**Pseudomonas aeruginosa** AlgR Phosphorylation Modulates Rhamnolipid Production and Motility

Yuta Okkotsu, a Prince Tiekua, b Liam F. Fitzsimmons, a Mair E. Churchill, b Michael J. Schurr a

Department of Microbiology, University of Colorado School of Medicine, Aurora, Colorado, USA; Department of Pharmacology, University of Colorado School of Medicine, Aurora, Colorado, USA

AlgR is a key **Pseudomonas aeruginosa** transcriptional response regulator required for virulence. AlgR activates alginate production and twitching motility but represses the Rhl quorum-sensing (QS) system, including rhamnolipid production. The role of AlgR phosphorylation is enigmatic, since phosphorylated AlgR (AlgR-P) is required for twitching motility through the **fimU** promoter but is not required for the activation of alginate production. In order to examine the role of AlgR phosphorylation **in vivo**, a PAO1 **algRD54E** strain (with **algR** encoding a D-to-E change at position 54), which constitutively activates **fimU** transcription and exhibits twitching motility, was created. A corresponding PAO1 **algRD54N** strain (with **algR** encoding a D-to-N change at position 54) that does not activate **fimU** or twitching motility was compared to PAO1, PAO1 **algRD54E**, PAO1 **ΔalgZ** (deletion of the **algZ** [**fimS**] gene, encoding a putative histidine kinase), and PAO1 **ΔalgR** for swarming motility, rhamnolipid production, and **rhlA** transcription. PAO1 and PAO1 **algRD54E** produced approximately 2-fold-higher levels of rhamnolipids than PAO1 **algRD54N** and PAO1 **ΔalgZ**, thereby indicating that phosphorylated AlgR is required for normal rhamnolipid production. Examination of purified AlgR, AlgR-P, AlgR D54N, and AlgR D54E showed that AlgR-P and AlgR D54E bound preferentially to the **fimU** and **rhlA** promoters. Additionally, AlgR-P bound specifically to two sites within the **rhlA** promoter that were not bound by unphosphorylated AlgR. Taken together, these results indicate that phosphorylated AlgR-P has increased affinity for the **rhlA** promoter and is required for the coordinate activation of twitching motility, rhamnolipid production, and swarming motility in **P. aeruginosa**.

**Pseudomonas aeruginosa** is an opportunistic human pathogen capable of causing fatal infections in patients with a defective immune system, such as those with AIDS, burn wounds, or cystic fibrosis (CF) (1–3). The majority of CF patients have chronic **P. aeruginosa** pulmonary infections recalcitrant to antibiotic treatment and immune clearance from the lungs. One of the virulence factors produced by **P. aeruginosa** that exacerbate chronic infection is the exopolsaccharide alginate (4). Alginate biosynthesis and its export by **P. aeruginosa** are tightly controlled and require a large number of genes encoding enzymes and export proteins (4–6). Motility also plays a significant role in the pathogenesis of **P. aeruginosa** (7, 8) and facilitates the colonization of the host as well as biofilm formation (9, 10). **P. aeruginosa** exhibits three major forms of appendage-mediated motility: (i) flagellum-based swimming motility in an aqueous environment, usually assessed on low agar concentrations (0.3% [wt/vol]) **in vitro**, (ii) type IVa pili-associated twitching motility, evaluated on solid surfaces (1% [wt/vol] agar) or at the interstitial surface between the agar and the plastic or glass (11), and (iii) swarming motility, which requires both flagella and type IV pili and is observed on semisolid surfaces (0.5 to 0.7% [wt/vol] agar) (12, 13). Swarming motility also uses biosurfactants produced by the bacteria, such as rhamnolipids and 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), which decrease the surface tension between the bacterial cells and the surface (14, 15). In addition to swimming, twitching, and swarming, **P. aeruginosa** exhibits sliding/spreading motility on semisolid surfaces in the absence of both flagella and type IV pili (16). Murray and Kazmierczak have shown that rhamnolipid production is required for sliding motility, and this phenotype responded to some of the same environmental cues as swarming motility (16).

Signal transduction through two-component regulatory systems (TCs) is an important mechanism by which microorganisms detect environmental stimuli and establish an adaptive response. Typical bacterial TCs consist of a “sensing” histidine kinase (HK), which detects the stimulus and modulates the signal pathway, and a “receiving” response regulator (RR), which directs the output of the signal. To date, 58 RRs, 59 sensor HKs, and 7 sensor/ regulator hybrids have been annotated in the **P. aeruginosa** PAO1 genome (17). The capacity of this organism to detect and adapt to various environments has been attributed to its large number of two-component systems (18). One TC of **P. aeruginosa**, AlgZ (FimS)-AlgR, controls both alginate production and two different types of motility, twitching and swarming (13, 19–21). AlgR, a member of the LytTR family of two-component response regulators, was one of the first response regulators identified in **P. aeruginosa**. Under conditions where the AlgU (also known as AlgT, or $\sigma^{22}$)/MucA sigma factor/antisigma factor-dependent pathway is turned on, AlgR is required for full activation of the algD operon and the algC gene, encoding the alginate biosynthetic, modification, and transport enzymes that confer the mucoid phenotype (19, 22). AlgR is also essential for the transcription of the fimU operon in nonmucoid **P. aeruginosa** strains (20, 21). The N terminus of AlgR is homologous to the receiver (REC) domain of two-component regulators and has been phos-
phorylated in vitro by the Escherichia coli histidine protein kinase CheA and acetyl phosphate (AcP) (20, 24, 25). The role of AlgR phosphorylation is enigmatic, since it is not necessary for alginate production but is essential for twitching motility (20, 24, 25). AlgR can also act as a negative regulator of hydrogen cyanide (HCN) production in a nonmucoid background (26) and can repress rhlI and rhlA transcription in a biofilm-specific manner (28).

In order to understand the role of AlgR phosphorylation in P. aeruginosa, we created a phosphomimetic form of AlgR (27). We report here, for the first time, the generation and utilization of a phosphomimetic form of a P. aeruginosa response regulator. In this study, we further characterize the role of AlgR phosphorylation in P. aeruginosa gene expression by comparing PAO1 containing algRD54E, a constitutively “on” allele, to PAO1 containing algRD54N (strain WFPA8), encoding a constitutively “off” form of AlgR. Previous evidence has suggested that deletion of algR caused a dysregulation of the Rick quorum-sensing system through the derepression of the rhl and rhlA genes (28). We present evidence that AlgR phosphorylation coactivated rhamnolipid production and twitching motility, resulting in normal swarming motility. In contrast, the expression of the phosphodefective algRD54N allele in P. aeruginosa resulted in less rhamnolipid production and loss of swarming and twitching motility.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. All strains and plasmids used in this study are listed in Table 1. Pseudomonas strains were maintained on Pseudomonas isolation agar (PIA), and E. coli strains were maintained on Miller lysogeny broth (LB) agar (Difco). For antibiotic selection, 150 μg/ml gentamicin, 250 μg/ml tetracycline, or 300 μg/ml carbenicillin was added to PIA, and 100 μg/ml ampicillin, 15 μg/ml gentamicin, 10 μg/ml tetracycline, 50 μg/ml kanamycin, or 30 μg/ml chloramphenicol was added to LB agar, where appropriate. Overnight broth cultures were grown in LB with the antibiotic concentrations listed above unless otherwise indicated. For allelic exchange mutagenesis, Pseudomonas cultures were selected on VBMM (29), supplemented with 50 μg/ml gentamicin for positive selection or 7.5% sucrose for counterselection when needed. For rhamnolipid assays, cultures were grown in tryptic soy broth (TSB) (Difco) supplemented with 1% glycerol.

Genetic manipulations. All cloning techniques were performed using E. coli DH5α to propagate and maintain plasmids, unless otherwise indicated. To make the algR complementation vector, the algR coding region was PCR amplified from the PAO1 genome using primers algR-CdgRgn-KpnI-F and algR-CdgRgn-HindIII-R. This PCR product was digested with KpnI and HindIII and was ligated into pHERD30T (30) to generate pHERD30T-algR. The pHERD30T-algRD54N vector was generated in a similar manner using chromosomal DNA extracted from WFPA8 (25). To create pHERD30T-algRD54E, the coding encoding Asp 54 (GAT) in algR

P. aeruginosa encoding D54E, the codon encoding Asp 54 (GAT) in algR

resulted in less rhamnolipid production and loss of swarming and twitching motility.

The fimU::lacZ transcriptional fusion was created by ligating a 609-bp PCR product amplified from the PAO1 chromosome with the primer pair comprising fimU TF XbaI and fimU TF HindIII into mini-CTX-1 lacZ to generate CTX-1 fimU::lacZ. The rhlATF::lacZ transcriptional fusion was created by PCR amplifying 980 bp of the promoter region using the primer pair comprising rhlA TF BamHI F and rhlA TF PstI R to generate CTX-1 rhlATF::lacZ. The CTX-1 fimU::lacZ or CTX-1 rhlATF::lacZ plasmid was transferred to the plasmid mobilization strain Sm10 and was biparentally conjugated into P. aeruginosa strains. The vector backbone was resolved by introducing the pFLP2 vector into the strains containing CTX-1 fimU::lacZ or CTX-1 rhlATF::lacZ and was verified by PCR. To construct the AlgR protein expression plasmids, the algR, algR12 (algRDK54E), or algR7 (algRDK54N) allele was PCR amplified using primers algRSalfI and algRNNotI and was directionally subcloned into the pGEX4T-3 vector (GE Healthcare Life Sciences), generating plasmid pGEX4T-3algR, pGEX4T-3algRDK54E, or pGEX4T-3algRDK54N.

Expression studies. Transcriptional expression of the fimU and rhlA promoters was measured by a β-galactosidase assay as described previously (28, 32), with modifications. Cells were lysed using a sonic dismbrator (15 W) in breaking buffer (200 mM Tris HCl, 200 mM NaCl, 2% glycerol, 1 mM dithiothreitol [pH 7.6]). An 800-μl volume of Z-buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4·7H2O, 50 mM 2-mercaptoethanol [pH 7.0]) was mixed with 10 μg of total protein, and 200 μl of o-nitrophenyl-β-D-galactopyranoside (ONPG) solution (4 mg/ml) was added to initiate the reaction. Kinetic readings were measured as the optical density at 420 nm (OD420). The activity was expressed in microunits per milligram, where 1 U was defined as the enzymatic activity of β-galactosidase to convert 1 micromol ONPG to o-nitrophenol per min (33). Each strain was tested with at least three biological replicates.

For real-time PCR analysis, total RNA was extracted from cultures using RNeasy spin columns (Qiagen) and was treated with DNase I (New England BioLabs). cDNA was generated using SuperScript II reverse transcriptase (Invitrogen Life Science Technologies). Real-time PCRs were conducted in a LightCycler 480 instrument with LightCycler 480 Probes Master Mix (Roche Applied Science), and rhlA and rhlD transcripts were analyzed using appropriate primer pairs (Table 2). Data were analyzed using LightCycler 480 software, release 1.5.0 (Roche Applied Science). Relative expression was normalized to rpoD cDNA levels in each sample.

For AlgR protein expression, 100-ml flasks were inoculated with 5.7 × 106 to 7.5 × 106 CFU. Collected cells were sonicated in phosphate-buffered saline (PBS) and were centrifuged (5 min, 16,000 × g) to pellet cell debris. Denatured samples were separated by SDS-PAGE. Separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Western blotting was performed as described previously (34) with modifications. Duplicate blots were probed either with an anti-AlgR rabbit serum (Open Biosystems) preabsorbed with a cell extract from an overnight culture of PAO1 ΔalgR or with an anti-OmlA antibody (a gift from Michael Vasili). A horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody and the ECL kit (GE Healthcare) were subsequently used to detect AlgR or OmlA proteins using chemiluminescence-based immunodetection. Blots were imaged with the ChemiDoc XRS system, and band intensities were quantified using Image Lab, version 2.0.1 (Bio-Rad), from biological replicates of three different time courses.

Purification of AlgR, AlgR D54N, and AlgR D54E. The pGEX4T-3algR, pGEX4T-3algRDK54N, and pGEX4T-3algRDK54E vectors were transformed into BL21 Rosetta cells (Novagen) and grown in LB supplemented with antibiotics. Cells were grown at 37°C until cultures reached an OD600 of 0.48, and protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 15°C. Cells were centrifuged (30 min, 8,000 × g) and were lysed in PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF) in a French pressure cell. Cell extracts were treated with 30 U/ml DNase, 1 mg/ml lysozyme, and Halt protease inhibitor (Thermo Scientific), centrifuged, and passed through a 0.2-μm-pore-size GD/X PVDF filter (Millipore), and supernatants con-
TABLE 1 Strains and plasmids used in this study

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Staining soluble glutathione S-transferase (GST)-AlgR fusion protein were bound to glutathione-Sepharose beads (GE Healthcare Life Sciences). Beads were fitted into a gravity column and were washed with PBS. The AlgR protein was cleaved from the GST tag with thrombin (10 U/ml column volume) for 18 h (GE Healthcare Life Sciences) before elution with PBS. All fractions were tested for protein expression and contamination by resolution on an SDS-PAGE gel and Coomassie blue staining (see Fig. S2 in the supplemental material). PBS was immediately replaced with storage buffer (20 mM Tris, 100 mM NaCl, 5 mM MgCl₂, 10% glycerol [pH 7.5]) using a stirred ultrafiltration cell (Millipore), and protein aliquots were stored at −80°C.

**AlgR biochemical studies.** Electrophoretic mobility shift assays (EMSA) were performed using purified AlgR proteins, as described previously (35). DNA fragments were prepared by PCR amplification of PAO1 chromosomal DNA with Tag polymerase (New England BioLabs) and the oligonucleotides listed in Table 2 for fimU and rhlA. Oligonucleotides were 5’-end labeled with [γ-32P]ATP (6,000 Ci/mmol) (PerkinElmer) with polynucleotide kinase (New England BioLabs). Excess radio-
nucleotide was removed with Illustra Microspin G-25 columns (GE Healthcare). The templates for the
rhlA SDM WT RB1 F and rhlA SDM WT RB2R), rhlAΔ1 (primers rhlA SDM delta RB1 F and rhlA SDM delta RB2R),
and rhlAΔ2 (primers rhlA SDM delta RB1 F and rhlA SDM delta RB2R) oligonucleotides were created using site-di-
rected mutagenesis of the pGEMT-htlAprom vector. Purified AlgR protein
was preincubated for 30 min at 37°C with nonradioactive acetyl phos-
phate (acetyl phosphate lithium potassium salt; Sigma) at the
concentrations indicated in Fig. 2 prior to DNA binding studies.

Binding assays were performed as described previously (35) with the
following modifications. Binding reaction mixtures were supplemented
with 2.5 ng/μl poly(dI·dC) (Sigma-Aldrich). Samples were loaded in a
native 6.5% polyacrylamide gel (10 mM Tris [pH 8.0], 0.38 mM glycine, 1
mM EDTA, 6.5% [2.67% bisacrylamide] acrylamide), applied to either a
phosphor screen or Kodak film, and imaged using Molecular Imager FX
and Quantity One software (Bio-Rad).

Motility assays. Subsurface twitching motility assays were performed as
described previously (36). Briefly, overnight cultures were inoculated in
the interstitial space between the plastic petri dish and the basal surface of
LB–1.0% Bacto agar and were incubated for 48 h at 37°C. Cultures were
stained with 0.5% crystal violet, and the diameter of the twitching zone
was measured. Surface twitching motility assays were performed as de-
scribed previously (37, 38). Cultures were grown in brain heart infusion
(BHI) medium (Difco) to an OD600 of 1.2 and were concentrated to 9 × 106
cells/ml in morpholinepropanesulfonic acid (MOPS) buffer (10 mM MOPS,
8.6 mM NaCl, 1 mM MgSO4, 11 mM dextrose, 0.5% glucose [pH 7.6], solidified with 1.5% Bacto
agar) and was incubated at 37°C for 24 h under 5% O2 (HERAcell 150;
Thermo Scientific). The twitching zone was imaged using a digital camera
and an Axiovert 25 inverted light microscope at ×100 magnification (10×
phase-contrast objective lens and 10× optical eyepiece) (Carl Zeiss).
Swarming motility was measured as described previously (39). Briefly,
stationary-phase cultures were concentrated by centrifugation and were
adjusted to an OD600 of 3.0 in PBS. Five microliters was spotted onto the
center of modified M9 medium agar (20 mM NH4Cl, 12 mM Na2HPO4,
22 mM KH2PO4, 8.6 mM NaCl, 1 mM MgSO4, 1 mM CaCl2·2H2O, 11
mM dextrose, 0.5% Casamino Acids, solidified with 0.5% Bacto agar)
and cultures were grown for 10 h at 30°C, followed by 20 h at room
temperature. For arabinose induction, volumes from a 20% stock solu-
tion of L-arabinose were added to swarming plates prior to solidifying.

Primer name 5’→3’ sequencea Gene(s)

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a 56-FAM, 5(6)-carboxyfluorescein; 3IABKFQ, 3’ Iowa Black FQ.
Doc XRS system (Bio-Rad), and the image contrast was adjusted using Photoshop, version 10.0 (Adobe).

**Rhamnolipid assays.** Rhamnolipid production was determined using three different assays: (i) an oscillo assay, (ii) thin-layer chromatography (TLC), and (iii) a hemolysis assay, as described previously (28, 40–43). To quantify the amount of rhamnolipid produced by each strain, the oscillo rhamnolipid assay was used (28, 42, 44). Bacterial cultures were grown at 37°C with shaking at 250 rpm in beveled flasks. Briefly, a 1-ml volume of samples was then collected and was centrifuged. Rhamnolipids were extracted from cell supernatants three times with 0.5 ml ethyl acetate. After ethyl acetate extraction, 1 ml of ethanol reagent (53% sulfuric acid, 0.2% orcinol) was added. Samples were first incubated at 80°C for 30 min and then cooled at room temperature for 15 min. Two hundred microliters of each sample was then added to a 96-well plate, and the absorbance at 522 nm was recorded by a Synergy 2 plate reader and was compared with a standard curve of rhamnose monohydrate. Total-rhamnolipid values were normalized to total-protein values of cell pellets measured by a Bradford assay.

TLC was performed as described previously (43). Briefly, samples were extracted as described for the oscillo assay. Ethyl acetate extracts were completely dried and were resuspended in 20 μl of 90% chloroform and 10% methanol. A 5-μl volume was added to a silica gel 60A thin-layer chromatography plate (Whatman) and was mobilized in a solvent system containing chloroform–methanol–acetic acid (65:15:2). The plate was incubated in a bath of 15% sulfuric acid in ethanol and was developed with a heat gun.

The hemolysis assay was performed as described previously, with a modification for 96-well plates (45). Horse blood in Alsever’s solution (Colorado Serum Company) was washed three times with PBS prior to use. Bacterial supernatants were obtained from 1 ml of 24-h cultures grown at 37°C, which was centrifuged for 5 min at 16,000 × g to pellet cells and separate supernatants. The supernatants were then heated at 95°C for 10 min, cooled, and serially diluted. Diluted supernatants were applied to a final blood concentration of 1% (vol/vol). PBS and water were used as negative and positive controls, respectively. Hemolysis reaction mixtures were incubated for 1 h at 37°C, and solubilized hemoglobin was separated from cell debris by centrifugation (10 min, 750 × g). Supernatants were transferred to new 96-well plates, and the absorbance of free hemoglobin was read as the OD542. The percentage of hemolysis was calculated as |

\[
\text{absorbance of the sample} - \text{absorbance of the blank}/\text{absorbance of the positive control} \times 100.
\]

**RESULTS**

**P. aeruginosa fimU expression increased in PAO1 algRD54E.** In order to elucidate the role of AlgR phosphorylation in *P. aeruginosa*, a phosphomimetic form of AlgR was constructed for comparison with an inactive, phosphodefective form of the protein in the PAO1 background. In previous studies with NtrC from *Salmonella enterica*, Ompr and RcsB from *E. coli*, and HupR from *Rhodobacter capsulatus*, a substitution of glutamate for the phosphoaccepting aspartate residue (D→E), located in the CheY-like REC domain, resulted in constitutive activation of the response regulators by mimicking the structure of the phosphoaspartyl form of the proteins (27, 46–49).

Transcription of the fimU operon was previously found to be dependent on the AlgR protein, and a strain expressing a phosphodefective form of AlgR (AlgR D54N) was incapable of expressing the genes in the operon and had abrogated twitching motility (25, 50, 51). These results indicate that the phosphoaccepting aspartate of AlgR is located at residue 54 (25). Since AlgR phosphorylation is required for fimU expression (25, 50, 51), a chromosomal fimU: lacZ reporter was constructed in order to determine if the algRD54E mutation expressed the phosphomimetic form of AlgR. It was hypothesized that AlgR D54E would activate transcription, while AlgR D54N would not.

By use of site-specific recombination via allelic exchange, the algR12 allele (encoding AlgR D54E, and therefore referred to here as the algRD54E allele) was introduced into the PAO1 chromosome to create PAO1 algRD54E (Fig. 1A). To study the effects of strains expressing different forms of AlgR, the fimU::lacZ transcriptional reporter was introduced into backgrounds containing the following alleles: algR + (PAO1), ΔalgR (PSL317), algR7 (encoding AlgR D54N, and therefore referred to here as algRD54N) (WFP48), or algRD54E (PAO1 algRD54E). The transcriptional activity of fimU in strains PAO1 ΔalgR and PAO1 algRD54N was greatly decreased, whereas activity from the same promoter in strain PAO1 algRD54E showed a 15-fold increase over that in PAO1 (Fig. 1B). These data again supported the hypothesis that AlgR D54E activated the transcription of the fimU promoter. It was also hypothesized that AlgR D54E should be a phosphomimetic in the absence of its putative cognate histidine kinase, AlgZ (FimS). In order to test this hypothesis, the algRD54E allele was introduced into the ΔalgZ background to create strain PAZ algRD54E (ΔalgZ algRD54E). As shown in Fig. 1B, fimU::lacZ reporter activity was significantly increased (36-fold) in the ΔalgZ algRD54E background.

In order to eliminate the possibility that the fimU promoter activities in PAO1 algRD54E and PAZ algRD54E were due to elevated protein expression, Western blot analysis was performed using a polyclonal anti-AlgR antibody to measure AlgR expression levels. AlgR protein levels for the algR +, algRD54E, and ΔalgZ backgrounds were comparable, and a slightly smaller amount was expressed in the algRD54E strain than in the wild type (WT). Unexpectedly, strain PAZ algRD54E did show a significant increase in AlgR protein expression over that in wild-type PAO1 (Fig. 1C and D). This increased protein expression may explain the large increase in fimU::lacZ transcriptional activity in this mutant. Overall, these results indicated that AlgR D54E was most likely locked in the phosphomimetic conformation in vivo and activated the fimU promoter independently of phosphorylation.

**Acetyl phosphate phosphorylated AlgR but not AlgR D54E or AlgR D54N.** It was then proposed that the increased fimU transcriptional activity observed in the strain expressing AlgR D54E was due to the recombinant protein being locked in the active conformation. Previously, AlgR autophosphorylated in the presence of acetyl phosphate (AcP) and carbamoyl phosphate (24). Additionally, previous studies showed that phosphorylation is specific to the aspartate residue (D54), where *E. coli* CheA acted as a histidine kinase to phosphorylate AlgR, but not AlgR D54N, with [γ-32P]ATP as the phosphate donor (25). However, the phosphomimetic AlgR D54E has not been tested to date, and its phosphoaccepting capacity was examined using AcP.

In order to examine the autokinase properties of AlgR or its isoforms for the *in vitro* studies, all three proteins were overexpressed and purified in *E. coli*, using a GST-tagged expression system (see Fig. S1A in the supplemental material). An *in vitro* phosphorylation reaction was then performed as previously described (52); it showed that only wild-type AlgR, and not AlgR D54E or AlgR D54E, was phosphorylated by radiolabeled [γ-32P]ATP generated (see Fig. S2 in the supplemental material). These data indicated that only wild-type AlgR was phosphorylated *in vitro* by the phosphodonor AcP and that Asp 54 was required for the phospholabeling.
Phosphorylated AlgR and AlgR D54E bound specifically to the fimU promoter. The transcriptional activity of fimU: lacZ in the presence of AlgR D54E was higher than that with wild-type AlgR (Fig. 1 and data not shown). Therefore, it was hypothesized that AlgR-P (in vitro-phosphorylated AlgR) or AlgR D54E would have DNA binding characteristics different from those of AlgR. In order to test this hypothesis, electrophoretic mobility shift assays (EMSAs) were performed. Previous studies on the binding of AlgR to the fimU promoter showed the presence of two AlgR binding sites (ABSs) within the intergenic region of fimT and fimU (50). Therefore, an 82-bp fimU promoter fragment containing both of the AlgR binding sites identified was tested for the effect of increasing amounts of AcP on the binding of AlgR to this fimU promoter fragment (Fig. 2A). Elevating the molar ratio of AcP to AlgR increased AlgR binding (Fig. 2B, lanes 3 to 8), where a 20:1 molar ratio was the minimum ratio required for binding (Fig. 2B, lane 6). The interaction of AlgR-P with fimU DNA was specific, since the addition of unlabeled fimU DNA significantly decreased this interaction (Fig. 2B, lanes 9 to 12), and AlgR-P did not bind to the pscEF promoter (Fig. 2C, lanes 11 to 13). These results indicated that AlgR-P might have a higher affinity for fimU DNA than unphosphorylated AlgR.

Next, the binding of AlgR to fimU DNA was compared with that of AlgR-P. Based on empirical evidence that a 20× excess of AcP was required for binding (Fig. 2B), we performed binding assays with a 500× molar excess of AcP. As shown in Fig. 2C, lanes 6 to 9, AlgR pretreated with AcP (AlgR-P) showed a higher level of binding than AlgR by itself (Fig. 2C, lanes 2 to 5). This result supported the hypothesis that phosphorylation directly affected the affinity of AlgR for the fimU promoter DNA. At higher protein/DNA ratios, an additional band was detected above the prominent main band (Fig. 2C, lane 9). The concentrations of

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**FIG 1** AlgR D54E increased fimU transcription. (A) Diagram showing the genotypes of the following strains: PAO1 (algR), PSL317 (ΔalgR), WFPA8 (algRD54E), PAO1 algRD54E, PAZ (ΔalgZ), and PAZ algRD54E (ΔalgZ algRD54E). The dashed regions represent the deletion of the gene indicated. (B) The strains diagramed in panel A were tested for fimU transcriptional activity. Strains contained a fimU: lacZ transcriptional reporter in the chromosomal attB locus. β-Galactosidase activities from 21-h LB cultures grown at 37°C were assayed. Significant differences from the wild type by Student t tests are indicated (***, P < 0.001). Results are means for three replicates. (C) Western blotting was performed using AlgR- and OmlA-specific antibodies. Sixteen-hour cultures of strains without fusion reporters were grown in LB at 37°C and were normalized to equivalent CFU/ml. Cells were sonicated, and proteins were separated by SDS-PAGE. (D) AlgR protein levels of strains relative to those of wild type were quantified by measuring the intensities of the Western blot bands, normalized to OmlA, as described in Materials and Methods. Results were compared with those for the wild type by one-way analysis of variance with the Bonferroni posttest. N.D., not determined; n.s., not significant. Asterisks indicate P values of 0.05 to 0.01 (*) or 0.01 to 0.001 (**). Results are means from three replicates.
**FIG 2** Phosphorylated AlgR and AlgR D54E bound to the **fimU** promoter. The effect of in vitro phosphorylation of AlgR on binding to an 82-bp **fimU** DNA fragment was compared with the binding of purified AlgR D54N or AlgR D54E using EMSA. (A) Diagram of the 82-bp fragment used in EMSAs and its location within the **fimT-fimU** intergenic region. (B) Increasing amounts of the phosphate donor, AcP, were added to the AlgR protein in order to determine the effect of phosphorylation on DNA binding. Wild-type AlgR protein was pretreated with AcP for 30 min prior to binding reactions. Lanes 1 to 8, **fimU** DNA alone (lane 1) or in the presence of AlgR without AcP (lane 2) or with increasing concentrations of AcP (lanes 3 to 8). Lanes 9 to 12, the AlgR-P–DNA complex was competed with the addition of nonradiolabeled **fimU** DNA. (C) Phosphorylated AlgR protein was compared with untreated AlgR for DNA binding. Purified AlgR protein was pretreated either with water only or with AcP (500× molar excess of protein) prior to binding reactions. Lanes 1 to 10, **fimU** DNA incubated with a no-protein control (lane 1), increasing concentrations of AlgR (lanes 2 to 5) or AlgR-P (lanes 6 to 9), or 5 mM AcP only (lane 10). Lanes 11 to 13, 5 nM **pscEF** DNA incubated with a no-protein control (lane 11), 2.5 mM AlgR (lane 12), or 2.5 mM AlgR-P (lane 13). (D) Purified AlgR D54N or AlgR D54E protein was tested for binding to **fimU** DNA. Lane 1, no-protein control; lanes 2 to 5, increasing concentrations of AlgR D54N; lanes 6 to 9, increasing concentrations of AlgR D54E (lanes 6 to 9). Open arrowheads, unbound DNA; filled arrowheads, AlgR-DNA complexes. All concentrations given in the figure are final binding reaction concentrations.
AlgR or AlgR-P that bound to fimU DNA did not bind to the nonspecific pscEF control DNA (Fig. 2C, lanes 11 to 13), indicating that the interaction of AlgR with fimU DNA was specific.

In order to verify that the AlgR isoforms AlgR D54E and AlgR D54N mimicked phosphorylated and unphosphorylated AlgR, respectively, these purified proteins were tested on the same fimU promoter fragment used in the experiments described above. As shown in Fig. 2D, AlgR D54E had higher affinity for DNA than AlgR D54N. Interestingly, the AlgR D54E protein did not form a higher-order shift (Fig. 2D, lane 9) such as that observed for AlgR-P (Fig. 2C, lane 9).

We further characterized the melting temperatures of the AlgR D54E and AlgR D54N isoforms in order to determine if the Asp mutation had an effect on protein stability. Although both isoforms displayed a 10°C decrease in melting temperature from that of wild-type AlgR (see Fig. S1B in the supplemental material), only the PAO1 algRD54E strain transcriptionally activated the fimU promoter (Fig. 1), and AlgR D54E bound the fimU promoter by EMSA (Fig. 2D). In summary, the binding of AlgR to the fimU promoter was dependent on AlgR phosphorylation, and the strains expressing the phosphodefective and phosphomimetic AlgR isoforms have potential use in characterizing the role of AlgR phosphorylation in P. aeruginosa.

The twitching motility phenotypes of PAO1 algRD54E and PAO1 were identical. The fimU promoter is upstream of six open reading frames (ORFs) (fimU–pilVWXY1E), which encode pilpil and a calcium-dependent retraction protein required for the normal assembly and function of the type IVa pilus apparatus (36, 53–56). Since the expression of the fimU operon directly affects type IVa pilus function, the twitching motility phenotype in the context of the algR and algZ mutants was tested. The wild-type (algR+), $\Delta_{algR}$, algRD54N, algRD54E, $\Delta_{algZ}$, and $\Delta_{algZ}$ algRD54E strains were examined for subsurface (Fig. 3A) and surface-associated (Fig. 3B) twitching motility. In both assays, the size of the twitching zone for PAO1 algRD54E was comparable to that for wild-type PAO1, indicating that despite increased fimU expression in the algRD54E strain, there was no net effect on normal twitching motility. As expected, the $\Delta_{algR}$ and algRD54N backgrounds were defective in twitching motility due to decreased fimU operon transcription, a finding that corroborated with previous studies (25, 30, 51). Motility was also decreased in the $\Delta_{algZ}$ background, as predicted (21), since deletion of the cognate histidine kinase would render intracellular AlgR mostly unphosphorylated.

It was also considered possible that introduction of the algRD54E allele into the $\Delta_{algZ}$ background would restore twitching motility. Yet twitching motility was mostly, but not completely, restored in strain PAZ algRD54E, compared to that in the wild type, by the subsurface and surface assays (Fig. 3A and B). Western blots showed a significant increase in AlgR protein expression (Fig. 1C and D), accompanied by a significant increase in fimU::lacZ activity (Fig. 1B). Previously, it was shown that the expression of the genes in the fimU operon is tightly regulated, and overproduction of any of those genes could contribute to the loss of type IV pilus function (56). Consequently, our observations are consistent with the view that overexpression of the genes encoded in the fimU operon, and the twitching motility defect observed in strain PAZ algRD54E, may be explained by the 12-fold increase in AlgR protein production (Fig. 1D) and the corresponding 36-fold increase in fimU expression (Fig. 1A).

AlgR phosphorylation was required for swarming motility. Previously, an algR deletion mutant showed a 4-fold reduction in swarming motility (13, 28). Since twitching motility was affected, the effects of the phosphorylation status of AlgR on swarming motility were examined. Deletion of algR or algZ in PAO1 resulted in a swarming phenotype that lacked discernible dendrites (or tendrils) (Fig. 3C). In fact, when the pilA::Tc background was tested, it displayed a phenotype similar to that of the $\Delta_{algR}$ or $\Delta_{algZ}$ background, possibly indicating that AlgR affected swimming motility through its impact on type IV pilus function (Fig. 3C).

Swarming motility was also altered in the strain expressing phosphodefective algRD54N, where the swimming zone was smaller than that of either the wild-type strain PAO1 or PAO1 algRD54E, with no discernible dendrites. Lastly, PAO1 algRD54E reproduced the swarming phenotype of the wild-type strain, indicating that the phosphomimetic/phosphorylated form of AlgR is required for the swimming phenotype.

Swarming motility is thought also to be coordinated and affected by flagellum-mediated motility and the production of biosurfactants [β-β-(β-β-hydroxyalkanoyloxy)alkanoic acid (HAA), monohammolipids, and dirhamnolipids] (14, 15, 57, 58). On the other hand, swimming motility is mediated primarily by a single polar flagellum and does not require rhamnolipid production (14). To eliminate the possibility that the defect in swarming motility was due to AlgR- or AlgZ-mediated regulation of flagella, swimming motility assays were performed. Deletion or mutation of algR did not affect swimming motility (see Fig. S3 in the supplemental material), indicating that a flagellar defect was not responsible for the peculiar swimming motility phenotypes. Although there was a discernible decrease in swimming in the $\Delta_{algZ}$ or $\Delta_{algZ}$ algRD54E background, this phenotypic effect was small compared to that for the flic::Gm negative control.

Expression of the rhlA gene decreased in the phosphodefective AlgR background. The results from the swimming and swarming motility assays using the algR deletion, phosphodefective, and phosphomimetic mutants suggested that there was a flagellum-independent dysregulation of the swimming phenotype in the strain expressing phosphodefective AlgR (AlgR D54N). Both flagella and rhamnolipids, as well as a number of other factors (14, 58), are required for the swimming phenotype across many Pseudomonas strains (59). We observed previously that AlgR repressed rhamnolipid production in the context of a 6-day biofilm (28). Therefore, it is possible that AlgR phosphorylation affects swimming motility through the regulation of rhamnolipid production.

In order to test whether the difference in swarming motility was due to AlgR regulation of the genes encoding the rhamnolipid biosynthesis genes, transcriptional analyses were performed on the rhlA gene. Previously, AlgR was found to repress the rhlAB genes in a biofilm-specific manner and to exert its regulation specifically at the rhlA promoter (28). The rhlA promoter is also rhl quorum-sensing dependent, activated by the N-butanoyl-l-homoserine lactone (C4-HSL)-sensing RhlR regulator (60), and a strain containing a rhlR-null allele was used as a negative control for rhlA expression. A transcriptional reporter of the rhlA promoter was constructed and placed in the wild-type, $\Delta_{algR}$, algRD54N, algRD54E, $\Delta_{algZ}$, or rhlR::Tn501 background (Fig. 4A). In addition, RNA was isolated, and rhlA transcript levels were measured by quantitative reverse transcription-PCR (qRT-PCR) (Fig. 4B). In agreement with the rhamnolipid production results (see below), rhlA promoter activity was decreased in the
AlgR phosphorylation modulates rhamnolipids. AlgR Phosphorylation Modulates Rhamnolipids

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**FIG 3** Effects of different algR alleles on twitching and swarming motility. (A) Subsurface twitching motilities were analyzed at 48 h after inoculation in LB–1% agar, and the average diameters of twitching zones were measured. Strains PAO1 (algR<sup>+</sup>) (WT), PSL317 (ΔalgR), WFPA8 (algRD54N), PAO1 algRD54E, PAZ (ΔalgZ), and PAZ algRD54E (ΔalgZ algRD54E) were analyzed. PAOA1 (pilA::Tc) was used as a negative control. Statistical analysis was performed by one-way analysis of variance. n.s., not significant; ***, P < 0.001. (B) The strains for which results are shown in panel A were tested for surface-associated twitching motility in a 5% O<sub>2</sub> incubator. Bacteria were inoculated onto the surface of M9 minimal medium. Cells were imaged at 24 h postinoculation. Arrows mark the extent of the twitching front. Bar, 1 mm. (C) Strains for which results are shown in panels A and B were tested for swarming motility. As additional controls, PAO1 ΔrhlA and PAO1C (fliC::Gm) were examined. Bar, 1 cm.

**algRD54N or ΔalgZ background strains.** As expected, expression in the rhlR::Tn501 and ΔrhlA mutants was diminished.

**Rhamnolipid production decreased in the phosphodefective AlgR background.** To test whether rhamnolipid production was affected by AlgR phosphorylation, rhamnolipid production was measured in the PAO1 (wild-type) and PAO1 ΔalgR strains and in the mutant strains expressing the phosphodefective and phosphomimetic forms of AlgR. Measurements were conducted in three separate ways: (i) thin-layer chromatography (TLC) (Fig. 5A), (ii) an orcinol-based colorimetric assay (Fig. 5B), and (iii) a hemolysis assay (Fig. 5C). Both the TLC and orcinol assays of the corresponding cell-secreted supernatants showed lower levels of total rhamnolipid production in the algRD54N or ΔalgZ background than in the wild type (Fig. 5A and B). In addition, heat-stable hemolytic activity was abrogated in heat-treated supernatants from the algRD54N or ΔalgZ background (Fig. 5C) (45). In contrast, strain PAO1 algRD54E was comparable to the wild type in all assays. The ΔalgR background, on the other hand, produced slightly decreased rhamnolipid levels in two of the three quantitative measurements. We were also able to replicate these results by
measuring rhamnolipid production in a colorimetric plate assay (cetyltrimethylammonium bromide [CTAB]/methylene blue assay) (data not shown). These data support the hypothesis that AlgR phosphorylation is required for full production of rhamnolipids.

Phosphorylated AlgR bound differentially to the rhlA promoter. Because the phosphomimetic and phosphodefective AlgR mutants showed effects on rhamnolipid production, the question of whether phosphorylation of AlgR might alter its binding to the rhlA promoter was considered. AlgR has been shown previously to bind to a DNA fragment spanning positions −804 to −622 relative to the transcriptional start site (Fig. 6A, fragment 1, AlgR binding site 1), with specific binding to a 9-bp consensus sequence found 702 bp upstream of the transcriptional start site. A second binding site was also discovered previously by homology, but purified AlgR did not bind to this site (28). Due to the apparent distance between the rhlA transcriptional start site and the AlgR binding sites, EMSAs were performed to determine if additional AlgR binding sites exist within the rhlA promoter. Six rhlA promoter fragments, spanning 1.1 kb, were PCR amplified and radiolabeled (Fig. 6A). Both AlgR and phosphorylated AlgR (AlgR-P) displayed binding to fragment 1 (Fig. 6B and C), and phosphorylated AlgR (AlgR-P) displayed binding to fragment 5 (Fig. 6B and E).

rhlA fragment 1 was further examined using a range of concentrations of AlgR or AlgR-P (Fig. 6C). Phosphorylated AlgR displayed a third bound form (Fig. 6C, top black arrow) not observed with the nonphosphorylated form of the protein (AlgR-P versus AlgR, Fig. 6C). This result was also recapitulated with the AlgR D54N and AlgR D54E isoforms (data not shown). These results suggest cooperativity between multiple binding sites within the fragment tested. In order to dissect these binding characteristics further, either one or both of the AlgR binding sites were mutated (Fig. 6D). Deletion of the highly conserved AlgR binding site 1 resulted in decreased AlgR affinity for the DNA fragment, while AlgR-P was still able to bind (Fig. 6D, Δ1). Deletion of both binding sites (Δ1Δ2) resulted in loss of binding altogether, indicating that AlgR binding site 2 is a possible binding site only in the presence of AlgR-P. However, mutation of AlgR binding site 2 (Δ2) did not affect binding, corroborating previous results suggesting that this may not be a binding site (28). Additionally, a fragment proximal to the transcriptional start site of rhlA (−192 to −36 relative to the transcriptional start site) resulted in protein binding. AlgR-P displayed higher affinity than AlgR for fragment 5 DNA (Fig. 6B and E). These results suggest that multiple binding sites may be involved in regulating the rhlA promoter, which depends on the phosphorylation-dependent affinity of the AlgR protein.

Expression of rhlAB restored the swarming motility phenotype in the algR D54N background. Based on the observations that the strain expressing algRD54N had a defective swarming motility phenotype and decreased rhamnolipid production under a range of conditions, we examined whether the swarming motility defect was due to its decreased rhamnolipid production. Therefore, a vector containing the rhlAB genes with an arabinosinate-inducible promoter was constructed. The pHERD30T or p30TrhlAB vector was cloned into the ΔrhlA or algRD54N background. As shown in Fig. 7A, induction with increased arabinose concentrations was associated with increased rhamnolipid production in the ΔrhlA background by TLC (Fig. 7A, lanes 3 to 5). The addition of 0.5% arabinose to the algRD54N background harboring p30TrhlAB increased the production of monorhamnolipids (Fig. 7A, lane 7) over that with no arabinose (Fig. 7A, lane 6).

Because rhamnolipid production was restored by the expression of the rhlAB genes, swarming motility was tested in the PAO1 ΔrhlA background containing the p30T rhlAB vector. Increased amounts of arabinose in the M9 medium restored the dendrites and the swarming motility for the ΔrhlA strain harboring p30TrhlAB, but not for the ΔrhlA strain carrying the empty-vector control (Fig. 7B). Additionally, swarming motility was restored in the algRD54N background harboring p30T rhlAB, but not for the same strain harboring the vector alone (Fig. 7C). The dendrites were again restored with increased concentrations of arabinose, supporting the hypothesis that a defect in rhamnolipid production contributes to the swarming motility defect observed in the algRD54N background.

**DISCUSSION**

Phosphomimics have not yet been tested in response regulators containing the AlgR/AgrA/LytR family of DNA binding domains. As summarized and suggested by Smith et al. (61), we replaced the
algR phosphorylation was required for maximal rhamnolipid production. (A) Thin-layer chromatography. Strains PAO1 (WT), PSL317 (ΔalgZ), /H9004 were grown for 16 h in TSB plus 1% glycerol. Mono, monorhamnolipid band; Di, dirhamnolipid band. (B) Strains for which results are shown in panel A were tested for rhamnolipid production by use of the orcinol colorimetric assay and comparison with a standard curve (see Materials and Methods). Values were normalized to total-protein values. (C) The extent of hemolysis due to rhamnolipid production was measured. Cultures were grown for 24 h in a phosphate-limiting medium (tryptic soy broth supplemented with 1% glucose), and cell-free supernatants were heat treated to denature polypeptide-based hemolysins. Serial dilutions were applied to 1% horse red blood cells for 1 h at 37°C, and the percentage of hemolysis was calculated based on total and no hemolysis. Statistical analysis was performed by one-way analysis of variance. n.s., not significant. Asterisks indicate P values of 0.05 to 0.01 (*), 0.01 to 0.001 (**), or <0.001 (***)..

**FIG 5** AlgR phosphorylation was required for maximal rhamnolipid production. (A) Thin-layer chromatography. Strains PAO1 (WT), PSL317 (ΔalgR), WFPA8 (algRD54N), PAO1 algRD54E, PAZ (ΔalgZ), and PAO1 ΔrhlA (ΔrhlA) were grown for 16 h in TSB plus 1% glycerol. Mono, monorhamnolipid band; Di, dirhamnolipid band. (B) Strains for which results are shown in panel A were tested for rhamnolipid production by use of the orcinol colorimetric assay and comparison with a standard curve (see Materials and Methods). Values were normalized to total-protein values. (C) The extent of hemolysis due to rhamnolipid production was measured. Cultures were grown for 24 h in a phosphate-limiting medium (tryptic soy broth supplemented with 1% glucose), and cell-free supernatants were heat treated to denature polypeptide-based hemolysins. Serial dilutions were applied to 1% horse red blood cells for 1 h at 37°C, and the percentage of hemolysis was calculated based on total and no hemolysis. Statistical analysis was performed by one-way analysis of variance. n.s., not significant. Asterisks indicate P values of 0.05 to 0.01 (*), 0.01 to 0.001 (**), or <0.001 (***)..

AlgR phosphorylation was required for maximal rhamnolipid production. We utilized the fimU promoter and the corresponding twitching motility phenotype as functional readouts for AlgR D54E (phosphomimetic) or AlgR D54N (phosphodefective) activity. The expression of the algRD54E allele from a plasmid (data not shown) or in a single copy from the chromosome (Fig. 1B) activated fimU promoter transcription and displayed wild-type twitching motility (Fig. 3A and B). Conversely, the PAO1 ΔalgZ and PAO1 algRD54N strains had decreased fimU transcriptional activity (Fig. 1B) and abrogated twitching motility (Fig. 3A and B). FimU, PilV, PilW, PilX, and PilE are required for surface pilation and most likely interact with PilA to stabilize the pilus fiber (56). Our results supported previous observations that phosphorylated AlgR is required for the expression of the fimU-pilVWXY1E operon and normal twitching behavior (25, 50).

Furthermore, we showed that AlgR D54E activity was independent of the histidine kinase AlgZ. The ΔalgZ algRD54E background activated fimU promoter activity (Fig. 1B) and twitching motility in vivo (Fig. 3A and B). This idea was supported in vitro, where AlgR D54E (or AlgR D54N) could not be phosphorylated with radiolabeled acetyl phosphate (see Fig. S2A in the supplemental material). There was a 12-fold increase in AlgR D54E protein expression in the ΔalgZ algRD54E background (Fig. 1D), which potentially explains the elevated fimU reporter activity (Fig. 1B). We now know the reason for elevated AlgR expression in this background. The rationale for deleting the algZ region, as noted in the discussion of Fig. 1A, was to avoid affecting the previously identified transcriptional start sites/promoters for algR. It is possible that this mutation, in combination with the algRD54E allele, increased AlgR expression. A proper stoichiometric expression of the minor pilins is essential for the formation of pilus strands and for functional twitching motility (56). The increased amounts of AlgR D54E resulting in the 36-fold elevation of fimU expression would likely disrupt the function of the type IVa pilus complex and result in the aberrant twitching motility phenotype observed for the ΔalgZ algRD54E strain (Fig. 3A and B). It was noted previously that overexpression of individual pilins in their respective pilin deletion backgrounds abrogated twitching motility but that overexpression of individual pilins in a wild-type strain expressing the fimU-pilVWXY1E2E genes showed only a modest decrease in twitching (56). Our result with PAZ algRD54E is agreement with this observation, since this strain retains the fimU operon genes. Taken together, these results are in agreement with previous observations for other organisms with different response regulators, where an aspartate-to-glutamate mutation results in activation of OmpR D55E (48), NtrC D54E (27, 49), and RcsB D56E (47) at their target promoters in a kinase-independent manner.

We provided evidence that AlgR phosphorylation appears to modulate rhamnolipid production. We demonstrated that rhlA expression (Fig. 4) and rhamnolipid production (Fig. 5) levels were decreased in the algRD54N and ΔalgZ backgrounds but were unaffected in the algRD54E background. We also observed AlgR-dependent effects on swarming motility. The WT and algRD54E backgrounds exhibited discernible dendrites, but the ΔalgR, algRD54N, and ΔalgZ backgrounds displayed noticeable swarming motility defects (Fig. 3C). The most likely explanation for the different swarming phenotypes observed for the ΔalgR and algRD54N backgrounds is that the strain carrying the algRD54N allele produced less rhamnolipid than the strain with the ΔalgR.
This decrease, in combination with abrogated twitching motility (Fig. 2), likely decreased overall swarming motility in the algR\textsuperscript{D54N} strain. Following this logic, we expected to see similar swarming phenotypes for PAO1\textsuperscript{algR\textsuperscript{D54N}} and PAO1\textsuperscript{algZ}. However, PAO1\textsuperscript{algZ} displayed a swarming phenotype similar to that of PAO1\textsuperscript{algR\textsuperscript{D54N}}. PAO1\textsuperscript{algR\textsuperscript{D54N}} expresses AlgR\textsuperscript{D54N} protein, which is unable to be phosphorylated, while the AlgR protein expressed in PAZ may be phosphorylated by other intracellular phosphodonsors. We currently do not know if or when AlgR may be phosphorylated in the absence of AlgZ. If this is

![Diagram of fragments used for EMSAs, showing their locations on the rhlA promoter.](https://www.example.com/image.png)

**FIG 6** Phosphorylated AlgR recognized three different binding sites within the rhlA promoter. (A) Diagram of fragments used for EMSAs, showing their locations on the rhlA promoter. The locations of the previously identified AlgR binding sites and sequences are indicated (28): the consensus AlgR binding site 1 (sense strand, 5’-CCGTTCGTC) and AlgR binding site 2 (antisense strand, 5’-CCGTGCTCC). (B) EMSAs using AlgR or acetyl phosphate-treated AlgR on DNA fragments corresponding to those diagramed in panel A. The concentrations of proteins in binding reaction mixtures are indicated. Controls contain rhlA fragments only (lanes 1, 5, 9, 13, 17, and 21). (C) Further examination of rhlA fragment 1, using AlgR or AlgR-P. Control lanes (lanes 1 and 10) contained rhlA fragment 1 only. For AlgR-P, AlgR was preincubated with a 10× molar ratio of acetyl phosphate prior to the binding reaction. Increasing concentrations of AlgR (lanes 2 to 9) and AlgR-P (lanes 11 to 18) were used (1, 5, 10, 50, 125, 250, 500, and 1,250 nM protein, respectively) in the presence of 5 nM radiolabeled DNA. Black arrowheads indicate the locations of AlgR-bound DNA; open arrowheads indicate the locations of unbound DNA. (D) Either AlgR binding site 1 (Δ1), AlgR binding site 2 (Δ2), or both (Δ1Δ2) were mutated (see Materials and Methods), and strains were tested for AlgR or AlgR-P binding. A 25:1 molar ratio of protein to DNA was used. (E) Binding of increasing concentrations of AlgR or AlgR-P protein to fragment 5. Control lanes (lanes 1 and 8) contained rhlA fragment 5 only. AlgR (lanes 2 to 7) and AlgR-P (lanes 9 to 14) concentrations were increased (1, 5, 10, 50, 125, and 250 nM protein, respectively) in the presence of 5 nM radiolabeled DNA.
the case, under certain conditions, AlgR could potentially increase rhamnolipid production and partially reverse the swarming defect. For the PAO1 \( \text{algZ algR} \quad \text{D54E} \) mutant, we expected to observe a phenotype resembling that of the wild type or PAO1 \( \text{algR} \quad \text{D54E} \). Instead, however, we observed a decrease in overall swarming. Rhamnolipid production appears to be finely regulated by AlgR levels; \( \text{algR} \) expressed from a plasmid repressed rhamnolipid production (data not shown). Since AlgR \( \text{D54E} \) expression is increased (Fig. 1C), it is possible that this reduced swarming in PAO1 \( \Delta \text{algZ algRD54E} \) is due to repression of rhamnolipids.

While the single polar flagellum helps to propel the organisms, the products of the RhlA, RhlB, and RhlC enzymes (HAA, monorhamnolipids, and dirhamnolipids) coordinate the formation of the dendrite phenotype in swarming cells (14). Since the \( \text{algR} \) mutants showed no swimming defects (see Fig. S3 in the supplemental material), we opine that the swarming defect is due to a dysregulation in rhamnolipid production. In fact, when \( \text{rhlAB} \) was expressed from a plasmid in the \( \text{algRD54N} \) background, dendrite formation was partially restored (Fig. 7). These results indicate an AlgR phosphorylation-dependent regulation of swarming motility through rhamnolipid expression.

We have not eliminated the possibility that AlgR phosphorylation also regulates swarming motility indirectly. Swarming motility is a highly complex phenotype that is regulated by a number of factors (62–65). Recently, type IVa pili have been shown to control swarming motility through cyclic di-GMP (c-di-GMP) levels (66). Low intracellular c-di-GMP levels are required for swimming and swarming motility (67). Deletion of \( \text{bifA} \), encoding a phosphodiesterase, results in a swimming defect due to a buildup of intracellular c-di-GMP (55). Deletion of \( \text{PilY1} \), \( \text{PilX} \), or \( \text{PilW} \) in a \( \text{bifA} \) deletion strain alleviates the swimming defect found with \( \text{bifA} \) deletion (55, 66) without changing intracellular c-di-GMP levels (55), indicating that PilY1 suppresses swimming specifically through BifA (66). Overexpression of PilY1 represses swimming, but not in a strain with a deletion of \( \text{sadC} \) (encoding a diguanylate cyclase that increases c-di-GMP levels), suggesting a role for PilY1 in directly or indirectly regulating c-di-GMP levels through SadC (66). While we observed no transcriptional effects on \( \text{sadC} \) or \( \text{bifA} \) in our microarrays (unpublished data), and no change in swarming motility in the \( \text{algRD54E} \) background (Fig. 3), it would be interesting to explore if and how AlgZ/R might affect c-di-GMP levels.

We suggest AlgR phosphorylation as a mechanism by which AlgR coordinates the transcription of various genes, including the \( \text{fimU} \) promoter (Fig. 2) and the \( \text{rhlAB} \) promoters (Fig. 8). Phosphorylation of AlgR appears to increase its binding affinity for these lower-consensus binding sites to enable promoter activation. We supported this hypothesis by prephosphorylating AlgR with acetyl phosphate and observing increased AlgR binding to \( \text{fimU} \) (Fig. 2B and C) and \( \text{rhlA} \) (Fig. 6B, C, and E) DNA. Compared
to the highest-affinity binding site from the algD promoter (5′-C CGTTGTC), fimU ABS1 (5′-CGGTTGGCC) has two mismatches and fimU ABS2 (5′-CCCTCGGGGC) has four mismatches (50) (underlined bases differ from those in the canonical AlgR binding site). Previous work on algC ABS2 (5′-CGGTTGGTC), algC ABS3 (5′-CCGGTTGTC), and algD ABS3 (5′-CGGTTGGTC) showed that mismatches in the binding site resulted in lowered AlgR affinity (68, 69), indicating that the sequence is essential for protein binding. In support of this idea, AlgR or AlgR-P displayed higher affinity to rhlA fragment 1 DNA (containing the conserved rhlA ABS1 [5′-CGGTTGTC]) (Fig. 6C) than to fimU promoter DNA (containing the less-conserved binding sites) (Fig. 2C).

The AlgR phosphorylation state may also modulate rhlAB expression as a function of the AlgR binding site location and binding affinities (Fig. 8). The algD and algC promoters contain AlgR binding sites that are found hundreds of bases apart from each other (22, 68, 70, 71). In the algD promoter, two identical ABSs are located −471 bp and −396 bp upstream, and one ABS is located −45 bp upstream, of the transcriptional start site (70, 71). DNA looping has been proposed as a mechanism for algD regulation (68). Additionally, an ABS in the algC promoter has been reported to function as an enhancer element for algC transcription (72). This idea is reminiscent of findings for other response regulators, such as OmpR, where cooperative binding of both proximal and distal binding sites in the ompF and ompC promoters influence how OmpR-P regulates the transcription of both promoters (48, 73). In this study, we observed multiple protein-DNA complexes with rhlA fragment 1 (Fig. 6C). We do not currently know the nature of these complexes and if or how AlgR is oligomerizing on the DNA. The facts that AgrA-type response regulators bind imperfect, direct 9-bp repeats separated by 12 bp (74) and that AlgR requires both binding sites to shift rhlA fragment 1 at this concentration (Fig. 6D, ΔΔ2) suggest that AlgR may be binding to multiple sites in rhlA fragment 1. However, even though the sequence for rhlA AlgR binding site 2 (5′-CGGTTGTC) is similar to the known AlgR binding sequence (5′-CGGTTGGTC) and is located at the appropriate distance for AgrA-type regulators (10 bp), our data also confirm previous results suggesting that rhlA AlgR binding site 2 is most likely not a real binding site (Fig. 6D, Δ2) (28). When EMSAs were performed with AlgR-P, the deletion of rhlA ABS1 (the highly conserved site) in rhlA fragment 1 (Fig. 6D, Δ1) resulted in a higher shift (top black arrowhead) than that seen for WT rhlA fragment 1. These large shifts were also present when AlgR-P (Fig. 6C, lanes 17 and 18), but not AlgR (Fig. 6C, lanes 8 and 9), was added in large amounts. Additional experiments are needed to elucidate how this occurs and whether it is biologically relevant.

In this study, a second region within rhlA fragment 5 was found to bind phosphorylated AlgR (Fig. 6E). In attempts to determine this third binding site, a probable AlgR binding sequence in rhlA fragment 5 (5′-CCGCCGTC [underlined bases differ from those in the canonical AlgR binding site]) was mutated, and the mutant was investigated for binding and transcriptional activity (data not shown). We surmised that the binding site, located adjacent to the RhlR-binding lux box, is reminiscent of those found in class I activation of promoters, where an activator binds to a target located upstream of the −35 element (75). However, we eliminated this site as a possible AlgR binding site, because a mutation of this site (5′-CCGCCGTC to 5′-ttaCCGCC) did not affect AlgR binding by EMSA (data not shown). By use of a transcriptional fusion, mutation of this site did decrease rhlA transcription, but this effect is most likely due to disruption of RhlR binding (data not shown) (76). Therefore, further investigation of this third binding site is necessary. Considering that there may be a third AlgR binding site located within rhlA fragment 5, and considering that two other AlgR binding sites are located on rhlA, it would not be unreasonable to suggest that a type of AlgR regulation similar to that found on the algD promoter may occur with the rhlA promoter (Fig. 8). We also propose that the binding affinities for the ABSs may affect whether the promoter is AlgR phosphorylation dependent or not (Fig. 8). This is supported by the fact that the algD promoter, containing two consensus AlgR binding sites (algD RB1 and algD RB2 [5′-CGGTTGGTC]) (68), does not require AlgR phosphorylation for its transcription (20, 77), while fimU requires AlgR phosphorylation for activation (50).

Our lab has demonstrated previously that AlgR represses rhlAB expression in the context of a 6-day biofilm, since the PA01 ΔalgR
strain showed increased rhlA expression and rhamnolipid production (28). Therefore, we initially expected to observe AlgR repression of rhlA transcription. In fact, our lab previously demonstrated that stationary-phase cultures did not display differences in rhlA expression (28), suggesting that AlgR repression of the rhlA promoter occurs predominantly in a late-stage biofilm. Under our experimental conditions, we observed that rhlA expression and rhamnolipid production in the PAO1 ΔalgR background were unchanged or decreased from those of the wild type (Fig. 4 and 5). This result may be due to the fact that in the absence of AlgR, a modulatory component on the rhlA promoter is absent, and rhlA expression is reliant on other regulators to activate this promoter. It was also necessary to perform multiple assays to confirm the rhamnolipid phenotype, because the differences in production were subtle. The discrepancy between PAO1 algRD54N and PAO1 ΔalgR results (Fig. 5) among the three rhamnolipid assays is likely due to different experimental parameters. For the hemolysis assay, the cell supernatant was collected after 24 h, potentially allowing increased rhamnolipid production in PAO1 ΔalgR. We previously measured rhamnolipid production in stationary phase and showed no difference in rhamnolipid production, since both of these scenarios require type IVa pili and AlgR has not been determined. We speculate that AlgR-P is a modulatory component on the rhlA promoter and increases in C4-HSL levels in the presence of the algR strain and the algRD54N strain showed increased rhamnolipid expression (28), suggesting that AlgR phosphorylation-dependent effect on virulence, since both genes within the fimU operon (and twitching motility) and rhamnolipid production are virulence determinants and are required for pathogenicity (86–89).

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