Pseudomonas aeruginosa AlgU Contributes to Posttranscriptional Activity by Increasing *rsmA* Expression in a *mucA22* Strain

Sean D. Stacey, Christopher L. Pritchett

Department of Health Sciences, East Tennessee State University, Johnson City, Tennessee, USA

ABSTRACT

Pseudomonas aeruginosa thrives in multiple environments and is capable of causing life-threatening infections in immunocompromised patients. RsmA is a posttranscriptional regulator that controls virulence factor production and biofilm formation. In this study, we investigated the expression and activity of *rsmA* and the protein that it encodes, RsmA, in *P. aeruginosa mucA* mutant strains, which are common in chronic infections. We determined that AlgU regulates a previously unknown *rsmA* promoter in *P. aeruginosa*. Western blot analysis confirmed that AlgU controls *rsmA* expression in both a laboratory strain and a clinical isolate. RNase protection assays confirmed the presence of two *rsmA* transcripts and suggest that RpoS and AlgU regulate *rsmA* expression. Due to the increased amounts of RsmA in *mucA* mutant strains, a translational leader fusion of the RsmA target, *tssA1*, was constructed and tested in *mucA*, *algU*, *retS*, *gacA*, and *rsmA* mutant backgrounds to examine posttranscriptional activity. From these studies, we determined that RsmA is active in *mucA22* mutants, suggesting a role for RsmA in *mucA* mutant strains. Taken together, we have demonstrated that AlgU controls *rsmA* transcription and is responsible for RsmA activity in *mucA* mutant strains. We propose that RsmA is active in *P. aeruginosa mucA* mutant strains and that RsmA also plays a role in chronic infections.

IMPORTANCE

P. aeruginosa causes severe infections in immunocompromised patients. The posttranscriptional regulator RsmA is known to control virulence and biofilm formation. We identify a new *rsmA* promoter and determine that AlgU is important in the control of *rsmA* expression. Mutant *mucA* strains that are considered mucoid were used to confirm increased *rsmA* expression from the AlgU promoter. We demonstrate, for the first time, that there is RsmA activity in mucoid *P. aeruginosa* strains. Our work suggests that RsmA may play a role during chronic infections as well as acute infections.

Pseudomonas aeruginosa produces a myriad of virulence factors and can cause both acute and chronic infections (1, 2). To survive and persist, *P. aeruginosa* must coordinate gene expression in response to changing environmental conditions. Global regulatory networks respond to changing environmental conditions to allow the physiological changes necessary for survival to be made. The *P. aeruginosa* genome encodes many global regulatory systems, including posttranscriptional regulators, such as RsmA. RsmA is important in regulating several virulence factors involved in acute and chronic infections (3).

Acute infections are characterized by motility and expression of the type III secretion system (4). RsmA positively regulates both motility and type III secretion system expression (4–6). A defining characteristic of chronic infections is biofilm formation, which includes the production of exopolysaccharides, such as alginate, and the type VI secretion system (T6SS) (7, 8). RsmA is a negative regulator of the exopolysaccharide gene *psl* and the first hemolysin-coregulated secretion island type VI secretion system (HSI-I T6SS) (5, 8, 9). A characteristic of some *P. aeruginosa* isolates causing chronic infections, such as those found in cystic fibrosis patients, is alginate overproduction, which is the result of the extracytoplasmic sigma factor AlgU (7, 10).

AlgU is normally sequestered at the cytoplasmic membrane by the anti-sigma factor MucA (7, 10). MucA prevents AlgU from binding and activating target promoters (7, 10). Once liberated from MucA, AlgU activates a variety of genes, including those important for biosynthesizing alginate (7, 10–13). Strains overproducing alginate are termed mucoid. *P. aeruginosa* strains that

initially colonize cystic fibrosis patients frequently evolve into strains that overproduce alginate, often because of mutations in the *mucA* gene (7, 10). A previous study found that a *mucA* mutant has increased *rsmA* expression (14). Because *mucA* mutations lead to hyperactive AlgU (7, 10), we hypothesized that AlgU might regulate *rsmA* transcription.

RsmA is a global posttranscriptional regulator that regulates several mRNAs involved in virulence (5, 6). RsmA positively regulates type III secretion and type IV pilus production, both of which are important virulence factors for acute infections (6, 14). In the case of biofilm formation, RsmA inhibits exopolysaccharide production (5, 9). Therefore, RsmA is important in determining what virulence factors are expressed by *P. aeruginosa*. RsmA activity is regulated by two small RNAs, RsmY and RsmZ, that bind RsmA and prevent RsmA from binding target mRNAs (15). RsmY and RsmZ sequester RsmA, allowing the production of exopolysaccharides and other RsmA target mRNAs (9, 16). Increased

Received 5 February 2016 Accepted 12 April 2016

Accepted manuscript posted online 18 April 2016

Citation Stacey SD, Pritchett CL. 2016. *Pseudomonas aeruginosa* AlgU contributes to posttranscriptional activity by increasing *rsmA* expression in a *mucA22* strain. J Bacteriol 198:1812–1826. doi:10.1128/JB.00133-16.

Editor: G. A. O'Toole, Geisel School of Medicine at Dartmouth

Address correspondence to Christopher L. Pritchett, pritchettc@etsu.edu.

This article is dedicated to Joseph Albright.

Copyright © 2016, American Society for Microbiology. All Rights Reserved.

RsmY and RsmZ levels or decreased RsmA activity can allow biofilm formation to occur. The expression of *rsmY* and *rsmZ* is positively controlled by RsmA, GacA, and LadS and negatively controlled by RetS (16–18). RsmA binds to highly conserved GGA motifs. Usually, one of the GGA motifs is found in a stem-loop structure formed near the ribosome-binding site (19). Multiple copies of the same GGA motif are also found in the inhibitory small RNAs RsmY and RsmZ (20, 21).

Although RsmA is important for virulence and global gene expression (5, 22, 23), little is known regarding the genetic controls governing *rsmA* expression. Currently, only a single *rsmA* promoter region, which resembles a σ^{70} (RpoD) promoter, has been identified in *P. aeruginosa* (24–26). A recent chromatin immunoprecipitation sequencing study showed that both RpoD and RpoS likely control *rsmA* expression (27). However, another study found similar RsmA levels in both an *rpoS* mutant and the wild-type strain (9). Early studies found that optimal *rsmA* expression requires GacA and that *rsmA* expression increases throughout growth (28), findings which support a role for RpoS in controlling *rsmA* expression. These seemingly contradictory data have not yet been clarified.

Given the importance of RsmA in controlling *P. aeruginosa* virulence and biofilm formation, there is a critical need to understand *rsmA* expression. In this study, we determined the genetic elements controlling *rsmA* expression. We identified a new promoter region and determined that AlgU and RpoS contribute to *rsmA* expression. Importantly, we demonstrate that RsmA is active in *P. aeruginosa* *muca* mutants. This work suggests that RsmA has a role in regulating gene expression in *muca* mutants and that AlgU is an important activator of *rsmA* expression.

MATERIALS AND METHODS

Strains, plasmids, and media. The strains used in this study are presented in Table 1. *Escherichia coli* strains were maintained on LB (Difco) plates or broth without or with antibiotics, as appropriate. *Pseudomonas aeruginosa* strains were grown on *Pseudomonas* isolation agar (PIA), LB medium, or Vogel-Bonner minimal medium (VBMM) supplemented with the appropriate concentrations of antibiotics. For *E. coli*, antibiotics were used at the following concentrations when appropriate: 10 μ g/ml tetracycline, 15 μ g/ml gentamicin, 100 μ g/ml ampicillin, and 35 μ g/ml kanamycin. For *Pseudomonas* strains, antibiotics were used at the following concentrations: 150 μ g/ml gentamicin, 50 μ g/ml tetracycline, and 300 μ g/ml carbenicillin. For allelic exchange, sucrose was supplemented at 10% in YT (1% tryptone and 0.5% yeast extract) medium.

Strain construction. All PCR products were amplified from *P. aeruginosa* PAO1, unless otherwise noted, using Q5 polymerase (New England BioLabs [NEB], Ipswich, MA) and primers listed in Table 2. Crossover PCR (splicing by overhang extension PCR) (29) was used to construct deletion mutations and to clone sequences into the suicide vector pEX18Tc or pEX18Gm (30). All cloned constructs were confirmed via sequencing. *P. aeruginosa* strains were conjugated with *E. coli* as a donor strain and the pRK2013-containing helper strain (31). Conjugations were performed overnight on LB plates at 37°C, and the conjugants were plated on the appropriate selective medium to obtain single-crossover mutants. Merodiploids were grown without selection and then screened for sucrose sensitivity on YT medium–10% sucrose plates. Mutations were confirmed using PCR with primers containing the suffix intF and intR in Table 2 and sequencing of the resulting PCR fragment. Hemagglutinin (HA) tagging of the proteins was accomplished using primers containing the HA tag at the 3' end of the gene, and HA was introduced as described above using the suicide vector pEX18Gm.

Transcriptional and translational leader fusion analysis. Upstream DNA fragments containing promoter regions were generated by using the

primers listed in Table 2 in conjunction with Q5 polymerase (NEB, Ipswich, MA). PAO1 genomic DNA was used as the template. PCR products were cloned into pCR2.1 and then subcloned into miniCTXlacZ using the restriction enzymes HindIII/BamHI, HindIII/EcoRI, or KpnI/BamHI (New England BioLabs, Ipswich, MA). To construct *rsmA* transcriptional fusions, the primer pair rsmAHindIII/rsmAEcoRIR was used and the product was inserted into the SalI/EcoRI sites of miniCTXlacZ using T4 DNA ligase (NEB, Ipswich, MA). The transcriptional fusion PE3-lacZ was constructed using the primers rsmAPE3F and rsmAPE3R and cloning into the KpnI/EcoRI site of miniCTXlacZ. The transcriptional fusion TF2-lacZ was constructed using primer pair rsmAPE1F/rsmAEcoRIR and cloned into miniCTXlacZ. The translational fusion vector CTXlacZCP was constructed by amplifying the *lacZ* gene using the primers listed in Table 2 in order to delete the *lacZ* ribosome-binding site and the translational initiation codon. The *tssA1* leader fusion was constructed using the *lacUV5* promoter in the 5' primer (PA0082FUV5) and primer PA0082ScaIR to amplify the *tssA1* untranslated region on the basis of the data from the study of Brencic and Lory (5) and cloned into CTXlacZCP. The fusion constructs were confirmed by sequencing and conjugated into *P. aeruginosa* strains by triparental conjugation. Strains were selected for tetracycline resistance and then conjugated with pFLP2 to remove vector sequences (30). Strains were selected for carbenicillin resistance, grown overnight without selection, and plated on YT medium with 10% sucrose to select for the loss of pFLP2. Individual colonies were patch plated onto VBMM supplemented with 300 μ g/ml carbenicillin and PIA to ensure the loss of pFLP2. To confirm the presence of the fusion constructs, PCR was performed using the forward primer used to construct the fusion and the reverse primer lacZRforTF (Table 2). β -Galactosidase activity was determined by incubating cell extracts with *o*-nitrophenyl- β -D-galactopyranoside (4 mg/ml) as described by Miller (32). A strain carrying the empty vector, miniCTXlacZ, was assayed, and the value for this background (28 Miller units) was subtracted from all transcriptional fusions. All mucoid strains were confirmed to be mucoid at the end of each experiment by plating on PIA plates and making sure that all colonies were mucoid. Experiments were performed in triplicate at least three times.

Site-directed mutagenesis. Phosphorylated primers rsmASDAlgU and rsmASDAlgUR were used in an inverse PCR with the PE3-lacZ transcriptional fusions as the templates. The predicted AlgU-dependent promoter in the –35 consensus sequence was mutated from GAACTT to GAATTC. The successful inverse PCR product was self-ligated, transformed into NEB5 α cells (NEB, Ipswich, MA), and screened by plasmid digestion with EcoRI digestion and sequencing. The constructs obtained by site-directed mutagenesis were conjugated into *Pseudomonas* strains as described above.

Western blot analysis. *P. aeruginosa* strains were grown in LB broth at 37°C for various times. The bacteria were collected by centrifugation, resuspended in sterile phosphate-buffered saline, and lysed by sonication. Total protein concentrations were quantified by the Bradford protein assay (Bio-Rad, Carlsbad, CA). Cell extracts (5 μ g) were separated by 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membranes were blocked and probed using a 1:2,500 dilution of anti-HA monoclonal antibody (Thermo Fisher, Pittsburgh, PA), followed by a 1:30,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin antibody (33). Arbitrary units were determined by standardizing the Western blots to the Coomassie-stained SDS-polyacrylamide gel of each strain using total protein analysis as described previously (34, 35). Detection was performed using an ECL Plus kit (Thermo Fisher, Pittsburgh, PA) and chemiluminescence detection (ProteinSimple, Santa Clara, CA). The blots and Coomassie-stained SDS-polyacrylamide gels were analyzed with ImageJ software. All Western blot analyses were repeated at least three times.

Primer extension. Total RNA (15 μ g) from *P. aeruginosa* was isolated using a Pure-Link RNA minikit (Life Technologies, Grand Island, NY). Radiolabeled primers rsmAPE1 and rsmAPE3 (Table 2) were used in a reverse transcription reaction in a ThermoScript reverse transcription-

TABLE 1 Strains, plasmids, and constructs used in this study

Strain, plasmid, or construct	Genotype or relevant property(ies)	Reference or source
<i>E. coli</i>		
NEB5α	<i>fhuA2 Δ(argF-lacZ)U169 phoA glnV44 φ80dlacZΔM15 gyrA96</i>	New England BioLabs
SM10	<i>thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km^r</i>	56
pRK2013 strain	Helper strain	31
<i>P. aeruginosa</i>		
PAO1	Wild type	58
<i>ΔalgU</i> mutant	<i>ΔalgU</i>	57
<i>mucA22</i> mutant	<i>mucA22</i>	59
<i>mucA22 ΔalgU</i> strain 2192	<i>mucA22 ΔalgU</i>	This study 45
383		45
<i>ΔrsmA</i> mutant	<i>rsmA</i> nonpolar deletion	This study
<i>mucA22 ΔrsmA</i> mutant	<i>rsmA</i> nonpolar deletion in <i>mucA22</i> strain	This study
PAO1 TF1- <i>lacZ</i>	<i>rsmA</i> transcriptional fusion strain	This study
<i>ΔalgU</i> TF1- <i>lacZ</i> strain	<i>rsmA</i> transcriptional fusion strain	This study
<i>mucA22</i> TF1- <i>lacZ</i> strain	<i>rsmA</i> transcriptional fusion strain	This study
<i>mucA22 ΔalgU</i> TF1- <i>lacZ</i> strain	<i>rsmA</i> transcriptional fusion strain	This study
2192 TF1- <i>lacZ</i>	<i>rsmA</i> transcriptional fusion strain	This study
383 TF1- <i>lacZ</i>	<i>rsmA</i> transcriptional fusion strain	This study
383-HA	Nonmucoid clinical isolate with <i>rsmA</i> -HA allele	This study
2192-HA	Mucoid clinical isolate with <i>rsmA</i> -HA allele	This study
2192-HA <i>ΔalgU</i>	2192 with <i>rsmA</i> -HA allele and <i>algU</i> deletion	This study
2192 <i>ΔalgU</i> TF3- <i>lacZ</i>	<i>rsmA</i> transcriptional fusion strain	This study
PAO1 TF3- <i>lacZ</i>	<i>rsmA</i> transcriptional fusion strain	This study
<i>ΔalgU</i> TF3- <i>lacZ</i> strain	<i>rsmA</i> transcriptional fusion strain	This study
<i>mucA22</i> TF3- <i>lacZ</i> strain	<i>rsmA</i> transcriptional fusion strain	This study
<i>mucA22 ΔalgU</i> TF3- <i>lacZ</i> strain	<i>rsmA</i> transcriptional fusion strain	This study
PAO1 TF3USDM- <i>lacZ</i>	Mutant <i>rsmA</i> transcriptional fusion strain	This study
<i>ΔalgU</i> TF3USDM- <i>lacZ</i> strain	Mutant <i>rsmA</i> transcriptional fusion strain	This study
<i>mucA22</i> TF3USDM- <i>lacZ</i> strain	Mutant <i>rsmA</i> transcriptional fusion strain	This study
<i>mucA22 ΔalgU</i> TF3USDM- <i>lacZ</i> strain	Mutant <i>rsmA</i> transcriptional fusion strain	This study
PAO1 TF2- <i>lacZ</i>	<i>rsmA</i> transcriptional fusion	This study
<i>mucA22</i> TF2- <i>lacZ</i> strain	<i>rsmA</i> transcriptional fusion	This study
<i>ΔalgU</i> TF2- <i>lacZ</i> strain	<i>rsmA</i> transcriptional fusion	This study
<i>mucA22 ΔalgU</i> strain	<i>rsmA</i> transcriptional fusion	This study
PAO1-HA	PAO1 with epitope-tagged RsmA	This study
<i>ΔalgU</i> -HA mutant	<i>ΔalgU</i> mutant with epitope-tagged RsmA	This study
<i>mucA22</i> -HA mutant	<i>mucA22</i> mutant with epitope-tagged RsmA	This study

TABLE 1 (Continued)

Strain, plasmid, or construct	Genotype or relevant property(ies)	Reference or source
<i>mucA22 ΔalgU</i> -HA mutant	<i>mucA22 ΔalgU</i> strain with epitope-tagged RsmA	This study
PAO1-HA/pTJ1	PAO1 with <i>rsmA</i> -HA allele and vector pTJ1 alone	This study
PAO1-HA/pAlgU	PAO1 with <i>rsmA</i> -HA allele and <i>algU</i> under control of pBAD promoter	This study
PAO1	Leader/translational fusion	This study
<i>lacUV5-tssA1-lacZ</i>		
PAO1 <i>ΔalgU</i>	Leader/translational fusion	This study
<i>lacUV5-tssA1-lacZ</i>		
PAO1 <i>ΔgacA</i>	Leader/translational fusion	This study
<i>lacUV5-tssA1-lacZ</i>		
PAO1 <i>ΔretS</i>	Leader/translational fusion	This study
<i>lacUV5-tssA1-lacZ</i>		
PAO1 <i>ΔrsmA</i>	Leader/translational fusion	This study
<i>lacUV5-tssA1-lacZ</i>		
PAO1 <i>mucA22</i>	Leader/translational fusion	This study
<i>ΔgacA</i>		
<i>lacUV5-tssA1-lacZ</i>		
PAO1 <i>mucA22 ΔretS</i>	Leader/translational fusion	This study
<i>lacUV5-tssA1-lacZ</i>		
PAO1 <i>mucA22 ΔalgU</i>	Leader/translational fusion	This study
<i>lacUV5-tssA1-lacZ</i>		
PAO1 <i>mucA22 ΔrsmA</i>	Leader/translational fusion	This study
<i>lacUV5-tssA1-lacZ</i>		
Plasmids and constructs		
pEX18Tc	Allelic exchange vector	30
pEX18Gm	Allelic exchange vector	30
<i>ΔrsmA</i> pEX18Tc	Allelic exchange for <i>rsmA</i> nonpolar deletion	This study
<i>ΔalgU</i> pEX18Tc	Allelic exchange for <i>algU</i> nonpolar deletion	57
<i>ΔrpoS</i> pEX18Tc	Allelic exchange for <i>rpoS</i> nonpolar deletion	This study
<i>rsmA</i> -HA pEX18Gm	Allelic exchange for <i>rsmA</i> -HA allele	This study
miniCTX <i>lacZ</i>	Transcriptional fusion vector	38
TF1- <i>lacZ</i> miniCTX	Transcriptional fusion	This study
TF3- <i>lacZ</i> miniCTX	Transcriptional fusion	This study
TF3USDM- <i>lacZ</i> miniCTX	Site-directed mutagenesis transcriptional fusion	This study
TF2- <i>lacZ</i>	Transcriptional fusion	This study
pTJ1	Integrating vector used for overexpression	43
<i>algU</i> pTJ1 (pAlgU)	<i>algU</i> complementation/overexpression	This study

PCR system (Life Technologies, Grand Island, NY). A temperature of 55°C was used for the extension. The same primer used for primer extension was also used in a sequencing reaction with the *rsmA* upstream sequence in pGEM-T Easy (Promega, Madison, WI) and a Sequenase 7-deaza-dGTP kit (Affymetrix, Cleveland, OH). The products of both the primer extensions and the sequencing reactions were run on a 6% acrylamide–8 M urea gel. The gel was dried, and the extension products were

TABLE 2 Primers used in this study

Primer name	Sequence	Use ^a
rsmARHindIII	GCGCAAGCTTCACGCGAATATTTTCAGGACAAC	TF
rsmAFHindIII	GCGCAAGCTTCGGCAACATCACCACGCTGG	Mutant construction
rsmARXbaI	GCGCTCTAGAGCAGGTGATCCTGCAGACC	Mutant construction
rsmASOEF	GCGTGAGGAGAAAGGAATGCTGCAT TAATTTTTATCTAATTTTCCCTTTGCG	Mutant construction
rsmASOER	GCAAAGGGAATAATAGATAAAAAATT AATGCAGCATTCCTTTCTCCTCACGC	Mutant construction
algUKOF	GCGCGAGCTCGACAGCAACTCGATGTTCCGGTC	Mutant construction
algUKOR	GCGCTCTAGAGTCGTACCAGGAAGCCAGCTG	Mutant construction
algUTJ1F2	GCGCCCATGGGAATGCTAACCCAGGAACAGGATCA	Complementation
algU HindIIIIR	GCGCAAGCTTTTCAGGCTTCTCGCAACAAAGGCTGCA	Complementation
algRTJ1F2	GCGCCCATGGGAATGAATGTCCTGATTGTCGATGAC	Overexpression
rpoS EcoRIF	GCGCGAATTCGATGGCACTCAAAAAAGAAGGGCC	Mutant construction
rpoS HindIIIIR	GCGCAAGCTTTCACTGGAACAGCGCGTCACT	Mutant construction
rpoS intF	CGTCGCTGACCGGGAGTTCG	PCR check
rpoS intR	CATTTATCTACTTAGGCTCACACG	PCR check
rsmASDMcheckF	GCCAAGGTTTCCATCGTCGG	PCR check
rsmAHAF	TACCCATACGATGTTCCAGATTACGCTTAATTTTTATCTAATTTTCCCTTTGCG	HA allele construction
rsmAHAR	TTAAGCGTAATCTGGAACATCGTATGGGTAATGGTTTGGCTCTTGATCTTTCTC	HA allele construction
rsmASDMAlgUF	CAGAATTCTTCATTCCGGCGGGACTG	SDM
rsmASDMAlgUR	CCAAACGGTCAAAAACGACAAAAG	SDM
rsmAPE1F	GCGCGGTACCAAGGATCGCGCTCTTGATTTCTGCGGATCCGCCGCCATTCTT	TF
rsmAPE3F	GCGCGGTACCGCTGACAGGCGAAAGGCG	TF/RPA
rsmAPE3R	GCGCGGATCCTCACCCAGTATTGACCAAGTCC	TF
rsmAEcoRIR	GCGCGAATTCACGAGTCAGAATCAGCATTCCTTTC	TF
rsmAPE1	GCGCGGTTGACGCCGATGCGC	Primer extension
rsmAPE3	CACGCGAATATTTTCAGGACAACAGTCTG	Primer extension
rsmAPE4	GCGCGATCCTTCACCCAGTATTGACCAAGTCC	Primer extension
gacAF	GCGCGAGCTCGAGGCGACCTTGCGTCATTC	Mutant construction
gacAR	GCGCTCTAGACGGCGATCGCCGAAACCAG	Mutant construction
gacASOEF-2	GCGACGAGGTGCAGCGTGATTGTGAATTCGCCAGCTAGATGAGCG	Mutant construction
gacASOER-2	CGCTCATCTAGCTGGCGGAATTCACAATCACGCTGCACCTCGTCGC	Mutant construction
gacAintF	CCAGGTGCTTGCGCTTTAC	PCR check
gacAintR	CCACGTAGAGAAGCTTGCGC	PCR check
retSFSaI	GCGCGAGCTCGAACGTCGCCATCGACGTGC	Mutant construction
PA0082FUV5	GCGCAAGCTTTTACACTTTATGCTTCCGGCTCGTATAATGTGTGGAGTCATCCAATATTTCATCAATGGC	LF
retSRXbaI	GCGCTCTAGAGCAGGTCTGGCTATGCCGG	Mutant construction
retSSOEF	GACTTCGCCGTGGTACGGCTTTCTGAGGGCAGCGACGTGCT	Mutant construction
retSSOER	AGCACGTCGCTGCCCTCAGGAAAGCCGTACCACGGCGAAGTC	Mutant construction
retSintF	CAAGGTCGAGGCGACCTGGG	PCR check
retSintR	GAATAACCGCGTGCGGTTATCC	PCR check
lacZScaIF	GCTATGACCATGATTAGTACTGATTCACTGGCCGTC	TLF vector
lacZRXhoI	CCCCTCGAGCAGACATGGCCTGCCCGGTTATTA	TLF vector
lacZRforTF	GATGTGCTGCAAGGCGATTAAG	SEQ
pHERDSF	ATCGCAACTCTCTACTGTTTCTC	SEQ
rsmASDMAlgRF	GAATTCGGCAGGAACCTTCATTCGGGC	RPA
rsmARPAR	TAATACGACTCACTATAGGGAGAGATAAAAAATTAATGGTTTGGCTCTTG	RPA

^a Abbreviations: TF, transcriptional fusion, SDM, site-directed mutagenesis; TLF, translational fusion, LF, leader fusion, SEQ, sequencing primer; RPA, RNase protection assay.

detected using autoradiography. Primer extension results were confirmed using two different primers a total of four times.

In vitro transcription and RPA. The *rsmA* probe for the RNase protection assay (RPA) was synthesized using a PCR product generated with primers that incorporated a T7 promoter sequence at the 5' end and a MAXIscript T7 kit (Life Technologies). The reverse primer containing the T7 promoter sequence contained a nonhomologous sequence to discriminate between the full-length probe and protected fragments. The primers rsmASDMAlgRF and rsmARPAR were used to produce the PCR product. The probes were labeled with UTP by the use of biotin-16-UTP (Life Technologies) at a ratio of 4:6 and were gel purified after *in vitro* transcription. Twenty micrograms of total RNA from each *P. aeruginosa* strain was precipitated with 800 pg of labeled

probe and resuspended in hybridization buffer {80% formamide, 400 mM NaCl, 40 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid); pH 6.4], 1 mM EDTA}, heated at 95°C for 5 min, and incubated overnight at 42°C. Negative controls consisting of the probe incubated with 10 µg *Saccharomyces cerevisiae* yeast RNA were also included. Digestion of unhybridized RNA was performed using RNase A at 25 µg/ml and RNase T1 at 10 U/ml. A biotinylated RNA ladder (Kerafast) was also used to determine the size of the protected probe fragments. The products of the reactions were run on a 5% acrylamide–8 M urea gel, and the gels were transferred to a positively charged membrane (Hybond N+; Amersham Biosciences) using a semidry blotting system and 0.5× Tris-borate-EDTA buffer (Hoefer, Holliston, MA). Nucleic acid was UV cross-linked, and the probes were detected using a chemiluminescent nucleic acid detection

FIG 1 Depiction of the *rsmA* genomic region, the promoters controlling *rsmA* expression, and the reagents used in this study. (A) Schematic representation of the *rsmA* genomic region. The sequences below the schematic represent those of the two potential promoters. The bent arrows above the sequence indicate the transcriptional start sites identified by primer extension analysis. The primers used in the primer extension experiments are identified by bent arrows below the genomic schematic. The potential promoters are indicated by a line underneath the sequence and are in bold. Potential sigma factor consensus sequences are indicated below the sequence. The probe used for RNase protection assays is indicated below, and position numbers are relative to the *rsmA* translational start site. (B) The *rsmA-lacZ* transcriptional fusions constructed are indicated. Arrows indicate the primers used in the construction of the transcriptional fusions. The position numbers above the arrows are in relation to the *rsmA* translational start codon. The X indicates the site-directed mutagenesis of the putative AlgU -35 consensus sequence.

module (Thermo Scientific). After washing, the blot was incubated for 5 min and developed using a FluoroChem M system (ProteinSimple). RNase protection assays were performed at least three times.

Overexpression/complementation studies. In order to overexpress *algU*, the coding sequence was amplified (Table 2) and cloned into the integrating vector pTJ1 for complementation or overexpression (36). Trimethoprim-resistant colonies were picked, cultured overnight, and diluted 1,000-fold on the following day. The presence of the gene of interest was confirmed using primers pTJ1R and either algUTJ1F2 or algRTJ1F2. Cultures requiring induction used 1% arabinose and were grown for 8 h in broth or overnight on agar plates.

Statistical analyses. Statistical analyses were performed using GraphPad Prism (version 6.0) software (GraphPad Software, La Jolla, CA). The results of transcriptional and translational fusions and densitometry analysis were compared using a one-way analysis of variance (ANOVA) with Tukey's posttest. ImageJ software was used for Western blot analysis.

RESULTS

AlgU hyperactivity significantly contributes to *rsmA* expression in *mucA* mutant strains. Previous studies using an *rsmA* translational fusion and/or Western blot analyses found that RsmA levels increase throughout the growth phases (5, 21, 28), which suggests a role of the stationary-phase sigma factor RpoS (37). Another study using a transcriptional fusion indicated that a *mucA* strain has increased *rsmA* expression (14). Because the alternative sigma

factor AlgU is available in strains lacking functional MucA, AlgU has increased activity in this background, and we postulated that AlgU might play a role in *rsmA* expression. To further understand *rsmA* regulation at a transcriptional level, an *rsmA* transcriptional fusion, TF1-*lacZ*, was constructed by cloning the entire intergenic region between *rsmA* and *lysC* in the transcriptional fusion vector miniCTXlacZ (Fig. 1) (38). Due to posttranscriptional *rsmA* autoregulation (39), it was important that only transcription be monitored. The miniCTXlacZ vector allows insertion of single-copy *lacZ* fusion integrants and contains an RNase III processing site to ensure that transcription is uncoupled from posttranscriptional effects (38, 40). In wild-type strain PAO1, the TF1-*lacZ* fusion had β -galactosidase activity of ~ 380 Miller units at 8 h (Fig. 2A). This result indicates that *rsmA* was expressed at low levels at this time point. The fusion was also tested at additional time points, including 16 h, and there was little change in TF1-*lacZ* activity (data not shown). In a Δ algU strain, the TF1-*lacZ* transcriptional fusion had no decrease in TF1-*lacZ* activity at 8 h (Fig. 2A). A Δ rpoS mutant was constructed and assayed for *rsmA* expression. There was no significant decrease in β -galactosidase activity at either 8 or 16 h (Fig. 2A). A Δ algU Δ rpoS strain was constructed, and the TF1-*lacZ* transcriptional fusion was assayed. No change in TF1-*lacZ* activity was seen in the double mutant. A

FIG 2 AlgU activates *rsmA* expression in *mucA* mutants. (A) Analysis of TF1-*lacZ* in mutant strains at 8 h of growth in LB broth. The TF1-*lacZ* transcriptional fusion was inserted into the *attB* site in the chromosomes of the indicated strains. Transcriptional fusion values are indicated as β -galactosidase activity. The values are the result of subtraction of the value for PAO1 containing the vector (miniCTX*lacZ*) alone (28 Miller units) from all test values. Fusion assays were all conducted at least three times. Determination of all significant differences from the results for the wild type was performed using a one-way analysis of variance and Tukey's posttest. **, $P = 0.01$; ***, $P < 0.001$. (B) (Top) Western analysis of RsmA in *algU* mutant strains. An *rsmA*-HA allele was constructed and introduced into various strains via homologous recombination, the strains were grown for 8 h, and whole-cell lysates were probed with anti-HA antibody. A single band was detected in all Western blots using the strains containing the *rsmA*-HA allele. A representative Western blot is shown. Western blot analysis was completed at least three times. The strains assayed are listed below the bottom panel. PAO1 without the *rsmA*-HA allele was run as a negative control (data not shown). (Bottom) Densitometry analysis was performed for each strain. Densitometry values, determined using ImageJ software, were standardized by dividing the intensity of the band in the Western blot by the intensity of the total protein in each lane stained with Coomassie. The result for PAO1 was set equal to 1, and the values for the other strains were normalized using the value for PAO1 as a reference. A one-way ANOVA with Tukey's posttest was used to determine statistical significance. ***, $P < 0.0001$.

Δ *algR* mutant had significantly increased *rsmA* expression at 8 h (Fig. 2A). A previous study indicated that AlgR activates *rsmA* expression (14), but the strain used, PA103, and the growth conditions differed from those used in our study. Our results suggest that *rsmA* is expressed at low levels in wild-type strain PAO1 and its mutant derivatives.

To further investigate the role of AlgU in controlling *rsmA* expression, the level of TF1-*lacZ* fusion expression was measured

in a *mucA22* strain as well as the isogenic *mucA22* Δ *algU* nonmucoid derivative. As shown in Fig. 2A, a *mucA22* strain had a >3-fold increase in *rsmA* expression compared to that in wild-type strain PAO1. In the *mucA22* Δ *algU* strain, *rsmA* transcriptional activity was reduced to the levels in wild-type strain PAO1 (Fig. 2A), confirming that AlgU does control *rsmA* expression. The analysis of the TF1-*lacZ* transcriptional fusion suggests that AlgU does play a role in regulating *rsmA* expression, but this effect is seen only when *mucA* is mutated and not able to sequester AlgU. In addition, RpoS does not regulate *rsmA* under these conditions.

To determine if RsmA protein levels mirrored the findings of *rsmA* expression analysis, we constructed a hemagglutinin (HA)-tagged *rsmA* allele that was introduced into strains by allelic exchange. As shown in Fig. 2B, the *mucA22* mutant had significantly higher RsmA levels than wild-type strain PAO1. Densitometry analysis indicated that there was a significant decrease in RsmA levels in the Δ *algU* mutant compared to those in wild-type strain PAO1 (Fig. 2B). The *mucA22* Δ *algU* mutant showed a drastic decrease in RsmA levels compared to those in the *mucA22* strain (Fig. 2B). The results of Western blot analysis suggested that AlgU is required for increased *rsmA* expression in *mucA* mutant backgrounds.

Identification of a second *rsmA* promoter. Because the sigma factor AlgU played a role in *rsmA* expression, the upstream region of *rsmA* was inspected for possible promoter sequences. A potential AlgU consensus sequence is located upstream of *rsmA* (Fig. 1A). To determine if this AlgU promoter is functional, we performed primer extension analysis. Total RNA was reverse transcribed using a radiolabeled primer (rsmAPE1) specific for a sequence located within the *rsmA* coding region, and extension products were detected on a denaturing polyacrylamide gel. Primer extension indicated that there were two transcriptional start sites (Fig. 3A), which suggests that *rsmA* has an additional promoter. The start site most distal to the coding region was identified as an A residue located 86 bp upstream of the predicted *rsmA* translational start codon. The first and closest transcriptional start site was a G residue located 40 bp upstream of the *rsmA* coding region. Primer extension analysis with an additional radiolabeled primer, rsmAPE3, produced a single extension product and confirmed the presence of the A residue identified to be a transcriptional start site 86 bp upstream of *rsmA* (data not shown). The second transcriptional start site was independently identified four times. Primer extension analysis with a third primer, rsmAPE4, whose sequence overlapped the most distal transcriptional start site, failed to produce an extension product, suggesting that *rsmA* has only two active promoters under the conditions tested.

Sequences upstream of the identified transcriptional start sites were examined for potential promoters (Fig. 1A). Three previous studies identified using transcriptome sequencing (25, 26) and 5' rapid amplification of cDNA ends (24) a transcriptional start site that is 40 nucleotides upstream of the *rsmA* translational start codon. The sequence of the proximal promoter identified by our primer extension analysis resembled a σ^{70} (RpoD) promoter sequence. Therefore, the proximal promoter that we identified was the same promoter previously identified, validating the findings of our primer extension analysis.

The second transcriptional start site has two possible promoter consensus regions upstream. A potential AlgU consensus sequence GAACCTT in the -35 region (Fig. 1A) (11) and a potential RpoS -10 region are located upstream of this transcriptional start site. The potential RpoS consensus sequence CAATACT differs

FIG 3 Identification of a second *rsmA* promoter region controlled by AlgU. (A) Primer extension analysis was performed to identify the transcriptional start sites of *rsmA*. Total RNA was reverse transcribed using a radiolabeled primer, *rsmA*PE1 (Table 2), and run alongside the products of the sequencing reactions on a denaturing polyacrylamide gel. Lanes G, A, T, and C, the sequencing ladder generated using the same primer used in the reverse transcription reaction; lane 1, a control lane with total RNA without reverse transcriptase; lane 2, reverse-transcribed PAO1 RNA. Arrows, extension products. (B) β -Galactosidase activity of chromosomal transcriptional fusion TF2-*lacZ* in the indicated strains tested in triplicate after incubation in LB broth for 8 h at 37°C as described in the legend to Fig. 2A. The value for PAO1 carrying the vector alone was subtracted from the value for each strain. (C) β -Galactosidase activity of chromosomal transcriptional fusions of TF3-*lacZ* in the indicated strains tested in triplicate after incubation in LB broth for 8 h at 37°C. The value for PAO1 carrying the vector alone was subtracted from the value for each strain. Transcriptional fusion assays were performed at least three times. All significant differences from the wild type were determined using a one-way analysis of variance and Tukey's posttest. ***, $P < 0.001$.

from the RpoS consensus sequence CTATACT by 1 nucleotide (41). *E. coli* RpoS promoters frequently have an extended structure, denoted by a G/T residue directly upstream of the conserved -10 region (42). The potential RpoS promoter upstream of *rsmA* also follows this extended structure (Fig. 1A). The potential RpoS -10 region overlaps the potential AlgU -10 region (Fig. 1A). On the basis of the results of primer extension and sequence analyses, *rsmA* has a second promoter that is controlled by AlgU and/or RpoS.

AlgU controls the distal *rsmA* promoter. Because two *rsmA* transcripts were detected by primer extension (Fig. 3A), this led us to test whether *rsmA* has two promoters. Two transcriptional fusions were constructed using the data generated by primer extension analysis (Fig. 1) to confirm the promoter activity of these regions. The activity of PAO1 carrying the vector alone (28 Miller units) was subtracted from the values obtained. A transcriptional fusion, TF2-*lacZ*, was constructed by deleting the putative distal promoter. TF2-*lacZ* has the 5' end at position -74 in relation to the *rsmA* translational start site and extends 18 nucleotides into the *rsmA* coding region (Fig. 1B). The transcriptional fusion TF3-

lacZ was constructed using the intergenic region from *lysC* to the distal transcriptional start site at position $+1$ as the 3' end of the fusion and therefore contains only the putative distal promoter (Fig. 3B). When these fusions were analyzed in wild-type strain PAO1, both fusions produced β -galactosidase activity (Fig. 3B and C), but the level of expression was very low. The values for both the TF2-*lacZ* and TF3-*lacZ* fusions were much lower than the value for TF1-*lacZ*, suggesting that the entire region is necessary for optimal *rsmA* expression.

The TF2-*lacZ* transcriptional fusion was constructed and tested to determine the activity of the proximal promoter. The TF2-*lacZ* fusion did not have increased reporter activity in the *mucA22* strain (Fig. 4B). When *algU* was deleted in either background, no decrease in reporter activity was detected, indicating that this promoter was constitutively active at low levels in the strains analyzed (Fig. 4C).

To determine if AlgU affected the distal promoter that we identified, the TF3-*lacZ* fusion was analyzed in wild-type strain PAO1 and a Δ *algU* mutant. The activity of the TF3-*lacZ* fusion in PAO1

FIG 4 AlgU overexpression and complementation confirm the AlgU regulation of *rsmA*. (A and B) β -Galactosidase activity of the TF3-*lacZ* (A) and Δ *algU* TF3-*lacZ* (B) constructs. (A) The *algU* gene was cloned into the integrating vector pTJ1 and induced with 1% arabinose. Strains were grown in LB broth for 4 h and induced with arabinose for 4 h. PAO1 with the vector alone (pTJ1) was used as a negative control. Data from triplicate experiments were averaged after subtraction of the value for PAO1 carrying the vector alone (28 Miller units). (B) Complementation of the Δ *algU* strain containing the transcriptional fusion TF3-*lacZ* was performed as described in the legend to panel A. (C) Overexpression of *algU* increased RsmA protein levels. PAO1-HA/pTJ1 was the PAO1 strain with the *rsmA*-HA allele and the vector alone. PAO1-HA/pAlgU contained the *rsmA*-HA allele and overexpressed *algU* when arabinose was added. ImageJ software was used for densitometry analysis, as described in the legend to Fig. 2B. (Top) A representative Western blot. Western blot analysis was performed at least three times. (Bottom) Densitometry results for the Western blots.

was low, and no difference in activity was seen when it was tested in a Δ *algU* mutant at 8 h. However, when the activity of the TF3-*lacZ* fusion was tested in a *muca22* strain, there was an $\sim$ 8-fold increase (Fig. 3C). The activity of the TF3-*lacZ* transcriptional fusion in a *muca22* Δ *algU* strain was significantly reduced $\sim$ 8-fold (Fig. 3C). These results show that the promoter contained in the TF3-*lacZ* fusion is AlgU dependent.

Overexpression and complementation of *algU* increase *rsmA* expression. For overexpression of *algU*, we used an arabinose-inducible promoter to control *algU* expression (43). As shown in Fig. 4A, there was a statistically significant increase in TF3-*lacZ* activity when *algU* was induced with arabinose in strain PAO1. The results suggest a role for AlgU in activating *rsmA* expression.

Additionally, the Δ *algU* mutant containing the TF3-*lacZ* transcriptional fusion was complemented with pAlgU. When 1% arabinose was used to induce *algU* expression, TF3-*lacZ* activity was also increased (Fig. 4B). Complementation and overexpression resulted in an increase in TF3-*lacZ* activity. From these results, we conclude that AlgU is necessary for increased *rsmA* expression.

To determine if RsmA protein levels matched those of the transcriptional fusions, we used the PAO1 *rsmA*-HA strain containing epitope-tagged *rsmA*. When *algU* expression was induced by the addition of arabinose, there was a significant increase in RsmA

levels (Fig. 4C). On the basis of this result, it was determined that *algU* overexpression is able to increase *rsmA* transcription.

Site-directed mutagenesis of the putative AlgU-binding site in the distal promoter reduces *rsmA* expression. Site-directed mutagenesis was performed on the TF3-*lacZ* transcriptional fusion to determine if the putative AlgU consensus sequence was required for increasing *rsmA* expression. The putative AlgU -35 sequence GAACTT was converted to GAATTC (underlined nucleotides indicate changes introduced) (Fig. 5). There was a >3 -fold decrease in the level of transcription in the *muca22* strain with the mutagenized AlgU promoter (Fig. 5). The results of the site-directed mutagenesis experiment demonstrate the necessity of the conserved AlgU -35 sequence and strongly suggest that AlgU directly controls *rsmA* transcription by binding the distal *rsmA* promoter.

AlgU regulation of *rsmA* is conserved. To determine if AlgU control over *rsmA* is a conserved mechanism in other *P. aeruginosa* strains, we investigated the role of AlgU in the clinical strains 2192 and 383. These strains were isolated from a longitudinal study (44) and have been determined to be isogenic (45). Strain 2192 is mucoid and has a *muca* mutation (46). We took a genetic approach and assayed our transcriptional fusions TF1-*lacZ* and TF3-*lacZ* to determine the role of AlgU in activating *rsmA* expression in these strains (Fig. 6). Strain 2192 had an $\sim$ 3-fold increase in the amounts of both the TF1-*lacZ* fusion and the TF3-*lacZ*

FIG 5 The potential AlgU-binding site is required for increased *rsmA* expression in a *mucA22* mutant. The TF3-*lacZ* transcriptional fusion was engineered with two mutations in the potential AlgU –35 consensus sequence (indicated by asterisks above the indicated nucleotides in the sequence above the graph). The transcriptional start site (the 3' end of the TF3-*lacZ* fusion) is indicated in bold. USDM indicates the fusion containing the mutation of the conserved AlgU –35 consensus sequence. β -Galactosidase activity was determined as described in Materials and Methods. Data from triplicate experiments were averaged after subtraction of the value for PAO1 carrying the vector (28 Miller units). A one-way ANOVA with Tukey's posttest was used to determine statistical significance. ***, $P < 0.0001$.

fusion compared to those in nonmucoid strain 383 (Fig. 6A and B). These results demonstrate that the AlgU-dependent promoter activity is increased in strain 2192 compared to that in its nonmucoid counterpart, strain 383. A 2192 Δ algU mutant was constructed, and assays for the expression of the TF1-*lacZ* and TF3-*lacZ* fusions were performed to confirm a role for AlgU in other *P. aeruginosa* strains. As shown in Fig. 6A and B, there was a statistically significant decrease in the levels of TF1- and TF3-*lacZ* expression, supporting the role of AlgU in regulating the distal promoter. To confirm the transcriptional fusion results, the levels of RsmA in the clinical strains were determined using the *rsmA*-HA allele. RsmA levels were increased in strain 2192 compared to those in the nonmucoid 383 parent strain (Fig. 6C), suggesting that AlgU activated *rsmA* expression in strain 2192. The 2192 Δ algU mutant had a significant decrease in the amount of RsmA compared to that in 2192 (Fig. 6C). On the basis of these results, it is likely that AlgU activates *rsmA* expression in multiple strains.

RpoS involvement in the control of *rsmA*. There are conflicting data regarding RpoS control of *rsmA* expression (9, 27). The potential RpoS-binding site upstream of *rsmA* implicated RpoS in the control of *rsmA* (Fig. 1A). However, the TF1-*lacZ* transcriptional fusion analysis did not demonstrate a decrease in *rsmA* expression in a Δ rpoS mutant. Because sigma factor competition on the distal promoter could have influenced our ability to detect RpoS activity on the *rsmA* promoter (47), we tested a double mutant, the Δ algU Δ rpoS mutant (Fig. 1A). No further decrease in TF1-*lacZ* activity in the Δ algU Δ rpoS mutant was detected. To further define the role of RpoS in controlling *rsmA* expression, we tested the TF2- and TF3-*lacZ* fusions for activity in either Δ rpoS strains or Δ algU Δ rpoS strains (Fig. 7). Interestingly, both a Δ rpoS

mutant and a Δ algU Δ rpoS mutant had significantly higher levels of activity when the TF2-*lacZ* promoter was used (Fig. 7A), suggesting that RpoS has a negative effect on transcription from the proximal *rsmA* promoter. No significant change was found when the TF3-*lacZ* transcriptional fusion was used in the Δ rpoS or Δ algU Δ rpoS mutant strains (Fig. 7B). From these results, the role of RpoS in regulating *rsmA* is inconclusive.

RpoS and AlgU are necessary for *rsmA* expression from the distal promoter. RNase protection assays (RPAs) were performed using an RNA probe specific for a region spanning the entire *rsmA* coding region and 129 bp upstream of the *rsmA* gene (Fig. 1A) to more directly analyze *rsmA* transcripts and better understand the role of AlgU and RpoS in controlling *rsmA* expression. The probe also includes non-template-encoded nucleotides to distinguish the full-length probe from protected fragments. As shown in Fig. 8, the RPAs confirmed the findings of our primer extension and transcriptional fusion analysis by detecting two *rsmA* transcripts in wild-type strain PAO1. The expected fragments were 281 bp and 235 bp, on the basis of the probe used. This matched what was seen in the RPAs (Fig. 8). Interestingly, the Δ algU mutant contained the same two transcripts as wild-type strain PAO1. When the Δ rpoS mutant was analyzed, both transcripts were detected, with the amount of the first transcript resulting from the amount of the proximal promoter being increased. A Δ algU Δ rpoS strain was also analyzed, and the larger transcript resulting from the distal promoter completely disappeared, demonstrating that both AlgU and RpoS contribute to transcription from the distal promoter (Fig. 8). The *mucA22* strain had both transcripts as well, with both transcripts having greater intensities. The highest band in the Δ rpoS and *mucA22* strains was likely undigested probe.

Three controls were included in the RPA. Two of the controls were the probe incubated with yeast RNA; one of these was treated with RNase, and the other was not. As shown in Fig. 8, RNase treatment completely degraded the probe, which indicates that the probe did not bind nonspecifically. The intact probe can be seen at ~330 bp in the lane in Fig. 8 labeled “probe alone – RNase,” which is consistent with the additional nucleotides that are non-template encoded. A Δ rsmA mutant was constructed and used as an additional control. In the Δ rsmA mutant, there was only one discernible protected probe fragment at ~150 bp. On the basis of how the Δ rsmA mutant was constructed, a protected fragment of 144 bp was expected. The results obtained with the Δ rsmA strain support the suggestion that there are only two *rsmA* transcripts, at least under the conditions used. Altogether, the results of the RPA suggested that there are two *rsmA* transcripts and that both AlgU and RpoS are involved in controlling the distal promoter region, suggesting overlapping promoters.

AlgU control of *rsmA* expression is responsible for RsmA activity in a *P. aeruginosa mucA22* strain. Because AlgU increased RsmA levels, we hypothesized that RsmA activity was also increased in a *mucA22* strain. We analyzed a variety of mutant strains, including strains that should alter the activity of RsmA on the basis of the current RsmA paradigm (5). RetS interferes with GacA phosphorylation, and mutation of *retS* should lead to increased production of the small RNAs RsmY and RsmZ (18, 48). Therefore, the level of RsmA activity should be reduced. In contrast, mutation of *gacA* should lead to a reduction in the amounts of the small RNAs RsmY and RsmZ (49). In this case, increased RsmA activity should be observed. Mutants with mutations in the

FIG 6 Clinical *mucA* mutant strains have increased *rsmA* expression. (A and B) β -Galactosidase activities of the TF1-*lacZ* (A) and TF3-*lacZ* (B) transcriptional fusions. (A) The β -galactosidase activity of the TF1-*lacZ* transcriptional fusion was assayed in clinical isolates 383 and 2192 and a 2192 Δ algU strain after 8 h, as described in the text. Data from triplicate experiments were averaged after subtraction of the value for PAO1 carrying the vector alone (28 Miller units). (B) The β -galactosidase activity of the TF3-*lacZ* transcriptional fusion was analyzed in strains 383, 2192, and 2192 Δ algU at 8 h, as described in the text. Data from triplicate experiments were averaged after subtraction of the value for PAO1 carrying the vector alone (28 Miller units). A one-way ANOVA with Tukey's posttest was used to determine statistical significance. ***, $P < 0.0001$. (C) RsmA levels are also increased in clinical *mucA* mutants. The *rsmA*-HA allele was exchanged for the wild-type *rsmA* allele in clinical isolates 383 and 2192. Whole-cell lysates were produced and probed with anti-HA antibodies. PAO1 was used as a negative control (data not shown). The results for PAO1-HA and *mucA22*-HA are shown for reference. (Top) A representative Western blot. (Bottom) Results of densitometry analysis of the blot performed using ImageJ software. Data are in arbitrary units and were produced from three Western blots. Densitometry analysis was performed as described in the legend to Fig. 2B.

gacA and *retS* genes were constructed in both the PAO1 and *mucA22* strain backgrounds.

RsmA has previously been shown to affect the production of the exopolysaccharide genes *pel* and *psl* (9, 16, 50). However, no studies have investigated the involvement of RsmA in alginate production. We hypothesized that if RsmA inhibits biofilm formation, then RsmA would inhibit alginate production. Interestingly, the *mucA22* Δ *gacA*, *mucA22* Δ *retS*, and *mucA22* Δ *rsmA* mutants still overproduced alginate (data not shown), and we conclude that RsmA does not affect alginate production.

RsmA posttranscriptional activity was tested using a *tssA1* leader/translational fusion containing the *lacUV5* promoter and the *tssA1* untranslated sequence, based on the study of Brencic and Lory (5) (Fig. 9A). Due to the low level of activity of this reporter, the data are presented as a percentage of the wild-type activity, where the values were normalized to those for the PAO1 strain (12 Miller units). The *mucA22* strain had fusion activity decreased ~3-fold compared to that of PAO1 (Fig. 9B). There was no difference in activity between PAO1 and a Δ algU strain when the leader

fusion was used (Fig. 9B). In the case of a *mucA22* Δ algU strain, there was a 5.5-fold increase in leader fusion activity compared to that in the *mucA22* strain (Fig. 9B). These results suggest that RsmA is active in the *mucA22* strain background and this is due to AlgU regulation of *rsmA*.

Further confirmation of RsmA activity in the *mucA22* strain was carried out using mutants with mutations in *gacA* and *retS*, as described above. While the differences in activity were not significant by statistical analysis, there was no activity from a *mucA22* Δ *gacA* strain, even after the reaction was allowed to proceed overnight. This result would be expected if RsmA activity were increased due to the absence of the small RNAs RsmY and RsmZ (49). As there was an increased amount of RsmA in the *mucA22* strain due to AlgU activation of the distal promoter, the difference between the *mucA22* strain and the *mucA22* Δ *gacA* strain should not be so different if our hypothesis is correct (Fig. 10). The leader fusion also had increased activity in both the *mucA22* Δ *rsmA* and the *mucA22* Δ *retS* strains compared to that in the *mucA22* strain (3-fold and 7-fold, respectively) (Fig. 9B).

FIG 7 RpoS is not a significant regulator of *rsmA* expression. (A) β -Galactosidase activity of the TF2-lacZ transcriptional fusion in the denoted strains. Data from triplicate experiments were averaged after subtraction of the value for PAO1 carrying the vector alone (28 Miller units). (B) β -Galactosidase activity of the TF3-lacZ transcriptional fusion in the denoted strains. β -Galactosidase activity was determined as described in the text. Data from triplicate experiments were averaged after subtraction of the value for PAO1 carrying the vector alone (28 Miller units). Significant differences from the results for the wild type were determined using a one-way analysis of variance and Tukey's posttest. ***, $P < 0.001$.

These results also indicate the RsmA activity in the *mucA22* strain background. The increased activity of the leader fusion indicates that the absence of RsmA or an abundance of RsmY and RsmZ due to the absence of RetS allows greater expression from the leader fusion. Altogether, these data suggest that RsmA activity is present in a *mucA22* strain.

DISCUSSION

RsmA is considered an important posttranscriptional regulator involved in the positive regulation of acute virulence factors, such as the type III secretion system and motility (5, 14). Little is known of the transcriptional regulation of *rsmA*. Initially, we undertook studies to determine how *rsmA* was regulated in *P. aeruginosa*. We demonstrate that AlgU activates *rsmA* expression and increases RsmA levels in a *mucA22* mutant and a clinical isolate. Analysis of a leader fusion to assess the posttranscriptional activity of the RsmA target, *tssA1* (5), led us to discover RsmA activity in the *mucA22* strain. To our knowledge, this is the first demonstration of RsmA activity in a *mucA* mutant strain of *P. aeruginosa*. Our work demonstrates that increased *rsmA* expression due to AlgU activation leads to changes in the RsmA regulon. We hypothesize that RsmA plays an important role during *P. aeruginosa* chronic

FIG 8 RPAs confirm the presence of two *rsmA* transcripts. RPAs of the indicated strains were performed after RNA was harvested from cultures grown in LB broth for 8 h at 37°C. Twenty micrograms of total RNA from the indicated strains was hybridized with 800 pg of biotinylated probe and subjected to RNase treatment. After RNase inactivation, RNA was precipitated from the samples and run on a 5% denaturing polyacrylamide gel, transferred to a nylon membrane, UV cross-linked, and developed using chemiluminescent detection. The undigested probe is indicated by the large arrow at the top. The protected *rsmA* probe is indicated by the three smaller arrows. Shown is a result representative of results from three RNase protection assays. Ladder, molecular size markers.

infection and that there are additional RsmA targets in *mucA* mutant strains.

One of the goals of this study was to determine how *rsmA* expression is regulated. Previous studies have raised questions about how *rsmA* is regulated in *P. aeruginosa* (9, 14, 24). In *E. coli*, five promoters control *csrA* (the orthologue of *rsmA*) expression (51). Only one *rsmA* promoter was previously identified in *P. aeruginosa* (24–26), and a previous study indicated that AlgR plays a role in regulating *rsmA* expression (14). Using primer extension, a new transcriptional start site was identified, indicating a second *rsmA* promoter (Fig. 2). Both transcriptional fusion analysis and RPA confirmed the presence of a second promoter (Fig. 3 and 8). Overexpression and Western blot analyses support a role of AlgU in controlling *rsmA* expression (Fig. 1E and 4). However, when *MucA* is functional, such as in PAO1, it is difficult to detect changes in *rsmA* expression. This explains the low levels of activity of the transcriptional reporters (Fig. 1A and 2). The role of RpoS

FIG 9 AlgU activation of *rsmA* leads to changes in *tssA1* expression. (A) Schematic of the genomic region of the RsmA-controlled genes *tssA1* and *fhlA1*. Below the genomic schematic is a depiction of the leader fusion that was constructed. The position numbers above the fusion construct indicate those of the primers used relative to the *tssA1* translational start codon. (B) Analysis of a TssA1 leader translational fusion driven by the *lacUV5* promoter in wild-type and mutant derivative strains. All assays were repeated at least three times in triplicate. Data were normalized to the mean for PAO1. PAO1 activity with the *lacUV5-tssA1-lacZ* fusion was 12 Miller units. A one-way ANOVA with Tukey's posttest was used to determine statistical significance. ***, $P < 0.0001$.

in controlling *rsmA* expression is somewhat less clear. While we were not able to detect RpoS regulation of *rsmA* by transcriptional fusion analysis (Fig. 7), the results of RPA suggested that RpoS does play a role (Fig. 8). Further studies are needed to determine the significance of RpoS in controlling *rsmA* expression. A previous study identified AlgR to be a regulator of *rsmA*, *rsmY*, and *rsmZ* (14). It is possible that AlgR and AlgU work together in regulating *rsmA* in *mucA* mutant strains. It is clear that *rsmA* regulation is complex, but this work solidifies the role of AlgU as an *rsmA* activator.

Our finding that AlgU controls *rsmA* expression is bolstered by the analysis of *rsmA* in the clinical strains 2192 (mucoid) and 383 (nonmucoid) (45). These data indicate higher levels of RsmA in the *muca* mutant strain 2192 (Fig. 6). The findings of our regulation studies are consistent with a previous observation that a *muca* mutant had increased levels of *rsmA* expression (14). Thus, our work implicates RsmA to be necessary for the persistence of *P. aeruginosa* during chronic infections, because *muca* mutants are found at later time points during infection. This is consistent with the requirement of *Helicobacter pylori*, which requires CsrA (the RsmA orthologue) for persistence in the stomach (52). Whether RsmA participates in regulating virulence factors in *muca* mutant strains is currently not known and is an unexplored area.

After finding that AlgU, an important sigma factor in chronic infections (7), regulates *rsmA* expression, we asked if the increased RsmA levels have activity in *mutA* mutant strains. Our results support a model (Fig. 10) where RsmA activity in the *mutA* mutant background is due to AlgU. First, using the *tssA1* leader fu-

FIG 10 Model depicting the expression and activity of *rsmA* and the protein that it encodes, RsmA, in *mucA* mutant strains. Two transcripts of different sizes are produced. AlgU and RpoS control the longer transcript originating from the distal promoter. In the *mucA* strain background, AlgU increases the level of transcription from the distal promoter, but both transcripts are increased in amount either by transcription initiation at both promoters or by posttranscriptional processing of the longer message. Due to increased *rsmA* transcription, there is an increased amount of RsmA in *mucA* mutants. Transcriptional start sites are represented by bent arrows. The left and right bent arrows indicate the distal and proximal promoters, respectively. The +1 indicates the putative translational *rsmA* start site. RsmA levels increase, preventing translation of *tssA1* and therefore production of the HSI-I T6SS. The dashed curved arrow on the left side indicates that AlgU either directly or indirectly leads to the activation of the *tssA1* operon. RetS and GacA are depicted on the right to show how they are incorporated in the RsmA scenario. GacA, when phosphorylated by GacS, activates the expression of two small RNAs, RsmY and RsmZ, that bind and prevent RsmA from performing posttranscriptional regulation. RetS interferes with GacA phosphorylation by physically binding GacS and allows RsmA to regulate expression of mRNAs. The small RNAs RsmY and RsmZ bind and prevent RsmA activity.

sion, the *mucA22* strain showed decreased activity, indicating increased posttranscriptional regulation compared to that in PAO1. Second, the *mucA22* Δ *algU* strain had increased leader fusion activity compared to that of the *mucA22* strain, which supports our conclusion that increased *rsmA* expression leads to increased RsmA activity. This result also demonstrates the importance of AlgU control of *rsmA* expression. Third, the increase in the fusion activity in the *mucA22* Δ *rsmA* strain compared to that in the *mucA22* strain was greater than the increase in the Δ *rsmA* mutant of PAO1. Further support for RsmA activity in *mucA* mutant strains comes from the increased leader fusion activity of the *mucA22* Δ *rsmA* and *mucA22* Δ *retS* strains compared to that of the *mucA22* strain. An alternative explanation of our data is that some other form of posttranscriptional regulation occurs independently of RsmA. However, the *gacA* and *retS* mutants are known to regulate RsmA activity, even though *rsmA* is expressed in these strains (data not shown). We do not favor this alternative hypothesis, as previous studies have determined that GacA controls only *rsmY* and *rsmZ* expression (21, 49). Another posttranscriptional regulator, RsmF/RsmN, is also known to control *tssA1* expression (24, 53). This could possibly have affected the fusion results found in the present study as well and may explain the increased expression of the *tssA1* leader fusion in the *mucA22* Δ *retS* strain. Regardless, the results still indicate increased posttranscriptional regulation of *tssA1* in a *mucA22* strain.

The results of a proteomic study of the isogenic clinical strains 383 and 2192 also support our findings. In this study, the mucoid strain 2192 has decreased amounts of HSI-I T6SS components, whereas the nonmucoid strain has increased HSI-I T6SS components (54). If the level of RsmA was increased due to AlgU in mucoid strain 2192, as presented here (Fig. 6), then the proteomic analysis suggests that RsmA is responsible for the decreased amounts of HSI-I T6SS components in mucoid strain 2192. We are currently examining RsmA activity in other clinical isolates.

However, a previous study did not find that RsmA posttranscriptionally regulates *tssA1* in a *mucA* mutant (14). A possible reason for the discrepancy between the findings of that study and those of our study is that different strains and growth conditions were used. In addition, the previous study found that *rsmY* and *rsmZ* expression are increased in a PA103 *mucA* mutant (14). We analyzed *rsmY* and *rsmZ* expression using transcriptional fusions and also found the same result (data not shown). It is not known how RsmA functions if RsmY and RsmZ levels are increased, and this requires further investigation. One possibility is that the promoter fusions do not accurately monitor the levels of the small RNAs. We are directly measuring RsmY and RsmZ levels in a *mucA* mutant strain to determine if this is the case. If RsmY and RsmZ levels are increased and there is still RsmA activity, then we speculate the higher-affinity target mRNAs present compete with RsmY and RsmZ. Alternatively, other factors that appropriately direct RsmA to its targets, even in the presence of RsmY and RsmZ, exist.

The *P. aeruginosa* *mucA* mutant strains provide a snapshot of some of the bacteria found in the lungs of patients with cystic fibrosis during chronic infection. The use of *mucA* mutant strains may help provide an understanding of the factors required for persistence and adaptation in the lung environment. For instance, the overproduction of alginate by *mucA* mutant strains provides a competitive advantage by sequestering free radicals, preventing complement attack, and inhibiting phagocytosis (55). Our data

suggest that RsmA also plays a role in chronic infections, such as those that occur in the lungs of cystic fibrosis patients. The RsmA regulon may have additional targets in *mucA* mutant backgrounds that have not yet been discovered. An understanding of RsmA in both acute and chronic infections may provide new ways to combat *P. aeruginosa*.

ACKNOWLEDGMENTS

We thank Joseph and Julia Albright for providing equipment and supplies for these studies. We thank Joanna Goldberg (Emory University) for plasmid construct pTJ1. We thank Laraine Powers and David Hurley (ETSU) for critical review of the manuscript. We thank Eric Jones for expert technical assistance.

FUNDING INFORMATION

This work, including the efforts of Chris L. Pritchett, was funded by Research and Development Committee, ETSU (RDC12-012M and RDC13-005M).

REFERENCES

1. Sadikot RT, Blackwell TS, Christman JW, Prince AS. 2005. Pathogen-host interactions in *Pseudomonas aeruginosa* pneumonia. *Am J Respir Crit Care Med* 171:1209–1223. <http://dx.doi.org/10.1164/rccm.200408-1044SO>.
2. Driscoll JA, Brody SL, Kollef MH. 2007. The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections. *Drugs* 67:351–368. <http://dx.doi.org/10.2165/00003495-200767030-00003>.
3. Vakulskas CA, Potts AH, Babitzke P, Ahmer BM, Romeo T. 2015. Regulation of bacterial virulence by Csr (Rsm) systems. *Microbiol Mol Biol Rev* 79:193–224. <http://dx.doi.org/10.1128/MMBR.00052-14>.
4. Coggan KA, Wolfgang MC. 2012. Global regulatory pathways and cross-talk control *Pseudomonas aeruginosa* environmental lifestyle and virulence phenotype. *Curr Issues Mol Biol* 14:47–70.
5. Brencic A, Lory S. 2009. Determination of the regulon and identification of novel mRNA targets of *Pseudomonas aeruginosa* RsmA. *Mol Microbiol* 72:612–632. <http://dx.doi.org/10.1111/j.1365-2958.2009.06670.x>.
6. Burrows E, Bayse C, Adams C, O'Gara F. 2006. Influence of the regulatory protein RsmA on cellular functions in *Pseudomonas aeruginosa* PAO1, as revealed by transcriptome analysis. *Microbiology* 152:405–418. <http://dx.doi.org/10.1099/mic.0.28324-0>.
7. Govan JRW, Deretic V. 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol Rev* 60:539–574.
8. Mougous JD, Cuff ME, Raunser S, Shen A, Zhou M, Gifford CA, Goodman AL, Joachimiak G, Ordonez CL, Lory S, Walz T, Joachimiak A, Mekalanos JJ. 2006. A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science* 312:1526–1530. <http://dx.doi.org/10.1126/science.1128393>.
9. Irie Y, Starkey M, Edwards AN, Wozniak DJ, Romeo T, Parsek MR. 2010. *Pseudomonas aeruginosa* biofilm matrix polysaccharide Psl is regulated transcriptionally by RpoS and post-transcriptionally by RsmA. *Mol Microbiol* 78:158–172. <http://dx.doi.org/10.1111/j.1365-2958.2010.07320.x>.
10. Ramsey DM, Wozniak DJ. 2005. Understanding the control of *Pseudomonas aeruginosa* alginate synthesis and the prospects for management of chronic infections in cystic fibrosis. *Mol Microbiol* 56:309–322. <http://dx.doi.org/10.1111/j.1365-2958.2005.04552.x>.
11. Firoved AM, Boucher JC, Deretic V. 2002. Global genomic analysis of AlgU (σ (E))-dependent promoters (sigmulon) in *Pseudomonas aeruginosa* and implications for inflammatory processes in cystic fibrosis. *J Bacteriol* 184:1057–1064. <http://dx.doi.org/10.1128/jb.184.4.1057-1064.2002>.
12. Firoved AM, Deretic V. 2003. Microarray analysis of global gene expression in mucoid *Pseudomonas aeruginosa*. *J Bacteriol* 185:1071–1081. <http://dx.doi.org/10.1128/JB.185.3.1071-1081.2003>.
13. Firoved AM, Ornatowski W, Deretic V. 2004. Microarray analysis reveals induction of lipoprotein genes in mucoid *Pseudomonas aeruginosa*: implications for inflammation in cystic fibrosis. *Infect Immun* 72:5012–5018. <http://dx.doi.org/10.1128/IAI.72.9.5012-5018.2004>.
14. Intile PJ, Diaz MR, Urbanowski ML, Wolfgang MC, Yahr TL. 2014. The AlgZR two-component system recalibrates the RsmAYZ posttranscrip-

- tional regulatory system to inhibit expression of the *Pseudomonas aeruginosa* type III secretion system. *J Bacteriol* 196:357–366. <http://dx.doi.org/10.1128/JB.01199-13>.
15. Lapouge K, Schubert M, Allain FH, Haas D. 2008. Gac/Rsm signal transduction pathway of gamma-proteobacteria: from RNA recognition to regulation of social behaviour. *Mol Microbiol* 67:241–253.
 16. Goodman AL, Kulasekara B, Rietsch A, Boyd D, Smith RS, Lory S. 2004. A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*. *Dev Cell* 7:745–754. <http://dx.doi.org/10.1016/j.devcel.2004.08.020>.
 17. Heurlier K, Williams F, Heeb S, Dormond C, Pessi G, Singer D, Camara M, Williams P, Haas D. 2004. Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 186:2936–2945. <http://dx.doi.org/10.1128/JB.186.10.2936-2945.2004>.
 18. Ventre I, Goodman AL, Vallet-Gely I, Vasseur P, Soscia C, Molin S, Bleves S, Lazdunski A, Lory S, Filloux A. 2006. Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. *Proc Natl Acad Sci U S A* 103:171–176. <http://dx.doi.org/10.1073/pnas.0507407103>.
 19. Schubert M, Lapouge K, Duss O, Oberstrass FC, Jelesarov I, Haas D, Allain FH. 2007. Molecular basis of messenger RNA recognition by the specific bacterial repressing clamp RsmA/CsrA. *Nat Struct Mol Biol* 14: 807–813. <http://dx.doi.org/10.1038/nsmb1285>.
 20. Valverde C, Lindell M, Wagner EG, Haas D. 2004. A repeated GGA motif is critical for the activity and stability of the riboregulator RsmY of *Pseudomonas fluorescens*. *J Biol Chem* 279:25066–25074. <http://dx.doi.org/10.1074/jbc.M401870200>.
 21. Kay E, Humair B, Denervaud V, Riedel K, Spahr S, Eberl L, Valverde C, Haas D. 2006. Two GacA-dependent small RNAs modulate the quorum-sensing response in *Pseudomonas aeruginosa*. *J Bacteriol* 188:6026–6033. <http://dx.doi.org/10.1128/JB.00409-06>.
 22. Mulcahy H, O'Callaghan J, O'Grady EP, Adams C, O'Gara F. 2006. The posttranscriptional regulator RsmA plays a role in the interaction between *Pseudomonas aeruginosa* and human airway epithelial cells by positively regulating the type III secretion system. *Infect Immun* 74:3012–3015. <http://dx.doi.org/10.1128/IAI.74.5.3012-3015.2006>.
 23. Mulcahy H, O'Callaghan J, O'Grady EP, Macia MD, Borrell N, Gomez C, Casey PG, Hill C, Adams C, Gahan CG, Oliver A, O'Gara F. 2008. *Pseudomonas aeruginosa* RsmA plays an important role during murine infection by influencing colonization, virulence, persistence, and pulmonary inflammation. *Infect Immun* 76:632–638. <http://dx.doi.org/10.1128/IAI.01132-07>.
 24. Marden JN, Diaz MR, Walton WG, Gode CJ, Betts L, Urbanowski ML, Redinbo MR, Yahr TL, Wolfgang MC. 2013. An unusual CsrA family member operates in series with RsmA to amplify posttranscriptional responses in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* 110:15055–15060. <http://dx.doi.org/10.1073/pnas.1307217110>.
 25. Dotsch A, Eckweiler D, Schniederjans M, Zimmermann A, Jensen V, Scharfe M, Geffers R, Haussler S. 2012. The *Pseudomonas aeruginosa* transcriptome in planktonic cultures and static biofilms using RNA sequencing. *PLoS One* 7:e31092. <http://dx.doi.org/10.1371/journal.pone.0031092>.
 26. Wurtzel O, Yoder-Himes DR, Han K, Dandekar AA, Edelheit S, Greenberg EP, Sorek R, Lory S. 2012. The single-nucleotide resolution transcriptome of *Pseudomonas aeruginosa* grown in body temperature. *PLoS Pathog* 8:e1002945. <http://dx.doi.org/10.1371/journal.ppat.1002945>.
 27. Schulz S, Eckweiler D, Bielecka A, Nicolai T, Franke R, Dotsch A, Hornischer K, Bruchmann S, Duvel J, Haussler S. 2015. Elucidation of sigma factor-associated networks in *Pseudomonas aeruginosa* reveals a modular architecture with limited and function-specific crosstalk. *PLoS Pathog* 11:e1004744. <http://dx.doi.org/10.1371/journal.ppat.1004744>.
 28. Pessi G, Williams F, Hindle Z, Heurlier K, Holden MT, Camara M, Haas D, Williams P. 2001. The global posttranscriptional regulator RsmA modulates production of virulence determinants and N-acylhomoserine lactones in *Pseudomonas aeruginosa*. *J Bacteriol* 183:6676–6683. <http://dx.doi.org/10.1128/JB.183.22.6676-6683.2001>.
 29. Higuchi R. 1989. Using PCR to engineer DNA, p 61–70. In Erlich HA (ed), *PCR technology: principles and applications for DNA amplification*. Stockton Press, New York, NY.
 30. Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 212:77–86. [http://dx.doi.org/10.1016/S0378-1119\(98\)00130-9](http://dx.doi.org/10.1016/S0378-1119(98)00130-9).
 31. Figurski DH, Helinski DR. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci U S A* 76:1648–1652. <http://dx.doi.org/10.1073/pnas.76.4.1648>.
 32. Miller JF. 1972. *Experiments in molecular genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
 33. Deretic V, Leveau JH, Mohr CD, Hibler NS. 1992. *In vitro* phosphorylation of AlgR, a regulator of mucoidy in *Pseudomonas aeruginosa*, by a histidine protein kinase and effects of small phospho-donor molecules. *Mol Microbiol* 6:2761–2767. <http://dx.doi.org/10.1111/j.1365-2958.1992.tb01455.x>.
 34. Aldridge GM, Podrebarac DM, Greenough WT, Weiler IJ. 2008. The use of total protein stains as loading controls: an alternative to high-abundance single-protein controls in semi-quantitative immunoblotting. *J Neurosci Methods* 172:250–254. <http://dx.doi.org/10.1016/j.jneumeth.2008.05.003>.
 35. Eaton SL, Roche SL, Llavero Hurtado M, Oldknow KJ, Farquharson C, Gillingwater TH, Wishart TM. 2013. Total protein analysis as a reliable loading control for quantitative fluorescent Western blotting. *PLoS One* 8:e72457. <http://dx.doi.org/10.1371/journal.pone.0072457>.
 36. Damron FH, McKenney ES, Barbier M, Liechti GW, Schweizer HP, Goldberg JB. 2013. Construction of mobilizable mini-Tn7 vectors for bioluminescent detection of gram-negative bacteria and single-copy promoter lux reporter analysis. *Appl Environ Microbiol* 79:4149–4153. <http://dx.doi.org/10.1128/AEM.00640-13>.
 37. Potvin E, Sanschagrin F, Levesque RC. 2008. Sigma factors in *Pseudomonas aeruginosa*. *FEMS Microbiol Rev* 32:38–55. <http://dx.doi.org/10.1111/j.1574-6976.2007.00092.x>.
 38. Becher A, Schweizer HP. 2000. Integration-proficient *Pseudomonas aeruginosa* vectors for isolation of single-copy chromosomal lacZ and lux gene fusions. *Biotechniques* 29:948–950, 952.
 39. Jean-Pierre F, Perreault J, Deziel E. 2015. Complex autoregulation of the post-transcriptional regulator RsmA in *Pseudomonas aeruginosa*. *Microbiology* 161:1889–1896. <http://dx.doi.org/10.1099/mic.0.000140>.
 40. Linn T, St Pierre R. 1990. Improved vector system for constructing transcriptional fusions that ensures independent translation of lacZ. *J Bacteriol* 172:1077–1084.
 41. Schuster M, Hawkins AC, Harwood CS, Greenberg EP. 2004. The *Pseudomonas aeruginosa* RpoS regulon and its relationship to quorum sensing. *Mol Microbiol* 51:973–985. <http://dx.doi.org/10.1046/j.1365-2958.2003.03886.x>.
 42. Typas A, Becker G, Hengge R. 2007. The molecular basis of selective promoter activation by the sigmaS subunit of RNA polymerase. *Mol Microbiol* 63:1296–1306. <http://dx.doi.org/10.1111/j.1365-2958.2007.05601.x>.
 43. Damron FH, McKenney ES, Schweizer HP, Goldberg JB. 2013. Construction of a broad-host-range Tn7-based vector for single-copy P(BAD)-controlled gene expression in gram-negative bacteria. *Appl Environ Microbiol* 79:718–721. <http://dx.doi.org/10.1128/AEM.02926-12>.
 44. Pier GB, Matthews WJ, Eardley DD. 1983. Immunochemical characterization of the mucoid exopolysaccharide of *Pseudomonas aeruginosa*. *J Infect Dis* 147:494–503. <http://dx.doi.org/10.1093/infdis/147.3.494>.
 45. Hanna SL, Sherman NE, Kinter MT, Goldberg JB. 2000. Comparison of proteins expressed by *Pseudomonas aeruginosa* strains representing initial and chronic isolates from a cystic fibrosis patient: an analysis by 2-D gel electrophoresis and capillary column liquid chromatography-tandem mass spectrometry. *Microbiology* 146(Pt 10):2495–2508.
 46. Mathee K, Narasimhan G, Valdes C, Qiu X, Matewish JM, Koehrsen M, Rokas A, Yandava CN, Engels R, Zeng E, Olavarietta R, Doud M, Smith RS, Montgomery P, White JR, Godfrey PA, Kodira C, Birren B, Galagan JE, Lory S. 2008. Dynamics of *Pseudomonas aeruginosa* genome evolution. *Proc Natl Acad Sci U S A* 105:3100–3105. <http://dx.doi.org/10.1073/pnas.0711982105>.
 47. Yin Y, Withers TR, Wang X, Yu HD. 2013. Evidence for sigma factor competition in the regulation of alginate production by *Pseudomonas aeruginosa*. *PLoS One* 8:e72329. <http://dx.doi.org/10.1371/journal.pone.0072329>.
 48. Goodman AL, Merighi M, Hyodo M, Ventre I, Filloux A, Lory S. 2009. Direct interaction between sensor kinase proteins mediates acute and chronic disease phenotypes in a bacterial pathogen. *Genes Dev* 23:249–259. <http://dx.doi.org/10.1101/gad.1739009>.

49. Brencic A, McFarland KA, McManus HR, Castang S, Mogno I, Dove SL, Lory S. 2009. The GacS/GacA signal transduction system of *Pseudomonas aeruginosa* acts exclusively through its control over the transcription of the RsmY and RsmZ regulatory small RNAs. *Mol Microbiol* 73:434–445. <http://dx.doi.org/10.1111/j.1365-2958.2009.06782.x>.
50. Sakuragi Y, Kolter R. 2007. Quorum-sensing regulation of the biofilm matrix genes (*pel*) of *Pseudomonas aeruginosa*. *J Bacteriol* 189:5383–5386. <http://dx.doi.org/10.1128/JB.00137-07>.
51. Yakhnin H, Yakhnin AV, Baker CS, Sineva E, Berezin I, Romeo T, Babitzke P. 2011. Complex regulation of the global regulatory gene *csrA*: CsrA-mediated translational repression, transcription from five promoters by *Esigma*(7)(0) and *Esigma*(S), and indirect transcriptional activation by CsrA. *Mol Microbiol* 81:689–704. <http://dx.doi.org/10.1111/j.1365-2958.2011.07723.x>.
52. Barnard FM, Loughlin MF, Fainberg HP, Messenger MP, Ussery DW, Williams P, Jenks PJ. 2004. Global regulation of virulence and the stress response by CsrA in the highly adapted human gastric pathogen *Helicobacter pylori*. *Mol Microbiol* 51:15–32.
53. Morris ER, Hall G, Li C, Heeb S, Kulkarni RV, Lovelock L, Silistre H, Messina M, Camara M, Emsley J, Williams P, Searle MS. 2013. Structural rearrangement in an RsmA/CsrA ortholog of *Pseudomonas aeruginosa* creates a dimeric RNA-binding protein, RsmN. *Structure* 21:1659–1671. <http://dx.doi.org/10.1016/j.str.2013.07.007>.
54. Rao J, Damron FH, Basler M, Digiandomenico A, Sherman NE, Fox JW, Mekalanos JJ, Goldberg JB. 2011. Comparisons of two proteomic analyses of non-mucoid and mucoid *Pseudomonas aeruginosa* clinical isolates from a cystic fibrosis patient. *Front Microbiol* 2:162. <http://dx.doi.org/10.3389/fmicb.2011.00162>.
55. Pier GB, Coleman F, Grout M, Franklin M, Ohman DE. 2001. Role of alginate O acetylation in resistance of mucoid *Pseudomonas aeruginosa* to opsonic phagocytosis. *Infect Immun* 69:1895–1901. <http://dx.doi.org/10.1128/IAI.69.3.1895-1901.2001>.
56. Miller VL, Mekalanos JJ. 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J Bacteriol* 170:2575–2583.
57. Damron FH, Qiu D, Yu HD. 2009. The *Pseudomonas aeruginosa* sensor kinase KinB negatively controls alginate production through AlgW-dependent MucA proteolysis. *J Bacteriol* 191:2285–2295. <http://dx.doi.org/10.1128/JB.01490-08>.
58. Holloway BW, Krishnapillai V, Morgan AF. 1979. Chromosomal genetics of *Pseudomonas*. *Microbiol Rev* 43:73–102.
59. Mathee K, Ciofu O, Sternberg C, Lindum PW, Campbell JJ, Jensen P, Johnsen AH, Givskov M, Ohman DE, Molin S, Hoiby N, Kharazmi A. 1999. Mucoid conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. *Microbiology* 145(Pt 6):1349–1357. <http://dx.doi.org/10.1099/13500872-145-6-1349>.