σ Factor and Anti- σ Factor That Control Swarming Motility and Biofilm Formation in *Pseudomonas aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa is capable of causing a variety of acute and chronic infections. Here, we provide evidence that *sbrR* (PA2895), a gene previously identified as required during chronic *P. aeruginosa* respiratory infection, encodes an anti- σ factor that inhibits the activity of its cognate extracytoplasmic-function σ factor, SbrI (PA2896). Bacterial two-hybrid analysis identified an N-terminal region of SbrR that interacts directly with SbrI and that was sufficient for inhibition of SbrI-dependent gene expression. We show that SbrI associates with RNA polymerase *in vivo* and identify the SbrIR regulon. In cells lacking SbrR, the SbrI-dependent expression of *muiA* was found to inhibit swarming motility and promote biofilm formation. Our findings reveal SbrR and SbrI as a novel set of regulators of swarming motility and biofilm formation in *P. aeruginosa* that mediate their effects through *muiA*, a gene not previously known to influence surface-associated behaviors in this organism.

IMPORTANCE

This study characterizes a σ factor/anti- σ factor system that reciprocally regulates the surface-associated behaviors of swarming motility and biofilm formation in the opportunistic pathogen *Pseudomonas aeruginosa*. We present evidence that SbrR is an anti- σ factor specific for its cognate σ factor, SbrI, and identify the SbrIR regulon in *P. aeruginosa*. We find that cells lacking SbrR are severely defective in swarming motility and exhibit enhanced biofilm formation. Moreover, we identify *muiA* (PA1494) as the SbrI-dependent gene responsible for mediating these effects. SbrIR have been implicated in virulence and in responding to antimicrobial and cell envelope stress. SbrIR may therefore represent a stress response system that influences the surface behaviors of *P. aeruginosa* during infection.

The Gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic human pathogen notorious for being the principal cause of morbidity and mortality in cystic fibrosis (CF) patients (1). In patients with CF, chronic pulmonary colonization by *P. aeruginosa* leads to chronic inflammation, progressive loss of lung function, and eventually respiratory failure and death (1). *P. aeruginosa* is also the fifth leading cause of nosocomial infections overall in the United States and is the second most common cause of ventilator-associated pneumonia (VAP) and catheter-associated urinary tract infections (CAUTI) (2, 3). In patients with VAP or CAUTI, *P. aeruginosa* grows as a biofilm on endotracheal tubes and catheters, respectively (4–6). In addition, *P. aeruginosa* is thought to persist as a biofilm in the CF lung (7). *P. aeruginosa* biofilms are associated with chronic infection and exhibit increased antibiotic resistance and resistance to clearance by the immune system (8). Thus, the ability to form biofilms contributes significantly to the clinical burden of *P. aeruginosa* infection.

In *P. aeruginosa*, growth as a biofilm is inversely regulated with a cooperative form of multicellular surface motility called swarming (9–12). Swarming motility is flagellum dependent and requires the secretion of surfactants regulated by quorum sensing (13–16). In addition, swarming motility correlates with increased expression of virulence factors and is associated with acute infection (9, 17). Several systems are known to mediate the transition from motile, swarming cells to cells growing as sessile biofilms, including cyclic di-GMP (c-di-GMP) signaling and the GacS/GacA two-component system (9, 11, 12).

PA2895, which we refer to here as *sbrR*, was identified in a signature-tagged mutagenesis (STM) screen as being required for persistence in a rat lung model of chronic *P. aeruginosa* respiratory

infection (18). Although SbrR has no significant homology to previously characterized proteins, *sbrR* is located in a putative bicistronic operon downstream of the PA2896 gene encoding a putative extracytoplasmic function (ECF) σ factor that we refer to here as SbrI (19). As a group, ECF σ factors are frequently cotranscribed with their own negative regulator, a transmembrane anti- σ factor (20, 21). Upon stimulation by the appropriate extracytoplasmic signal, the ECF σ factor is released from the anti- σ factor, allowing it to associate with RNA polymerase (RNAP) and activate expression of its regulon.

Here, we present evidence that SbrI and SbrR are an ECF σ and anti- σ factor pair. We identify the SbrIR regulon and show that SbrI and SbrR influence biofilm formation and swarming motility by controlling the expression of *muiA* (mucoidy inhibitor A) (PA1494). In particular, we show that cells lacking *sbrR* are unable to engage in swarming motility and exhibit increased biofilm for-

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mation due to the SbrI-dependent increase in *muiA* expression observed in these cells. We have named PA2896 and PA2895 SbrI and SbrR, respectively, as a result of the swarming- and biofilm-related phenotypes we observe in $\Delta sbrR$ mutant cells. SbrI and SbrR constitute a pair of regulators controlling swarming motility and biofilm formation in *P. aeruginosa* that mediate their effects through MuiA.

MATERIALS AND METHODS

Bacterial strains. *E. coli* DH5 α F'IQ (Invitrogen) was used as the recipient strain for all plasmid constructions. *E. coli* SM10 λ pir served as the conjugative donor for transferring plasmids into *P. aeruginosa* during strain construction. The *P. aeruginosa* strains used included PAO1 (provided by A. Rietsch) and PA14 (provided by L. Rahme). Bacterial cultures were routinely grown at 37°C in lysogeny broth (LB) or on plates containing LB solidified with 1.5% agar unless otherwise noted. When appropriate, gentamicin (30 μ g/ml) and carbenicillin (200 μ g/ml) were used for selection in *P. aeruginosa* cultures. A list of strains and plasmids used in this study is available in Table S1 in the supplemental material.

Plasmids and strains for TAP tag experiments. Plasmid pP30 Δ -PA2896-TAP was made by cloning an $\sim$ 300-bp fragment of DNA corresponding to a 3' portion of the *sbrI* gene into pP30 Δ -YTAP cut with HindIII and NotI; the portion of the PA2896 gene was cloned so that it was in frame with the DNA specifying the tandem-affinity purification (TAP) tag. pP30 Δ -SbrI-TAP was used to generate PAO1 SbrI-TAP, as previously described (22). Strain PAO1 RpoS-TAP was constructed in a similar way using the vector pP30 Δ -RpoS-TAP, which contains a portion of the *P. aeruginosa* *rpoS* gene fused in frame to DNA specifying the TAP tag. The PAO1 AceF-TAP strain, which expresses AceF-TAP and served as a control, has been described previously (22). Plasmid pP30 Δ FRT-SbrI-VSV-G was made by subcloning the HindIII/NotI *sbrI* fragment from pP30 Δ -SbrI-TAP into pP30 Δ FRT-MvaT-VSV-G to replace *mvaT* so that the 3' end of *sbrI* was in frame with the vesicular stomatitis virus G protein (VSV-G) tag (23). PAO1 β' -TAP SbrI-V was constructed in a similar manner by integrating pP30 Δ FRT-SbrI-VSV-G into the previously described strain PAO1 β' -TAP (24).

Reporter strain and plasmids for bacterial two-hybrid assays. Bacterial two-hybrid assays were performed with the *Escherichia coli* reporter strain KS1 (25); KS1 harbors on its chromosome the P_{lac} λ O_R2-62 test promoter driving expression of a linked *lacZ* reporter gene. Plasmids pAC λ cl32 and pBR α LN have been described previously (26) and were used to create fusions to the C terminus of λ cl and the C terminus of the α -linker, respectively. Plasmid pAC λ cl-SbrR-NTR encodes λ cl (residues 1 to 236) fused to residues 2 to 64 of SbrR from *P. aeruginosa* via a small linker composed of three alanine residues. Plasmid pAC λ cl-SbrR-NTR was made by cloning the appropriate NotI-BamHI-digested PCR product into pAC λ cl32 that had been digested with NotI and BstYI, thus placing expression of the λ cl-SbrR-NTR fusion protein under the control of the IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible *lacUV5* promoter. Plasmid pBR α -SbrI encodes residues 1 to 248 of the α subunit of *E. coli* RNA polymerase fused to residues 2 to 194 of SbrI from *P. aeruginosa* via a small linker composed of three alanine residues. Plasmid pBR α -SbrI was made by cloning the appropriate NotI-BamHI-digested PCR product into pBR α LN digested with NotI and BamHI, thus placing the α -fusion under the control of tandem *lpp* and IPTG-inducible *lacUV5* promoters. Plasmid pBR α encodes wild-type (WT) α under the control of tandem *lpp* and IPTG-inducible *lacUV5* promoters and has been described previously (25).

Promoter-*lacZ* fusion reporter strains. The PAO1 promoter-*lacZ* fusion reporter strains PAO1 *attB*:: P_{sbrI} -*lacZ*, PAO1 *attB*:: P_{muiA} -*lacZ*, and PAO1 *attB*:: P_{PA4495} -*lacZ* contain the putative promoter regions of *sbrI*, *muiA*, and PA4495, respectively, fused to the *lacZ* gene and integrated in single copy into the ϕ CTX locus in the PAO1 chromosome. The putative *sbrI* promoter region consisted of the 327-bp intergenic region upstream of the *sbrI* start codon (27). This region was amplified by PCR and cloned

into mini-CTX-*lacZ* as a BamHI/PstI fragment to generate mini-CTX- P_{sbrI} -*lacZ*. The upstream intergenic regions of *muiA* (122 bp) and PA4495 (275 bp) were also PCR amplified and cloned into mini-CTX-*lacZ* on HindIII/BamHI fragments to generate mini-CTX- P_{muiA} -*lacZ* and mini-CTX- P_{PA4495} -*lacZ*, respectively. The resulting plasmids were integrated in single copy into the ϕ CTX site to create reporter strains PAO1 *attB*:: P_{sbrI} -*lacZ*, PAO1 *attB*:: P_{muiA} -*lacZ*, and PAO1 *attB*:: P_{PA4495} -*lacZ* as previously described (28).

Construction of deletion mutant strains. The deletion construct for the *sbrR* gene was generated by amplifying regions $\sim$ 700 bp in length that flank *sbrR* in the PAO1 genome by PCR and then splicing the flanking regions together by overlap extension PCR. Due to a 4-bp overlap between the 3' end of *sbrI* and the 5' end of *sbrR*, the deletion was designed so that *sbrI* would not be disrupted by the *sbrR* deletion construct. The deletion was in frame and contained a 9-bp NotI linker sequence, 5'-GCGGCCG CC-3', separating the two flanking regions. The resulting PCR product was cloned on a HindIII/XbaI fragment into plasmid pEXG2 (29), yielding plasmid pEXG2- Δ *sbrR*. The PAO1 Δ *sbrR* and PA14 Δ *sbrR* deletion strains were generated with this plasmid by allelic replacement, as previously described (30). Plasmid pEXG2- Δ *sbrR* was also used to generate the Δ *sbrR* mutant reporter strains in a similar manner. The Δ *sbrIR* deletion construct was generated by amplifying the $\sim$ 700-bp 5' flanking region of *sbrI* in the PAO1 genome by PCR. The PCR product was digested with XbaI and NotI and cloned into pEXG2- Δ *sbrR* digested with XbaI and NotI, so that the 5'-flanking *sbrI* XbaI/NotI fragment replaced the 5' flanking region used for deleting *sbrR*, yielding plasmid pEXG2- Δ *sbrIR*. This plasmid was used to create the Δ *sbrIR* deletion strains, as previously described (30). The *muiA* deletion construct was made in a similar fashion to the *sbrR* deletion construct. Flanking regions $\sim$ 700 bp in length on either side of *muiA* in the PAO1 genome were amplified by PCR and spliced together by overlap extension PCR. The deletion was in frame and included the NotI linker, as described above. This PCR product was cloned into pEXG2 to generate pEXG2- Δ *muiA*. The resulting plasmid was used to delete *muiA*, as described above. Deletions were confirmed by PCR.

Tandem-affinity purification. Cells were grown at 37°C with aeration in 200 ml of LB in 1-liter flasks to an optical density at 600 nm (OD₆₀₀) of $\sim$ 1 and then harvested by centrifugation at 4°C. TAP was then performed, as described previously (29). The purified proteins were concentrated using Amicon Ultra-4 centrifugal-filtration units with a 10-kDa molecular-mass cutoff (Millipore), separated on 4 to 12% Bis-Tris NuPAGE gels (Invitrogen), and stained with Coomassie blue.

Western blots. Purified proteins and cell lysates were separated on 4 to 12% Bis-Tris NuPAGE (Invitrogen), and Western blotting was performed as described previously (22). The VSV-G tag was detected using polyclonal rabbit anti-VSV-G (Sigma-Aldrich) and peroxidase-conjugated goat anti-rabbit IgG antibodies (Sigma-Aldrich).

Bacterial two-hybrid assays. Cells were grown with aeration at 37°C in LB supplemented with kanamycin (50 μ g/ml), carbenicillin (100 μ g/ml), chloramphenicol (25 μ g/ml), and IPTG at the concentrations indicated. β -Galactosidase assays were performed as described previously (26). Assays were performed three times in duplicate on separate occasions, and a representative data set is shown. The values are averages based on one experiment; duplicate measurements differed by $<10\%$.

Construction of *sbrR*, *sbrI*, and *muiA* expression plasmids. To make expression plasmid pPSV38-SbrR, a DNA fragment containing the *P. aeruginosa* PAO1 *sbrR* coding sequence flanked by HindIII and BamHI sites was amplified by PCR and cloned into pPSV38 (31). pPSV38-SbrR directs IPTG-inducible synthesis of SbrI and confers resistance to gentamicin. The same process was used to generate expression plasmid pPSV38-SbrI. To construct pPSV38-SbrR-NTR, a DNA fragment encoding the first 64 residues of SbrR flanked by HindIII and BamHI sites was amplified by PCR and cloned into pPSV38. To make pHERD20T-MuiA, a DNA fragment containing the *P. aeruginosa* PA14 *muiA* coding sequence flanked by XbaI and PstI was amplified by PCR and cloned into

TABLE 1 DNA microarray results comparing fold changes in gene expression relative to WT PAO1

Gene no.	Gene name	Fold change ^a		
		PAO1 $\Delta sbrR$ pPSV38 vs. PAO1 pPSV38	PAO1 $\Delta sbrR$ pSbrR vs. PAO1 pPSV38	PAO1 $\Delta sbrIR$ pPSV38 vs. PAO1 pPSV38
PA0086	<i>tagJ1</i>	2.3	—	—
PA0089	<i>tssG1</i>	2.2	—	—
PA0167		—	−2.4	—
PA0171		2.5	—	—
PA0839		3.8	—	—
PA0971	<i>tolA</i>	3.5	—	—
PA0972	<i>tolB</i>	2.6	—	—
PA1202		13.0	14.0	—
PA1203		4.2	3.1	—
PA1493	<i>cysP</i>	2.8	—	—
PA1494	<i>muiA</i>	103.1	−2.2	−2.2
PA2432	<i>bexR</i>	14.2	9.5	—
PA2895	<i>sbrR</i>	—	6.0	−2.0
PA2896	<i>sbrI</i>	18.0	—	−2.4
PA3179		—	2.1	2.2
PA3569	<i>mmsB</i>	−9.7	—	—
PA3570	<i>mmsA</i>	−9.3	—	—
PA3876	<i>narK2</i>	2.8	6.9	4.9
PA3923		−5.4	—	—
PA4495		21.9	—	—
PA5172	<i>arcB</i>	−2.6	—	—
PA5315	<i>rpmG</i>	2.7	—	—
PA5435		−2.4	—	—
PA5445		2.5	—	—

^a —, no change in expression. pPSV38 is an empty-vector control.

pHERD20T (32). pHERD20T-MuiA directs the arabinose-inducible synthesis of MuiA and confers resistance to ampicillin.

Microarray experiments. Cells of PAO1 (pPSV38), PAO1 $\Delta sbrR$ (pPSV38), PAO1 $\Delta sbrR$ (pPSV38-SbrR), and PAO1 $\Delta sbrIR$ (pPSV38) were grown with aeration at 37°C in 200 ml LB supplemented with gentamicin (30 μ g/ml). Biological-duplicate cultures of each strain were inoculated at a starting OD₆₀₀ of 0.01 and grown to an OD₆₀₀ of ~0.5 (corresponding to the mid-logarithmic phase of growth). RNA isolation, cDNA synthesis, cDNA fragmentation, and labeling were performed as described previously (33). Labeled cDNA was hybridized to Affymetrix GeneChip *P. aeruginosa* genome arrays (Affymetrix), and GeneSpring GX (Agilent Technologies) was used to analyze data for statistically significant changes in gene expression. The genes with changes in expression of ≥ 2 -fold ($P \leq 0.01$) are listed in Table 1.

Reporter strain β -galactosidase assays. Cells were grown at 37°C with aeration in LB supplemented with gentamicin (30 μ g/ml). The cells were permeabilized with sodium dodecyl sulfate and CHCl₃ and assayed for β -galactosidase activity, as described previously (26). Assays were performed at least twice in biological triplicate. Representative data sets are shown.

Motility assays. Swimming motility and swarming motility assays were performed as previously described (34, 35). Agar plates for assessing swimming motility and swarming motility consisted of M8 medium (34, 35) supplemented with glucose, MgSO₄, Casamino Acids (CAA), and 0.3% agar for swim plates or 0.5% agar for swarm plates. Arabinose (0.1%) was added where indicated. Swim plates were stab inoculated from colonies grown overnight on LB agar plates. Swarm plates were inoculated with 3 μ l of liquid culture grown overnight in LB. Swim plates and swarm plates were incubated for ~20 h at 37°C. Quantification of swim and swarm zones was performed using ImageJ software. Experiments for testing swimming motility and swarming motility were performed in biolog-

ical triplicate or quadruplicate on three separate days. The data shown are aggregates of 3 separate experiments normalized to the WT. Subsurface twitching motility was assayed as described previously (36). Bacteria were stab inoculated through a layer of LB agar (1% agar) to the bottom of the petri dish. After incubation for ~24 h at 37°C, the twitching motility was examined by removing the agar and staining the attached cells with Coomassie blue (Sigma-Aldrich). Quantification of twitching motility was performed by measuring the maximum diameter in millimeters of the circular zones formed by attached cells. Twitching motility experiments were performed in quadruplicate on two separate occasions. The data shown represent the results of those experiments shown in aggregate and normalized to the WT. Statistical analysis of motility data was performed using Prism version 6.0 (GraphPad).

Biofilm formation assay. Biofilm formation in 96-well microtiter plates was assayed as previously described, with modifications (37). Overnight cultures grown in LB were used to inoculate fresh medium to a starting OD₆₀₀ of 0.1. The medium was supplemented with carbenicillin (200 μ g/ml) and 1% arabinose, as indicated; 100 μ l of each bacterial suspension was dispensed in quadruplicate into the wells of a Costar 96-well polyvinylchloride microtiter plate and incubated for 8 h at 37°C. Following incubation, the plates were washed twice with water, and adherent biofilms were stained with 150 μ l of 0.1% crystal violet for 15 min. Following staining, the plates were washed twice with water and allowed to dry overnight. The stained biofilms were solubilized with 200 μ l of 33% acetic acid, and absorbance at 595 nm was read with a Tecan Infinite 200 plate reader. Experiments were performed on at least two separate occasions. Representative results are shown.

Microarray data accession number. The data discussed here have been deposited in NCBI's Gene Expression Omnibus (38) and are accessible through GEO Series accession number GSE74917.

RESULTS

SbrI associates with RNA polymerase. *sbrR* was previously identified through a signature-tagged transposon mutagenesis screen as essential for the persistence of *P. aeruginosa* in a chronic respiratory infection model (18). *sbrR* is predicted to be a component of a bicistronic operon, together with *sbrI* (Fig. 1A). A 4-bp overlap in the coding sequences of *sbrR* and *sbrI* suggests strong trans-

FIG 1 SbrI interacts with RNAP and is more abundant in $\Delta sbrR$ mutants. (A) Putative *sbrIR* operon. (B) The β , β' , and α subunits of RNAP copurify with RpoS-TAP (lane 2) and SbrI-TAP (lane 3) but not with AceF-TAP (lane 1). The purified proteins were separated by SDS-PAGE and stained with Coomassie blue. AceF-CBP, RpoS-CBP, and SbrI-CBP indicate the purified proteins with the calmodulin binding protein (CBP) moiety that remains after cleaving the protein A moiety of the TAP tag during purification. (C) SbrR has a negative effect on SbrI-V protein abundance. Shown is an anti-VSV-G Western blot of WT PAO1 (lane 1), PAO1 SbrI-V (lane 2), and PAO1 $\Delta sbrR$ SbrI-V (lane 3).

lational coupling of these genes. While SbrR has no homology to any previously characterized proteins, SbrI is annotated as a probable ECF σ factor in the *Pseudomonas* Genome Database and shares significant sequence homology with other ECF σ factors (27). To determine if SbrI might function as a σ factor, we purified SbrI from cells of *P. aeruginosa* and asked whether subunits of RNAP copurified with it.

To facilitate the purification of SbrI from *P. aeruginosa*, we constructed a strain of PAO1 that synthesized SbrI with a TAP tag fused to its C terminus (SbrI-TAP) from its native chromosomal location. As a positive control for our ability to detect an association between a σ factor and RNAP, we also constructed a second strain that synthesized the stationary-phase-specific σ factor RpoS with a C-terminal TAP tag (RpoS-TAP). As a negative control, we used a previously constructed strain that synthesizes AceF (a subunit of pyruvate dehydrogenase that is not expected to interact with RNAP) with a TAP tag fused to its C terminus (AceF-TAP) (22). We then purified SbrI, RpoS, and AceF by TAP and analyzed those proteins that copurified by SDS-PAGE, followed by staining with Coomassie blue. Proteins with the expected molecular weights for the β , β' , and α subunits of RNAP copurify with both RpoS-TAP and SbrI-TAP but not the negative-control AceF-TAP (Fig. 1B). This suggests that SbrI associates with RNAP *in vivo*, consistent with its predicted function as a σ factor. SbrI appears as a doublet in Fig. 1B, suggesting it can exist as a high-molecular-weight and a low-molecular-weight species. It is unclear if this doublet represents SbrI processing or the use of an alternative translational start site or if there is any functional difference between the two forms. However, both forms copurify with β' -TAP (see Fig. S1 in the supplemental material), suggesting that both forms are capable of associating with RNAP.

SbrR negatively influences the abundance of SbrI. The genomic arrangement of *sbrI* and *sbrR* is consistent with that of an ECF σ factor and its cognate anti- σ factor. In the absence of their cognate anti- σ factors, autoregulated σ factors can become constitutively active, resulting in increased abundance of the σ factor. However, in some cases, ECF σ factors exhibit reduced activity and abundance in the absence of their cognate anti- σ factors, which are thought to stabilize and protect the σ factor from degradation (39, 40). Alternatively, anti- σ factors can also promote the proteolysis of their cognate σ factors (41). A comparison of the abundance of SbrI with a C-terminal VSV-G epitope tag (SbrI-V) in WT and Δ *sbrR* mutant cells revealed that SbrI-V (synthesized from its native locus) is more abundant in the absence of SbrR (Fig. 1C). This suggests that SbrR negatively influences the abundance of SbrI and that SbrR might inhibit the expression of *sbrI*.

Transcription from the *sbrI* promoter is negatively regulated by SbrR. After observing increased SbrI protein abundance in cells of the Δ *sbrR* mutant strain, we were interested in determining whether SbrR represses transcription from the *sbrI* promoter. To test this, we integrated a construct with the putative *sbrI* promoter region upstream of a *lacZ* reporter at the ϕ CTX phage attachment site on the PAO1 chromosome to generate the reporter strain PAO1 *attB::P_{sbrI}-lacZ*. We then created an in-frame Δ *sbrR* deletion in this strain to test the effects of SbrR on expression from the *sbrI* promoter. In cells of the Δ *sbrR* mutant reporter strain, β -galactosidase activity increased 45-fold relative to that observed in cells of the WT reporter strain (Fig. 2A, left graph), suggesting that SbrR inhibits transcription from the *sbrI* promoter. This increase was restored to WT levels by complementation with *sbrR* from a

plasmid (Fig. 2A, left graph). Cells of the Δ *sbrIR* double-mutant strain exhibited basal levels of β -galactosidase activity similar to that observed in cells of the WT reporter strain (Fig. 2A, left graph), suggesting that expression from the *sbrI* promoter is *sbrI* dependent. Ectopic expression of *sbrI* in cells of the Δ *sbrIR* mutant strain resulted in an increase in β -galactosidase activity (Fig. 2A), confirming the positive regulatory effect of SbrI on its own promoter. Expression of the *P_{sbrI}-lacZ* reporter was higher in cells of the Δ *sbrR* mutant than in cells of the Δ *sbrIR* double mutant, in which *sbrI* was under the control of a heterologous promoter (Fig. 2A). This difference might be explained by an SbrI-dependent positive-feedback loop that serves to amplify *sbrI* expression only when *sbrI* is under the control of its native promoter. Taken together, these results suggest that SbrR inhibits SbrI-dependent transcription and that *sbrI* is positively autoregulated, consistent with a model in which SbrI is an ECF σ factor that controls its own expression and SbrR is its cognate anti- σ factor.

The N-terminal region of SbrR inhibits the activity of SbrI. It has been shown that the N-terminal cytoplasmic region of ECF anti- σ factors can be sufficient for anti- σ factor activity (40, 42–44). SbrR is predicted to contain a single transmembrane α -helix from residues 65 to 87, with a cytoplasmic N-terminal domain and a periplasmic C-terminal domain (CTD), consistent with the membrane topology of other ECF anti- σ factors (Fig. 2C). To determine if the N-terminal region (NTR) of SbrR was capable of inhibiting SbrI-dependent gene expression, we truncated SbrR at the start of the predicted transmembrane domain to produce SbrR-NTR (residues 1 to 64) (Fig. 2B). The levels of β -galactosidase activity in the Δ *sbrR* reporter strain expressing SbrR-NTR are indistinguishable from those expressing full-length SbrR (Fig. 2A), which suggests SbrR-NTR contains the region of SbrR necessary for inhibiting SbrI activity. It further suggests that SbrR-NTR may contain a domain that is capable of interacting with SbrI.

The N-terminal region of SbrR interacts with SbrI. ECF anti- σ factors inhibit their cognate σ factors by binding to them directly and preventing their association with RNAP (42, 44–46). We have shown that both SbrR and SbrR-NTR inhibit SbrI-dependent gene expression (Fig. 2B), and we next sought to determine whether SbrR directly interacts with SbrI, using a bacterial two-hybrid system.

In this two-hybrid system, the detection of a protein-protein interaction relies on the observation that an interaction between a DNA-bound protein and a subunit of RNAP can result in transcription activation of a test promoter (25, 26, 47). In the version of the assay used here, contact between a protein fused to the α subunit of *E. coli* RNAP and another protein (or protein domain) fused to the λ C1 DNA-binding protein activates the transcription of a *lacZ* reporter gene situated downstream of an appropriate test promoter containing a λ C1 binding site (Fig. 2C).

To test whether SbrR could interact directly with SbrI, we created two compatible plasmids, one expressing SbrR-NTR (residues 2 to 64) fused to the C terminus of λ C1 and the other expressing an α fusion protein where the CTD of α has been replaced with full-length SbrI (residues 2 to 194). We then determined whether the resulting λ C1-SbrR-NTR fusion protein could activate transcription from the test promoter in cells that also synthesized the α -SbrI fusion protein. Plasmids directing the synthesis of the λ C1-SbrR-NTR and the α -SbrI fusion proteins were used to transform *E. coli* strain KS1, which harbors the *P_{lac}AO_R2-62* test promoter

FIG 2 SbrR is an anti- σ factor that directly interacts with SbrI and inhibits its activity. (A) β -Galactosidase activities of PAO1 P_{sbrI} -*lacZ*, PAO1 P_{muiA} -*lacZ*, and PAO1 P_{PA4495} -*lacZ* reporter strains. $\Delta sbrR$ and $\Delta sbrIR$ mutants were generated in each reporter strain and transformed with the indicated plasmids. pPSV38 is an empty-vector control. The error bars represent standard deviations between three biological replicates. (B) Schematic representation of SbrR and the location of its predicted transmembrane (TM) domain. (C) Schematic representation of the bacterial two-hybrid system. Interaction between the N-terminal region of SbrR (SbrR-NTR) fused to λ CI and SbrI fused to the α subunit of RNA polymerase activates transcription from the test promoter driving expression of *lacZ*. The diagram depicts the test promoter P_{lac} λ O_R2-62, which bears the λ operator O_R2 centered 62 bp upstream from the transcription start site of the *lac* core promoter. This test promoter is linked to *lacZ* and located on the chromosome. (D) Effect of λ CI-SbrR-NTR on transcription from P_{lac} λ O_R2-62 in the presence of the α -SbrI chimera. Cells harboring compatible plasmids directing the synthesis of the indicated proteins were grown in the presence of different concentrations of IPTG and assayed for β -galactosidase activity.

(depicted in Fig. 2C) linked to *lacZ* and integrated in single copy in the *E. coli* chromosome (26). We found that the λ CI-SbrR-NTR fusion protein strongly activated the transcription of the *lacZ* reporter in cells that also synthesized the α -SbrI fusion protein but not in cells that contained only WT α (Fig. 2D). Additional controls revealed that WT λ CI failed to activate expression of the *lacZ* reporter in the presence of the α -SbrI fusion protein or in the presence of WT α (Fig. 2D). These results suggest that SbrR and SbrI directly interact, consistent with the hypothesis that SbrR is the cognate anti- σ factor of SbrI.

Defining the SbrIR regulon. Next, we wanted to identify the genes controlled by SbrI and SbrR. Based on our results described above, which show that SbrR inhibits the expression of *sbrI* and that SbrI is positively autoregulated, we reasoned the SbrI regulon would be constitutively expressed in $\Delta sbrR$ mutant cells. Using DNA microarrays, we compared gene expression in $\Delta sbrR$ mutant cells, $\Delta sbrIR$ mutant cells, and WT cells containing an empty vector, together with $\Delta sbrR$ mutant cells containing a vector that expresses *sbrR*.

Compared to WT cells, the expression of 21 genes changed >2-fold in cells of the $\Delta sbrR$ mutant (Table 1). The expression of three genes in particular was strongly influenced by the deletion of *sbrR*.

Specifically, the expression of *muiA* (PA1494) was 103-fold higher in cells of the $\Delta sbrR$ mutant than in WT cells, while expression of PA4495 and *sbrI* was 22- and 18-fold higher, respectively, in cells of the $\Delta sbrR$ mutant than in the WT (Table 1). The effects of the $\Delta sbrR$ deletion on *muiA*, PA4495, and *sbrI* expression could be complemented by providing *sbrI* in *trans* from a plasmid (Table 1). Furthermore, upregulation of *muiA* and PA4495 did not occur in cells of the $\Delta sbrIR$ double mutant, suggesting that the upregulation of these genes observed in cells of the $\Delta sbrR$ single mutant is dependent upon SbrI (Table 1). Consistent with the results of our microarray analyses, the expression of putative *muiA* promoter- and PA4495 promoter-*lacZ* fusions was upregulated in cells of a $\Delta sbrR$ mutant compared to the WT (Fig. 2A). Moreover, this upregulation could be complemented by ectopic synthesis of SbrR or SbrR-NTR from a plasmid and was dependent upon SbrI (Fig. 2A). Taken together, our findings suggest that transcription from the P_{muiA} , P_{PA4495} , and P_{sbrI} promoters is positively regulated by the ECF σ factor SbrI and inhibited by SbrI's cognate anti- σ factor, SbrR.

Microarray analyses revealed that several genes exhibited differential expression in $\Delta sbrR$ mutant cells relative to WT cells that did not respond to complementation with pPSV38-SbrR, suggest-

FIG 3 Swimming, twitching, and swarming motility of WT and $\Delta sbrR$ mutant strains. (A) Relative diameters of twitching motility zones for the indicated PAO1 and PA14 strains normalized to the WT for each strain. *, $P = 0.02$; **, $P < 0.001$. (B) Relative diameters of swimming motility zones of the indicated PAO1 and PA14 strains normalized to the WT for each strain. **, $P < 0.001$. (C) Relative swarming motilities of the indicated PA14 strains. The plates were photographed, and the area of motile cells was quantified with ImageJ. **, $P < 0.001$. (D) Representative image of PA14 swarming motility in WT and $\Delta sbrR$ strains. Significant differences between strains were determined by t test. The error bars represent 95% confidence intervals.

ing SbrR may not control the expression of these genes (Table 1). Indeed, previous work in our laboratory may explain the differential regulation of three genes (PA1202, PA1203, and PA2432) that fit this profile. We have previously shown that expression of PA1202, PA1203, and PA2432 (*bexR*) is bistable and subject to the control of a bistable switch mediated by the transcription regulator BexR (48). Therefore, the differential expression of these genes may result from bistable BexR activity, rather than changes in expression attributable to SbrI activation. PA3876 (*narK2*) also exhibited higher expression levels in $\Delta sbrR$ mutant cells than in WT cells that were unaffected by complementation with pPSV38-SbrR (Table 1). The expression of *narK2* was also elevated in $\Delta sbrIR$ double-mutant cells relative to the WT (Table 1), suggesting that the observed changes in *narK2* expression are independent of SbrI.

Several genes were found to be downregulated in $\Delta sbrR$ mutant cells relative to the WT. In particular, the *mmsAB* operon was found to be downregulated roughly 9-fold in $\Delta sbrR$ mutant cells relative to the WT (Table 1). MmsA and MmsB are enzymes involved in valine metabolism (49). The *mmsAB* operon is positively regulated by the divergently transcribed AraC-like transcription regulator MmsR (49); however, no changes in *mmsR* expression were observed by DNA microarray. These findings suggest that although SbrR functions principally as a negative regulator of the *muiaA*, PA4495, and *sbrI* genes, SbrR might also exert positive effects on the expression of some genes.

$\Delta sbrR$ mutants exhibit a severe swarming motility defect. *P. aeruginosa* is capable of several types of motility, including twitching, swimming, and swarming. Twitching motility is a form of surface motility and is mediated by type IV pili (50). Swimming motility occurs in low-viscosity liquid environments and is a unicellular behavior dependent upon flagella and the chemotaxis system (13). Swarming is a form of cooperative multicellular motility

that occurs on hydrated surfaces and in viscous liquid environments (13). In *P. aeruginosa*, swarming motility is dependent upon flagella and secreted surfactants, the production of which is controlled by quorum sensing (14, 15). On agar plates, WT *P. aeruginosa* colonies grown overnight expand their borders via surface motility (51). When we compared our WT and $\Delta sbrR$ mutant strains, we noticed colonies of $\Delta sbrR$ mutant cells were slightly smaller (data not shown), suggesting these cells might have a motility defect. To determine if SbrR influences motility, we compared twitching, swimming, and swarming motilities in WT and $\Delta sbrR$ mutants of *P. aeruginosa* strains PAO1 and PA14.

Compared to the WT, PAO1 $\Delta sbrR$ and PA14 $\Delta sbrR$ mutants exhibit reductions in twitching motility of 14% and 20%, respectively (Fig. 3A). These findings suggest that although $\Delta sbrR$ mutants have reduced twitching motility, cells of these mutants continue to produce functional type IV pili and engage in twitching motility.

Next, we tested the swimming motility of our strains. PAO1 $\Delta flgK$ and PA14 $\Delta flgK$ mutants that do not produce flagella were unable to swim from the point of inoculation (data not shown). Compared to the WT, PAO1 and PA14 $\Delta sbrR$ mutants exhibited reductions in swimming motility of 12% and 19%, respectively (Fig. 3B). Thus, swimming motility is only slightly reduced in $\Delta sbrR$ mutant cells, suggesting that these mutant cells continue to engage in chemotaxis and continue to produce functional flagella. Fluorescent staining of WT PAO1 and PAO1 $\Delta sbrR$ revealed that both WT and mutant strains produce normal flagella (data not shown).

To test whether cells of our $\Delta sbrR$ mutant strains exhibited a swarming defect, we inoculated cells from overnight cultures onto plates containing a minimal medium solidified with 0.5% agar. WT PA14 is a robust swarmer, forming colonies with dendrites that extend radially from the point of inoculation in an irregular

FIG 4 MuiA inhibits swarming motility and enhances biofilm formation. (A) Swarming motility inhibition in $\Delta sbrR$ mutants is dependent upon *sbrI* and *muiA*. Representative images of swarming plates are shown above the quantification of the area of each strain's swarm relative to WT PA14. (B) Swarming motility is inhibited by ectopic expression of *muiA*. Cells were grown in the presence of 0.1% arabinose to induce expression of *muiA*. (C) Enhanced biofilm formation in PA14 $\Delta sbrR$ mutants is dependent upon *sbrI* and *muiA*. (D) Ectopic expression of *muiA* results in enhanced biofilm formation. Cells were grown in the presence of 1% arabinose to induce expression of *muiA*. The error bars represent 95% confidence intervals.

starburst (Fig. 3D). In contrast to our WT PA14 strain, cells of our WT PAO1 strain did not swarm appreciably under these conditions, precluding an analysis of swarming motility in this strain background (data not shown). When we examined our PA14 $\Delta sbrR$ mutant strain, we discovered it does not spread from the point of inoculation and is completely defective for swarming motility (Fig. 3C and D). This suggests that SbrR promotes swarming motility or that SbrR represses an inhibitor(s) of swarming motility.

The effect of SbrR on swarming motility is dependent upon SbrI and MuiA. Given SbrR's role as an anti- σ factor, we reasoned that constitutive activation of SbrI might be responsible for the motility defect in $\Delta sbrR$ mutant cells. To determine if the swarming defect in PA14 $\Delta sbrR$ mutants was dependent upon *sbrI*, we constructed the double mutant PA14 $\Delta sbrIR$. Swarming motility of cells of the PA14 $\Delta sbrIR$ strain was restored to WT levels (Fig. 4A), demonstrating that SbrI is necessary for swarming inhibition in PA14 $\Delta sbrR$ mutant cells.

Next, we reasoned that SbrI-dependent inhibition of swarming motility in PA14 $\Delta sbrR$ mutants was likely due to the constitutive expression of a gene(s) in the SbrI regulon. The three most highly upregulated genes in the cells of the $\Delta sbrR$ mutant in our microarrays were *muiA*, PA4495, and *sbrI* itself (Table 1). MuiA and PA4495 have not previously been implicated in swarming motility. However, ectopic expression of *muiA* has been shown to suppress alginate overproduction in mucoid strains that retain WT MucA (52). Neither MuiA nor PA4495 has any significant homology to any previously characterized proteins, but both proteins are predicted to contain N-terminal secretion signals and have been

found experimentally in the periplasm (53). To test whether MuiA or PA4495 contributed to the inhibition of swarming motility exhibited by the $\Delta sbrR$ mutant strain, we generated PA14 $\Delta sbrR$ $\Delta muiA$ double mutants and PA14 $\Delta sbrR$ Δ PA4495 double mutants. Unlike the cells of a $\Delta sbrR$ single mutant, cells of a $\Delta sbrR$ $\Delta muiA$ mutant did not exhibit a swarming motility defect and instead resembled WT PA14 with respect to their ability to swarm (Fig. 4A). This finding indicates that MuiA is necessary for swarming inhibition in $\Delta sbrR$ mutant cells. The PA14 $\Delta sbrR$ $\Delta muiA$ double-mutant strain could be complemented by ectopic expression of *muiA*, which restored swarming inhibition (Fig. 4B). In contrast, the PA14 $\Delta sbrR$ Δ PA4495 double-mutant strain exhibited a swarming motility defect similar to that of the PA14 $\Delta sbrR$ single mutant (see Fig. S2 in the supplemental material), suggesting PA4495 is not necessary for the inhibition of swarming motility in $\Delta sbrR$ mutant cells. Together, these results suggest that the inhibition of swarming motility in $\Delta sbrR$ mutant cells is the result of increased SbrI-dependent expression of MuiA.

Expression of *muiA* is sufficient for the inhibition of swarming motility. In cells of the $\Delta sbrR$ mutant, the expression of *muiA*, PA4495, and *sbrI* is increased (Table 1) and swarming motility is inhibited (Fig. 4A and B). Moreover, MuiA is necessary for the inhibition of swarming motility in the $\Delta sbrR$ mutant strain (Fig. 4A). Therefore, we wondered whether ectopic expression of *muiA* would suffice to inhibit swarming motility or if MuiA-mediated inhibition of swarming motility in the $\Delta sbrR$ mutant strain required activation of the entire SbrI regulon. To test this, we transformed WT PA14 cells with the same *muiA*-expressing plasmid used to complement the PA14 $\Delta sbrR$ $\Delta muiA$ double mutant

(pHERD20T-MuiA) and an empty-vector control (pHERD20T). Swarming motility was inhibited to levels comparable to that of the $\Delta sbrR$ mutant strain in WT PA14 cells transformed with pMuiA (pHERD20T-MuiA) but not in WT PA14 cells transformed with the empty-vector control (pHERD20T) (Fig. 4B). Thus, ectopic expression of *muiA* is sufficient for the inhibition of swarming motility.

$\Delta sbrR$ mutant cells exhibit enhanced biofilm formation. The surface-associated behaviors of swarming motility and biofilm formation are inversely coregulated in *P. aeruginosa* PA14 (9–12). That is, strains that exhibit increased swarming motility produce less biofilm, while strains that produce increased levels of biofilm exhibit reduced or inhibited swarming motility. Given this relationship, we next asked whether PA14 $\Delta sbrR$ mutant cells were altered with respect to their ability to form biofilms. Following 8 h of static growth, cells of the nonswarming PA14 $\Delta sbrR$ mutant strain formed ~2-fold more biofilm than cells of the WT strain (Fig. 4C). This finding suggests that SbrR reduces biofilm formation or inhibits factors that facilitate biofilm formation.

The effect of SbrR on biofilm formation is dependent on SbrI and MuiA. Next, we tested the PA14 $\Delta sbrIR$ double-mutant strain to determine if the increase in biofilm formation in the $\Delta sbrR$ mutant strain was SbrI dependent. PA14 $\Delta sbrIR$ formed biofilms at levels similar to that of the WT (Fig. 4C), suggesting that in addition to inhibiting swarming motility, increased expression of the SbrI regulon in the $\Delta sbrR$ mutant strain also promotes biofilm formation.

Our previous finding that the swarming motility defect of the $\Delta sbrR$ mutant strain was MuiA dependent led us to ask whether MuiA was also required for enhanced biofilm formation in the $\Delta sbrR$ strain. Cells of a PA14 $\Delta sbrR \Delta muiA$ mutant strain formed biofilms at levels similar to those of the WT (Fig. 4C), indicating MuiA is necessary for the enhanced biofilm formation observed in the $\Delta sbrR$ mutant strain. In addition, biofilm formation in PA14 $\Delta sbrR \Delta muiA$ mutants could be restored to $\Delta sbrR$ mutant levels by expressing *muiA* from a plasmid (Fig. 4D). These findings suggest that enhanced biofilm formation in the $\Delta sbrR$ mutant strain may be the result of increased SbrI-dependent expression of *muiA*. Lastly, while MuiA is required for increased biofilm formation in the $\Delta sbrR$ mutant strain, the PA14 $\Delta muiA$ mutant strain formed biofilms at WT levels (Fig. 4C). This indicates MuiA is not required for biofilm formation in WT cells of *P. aeruginosa* PA14 under the conditions of our experiments.

Expression of *muiA* is sufficient to promote biofilm formation. Ectopic expression of *muiA* in WT PA14 results in a swarming motility defect equivalent to that observed in cells of the $\Delta sbrR$ mutant strain (Fig. 4B), suggesting it is sufficient for the inhibition of swarming motility. In light of this observation and the inverse relationship between swarming motility and biofilm formation, we next asked whether ectopic expression of *muiA* was also sufficient to enhance biofilm formation. In WT PA14 cells transformed with a *muiA* expression construct but not those transformed with an empty vector, there is an increase in biofilm formation that is comparable to that seen in $\Delta sbrR$ mutants (Fig. 4D), demonstrating that ectopic expression of *muiA* is sufficient to promote biofilm formation. Taken together, our findings suggest that in $\Delta sbrR$ mutant cells, constitutive activation of SbrI results in high levels of *muiA* expression, which enhances biofilm formation via an unknown mechanism.

DISCUSSION

We have presented evidence that SbrI and SbrR constitute a σ factor and anti- σ factor pair, with the N-terminal portion of SbrR interacting directly with SbrI. Cells lacking SbrR are defective for swarming motility and exhibit enhanced biofilm formation as a result of the SbrI-dependent increase in *muiA* expression that occurs in these cells. SbrR and SbrI represent a novel set of regulators of swarming motility and biofilm formation in *P. aeruginosa* that mediate their effects through MuiA, a protein not previously known to influence either of these processes.

The SbrIR regulon. Our transcription-profiling experiments identified a relatively small number of genes that appear to be negatively controlled by SbrR and that are expressed in an SbrI-dependent manner. In particular, the putative *sbrIR* operon, *muiA*, and PA4495 exhibited the largest changes in expression in cells of the $\Delta sbrR$ anti- σ factor mutant relative to the WT (Table 1). Using promoter-*lacZ* fusion reporter strains, we demonstrated that SbrR negatively regulates transcription from the promoters of these genes. We also showed that the increase in transcription that occurs from these promoters in the absence of SbrR is dependent upon SbrI. These findings support the idea that SbrR is an anti- σ factor specific for SbrI and are consistent with those of a previous study that reported PA2896 (SbrI)-dependent expression of *muiA* (PA1494) and PA4495 in response to the overexpression of the periplasmic protease *ctpA* (19).

By aligning the transcription start sites of *sbrI*, *muiA*, and PA4495 derived from transcriptome sequencing (RNA-seq) studies, Seo and Darwin identified putative –10 and –35 promoter elements with the sequence TAACCCG-N₁₆-CGTCTCA-N₆-A (+1) (19). Using the Find Individual Motif Occurrences (FIMO) program to search for this putative SbrI-dependent promoter sequence in the PAO1 and PA14 genomes revealed statistically significant matches to this consensus only in the promoters of *sbrI*, *muiA*, and PA4495 (false-discovery rate [*q*] ≤ 0.01) (data not shown). The *sbrI*, *muiA*, and PA4495 promoters may therefore be the only ones that are recognized directly by RNAP-containing SbrI. We note that the SbrIR regulon defined here is not unusually small for ECF σ factors, which frequently control the expression of relatively small sets of genes (20).

Taken together, our results suggest a model in which the anti- σ factor SbrR binds to SbrI and sequesters it at the membrane, preventing it from associating with RNAP (Fig. 5). In response to an unknown extracytoplasmic signal, SbrI is released from SbrR and associates with RNAP, resulting in expression of the SbrI regulon (Fig. 5). The SbrI regulon likely consists of the putative *sbrIR* operon, *muiA*, and PA4495. SbrI-dependent expression of SbrI results in a positive-feedback loop, amplifying the expression of the SbrI regulon. *muiA* and PA4495 are expressed at high levels and exported to the periplasm, leading to *muiA*-dependent inhibition of swarming motility and enhanced biofilm formation. Previous work has shown that the expression of *sbrI*, *muiA*, and PA4495 becomes elevated following osmotic shock (54), following treatment with the cell wall-inhibitory antibiotic D-cycloserine (55), and following ectopic expression of the periplasmic protease CtpA, which leads to disruption of the cell envelope (19). We suggest that cell envelope stress might be sensed by SbrR, leading to SbrI activation, expression of *muiA*, and transition from motile swarming cells to growth as a biofilm (Fig. 5). It is also possible

FIG 5 Model of the SbrIR regulon. SbrR is an anti- σ factor that binds to SbrI and inhibits its activity. In response to an unknown signal, SbrI is released from SbrR, resulting in increased expression of the SbrI regulon. Increased expression of *muiA* results in the inhibition of swarming motility and enhanced biofilm formation. OM, outer membrane; IM, inner membrane.

that CtpA is capable of directly degrading SbrR, resulting in the activation of SbrI (19).

SbrR and SbrI control swarming motility and biofilm formation in *P. aeruginosa* PA14 through MuiA. The *muiA* gene was the most highly upregulated member of the SbrI regulon in Δ *sbrR* mutant cells relative to the WT (Table 1), and expression from the *P_{muiA}* promoter was shown to be SbrI dependent (Fig. 2A) (19). We have shown that increased SbrI-dependent expression of *muiA* in Δ *sbrR* mutant cells inhibits swarming motility and enhances biofilm formation and that ectopic expression of *muiA* in WT cells has the same effect.

Swarming motility in PA14 is dependent upon flagella and secreted surfactants (14, 15). As demonstrated by our swimming assays, PA14 Δ *sbrR* mutants are capable of chemotaxis and produce functional flagella (Fig. 3B). On the 0.5% agar plates used to observe swarming, surfactant production can be observed as an area of wetness surrounding surfactant-producing colonies (15, 16). We observed this region surrounding nonswarming PA14 Δ *sbrR* mutant colonies, suggesting the strain continues to produce surfactants (data not shown). These findings suggest the MuiA-dependent swarming motility defect in PA14 Δ *sbrR* cells is unlikely to be caused by a defect in either flagella or surfactant production.

MuiA has previously been shown to inhibit the mucoid phenotype of certain clinical isolates in which alginate is overproduced due to the presence of mutations that activate AlgW (52). However, we think it unlikely that MuiA inhibits swarming motility and promotes biofilm formation in PA14 through an inhibitory effect on alginate production. Indeed, MuiA does not appear to be a general inhibitor of alginate production, e.g., MuiA did not detectably influence the production of alginate in cells that produce truncated versions of MucA (52). Furthermore, alginate does not appear to be produced by WT PA14 during growth as a biofilm (56).

In addition to MuiA, several other systems have been shown to exert reciprocal control over swarming motility and biofilm formation in *P. aeruginosa*, including c-di-GMP and the GacS/GacA two-component system (9, 11, 12). We note that the abundance of the *muiA* and PA4495 transcripts is elevated in cells in which the GacS/GacA system is artificially activated through deletion of *retS* (9). The GacS/GacA system functions by activating the expression of genes encoding the small RNAs RsmY and RsmZ (57). These small RNAs in turn sequester the RNA-binding protein RsmA that binds many mRNAs directly to influence their translation or abundance (or both) (58). Neither *sbrI* nor *sbrR* transcript abundance was altered in cells of a *retS* mutant relative to the WT (9), suggesting that the *muiA* and PA4495 transcripts may be direct targets of RsmA. Future work will be aimed at identifying the mechanism by which MuiA represses swarming motility and enhances biofilm formation in *P. aeruginosa* PA14.

Connections to virulence. A signature-tagged mutagenesis screen identified *sbrR* (PA2895) as a gene required during chronic respiratory infection in a rat lung model (18). Interestingly, a significant portion of the mutants identified in this screen exhibited impaired swarming motility, indicating that swarming motility is an important virulence determinant in this model of chronic respiratory infection (18). Although an *sbrR* (PA2895) mutant in *P. aeruginosa* strain PA103 was previously shown to be defective for protease secretion, we do not observe any protease secretion defect in cells of our PAO1 Δ *sbrR* or PA14 Δ *sbrR* mutant cells (data not shown). The results presented here suggest the virulence defect of *sbrR* mutants in the rat lung model of chronic respiratory infection could be explained in whole or in part by their swarming motility defect, or possibly through the misregulation of either swarming motility, biofilm formation, or both. Recent transposon-sequencing (Tn-Seq) analyses indicated that SbrI is required for colonization of the murine gastrointestinal (GI) tract in a neutropenic model of acute infection (59). SbrIR may therefore play important regulatory roles during both chronic and acute infections.

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