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MEETING PRESENTATION

# H-NS Family Members MvaT and MvaU Regulate the *Pseudomonas aeruginosa* Type III Secretion System

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**ABSTRACT** *Pseudomonas aeruginosa* is an opportunistic Gram-negative pathogen capable of causing severe disease in immunocompromised individuals. A major *P. aeruginosa* virulence factor is the type III secretion system (T3SS). The T3SS is used to translocate effector proteins into host cells, causing cytotoxicity. The T3SS is under the transcriptional control of the master regulator ExsA. ExsA is encoded in the *exsCEBA* operon and autoregulates transcription via the $P_{exsC}$ promoter. There is also a Vfr-dependent promoter ($P_{exsA}$) located in the intergenic region between *exsB* and *exsA*. A previous chromatin immunoprecipitation (ChIP)-on-chip experiment identified strong binding signatures for MvaT and MvaU in the intergenic region containing the $P_{exsA}$ promoter. MvaT and MvaU are DNA-binding histone-like nucleoid-structuring proteins that can repress gene expression. As predicted from the previous ChIP data, purified MvaT specifically bound to the $P_{exsA}$ promoter region in electrophoretic mobility shift assays. Whereas disruption of *mvaT* or *mvaU* by either transposon insertion or clustered regularly interspaced short palindromic repeat interference (CRISPRi) derepressed $P_{exsA}$ promoter activity and T3SS gene expression, overexpression of MvaT or MvaU inhibited $P_{exsA}$ promoter activity. Disruption of *mvaT*, however, did not suppress the Vfr requirement for $P_{exsA}$ promoter activity. Mutated MvaT/MvaU defective in transcriptional silencing exhibited dominant negative activity, resulting in a significant increase in $P_{exsA}$ promoter activity. Because no effect of MvaT or MvaU on Vfr expression was detected, we propose a model in which the primary effect of MvaT/MvaU on T3SS gene expression is through direct silencing of the $P_{exsA}$ promoter.

**IMPORTANCE** Global regulatory systems play a prominent role in controlling the *P. aeruginosa* T3SS and include the Gac/RsmA, c-di-GMP, and Vfr-cAMP signaling pathways. Many of these pathways appear to directly or indirectly influence *exsA* transcription or translation. In this study, the histone-like proteins MvaT and MvaU are added to the growing list of global regulators that control the T3SS. MvaT and MvaU bind AT-rich regions in the genome and silence xenogeneic genes, including pathogenicity islands. The T3SS gene cluster has been horizontally transmitted among many Gram-negative pathogens. Control by MvaT/MvaU may reflect a residual effect that has persisted since the initial acquisition of the gene cluster, subsequently imposing a requirement for active regulatory mechanisms to override MvaT/MvaU-mediated silencing.

**KEYWORDS** ExsA, H-NS, MvaT, MvaU, *Pseudomonas aeruginosa*, type III secretion system

Members of the histone-like nucleoid-structuring protein (H-NS) family of proteins are found in many Gram-negative bacteria and function as global regulators of gene expression. Most studies of H-NS have been performed in *Escherichia coli*. *E. coli* H-NS serves a regulatory role and binds to hundreds of loci, repressing over a thousand different genes (1). Many of the repressed genes were acquired horizontally (1).

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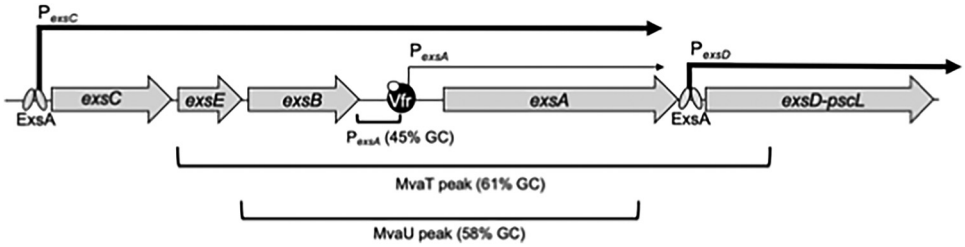


**FIG 1** Diagram of the T3SS regulatory locus. Promoter regions that control transcription of the *exsCEBA* operon ($P_{exsC}$), *exsA* ($P_{exsA}$), and the *exsD* to *pscL* operon ($P_{exsD}$) are indicated by arrows. Brackets indicate the MvaT and MvaU binding regions, based on data from Castang et al. (22), and the region used for the $P_{exsA}$ EMSA probe (Fig. 4). Percent GC content is indicated for each bracketed region.

Additionally, H-NS can play a structural role by bridging and compacting DNA (2–4). H-NS and related proteins consist of a coiled-coil amino-terminal dimerization domain and a carboxy-terminal DNA-binding domain connected by a flexible linker (5–10). The central area of the protein (part of the amino-terminal domain and linker) is involved in mediating formation of higher-order oligomers consisting of more than two dimers (8, 11). Using these domains, H-NS binds to curved AT-rich DNA and then oligomerizes across adjacent DNA to repress gene expression (12). These curved AT-rich regions are often found in promoter regions and xenogeneic DNA (1, 13–16). Such xenogeneic loci include prophages and pathogenicity islands, which have a lower GC content compared to GC-rich Gram-negative genomes. The H-NS-DNA nucleoprotein complex then silences target genes either by occluding RNA polymerase (RNAP) from the promoter or by trapping RNAP between two bridged sections of DNA (17, 18). Transcription factors can compete with H-NS for promoter regions to initiate transcription. Additionally, bridged DNA is refractory to elongating RNAP and can force transcriptional pausing, but RNAP can displace H-NS linearly bound to DNA (19).

H-NS family members are also found in nonenteric Gram-negative bacteria, including the pseudomonads. *Pseudomonas aeruginosa* is an opportunistic Gram-negative pathogen capable of causing severe disease in immunocompromised individuals. *P. aeruginosa* encodes two H-NS family proteins, MvaT and MvaU (20, 21). Like other H-NS-like proteins, MvaT and MvaU preferentially bind curved AT-rich DNA (22, 23) and have the same protein domains as *E. coli* H-NS (24). MvaT was originally described as a global regulator of virulence gene expression (25). MvaT and MvaU have overlapping regulons (22) and can interact with each other to form homomeric and/or heteromeric filaments (24). MvaT and MvaU also negatively regulate their own transcription and transcription of each other (24, 26).

MvaT controls the expression of at least 150 genes, including those encoding virulence factors and surface structures, such as fimbriae (26). In the *P. aeruginosa* reference strain PAO1, MvaT and MvaU silence prophage genes, preventing activation and lysis by the Pf4 phage (27). Due to this essential function, PAO1 can tolerate deletion of either *mvaT* or *mvaU* but not both (27). MvaT and MvaU directly bind to more than 100 loci, encoding 394 and 311 genes, respectively (22). These loci are AT-rich and mostly overlap for MvaT and MvaU binding (22). One region bound by both MvaT and MvaU is located in the major regulatory locus of the type III secretion system (T3SS) (22). The T3SS is a molecular machine found in many Gram-negative bacteria that functions to translocate effector proteins into the eukaryotic host cell cytosol. The *P. aeruginosa* T3SS translocates exotoxins (ExoS, ExoT, ExoU, and/or ExoY) that manipulate host cell signaling and/or cause cytotoxicity (28).

The T3SS is under the transcriptional control of the master regulator ExsA (29). A schematic of the *exsA* locus is shown in Fig. 1. ExsA is encoded in an operon beginning with *exsC* and autoregulates its transcription via the $P_{exsC}$ promoter (29). ExsA is also transcribed from another promoter, $P_{exsA'}$ which is located in the intergenic region

between *exsB* and *exsA* (30). The $P_{exsA}$ promoter is under the transcriptional control of the global virulence regulator Vfr, a cAMP-binding protein (30). Transcriptional regulators Fis (31) and VqsM (32) have also been reported to have positive effects on *exsA* transcription. Castang et al. (22) reported binding signatures for MvaT and MvaU in the T3SS locus, centered around the $P_{exsA}$ promoter (Fig. 1). In this study, we demonstrate that disruption of *mvaT* or *mvaU*, by clean deletion, transposon (Tn) insertion, or clustered regularly interspaced short palindromic repeat interference (CRISPRi), increases T3SS gene expression. Conversely, overexpression of *mvaT* or *mvaU* decreases T3SS gene expression. Deletion of *mvaT* does not suppress the requirement of Vfr for transcription from the $P_{exsA}$ promoter. Finally, MvaT specifically binds to the $P_{exsA}$ promoter *in vitro*. We conclude that MvaT and MvaU inhibit the T3SS by directly silencing Vfr-dependent transcription from the $P_{exsA}$ promoter.

## RESULTS

**Tn insertion *mvaT* and *mvaU* mutants have increased T3SS gene expression.** A previous study reported chromatin immunoprecipitation (ChIP)-on-chip binding signatures for MvaT and MvaU in the T3SS locus, centered around the 297-bp *exsB-exsA* intergenic region (22) (Fig. 1). More recently, the same intergenic region was shown to contain an *exsA*-specific promoter ($P_{exsA}$) (30, 32). We hypothesized that MvaT and MvaU control T3SS gene expression by modulating $P_{exsA}$ promoter activity. To test this, we first used the *mvaT*::Tn and *mvaU*::Tn mutants from the *P. aeruginosa* PA14 nonredundant Tn insertion library (33). The *exsA*::Tn mutant, which is completely defective for T3SS gene expression, was used as a negative control. The Tn insertion site in each mutant was verified by PCR (see Fig. S1 in the supplemental material). Growth curves determined in liquid culture (LB) at 37°C indicated that the Tn insertions had no significant effect on growth (Fig. S2A). MvaT and MvaU were shown previously to negatively regulate production of pyocyanin (22, 25, 34). Consistent with this, the PA14 *mvaT* and *mvaU* Tn mutants produced 1.5- to 1.7-fold more pyocyanin than the wild-type (wt) PA14 strain (Fig. 2A).

To monitor T3SS gene expression, we used two transcriptional reporters. The $P_{exsD}$-*lacZ* reporter measures activity from the ExsA-dependent $P_{exsD}$ promoter (35), and the $P_{exsA}$-*lacZ* reporter measures activity from the Vfr-dependent $P_{exsA}$ promoter (30). Strains were cultured under noninducing (high $Ca^{2+}$ levels, without EGTA) and inducing (low $Ca^{2+}$ levels, with EGTA) conditions for T3SS gene expression and assayed for $\beta$-galactosidase activity. Under noninducing conditions, $P_{exsD}$-*lacZ* reporter activity was minimal in wt PA14 and each of the Tn insertion strains (Fig. 2B). Reporter activity increased 8-fold in wt PA14 cultured under inducing conditions and remained defective in the *exsA* mutant. Disruption of either *mvaT* or *mvaU* resulted in an ~2-fold increase in reporter activity, relative to wt PA14 cultured under inducing conditions. Similarly, $P_{exsA}$-*lacZ* transcriptional reporter activity increased above wt levels when either *mvaT* or *mvaU* was disrupted (Fig. 2B). Whereas the *mvaT*::Tn mutant demonstrated increased $P_{exsA}$-*lacZ* activity under both noninducing and inducing conditions, activity was significantly increased for the *mvaU*::Tn mutant only under inducing conditions. Consistent with the increase in $P_{exsA}$-*lacZ* reporter activity, ExsA levels were elevated in the whole-cell lysate fractions of the *mvaT*::Tn and *mvaU*::Tn mutants (Fig. 2C). The increase in ExsA protein levels also resulted in increased T3SS activity, as shown by elevated levels of ExoU and PcrV in the culture supernatant fractions of the *mvaT*::Tn and *mvaU*::Tn mutants (Fig. 2C). ExoU and PcrV are secreted T3SS substrates. From these data, we conclude that disruption of *mvaT* or *mvaU* derepresses T3SS gene expression in *P. aeruginosa* strain PA14.

**Overexpression of *mvaT* or *mvaU* inhibits T3SS gene expression in strain PA14.** Based on the finding of elevated T3SS gene expression in the *mvaT* and *mvaU* Tn mutants, we hypothesized that increased MvaT or MvaU expression would inhibit T3SS gene expression. To test this, we constructed plasmids to express *mvaT* or *mvaU* from an arabinose-inducible promoter. PA14 strains were cultured under T3SS-inducing conditions in the presence of 0.02% or 0.1% arabinose to induce expression of MvaT or

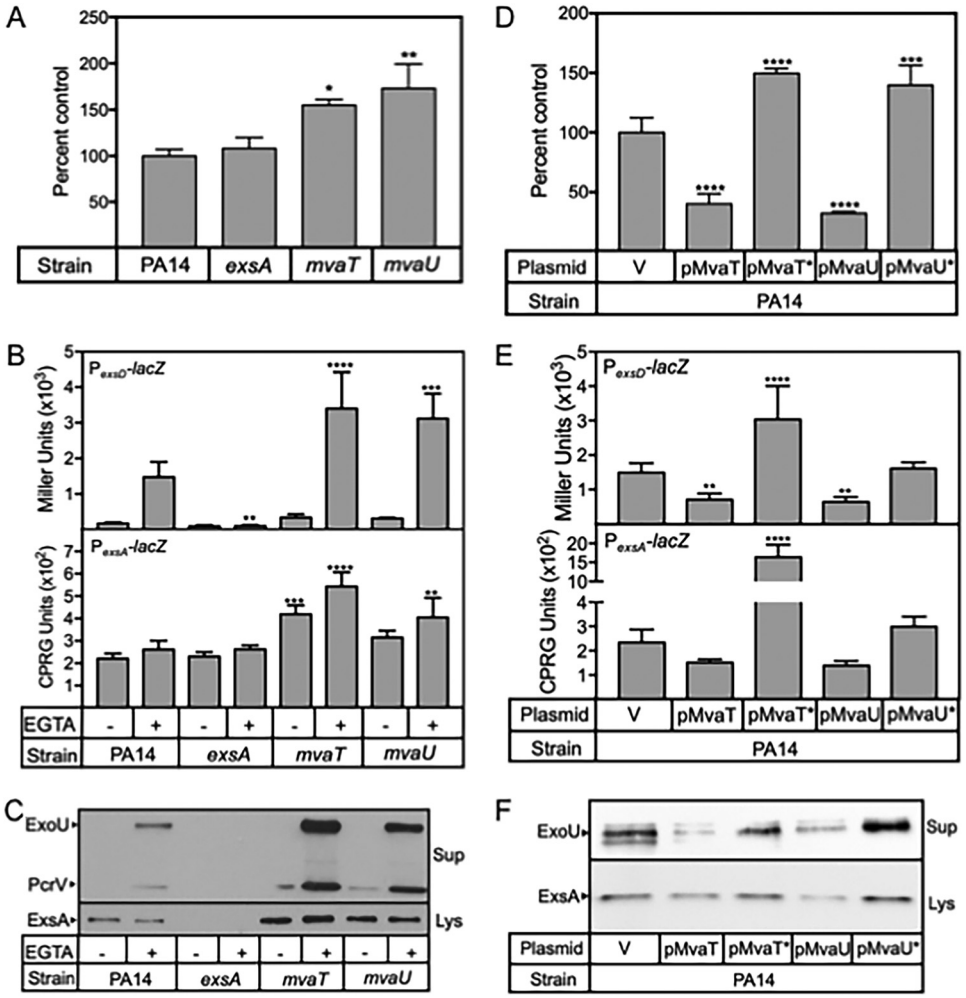


**FIG 2** MvaT and MvaU inhibit pyocyanin production and T3SS gene expression in strain PA14. The wt PA14 strain and *exsA*, *mvaT*, and *mvaU* Tn insertion mutants (A to C) and wt PA14 carrying MvaT and MvaU expression plasmids (D to F) were cultured at 37°C and assayed for pyocyanin production (A and D) and T3SS gene expression (B, C, E, and F). MvaT and MvaU expression from $P_{BAD}$ was induced by addition of 0.02% arabinose (pMvaT and pMvaT*) or 0.1% arabinose (vector control [V], pMvaU, and pMvaU*). (A and D) The pyocyanin data are relative to the wt strain or the vector strain, normalized to 100%. (B and E) The indicated strains carrying the $P_{exsA}$-*lacZ* or $P_{exsD}$-*lacZ* transcriptional reporter were cultured under noninducing or inducing (with EGTA) conditions for T3SS gene expression and were assayed for $\beta$-galactosidase activity. (C and F) Supernatant (Sup) and lysate (Lys) samples from the same cultures were immunoblotted for ExoU, PcrV, and ExsA. Error bars represent the standard deviations of at least three biological replicates. The indicated statistical differences are relative to wt PA14 (A to C) or the vector control (D to F). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

MvaU, respectively. Different arabinose concentrations were used to minimize growth defects resulting from MvaT or MvaU overexpression (Fig. S2B). As a control, we measured pyocyanin levels, observing a significant decrease when either MvaT or MvaU was overexpressed (Fig. 2D). As expected, overexpression of either *mvaT* or *mvaU* inhibited $P_{exsD}$-*lacZ* reporter activity compared to the vector control (Fig. 2E). Overexpression of *mvaT* or *mvaU* reproducibly decreased $P_{exsA}$-*lacZ* activity but not to a level of statistical significance ($P = 0.85$ and $P = 0.77$, respectively). Consistent with the transcriptional reporter data, ExsA and ExoU protein levels were reduced upon MvaT or MvaU expression (Fig. 2F).

MvaT and MvaU share 79% amino acid similarity and bind DNA as homodimers (24). MvaT and MvaU can also interact to form mixed heterodimers (9, 24). Maximal transcription silencing requires formation of higher-order oligomers (9). Previous studies found that a F36S MvaT point mutant bound DNA as a homodimer but could not form higher-order oligomers (9, 36). The MvaT F36S mutant has a dominant negative

effect on *cupA* transcription *in vivo* (9). The F36 residue is conserved in MvaU. We hypothesized that MvaT and MvaU F36S mutants would no longer repress pyocyanin production or T3SS gene expression. Whereas overexpression of wt MvaT/MvaU inhibited pyocyanin production (Fig. 2D) and $P_{exsD}$-*lacZ* reporter activity, MvaT F36S (pMvaT*) and MvaU F36S (pMvaU*) lacked inhibitory activity (Fig. 2E). Rather than inhibition, expression of MvaT F36S (pMvaT*) or MvaU F36S (pMvaU*) significantly increased pyocyanin production. We also observed significant increases in $P_{exsD}$-*lacZ* and $P_{exsA}$-*lacZ* activity upon MvaT F36S expression, consistent with the previous report of dominant negative activity (9). That activity is even more dramatic than the effect of disrupting either *mvaT* or *mvaU* (Fig. 2B) and likely results from nonproductive interaction of MvaT F36S with wt MvaT and/or MvaU. We did not observe a dramatic increase in T3SS protein levels by Western blotting, although there was a slight increase in ExoU secretion when MvaU F36S was expressed (Fig. 2F). From these data, we conclude that MvaT and MvaU overexpression represses T3SS gene expression in strain PA14.

**CRISPRi knockdown of *mvaU* in a Δ*mvaT* background stimulates T3SS gene expression in strain PA103.** To verify our findings from the PA14 Tn mutants, we constructed unmarked in-frame deletion mutants of *mvaT* and *mvaU* in *P. aeruginosa* strain PA103. Attempts to construct a double mutant were unsuccessful, which supports the prior conclusion that loss of both *mvaT* and *mvaU* is lethal (22, 27). Similar to our findings with strain PA14, deletion of *mvaT* or *mvaU* significantly derepressed $P_{exsD}$-*lacZ* and $P_{exsA}$-*lacZ* reporter activity in strain PA103 when cells were cultured under inducing conditions (with EGTA) (Fig. 3A). Deletion of *mvaT* also increased $P_{exsD}$-*lacZ* reporter activity more than 4-fold compared to the wt PA103 strain under noninducing conditions (without EGTA).

Our finding that deletion of either *mvaT* or *mvaU* leads to elevated $P_{exsA}$-*lacZ* reporter activity suggests that both participate in the silencing of $P_{exsA}$ promoter activity. Testing this prediction requires measurement of $P_{exsA}$-*lacZ* reporter activity in the absence of both *mvaT* and *mvaU*. Because mutation of both *mvaT* and *mvaU* appears to be lethal, we utilized CRISPRi (37, 38) to repress *mvaU* transcription in wt *P. aeruginosa* and the *mvaT* mutant. As a control for the efficacy of the CRISPRi system, we targeted *exsC*, which is essential for T3SS gene expression (39). Targeted repression of *exsC* greatly diminished (60-fold) $P_{exsD}$-*lacZ* activity compared to the vector control (Fig. 3B), and targeting of *mvaU* led to a modest but reproducible increase in $P_{exsD}$-*lacZ* activity. Next, we evaluated $P_{exsA}$-*lacZ* reporter in wt PA103 and the Δ*mvaT* strain while targeting *mvaU* (Fig. 3C). Whereas depletion of *mvaU* in the wt background had no significant effect on $P_{exsA}$-*lacZ* activity, targeting of *mvaU* for CRISPRi depletion in the Δ*mvaT* mutant resulted in a significant increase over the value seen for the vector control. From these data, we conclude that MvaT and MvaU function together to control $P_{exsA}$ promoter activity.

**MvaT specifically binds the *exsA* promoter *in vitro*.** MvaT and MvaU preferentially interact with AT-rich DNA (22, 23). The $P_{exsA}$ promoter region (Fig. 1) has an AT content of 55%, which is significantly higher than the average AT content of the *P. aeruginosa* genome (~32%). To confirm that MvaT binds the *exsA* promoter region, we performed electrophoretic mobility shift assays (EMSAs) with recombinant histidine-tagged MvaT. We were unable to purify histidine-tagged MvaU because overexpression severely inhibited *E. coli* growth. Incubation of 150 nM MvaT with labeled $P_{exsA}$ promoter probe resulted in the formation of a MvaT-$P_{exsA}$ complex (Fig. 4A). To demonstrate specific binding of MvaT to the $P_{exsA}$ promoter region, we performed competition experiments by radiolabeling the $P_{exsA}$ promoter probe and adding excess unlabeled DNA to compete for MvaT binding. As a positive control, we first added an excess of unlabeled $P_{mvaU}$ promoter probe, a known MvaT target. Addition of a 25-fold molar excess of the $P_{mvaU}$ probe interfered with the MvaT-$P_{exsA}$ interaction, and a 125-fold excess completely eliminated observable MvaT binding. Similarly, addition of an unlabeled $P_{exsA}$ promoter fragment resulted in a dose-dependent loss of binding comparable to that

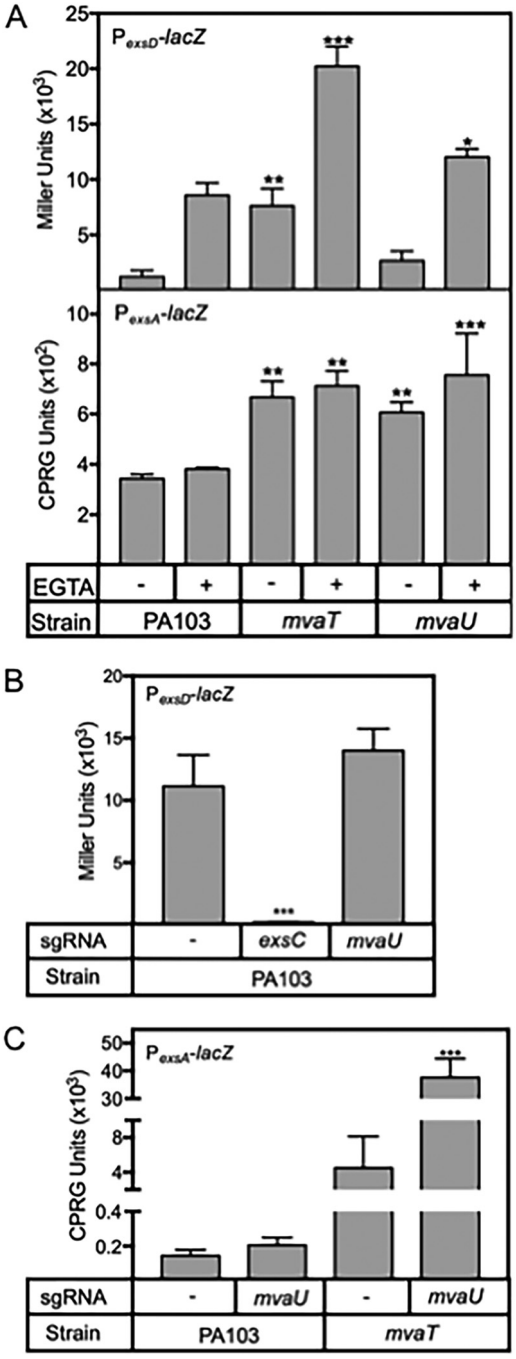


**FIG 3** MvaT inhibits the T3SS in strain PA103. (A) The indicated strains carrying the $P_{exsA}$-*lacZ* or $P_{exsD}$-*lacZ* reporter were cultured at 37°C under noninducing (without EGTA) or inducing (with EGTA) conditions and were assayed for β-galactosidase activity. (B and C) PA103 strains carrying $P_{lac}$-*dcas9*, the $P_{exsD}$-*lacZ* (B) or $P_{exsA}$-*lacZ* (C) reporter, and sgRNA expression plasmids or a vector control (−) were cultured at 37°C under inducing conditions for expression of T3SS genes and *dcas9* and were assayed for β-galactosidase activity The indicated statistical differences are relative to the wt PA103 control (A) or the vector control for each strain (B and C). Error bars represent standard deviations across at least three biological replicates. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

observed with the $P_{mvaU}$ competitor. As a negative control, we added a 125-fold excess of a nonspecific *algD* probe, and we observed no disruption of MvaT-$P_{exsA}$ binding. The experiment was also performed in reverse, by radiolabeling $P_{mvaU}$ and titrating unlabeled $P_{mvaU}$ or $P_{exsA}$ promoter probe (Fig. S3). Once again, $P_{mvaU}$ and $P_{exsA}$ competed for MvaT binding, whereas the nonspecific *algD* probe did not.

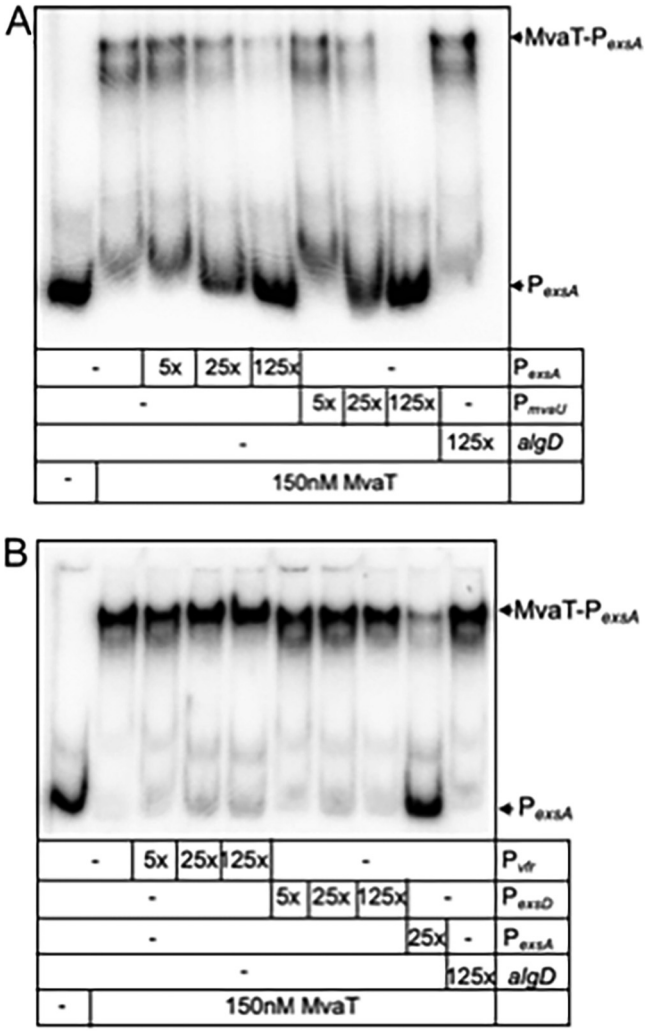


**FIG 4** His-tagged MvaT directly interacts with the *exsA* promoter region *in vitro*. MvaT (150 nM) was incubated for 15 min at 25°C with 0.125 nM radiolabeled $P_{exsA}$ promoter probe and increasing concentrations (the indicated fold molar excess) of unlabeled DNA (A, $P_{mvaU}$ or $P_{exsA}$; B, $P_{vfr}$ or $P_{exsD}$). Unlabeled $P_{mvaU}$ and *algD* were used as positive and negative controls, respectively. Arrows indicate the positions of unbound $P_{exsA}$ and the MvaT-$P_{exsA}$ promoter probe complexes. Representative data from three replicate experiments are shown.

**MvaT and MvaU regulate T3SS gene expression through direct repression of the $P_{exsA}$ promoter.** Translation of ExsA is stimulated by the RNA-binding protein RsmA, through an unknown mechanism (Fig. 5A) (40, 41). When RsmA is sequestered by the small noncoding RNAs RsmY and RsmZ, ExsA is less efficiently translated (41). Previous studies found that *rsmZ* transcription is repressed by MvaT (22, 42). In agreement with those data, we found that $P_{rsmZ}$-*lacZ* and $P_{rsmY}$-*lacZ* reporter activities were both decreased by MvaT or MvaU overexpression in wt strain PA103 compared to the vector control (Fig. 5B) and that deletion of either *mvaT* or *mvaU* resulted in elevated $P_{rsmZ}$-*lacZ* and $P_{rsmY}$-*lacZ* reporter activity. These findings, however, are opposite of the results expected if MvaT/MvaU effects on RsmYZ overexpression affect T3SS gene expression, because liberating RsmA should increase ExsA expression and T3SS gene expression. Thus, the effect of MvaT/MvaU on T3SS gene expression is expected to function independently from the Rsm system.

The previously determined ChIP-on-chip signature for the *exsB-exsA* intergenic region extended downstream to include the *exsD* promoter region (located just downstream of *exsA*) (Fig. 1) (22). In addition to upstream inhibition of $P_{exsA}$ promoter activity

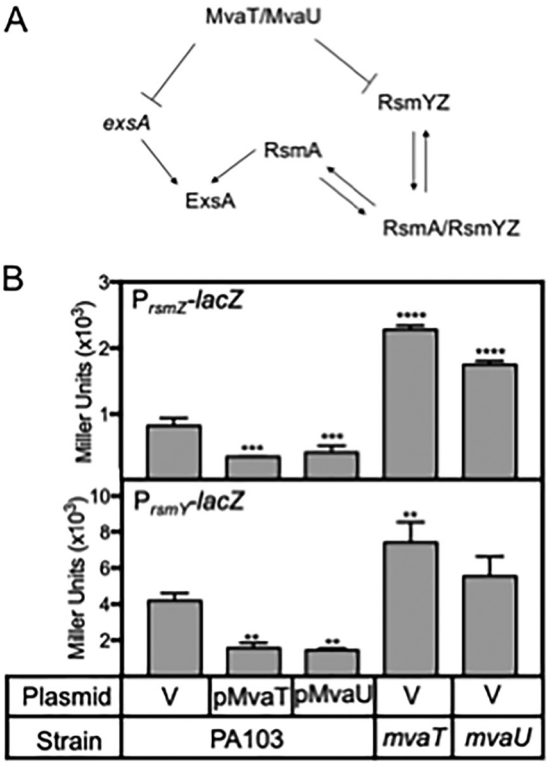


**FIG 5** (A) The schematic shows MvaT/MvaU regulation of *exsA*. (B) MvaT and MvaU repress $P_{rsmZ}$-*lacZ* and $P_{rsmY}$-*lacZ* reporter activity. Strains carrying the $P_{rsmY}$-*lacZ* or $P_{rsmZ}$-*lacZ* reporter were cultured at 37°C under T3SS-inducing conditions and were assayed for $\beta$-galactosidase activity. Error bars represent the standard deviations of three biological replicates. The indicated statistical differences are relative to the wt PA103 vector control. **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

(and ExsA synthesis) by MvaT/MvaU, our observation of reduced $P_{exsD}$-*lacZ* transcriptional reporter activity (Fig. 2 and 3) raised the possibility of a direct effect on the $P_{exsD}$ promoter. To examine this, we performed competition experiments with unlabeled $P_{exsD}$ promoter probe and radiolabeled $P_{exsA}$ (Fig. 4B) promoter probe; we observed no disruption of the MvaT-$P_{exsA}$ interaction with a 125-fold excess of $P_{exsD}$. These data indicate that MvaT does not bind directly to the $P_{exsD}$ promoter.

The $P_{exsA}$ promoter is under the transcriptional control of the cAMP-binding protein Vfr (30). Vfr autoregulates its own transcription from the $P_{vfr}$ promoter (43, 44). To determine whether MvaT influences *vfr* transcription, competition experiments were performed with an excess of unlabeled $P_{vfr}$ promoter probe, but no evidence of MvaT binding was observed (Fig. 4B). Additionally, immunoblots revealed no changes in Vfr expression upon MvaT or MvaU overexpression in strains PA14, PA103, and PAK (Fig. 6). The effects of MvaT and MvaU on $P_{exsA}$ promoter activity, therefore, are direct and do not involve alteration of *vfr* expression.

**Vfr activation of T3SS gene expression is not dependent on MvaT.** We previously proposed that Vfr activates the $P_{exsA}$ promoter through recruitment of RNAP (30). The involvement of MvaT/MvaU suggested another potential mechanism accounting for the Vfr requirement. Rather than direct activation through recruitment of RNAP, Vfr could indirectly stimulate transcription by displacing MvaT/MvaU from the promoter region, thus relieving their inhibitory effects. To test this, we generated a *vfr mvaT* double mutant in strain PA103 and measured $P_{exsD}$-*lacZ* and $P_{exsA}$-*lacZ* reporter activity and ExsA expression under inducing conditions (Fig. 7). Whereas deletion of *vfr* resulted in significant decreases in ExsA synthesis and $P_{exsD}$-*lacZ* and $P_{exsA}$-*lacZ* reporter activities, deletion of *mvaT* increased activity 1.7- to 2.5-fold over wt levels. The phenotypes of the *vfr* single mutant and the *vfr mvaT* double mutant were identical, showing that

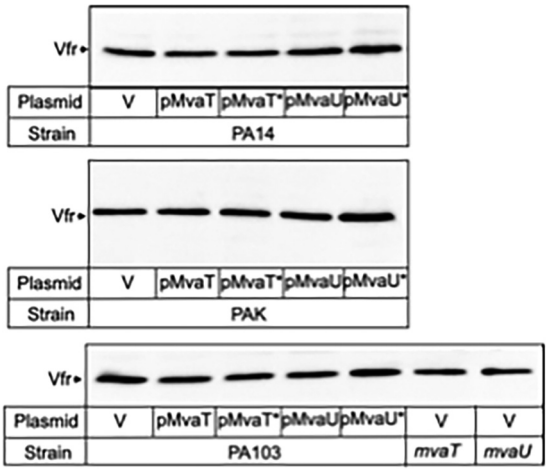


**FIG 6** MvaT does not affect Vfr levels. Strains carrying the indicated expression plasmids were cultured at 37°C under inducing conditions (with EGTA) and were assayed for Vfr production by immunoblotting. MvaT/MvaU expression was induced from $P_{BAD}$ with either 0.02% arabinose (pMvaT and pMvaT* in strain PA14) or 0.1% arabinose (vector control [V], pMvaU, pMvaU*, and all plasmids in PAK).

*vfr* is epistatic to *mvaT*, that Vfr is required even in the absence of *mvaT*, and that Vfr functions as a direct activator of the $P_{exsA}$ promoter.

**Control of T3SS gene expression by MvaT/MvaU in strain PAK.** Strains PA14 and PA103 are ExoU positive and ExoS negative and are representative of only ~30% of sequenced *P. aeruginosa* genomes (45); the remaining ~70% are ExoU negative and ExoS positive. To ensure that our findings with MvaT/MvaU are translatable to ExoU-negative and ExoS-positive strains, we performed CRISPRi and MvaT/MvaU expression studies in strain PAK. Depletion of *mvaT* or *mvaU* by CRISPRi resulted in a significant increase in $P_{exsD}$-*lacZ* reporter activity (Fig. S4A). The effects of MvaT or MvaU overex-

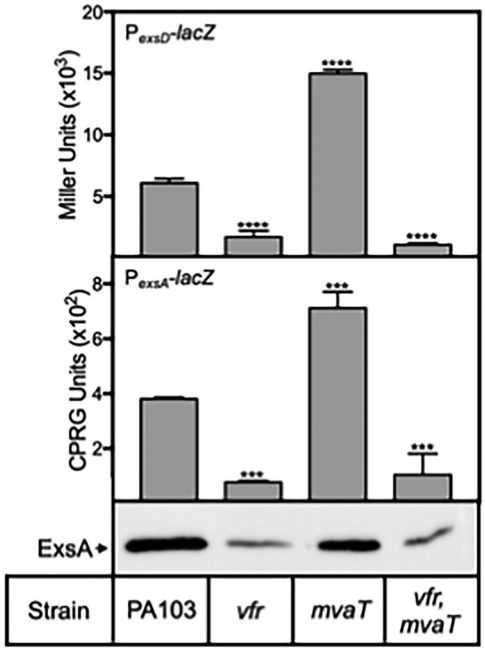


**FIG 7** Vfr is required for ExsA transcription from $P_{exsA}$ irrespective of MvaT. Strains carrying the $P_{exsA}$-*lacZ* or $P_{exsD}$-*lacZ* reporter were cultured at 37°C under inducing conditions (with EGTA) and assayed for $\beta$-galactosidase activity and ExsA synthesis. The immunoblot is representative of two biological replicates. Error bars represent the standard deviations of at least three biological replicates. The indicated statistical differences are relative to the wt PA103 control. ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

pression, however, were more modest than those observed in strains PA14 and PA103, with significance being observed only for $P_{exsD}$-*lacZ* reporter activity and ExoS production upon MvaT expression. Overexpression of the dominant negative MvaT F36S in strain PAK significantly increased $P_{exsD}$-*lacZ* and $P_{exsA}$-*lacZ* activity compared to the vector control, and MvaT F36S significantly increased $P_{exsD}$-*lacZ* activity. From these data, we conclude that MvaT and MvaU show common trends toward inhibition of $P_{exsA}$ promoter activity in strains PA14, PA103, and PAK, with some minor strain variability in the overall levels of activity.

## DISCUSSION

A previous study using ChIP-on-chip found that the H-NS-like proteins MvaT and MvaU bind *in vivo* to the *exsB-exsA* intergenic region, which is now known to contain the $P_{exsA}$ promoter (22, 30). In this study, we confirm specific binding of MvaT to the Vfr-dependent $P_{exsA}$ promoter region *in vitro* and repression of $P_{exsA}$ promoter activity *in vivo*. MvaT and MvaU are global regulators that bind to more than 100 chromosomal loci in *P. aeruginosa* (22). While our data show specific MvaT/MvaU-mediated repression of *exsA* transcription, control of T3SS gene expression is complex and involves numerous additional regulators (30–32, 41, 46–53). Thus, we also considered potential indirect effects of MvaT/MvaU on T3SS gene expression. Neither MvaT nor MvaU was previously reported to bind the $P_{vfr}$ promoter region (22). This is consistent with our EMSA data and immunoblots showing no MvaT binding to the $P_{vfr}$ promoter region or effects on Vfr expression (Fig. 4B and 6). The ChIP-on-chip signature for MvaT in the *exsB-exsA* intergenic region was broad and extended downstream of *exsA* to include the $P_{exsD}$ promoter region (Fig. 1) (22). The $P_{exsD}$ promoter controls transcription of the 12-gene *exsD-pscL* operon (54). Because most of those genes are structural subunits of the T3SS and T3SS gene expression is intimately linked to secretory activity through the ExsACDE partner-switching mechanism, reduced expression of secretory genes results in feedback inhibition of T3SS gene expression (35, 39, 54). Nevertheless, our data indicate that MvaT does not directly bind to the $P_{exsD}$ promoter (Fig. 4B). It remains possible that some indirect silencing of the $P_{exsD}$ promoter occurs through MvaT/MvaU binding to the $P_{exsA}$ promoter, with subsequent oligomerization that extends downstream to the $P_{exsD}$ promoter. MvaT also regulates transcription of the small noncoding RNA *rsmZ* (22, 42) and, as reported herein, *rsmY* (Fig. 5B). RsmY and RsmZ function by sequestering the RNA-binding protein RsmA, which appears to promote ExsA translation (41). Reduced RsmY/RsmZ expression should increase RsmA availability and stimulate T3SS gene expression (41). Our finding that MvaT and MvaU inhibit both RsmY/RsmZ expression (Fig. 5B) (42) and T3SS gene expression (Fig. 2 and 3; also see Fig. S4 in the supplemental material) is opposite to the expected result. These combined data suggest that the primary influence of MvaT/MvaU on T3SS gene expression is independent of Vfr and the Rsm system and instead involves direct effects on the Vfr-dependent $P_{exsA}$ promoter. Involvement of other MvaT/MvaU-controlled genes that contribute to T3SS gene expression, however, remains a possibility.

H-NS-mediated repression of T3SS gene expression is a common theme. In *Yersinia pseudotuberculosis*, the small histone-like protein YmoA negatively regulates the transcription of *lcrF*, the primary activator of the T3SS (55). Deletion of *E. coli* H-NS is required for high-level expression of the *Yersinia* T3SS in *E. coli* (56). In enteropathogenic and enterohemorrhagic *E. coli*, H-NS silences expression of the T3SS-associated locus for enterocyte attachment and effacement (57, 58). The primary regulator of the *E. coli* T3SS (Ler) activates transcription by antagonizing H-NS-mediated silencing (57, 59). Involvement of H-NS in repression of T3SS gene expression is also seen in *Salmonella enterica*, *Shigella flexneri*, and the plant pathogen *Xanthomonas oryzae* (60–62).

In *Vibrio parahaemolyticus*, T3SS-1 is controlled by an *exsA* homolog (63). Whereas H-NS inhibits transcription of *V. parahaemolyticus exsA*, HlyU activates transcription by antagonizing the inhibitory activity of H-NS (64). A similar relationship may exist in *P. aeruginosa*. Vfr (30), Fis (31), and VqsM (32) are positive regulators of the $P_{exsA}$ promoter

region. Using a double mutant lacking both *vfr* and *mvaT*, we found that Vfr was required for $P_{exsA}$ promoter activity irrespective of whether MvaT was present or absent (Fig. 7). Although we have excluded Vfr, Fis or VqsM may activate *exsA* transcription through H-NS antagonism. There may also be differential or conditional requirements for factors acting positively at the $P_{exsA}$ promoter. Transcription of *rsmZ* is positively controlled by GacA and BswR and is repressed by MvaT (42, 65, 66). Whereas activation by GacA is essential for *rsmZ* expression, activation by BswR is required only when MvaT is present (66). It remains possible that the requirement for VqsM and/or Fis is conditional, depending on the MvaT/MvaU status in the cell.

MvaT and MvaU bind to similar regions of the genome, share considerable overlap in their respective regulons, and are thought to play redundant roles in gene silencing (22). Our data are consistent with MvaT and MvaU redundancy, as disruption of either one by Tn insertion, deletion, or CRISPRi generated similar effects on T3SS gene expression, although some minor strain-to-strain variation was noted. Previous reports found MvaT to play a more dominant role, as some phenotypes are more pronounced for the *mvaT* mutant (24, 26, 42). This may reflect differences in the relative affinities of MvaT and MvaU for some promoter regions. There is also the potential for compensatory effects because MvaT and MvaU repress the transcription of one another (24). Therefore, deletion of either one results in elevated expression of the other, which probably accounts for the relatively modest effects (2-fold at best) on T3SS gene expression we observed for the individual deletion mutants. Redundancy is also consistent with the dramatic increase in $P_{exsA}$-*lacZ* reporter activity observed for the *mvaT* mutant when *mvaU* function was disrupted by CRISPRi (Fig. 3C) or upon overexpression of the dominant negative form of MvaT in wt cells (Fig. 2E; also see Fig. S4B), which likely interferes with both MvaT and MvaU function. Whereas the PAO1 *mvaT mvaU* double mutant is nonviable due to the reactivation of a prophage repressed by MvaT/MvaU (27), the same prophage is not found in strain PA103. This might account for the lack of a growth effect following CRISPRi of *mvaU* in the PA103 *mvaT* mutant (Fig. S5).

Although silencing of T3SS gene expression by MvaT/MvaU is evident, the role of silencing is not entirely clear. *In vitro*, the DNA-binding capabilities of MvaT/MvaU are moderately responsive to changes in temperature, osmolarity, and pH, but the formation of rigid filaments is not affected (36, 67). While it is possible that these changes are responsible for MvaT/MvaU regulation, a more likely explanation may lie in the underlying role for MvaT/MvaU in the silencing of xenogeneic DNA acquired by horizontal gene transfer (22, 23). Phylogenetic studies indicate that T3SSs are acquired by horizontal gene transfer (68). Whereas T3SS gene expression confers a fitness advantage under certain conditions, expression is probably disadvantageous under many other conditions, owing to energetic and other considerations. For this reason, T3SS gene expression in all microorganisms is very tightly controlled. As proposed by others, silencing of xenogeneic DNA by MvaT/MvaU likely drives evolution by permitting the acquisition of gene clusters without incurring potential fitness defects (69). This trait may drive further evolution by then imposing a requirement on the new carrier to evolve mechanisms to appropriately control expression in such a manner that the selective advantage conferred by the trait is realized.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The bacterial strains used in this study are provided in Table S1 in the supplemental material. Cloning was performed using *E. coli* DH5$\alpha$ maintained on LB agar supplemented with gentamicin (15 $\mu$g/ml), kanamycin (50 $\mu$g/ml), or tetracycline (12 $\mu$g/ml), as required. MvaT was purified from Tuner DE3 *E. coli* maintained on LB supplemented with kanamycin (50 $\mu$g/ml) and induced with 1 mM isopropyl-$\beta$-D-thiogalactopyranoside (IPTG). *P. aeruginosa* strains were maintained on Vogel-Bonner minimal (VBM) medium supplemented with gentamicin (80 $\mu$g/ml), carbenicillin (300 $\mu$g/ml), or tetracycline (50 $\mu$g/ml). $P_{lac}$-*dcas9* was induced with 1 mM IPTG. For growth curves, *P. aeruginosa* strains were grown overnight at 37°C, with shaking, in liquid LB with appropriate antibiotics. The following day, cultures were diluted to an optical density at 600 nm ($OD_{600}$) of 0.01 in LB with the appropriate antibiotic and arabinose or IPTG, as described in the figure legends. Cultures were grown for

growth curves at 37°C in a 96-well plate in a Tecan Infinite M200 microtiter plate reader. Measurements at $OD_{600}$ were taken at time zero and every 30 min over a 24-h period.

**Plasmid and strain construction.** Plasmids, details of plasmid construction, and primers (Integrated DNA Technologies, Coralville, IA) are listed in Tables S2 and S3. Plasmids were introduced into *P. aeruginosa* by electroporation (70) or conjugation from *E. coli* strain Sm10 (71). For reporter fusions (CTXΦ *att* site) and the *dcas9* expression plasmid (Tn*7* insertion site), pFLP2 was then introduced by either electroporation or conjugation, and pFLP2 was resolved on yeast-tryptone medium supplemented with 10% sucrose. PA103 deletion strains were constructed using homologous recombination. The Tn insertion mutants (33) were verified using primer pairs flanking *exsA* (primers 155609005 and 155609008), *mvaT* (primers 161015209 and 161015210), or *mvaU* (primers 161015211 and 161015212).

The *mvaT* deletion plasmid (pAM254) was generated by Gibson cloning of the Δ*mvaT* allele from the PAO1 Δ*mvaT* strain (24) by PCR using primers 129746433 and 129746434 and cloning into the pEXG2Tc allelic exchange vector. MvaT and MvaU expression vectors were constructed by Gibson cloning of PCR products into pJN105. pEAW025 and pEAW026 were constructed by site-directed mutagenesis with the indicated primers (Table S2) and *Pfu* Turbo polymerase. All constructs were confirmed by Sanger sequencing by the Iowa Institute of Human Genetics Genomics Division at the University of Iowa.

**CRISPRi.** CRISPRi was implemented using pBx-Spas-sgRNA-Gm (Addgene plasmid 105232), pUC18-mini-Tn7T-Plac-dCas9 ($P_{lac}$) (Addgene plasmid 105235) (38), and pTNS2 (Addgene plasmid 64968) (72). The base-pairing regions for the single guide RNAs (sgRNAs) were designed as described by Larson et al. (73). The sgRNA plasmids were generated by designing FastCloning (74) primers for PCR around pBx-Spas-sgRNA-Gm, with 5′ extensions consisting of the sgRNA base-pairing region for each targeted gene (Table S3). The PCR products were treated with DpnI (New England Biolabs) and introduced into DH5α *E. coli* by heat shock. The plasmids were confirmed by Sanger sequencing. pUC18-mini-Tn7T-Plac-dCas9 was integrated into the Tn*7* site using the helper plasmid pTNS2 (72). The gentamicin marker was removed using pFLP2 (75), and the sgRNA plasmids were then transformed into the resulting strains. For all assays, *dcas9* expression was induced by adding 1 mM IPTG. All sgRNAs targeted the template strand 5′ untranslated region of the transcript or the first third of the gene (38).

**Pyocyanin measurements.** *P. aeruginosa* strains were grown overnight at 37°C, with shaking, in liquid LB with appropriate antibiotics. The following day, cultures were diluted to an $OD_{600}$ of 0.1 in LB with the appropriate antibiotic and arabinose, as described in the figure legends. Cultures were grown at 37°C until the $OD_{600}$ reached ~1.2. Pyocyanin levels was measured at 691 nm, as described previously (76).

**β-Galactosidase assays.** *P. aeruginosa* strains were grown overnight at 37°C, with shaking, in liquid LB with appropriate antibiotics. The following day, cultures were diluted to an $OD_{600}$ of 0.1 in tryptic soy broth with 1% glycerol, 100 mM monosodium glutamate (MSG), 2 mM EGTA, the appropriate antibiotic, and arabinose or IPTG, as described in the figure legends. Cultures were grown at 37°C, with shaking, and were harvested when the $OD_{600}$ reached ~1.0. β-Galactosidase activity was measured with the substrate *o*-nitrophenyl-β-galactoside (ONPG) ($P_{exsD}$-*lacZ* and $P_{rsmZ}$-*lacZ*) or chlorophenol red-β-D-galactopyranoside (CPRG) ($P_{exsA}$-*lacZ*), as described previously (30, 39). Miller and CPRG units are reported as averages of at least three experiments, with error bars representing the standard deviations. Statistical significance was determined by one-way analysis of variance (ANOVA) or an unpaired *t* test, using GraphPad Prism.

**Immunoblots.** Whole-cell fractions were prepared by growing *P. aeruginosa* strains as for the β-galactosidase assays and harvesting 1.25 ml when the $OD_{600}$ reached ~1.4. Whole-cell and supernatant fractions were separated by centrifugation (13,000 rpm for 5 min). Culture pellets were suspended in 250 μl of loading buffer and lysed by sonication. Supernatant proteins were precipitated by the addition of trichloroacetic acid to a final concentration of 13% and overnight incubation at 4°C. Proteins were sedimented by centrifugation (13,000 rpm for 5 min), washed with acetone, and suspended in either 15 or 30 μl of 2× loading buffer (PA14 or PAK, respectively). Cell fractions were separated by 12% SDS-PAGE and analyzed by immunoblotting with primary antibodies to Vfr (1:1,000), ExsA (1:1,000), ExoU (1:10,000), PcrV (1:10,000), and/or ExoS (1:10,000), in Tris-buffered saline with 5% skim milk and 0.1% Tween 20. Secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG antibody) was used at a 1:8,000 dilution. Blots were imaged on film (Fig. 1) or with an Azure Biosystems Sapphire imager (all other figures), using SuperSignal West Pico Plus substrate (Thermo Scientific).

**EMSAs.** DNA promoter probes were generated by PCR as follows: $P_{exsA}$ (255 bp), primers 126323954 and 126323955; $P_{mvaU}$ (388 bp), primers 165186174 and 165186175; $P_{exsD}$ (230 bp), primers 164768881 and 164768882; $P_{vfr}$ (405 bp), primers 165186172 and 165186173; *algD* (163 bp), primers 85333731 and 85333730). The probes were purified using a MinElute gel purification kit (Qiagen), end-labeled (1 pmol) with 10 μCi of [γ-$^{32}$P]ATP (Perkin Elmer), and purified using a Qiagen QiaQuick PCR purification kit.

MvaT-His was purified from BL21 Tuner *E. coli* as described previously for ExsA (77), with the following modifications. Harvested cells were suspended in 16 ml binding buffer (20 mM Tris [pH 7.9], 500 mM NaCl, 5 mM imidazole, 0.5% Tween 20) supplemented with cOmplete mini protease inhibitor cocktail (Sigma-Aldrich). Cells were lysed with two passes through a microfluidizer. The cleared lysates were purified using nickel affinity chromatography. The nickel column was washed with 10 ml of binding buffer followed by 20 ml of wash buffer (20 mM Tris [pH 7.9], 500 mM NaCl, 60 mM imidazole, 0.5% Tween 20). Protein was then eluted in 1-ml fractions with elution buffer (20 mM Tris [pH 7.9], 500 mM NaCl, 1 M imidazole, 0.5% Tween 20). Fractions were assayed for protein content with the bicinchoninic acid (BCA) assay and analyzed using 12% SDS-PAGE. Peak fractions were pooled and dialyzed overnight at 4°C in 4 liters of dialysis buffer (20 mM Tris [pH 7.9], 500 mM NaCl, 1 mM dithiothreitol [DTT], 0.5%

Tween 20). The dialysate was cleared of aggregates by centrifugation (100,000 × *g* for 1 h at 4°C) and assayed for protein content, and aliquots were flash frozen in liquid nitrogen and stored at −80°C.

Competition EMSAs were performed as follows. Radiolabeled DNA probe (0.125 nM) in H-NS binding buffer [20 mM Tris (pH 7.5), 100 mM KCl, 1 mM DTT, 10% glycerol, 0.25 ng/μl poly(dI-dC), 100 μg/ml bovine serum albumin] was mixed with 1 μl of unlabeled DNA in a 19-μl reaction volume for 5 min at room temperature. MvaT (diluted in H-NS binding buffer) was added to a final volume of 20 μl, and the mixture was incubated for 15 min at room temperature. Each sample received 1 μl of loading dye (0.2% xylene cyanol, 50% glycerol) and was analyzed on a 5% polyacrylamide glycine gel (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 384 mM glycine) at 4°C. Gels were imaged with a FLA-7000 PhosphorImager (Fujifilm) and analyzed using MultiGauge v3.0 software (Fujifilm) or an Azure Biosystems Sapphire imager.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00054-19.

**SUPPLEMENTAL FILE 1**, PDF file, 1.1 MB.

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