.5	0.035	Arginine deiminase
PA5261	*algR*	13.8	<0.001	Alginate biosynthesis regulatory protein AlgR
PA5497		−3.2	0.004	Hypothetical protein
PA5506		−4.4	0.022	Hypothetical protein
PA5507		−5.4	0.006	Hypothetical protein
PA5508		−5.6	0.030	Probable glutamine synthetase
PA5509		−7.3	0.021	Hypothetical protein

[a] Fold activation was determined by comparing the transcription of PAO1 to that of its isogenic *algR* mutant PSL317. Five replicates of each strain were used for the comparison. Genes whose transcription was higher in PAO1 than in PSL317 were called AlgR activated and are represent by a positive fold activation value. Genes whose transcription was higher in PSL317 than in PAO1 were called AlgR repressed and are represented with a negative fold activation value. Only genes with a statistically significant difference in transcription ($P < 0.05$) between PAO1 and PSL317 and a greater-than-threefold AlgR activation or repression are shown.

[b] *P* values were determined by Student's *t* test with Data Mining Tools (Affymetrix).

[c] Protein descriptions are from the *Pseudomonas* Genome Project website (www.pseudomonas.com).

[d] RND, resistance-nodulation-division.

pression (37). Additionally, overexpression of *algR* in PAO1 (the nonmucoid background) renders PAO1 avirulent in a mouse septicemia model (32), thus indicating that the relative amounts of AlgR may be critical for gene regulation. In order to determine how these levels of AlgR affect gene expression, we compared the expression profiles of PAO1 and PAO1 overexpressing *algR* from the plasmid pCMR7. Our analysis indicated that overexpression of AlgR activated the expression of 312 genes and repressed the transcription of 573 genes (Fig. 4). The majority (41%) of these genes were uncharacterized or hypothetical genes. A large number of transcriptional regulators and two-component regulatory systems were identified as AlgR regulated, both activated and repressed, when AlgR was overexpressed. Thus, the large number of genes that were identified as AlgR regulated is likely in part due to indirect AlgR regulation through a second transcriptional regulator.

The most highly AlgR-regulated genes were identified by combining genes whose transcription was either activated or repressed threefold by AlgR during overexpression and in mid-logarithmic or stationary phase (Table 4). Only 16 genes met these criteria (Table 4). Only one gene, *PA1048*, was shared between the lists of highly AlgR-regulated genes in stationary phase and during AlgR overexpression. The remaining genes were highly regulated by AlgR in mid-log growth and during AlgR overexpression. AlgR repressed the transcription of 11 genes during both mid-log growth and AlgR overexpression, indicating that the presence of AlgR may be enough to repress transcription.

Identification of AlgR-regulated twitching motility genes in *P. aeruginosa*. AlgR regulates twitching motility (59); however, the specific pilus gene(s) that AlgR controls has not been identified. We compared the *P. aeruginosa* Affymetrix GeneChip expression profiles of mid-log- and stationary-phase-grown PAO1 and PSL317 (Δ*algR*) and of PAO1 and a PAO1 AlgR-overproducing strain in order to identify known pilus genes that may be AlgR regulated. Surprisingly, our initial analysis comparing PAO1 and PSL317 in the mid-log growth phase identified no known pilus genes. However, the expression profiles of PAO1 and PSL317 grown to early stationary phase did identify a single operon consisting of the genes *fimU*,

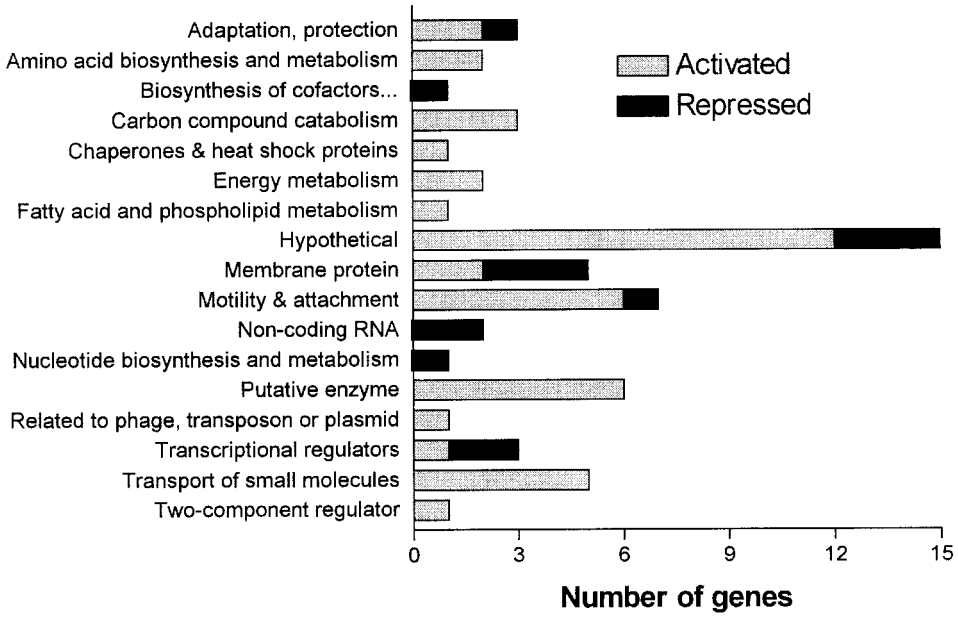

FIG. 3. Functional classes of AlgR-regulated genes from early stationary phase. The genes were identified as either activated or repressed by AlgR with use of the comparison of gene expression in PAO1 and in PSL317 (Δ*algR*) grown as described in Materials and Methods. All genes that had a significant difference in expression ($P < 0.05$ as determined by *t* test) are included. Functional classes were determined using the *Pseudomonas* Genome Project website (www.pseudomonas.com) on 14 January 2004.

pilV, *pilW*, *pilX*, *pilY1*, and *pilY2* as potentially activated by AlgR (Table 5). The fold changes varied from 8.46 for *pilX* to 2.20 for *fimU* and *pilV*.

Since *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, and *pilY2* were identified as among the most highly activated AlgR-regulated genes, the entire operon consisting of *fimT*, *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, *pilY2*, and *pilE* was cloned into the plasmid pVD*tac*39 (pVD*tac*PIL) and tested for its ability to complement the twitching motility defect in the *algR* deletion strain PSL317. PSL317 harboring the plasmid pVD*tac*PIL had approximately the same zone of twitching motility as did PAO1 (Fig. 5A and B). A Western blot assay to detect AlgR in PAO1, PSL317, and PSL317 harboring pVD*tac*PIL confirmed that the complementation of the twitching motility defect was due to the genes introduced on the plasmid and not due to an effect by AlgR (Fig. 5C). While PAO1 did produce a statistically larger twitching motility zone (Fig. 5B), the near-complete complementation of the twitching motility defect indicates that the genes included in this operon are the genes responsible for the twitching motility defect seen in an *algR* mutant.

TABLE 3. Genes that had a greater-than-threefold AlgR activation or repression in PAO1 during early stationary phase

PA no.	Gene name	Fold activation[a]	*P* value[b]	Protein description[c]
PA0694	*exbD2*	−4.3	0.030	Transport protein ExbD
PA1048		5.0	0.033	Probable outer membrane protein precursor
PA1072	*braE*	3.6	0.05	Branched-chain amino acid transport protein BraE
PA1106		4.5	0.002	Hypothetical protein
PA1184		−3.8	0.046	Probable transcriptional regulator
PA1355		4.3	0.040	Hypothetical protein
PA2555		3.0	0.046	Probable AMP-binding enzyme
PA3472		3.0	0.025	Hypothetical protein
PA3501		4.6	0.036	Hypothetical protein
PA3709		4.2	0.029	Probable major facilitator superfamily transporter
PA4501		3.5	0.009	Probable porin
PA4552	*pilW*	4.9	0.020	Type 4 fimbrial biogenesis protein PilW
PA4553	*pilX*	8.5	0.008	Type 4 fimbrial biogenesis protein PilX
PA4554	*pilY1*	3.4	0.044	Type 4 fimbrial biogenesis protein PilY1
PA5261	*algR*	16.0	0.016	Alginate biosynthesis regulatory protein AlgR

[a] Fold activation was determined by comparing the transcription of PAO1 to its isogenic *algR* mutant PSL317. Three replicates of each strain were used for the comparison. Genes whose transcription was higher in PAO1 than in PSL317 were called AlgR activated and are represented by a positive fold activation value. Genes whose transcription was higher in PSL317 than in PAO1 were called AlgR repressed and are represented with a negative fold activation value. Only genes with a statistically significant difference in transcription ($P < 0.05$) between PAO1 and PSL317 and a greater-than-threefold AlgR activation or repression are shown.

[b] *P* values were determined by Student's *t* test with Data Mining Tools (Affymetrix).

[c] Protein descriptions are from the *Pseudomonas* Genome Project website (www.pseudomonas.com).

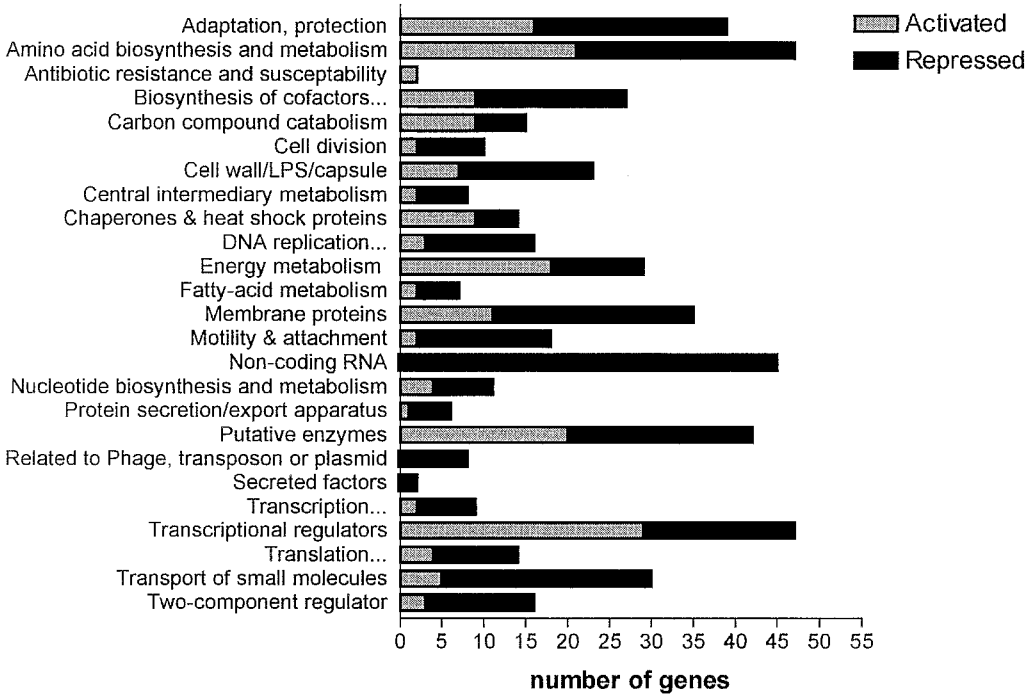

FIG. 4. Functional classes of AlgR-regulated genes when AlgR was overexpressed from the plasmid pCMR7. The genes were identified as either activated or repressed by AlgR by use of the comparison of gene expression in PAO1 and in PAO1 overexpressing AlgR grown as described in Materials and Methods. All genes that had a significant difference in expression ($P < 0.05$ as determined by *t* test) are included. Functional classes were determined using the *Pseudomonas* Genome Project website (www.pseudomonas.com) on 14 January 2004.

AlgR regulates HCN production in *P. aeruginosa*. Since our transcriptional profiling analyses revealed that AlgR may be acting as a repressor of transcription, we chose to examine the transcription of two genes repressed by AlgR. One of the genes selected was *hcnA*, the first gene in the *hcnABC* operon that encodes the HCN synthase (29). This operon was selected for several reasons. First, Firoved and Deretic demonstrated that *hcnA* is activated in mucoid *P. aeruginosa* (19). Because AlgU

TABLE 4. Genes that had a greater-than-threefold AlgR activation or repression when AlgR was overexpressed and in either mid-log growth or early stationary phase

PA no.	Gene name	Fold activation[a]			Protein description[b]
		Mid-log phase	Stationary phase	Overexpression	
PA1048			5.0	−5.5	Probable outer membrane protein precursor
PA1414		−9.4		−17.1	Hypothetical protein
PA1546	*hemN*	−5.0		−6.7	Oxygen-independent coproporphyrinogen III oxidase
PA1555		−7.4		−12.4	Probable cytochrome *c*
PA1556		−8.3		−9.3	Probable cytochrome *c* oxidase subunit
PA1557		−4.4		−3.0	Probable cytochrome oxidase subunit (cbb_3 type)
PA2119		−7.5		−4.0	Alcohol dehydrogenase (Zn dependent)
PA2493	*mexE*	7.5		−9.3	RND[c] multidrug efflux membrane fusion protein MexE precursor
PA2494	*mexF*	17.3		−7.6	RND multidrug efflux transporter MexF
PA3309		−6.8		−9.6	Conserved hypothetical protein
PA4067	*oprG*	−8.0		−8.9	Outer membrane protein OprG precursor
PA4348		−4.4		−13.3	Conserved hypothetical protein
PA4352		−4.5		−4.3	Conserved hypothetical protein
PA4613	*katB*	3.2		5.0	Catalase
PA5171	*arcA*	−6.5		−10.5	Arginine deiminase
PA5261	*algR*	13.8	16.0	−8.0	Alginate biosynthesis regulatory protein AlgR

[a] Fold activation is defined in Table 2. The expression of all the genes listed was statistically different ($P < 0.05$ as determined by *t test*), and greater than threefold different in the mid-log phase is the comparison of transcription in mid-log-phase-grown PAO1 to that in PSL317 (Δ*algR*). Stationary phase is the comparison of transcription in stationary-phase-grown PAO1 to that in PSL317 (Δ*algR*). Overexpression is the comparison of transcription in mid-log-phase-grown PAO1 to that in PAO1 overexpressing AlgR from the plasmid pCMR7.

[b] Protein descriptions were taken from the *Pseudomonas* Genome Project website (www.pseudomonas.com) on 14 January 2004.

[c] RND, resistance-nodulation-division.

TABLE 5. Known pilus genes identified as activated by AlgR

PA no.	Gene name	Fold activation	
		PAO1/PAO1(pCMR7) (overexpression[a])	PAO1/PSL317 (stationary phase[b])
PA0395	*pilT*	1.34	
PA0410	*pilI*	1.15	
PA3805	*pilF*		
PA4527	*pilC*	2.40	
PA4550	*fimU*		2.20
PA4551	*pilV*		2.20
PA4552	*pilW*		4.86
PA4553	*pilX*		8.46
PA4554	*pilY1*		3.43
PA4555	*pilY2*		2.22
PA5040	*pilQ*	2.38	
PA5042	*pilO*	2.38	
PA5043	*pilN*	1.24	
PA5044	*pilM*	2.3	

[a] Fold activation in PAO1 over that in PAO1(pCMR7) grown to mid-log phase as described in Materials and Methods.
[b] Fold activation in PAO1 over that in PSL317 grown to stationary phase as described in Materials and Methods.

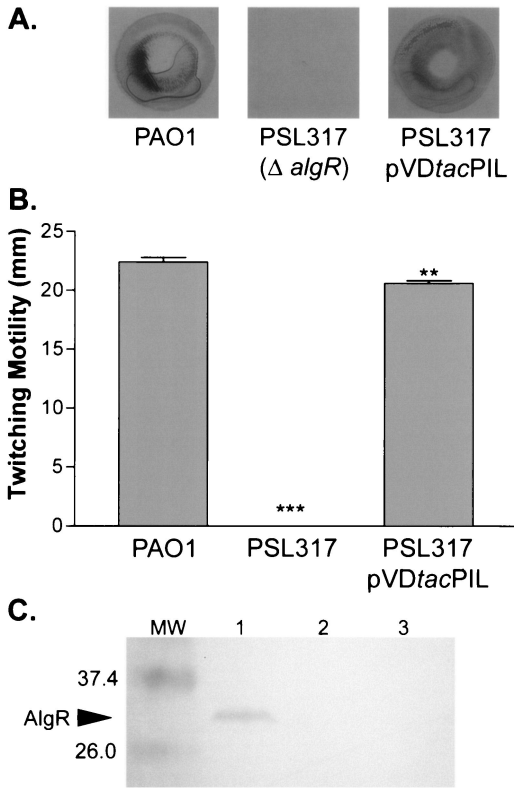

FIG. 5. Expression of *fimT*, *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, *pilY2*, and *pilE* in *trans* complemented the twitching motility defect in the *algR* mutant PSL317. (A) Representative photographs of the twitching motility of the wild-type strain PAO1, the *algR* mutant PSL317 (Δ*algR*), and PSL317 containing the complementation plasmid pVD*tac*PIL showing the zone of twitching motility. (B) Quantified twitching motility zones for PAO1, PSL317, and PSL317(pVD*tac*PIL) ($n = 5$ for all strains). No twitching motility was observed for PSL317 on any replicate. Error bars represent standard errors. **, $P < 0.01$; ***, $P < 0.0001$. (C) Western blot analysis of AlgR production in PAO1 (lane 1), PSL317 (lane 2), and PSL317(pVD*tac*PIL) (lane 3). Equivalent amounts of total protein (25 μg) were separated on a 4 to 20% gradient sodium dodecyl sulfate-polyacrylamide gel. The proteins were blotted and probed with an anti-AlgR monoclonal antibody (see Materials and Methods). MW, molecular weight standards (weights are given at left in thousands).

increases *algR* expression in mucoid *P. aeruginosa* (37), their results may indicate that increased *hcnA* transcription in mucoid *P. aeruginosa* may be due to AlgR. Second, the transcriptional regulation of *hcnA* has been well studied. Two transcriptional start sites for *hcnA* (T1 and T2) have been identified (45) (Fig. 6B), and a number of transcription factors including ANR (29), LasR and RhlR (45) and the *rsmA* product (46) regulate the expression of *hcnA*. Third, the HCN synthase produces an assayable end product, HCN, allowing confirmation of transcriptional expression.

Analysis of our data from the Affymetrix GeneChip on PAO1 and PSL317 grown to mid-log phase indicated that AlgR repressed the transcription of the *hcnABC* operon by approximately threefold (*hcnB* = 3.0). To confirm AlgR dependence of the *hcnA* promoter, an S1 nuclease protection assay was performed comparing transcription in PAO1 and PSL317 (Δ*algR*) grown under the same conditions that were used in the transcriptional profiling experiments. The results of the S1 nuclease protection assay show that AlgR repressed both the T1 promoter and the T2 promoter of *hcnA* under these growth conditions (Fig. 6A).

A quantitative HCN assay was performed on PAO1 and PSL317 to determine if the difference in transcription of the *hcnABC* operon corresponds to a difference in HCN production. The results of that assay demonstrated that PSL317 produced 1,479.4 μM HCN while PAO1 produced only 445.0 μM HCN, a 3.3-fold difference in HCN production. Complementation of *algR* in *trans* returned the production of HCN to near-wild-type levels (Fig. 6C). These data indicate that the differences in transcription caused by AlgR repression of both *hcnA* promoters result in an equivalent decrease in HCN produced by PAO1 compared to that for PSL317. Moreover, overexpression of AlgR eliminated HCN production in PAO1, indicating further that AlgR repressed *hcnA* expression (Fig. 6C). This series of experiments demonstrated that AlgR repressed transcription of *hcnA* and that mutations in *algR* resulted in increased HCN production in PAO1.

AlgR regulates expression of PA1557, a putative cbb_3-type cytochrome. Our transcriptional profiling analyses revealed that AlgR may be acting as a repressor. We therefore chose to examine the transcription of a second gene, *PA1557*, repressed by AlgR. ORF *PA1557*, followed by *PA1556* and *PA1555*, comprises a putative cbb_3-type cytochrome oxidase operon that shows the highest homology to the nitrogen fixation operon *fixNOQP* of *Bradyrhizobium japonica* (47). According to sequence analysis (www.pseudomonas.com) *P. aeruginosa* contains two operons (*PA1557* to *PA1555* and *PA1554* to *PA1552*) with homology to *fixNOP* with both of the *P. aeruginosa* operons missing *fixQ*. It appears that only the *PA1557* to *PA1555* operon was AlgR regulated (Tables 2 and 4). We confirmed AlgR dependence of the uncharacterized ORF *PA1557* in two different conditions, the PAO1 at mid-log growth phase and the AlgR overexpression condition. A primer extension experiment was performed to map the transcriptional start sites of

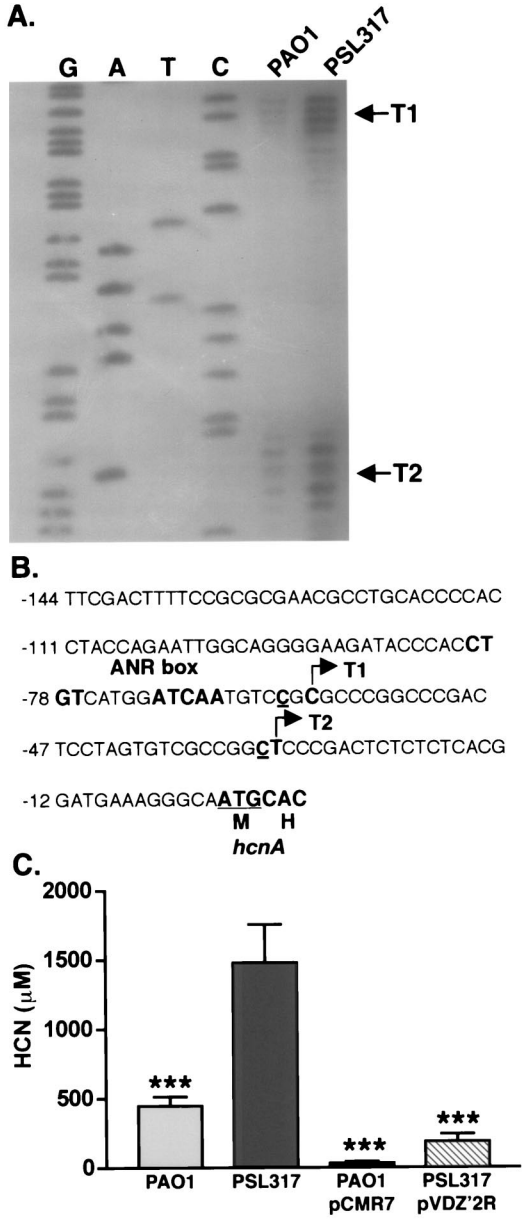

FIG. 6. AlgR regulates *hcnA* transcription and HCN production. (A) An S1 nuclease protection analysis of *hcnA* promoter activity in strains PAO1 and PSL317 (Δ*algR*) showing AlgR repression of the two previously identified transcriptional start sites, T1 and T2 (45). (B) The *hcnA* promoter sequence, highlighting the positions of the T1 and T2 transcriptional start sites, the previously identified ANR box (45), and the translational start site. Arrows indicate the positions of the previously published T1 and T2. The underlined bases indicate the mapped transcriptional start site seen in the S1 nuclease protection assay (panel A). (C) HCN production from strains PAO1, PSL317 (Δ*algR*), PAO1(pCMR7) (AlgR overexpression), and PSL317(pVDZ′2R) (*algR* complementation) correlates with the difference in *hcnA* transcription. ***, $P < 0.001$ compared to PSL317 as determined by the Tukey-Kramer multiple comparison test.

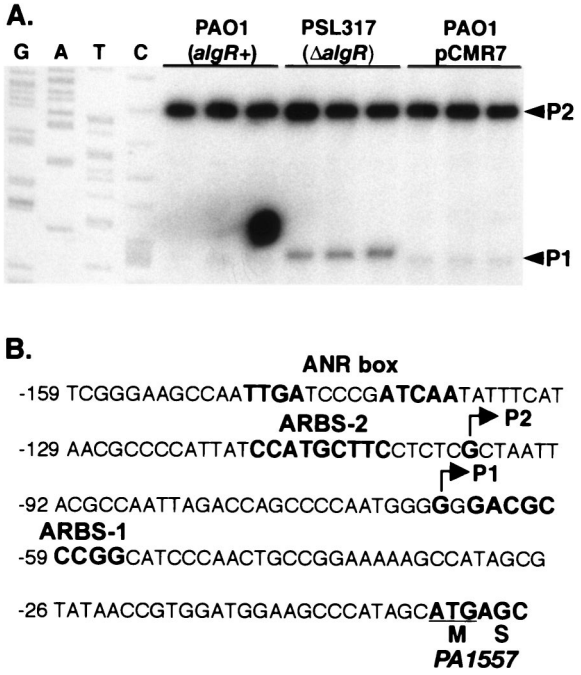

FIG. 7. The P1 promoter of *PA1557* is AlgR dependent. (A) The primer extension analysis of the *PA1557* promoter in PAO1, PSL317 (Δ*algR*), and PAO1 overexpressing AlgR from the plasmid pCMR7 (PAO1 pCMR7) identified two promoters, P1 and P2. The GATC lanes comprise a sequencing ladder generated from the same primer that was used in the primer extension. (B) Sequence map of the *PA1557* promoter indicating the positions of the P1 and P2 promoters relative to the putative translational start site (underlined) in addition to the position of two putative AlgR binding sites (ARBS-1 and ARBS-2) and a putative ANR box.

PA1557, which revealed the presence of two transcriptional starts for *PA1557*, P1 and P2 (Fig. 7), with the P1 promoter of *PA1557* appearing to be the promoter that is repressed by AlgR in both conditions. These results are in agreement with the differences in expression observed in the transcriptional profiling experiments.

DISCUSSION

In this study we have identified four new roles for the *P. aeruginosa* transcriptional regulator AlgR: (i) AlgR can repress gene transcription; (ii) AlgR activates the *fimTU-pilVWXY1Y2E* operon, the expression of which is sufficient to correct the twitching motility defect in an *algR* mutant; (iii) AlgR regulates HCN production; and (iv) AlgR controls transcription of the putative cbb_3-type cytochrome PA1557. Additionally, this study has given some insight into the mechanisms of AlgR regulation. AlgR regulates the transcription of the alginate genes *algD* and *algC* by directly binding to three sites within the *algD* promoter (39, 40) and the *algC* promoter (21, 63) (Fig. 8A). The position of the AlgR binding site varies greatly between the *algD* and *algC* promoters, ranging from −476 to −37 in the *algD* promoter and −99 to +402 in the *algC* promoter. An alignment of the published AlgR binding sites reveals the first base to be poorly conserved between the published sites. Removing the first base and using only the 9-bp sequences (shown in boldface in Fig. 8A) to search for putative AlgR binding sites in the promoter regions (defined as −873 to +402 relative to the translational start site) of genes found to be highly AlgR dependent (Table 4) revealed only six

A.

algD RB1	-476	CGCTA	**CCGTTCGTC**	CTCCC -458
algD RB2	-400	CTCAA	**CCGTTCGTC**	TGCAA -382
algD RB3	-32	TCCGG	**CGGTTTGTC**	CCGCT -50
algC ABS1	-99	CTCAC	**CCGTTCGTC**	GCTGT -81
algC ABS2	+156	CAGAT	**CCGTTGTTC**	CAGAA +174
algC ABS3	+384	GCGAA	**CCGTGCGTC**	GCTGT +402

B.

lon ARBS	+65	TCATC	**CCGTTGTTC**	GTCGG +82
PA2828 ARBS	+357	CGTCG	**CCGTTGTTC**	AGCAG +339
ibpA ARBS	-165	AACGA	**CCGTTCGTC**	GTGCT -183
PA4625 ARBS	-144	GGCGA	**CCGTTCGTC**	GGAGT -162
hslV ARBS	-82	CACCG	**CCGTGCGTC**	GGAAC -101
PA5475 ARBS	-131	GCATG	**CCGTGCGTC**	GGCTC -112
AlgR consensus sequence:			**CCGTKBKTC**	

C.

PA1557 ARBS-1	-51	GGATG	**CCGgGCGTC**	CCCCC -69
PA1557 ARBS-2	-118	ATTAT	**CCaTGCTTC**	CTCTC -100
hcnA ARBS-1	-395	ACCAC	**CCGTcGTTC**	CGTCA -413
hcnA ARBS-2	+117	CAATG	**CCGTGGGcC**	TGCGC +135
pilV ARBS-1	-65	GGCCC	**CCGTcGGTC**	TTGCC -83
pilV ARBS-2	-125	GGGTA	**CCGTTGGcC**	GTGAA -143
pilV ARBS-3	-566	CAGGC	**CCGTTTGgC**	ATGCT -528

FIG. 8. Alignment of AlgR binding sites of AlgR-regulated genes. (A) Alignment of the known AlgR binding sites from the *algD* (39, 40) and *algC* (21, 63) promoters. (B) Alignment of putative AlgR binding sites within the promoter regions of *lon* (*PA1803*), *ibpA* (*PA3126*), *hslV* (*PA5053*), *PA2828*, *PA4625*, and *PA5475*. The position of the AlgR binding site (in boldface) is relative to the putative translational start site. (C) Alignment of putative AlgR binding sites of *PA1557*, *hcnA*, and *pilV* promoter regions. Mismatched bases in the AlgR binding site are indicated by lowercase letters. The AlgR consensus sequence is a composite of the known AlgR binding sequences within the *algD* and *algC* genes. B, G, C, or T; K, G or T.

genes with *algC* or *algD* AlgR binding sites within their promoter (Fig. 8B). This result may indicate that (i) the AlgR binding sequence may only be partially known or (ii) the activated form of AlgR may bind to a different sequence. In support of the first possibility, AlgR has been proposed to be a member of a family of transcriptional regulators that may bind to imperfect direct repeats of the sequence pattern [TA][AC][CA]GTTN[AG][TG], suggesting that AlgR may be binding as a dimer to the promoters that it regulates (41). On the other hand, the affinity of AlgR for the *algC* ABS3 binding site is enhanced when AlgR is phosphorylated (61).

Three characterized genes with *algD* or *algC* consensus AlgR binding sites in their promoters that showed AlgR dependence in our transcriptional profiles were *ibpA*, *lon*, and *hslV*, all potentially involved in the stress response of *P. aeruginosa*. The AlgR regulation of these three stress response genes not only increases the global relevance of the AlgR regulon but is also in accordance with other evidence that AlgR may play a role in stress. The expression of AlgR has been shown to be activated by the extreme heat shock sigma factor AlgU (37). In *Escherichia coli* IbpA (along with other small heat shock proteins) works in conjunction with the DnaK-DnaJ-GrpE and the GroEL-GroES systems to manage environmental stress, but IbpA appears to be dispensable (57). It is interesting that *dnaK* also showed a twofold repression by AlgR in the AlgR overexpression experiment, but the *dnaK* promoter does not contain a putative AlgR binding sequence. Since *P. aeruginosa ibpA* has not been characterized and has only 67% homology with *E. coli ibpA*, the effects of the differing primary structures on IbpA function in the stress response of *P. aeruginosa* are unknown. It is possible that some of the genes that showed AlgR dependence but do not have an identifiable AlgR binding site could be regulated indirectly through proteolytic degradation of a transcription factor. Consistent with the hypothesis that AlgR may regulate *P. aeruginosa* stress genes, we have shown previously that the *algR* mutant strain PAO700 (*algR*::Gm^r) was found to be more resistant to hydrogen peroxide, myeloperoxidase, and human neutrophils (31). We discovered two potential AlgR-regulated genes, *katA* and *katB*, from our global expression analysis that may account for the resistance phenotypes observed. The transcriptional profile of AlgR-dependent genes indicated that *katA* was repressed 1.80-fold by AlgR while *katB* was activated 3.16-fold by AlgR. *P. aeruginosa* contains at least three catalase genes: *katA*, *katB*, and *katC* (33). KatA is constitutively active and provides the major catalase activity in all phases of growth (8). In addition, the expression of KatA can be induced by oxidative stress (8). The expression of KatB is low under normal growth conditions but is activated in response to oxidative stress (8). Therefore, analysis of the AlgR transcriptional profile indicates that AlgR represses the expression of KatA, the "housekeeping" and most highly expressed catalase, which may result in the *algR* mutant producing more catalase than the wild type does. This may be one potential explanation for the observation that PAO700 was more resistant to H_2O_2 than was PAO1 (32).

Interestingly, another microarray study comparing the gene expression of *P. aeruginosa* strains PAK and PAK *algZ* (*fimS*) also identified *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, *pilY2*, and *pilE* as among the most AlgZ-dependent genes (M. Wolfgang, personal communication). Mutations in either *algZ-fimS* or *algR* result in elimination of the twitching motility phenotype (59). The *fimU-pilVWXY1Y2* operon does contain two putative AlgR binding sites, although neither is identical to the *algC* or *algD* consensus AlgR binding sites. One putative AlgR binding site is located 79 bp from the 3′ end of *fimU*, directly upstream of *pilV* (Fig. 8C). The studies that characterized *pilV* identified a strong *pilV* promoter along with a second, weaker *pilV* promoter within the coding region of *fimU* (2, 3). Therefore, this putative AlgR site could be in the promoter region of *pilV*, indicating that AlgR could directly regulate *pilV* expression. Since the translational starts and stops for *pilV*, *pilW*, and *pilX* overlap (2, 3), it is possible that all three genes are regulated by the two potentially AlgR-dependent *pilV* promoters. The other putative AlgR binding site is located 947 bp into the coding region of *pilY1*. Due to the long distance of this site from an intergenic region, the potential function of this site is unknown. The AlgR overexpression transcriptional profile also identified an additional eight known pilus genes as potentially activated by AlgR (Table 5), including nearly the entire *pilMNOPQ* operon (only *pilP* did not show differential expression). Interestingly, *pilMNOPQ* mutants have been described as able to produce equivalent amounts of pilin but unable to express it on the cell surface as determined by electron microscopy and phage PO4 sensitivity assays (34). This is the same phenotype reported for an *algR* mutant (59). However, there are no putative AlgR binding sites in or near the promoter

regions of most of the genes in the *pilMNOPQ* operon. There is one AlgR binding site identical to the *algD* RB1 site within the *pilQ* coding region, but it is 582 bp from the 3′ end of *pilQ*. The lack of AlgR binding sites near the 5′ end of this operon indicates that the *pilMNOPQ* operon is likely indirectly AlgR regulated. None of the promoter regions of the other genes, *pilT*, *pilI*, or *pilC*, contains a putative AlgR binding site, again indicating indirect AlgR regulation.

Other studies have shown that *P. aeruginosa* produces HCN in infected burn eschar of human patients and that HCN was detectable in the viscera upon postmortem analysis (25). A more recent study has identified HCN produced by *P. aeruginosa* as a primary virulence factor for the paralytic killing of *Caenorhabditis elegans* by *P. aeruginosa* (23), and other studies have shown HCN to be an inhibitor of fungal growth in plant leaf and root infection (20, 58). These studies indicate that the HCN produced by *P. aeruginosa* may affect host cells and may be important in virulence. 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 (45). Currently, five *hcnA* regulatory proteins have been identified: GacA (48), ANR (48, 64), LasR and RhlR (45), and RsmA (46). The global regulator GacA positively controls HCN synthesis as well as other secondary metabolites and exoenzymes (48). *P. aeruginosa* mutants with mutations in *gacA* or *anr* produce very little HCN (48, 64). LasR and RhlR are quorum-sensing regulators required for transcription of the *hcnA* promoter (45). 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 (44). Since our data suggest that AlgR is affecting T1 and T2 transcription, AlgR is yet another transcriptional regulator involved with *hcnA* expression, indicating that AlgR and LasR and/or RhlR and ANR coordinately regulate this promoter. In support of this possibility, analysis of the *hcnA* promoter reveals a putative AlgR binding site from −400 to −408 bp upstream of the translational start site (Fig. 8C). This site (CCGTCGTTC) differs by only one base from the ABS2 site of *algC* (63) (Fig. 8A and C), indicating that AlgR may bind directly to the *hcnA* promoter to regulate its transcription.

The promoter of *PA1557* shares some very interesting features with the promoter of *hcnA*. The first is that both promoters contain putative AlgR binding sites (Fig. 7B and 8C). Another similarity between the two promoters is that they contain a putative ANR binding site (Fig. 6B and 7B). In addition to *hcnA* and *PA1557*, two other genes that were AlgR dependent in the transcriptional profiling analysis (Tables 2 and 4) are known to be ANR dependent. The *arcDABC* operon, which encodes the anaerobic arginine deiminase enzymes (22), and *hemN*, which encodes the oxygen-independent coproporphyrinogen III oxidase (49), are both ANR dependent. None of the conditions that we examined by transcriptional profiling revealed that *anr* was AlgR regulated. The possible mechanism of AlgR and ANR coregulation is unknown, but the number of promoters (*hcnA*, *PA1557*, *arcD*, and *hemN*) regulated by both suggests more than coincidental regulation.

The fact that only 6 out of the 53 genes that show AlgR regulation in two out of the three conditions tested possess a known AlgR binding site indicates that the mechanism of AlgR regulation is more complex than originally thought. A relatively small number of AlgR-regulated genes without AlgR binding sites would have been expected due to indirect effects through other transcriptional regulators, but those regulators would have been expected to possess AlgR binding sites. However, none of the transcriptional regulators on the list of the genes most regulated by AlgR (Table 4) contain AlgR binding sites. The Lon and HslVU proteases certainly could account for a portion of the genes indirectly regulated by AlgR, but it is unlikely that the astounding numbers of genes that show indirect AlgR dependence are through these two proteases alone. The distinct possibility exists that AlgR is capable of binding to additional sequences that have yet to be elucidated. Most of the work describing the AlgR binding site was done in vitro using AlgR purified from *E. coli* (39, 40) or using crude cell extracts of *P. aeruginosa* that overexpresses AlgR (21, 63). Therefore, the effects of posttranslational modification were not taken into account. Further studies are warranted to discern the role of AlgR posttranslational modifications and the ability of AlgR to switch from a repressor to an activator in control of *P. aeruginosa* gene transcription.

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